Title
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Permalink
https://escholarship.org/uc/item/4821q22s

Journal
Genetics, 199(2)

ISSN
0016-6731

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Publication Date
2015-02-01

DOI
10.1534/genetics.114.172361

Peer reviewed
Rapid and Precise Engineering of the *Caenorhabditis elegans* Genome with Lethal Mutation Co-Conversion and Inactivation of NHEJ Repair

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ABSTRACT As in other organisms, CRISPR/Cas9 methods provide a powerful approach for genome editing in the nematode *Caenorhabditis elegans*. Oligonucleotides are excellent repair templates for introducing substitutions and short insertions, as they are cost effective, require no cloning, and appear in other organisms to target changes by homologous recombination at DNA double-strand breaks (DSBs). Here, I describe a methodology in *C. elegans* to efficiently knock in epitope tags in 8–9 days, using a temperature-sensitive lethal mutation in the *pha-1* gene as a co-conversion marker. I demonstrate that 60mer oligos with 29 bp of homology drive efficient knock-in of point mutations, and that disabling nonhomologous end joining by RNAi inactivation of the *cku-80* gene significantly improves knock-in efficiency. Homology arms of 35–80 bp are sufficient for efficient editing and DSBs up to 54 bp away from the insertion site produced knock-ins. These findings will likely be applicable for a range of genome editing approaches in *C. elegans*, which will improve editing efficiency and minimize screening efforts.

KEYWORDS oligonucleotide-mediated homologous recombination; CRISPR/Cas9; nonhomologous end joining; *pha-1*; co-conversion

SEQUENCE-SPECIFIC nucleases are a critical tool for manipulation of DNA sequences. The bacterial type II clustered regularly interspaced short palindromic repeats (CRISPR) system, which normally protects against viral DNA and provides a memory of exposure (Jinek et al. 2012), has recently revolutionized genome editing in multiple organisms (Cong et al. 2013; DiCarlo et al. 2013; Gratzi et al. 2013; Hwang et al. 2013; Li et al. 2013; Ran et al. 2013; Gratzi et al. 2014; Nakanishi et al. 2014). For genome editing, the system has been simplified to two components: the Cas9 nuclease, which generates DNA double-strand breaks (DSBs), and a chimeric small guide RNA (sgRNA) that fills the function of two small RNAs in the native bacterial system (Cong et al. 2013). Specific genomic sequences are targeted by the 5’-most 15–20 bp of the sgRNA through the formation of an RNA:DNA hybrid (Jinek et al. 2012; Mali et al. 2013). An NGG motif (protospacer adjacent motif, PAM) must immediately follow the target sequence in the genome (Jinek et al. 2012; Ran et al. 2013). This PAM directs Cas9 to cleave the DNA 3 bp 5’ to the PAM (Jinek et al. 2012). Depending on the desired experimental outcome, one can select for error-prone repair by pathways such as nonhomologous end joining (NHEJ) to generate insertion–deletion (indel) mutations, or homologous recombination to knock in specific sequences.

Initial genome editing methods in *Caenorhabditis elegans* harnessed excision of a Tc or Mos transposon to generate a DSB, and a plasmid repair template to knock in (Plasterk and Groenen 1992; Robert and Bessereau 2007; Frøkjær-Jensen et al. 2008; Frøkjær-Jensen et al. 2012), or delete (Frøkjær-Jensen et al. 2010) desired sequences through homologous recombination. These methods are robust, but the relative rarity of the editing event requires use of a selectable marker, such as *unc-119* rescue or antibiotic resistance (Frøkjær-Jensen et al. 2008; Giordano-Santini et al. 2010), and a transposon site is ideally needed within 1–2 kb of the desired edit (Robert and Bessereau 2007). Zinc finger and transcription activator-like effector nucleases (Wood et al. 2011) and CRISPR/Cas9 have allowed for similar efficient editing without the constraint of transposon insertions. In particular, the ease and rapidity of generating new sgRNAs for the CRISPR/Cas9 system means that transgenic strains can be created precisely and rapidly and any endogenous
NGG sequence can theoretically be targeted. Several CRISPR/Cas9 systems have been described, each with individual strengths and weaknesses (Waaijers and Boxem 2014). Cas9 can be delivered by micro-injection of in vitro transcribed mRNA (Chiu et al. 2013; Lo et al. 2013), pure protein (Cho et al. 2013), or plasmid DNA (Chen et al. 2013b; Dickinson et al. 2013; Friedland et al. 2013; Katic and Grosshans 2013; Waaijers et al. 2013). Similarly, the sgRNAs can be introduced by in vitro transcription, which does not require polyA tailing or 5’ methyl cap addition (Chiu et al. 2013; Cho et al. 2013; Lo et al. 2013), or driven by RNA polymerase III promoters such as U6 (Chen et al. 2013b; Dickinson et al. 2013; Friedland et al. 2013; Katic and Grosshans 2013; Waaijers et al. 2013) or rpr-1 (Chiu et al. 2013). Most groups use the chimeric sgRNA, though a previous report described higher in vitro nuclease activity using the two separate bacterial small RNAs (Lo et al. 2013). Multiple groups have developed protocols for both knockouts and knock-ins. Knock-ins have been primarily generated through efficient selection schemes based on the earlier Mosl-mediated single-copy transgene insertion methods using genetic markers such as unc-119 (Dickinson et al. 2013), drug resistance markers (Chen et al. 2013b), or fluorescence (Tzur et al. 2013). Typically, plasmid repair templates with 1 kb or more of homology flanking the insert have been used (Chen et al. 2013b; Dickinson et al. 2013; Tzur et al. 2013; Kim et al. 2014).

Recently, several reports have described methods to introduce single-basepair changes, small epitopes, and larger tags such as GFP without the need for selectable markers; these approaches either directly screened all F1 progeny from co-injection marker positive animals (Paix et al. 2014) or employed a co-CRISPR/co-conversion approach where selection for one editing event resulted in an enrichment for edits at unrelated loci (Arribere et al. 2014; Kim et al. 2014). Direct screening of F1’s allows editing without introduction of additional mutations, but is more labor intensive, while co-CRISPR/co-conversion allows for identification of editing events while minimizing hands-on screening, but requires outcrossing or meiotic segregation of the marker allele. Co-CRISPR selects for mutation in the unc-22 gene, with mutant homozygotes identified in the F2 progeny of co-injection marker positive animals, or less frequently in the F1 progeny (Kim et al. 2014), though haploinsufficiency of the unc-22 locus in 1% nicotine should allow for identification of unc-22 mutant homozygotes in F1 animals (Moerman and Baillie 1979). Co-conversion selects for knock-in of dominant alleles in the dpy-10, sqt-1, or rol-6 genes in the F1 progeny of injected animals (Arribere et al. 2014).

The goal of this study was to test whether repair of a temperature-sensitive lethal point mutation could be used as an alternate co-conversion marker, as such an approach could in theory provide robust selection, minimal screening, and no requirement for outcrossing or meiotic segregation of marker alleles/mutations. I focused on using single-stranded oligonucleotides (oligos) as a template as they have been successfully used in a range of model organisms (Igoucheva et al. 2001; Storici et al. 2003; Chen et al. 2011; Bedell et al. 2012; DiCarlo et al. 2013), are cost effective, and require no cloning. In C. elegans, oligonucleotides have been used to introduce single-base changes (Arribere et al. 2014; Zhao et al. 2014), inactivate genes by introducing premature stop codons or deleting sequences (Lo et al. 2013; Paix et al. 2014), or insert small protein epitopes (Lo et al. 2013; Paix et al. 2014). Introduction of epitopes seamlessly into endogenous loci has numerous experimental uses: chromatin immunoprecipitation, purification of protein complexes followed by mass spectrometry, and detection of proteins by immunofluorescence or immunoblotting. Furthermore, optimizing insertion of epitope tags using oligonucleotide templates is almost certain to be applicable to single-base editing. Here, I describe a robust, cost effective, widely applicable method using pha-1 co-conversion and inactivation of NHEJ repair to rapidly and precisely engineer the C. elegans genome using oligo-templated repair with the CRISPR/Cas9 system.

Materials and Methods

Genetics

The following strains were used in this study: N2(WT), GE24 pha-1(e2123) III, RB873 lig-4(ok716) III, which were provided by the Caenorhabditis Genetics Center. N2 animals and lig-4 mutants were propagated at 20°, while pha-1(e2123) mutants were propagated at 15°. Animals were maintained on nematode growth medium seeded with Escherichia coli OP50 (Brenner 1974). For later injection experiments, pha-1(e2123) mutants were maintained on HB101. Growth conditions for each experiment are indicated in figure and table legends. Strains generated for this study are listed in Supporting Information, File S1, Table S1.

Microinjection

Mixtures of plasmids and oligos were microinjected into the gonad of young adult animals. Plasmids were purified using a Qiagen midiprep kit. Oligos were resuspended in TE buffer and working stocks of 2 μg/μl were made with nuclease-free dH2O. Repair template oligos are listed in Table S2. For PCR-generated Pu6::sgRNA templates, PCR products were purified from 100-μl reactions and concentrated 10-fold using a DNA Clean and Concentrator kit (Zymo Research, no. D4004).

MfeI deletion assay

Wild-type (WT) animals were microinjected with 50 ng/μl of pJW1138 or pJW1236 [klp-12 targeting CRISPR/Cas9 plasmids with original and flipped plus extended (F+E) sgRNAs, respectively], 10 ng/μl myo-2::tdTomato co-injection marker, and 40 ng/μl of pBluescript DNA. Marker positive F1 animals were picked into 30 μl of M9 + gelatin in a 96 well. Concentrated OP50 food (30 μl; see the “PCR-based knock-in screening” protocol (File S1) in the for recipe) was then added and F1’s were incubated for 3–4 days at 25° to allow progeny to develop. Worms were lysed and genotyped as

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described in the PCR-based knock-in screening section (see Supporting Information). To each 10 μl PCR, 5 μl of MfeI-HF (NEB, R3589L) digestion mixture (1 μl 10× CutSmart buffer, 3.5 μl dH2O, 0.5 μl MfeI-HF) was added. The reaction was mixed, incubated at 37° for 1 hr, and then resolved on a 1.5% TAE-agarose gel.

**Temperature-sensitive pha-1 co-conversion screening**

For temperature-sensitive pha-1 [pha-1(ts)] co-conversion experiments, animals were microinjected with 60 ng/μl of pJW1285 (pha-1 targeting) CRISPR/Cas9 plasmid, 60 ng/μl of either pJW1285 (nhr-23 PAM no. 1 targeting) or pJW1268 (nhr-23 PAM no. 2 targeting) Cas9 plasmid and 50 ng/μl of the appropriate repair oligos. In experiments where a co-injection marker was included, myo-2::tdTomato was used at 10 ng/μl. PCR-derivable Pu6::sgRNA templates were injected at a concentration of 25 ng/μl along with 50 ng/μl of pJW1285 (pha-1 targeting CRISPR/Cas9) and 50 ng/μl of the appropriate repair oligos. Injected adults grown at the permissive temperature (15°) were singled into wells of a 24-well plate containing NGM-lite agar and seeded with OP50, shifted to 25°, and incubated for 3–4 days. Rescued F1’s (L3s to adults) were the only animals other than the P0 animals observed in wells. These F1 rescues were singled onto individual plates and incubated for two days to allow progeny development. The parental F1 animals were then genotyped by restriction digestion, as described below, to identify animals with a 2×FLAG insertion. The remaining PCR product was purified using a DNA Clean and Concentrator kit (Zymo Research) and then sequenced using knock-in specific primers (Table S3). To recover homozygotes for sequence-verified knock-ins, 12–24 F2 progeny were singled onto individual plates, incubated at 25° for 2 days, and the parental F2 was genotyped to confirm the 2×FLAG insertion. These progeny were also genotyped for pha-1(ts) repair by PCR and CEL-1 digestion followed by sequencing of candidate repair homozygotes.

**Genotyping PCRs and restriction digestion**

Single F1 animals were picked into 10 μl of single-worm lysis buffer [10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.45% IGEPAI, 0.45% Tween-20] containing 1 mg/ml proteinase K. Tubes were incubated on dry ice for 15 min, 62° for 1 hr, and then heated to 95° for 20 min to inactivate the proteinase K. Genotyping PCRs were performed using Phusion polymerase (NEB, no. M0530S) and High Fidelity buffer; 1/10 volume of lysate was used as template in the PCR. For identification of knock-in events in the F1, 30-μl PCRs were performed. Five microliters of this PCR was removed and BamHI digested by adding 10 μl of digestion mix per PCR (1 μl 10× NEBuffer 3, 0.5 μl BamHI, and 8.5 μl dH2O) and digesting for 1 hr at 37° before resolving on a 1.5% TAE-agarose gel. CEL-1 was purified from four heads of nonorganic celery (Safeway) with the celery juice extracted with a BJE510XL 900W juicer (Breville). Purification was performed as described (Yang et al. 2000) to the end of dialysis in step 1, as described by Lo et al. (2013). For CEL-1 digestions, 10 μl of enzyme mix (2 μl CEL-1 + 2 μl 5xPhusion High Fidelity buffer + 6 μl dH20) was added to the 5-μl PCR and incubated at 42° for 1 hr prior to resolving on a 1.5% TAE-agarose gel, as described above. Oligos used for genotyping are listed in Table S3.

**Vector generation**

pJW1138 (klp-12 targeting CRISPR/Cas9) and pJW1185 (nhr-25 targeting CRISPR/Cas9) were derived from pDD162 using a Q5 Mutagenesis kit (NEB, no. E0554S) as previously described (Dickinson et al. 2013). sgRNA(F+E) was synthesized as a gene fragment (IDT gBlock; sequence in Table S4) with a Y61A9LA.1 targeting sequence (Friedland et al. 2013) and introduced into a pDD162-derived vector by Gibson cloning (NEB, no. E5510S) to generate pJW1219. pJW1236 (klp-12 targeting CRISPR/Cas9), pJW1254 (nhr-23 PAM no. 1 targeting CRISPR/Cas9), pJW1268 (nhr-23 PAM no. 2 targeting CRISPR/Cas9) and pJW1285 (pha-1 targeting CRISPR/ Cas9) were derived from pJW1219 [CRISPR/Cas9 with sgRNA (F+E)] through Q5 mutagenesis. The nhr-23, nhr-25, and pha-1 PAMs were manually chosen by searching for an NGG sequence in either strand close to the desired insertion site; these sgRNA target sites were then checked for specificity using the http://crispr.mit.edu website. All target sites scored >90 with no off-target sites in genes. The Pu6::sgRNA template sequence was deleted from the pJW1219 vector using Q5 mutagenesis to generate pJW1259. All plasmids (standard vector propagation, and those generated by Gibson assembly, Q5 site-directed mutagenesis, or TOPO-blunt cloning) were transformed into PEG/DMSO DH5 alpha competent cells (protocol in File S1) made in house. pJW1219, pJW1259, pJW1285, pJW1310, and pJW1311 are available through AddGene.

**Generation of U6 promoter::sgRNA templates by PCR**

The U6 promoter and chimeric sgRNA(F+E) template were amplified from pJW1219 and cloned into the pCR-Blunt II-TOPO vector (Invitrogen, no. K2800-20) to generate pJW1310 and pJW1311, respectively. The U6 promoter was then PCR amplified from pJW1310 with oligos 1787 and 1788, while the sgRNA template was amplified from pJW1311 with oligo 1790 and a target-specific 60mer that contained 20 bp of homology to the U6 promoter, 20 bp of new sgRNA target sequence, and 20 bp of homology to the sgRNA template (Table S3). New sgRNA template primers can be made by replacing the N20 in the following sequence with 20 bp of target specific sequence: 5’-ctctctatgccgagtagcttcg(N20)gtttaagactagtggc-3’. The U6 promoter and sgRNA template PCRs were mixed (0.5 μl each per 100 μl PCR reaction) and amplified using the external primers (1787 and 1790; Table S3). The cycling parameters were: (i) 98° denaturation; (ii) 35 cycles of 98° for 10 sec, 61° for 30 sec, 72° for 20 sec; and (iii) 72° for 1-min final extension. To generate more Pu6::sgRNA templates, the fused product was used in a 100-μl nested reaction with primers 1793 and 1794 (Table S3).
RNAi

Feeding RNAi was performed as described (Kamath et al. 2001; Ward et al. 2014). Four gravid *pha-1(ts)* adults were placed on 6-cm plates freshly seeded with HT115 bacteria expressing control or *cku-80* dsRNA, obtained from the Ahrringer library (Kamath et al. 2003). Adults were ready to inject on the RNAi plates 4–6 days later. For each experiment *pha-1(ts)* mutants were also put on plates seeded with bacteria expressing *nhr-25* dsRNA. RNAi efficacy was confirmed by observing molting defects, protruding vulvae, abnormal germlines, and sterility arising from *nhr-25* inactivation (Asahina et al. 2000; Gissendanner and Sluder 2000; Brooks et al. 2003; Chen et al. 2004).

Immunoblotting

Lysates were generated as described in File S1 and resolved on Mini-PROTEAN TGX stain-free 4–15% gradient gels (Bio-Rad, no. 456-8086). Stain-free gels (Bio-Rad) contain a compound evenly distributed in the precast acrylamide gel that reacts with tryptophan following UV exposure and gives a strong fluorescent signal that can be used to stain for total-protein levels in acrylamide gels, monitor transfer in immunoblotting, and serve as a loading control. Following resolution, the stain-free compound was activated (Posch et al. 2013) and proteins were transferred to an Immobilon FL PVDF membrane (Millipore, no. IFPLO0010) at 100 V for 60 min. Total protein, pre- and post-transfer, was monitored using the stain-free fluorophore as described (Posch et al. 2013). Stain-free imaging of total protein was used to confirm equal loading. The blots were sequentially probed with anti-FLAG (1:1000) (M2 clone, Sigma, no. F1504), and sheep antimouse–HRP conjugate (1:5000) (GE Healthcare, no. RPN4201). WesternBright Sirius HRP substrate (Advanta, no. K-12043-C20) and ECL Prime (GE Healthcare, no. RPN2232) were used to develop the blots in Figures 2 and 5, respectively. All blots were imaged using a ChemiDoc MP imaging system (Bio-Rad).

Accession codes

The accession codes are as follows: pJW1219 [Cas9-sgRNA(F+E) targeting site in Y61A91A.1, AddGene plasmid 61250]; pJW1259 [Cas9 plasmid with sgRNA deleted, AddGene plasmid 61251]; pJW1285 [Cas9-sgRNA(F+E) targeting site in *pha-1*, AddGene plasmid 61252]; pJW1310 (U6 promoter template vector; AddGene plasmid 61253); and pJW1311 [sgRNA(F+E) template vector, AddGene plasmid 61254].

Additional methods are described in Supporting Information.

Results and Discussion

*sgRNA(F+E) displays increased activity relative to the original sgRNA in deleting an MfeI restriction site in the klp-12 gene*

Initial reports describing use of oligos to introduce single-base changes reported edits in 0.7–3.5% of the *F*1 progeny screened (Zhao et al. 2014). Recent work in mammalian cells reported that a modified sgRNA(F+E), with an extended Cas9 binding structure and removal of a potential PolIII terminator by an A-U basepair flip, exhibits improved activity (Chen et al. 2013a). I therefore tested whether this modified sgRNA(F+E) displayed increased activity in *C. elegans*, as a potential tool to improve editing efficiency. The modified sgRNA was introduced into the pDD162 CRISPR/Cas9 plasmid (Dickinson et al. 2013), and to evaluate sgRNA activity, deletion of an MfeI restriction site in the *klp-12* gene (Friedland et al. 2013) was used as a readout (Figure S1). sgRNA(F+E) produced a significant increase in deletion of the MfeI site in animals positive for the co-injection marker (Table 1), and was used for all subsequent experiments.

*pha-1(ts) co-conversion increases knock-in efficiency with minimal handling*

Having increased sgRNA activity, I next turned to improving the screening process. Two recent reports describe that selection for a visible phenotype produced by a CRISPR/Cas9 triggered editing event (i.e., *unc-22* mutation or *dpyp-10(cn64)* knock-in) results in an increase in knock-out and knock-in efficiencies at other genomic loci (Arribere et al. 2014; Kim et al. 2014). These approaches can be used in any genetic background, but require outcrossing or meiotic segregation of the selective mutation. My aim was to develop a stringent system with minimal handling and no outcrossing or meiotic segregation of selection markers required. *pha-1(e2123)* is a temperature-sensitive embryonic lethal mutation; mutants are viable at 15°C, but display embryonic and early larval lethality when cultivated at 25°C (Schnabel et al. 1991; Granato et al. 1994). *pha-1(e2123)* has been previously used to select and propagate extrachromosomal arrays carrying a *pha-1(+)* marker (Granato et al. 1994). *pha-1(ts)* mutants display a low spontaneous reversion frequency of 2.5 × 10⁻³ per haploid genome (Schnabel et al. 1991); the stringency of this selection was confirmed by plating 144 gravid adults and shifting them to 25°C; no viable progeny were produced, only dead eggs and arrested larvae.

I selected a CRISPR/Cas9 target site that would produce a DSB 17 bp 3' from the ec2123 point mutation and designed an 80mer repair oligo with the PAM silently mutated to prevent recleavage of the site in an edited animal (Figure 1A). Twelve WT animals were injected with the CRISPR/Cas9 plasmid, a co-injection marker, and a commercially purchased, PAGE purified repair 80mer. Eight rescued *F*1’s were recovered, all heterozygotes for *pha-1(ts)* repair (data not shown). Although all eight *F*1’s were positive for the co-injection marker, in later experiments, I also observed *pha-1(ts)* rescue in marker-negative animals. Thus, as previously observed (Arribere et al. 2014; Zhao et al. 2014), heritable transgenesis is not a prerequisite for efficient editing. Oligo-mediated repair of *pha-1(ts)* allowed recovery of repair heterozygotes, and the complete penetrance of the ec2123 embryonic lethality meant that only *F*2 heterozygotes (i.e., rescued animals) would develop, making screening extremely rapid.

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Table 1 sgRNA(F+E) has increased activity relative to the original sgRNA in deleting an MfeI restriction site in the klp-12 gene

| Genotype | Guide RNA | % deletion (± 95% CI) | N |
|----------|-----------|-----------------------|---|
| WT       | Original sgRNA | 54 (±2.73)            | 157b |
| WT       | sgRNA(F+E)   | 83 (±0.31)            | 85c |

N = number of co-injection marker positive F1 progeny screened.

a (Number of animals with loss of MfeI restriction site/total number of co-injection marker positive animals screened) × 100; CI, confidence interval; 95% confidence interval, Zα/2 = 1.96σ/n. *Two-tailed t-test comparing original sgRNA to sgRNA F+E, P = 0.043.

b From four independent injections.

c From two independent injections.

A homozygote for pha-1(ts) repair had no significant difference in brood size compared to a WT control (Table S5; two-tailed t-test, P = 0.75).

I next tested whether pha-1(ts) repair could be used to enrich for knock-in of a 2×FLAG oligo into the 3′ end of the nhr-23 gene (Figure 1B), a nuclear hormone receptor involved in molting and embryonic development (Kostrouchova et al. 1998, 2001). I identified two potential PAMs near the desired insertion site that could be silently mutated (Figure 1B). I designed a 200mer oligo to insert a 2×FLAG epitope just before the stop codon. As half of sgRNAs have been reported to fail in C. elegans (Kim et al. 2014), I mutated two PAMs in the oligo to allow knock-in attempts with different sgRNAs. An 18-bp linker sequence encoding the flexible linker peptide glycine-serine-4xglycine (GSGGGG; Figure 1B) was included to spatially separate the 2×FLAG tag from the NHR-23 C terminus, potentially facilitating accessibility of the tag for immunoprecipitation. Additionally, the tag encoded a BamHI restriction site, which was used for diagnostic restriction digestion in PCR-based screening (Figure 1B). I injected a CRISPR/Cas9 plasmid targeting pha-1, an nhr-23 CRISPR/Cas9 construct targeting the PAM nearest the stop codon (PAM no. 1; Figure 1B), the 80mer pha-1(ts) repair oligo (Figure 1A), and the 200mer nhr-23:2×FLAG oligo (Figure 1B); this oligo was not PAGE purified to test whether high variability was a necessary cost. From 16 viable injected P0 animals, nine pha-1(ts) rescued F1 progeny were obtained (Figure 1D). These animals were plated, allowed to self-fertilize, and then single-worm genotyping was performed (Figure 1C). Two of these nine animals were heterozygous for a potential 2×FLAG insertion by diagnostic digest, of which one was a correct insertion; the other candidate had a frameshift in the 2×FLAG epitope. F2 progeny of the animal carrying the precise 2×FLAG knock-in were singled and a homozygote for both the insertion and the repaired pha-1(ts) allele was obtained; both knock-ins were confirmed by sequencing. Broodsize analysis of a representative knock-in confirmed viability with no phenotype, indicative of nhr-23 loss of function (i.e., molting defects or high embryonic lethality) (Table S5).

To distinguish between pha-1(ts) repair homozygotes and heterozygotes without sequencing, I used CEL-1 digestion of PCR products (Figure S2). CEL-1 is a celery endonuclease that recognizes and cleaves mismatches in double-stranded DNA (dsDNA) formed from single nucleotide polymorphisms or small insertions or deletions (Yang et al. 2000; Wood et al. 2011). In this assay, PCRs from repair heterozygotes will lead to a digestion product (Figure S2). Normally, the absence of digestion could indicate that the animals were homozygous for either the ts allele or the repaired allele. However, growth at the restrictive temperature eliminates all ts homozygotes; therefore, all undigested PCR products are repair homozygotes. The genotypes predicted by CEL-1 digestion were confirmed by sequencing. CEL-1 or other mismatch cutting nucleases such as mung bean nuclease or T7E1 represent an efficient method to monitor single-basepair changes and help reduce the number of animals that need to be sequenced to identify homozygous knocked-in point mutations.

Short oligos are effective templates for gene conversion

The stringency of pha-1 co-conversion offered a powerful tool to optimize oligo editing parameters. As much experimental effort was spent isolating homozygotes to confirm correct epitope insertion, I designed primers that allowed sequencing of the 2×FLAG tag in heterozygotes. These primers bound the insertion junction, with the two 3′-most bases binding to the inserted sequence (Figure S3). In some cases, poor sequencing quality made it difficult to confirm the sequence in the center of the epitope; in these cases, a separate PCR was performed using the epitope-specific primer and an external primer and sequencing was performed on this purified PCR product using the external primer. Screening in heterozygotes greatly reduced hands-on effort required to identify correct insertions.

I next tested whether oligo length (200mer, 80mer, and 60mer) affected repair efficiency. The 80mer was PAGE purified, but the 200mer and 60mers were not. These oligos were all sense in relation to the pha-1 coding strand. The 80mer and 200mer produced similar numbers of pha-1 rescued F1's (9 and 12, respectively) and with the 80mer producing one nhr-23:2×FLAG insertion and the 200mer producing four insertions (Figure 1D). Interestingly, I observed a high percentage of males in the pha-1(ts) repaired F1, generated using the 200mer (Table S6). Despite the 60mer only producing three pha-1 rescues, two of those F1 also had knock-ins at the nhr-23 locus. One knock-in was incomplete and produced a frameshift; the other had an extra insertion after the 2×FLAG tag, but frame was maintained. I confirmed the expression of the 2×FLAG tag by immunoblotting in five knock-in lines carrying a precise insertion of the 2×FLAG tag, as well as a line that contained an imprecise insertion. All five precise knock-ins expressed the FLAG tag, whereas no band was seen for the strain carrying the frameshifted FLAG tag (Figure 2). pha-1(ts) co-conversion allowed isolation of sequence-verified homozygotes for a knock-in event within 8–9 days.

Additional repair template considerations

I then examined whether oligo polarity had an impact on editing, using a PAGE purified, antisense version of the 80mer
pha-1(ts) repair oligo. The sense oligo was homologous to the coding DNA strand, whereas the antisense oligo was homologous to the template DNA strand. The antisense oligo produced the fewest knock-ins per viable P0 animal of all conditions tested, but still had a high rate of nhr-23::23FLAG knock-in per rescued F1 (Figure 1D). Thus, it appeared to be a poor repair template for pha-1(ts) repair, but once this repair event was selected could still lead to enrichment in nhr-23::23FLAG knock-in. To further explore this variable, I tested whether nhr-23::23FLAG could be knocked in using an antisense 200mer. Using a sense pha-1 200mer, five rescued F1’s were obtained from 57 viable P0 animals, but no nhr-23::23FLAG knock-ins were detected (Figure 1D). Including the antisense 200mer with the sense 200mer did result in a decrease in knock-in efficiency (1/5 vs. 1/9), but more animals need to be screened to determine if this effect is significant (Figure S4). Interestingly, when annealed nhr-23::23FLAG sense and antisense oligos were injected, no knock-ins were recovered, indicating that dsDNA is not a better template than single-stranded DNA (ssDNA) and may be less effective (Figure S4). These data suggest that the strand to which oligo homology is derived could be an important parameter for editing efficiency and that ssDNA may be more effective than dsDNA for epitope knock-in. However, these inferences must be tested with many additional combinations of sgRNAs, loci, and repair templates to assess their generality.

**Growth on HB101 suppresses pha-1(ts) low brood size and sterility**

A number of injected P0s in these experiments were sterile. This sterility could have been a consequence of injection trauma or general sickness of pha-1(ts) mutants. To discriminate
between these possibilities, I shifted adult *pha-1(ts)* mutants to 25°C to mimic the selection protocol. Interestingly, these animals displayed sterility (1/24 P0) and low brood sizes (<10 eggs) in 6 of 24 P0s. Growth on HB101 has been shown to suppress the slow growth rate of *eat-2 (ad465)* and *eat-5 (ad1402)* mutants, which have defective pharyngeal pumping (Shtonda and Avery 2006). Moreover, *unc-119(ed3)* strains are frequently grown on HB101 in genome editing protocols to ameliorate sickness of that strain (Frøkjær-Jensen et al. 2010; Dickinson et al. 2013). I therefore tested the effect of HB101 growth on *pha-1(ts)* mutants by propagating animals for several generations on HB101 at 15°C, then picking adult animals and shifting them to 25°C. Growth on HB101 suppressed the low brood size and sterility observed during growth on OP50. *pha-1(ts)* mutants grown on HB101 and then shifted to OP50 at L4 had brood sizes of 127 ± 34 (Table S5) with 0% viable progeny. Subsequently, the *pha-1(ts)* strain was maintained on HB101 at 15°C prior to micro-injection.

**A PCR fusion method to rapidly generate new sgRNA templates**

Having demonstrated that oligo-mediated repair of the *pha-1(e2123)* mutation can be used to efficiently enrich for knock-ins at other loci, I next turned to optimizing sgRNA delivery. Generation of new sgRNA templates for plasmid-based CRISPR/Cas9 systems typically involves site-directed mutagenesis (Dickinson et al. 2013) or cloning using oligonucleotides (Waaijers et al. 2013) or PCR products (Friedland et al. 2013; Kim et al. 2014). A major impediment to the success of a CRISPR/Cas9 experiment is the efficiency of the sgRNA; Kim et al. (2014) report that half of their tested sgRNAs fail. Given that efficient promoter::GFP reporters can be generated by fusion PCR (Hobert 2002), I tested whether the same approach could be used to express sgRNAs from the U6 promoter. This approach would allow rapid screening of sgRNAs, as PCR templates could be injected on the same day of amplification without need for cloning or transformation. For cost efficiency, four PCR primers were used, of which only one is changed to generate a new P*U6*::sgRNA template (Figure 3A). As a proof of principle, I generated P*U6*::sgRNA template fusions targeting *pha-1* and *nhr-23* PAM no. 1, and deleted the sgRNA template sequence from the pJW1219 Cas9/CRISPR plasmid. Injecting this Cas9 plasmid (pJW1259), the PCR-generated *pha-1* and *nhr-23* P*U6*::sgRNA templates, the *pha-1(ts)* sense 80mer repair template, and the *nhr-23:2*FLAG sense 200mer, efficient knock-in at both loci with comparable frequency to the corresponding plasmid-based P*U6*::sgRNA templates was observed (Figure 3B). Fusion PCR thus allows rapid, cost-effective generation of sgRNA templates driven by the U6 promoter.

**DSBs up to 54 bp from the insertion site can generate nhr-23::2×FLAG knock-ins**

During my initial design of the nhr-23::2×FLAG repair oligo, I mutated two potential PAMs, as it has been reported that half of all sgRNAs fail; this single oligo would allow testing of two separate sgRNAs (Kim et al. 2014). While analyzing the sequence of the 22 candidate nhr-23::2×FLAG knock-ins generated using sgRNAs targeting PAM no.1 and identified by BamHI digest (Figure 1D, Figure 3B, Table 2), I observed co-conversion of the PAM no. 2 silent mutation in six lines.

**Figure 2** Detection of NHR-23::2×FLAG in precise knock-ins. Four micrograms of protein from synchronized gravid adults of the indicated strains was analyzed by immunoblotting with anti-FLAG. KRY48 contains a frameshift in the 2×FLAG tag and thus does not express the epitope. Stain-free (Bio-Rad) analysis of total protein on the blot is provided as a loading control. Marker size (in kilodaltons) is provided.
These co-conversion events suggested that pha-1(ts) selection could be used to explore gene conversion track lengths and effectiveness of DSB position relative to the insertion site.

The silent point mutations in PAMs no. 1 and no. 2 were 29 bp apart, which agrees with my observation that a 60mer with 29 bp of homology can be used to efficiently knock-in point mutations (Figures 1D and Figure 4A). For these next experiments, I sequenced all pha-1(ts) rescued F1’s. I first tested whether an sgRNA targeting PAM no. 2 could be used to knock in the 2×FLAG epitope (Figure 4A). I designed a modified version of the 200mer used for the above experiment that also carried three silent mutations to disrupt the sgRNA binding to the PAM no. 3 target site, as well as the mutations in PAMs no. 1 and no. 2 (Figure 4A). A fourth potential PAM (no. 4) was left intact in the repair template, as inactivation would leave only 10 bp of 5’ homology; this sgRNA was inactive (Figure 4, A and B). Using an sgRNA targeting PAM no. 3 to generate a DSB, two of eight pha-1(ts)-rescued F1’s carried the silent mutation in PAM no. 3; the others all had wild-type sequence, suggesting that this may be an inefficient sgRNA (Figure 4B, Table S7). Of the two animals with mutations in PAM no. 3, both had the PAM no. 2 inactivating mutation, and one had the PAM no. 1 mutation and insertion of the 2×FLAG tag (Table S7). Though there was a 1-bp deletion within the tag, this experiment demonstrates that a DSB 54 bp from an insertion site can be used for introduction of epitopes, and that the presence of mutations in a stretch of homology does not prevent gene conversion; five point mutations in a 55-bp stretch were introduced into the genome along with the 65 bp of 2×FLAG tag (with the 1-bp deletion). Animals were not tracked to identify originating P0s; however, for the PAM no. 1 knock-in experiment, the six animals with PAM no. 2 mutation co-conversion were isolated from six different injections, and thus represent independent events (Figure 4B). Knock-in efficiency appeared higher the closer the DSB was to the insertion site, but sgRNA efficiency could also play a role in this observation.

Homology arms of 35 bp are sufficient for oligo-templated insertion of a 2×FLAG tag

Exploring DSB position relative to the insertion site demonstrated that the stringency of pha-1(ts) co-conversion combined with the speed and ease in recovering editing events allowed rapid testing of editing parameters. Interestingly, the 200mer used for the PAM no. 3 knock-in experiments only had 29 bp of homology 5’ to the DSB (Figure 4, A and B), suggesting that homology arms could be relatively short. Efficient repair of the pha-1(ts) allele was observed using a 60mer with 29 bp of homology (Figure 1D). Furthermore, recent data using PCR-derived dsDNA repair templates demonstrated...
that 30- to 60-bp homology arms were optimal, with knock-in efficiency actually decreasing with longer homology arms (Paix et al. 2014). To test the ideal length of homology for oligo-mediated insertion of epitopes, I designed nhr-23::2×FLAG oligos with 35 bp, 25 bp, or 15 bp of homology and tested their ability to introduce the 2×FLAG epitope using a DSB generated by an sgRNA targeting PAM no. 1. Knock-in efficiency using these homology arms was compared to pooled data from the pha-1(ts) and nhr-23::2×FLAG sense oligo experiments (Figure 1D and Figure 3B). This 200mer sense nhr-23::2×FLAG oligo (Figure 1B) contained 54 bp of homology 3′ to the insertion site and 76 bp of homology 5′ to PAM no. 1 (76/54). With the 35-bp homology arms, two animals from 9 rescued F1′s had correct insertion of the 2×FLAG epitope (Figure 4C). This 22% knock-in per F1 screened (2 of 9) is comparable to the 26% efficiency (7 of 27) observed using the 76/54 oligo (Figure 4C). The knock-in rate per successfully injected P0 was also comparable for the 35-bp and the 76/54-bp homology arms (4.76% 2/42 vs. 6.86% 7/102]). The 15- and 25-bp homology arm produced fewer pha-1(ts) rescues per successfully injected P0, and only the 25-bp homology arms could produce a 2×FLAG insertion, though this knock-in carried a point mutation (Figure 4C). These data suggest that, similar to dsDNA templates, homology arms of 35–80 bp are ideal for oligo-mediated editing.

**Inactivation of NHEJ repair increases knock-in efficiency**

In *Drosophila*, increased homologous recombination efficiency can come from inactivation of NHEJ (Beumer et al. 2008; Bottcher et al. 2014). I therefore used the pha-1(ts) system to test whether NHEJ inactivation impacted knock-in by homologous recombination. NHEJ mutants may have additional background mutations due to compromised repair of endogenous DSBs and would require additional outcrossing. I therefore tested the effect on knock-in frequency of temporary inactivation of the *C. elegans* homolog of Ku80 (cku-80), part of a heterodimer that binds the end of DSBs in NHEJ, and which had reported RNAi phenotypes (Dmitrieva et al. 2005; McColl et al. 2005). pha-1 and nhr-23 CRISPR/Cas9 plasmids, the pha-1 sense 80mer repair oligo, and the nhr-23 sense 200mer repair oligo were injected into pha-1(ts) mutants treated with control or cku-80 RNAi. Control RNAi produced one pha-1(ts) rescued F1, which also carried a sequence-confirmed nhr-23 knock-in event (Table 2). However, inactivation of cku-80 by RNAi produced an increase in both pha-1(ts) rescue (10 F1’s) and nhr-23::2×FLAG co-knock-ins recovered (n = 5) (Table 2). These experiments suggested that NHEJ inactivation boosts oligo-mediated knock-in efficiency.

To confirm that pha-1(ts) co-conversion, PCR-generated sgRNAs templates, and NHEJ inactivation were effective on other loci, I attempted to introduce a 2×FLAG epitope in nhr-25, a broadly expressed nuclear hormone receptor that regulates several developmental and physiological programs (Asahina et al. 2000; Gissendanner and Sluder 2000; Chen et al. 2004; Asahina et al. 2006; Mullaney et al. 2010; Ward et al. 2013, 2014). I selected the 3′ end of nhr-25 as there are two isoforms that share a common 3′ end, but differ in promoter use; thus, targeting the 3′ end of nhr-25 with the 2×FLAG should in principle allow for labeling of all known nhr-25 isoforms. I designed a 175mer repair template with six silent point mutations in the 20-bp sequence preceding the PAM, as the PAM could not be silently mutated (Figure S5, A and B). As with the nhr-23::2×FLAG construct, an 18-bp spacer encoding with a BamHI site was also included (Figure S5, A and B). This oligo contained 41 bp of homology 5′ to the mutated sgRNA target sequence and 43 bp of 3′ homologous sequence. pha-1(ts) mutant animals grown on either control or cku-80 RNAi were injected with a pha-1(ts) CRISPR/Cas9 plasmid, an nhr-25 Pu6::sgRNA template PCR product, and pha-1(ts) and nhr-25::2×FLAG repair oligos. Seven pha-1(ts) rescues were recovered from the control RNAi-treated animals and 36 from the cku-80 RNAi-treated animals (Table 2). No knock-in candidates were recovered from the control RNAi injection, but five were recovered

| P0 strain | pha-1 oligo | Repair oligo | Viable injected P0 | pha-1 rescued F1 | P0 with rescued F1 | PCR hits | Knock-ins | Knock-ins/F1 rescue (%) | Knock-ins/P0 rescue (%) |
|-----------|-------------|--------------|--------------------|-----------------|-------------------|----------|------------|-------------------------|------------------------|
| pha-1(ts); control(RNAi) | 80mer nhr-23::2×FLAG | 10 | 1 | 1 | 1 | 100.0 | 1.0 |
| pha-1(ts); cku-80(RNAi) | 80mer nhr-23::2×FLAG | 16 | 10 | 6 | 6 | 5 | 50.0 | 3.1 |
| pha-1(ts); control(RNAi) | 200mer nhr-25::2×FLAG | 21 | 7 | 4 | 0 | 0 | 0.0 |
| pha-1(ts); cku-80(RNAi) | 200mer nhr-25::2×FLAG | 22 | 36 | 12 | 5 | 4 | 11.1 | 18.2 |
| pha-1(ts); control(RNAi) | 200mer nhr-23::3×FLAG | 34 | 7 | 4 | 1 | 1 | 14.3 | 3.0 |
| pha-1(ts); cku-80(RNAi) | 200mer nhr-23::3×FLAG | 13 | 5 | 3 | 2 | 2 | 40.0 | 15.4 |
| pha-1(ts); control(RNAi) | 200mer nhr-25::3×FLAG | 16 | 10 | 6 | 6 | 5 | 50.0 | 3.1 |
| pha-1(ts); cku-80(RNAi) | 200mer 2×FLAG::smo-1 l1g-4 stop | 15 | 29 | 7 | 14 | 11 | 38.0 | 73.3 |

Summary of pha-1(ts) coselction experiments testing NHEJ inactivation. The RNAi treatment of the injected P0 animals is indicated. Viable P0 are the number of injected animals that produced eggs; a variable number of animals are sterile in each experiment. The length of the pha-1(ts) sense repair oligo is provided. For nhr-23::2×FLAG experiments, animals were injected with 60 ng/μl each of the pha-1 and nhr-23 CRISPR/Cas9 plasmids, and 50 ng/μl each of the pha-1(ts) repair and nhr-23::2×FLAG (sense) oligos. For the remaining experiments, animals were injected with 50 ng/μl of the pha-1 CRISPR/Cas9 plasmid, 25 ng/μl of appropriate Pu6::sgRNA template PCR product, and 50 ng/μl each of the pha-1(ts) repair oligo and knock-in oligo.

Pre-RNAi diet = OP50 *E. coli.*

Pre-RNAi diet = HB101 F. coli.
from the cku-80 RNAi-treated animals, of which four were correct insertions of the 2×FLAG tag (Table 2). Strikingly, one of these animals was an F1 homozygous knock-in. These strains were viable and did not display defects associated with nhr-25 inactivation (protruding vulvae, molting defects, embryonic lethality; see Table S5 for a representative brood size). I confirmed expression of the 2×FLAG tag in an outcrossed, representative line (Figure 5B).

**Multiplexed editing using pha-1(ts) co-conversion and cku-80 RNAi**

Given the efficient editing observed in cku-80 RNAi-treated animals and the 35–80 bp of homology sufficient for oligo-templated repair, I next tested whether I could introduce larger epitopes using pha-1(ts) co-conversion. As Kim et al. (2014) had reported generating mutations in both avr-14 and avr-15 in one unc-22 co-CRISPR experiment, I attempted to knock in 84-bp 3xFLAG tags containing the GSGGGG spacer into both nhr-23 and nhr-25 in one injection experiment (Figure S5A). From 13 successfully injected P0 animals treated with cku-80 RNAi, two precise nhr-23::3xFLAG lines were obtained (Table 2). A viable nhr-25::3xFLAG line that expressed the 3xFLAG tag, but had a 51-bp duplication in the 3′ UTR was also obtained; as the tag was expressed and the strain was viable, this line was scored as a correct knock-in (Table 2, Table S5). A single knock-in animal was

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**Figure 4** DSBs up to 54 bp from an insertion site, and 35-bp homology arms can be used for oligo-templated repair in nhr-23. (A) Schematic of the nhr-23 3′ end, indicating the stop codon (blue text), four PAMs tested (red text), and position of the DSBs (scissor). Mutations used to inactivate PAMs in repair templates are provided in the nhr-23(PAM MUT) sequence and indicated by the vertical lines between the (+) and (PAM MUT) sequence. (B) Testing effect of DSB position on nhr-23::2×FLAG epitope knock-in efficiency. The PAMs for the sgRNAs used to generate the DSB and mutations used to inactivate the PAMs in the repair templates are provided in A. Animals were co-injected with 50 ng/μl of pha-1 targeting CRISPR/Cas9 plasmid, 50 ng/μl each of the pha-1-ts) repair oligo and a 200mer sense nhr-23::2×FLAG repair oligo, and either 50 ng/μl of nhr-23 targeting CRISPR/Cas9 plasmid (PAMs no. 1 or no. 2), or 25 ng/μl of PU6::sgRNA template PCR product (PAMs no. 3 and no. 4). For the PAM no. 1 and no. 2 sgRNA experiments, the repair template carried mutations in both PAMs. For the PAM no. 3 and no. 4 experiments, the repair template carried mutations in both PAMs. For the PAM no. 3 and no. 4 experiments, the repair template carried mutations in both PAMs. For the PAM no. 3 and no. 4 experiments, the repair template carried mutations in both PAMs.

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**Table 2**

| Homology arms (bp) | Viable injected P0 | pha-1 rescued P1 | P0 with rescued F1 | PCR hits | Knockins | % Knockins/pha-1 rescue | % Knockins/P0 |
|-------------------|--------------------|-----------------|--------------------|----------|-----------|--------------------------|-------------|
| 76/54             | 102                | 27              | 19                 | 10       | 7         | 25.9%                    | 6.9%        |
| 35/35             | 42                 | 9               | 6                  | 2        | 2         | 22.2%                    | 4.8%        |
| 25/25             | 34                 | 1               | 1                  | 1        | 0         | 0.0%                     | 0.0%        |
| 15/15             | 21                 | 2               | 1                  | 1        | 0         | 0.0%                     | 0.0%        |

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**Table S5**

| PAM | Insertion site | FLAG tag | Insertion site |
|-----|----------------|----------|----------------|
| 1   | 9 bp           | 212      | 22            |
| 2   | 29 bp          | 16       | 1             |
| 3   | 54 bp          | 13       | 1             |
| 4   | 67 bp          | 20       | 0             |

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**Figure S5A**

A viable nhr-25::3xFLAG line that expressed the 3xFLAG tag, but had a 51-bp duplication in the 3′ UTR was also obtained; as the tag was expressed and the strain was viable, this line was scored as a correct knock-in (Table 2, Table S5). A single knock-in animal was...
obtained from 34 successfully injected P0 animals grown on control RNAi (Table 2). This F1 animal was homozygous for an nhr-25::3xFLAG insertion and heterozygous for an nhr-23::3xFLAG insertion (Table 2). Viability was confirmed for representative nhr-23::3xFLAG and nhr-25::3xFLAG lines and for the nhr-23::3xFLAG; nhr-25::3xFLAG double knock-in line (Table S5). No defects consistent with nhr-23 or nhr-25 inactivation were observed, though the double knock-in did have a lower brood size (Table S5). I confirmed FLAG epitope expression in outcrossed nhr-23::3xFLAG lines and nhr-25::3xFLAG lines (Figure 5, A and B). The 3xFLAG epitope lines displayed a marked increase in band intensity in immunoblots compared to 2xFLAG lines, for both NHR-23 and NHR-25 (Figure 5, A and B). Notably, the nhr-23::2xFLAG epitope was not detectable in this experiment (Figure 5A), as a more potent ECL substrate was required for detection by immunoblotting (Figure 2); this more potent ECL substrate may explain the nonspecific band observed in Figure 2, but not in Figure 5A. I also observed extra bands in the 3xFLAG lines, which would be consistent with NHR-23 and NHR-25 isoforms, though they could also represent degradation products (Figure 5, A and B). Comparing all NHEJ inactivation experiments, growing P0 animals on cku-80 RNAi produced a significant increase in the number of knock-ins recovered per injection experiment (two-tailed t-test \( P = 0.01 \)) and percentage of knock-ins per viable P0 (two-tailed t-test \( P = 0.016 \)). No significant difference was observed in the number of P0 animals with rescued F1 progeny (two-tailed t-test \( P = 0.16 \)) or in the percentage of knock-ins per rescued F1 (two-tailed t-test \( P = 0.85 \)). Recent manuscripts have described a “jackpot” phenomenon (Arribere et al. 2014; Paix et al. 2014), where the majority of edits come from a small number of P0 animals. There may be a trend of “richer jackpots” in cku-80 RNAi-treated animals with 2.6 rescued F1/s/P0 producing F1 rescues (\( \geq 0.44 \); 95% confidence interval) vs. 1.6 (\( \geq 0.50 \); 95% confidence interval) in control RNAi-treated animals. This difference was not significant in a two-tailed t-test (\( P = 0.19 \)) potentially due to the large variation in number of F1 rescues and knock-ins produced across assays, and this observation will require further exploration.

**PAGE purification of oligos is not necessary, but increases editing efficiency**

The nhr-25::3xFLAG oligo appeared to be a poor repair template, as it yielded lower knock-in efficiency in comparison to the nhr-25::2xFLAG template, and complex insertions were observed in two nhr-25::3xFLAG candidate knock-in lines. Although experiments demonstrated that PAGE purification was not necessary, the nhr-25::3xFLAG template was ideal to test whether PAGE purification nonetheless increased knock-in efficiency or fidelity. Resolving unpurified and purified oligos on a denaturing TBE-Urea gel revealed that the unpurified oligo preparation contained large amounts of incorrectly sized product (Figure S6A). *pha-1*(ts) animals grown on control or *cku-80* RNAi were injected with the *pha-1* targeting CRISPR/Cas9 vector, an *nhr-25* sgRNA template PCR product, a *pha-1* repair oligo, and either purified or unpurified *nhr-25::3xFLAG* 175mers. Interestingly, this experiment demonstrated that PAGE purification resulted in higher rates of knock-ins per F1 and per viable P0 (Figure S6B). Comparing all experiments using the *nhr-25::3xFLAG* oligos (Table 2, Figure S6C), both knock-in candidates obtained using the purified oligo were precise insertions, whereas two of the four candidates generated with the unpurified oligo contained additional sequence inserted (Figure S6C). These experiments suggested that one can either opt for an increased knock-in rate with PAGE purified oligos or avoid this cost and inject/screen more animals.

**Efficient editing at other genomic loci and rapid testing of sgRNA activity using *pha-1*(ts) selection and NHEJ inactivation**

Finally, I wished to test these editing parameters on other genes to test their general applicability and robustness. Based on my previous work on NHR-25 sumoylation (Ward et al. 2013), I chose a previously described weakly efficient sgRNA (Kim et al. 2014) that cleaved 19 bp 3’ to the start codon of the single *C. elegans* SUMO gene, smo-1 (Broday et al. 2004). I designed a 175mer repair oligo to create a SMO-1 N-terminal 2xFLAG fusion (Figure S5, C and D). I also designed two overlapping sgRNAs to target the NHEJ gene, lig-4, and a 60mer oligo as the homologous recombination template to insert a stop codon (oligos were unpurified). From nine successfully injected P0 animals grown on cku-80 RNAi and injected with PCR-derived sgRNA templates, 27 *pha-1*(ts) rescued F1 animals were obtained. Fourteen of these F1’s carried knock-ins in smo-1, of which 11 were precise (Table 2). I confirmed the expression of the 2xFLAG epitope in a representative line (KRY82) by immunoblotting; signal was observed over a large range of molecular weights, as expected from SUMO conjugation to hundreds of substrates in *C. elegans* (Kaminsky et al. 2009) (Figure 5C). Although viable, 2xFLAG:smo-1 homozygotes displayed partially penetrant phenotypes consistent with smo-1 reduction of function, such as small body size and protruding vulvae. Thus, epitope placement may need to be optimized in smo-1, though the creation of a viable hypomorph will be a useful reagent for the community. The lig-4 sgRNAs failed, as no knock-ins or mutations were obtained in lig-4, demonstrating that this approach also allows rapid testing of sgRNA efficacy, similar to Kim et al. (2014) (Table 2). Together, these results demonstrate that a co-conversion approach using a temperature-sensitive mutant allele, PCR-generated sgRNA templates, and NHEJ inactivation by RNAi provide a flexible, robust platform to recover genome editing events.

**pha-1*(ts) coselection increases screening efficiency**

Based on recent co-CRIPSR/co-conversion reports (Arribere et al. 2014; Kim et al. 2014), and the frequency at which relatively rare *pha-1*(ts) repair events were associated with edits at other loci, it was highly probable that *pha-1*(ts)
coselection increases screening efficiency. Prior to developing pha-1(ts) co-conversion, I had attempted to introduce 2×FLAG tags onto the 3′ end of nhr-23 and nhr-25 and recover knock-ins through direct screening of F1 progeny, similar to the approach described by Paix et al. (2014) (see Supporting Information for detailed methods). No knock-ins were recovered from 380 screened WT F1’s (Table S8). I also tested the effect of NHEJ inactivation by injecting into lig-4(ok716) mutants; DNA ligase 4 (lig-4) encodes the enzyme that seals DSBs in canonical NHEJ, and ok716 is an out-of-frame deletion that removes the catalytic ligase domain and is predicted to result in a premature stop codon (Clejan et al. 2006). A single knock-in was recovered from 768 screened lig-4 mutant F1’s (Table S8); after outcrossing the lig-4 mutation, this nhr-25::2×FLAG line had a brood size equivalent to a knock-in produced by pha-1(ts) coselection (Table S5). In comparable pha-1(ts) coselection experiments, no knock-ins were recovered from 7 F1 laid by control RNAi treated P0s and four knock-ins were recovered from 36 F1’s laid by cku-80 RNAi-treated P0 (Table 2).

For nhr-23::2×FLAG knock-in experiments, I used a knock-in-specific PCR screening approach; as in optimization experiments, it detected knock-in DNA diluted 1:1280 with WT DNA, whereas diagnostic restriction digestion of PCR products could only detect up to a 1:20 dilution of knock-in DNA with WT DNA (Figure S7). No knock-ins were recovered from 200 WT F1 animals or 840 lig-4 mutant F1’s (Table S8). In comparison, pha-1(ts) coselection using a similar sense nhr-23::2×FLAG repair template yielded seven knock-ins from 27 screened pha-1(ts) rescued F1’s (Figure 4C). Comparing all similar nhr-23::2×FLAG and nhr-25::2×FLAG direct selection and pha-1 co-conversion experiments (i.e., excluding antisense oligo and NHEJ inactivation experiments) demonstrated a significant increase in screening efficiency compared to direct F1 screening (two-tailed t-test, P = 0.01).

**Conclusions**

Here, I demonstrate that selection for repair of a temperature-sensitive point mutation using an oligonucleotide template can be used to efficiently select for knock-in at other loci in as quickly as 8–9 days. Several findings of this work should be applicable for a range of co-conversion/co-CRISPR approaches.

First, the increased sgRNA(F+E) activity (Table 1) may improve knock-in and knock-out efficiencies and reduce the number of sgRNAs that one must test to identify an active sgRNA. Second, for introduction of single-basepair changes, efficient editing was observed using 60mers, 80mers, and 200mers. There may be a slight improvement in knock-in efficiency with longer repair templates, but using shorter oligos still produces repair events at a high rate. These data are consistent with the 29-bp repair track length observed using the second silent PAM mutation in the nhr-23::2×FLAG repair oligo and the efficient nhr-23::2×FLAG knock-ins obtained using 35 bp of homology (Figure 4). This optimal homology for oligo-mediated editing is similar to that reported for dsDNA templates (Paix et al. 2014). The 84-bp 3xFLAG nhr-23 and nhr-25 knock-ins is a larger insertion than described by several recent reports (Lo et al. 2013;
Arribere et al., 2014; Paix et al., 2014; Zhao et al., 2014); a 66-bp 3xFLAG insertion in the nos-1 gene is the most comparable edit (Paix et al., 2014). Based on the minimal homology length of 35 bp and current oligo synthesis limit of 200 bp, it may be possible to knock in sequences of up to 130 bp using oligo templates. Third, I demonstrated that oligos do not need to be PAGE purified, as efficient editing was observed using unpurified 60mers and 200mers to edit the pha-1(ts) allele and to introduce the nhr-23::2×FLAG epitope (Figure 1D). However, PAGE purified oligos did result in increased knock-in efficiency and fewer imprecise knock-ins (Figure S6). Thus, an investigator can choose between the additional cost for the increased editing efficiency or simply inject more P0 animals and screen more F1’s. It is very important to note that there is likely wide variation in quality of unpurified oligos between different suppliers. Different preparations may contain different inhibitors or cytotoxic compounds and one should confirm the efficiency of unpurified oligos from a new supplier by testing a control such as pha-1(ts) rescue or dpy-10(gf) knock-in (Arribere et al., 2014). Fourth, PCR-generated PU6::sgRNA templates allow rapid production of new sgRNAs without need for cloning. Moreover, the robust sgRNA activity observed when injecting linear dsDNA templates enables other technologies for sgRNA template production such as gene synthesis or oligonucleotide arrays (Bassik et al., 2009; Gilbert et al., 2014), which could be used to create pooled sgRNA template libraries. Injecting multiple PCR-generated PU6::sgRNA templates allows several editing experiments to be performed simultaneously. From single-injection experiments, I was able to knock 3xFLAG tags into both the nhr-23 and nhr-25 loci or generate 2×FLAG:: smo-1 lines while also determining that the lig-4 sgRNAs were inactive (Table 2). Finally, the observation that inactivation of NHEJ can lead to improved knock-in rates can be adapted for any HR-based editing system, though it would reduce the efficiency of systems that rely on coselection of CRISPR-generated mutations as a marker. The increased knock-in efficiency observed following NHEJ inactivation was surprising and suggests that at least some knock-ins must be occurring outside in the germline; previous work has shown that NHEJ is actively suppressed in meiosis to ensure faithful repair of DSBs by homologous recombination (Adamo et al., 2010). Determining the cell types in which knock-ins are occurring and the DNA repair pathways involved could lead to improvements in editing efficiency.

It was intriguing that, for pha-1 and nhr-23, higher editing efficiency was observed using oligos with homology to the coding strand. In these experiments, the sgRNA for pha-1 recognized the coding strand, while the nhr-23 PAM no. 1 sgRNA recognized the template strand (Figure 1D, Table S9). Although numerous explanations could be invoked for a polarity bias (sgRNA sequestration of the oligo, oligo:mRNA hybridization, different secondary structures in a given oligo and its reverse complement, etc.), it is unclear whether my data reflect a biologically meaningful trend or a chance observation. Best practice should be to clearly report the strand to which the sgRNA binds and the strand to which the oligo contains homologous sequence (Table S9). A metaanalysis of many more combinations of sgRNAs and oligos of differing polarities at a large number of genes is required to definitively determine whether oligo polarity is an important parameter. If one fails to obtain oligo-templated knock-ins when using a sgRNA with confirmed activity, then it may be worth testing the complement of the oligo.

The pha-1(ts) approach is distinct from reported co-CRISPR and co-conversion methods (Arribere et al., 2014; Kim et al., 2014) in that it starts with a mutant animal and results in restoration of a wild-type animal. In contrast, the other two approaches can be used with any strain, but require outcrossing or meiotic segregation of the selection marker. pha-1(ts) co-conversion may be advantageous as a marker in cases where the desired insertion site is linked to the selectable marker site. Arribere et al. (2014) demonstrated iterative editing using dpy-10(gf) co-conversion. pha-1(ts) co-conversion should allow for iterative editing events, though not as elegantly as the Arribere approach. pha-1(ts) F1 rescues have all been heterozygous for the repair event. By shifting to the permissive temperature (15°) after generating homozygous animals of a desired knock-in, one could reisolate the pha-1(ts) allele and perform another round of editing; this pha-1(ts) reisolation would require an additional 5 days. Alternatively, one could simply cross the pha-1(ts) allele back into an edited strain.

The optimizations I report make oligo-mediated editing efficient, cost effective, and can be applicable to any CRISPR-mediated editing system. The development of three distinct variations of coediting selection (unc-22 mutation; Kim et al., 2014), rol-6(gf)/sqt-1(gf)/dpy-10(gf) knock-in (Arribere et al., 2014), and pha-1(ts) repair (this article) highlight the robustness of this method to select for genome editing events. The recent description of PCR-derived dsDNA templates (Paix et al., 2014) will make these approaches even more powerful; the abundance of potential genetic markers in other model organisms should make these widely applicable approaches. A current challenge in cell-culture-based systems has been the laborious recovery of rare knock-in events. Coselection markers such as oligo-mediated repair of mutated GFP or drug resistance cassettes, or inactivation of hypoxanthine phosphoribosyl transferase, could yield similar improvements in editing event recovery in these systems.

Acknowledgments

I thank Teresita Bernal, Soledad de Guzman, and Greg Wright for experimental assistance; Dan Dickinson, Te-Wen Lo, Barbara Meyer, Rob Nakamura, Axel Bethke, and members of the Yamamoto lab for helpful discussions and technical advice; Lindsey Pack, Gabriela Monsalve, Debbie Thurtle, Elizabeth Silva, Stefan Taubert, Kaveh Ashrafi, and Keith Yamamoto for advice and comments on the manuscript; and the reviewers for their many excellent comments and experimental suggestions. Some strains were provided by the CGC, which is funded by National Institutes of Health.
(NIH) Office of Research Infrastructure Programs (P40 OD010440). J.D.W. was supported by the National Institute of General Medical Sciences of the NIH under award no. K99GM107345. Additional support was from NIH (CA20535) and U.S. National Science Foundation (MCB 1157767) awards to K. Yamamoto. The content is solely the responsibility of the author and does not necessarily represent the official views of the NIH.

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Communicating editor: O. Hobert
**Figure S1  Representative klp-12 deletion experiment.** Injection of 50 ng/µl of CRISPR/Cas9 plasmid targeting a previously described PAM in the klp-12 locus (FRIEDLAND et al. 2013) results in deletion of an adjacent MfeI restriction site. WT and mutated MfeI digested PCR products are indicated. Products were run on a 1.5% TAE-agarose gel and the 1KB+ (Invitrogen) size standard is provided. klp-12 genotype inferred by MfeI digestion is indicated. WT animals have complete MfeI digestion of the PCR product, mutant homozygotes have no digestion of the PCR product, and heterozygotes have a mixture of digested and undigested PCR products.
Figure S2  Representative genotyping of the progeny of an nhr-23::2xFLAG knock-in heterozygote. (A) BamH1 digestion to genotype nhr-23::2xFLAG knock-in. Twenty-four progeny from an nhr-23::2xFLAG knock-in heterozygote were plated out, allowed to lay progeny, and the parental animal was genotyped. Candidate homozygotes are indicated by i, ii, and iii. (B) CEL-1 digestion of PCR products amplifying the pha-1(ts) repair site. CEL-1 cuts mismatches. (i and iii) are pha-1 repair heterozygotes, as indicated by the digestion product. (ii) is a pha-1(ts) repair homozygote. pha-1 and nhr-23::2xFLAG genotypes were confirmed by sequencing of the PCR products. The 1KB+ (Invitrogen) size standard is provided in A and B.
**Figure S3  Oligo design to sequence FLAG knock-in heterozygotes.** Design of oligos to sequence into 5’ end (A) and 3’ end (B) of *nhr-23* 2x and 3x FLAG knock-ins. The non-coding strand of *nhr-23* (+) is shown paired with the sequencing oligo. The stop codon (blue text), PAM #1 (red text), and a portion of the 2xFLAG knock-in sequence (orange text) are indicated. The oligo is designed to bind the genomic sequence at the insertion site with the last two bases binding bases in the insert. In cases where sequence is too poor to confirm correct insertion of an epitope, an additional round of PCR can be performed using one of the insert-specific oligos and an external primer that binds in the genomic sequence. Purification of this product followed by sequencing using the primer that binds in the genomic sequence provides the entire epitope sequence. In events where 2 bp of knock-in sequence is not sufficient to confer specificity, increasing the knock-in specific homology will correct the problem at the expense of sequencing coverage of the knock-in.
dsDNA is not a more effective template than ssDNA for introduction of a 2xFLAG epitope at the 3’ end of nhr-23. (A) 50 ng/µl of sense of and antisense nhr-23::2xFLAG oligos in annealing buffer (TE buffer with 50 mM NaCl) were either annealed by heating to 95°C for two minutes and then slowly cooling to 25°C over 30 minutes in a thermocycler, or mock annealed (kept at 25°C). Annealing was confirmed by resolving the annealed and mock annealed oligos on a 4% TAE-agarose gel and staining with GelRed. The 1KB+ (Invitrogen) size standard is provided. (B) Table comparing the knock-in efficiencies of sense oligos, and either mock annealed or annealed sense+antisense nhr-23::2xFLAG 200mers. The sense 200mer data is pooled from all experiments using pha-1(ts) sense oligos and nhr-23::2xFLAG sense 200mers (Figures 1 and 3). A control where the sense oligo was injected in annealing buffer was performed to ensure that the buffer did not affect knock-in efficiency.
Figure S5  FLAG tag sequences and schematic of insertion sites in nhr-25 and smo-1. (A) DNA and amino acid sequence of 2x and 3x FLAG epitopes used in nhr-23 and nhr-25 editing experiments. A GSGGGG amino acid linker sequence precedes the epitope; a BamHI site is encoded in this linker sequence for genotyping by restriction digestion. (B) Sequence of the nhr-25 genomic locus targeted. The PAM (red text), sgRNA target sequence, stop codon (blue text), and position of the DSB are indicated. The amino acid sequence of the targeted locus is provided. The bases mutated in the oligo template to inactivate the PAM (nhr-25(PAM MUT)) are in uppercase font in the sgRNA target sequence, with the corresponding WT bases in nhr-25(+). (C) DNA and amino acid sequence of the 2x FLAG epitope used in smo-1 editing experiments. A glycine-serine dipeptide linker encoding a BamHI site for diagnostic restriction digestion follows the epitope. (D) Sequence of the smo-1 genomic locus targeted. The PAM (red text), sgRNA target sequence, start codon (purple text), and position of the DSB are indicated. The amino acid sequence of the targeted locus is provided. The bases mutated in the oligo template to inactivate the PAM (smo-1(PAM MUT)) are in uppercase font in the sgRNA target sequence, with the corresponding WT bases in smo-1(+).
Figure S6  PAGE purification of oligos results in increased knock-in efficiency. (A) Comparison of PAGE purified and unpurified nhr-25::3xFLAG oligos. 200 ng of oligos were resolved on a denaturing 8% TBE-Urea polyacrylamide gel and stained with SYBR Gold. A 100 bp ladder (Invitrogen; sizes in bp) is provided as a standard. (B) Comparison of knock-in efficiency of unpurified and PAGE purified oligos in animals grown on HB101 and then transferred to either control RNAi or ck-80 RNAi. (C) Comparison of all experiments using unpurified vs PAGE purified nhr-25::3xFLAG oligos.
Figure S7 Detection of knock-ins by knock-in specific PCR and diagnostic restriction digestion. (A) For nhr::23::2xFLAG direct screening, a knock-in specific PCR approach was developed to minimize the number of PCRs required to identify knock-ins. Using the nhr-25::2xFLAG strain generated by direct selection (Table S8) as a control, oligos were designed to bind within the inserted sequence (oligo #1715) and outside of the insertion area. nhr-25::2xFLAG knock-in lysate was diluted as indicated with WT lysate and used as template in a knock-in specific genotyping PCR. No product was detected in the absence of nhr-25::2xFLAG lysate and knock-in product could be detected across the dilution range, to 1:1280. (NTC; no template control). (B) Identification of nhr-25:2xFLAG knock-in by diagnostic restriction digest. nhr-25::2xFLAG lysate was diluted and used in genotyping PCR as in (A). The product was then digested by BamHI to detect knock-ins. Knock-in product could be detected up to a 1:20 dilution. The 1KB+ (Invitrogen) size standard is provided in A and B.
PCR-based knock-in screening

For direct screening of nhr-25::2xFLAG knock-ins, WT animals or lig-4(ok716) mutants were injected with 100 ng/μl of pJW1185 (nhr-25 targeting CRISPR/Cas9), 10 ng/μl of a myo-2::tdTomato co-injection marker, and 100 ng/μl of a nhr-25::2xFLAG 135 mer (oligo #1580, Table S2). P0 animals were plated in single wells of 12-well plates containing NGM-lite agar seeded with OP50 E. coli. Following incubation at 25°C for three days, wells were scored for the presence of marker positive F1 progeny. From these wells, the marker positive F1 were picked off and discarded and 1 ml of M9+gelatin was added. This step was performed because previous reports suggested that edits occurred in marker negative F1 (Zhang et al. 2014). Marker negative F1s from these wells were pipetted into 30 μl of M9+gelatin in a 96-well plate; four worms were pipetted into each well. A multichannel pipette was used to add 30 μl of 2xOP50 food to each well. This food was made by inoculating a one liter culture of LB+streptomycin (50 μg/ml) with a single colony of OP50, shaking for 16 hours at 225 rpm at 37°C, pelleting the culture by spinning at 4000 rpm, and resuspending in 10 ml of M9. For the 2xOP50 food, 5 ml of this concentrated OP50 was added to 45 ml of M9+gelatin containing 10 μg/ml cholesterol. Plates were parafilmed and incubated at 25°C for 3-4 days. Lysates were made by using a multichannel pipette to transfer 10 μl of worm culture to 10 μl of single-worm lysis buffer containing proteinase K in a 96-well plate. The lysates were then processed, and genotyping PCRs and BamHI digests performed as described in the “Genotyping PCRs and restriction digestion” section in the main-text Methods. For wells with a hit, in order to identify homozygotes, 24 F2 progeny were transferred to single wells of a 96-well plate and incubated and screened as above.

For pooled screening of nhr-23::2xFLAG knock-ins, WT animals or lig-4(ok716) mutants were injected with 50 ng/μl of pJW1254 (nhr-23 PAM #1 targeting CRISPR/Cas9), 5 ng/μl of a myo-2::tdTomato co-injection marker, and 50 ng/μl of a sense nhr-23::2xFLAG 199mer (oligo #1719, Table S2). P0 animals were plated and marker positive wells were identified as above. All progeny from marker positive wells were washed out with 1 ml of M9+gelatin and diluted to ~10 worms/30μl and this volume (30 μl) was plated in 96 wells using a repeat pipetter. The number of worms/well was averaged in two rows of the plate to confirm the estimated concentration of 10 worms/well. Food was added, the plates were incubated, and lysates made as described above. Knock-in specific PCRs were performed using an oligo that internally bound the 2xFLAG sequence (#1715) and an oligo that bound external to the knock-in sequence. Four rows were pooled for each PCR reaction with 0.5 μl of lysate from each row used in a 20 μl PCR. For wells with a hit, in order to identify homozygotes, 48-96 F2 progeny were transferred to single wells of a 96-well plate and incubated and screened as above.

Generation of lysates for immunoblotting

For the immunoblot in Figure 2, animals of the indicated genotype were synchronized by alkaline bleaching followed by plating overnight in the absence of food. Approximately 3000 arrested L1s were plated on 10 cm NGM-lite plates seeded with OP50 and incubated at 25°C for 48 hours, at which point the animals were gravid adults. Animals were washed off of the plates with M9+gelatin, pelleted and transferred to a 1.5 ml tube and washed four times with 1 ml...
of M9. The M9 was aspirated, leaving 150 µl and the pellet flash-frozen in liquid nitrogen and stored at -80°C. The pellet was resuspended by adding 150 µl 2xRIPA buffer (100 mM Tris-HCl, 900 mM NaCl, 2% NP-40, 1% Sodium deoxycholate and 0.2% SDS, pH 7.4) supplemented with Protease Inhibitor Cocktail Set III, EDTA-free (Calbiochem, #539134-1SET), 1mM PMSF, 10 µM MG-132 proteasome inhibitor (Caymon, #10012628), and 1 mM DTT. Worms were lysed by three cycles of sonication on ice (10 sec, 20% amplitude). Debris was pelleted by centrifugation at 14,000 rpm for 10 minutes at 4°C and protein concentration was determined by a 660nm Protein Assay (Pierce). Four micrograms of total protein was resolved by SDS-PAGE using a Mini-PROTEAN TGX Stain-Free 4-15% gradient gel (Bio-Rad, #456-8086).

For the immunoblot in Figure 5, ten gravid adults were placed on 10 cm plates and incubated for four days at 25°C. Lysates were made by washing crowded, mixed stage animals off of these 10 cm plates in M9+gelatin, pelleting at 700xg for two minutes, and transferring to a 1.5 ml tube. The worms were washed four additional times with 1 ml of M9+gelatin. The M9+gelatin was aspirated to just above the worm pellet and the pellet was rapidly freeze-thawed three times (cycling between liquid nitrogen and a 42°C water bath) before 4x Laemml buffer was added to a final concentration of 1x. The samples boiled for 10 minutes, then the lysate was frozen for 15 minutes on dry ice and then boiled again for 10 minutes. Debris was pelleted by centrifugation at 14,000 rpm in a microcentrifuge for 5 minutes. Ten microlitres of lysate was resolved by SDS-PAGE on a Mini-PROTEAN TGX 4-15% gradient gel (Bio-Rad, catalog #456-1086) at run at 250 V.

**PEG/DMSO DH5a competent cells**

A single DH5 alpha colony from a freshly struck plate was used to inoculate a 5 ml LB culture and incubated overnight at 37°C shaking at 225 rpm. This culture was used to inoculate 500 ml of LB which was shaken at 37°C until an OD600 of 0.5-0.6 was reached. Cells were pelleted by centrifugation for 5 min at 2000 rpm, 4°C. Cells were gently resuspended in 25 ml of ice cold TSB buffer (LB pH 6.1, 10% PEG-3350, 5% DMSO, 10 mM MgCl₂, 10 mM MGSO₄), incubated on ice for 10 minutes, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C. To transform the cells, an aliquot was thawed on ice and the DNA to be transformed was mixed with 5xKCM (500 mM KCl, 150 mM CaCl₂, 250 mM MgCl₂) and dH₂O to a final volume of 100 µl at 1xKCM final concentration. An equal amount of cells was added, mixed by gentle inversion, and incubated on ice for 20 minutes. The mixture was then incubated at room temperature for 10 minutes before 1 ml of SOC or LB was added and the transformation was shaken for 1 hr at 37°C before plating on LB containing appropriate antibiotics.
| Strain name | Genotype |
|-------------|----------|
| KRY41       | lig-4(ok716) III; nhr-25[kry1[nhr-25::2xFLAG]] X |
| KRY42       | nhr-25[kry1[nhr-25::2xFLAG]] X |
| KRY46       | nhr-23[kry4[nhr-23::2xFLAG]]; pha-1[kry5[Y169C*e2123]] III |
| KRY47       | nhr-23[kry6[nhr-23::2xFLAG]]; pha-1[kry7[Y169C*e2123]] III |
| KRY48       | nhr-23[kry8[nhr-23::2xFLAG]]; pha-1[kry9[Y169C*e2123]] III |
| KRY49       | nhr-23[kry10[nhr-23::2xFLAG]]; pha-1[kry11[Y169C*e2123]] III |
| KRY50       | nhr-23[kry12[nhr-23::2xFLAG]]; pha-1[kry13[Y169C*e2123]] III |
| KRY51       | nhr-23[kry14[nhr-23::2xFLAG]]; pha-1[kry15[Y169C*e2123]] III |
| KRY52       | nhr-23[kry16[nhr-23::2xFLAG]]; pha-1[kry17[Y169C*e2123]] III |
| KRY53       | nhr-23[kry18[nhr-23::2xFLAG]]; pha-1[kry19[Y169C*e2123]] III |
| KRY54       | nhr-23[kry20[nhr-23::2xFLAG]]; pha-1[kry21[Y169C*e2123]] III |
| KRY55       | nhr-23[kry22[nhr-23::2xFLAG]]; pha-1[kry23[Y169C*e2123]] III |
| KRY56       | nhr-23[kry24[nhr-23::2xFLAG]]; pha-1[kry25[Y169C*e2123]] III |
| KRY57       | nhr-23[kry26[nhr-23::2xFLAG]]; pha-1[kry27[Y169C*e2123]] III |
| KRY58       | nhr-23[kry28[nhr-23::2xFLAG]]; pha-1[kry29[Y169C*e2123]] III |
| KRY59       | nhr-23[kry30[nhr-23::2xFLAG]]; pha-1[kry31[Y169C*e2123]] III |
| KRY60       | nhr-23[kry32[nhr-23::2xFLAG]]; pha-1[kry33[Y169C*e2123]] III |
| KRY64       | pha-1[kry34[Y169C*e2123]] III; nhr-25[kry35[nhr-25::2xFLAG]] X |
| KRY65       | pha-1[kry36[Y169C*e2123]] III; nhr-25[kry37[nhr-25::2xFLAG]] X |
| KRY66       | pha-1[kry38[Y169C*e2123]] III; nhr-25[kry39[nhr-25::2xFLAG]] X |
| KRY67       | pha-1[kry40[Y169C*e2123]] III; nhr-25[kry41[nhr-25::2xFLAG]] X |
| KRY70       | pha-1[kry42[Y169C*e2123]] III |
| KRY71       | pha-1[kry43[Y169C*e2123]] III |
| KRY72       | nhr-23[kry44[nhr-23::3xFLAG]]; pha-1[kry45[Y169C*e2123]] III |
| KRY73       | nhr-23[kry46[nhr-23::3xFLAG]]; pha-1[kry47[Y169C*e2123]] III |
| KRY74       | pha-1[kry48[Y169C*e2123]] III; nhr-25[kry49[nhr-25::3xFLAG]] X |
| KRY75       | nhr-23[kry50[nhr-23::3xFLAG]]; pha-1[kry51[Y169C*e2123]] III; nhr-25[kry52[nhr-25::3xFLAG]] X |
| KRY76       | nhr-23[kry6[nhr-23::2xFLAG]] I |
| KRY77       | nhr-23[kry44[nhr-23::3xFLAG]] I |
| KRY78       | nhr-23[kry50[nhr-23::3xFLAG]] I |
| KRY79       | nhr-25[kry35[nhr-25::2xFLAG]] X |
| KRY80       | nhr-25[kry52[nhr-25::3xFLAG]] X |
| KRY81       | smo-1[kry53[2xFLAG::smo-1]] I; pha-1[kry54[Y169C*e2123]] III |
| KRY82       | smo-1[kry55[2xFLAG::smo-1]] I; pha-1[kry56[Y169C*e2123]] III |
| Primer | Description | Sequence |
|--------|-------------|----------|
| 1580   | PAGE purified nhr-25::2x FLAG (sense 135mer) | aagccacatacacagctgtgcgtcagctagcagatccgagaggtgcggggattacaggtg acgcagataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 1719   | nhr-23::2xFLAG (sense 199mer; PAM #1 mutated) | tgcgtgcgtcagctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 1831   | nhr-23::2xFLAG (sense 200mer; PAM #1 and #2 mutated) | aaaaactcgaatctcagctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 1832   | nhr-23::2xFLAG (antisense 200mer; PAM #1 and #2 mutated) | gggtatcctctgacagctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 1899   | PAGE purified pha-1 repair (sense 80mer) | caaaatctcgaatctcagctagcagatccgagaggtgcggggactacaaagaccatgacggtgattataaagatcatgatatcgattacaaggatgacgatgacaagtgactgaatccatatatcatcatagttttatccatgttcctcttcccctatcccacaggtgctgagtcag |
| 1985   | PAGE purified pha-1 repair (antisense 80mer) | cttacatagtttatgattctgatttaagtatccatatagtttatcaggtgctgagtcag |
| 1986   | pha-1 repair (sense 60mer) | cttacatagtttatgatttaagtatccatatagttatatcaggtgctgagtcag |
| 1987   | pha-1 repair (sense 200mer) | cttacatagtttatgatttaagtatccatatagttatatcaggtgctgagtcag |
| 1989   | nhr-25::2xFLAG (sense 175mer; PAM mutated) | caggtgcccaatgctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 2014   | nhr-25::3xFLAG (sense 193mer; PAM mutated) | acgtcaggtgcccaatgctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 2015   | nhr-23::3xFLAG (sense 193mer; PAM #1 and #2 mutated) | ttccgaatctcagctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
| 2085   | nhr-25::3xFLAG (sense 193mer; PAM mutated)-PAGE purified | acctcaggtgcccaatgctagcagatccgagaggtgcggggattacaggtg gcagacgataaggattacagagtcagacagcgacagtaaagggctctgtcatagacgggctggaacacttc caa |
|    | nhr-23::2xFLAG (sense 140mer; 35 bp homology arms) | cctgccctctacaagagctattcactgcagatcgacctggatccggaggtggcggggattacaaggatgacgacgataagtgactgaatccatatcatatcatcaatagttttatccatgctctccttccctatccc |
|----|-------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| 2086 | nhr-23::2xFLAG (sense 120mer; 25 bp homology arms) | acaaaagagctttctcagatcgacctggatccggaggtggcggggattacaaggatgacgacgataagtgactgaatccatatcatatcatcaatagtcatccatgctctccttccctatccc |
| 2087 | nhr-23::2xFLAG (sense 100mer; 15 bp homology arms) | attcactgcagatcctggatccggaggtggcggggattacaaggatgacgacgataagtgactgaatccatatcatatcatcaatagtcatccatgctctccttccctatccc |
| 2088 | nhr-23::2xFLAG (sense 200mer; PAM #1, #2, and #3 mutated) | aaaaacctcgaatgtctgatccaacatcatctgaaaagcttcctgcactctacaaagagctattcactgcagatcgacctggatccggaggtggcggggattacaaggatgacgacgataagtgactgaatccatatcatatcatcaatagtcatccatgctctccttccctatccc |
| 2089 | lig-4 (sense 60mer; insert stop codon, delete part of exon 1) | agtagtggacgttccttcaacaagaagtttaaggatcctgtaagacaattgggccgaactattaca |
| 2099 | 2xFLAG::smo-1 (sense 175mer; PAM mutated) | ttcctttttcaaatctaatttggtttctcagagactccctcctataaaagatggattacaaggatgacgacgataaggattacaaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagcaccagggactcctgacgacgataaggatgacgacgataaggatcctgctgacgcagcagc
### Table S3: Oligos used for this study

| Primer | Description | Sequence |
|--------|-------------|----------|
| 1335   | PU6 primer for site-directed mutagenesis | caagacatctgcaatagg |
| 1349   | sgRNA template sequencing oligo | ctctgacatcagctcccg |
| 1432   | Generation of klp-12 CRISPR/Cas9 plasmid by Q5 mutagenesis (pJW1138; pair with oligo 1335) | atccacaagttacaattggGTTTTAGAGCTAGAAATA
|        |             | GCAAGT |
| 1436   | klp-12 genotyping-F | ccatcgaataatccatccagat |
| 1437   | klp-12 genotyping-R | gttgccttgaggtcgcag |
| 1582   | Generation of nhr-25 CRISPR/Cas9 plasmid by Q5 mutagenesis (pJW1185; pair with oligo 1335) | catacactgctgcatgacggatTTTAAGAGCTATGCTGGA
|        |             | GAAACAG |
| 1584   | nhr-25 C-terminal insert screening-F | agagaagaagactcggag |
| 1585   | nhr-25 C-terminal insert screening-R | tgtgagggtttggcactagg |
| 1586   | nhr-23 C-terminal insert screening-F | gtggcctggaagaatctacg |
| 1587   | nhr-23 C-terminal insert screening-R | aagagctactcctctgcaac |
| 1715   | FLAG-specific oligo for direct screening. Pair with oligo 1585 or 1587. | gggattacaaggaagacag |
| 1734   | Generation of nhr-23 CRISPR/Cas9 plasmid by Q5 mutagenesis (pJW1254; pair with oligo 1335) | aagagctactgcatgcagatTTTAAGAGCTATGCTGGA
|        |             | GAAACAG |
| 1763   | Generation of klp-12 CRISPR/Cas9 plasmid by Q5 mutagenesis (pJW1236; pair with oligo 1335) | atccacaagttacaattggGTTTAAGAGCTATGCTGGA
|        |             | GAAACAG |
| 1785   | deletion of PU6-sgRNA template in pJW1219 to generate pJW1259-F | cgcagttgtaaacaagggcacc |
| 1786   | deletion of PU6-sgRNA template in pJW1219 to generate pJW1259-R | cggagctgctatgtgacag |
| 1787   | PU6-F for generating pJW1310 and cloning PU6 for PCR-derived sgRNA templates | attggtcttggtgcttacg |
| 1788   | PU6-R for generating pJW1310 and cloning PU6 for PCR-derived sgRNA templates | caagacactgctgcaataggag |
| 1789   | sgRNA-F for generating pJW1311 | gtttaagctatgctggaac |
| 1790   | sgRNA-R for generating pJW1311 and cloning PU6::sgRNA templates | aaaaatagccgcatcagcag |
| 1793   | nested PU6-sgRNA template-F | aacgtgctgactgagaaacc |
| 1794   | nested PU6-sgRNA template-R | ggtgcattacgctgacag |
| 1898 | *pha-1* PU6-sgRNA template by PCR fusion (pair with oligo 1790) | cctctattgcagatgtctttGagataaactttaacatgtgagagat
| 1908 | *pha-1* genotyping-F | caatttgccagcattctagttg |
| 1909 | *pha-1* genotyping-R | tgcgcacactgaatcaggtc |
| 1988 | *nhr-25* PAM#2 PU6-sgRNA template (pair with oligo 1790) | cctctattgcagatgtctttGagataaactttaacatgtgagagat
| 1995 | Generation of *nhr-25* CRISPR/Cas9 plasmid by Q5 mutagenesis (pJW1308; pair with oligo 1335) | atacagtctgtgcctcagaGTGTTAAGACTATGCTGG GAAACAG |
| 2093 | *nhr-23* sgRNA PAM #4 PU6-sgRNA template by PCR fusion (pair with oligo 1790) | cctctattgcagatgtctttGagataaactttaacatgtgagagat
| 2097 | *lig-4* sgRNA#1 PU6-sgRNA template by PCR fusion (pair with oligo 1790) | cctctattgcagatgtctttGagataaactttaacatgtgagagat |
| 2098 | *lig-4* sgRNA#2 PU6-sgRNA template by PCR fusion (pair with oligo 1790) | cctctattgcagatgtctttGagataaactttaacatgtgagagat |
| 2104 | *smo-1* sgRNA#1 PU6-sgRNA template by PCR fusion (pair with oligo 1790); sgRNA is from (Kim et al. 2014) | cctctattgcagatgtctttGagataaactttaacatgtgagagat |
| 2114 | *nhr-23* FLAG specific: for sequencing into 5' end of 2xFLAG and 3xFLAG tags in heterozygotes | ttactgcagatcagcttgg |
| 2115 | *nhr-23* FLAG specific: for sequencing into 3' end of 2xFLAG and 3xFLAG tags in heterozygotes | atgatatatggagacttacct |
| 2117 | *nhr-25* FLAG specific: for sequencing into 5' end of 2xFLAG and 3xFLAG tags in heterozygotes | gtcacttatgacagacttact |
| 2118 | *nhr-25* FLAG specific: for sequencing into 3' end of 2xFLAG and 3xFLAG tags in heterozygotes | cctctatgacagacattact |
| 2127 | *smo-1* genotyping-F | cgctccgccagcaataagata |
| 2128 | *smo-1* genotyping-R | tgtgaaaggggtggatgggtg |
| 2129 | *lig-4* genotyping-F | ggcaagactcaagctggat |
| 2130 | *lig-4* genotyping-R | cccatctatgcgtgctccg |
| 2135 | *smo-1* FLAG specific: for sequencing into 5' end of 2xFLAG tag heterozygotes | ctccctgtataaaacgatgga |
| 2136 | *smo-1* FLAG specific: for sequencing into 3' end of 2xFLAG tag heterozygotes | tgtgcgcgtctgctacgag |
| GSGGGG-2xFLAG epitope (used in *nhr-23* and *nhr-25* editing) | ggcgggacagatggagcagagataagataaagagagtagtgcag |
| GSGGGG-3xFLAG epitope (used in *nhr-23* and *nhr-25* editing) | ggcgggacagatggagcagagataagataaagagagtagtgcag |
| 2xFLAG-GS epitope (used in *smo-1* editing) | gactgacgagataagagagtagtgcag |

For oligos 1828, 1898, 1988, 2093, 2097, 2098, and 2104, the underlined, lowercase sequence is the sgRNA target site. The uppercase G 1 bp 5' to the sgRNA target is the +1 base of the U6 transcript and the uppercase sequence 3' top the sgRNA target is a portion of the chimeric sgRNA.
For oligos 1432, 1582, 1734, 1829, 1897, and 1995 the lowercase sequence is the sgRNA target sequence, uppercase sequence is a portion of the chimeric sgRNA.
| Primer | Description | Sequence |
|--------|-------------|----------|
| 1643   | sgRNA(F+E)  | tataaacctcctatatgagatgtttgatgtgttagtcaattgtgtaaagagctagctgggaaacagcatagcaagtttaaatagcgtctggtttttttgtgaaatttctggcgtaaatagccgactgcccgcacc |
Table S5  Brood size analysis of indicated genotypes

| Strain | Genotype | Broodsize | P0 animals scored | Embryonic lethality (%) | Males (%) | Molting defects (%) |
|--------|----------|-----------|------------------|-------------------------|-----------|---------------------|
| N2     | WT       | 213±45   | 19               | 0.16                    | 0         | 0 (n=4458)         |
| GE24   | pha-1(e2123) III | 128±34 (n=1520) | 12               | 72.7                    | 0         | n/a                |
| KRY42b | nhr-25(kry1[nhr-25::2xFLAG]) X | 191±42 (n=3627) | 19               | 1.25                    | 0         | 0 (n=3627)         |
| KRY71  | pha-1(kry43[Y169C*e2123]) III | 210.7±30 (n=2528) | 12               | 1.19                    | 0         | 0 (n=2528)         |
| KRY49  | nhr-23(kry10[nhr-23::2xFLAG]) I; pha-1(kry11[Y169C*e2123]) III | 167.3±34 (n=1840) | 11               | 1.68                    | 0         | 0 (n=1840)         |
| KRY64  | pha-1(kry34[Y169C*e2123]) III; nhr-25(kry35[nhr-25::2xFLAG]) X | 146.8±35 (n=1762) | 12               | 2.27                    | 0.11      | 0 (n=1762)         |
| KRY72  | nhr-23(kry44[nhr-23::3xFLAG]) I; pha-1(kry45[Y169C*e2123]) III | 167.1±68 (n=1671) | 10               | 0.78                    | 0         | 0 (n=1671)         |
| KRY74  | pha-1(kry48[Y169C*e2123]) III; nhr-25(kry49[nhr-25::3xFLAG]) X | 180.8±48 (n=1671) | 12               | 2.00                    | 0         | 0 (n=2170)         |
| KRY75  | nhr-23(kry50[nhr-23::3xFLAG]) I;pha-1(kry51[Y169C*e2123]) III; nhr-25(kry52[nhr-25::3xFLAG]) X | 112.7±46 (n=1352) | 12               | 3.42                    | 0.15      | 0 (n=1352)         |

aall remaining progeny arrested as larvae.

bfrom direct screening approach. *lig-4(ok716)* removed by outcrossing; strain was outcrossed 6x.
Table S6  Males recovered in injection experiments

| P0 strain                     | Diet      | Expt. | pha-1 oligo | Repair oligo | Viable injected P0 | P0 with rescued F1 | ♂ n | ♂ PCR hit |
|-------------------------------|-----------|-------|-------------|---------------|---------------------|---------------------|-----|-----------|
| pha-1(ts)                     | OP50      | Figure 1D | 80mer sense | nhr-23::2xFLAG sense | 16 | 3 | 0 | 0 |
| pha-1(ts)                     | OP50      | Figure 1D | 80mer antisense | nhr-23::2xFLAG sense | 47 | 3 | 0 | 0 |
| pha-1(ts)                     | OP50      | Figure 1D | 60mer sense | nhr-23::2xFLAG sense | 11 | 3 | 0 | 0 |
| pha-1(ts)                     | OP50      | Figure 1D | 200mer sense | nhr-23::2xFLAG sense | 28 | 10 | 3 | 2 |
| pha-1(ts)                     | OP50      | Figure 1D | 200mer sense | nhr-23::2xFLAG antisense | 57 | 4 | 0 | 0 |
| pha-1(ts)                     | HB101     | Figure 3B | 200mer sense | nhr-23::2xFLAG sense | 27 | 3 | 0 | 0 |
| pha-1(ts); control(RNAi)      | OP50 then RNAi | Table 2 | 80mer sense | nhr-23::2xFLAG sense | 10 | 1 | 0 | 0 |
| pha-1(ts); cku-80(RNAi)       | OP50 then RNAi | Table 2 | 80mer sense | nhr-23::2xFLAG sense | 16 | 6 | 1 | 1 |
| pha-1(ts); control(RNAi)      | HB101 then RNAi | Table 2 | 200mer sense | nhr-25::2xFLAG sense | 21 | 4 | 1 | 0 |
| pha-1(ts); cku-80(RNAi)       | HB101 then RNAi | Table 2 | 200mer sense | nhr-25::2xFLAG sense | 22 | 12 | 3 | 0 |
| pha-1(ts); control(RNAi)      | HB101 then RNAi | Table 2 | 200mer sense | nhr-23::2xFLAG sense; nhr-25::3xFLAG | 34 | 4 | 0 | 0 |
| pha-1(ts); cku-80(RNAi)       | HB101 then RNAi | Table 2 | 200mer sense | nhr-23::2xFLAG sense; nhr-25::3xFLAG | 13 | 3 | 0 | 0 |
| pha-1(ts); cku-80(RNAi)       | HB101 then RNAi | Table 2 | 200mer sense | 2xFLAG::smo-1 lig-4 stop | 15 | 7 | 0 | 0 |
Table S7  Conversion events associated with different DSB positions for *nhr*-23::2xFLAG knock-ins

| sgRNA | DSB Distance from insert site | Sequenced animals | PAM#1 only | PAM#2 only | PAM#3 only | PAM#1+ PAM#2 | PAM#2+ PAM#3 | PAM#1+ PAM#2+ PAM#3 | PAM#1+ FLAG | PAM#1+ PAM#2+ FLAG | PAM#1+ PAM#2+ PAM#3+ FLAG |
|-------|------------------------------|-------------------|------------|------------|------------|--------------|--------------|----------------------|-------------|-------------------|-------------------------|
| PAM #1\textsuperscript{a} | 9 bp | 22\textsuperscript{b} | n/a | n/a | n/a | n/a | n/a | n/a | 16 | 6 | n/a |
| PAM #2 | 29 bp | 12\textsuperscript{c} | 0 | 2 | n/a | 0 | n/a | n/a | 0 | 1 | n/a |
| PAM #3 | 54 bp | 8\textsuperscript{d} | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1\textsuperscript{e} |

For the data presented in Figure 4, the number of animals with conversion events at the indicated PAMs, and number FLAG tag knock-in was presented. Here, a more detailed breakdown of the knock-in events is provided. There was no PAM #3 mutation present in the repair oligo used for the PAM #1 and PAM #2 sgRNA experiments.

\textsuperscript{a}Pooled from all 200mer *nhr*-23-2xFLAG injections (Figures 1 and 3, Table 2). As these animals were selected for based on a potential FLAG insertion, there were no “PAM only” gene conversion events that would be identified in this dataset.

\textsuperscript{b}Includes 14 precise insertions and 8 partial insertions

\textsuperscript{c}Of the 12 sequenced animals, nine had no knocked-in sequence

\textsuperscript{d}Of the eight sequenced animals, six had no knocked-in sequence

\textsuperscript{e}1 bp deletion in inserted 2xFLAG epitope
Table S8  *nhr-23::2xFLAG* and *nhr-25::2xFLAG* knock-in identification by direct screening

| P0 strain | Repair oligo     | Oligo polarity | Successfully injected P0 | F1 screened | PCR hits | Precise Knockins | % Knockins per F1 | % Knockins per P0 |
|-----------|------------------|----------------|--------------------------|-------------|----------|-----------------|------------------|------------------|
| WT        | *nhr-25::2xFLAG* | sense          | 12                       | 380         | 0        | 0               | 0.00             | 0                |
| *lig-4(ok716)* | *nhr-25::2xFLAG* | sense          | 10                       | 768         | 1        | 1               | 0.13             | 10               |
| WT        | *nhr-23::2xFLAG* | sense          | 2                        | 200         | 2        | 0               | 0                | 0                |
| *lig-4(ok716)* | *nhr-23::2xFLAG* | sense          | 4                        | 800         | 8        | 0               | 0                | 0                |

For the *nhr-25* experiments, animals were injected with 100 ng/µl of CRISPR/Cas9 plasmid targeting the same PAM used for the experiments described in Figure 5, 10 ng/µl of a *myo-2::tdTomato* co-injection marker, and 100 ng/µl of a 135mer *nhr-25::2xFLAG* repair oligo with 35 bp homology arms (oligo #1580), which was the synthesis size limit at the time. Injected P0 animals were singly plated, and plates lacking co-injection marker positive F1 progeny were discarded. As Zhao et al. (2014) had reported that only non-transgenic F1s contained knock-ins, marker-negative F1 were transferred into 96-well plates (four worms/well), allowed to self-fertilize, and potential knock-ins were identified by PCR and diagnostic *Bam*HI digestion, as in the *pha-1(ts)* co-selection experiments. Oligo polarity is with respect to the coding strand.

For *nhr-23* experiments, animals were injected with 50 ng/µl of a CRISPR/Cas9 plasmid targeting *nhr-23* PAM #1 (Figure 1), 10 ng/µl of a *myo-2::tdTomato* co-injection marker, and 100 ng/µl of a 199mer 2xFLAG repair oligo with the PAM mutated (oligos #1719). Wells containing marker-positive F1 progeny were identified, all animals from these wells were pooled, and 10 worms were plated per well of a 96-well plate. Following self-fertilization, a portion of the well was taken for genotyping and four rows were pooled for knock-in specific PCR using an oligo internal to the insert and an oligo external to the insert.
Table S9  Summary of strands to which sgRNAs bind, sgRNA activity, repair oligo strand homology, and repair oligo efficiency

| Gene       | sgRNA sequencea | Strand to which sgRNA bindsb | sgRNA activity | Repair oligo                        | Repair oligo strandc | Repair oligo efficiency |
|------------|-----------------|------------------------------|----------------|-------------------------------------|-----------------------|-------------------------|
| pha-1(e2123) | atgaataacctgatga acat(cgg) | coding                      | Y              | 60, 80 and 200 mer pha-1(ts) repair (oligos 1899, 1986, 1987) | coding                | High                    |
|            |                 |                              |                | 80mer pha-1(ts) repair (oligo 1985)                  | template              | Weak                    |
| nhr-23 PAM #1 | agacgcttagctgacagat(cgg) | template                    | Y              | 200mer nhr-23::2xFLAG (oligo 1831, 2 PAMs mut)       | coding                | High                    |
|            |                 |                              |                | 200mer nhr-23::2xFLAG (oligo 1832, 2 PAMs mut)       | template              | Weak                    |
|            |                 |                              |                | 193mer nhr-23::3xFLAG (oligos 2015 and 2085, 2 PAMs mut) | coding                | Moderate                |
| nhr-23 PAM #2 | agtaatagctctttgtaga(cgg) | coding                      | Y              | 200mer nhr-23::2xFLAG (oligo 1831, 2 PAMs mut)       | template              | Moderate                |
| nhr-23 PAM #3 | ggagacctctgctagcgtatg(cgg) | coding                      | Y (moderate)   | 200mer nhr-23::2xFLAG (oligo 2089, 3 PAMs mut)       | template              | Moderate                |
| nhr-23 PAM #4 | agatgttggatcagatt(cgg) | coding                      | N              | 200mer nhr-23::2xFLAG (oligo 2089, 3 PAMs mut)       | template              | n/a (sgRNA fail)        |
| nhr-25     | atacgctgtgcgtacg(tgg) | template                    | Y              | 175mer nhr-25::2xFLAG (oligo 1989; PAM mut)          | template              | Moderate                |
|            |                 |                              |                | 193mer nhr-25::3xFLAG (oligo 2014; PAM mut)-unpurified | template              | Weak                    |
|            |                 |                              |                | 193mer nhr-25::3xFLAG (oligo 2085; PAM mut)-PAGE purified | template              | Weak                    |
| smo-1      | ggcgatgatgcagcctcagatt(cgg) | template                  | Y              | 175mer 2xFLAG::smo-1 (oligo 2105; PAM mutated)       | template              | High                    |
| lig-4      | acgtctcagctcactcagatt(cgg) | template                  | N              | 60mer lig-4 stop/exon deletion (oligo 2099)           | template              | n/a (sgRNA fail)        |
|           | ttcgatctcagctcactcagatt(cgg) | template                  | N              |                                                    |                       |                         |

*a* sgRNA target sequence. The PAM sequence is provided in brackets.

*b* strand to which sgRNA binds. i.e. for a target sequence with an "NGG" PAM in the coding sequence, the sgRNA would bind to the template strand

*c* strand from which oligo homology is derived
Supplemental references

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