| Entry | Experimental purpose | Gene | Background | Editing | crRNA | Oligo (CrRNA) | sequence | Targeted sequence | Guide concentration | GFP+/RFP+ (CRISPR) | GFP+/RFP+ (sgRNA) | Buffer | Notes |
|-------|----------------------|-----|-----------|--------|-------|----------------|----------|-----------------|-------------------|-----------------|-----------------|--------|-------|
| 2A04  | Common crRNA        | TSB | 1        | siRNA  | dpy   | 82 (1/10)     | 5.2 (5.1) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2B04  | Immunofluorescence  | TSB | 1        | siRNA  | dpy   | 96 (2/10)     | 4.9 (4.6) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2A05  | Increase cell rate  | TSB | 1        | siRNA  | dpy   | 54 (2/10)     | 5.2 (5.1) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2B05  | Baseline            | TSB | 1        | siRNA  | dpy   | 54 (2/10)     | 5.2 (5.1) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2A06  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2B06  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2A07  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2B07  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2A08  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2B08  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2A09  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |
| 2B09  | CRISPR knock-in     | TSB | 24       | CRISPR | dpy   | 147 (2/10)   | 6.1 (6.4) | 10 (3/32) | 0.30             | 2.5 (2/8)       | 0.4 (1/5)       | 0.4     | no    |

Table 3: summary of all results

Also available for download as an Excel file at www.genetics.org/lookup/suppl doi:10.1534/genetics.115.179382/-/DC1

A. Paix et al.
# Table S2: List of plasmids

| Name     | Backbone | Insert                                         | Note                                           |
|----------|----------|-----------------------------------------------|------------------------------------------------|
| AP575-1  | pUC19    | 3Xflag::tagRFP::myc                           | no intron                                     |
| AP588-1  | pUC19    | myc::TEV::mTagGFP::TEV::3Xflag                | no intron                                     |
| AP582-1  | pUC19    | Ollas::mCherry::linker::H2B::V5               | no intron                                     |
| pCM1.35  | see Merritt et al. 2008 | GFP::H2B | see Merritt et al. 2008                  |
| AP625-1  | pUC19    | myc::TEV::3Xflag::eGFP::3Xflag::TEV::myc        | no intron                                     |
| AP682-1  | pUC19    | TEV::eGFP::myc::3Xflag                          | no intron                                     |
| AP334-2/5/6 | pDD162 | crRNA 728 target sequence                     | see Paix et al. 2014                          |
| AP568-1/2/3 | pDD162 | crRNA 589 target sequence                     | crRNA target sequence from Arribere et al. 2014 |
| nm2973   | see Fu et al. 2014 | Cas9::NLSv4o::His6 | see Fu et al. 2014 |
| crRNA name | Target gene | Gene-specific sequence | Location | % G/C | G at 3' end of the crRNA | C at 3' end of the crRNA | Sens/Antisens | Exp. | Efficiency of FP insertion (% Rollier, 3 best P0s) |
|------------|-------------|------------------------|----------|------|-------------------------|-------------------------|---------------|-----|----------------------------------|
| 589        | dpy-10      | gctaccatagggacaacagag | in ORF   | 60   | yes                     | no                      | AS            | All | NA                               |
| 728        | gtp-1       | ccacgaggtgtatgcag      | near STOP| 65   | yes                     | no                      | S             |     | 51.9% (AP78) and 70.2% (AP96)    |
| 723        | gh-1        | tctctcaagagagagagcagg | near STOP| 50   | no                      | yes                     | S             | AP106 | 47.5%                            |
| 699        | htp-3       | agaggaactcaagattttcg  | at STOP  | 40   | no                      | yes                     | S             | AP114 | 2.0%                             |
| 691        | gtp-1       | ggccttaccaaggataaga   | near ATG | 45   | no                      | no                      | S             | AP115 | 58.1% (crRNAs 691 and 728 were used for gene replacement) |
| 719        | gfp/egfp    | caacaactgaaggaaggcag   | near 3’ end of gfp/egfp | 45 | yes                     | no                      | AS            | AP105/105D | 26.5% (AP105) (crRNAs 719 and 720 were used for gene replacement) |
| 720        | gfp/egfp    | cacctaactgaacaagag     | near 5’ end of gfp/egfp | 30 | no                      | no                      | AS            | AP105/105D | 35.0% (was performed with gtp-1 co-edition) |
| 689        | fbf-2       | ggtagtacccggtgtgtaa    | at STOP  | 50   | no                      | no                      | S             | AP103 |                                |
| 772        | gtp-1       | tcgtagtagtgagccacagag  | near STOP| 70   | yes (GG)                | no                      | S             | AP92  | 20.2%                             |
| Gene | crRNA | Description | Sequence |
|------|-------|-------------|----------|
| *dpy-10* | 589 | Co-CRISPR misense mutation | ssODN from Arribere et al. 2014 (See Suppl. File S1) |
| *gtbp-1* | 728 | Ollas tag insertion at the STOP codon | GGCAGGTTGGTGGTGCAACGGAGAGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---Ollas---tagaatctgctctcttcctctctttgtaag |
| *gtbp-1* | 728 | mTagGFP::TEV::3Xflag insertion at the STOP codon | GGCAGGTTGGTGGTGCAACGGAGAGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---mTagGFP::TEV::3Xflag---tagaatctgctctcttttcctctctttgtaag |
| *gtbp-1* | 728 | mCherry insertion at cut | GGGTTGAGTGGTGAGTGGTGGAGGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---mCherry--- 
AGCGGTTTTCAAAAATGCAGGAC |
| *gtbp-1* | 772 | mCherry insertion at cut | CTTCCAGGTTGGTGGTGCAACGGAGAGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---mCherry--- 
AGCGGTTTTCAAAAATGCAGGAC |
| *gtbp-1* | 728 | eGFP insertion at cut | GGGTTGAGTGGTGAGTGGTGGAGGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---eGFP--- 
AGCGGTTTTCAAAAATGCAGGAC |
| *fbf-2* | 689 | tagRFP insertion at cut (STOP codon) | CAAATTTTCTTCTTCTCaGAGGGAGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---tagRFP--- 
AGCGGTTTTCAAAAATGCAGGAC |
| *GFP/eGFP* | 719 and 720 | Replacement by tagRFP | GGGAGAGAATCTTATCTAGTGGTGACGAATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---tagRFP--- 
AGCGGTTTTCAAAAATGCAGGAC |
| *glh-1* | 723 | tagRFP insertion at the STOP codon | CCAGGTGAGTTCTGACATGGCAGGCTCTGGATGGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---tagRFP--- 
tagaaacgagcacaattgataagtgctctctctcttaaatgc |
| *htp-3* | 699 | TEV::eGFP::myc::3Xflag insertion at the STOP codon | CCAGGTGAGTTCTGACATGGCAGGCTCTGGATGGATGGTAAGGGTTTCAAAAATGCAGGAC <br>AAAAT---TEV::eGFP::myc::3Xflag--- 
tagaaacgagcacaattgataagtgctctctctcttaaatgc |
| *gtbp-1* | 691 and 728 | *gtbp-1* replacement by GFP::H2B | aaATCGACGATAACCTGGCTTAAACCCAGAATGA---GFP::H2B--- 
tagaaacgagcacaattgataagtgctctctctcttaaatgc |

Lower case (non-coding), upper case (coding), red (silent mutations in the repair template to prevent Cas9 re-cutting), blue (insertion), bold (PAM sequence), underlined (crRNA sequence).
| JH number | Gene targeted | crRNA | Insert | Location | Recipient strain |
|-----------|---------------|-------|--------|----------|------------------|
| JH3324    | gtbp-1        | 728   | mCherry| near STOP codon (at the cut) | N2 |
| JH3314    | gtbp-1        | 728   | eGFP::3Xflag | near STOP codon (at the cut) | N2 |
| JH4000    | gtbp-1        | 728   | Ollas  | STOP codon | N2 |
| JH4001    | gtbp-1        | 728   | mTagGFP::TEV::3Xflag | STOP codon | N2 |
| JH4002    | gtbp-1        | 728   | eGFP | near STOP codon (at the cut) | N2 |
| JH4003    | fbf-2         | 689   | tagRFP | STOP codon | N2 |
| JH4004    | gtbp-1, fbf-2 | 728, 689 | eGFP for gtbp-1 and tagRFP for fbf-2 | near STOP codon (at the cut) of gtbp-1 and at the STOP codon of fbf-2 | N2 |
| JH4005    | gtbp-1        | 719 and 720 | eGFP replacement by tagRFP | Between 5'/3' ends of eGFP | JH3314 |
| JH4006    | deps-1        | 719 and 720 | GFP replacement by tagRFP | Between 5'/3' ends of GFP | JH3207 |
| JH4007    | glh-1         | 723   | tagRFP | STOP codon | N2 |
| JH4008    | htp-3         | 699   | TEV::eGFP::myc::3Xflag | STOP codon | N2 |
| JH4009    | gtbp-1        | 691 and 728 | gtbp-1 replacement by GFP::H2B | Between 5'/3' ends of gtbp-1 ORF | N2 |
File S1: Direct delivery CRISPR-HDR editing protocol for C. elegans
(Paix et al. 2015)

Protocol updates will be posted on the Seydoux lab website:
http://www.bs.jhmi.edu/MBG/SeydouxLab/

Protocol Overview

- Design crRNA(s) and a repair template for your gene of interest.
- Inject Cas9/crRNA/tracrRNA complexes and repair templates targeting your gene of interest and dpy-10.
- Identify broods with Rollers (first generation after injection).
- Screen Rollers (and their non-Roller siblings, if desired) for edits at your gene of interest
- See Section F for a positive control experiment – tagging gtbp-1 with GFP using dpy-10 co-CRISPR.

A. Preparation of reagents

Cas9

Recombinant Cas9::NLS can be purified from E. coli (see attached protocol File S2) or purchased from commercial sources.

tracrRNA

The universal tracrRNA is a structural RNA that links the crRNA to Cas9. The same tracrRNA is used for all experiments.

We order it from Dharmacon #U-002000-05/20/50 (http://dharmacon.gelifesciences.com/gene-editing/crispr-cas9/edit-r-tracrRNA/). The tracrRNA is 74nt long (Jinek et al. 2012):
AACAGCAUAGCAAGUAAAUAAGGCUAGUCCGUUAAUCAACUUGAAAAGUGGCACCGAGUCGGUGCUCUUUUUU

Upon receipt, briefly spin the tubes and reconstitute at 4µg/µl (0.17nmol/µl): add 29.8µl of Tris pH 7.5 to the 5nmol provided (U-002000-05). Other amounts of tracrRNA are available (U-002000-20/50). Store at -80°C.
crRNA

The crRNA consist of a 20nt gene-specific sequence followed by a universal sequence (GUUUUAGAGCUAUGCUGUUUUG) required to interact with the tracrRNA. The 20nt gene-specific sequence must lie upstream of a PAM sequence (NGG) in genomic DNA. DO NOT INCLUDE THE PAM IN THE crRNA!

Remember to look for PAM sites on both DNA strands – crRNAs can target either strand.

Not all crRNAs work well (Table S3). Desired features include (in order of importance) 1) cleavage site as close as possible to the edit site, 2) good sequence (see below) and 3) few off-target sites (we use the website http://crispr.mit.edu/ for off-target prediction; Hsu et al. 2013). If there are off-target sites, those sites should have 3 or more mismatches, preferentially close to the PAM.

In our hands, the most predictable determinant of guide RNA efficiency for HDR is distance between the cleavage site and the edit that you are trying to introduce. Optimal distance is <10 bases. We have obtained edits up to 30 bases away from the cleavage site, but the efficiency of edit incorporation drops by a factor of ~5-10.

Several recommendations for crRNA sequence have been reported (Farboud et al. 2015; Gagnon et al. 2014; Doench et al. 2014), and we try to follow them when possible. These recommendations are:

- 50 to 75% overall GC content
- GG or G, but no C, for the 3’ most residue(s) immediately upstream of the PAM

The Broad Institute website implements these recommendations for guide RNA scoring (http://www.broadinstitute.org/rnaí/public/analysis-tools/sgrna-design) (Doench et al. 2014).

Order your gene specific crRNA (20nt specific sequence + GUUUAGAGCUAUGCUGUUUUG) from Dharmacon (http://dharmacon.gelifesciences.com/gene-editing/crispr-rna-configurator/).

Also order the crRNA for dpy-10: GCUACCAUAGGCACCAGAG + GUUUAGAGCUAUGCUGUUUUG

Upon receipt, briefly spin the tubes and reconstitute at 8µg/µl (0.6nmol/µl): add 33.8µl of Tris pH 7.5 to the 20nmol provided. Store at -80°C.

Repair template design

Repair templates should contain ~35nt homology arms (Paix et al. 2014) (Table S4): sequences at the 5’ and 3’ end of the repair template that are homologous to sequences flanking the cut and edit in the genomic DNA. Ideally, flanking sequences should terminate with a C or G and contain good sequence diversity at their extremities (no hairpins).

The repair template should also contain mutations that make it resistant to re-cutting by Cas9/crRNA-tracrRNA complex after integration in the genome. These mutations can be 1) insertions that disrupt the crRNA sequence or separate the crRNA sequence from the PAM or 2) mismatches that disrupt the PAM or crRNA sequence (we typically create between 2 and 4 mismatches when disrupting the crRNA sequence, mutations closest to the PAM are the most effective) (Jinek et al. 2012).
Be careful to introduce only silent changes using codons that are used at a frequency similar to the original codon (http://www.genscript.com/cgi-bin/tools/codon_freq_table). If possible, avoid crRNAs that target non-coding sequences since mutations in these sequences could possibly affect regulatory (splicing, promoter) motifs.

For small edits, engineer a restriction site in your repair template to facilitate screening (see Paix et al. 2014). For insertions >20bp, we typically identify the edits by size shift in the PCR product. When inserting a fluorescent protein, it is possible to screen directly by visual examination of F1s or F2s if the pattern is known.

Recommendation for antigenic peptide tag sequences can be found in Paix et al. 2014.

**Repair template synthesis**

A. Small edits (<100nt):

Use single-stranded oligonucleotides (ssODNs, 200nt maximum size, 4nM ultramer, salt free) ordered from IDT. Reconstitute ssODN at 1µg/µl according to the amount provided by the manufacturer. Sense-strand ssODNs have been reported to work better (Katic et al. 2015).

The ssODN repair template for *dpy-10* is:

CACTTGAACTTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCATGCGGTGCCTATGGTAGCGGAGCTT
CACATGGCTTCAGACCAACAGCCTAT
- Use a working aliquot at 500ng/µl in H2O.

B. Large edits (100bp-2kb): Use PCR amplicons.

Note that this type of template may not work efficiently for inserts > 3kb (A. Paix, unpublished).

-Primer design: Design the primers so that they contain the desired homology arms (~35nt), mutations in the crRNA site(s) and sequences complementary to insert. Be sure to have a C or G at the 3’ end of the primers. Where possible, limit the size of the primers to less than 65nt in order to avoid primer contamination after PCR purification (see below). See Table S2 for available plasmids that can be used as templates to amplify fluorescent proteins and tags.

-PCR: Amplify the PCR template using this reaction mix: Mix 2µl of template plasmid (from a standard miniprep of 1.5ml bacterial culture), 2µl of forward and reverse primers (100µM stock), 194µl of H2O, 200µl of Phusion Master Mix 2X (NEB, #M0531L). Split the mix in 8 PCR tubes (50µl per tubes) and do a gradient PCR as follow:
---------
98°C, for 2min
---------
98°C, for 30s
60 to 72°C gradient, for 30s
72°C, for 45s
---------
Repeat 29 times
---------
72°C, for 10min
Hold at 10°C
---------

Add 10µl of 6X Orange loading dye (Bioworld, #10570024-1) to each tube and run 8µl of it on an agarose gel.

Pool the positive PCRs in one tube (up to 8) and purify them in one Qiagen minelute column (#28006), elution with 10µl of H2O. For most templates, the 8 annealing temperatures will give good yield and therefore we routinely pool the 8 reactions together. The expected yield ranges from 1 to 1.5µg/µl.

If the PCR primers are >65nt (typically when the edit is away from the cut), a second (nested) PCR step is necessary because the long primers will be present in the purified PCR pool at a concentration high enough to be toxic for injection. For the nested PCR, use forward and reverse primers of 18-22nt corresponding at the 5' end 3' ends of the template generated in the first PCR. Run a second PCR as before but with the following master mix (for 8 annealing temperatures of PCR): 0.8µl of 1st round of purified PCR, 2µl of forward and reverse primers, 195.2µl of H2O, 200µl of Phusion Master mix 2X.

B. Preparing injection mixes

I. One locus editing using home-made Cas9

Cas9 prep (10µg/µl): 5µl
tracrRNA (4µg/µl): 5µl
dpy-10 crRNA (8µg/µl): 0.4µl
**dpy-10 ssODN (500ng/µl):** 0.55µl

Targeted gene crRNA (8µg/µl): 1µl

PCR template (s) (several templates can be mixed): Up to 500ng/µl final in the mix

OR ssODN (s) (several templates can be mixed) (1µg/µl): 2.2µl total

KCl (1M): 0.5µl

Hepes pH7.4 (200mM): 0.75µl

H2O: add if necessary to reach a final volume of 20µl

---

**II. Multi-loci editing using home-made Cas9**

Cas9 prep (10µg/µl): 5µl

tracrRNA (4µg/µl): 6.7µl

dpy-10 crRNA (8µg/µl): 0.4µl

dpy-10 ssODN (500ng/µl): 0.55µl

Targeted gene crRNA1 (8µg/µl): 0.75µl

Targeted gene crRNA2 (8µg/µl): 0.75µl

PCR templates (to repair the cuts corresponding to crRNAs 1/2): Up to 500ng/µl final in the mix

OR ssODNs (to repair the cuts corresponding to crRNAs 1/2) (1µg/µl): 2.2µl total

KCl (1M): 0.5µl

Hepes pH7.4 (200mM): 0.75µl

H2O: add if necessary to reach a volume of 20µl

---

**III. Note for edits on chromosome 2**

For loci on LGII, the edits will be linked to the dpy-10(Rol) edit. If you prefer to recover edits that are unlinked to the dpy-10 edit, use 0.28µl of dpy-10 crRNA to maximize edits in non-Roller animals.

Alternatively, you can use rescue of pha-1(ts) mutation (LGIII) as an alternative co-CRISPR strategy as described in Ward, 2015.
IV. Injection mixes processing

Add each components of the injection mix in a 0.5ml tube (add Cas9 last). Place the 0.5ml tube in a 1.5ml eppendorf tube and spin for 2min at 13000rpm. Incubate at 37°C for 10-15min. Immediately load the injection needles and process to injection.

Note that the volume of the injection mixes can be decreased if necessary as long as the molarity of each component is maintained.

C. Injections, worm recovery and handling

Inject both arms of young adult hermaphrodites (with a few embryos). Be sure hermaphrodites are young enough to lay eggs for next two days. See WormBook for injection protocol: http://www.wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html

30min to 1h after injection, recover the injected hermaphrodites (P0s) as follows: Every 5-10min, add:

5µl / 5µl / 10µl /10µl /20µl /20µl / 40µl of 1X M9

Clone out the P0s onto NNGM plates (1 P0 per plate, at 20°C). Use fresh NNGM plates with a thin layer of OP50 bacteria at the center to facilitate screening for Rollers. It is important to avoid that the P0s touch the mineral oil on the injection pad because the oil will kill them. After 20-23 hours, transfer the P0s to second plate (again 1 P0 per plate). For experiments using large PCR repair templates, you may find that edits arise more frequently on the second-day plates (Paix et al. 2014).

Examine the F1s for Rollers 4-5 days after cloning the P0s. You should recover Rollers from ~30-70% of injected hermaphrodites. Determine the number of Roller F1s per P0 and select the 3 P0s giving the most Rollers. These are your “jackpot broods”.

Clone all the Roller F1s (from the jackpot broods or from all broods if you do not have that many). You can also clone non-Rollers (only from jackpot broods) if you prefer to isolate your edit without the dpy-10 edit (this is useful if your gene in on the same linkage group as dpy-10: LGII).

D. Screening for edit of interest

For the insertion of a fluorescent protein, if you know the expected pattern, you can screen the F1s (after you have allowed them to lay eggs) by placing them in a drop of M9 containing levamisole (1mM) under the 10X objective of a compound microscope. You can also use no coverslip if you want to recover the worms (in that case no need to clone them out first). Use 3 or 12-well microscope slides.
For smaller edits (antigenic tag or mutation), for each F1, pool 10 F2s in 15µl of lysis buffer and PCR the edited locus. Avoid picking bacteria with the F2s. The edits can be detected by a size-change or by restriction-enzyme digest if a restriction site was included in the rescue template. You can also PCR directly each F1, but we have found in practice that it is easier to PCR cohorts (10 or so) of F2s.

E. Strain establishment

We recommend recovering at least two independent edits (derived from different P0s) for each experiment.

If the edit was identified in a Roller worm, pick ~8 or more non-Roller F2/3s to separate the edit from dpy-10 and recover homozygous edits.

For fluorescent protein integration, check the segregation of the fluorescent signal in F3 worms derived from singled-out F2s to identify homozygous lines.

Sequence-verify the edits once the homozygous strains are established. Sequence at least the entire sequence that was present in your rescue template. You may also want to sequence possible off-target loci.

Remember that mutants may not be viable when homozygous. We have also isolated edits that cause dominant phenotypes in the F1 generation (dominant sterile or dominant maternal effect lethal/sterile).

Note that some tagged proteins are not fully functional - check the homozygous edited lines for brood size and viability at 20°C and 25°C.

F. Special applications of protocol

ORF replacement to obtain null allele and transcriptional reporter

Design crRNAs near the Start and Stop codons. Design repair template with homology arms that reach up to the cleavage sites, and are in frame with the ORF of the gene of interest, if any coding sequence remains after replacement. We recommend recoding any coding sequence remaining between the two cuts to force gene conversion of the entire template.

Make the GFP::H2B repair template as described in Repair template synthesis. Use pCM1.35 (Available at Addgene; Merritt et al. 2008) (Table S2) as a PCR plasmid template.

Process as described in Reagents for replacement of GFP (and eGFP variant) with tagRFP (or other FPs) for ORF replacement.
**Reagents for replacement of GFP (and eGFP variant) with tagRFP (or other FPs)**

crRNA GFP Nt (#720 in Table S3): CCAUCUAUUCAAACAAGAAU + GUUUAGAGCUAUGCUUUG

crRNA GFP Ct (#719 in Table S3): CAAAUCAGAAAGCAUG + GUUUAGAGCUAUGCUUUG

Primers pairs to generate template (from RFP containing-plasmid pAP575-1):

Forward primer (5’ to 3’, lower case indicating the homology arm sequence):
ggagaagaacctttcactggagttgcccattGTGTCTAAGGGCGAAGCTG

Reverse primer (5’ to 3’, lower case indicating the homology arm sequence):
gtaatcccagcagctgttacaaactcaagaaggacATTAAGTTTGTGCCCCAGTTTG

PCR condition: use annealing temperature of 63°C, elongation step of 45s, pAP575-1 as a plasmid template.

Injection mix:

- Cas9 home-made prep (10µg/µl): 5µl
- tracrRNA (4µg/µl): 6.7µl
- *dpy-10* crRNA (8µg/µl): 0.4µl
- *dpy-10* ssODN (500ng/µl): 0.55µl
- crRNA GFP Nt (8µg/µl): 0.75µl
- crRNA GFP Ct (8µg/µl): 0.75µl

PCR template for GFP (and eGFP variant) replacement: Up to 500ng/µl final in the mix

KCl (1M): 0.5µl

Hepes pH7.4 (200mM): 0.75µl

H2O: add if necessary to reach a volume of 20µl

Multi-colors replacement (using the same homology arms than the one specified above - lower case in primer sequences) (Chudakov et al. 2010) can also be performed.

Note that the same injection mix / injection needle can be used on different GFP tagged strains.
**Positive control experiment (to test protocol in your hands and/or activity of your home-made Cas9): Tag gtpb-1 with eGFP or mCherry using dpy-10 co-CRISPR.**

Prepare the repair PCR template as indicated in Reagents for *gtbp-1* eGFP and mCherry tagging and Repair template synthesis.

Make the injection mix as indicated in injection mixes, part I. Use the crRNA *gtbp-1* Ct (#728 in Table S3).

Inject 15-20 young adult N2 worms and recover as described in Worm recovery and handling. Pool the recovered worms (P0s) on one plate and incubate for 22-23h at 20°C (day 1). Clone the P0s to individual OP50 plates and incubate at 20°C for 4-5 days (day 2).

When the F1s reach the adult stage, check for Rollers. At least 3 P0s should give a high number of Rollers (>15) (Note that we do not count/examine Dumpy F1s since these are homozygous edits at the *dpy-10* locus). From those “jackpot broods”, screen the Rollers for fluorescent protein expression as described in Screening for edit of interest. 50% or more of the Rollers (at day 2) should be positive for fluorescence.

**Reagents for *gtbp-1* eGFP and mCherry tagging**

crRNA *gtbp-1* Ct (#728 in Table S3): CCACGAGGUGGUAGUGCGCAG + GUUUUAGAGCUAUGCUGUUUUG

Primers pairs to generate template (from eGFP containing-plasmid pAP682-1):

Forward primer (5’ to 3’, lower case indicating the homology arm sequence) for eGFP insertion: ggttcgggtggtgctccacgaggtggtatgcgcGTGAGTAAAGGAGAAGAAC

Reverse primer (5’ to 3’, lower case indicating the homology arm sequence) for eGFP insertion: cttctaattttgctccgcattttggaaaccgctCTTGTACAGCTCGTCCATGCC

Primers pairs to generate template (from mCherry containing-plasmid pAP582-1):

Forward primer (5’ to 3’, lower case indicating the homology arm sequence) for mCherry insertion: ggttcgggtggtgctccacgaggtggtatgcgcGTCTCAAAGGGTGAAGAAGATAAC

Reverse primer (5’ to 3’ lower case indicating the homology arm sequence) for mCherry insertion: cttctaattttgctccgcattttggaaaccgctCTTGATACAGCTCGTCCATGCC

PCR condition: use annealing temperature of 63°C, elongation step of 45s, pAP682-1 (eGFP) or pAP582-1 (mCherry) as a plasmid template. Do 8 PCR reactions and pool them (400µl total), purify on minelute column (see Repair template synthesis).
**Additional references**

Chudakov *et al.*, Fluorescent Proteins and Their Applications in Imaging Living Cells and Tissues

Doench *et al.*, Rational Design of Highly Active sgRNAs for CRISPR-Cas9-Mediated Gene Inactivation

Farboud and Meyer, Dramatic Enhancement of Genome Editing by CRISPR/Cas9 through Improved Guide RNA Design

Gagnon *et al.*, Efficient Mutagenesis by Cas9 Protein-Mediated Oligonucleotide Insertion and Large-Scale Assessment of Single-Guide RNAs

Hsu *et al.*, DNA Targeting Specificity of RNA-Guided Cas9 Nucleases

Jinek *et al.*, A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity

Katic, Xu, and Ciosk, CRISPR/Cas9 Genome Editing in Caenorhabditis Elegans

Merritt *et al.*, 3’ UTRs Are the Primary Regulators of Gene Expression in the C. Elegans Germline

Paix *et al.*, Scalable and Versatile Genome Editing Using Linear DNAs with Microhomology to Cas9 Sites in Caenorhabditis Elegans

Ward, Rapid and precise engineering of the Caenorhabditis elegans genome with lethal mutation co-conversion and inactivation of NHEJ repair
File S2: Cas9 preparation protocol
(Paix et al. 2015)

Protocol updates will be posted on the Seydoux lab website:
http://www.bs.jhmi.edu/MBG/SeydouxLab/

Purification of Cas9::NLS540::His6:

1. Transform DE3 GOLD (Agilent, #230132) cells with nm2973 plasmid (Fu et al. 2014) and plate on LB + 50µg/mL Carbenicillin.

2. Inoculate 25mL LB + 50 µg/mL Carbenicillin with bacteria from the fresh transformation and incubate at 37°C overnight.

3. Transfer 5mL of overnight culture to 1L LB + 0.1% glucose + 50 µg/mL Carbenicillin and grow at 25°C. Grow to OD600=~0.5.

3. Shift culture to 18°C for 15-25 minutes, then add IPTG to 0.2 mM. Incubate overnight.

4. Pellet culture and obtain wet weight. Resuspend at ~6 mL/g cells with Buffer A + protease inhibitor (Roche, #11836170001) + 1mM PMSF.

5. Sonicate 6 x 45s (setting 3 at 30%, 1 second pulse-2 second pause) with 1 minute cooling in between.

6. Spin lysate 30 minutes at 16000xg and transfer supernatant to a fresh tube.

7. Equilibrate a 5mL Ni-agarose (Qiagen, #30410) with column with Buffer A (at least 25mL).

8. Batch bind clarified lysate with Ni-agarose 45 minutes at 4°C.

9. Wash Ni-agarose column with 100mL of Buffer B.

10. Elute protein with Buffer C. Determine fractions that have Cas9 protein using Bradford assay or by running a small amount on SDS-PAGE gel. Pool fractions.

11. To remove contaminating DNA in the prep. Equilibrate a 5mL Q Sepharose (Sigma, #Q1126) column with 1M KCl (25mL, this charges the column). Then equilibrate Q Sepharose column with Buffer C (25mL).

12. Flow eluent (from step 11) over Q Sepharose column. Collect flow-through and dialyze into 1L Buffer D for 5 hours at 4°C. Transfer into 1L Buffer D and dialyze overnight.
13. Concentrate protein to ~10 mg/mL using a 100K centrifugal filter (Milipore, UFC910024). Aliquot and flash-freeze in liquid nitrogen. Store aliquots at -80°C. Typical yield is sufficient for 50-70 single-use aliquots (5μl aliquot, 10μg/μl Cas9).

**Buffers:**

Buffer A: 20mM Tris ph 8.0, 250 mM KCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP
Buffer B: 20mM Tris ph 8.0, 800 mM KCl, 20 mM imidazole, 10% glycerol, 1mM TCEP
Buffer C: 20mM Hepes ph 8.0, 500 mM KCl, 250 mM imidazole, 10% glycerol
Buffer D: 20mM Hepes ph 8.0, 500 mM KCl, 20% glycerol

**Purified Cas9::NLS\textsubscript{SV40}::His\textsubscript{6} resolved by SDS-PAGE:**

Recombinant Cas9::NLS\textsubscript{SV40}::His\textsubscript{6} was affinity purified using Ni-agarose (lane 1). Pooled eluent was flowed over Q sepharose to remove contaminating DNA bound to Cas9 (lane 2). Samples were resolved by SDS-PAGE and visualized by coomassie staining.

**Cas9 activity assay:**

We recommend testing your Cas9 preparation using the method outlined in the direct delivery protocol (File S1, Section F).