High Efficiency, Homology-Directed Genome Editing in *Caenorhabditis elegans* Using CRISPR-Cas9 Ribonucleoprotein Complexes

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ABSTRACT Homology-directed repair (HDR) of breaks induced by the RNA-programmed nuclease Cas9 has become a popular method for genome editing in several organisms. Most HDR protocols rely on plasmid-based expression of Cas9 and the gene-specific guide RNAs. Here we report that direct injection of *in vitro*–assembled Cas9-CRISPR RNA (crRNA) *trans*-activating crRNA (tracrRNA) ribonucleoprotein complexes into the gonad of *Caenorhabditis elegans* yields HDR edits at a high frequency. Building on our earlier finding that PCR fragments with 35-base homology are efficient repair templates, we developed an entirely cloning-free protocol for the generation of seamless HDR edits without selection. Combined with the co-CRISPR method, this protocol is sufficiently robust for use with low-efficiency guide RNAs and to generate complex edits, including ORF replacement and simultaneous tagging of two genes with fluorescent proteins.

KEYWORDS CRISPR-Cas9; genome editing; homology-directed repair; ribonucleoprotein complexes; *C. elegans*

THE CRISPR-Cas9 system is a bacterial adaptive immune system that has been harnessed as a powerful genome editing tool (Doudna and Charpentier 2014). Cas9 is a nuclease that functions with two small RNAs: CRISPR RNA (crRNA), which guides Cas9 to complementary target sequences, and *trans*-activating crRNA (tracrRNA), which binds to the crRNA and to Cas9 to form the ribonucleoprotein (RNP) complex (Deltcheva *et al.* 2011). For use in genome editing, the crRNA and tracrRNA are often combined into a single chimeric guide RNA (sgRNA) (Jinek *et al.* 2012). Expression of Cas9 and sgRNA in cells leads to cleavage of complementary genomic sequences. The double-strand breaks are repaired by endogenous cellular pathways, including end-joining mechanisms [*e.g.*, nonhomologous end joining (NHEJ) and theta-mediated end joining (TMEJ)] and homology-dependent repair (HDR) mechanisms (van Schendel *et al.* 2015). End joining typically introduces random insertions/deletions at the DNA break site, which can disrupt gene activity. HDR, in contrast, is a more precise repair process that uses a repair template. If the repair template contains edits flanked by sequences that are homologous to the cleavage site (homology arms), the edits will be incorporated by gene conversion. The high efficiency of Cas9 and the simplicity of guide RNA design have made it possible to develop end-joining protocols to systematically knock out genes (Hsu *et al.* 2014; Shah *et al.* 2015). In principle, scalable HDR protocols also could be used for systematic knock-ins of custom edits [such as green fluorescent protein (GFP)]. Unfortunately, current HDR protocols are inefficient (Hsu *et al.* 2014). First, most HDR protocols require cloning to create a repair template and a guide RNA expression vector for each gene to be targeted. Second, the efficiency of HDR typically is low, requiring the screening of large number of animals or the use of selection markers that are integrated alongside the desired edits (Dickinson *et al.* 2013, 2015).

Recently, we found in *Caenorhabditis elegans* that linear DNAs with homology arms as short as 35 bases can support the efficient incorporation of HDR edits (Paix *et al.* 2014). This finding simplifies the construction of donor templates.

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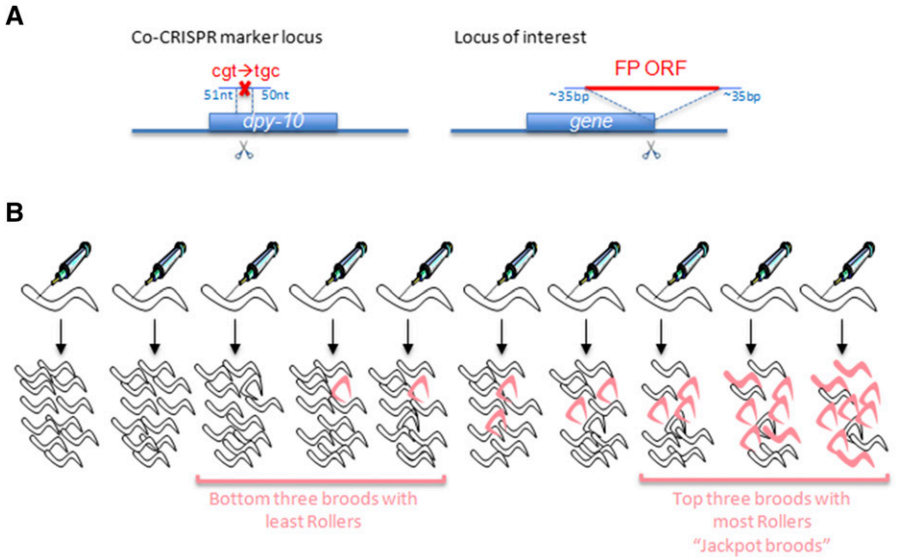

Figure 1 Co-CRISPR strategy. (A) We cotargeted (1) the *dpy-10* locus with a ssODN repair template to introduce a missense mutation leading to the dominant roller phenotype (Arribere *et al.* 2014) and (2) a second locus (gene of interest) with a PCR repair template to insert a fluorescent protein (FP) near the C-terminus. (B) Experimental outline. The gonads of 10–20 hermaphrodites are injected, and their broods are examined for the presence of rollers (*dpy-10* edits) and FP$^+$ animals. In typical experiments, >50% of hermaphrodites segregate rollers. Jackpot broods are the broods with the highest numbers of rollers. Edits at the gene of interest (pink) are found in both roller and nonroller worms, but only among broods that contain rollers.

For example, a donor template to introduce GFP can be synthesized using two 55-base oligos, each containing 35 bases that are homologous to the targeted locus and 20 bases that are homologous to GFP. The PCR amplicon is injected alongside plasmids coding for Cas9 and the sgRNA into the gonad of adult hermaphrodites, and their progeny are screened for GFP expression by visual inspection or by PCR screening. Using this method, we were able to recover GFP fusions at six of eight loci attempted. HDR frequencies were high enough to avoid the use of coselection makers but were still relatively low and variable (0.4–12%) (Paix *et al.* 2014). Other researchers also have reported difficulties in obtaining HDR edits, a problem that can be partially overcome by improvements in the design of guide RNAs and repair templates (Farboud and Meyer 2015; Katic *et al.* 2015).

Two additional factors are likely to also contribute to low edit frequency. First, the efficiency of expressing and assembling Cas9–sgRNA complexes from plasmids is not known and may be low given the tendency of *C. elegans* germ cells to silence foreign DNA (Kelly *et al.* 1997). In mouse embryos and mammalian cells, direct delivery of Cas9 complexes assembled *in vitro* has been reported to yield a higher frequency of edits (Lin *et al.* 2014; Aida *et al.* 2015; Liang *et al.* 2015). Injection of Cas9 complexes has been used in *C. elegans* to create edits by end-joining mechanisms (Cho *et al.* 2013) but has not yet been compared directly to plasmid delivery or used for HDR.

A second factor that contributes to the low edit frequency is that many injected hermaphrodites generate no edits. For reasons that remain unclear, we have found that edits tend to cluster among the progeny of a minority of injected mothers (jackpot broods) (Paix *et al.* 2014). Identification of jackpot broods before screening would eliminate the need to screen the broods of nonproductive hermaphrodites. Recently, three reports have described methods to enrich for desired edits by selecting or screening for editing at a second marker locus (Arribere *et al.* 2014; Kim *et al.* 2014; Ward 2015). In the method described by Arribere *et al.* (2014), a dominant mutation is introduced by HDR in the marker locus *dpy-10*, leading to a roller phenotype that is easily identified among the progeny of injected hermaphrodites.

Here we report the development of a new direct-delivery protocol that combines injection of *in vitro*–synthesized and –assembled Cas9-crRNA-tracrRNA complexes with the *dpy-10* co-CRISPR approach of Arribere *et al.* (2014) to identify jackpot broods. The direct-delivery protocol is entirely cloning-free and generates edits at frequencies ranging from 2 to 70% of F_1 progeny, a 10-fold improvement over our plasmid-based earlier method (Paix *et al.* 2014). This new protocol permits the use of inefficient sgRNAs that failed in our previous study and expands the repertoire of possible genome edits to include ORF swaps and fluorescent protein (FP) tagging of two genes at once.

Materials and Methods

Supplementary materials include protocols for direct-delivery editing (Supporting Information, File S1) and for Cas9 protein purification (File S2), as well as lists of plasmids (Table S2), crRNAs (Table S3), rescue templates (Table S4), and strains (Table S5).

Data availability

Strains and plasmids are available from the Caenorhabditis Genetics Center (CGC) and Addgene, or upon request.

Results and Discussion

Injection of Cas9 RNP complexes supports robust HDR

We developed a simple method to purify from *Escherichia coli* recombinant Cas9 [fused at its C-terminus with a nuclear localization sequence (Fu *et al.* 2014)] and assemble Cas9-crRNA-tracrRNA RNP complexes in the presence of repair templates and in a buffer suited for injection into *C. elegans*

Table 1 Optimization of Cas9, crRNA, and rescue template concentrations

Experiment number	Experiment	Insertion in *gtbp-1* (size)	Cas9 pmol/μl	Cas9 source	crRNA ratio (*gtbp-1*/*dpy-10*)	template ratio (*gtbp-1*/ *dpy-10*)	Percent P_0 with rollers (*n*)	Percent edits rollers (*n*)	Percent edits nonrollers (*n*)
AP80	Baseline	mCherry (700 bp)	20.6	Homemade	1 (15.7/15.7)	1.3 (0.56/0.43)	80% (15)	24.4% (131)	2.9% (134)
AP78	Increase crRNA ratio	mCherry (700 bp)	20.6	Homemade	2.5 (39.4/15.7)	1.3 (0.56/0.43)	50% (20)	51.9% (131)	17.8% (174)
AP111	Decrease Cas9 concentration	mCherry (700 bp)	4.1	Homemade	2.5 (39.4/15.7)	1.3 (0.57/0.43)	33% (18)	33.3% (12)	12.7% (212)
AP96	Increase template ratio	mCherry (700 bp)	15.5	Homemade	2.5 (29.6/11.8)	2.5 (1.13/0.44)	45% (11)	70.2% (84)	22.1% (122)
AP108	Lower crRNA concentrations	mCherry (700 bp)	15.5	Homemade	2.5 (11.1/4.4)	2.5 (1.13/0.44)	10% (10)	60% (10)	10% (40)
AP112	Plasmid delivery	mCherry (700 bp)	NA	Plasmid	2.5 (0.025/0.01)	1.3 (0.56/0.43)	11% (17)	83% (18)	14% (120)
AP60	Commercial Cas9	eGFP (800 bp)	4.1	Commercial	4.2 (39.4/9.4)	0.9 (0.40/0.43)	50% (30)	55% (89)	18.9% (37)
AP58	Commercial Cas9 + Increase template ratio	eGFP (800 bp)	4.1	Commercial	4.2 (39.4/9.4)	2.1 (0.91/0.43)	61.5% (25)	61.1% (54)	52.1% (46)

All experiments were conducted as described in Figure 1 by cotargeting *dpy-10* and *gtbp-1*. Concentrations are in picomoles per microliter. Percent P_0 with rollers, percent of injected hermaphrodites that segregated rollers (*n*= total number of injected hermaphrodites); Percent edits rollers, percent FP^+ edits at the *gtbp-1* locus among rollers from the top three broods with the most rollers (*n*= number of rollers screened for FP expression); Percent edits nonrollers, percent FP^+ edits at the *gtbp-1* locus among nonrollers from the top three broods with the most rollers (*n*= number of nonrollers screened for FP expression).

(File S1 and File S2). Cas9 also can be obtained from a commercial source, as in Cho *et al.* (2013). As in the co-CRISPR method of Arribere *et al.* (2014), we co-injected Cas9 complexes and repair templates targeting the marker gene *dpy-10* and a second target locus. *dpy-10* was repaired with a single-strand oligodeoxyribonucleotide (ssODN) that introduces a missense mutation in the *dpy-10* ORF (Arribere *et al.* 2014). The missense mutation causes a dominant roller phenotype that is easily spotted under a dissecting microscope. For the second locus, we first used *gtbp-1* (aka *K08F4.2*), a nonessential gene expressed in most tissues, which we targeted previously using plasmid delivery (Paix *et al.* 2014). We introduced a single cut near the C-terminus of *gtbp-1* and repaired the lesion with an ~700-base double-stranded PCR fragment containing the ORF for a fluorescent protein [red fluorescent protein (RFP) or GFP] flanked by 35-base arms that were homologous to sequences immediately surrounding the cut site (Figure 1).

For three experiments (Table 1 and Table S1, experiments AP58, AP60, and AP78), we examined the broods of all injected hermaphrodites by visual inspection for the presence of roller and fluorescent (FP^+) worms (Figure 2). We found that 50–61% of injected hermaphrodites segregated rollers (Table 1). Broods without rollers contained no FP^+ edits (experiment AP58: 0 of 62 F_1 progeny examined from five broods). Among broods with rollers, edits could be found in both rollers and their nonroller siblings (Table 1). For each experiment, we found that the frequency of FP^+ edits among the three broods that segregated the highest number of rollers was higher than that observed among the three broods with the lowest numbers of rollers (Figure 2), as also reported by Arribere *et al.* (2014). For simplicity, in subsequent experiments, we analyzed only the three broods with the highest number of rollers (jackpot broods) and ignored all other broods. We conclude that direct delivery of Cas9 RNP complexes supports robust HDR and, like plasmid delivery, generates jackpot broods that are easily identified using the *dpy-10* co-CRISPR marker (Arribere *et al.* 2014).

Increasing the target/marker ratio of crRNAs and repair templates increases HDR frequency at the target locus

When using equimolar amounts of crRNA and templates for *dpy-10* and *gtbp-1* (Table 1, experiment AP80), we obtained FP^+ edits at a higher frequency among rollers than among nonrollers (24 *vs.* 3%) (Table 1). For genes unlinked to *dpy-10*, edits of interest can be separated from the dominant *dpy-10(Rol)* edit by picking nonroller worms in the next generation. For genes linked to *dpy-10*, however, recovering the edit without the marker is more difficult, and it is therefore preferable to isolate the edit in nonroller F_1 progeny. We reasoned that increasing the levels of *gtbp-1* crRNA and template relative to that of *dpy-10* should increase the frequency of *gtbp-1* edits overall, including in the nonroller population. As expected, increasing the *gtbp-1*/*dpy-10* ratio of crRNA (experiments AP80 and AP78) and template (experiments AP78 and AP96) increased the number of GFP^+

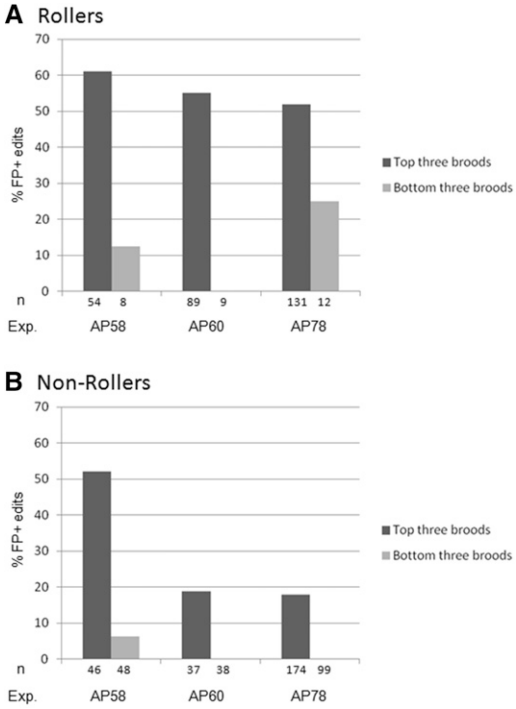

Figure 2 Jackpot broods. Broods with high numbers of *dpy-10* edits contain higher percentages of *gtbp-1* edits compared to broods with few *dpy-10* edits. For three separate experiments, we compared the frequency of FP$^+$ edits (insertion of GFP or RFP) at the *gtbp-1* locus among rollers (A) and nonrollers (B) derived from the top three broods with the highest numbers of rollers (jackpot broods) compared to the bottom three broods with the lowest numbers of rollers (as depicted in Figure 1). The frequency of *gtbp-1* edits is higher in the jackpot broods. For experiments AP58, AP60, and AP78, the top three jackpot broods contained 70.2 (33 of 47), 44.2 (49 of 111), and 71.6% (68 of 95) of all FP$^+$ edits, respectively. Numbers refer to the number of F_1 progeny screened.

edits (Table 1 and Table S1). We also found, however, that decreasing the concentration of *dpy-10* crRNA below 5 pmol/μl made it difficult to obtain enough rollers for analysis (experiment AP108). Similarly, the need to maintain relatively high overall concentrations of Cas9 complexes limits the amount of excess target template that can be added to the injection mix (see protocol in File S1 and Table 1, experiments AP78 and AP111). Under the best conditions tested (experiment AP58, high *gtbp-1/dpy-10* ratio for both the crRNAs and the repair templates), 61% of injected hermaphrodites segregated rollers, and the jackpot broods gave rise to 61% FP$^+$ edits among rollers and 52% FP$^+$ edits among nonrollers.

Direct injection of preassembled Cas9 RNP complexes generates more overall edits per injected hermaphrodite than plasmid delivery

The FP$^+$ edit frequencies shown in Table 1 are four- to five-fold higher than those obtained using plasmid delivery at the same locus without co-CRISPR (12%) (Paix *et al.* 2014) or at a different locus using co-CRISPR and longer homology arms (14%) (Arribere *et al.* 2014). To directly compare the efficiency of direct delivery *vs.* plasmid delivery, we injected plasmids expressing Cas9 and sgRNAs targeting *gtbp-1* and *dpy-10* with the same high *gtbp-1/dpy-10* ratio as in experiment AP78. Of 17 injected hermaphrodites, we obtained only two (11%) that segregated rollers, for a total of 18 rollers (Table 1, experiment AP112). This low roller frequency is consistent with that reported by Arribere *et al.* (2014). The frequency of FP$^+$ worms across the two roller broods, however, was high (83% among rollers, 14% among nonrollers). These findings confirm that plasmid delivery can generate jackpot broods with high edit frequencies. Direct delivery of preassembled Cas9 RNP complexes, however, yields a higher number of productive injections, *i.e.*, injected hermaphrodites that generate rollers (50 *vs.* 11%). In practice, this means that fewer hermaphrodites (∼10 instead of ∼50) need to be injected to obtain jackpot broods.

Application of the protocol to challenging edits

Low-efficiency sgRNAs: We reasoned that the higher efficiency of our protocol might make it possible to use low-efficiency sgRNAs. Previously, using plasmid delivery and no co-CRISPR, we failed to recover GFP edits at two loci, *glh-1* and *htp-3*, likely because the sgRNAs targeting those genes failed to support sufficient cutting to stimulate HDR (Paix *et al.* 2014). Both of these sgRNAs have a C at their 3′ ends, which is thought to be unfavorable (Doench *et al.* 2014). We retested the same sgRNAs and repair templates using complexes assembled *in vitro* at high target/*dpy-10* crRNA and template ratios. We obtained 47% edits for *glh-1* and 2% edits for *htp-3* (Table 2 and Table S1, experiments AP106 and AP114). A recent report has shown that guide RNAs that end in GG consistently yield good editing efficiencies in *C. elegans* (Farboud and Meyer 2015). We tested such a sgRNA at the *gtbp-1* locus and obtained 20% edits (Table 2, experiment AP92), a frequency comparable to that of the original sgRNA we used at that locus, which ends in a single G. We conclude that the higher efficiency of the new protocol expands the range of sgRNAs that can be used, although efficiencies still vary greatly (Table S3).

Insertion at a distance from Cas9-induced cleavage site: We previously noted that edits at a distance from the cleavage site (>10 bases) are incorporated less efficiently than edits closer to the cleavage site (Paix *et al.* 2014). We found that this observation still holds using the new protocol. We obtained 6% edits when inserting GFP in *gtbp-1* 28 bases away from the cleavage site (Table 2, experiment AP83) compared to 51% edits when inserted directly at the cleavage site under the same crRNA and template ratios (Table 1, experiment AP78). A low edit frequency also was observed when using a ssODN to insert a small tag at the same position away from the cleavage site (Table 2, experiment AP82). Increasing the ssODN concentration improved efficiency modestly (experiment AP95, 2 *vs.* 8.8%). We conclude that edits away from the cleavage site are incorporated less efficiently than edits close to the cleavage site, as reported previously

Table 2 Protocol performance with different types of edits

Experiment number	Edit	Target locus	Recipient strain	Size of insertion	Distance from cut	crRNA ratio (gof/*dpy-10*)	Template ratio (gof/*dpy-10*)	Percent edits rollers (*n*)	Percent edits nonrollers (n)
AP106	RFP insertion with low-efficiency guide RNA	*glh-1*	*wt*	750 bp	6	2.5 (29.6/11.8)	1.8 (0.83/0.44)	47.5% (101)	16.6% (120)
AP114	GFP insertion with low-efficiency guide RNA	*htp-3*	*wt*	900 bp	8	2.5 (29.6/11.8)	1.5 (0.70/0.44)	2% (198)	0.7% (282)
AP92	RFP insertion with GG guide RNA	*gtbp-1*	*wt*	700 bp	1	2.5 (39.4/15.7)	1.3 (0.56/0.43)	20.2% (69)	15.8% (126)
AP83	GFP insertion at distance from cut	*gtbp-1*	*wt*	800 bp	28	2.5 (39.4/15.7)	1.1 (0.48/0.43)	6.0% (82)	3.9% (76)
AP82	Ollas insertion at distance from cut	*gtbp-1*	*wt*	27 nt	28	2.5 (39.4/15.7)	1.1 (0.51/0.43)	2.1% (47)	0% (47)
AP95	Ollas insertion at distance from cut + increase ssODN template	*gtbp-1*	*wt*	27 nt	28	2.5 (39.4/15.7)	6 (2.58/0.43)	8.8% (34)	0% (34)
AP103	Tagging two genes at once (GFP/RFP)	*gtbp-1 and fbf-2*	*wt*	750 and 750 bp	1 and 1	1.8 (14.8/8.1) + 3.6 (29.6/8.1)	0.4 (0.20/0.44) + 0.9 (0.41/0.44)	28% (57)	7.1% (223)
AP115	Replacement of *gtbp-1* ORF with GFP::H2B	*gtbp-1*	*wt*	1300 bp	1 and 0	1.8 (22.2/11.8) + 1.8 (22.2/11.8)	0.9 (0.41/0.44)	58.1% (74)	7.5% (120)
AP105	Replacement of GFP with RFP	*gtbp-1*	*gtbp-1* ::*eGFP* ::*3Xflag*	750 bp	0 and 1	1.8 (22.2/11.8) + 1.8 (22.2/11.8)	1.3 (0.59/0.44)	26.5% (79)	2.7% (144)
AP105D	Replacement of GFP with RFP	*deps-1*	*deps-1* ::*GFP*	750 bp	0 and 1	1.8 (22.2/11.8) + 1.8 (22.2/11.8)	1.3 (0.59/0.44)	21.1% (52)	5.9% (135)

All experiments were conducted as described in Figure 1 by cotargeting *dpy-10* and one (or two) target loci as indicated. Concentrations are in picomoles per microliter. gof, gene of interest; Percent edits rollers, % FP^+ edits at the target locus among rollers in the top three broods with the most rollers (*n*= number of rollers screened); Percent edits nonrollers: % FP^+ edits at the target locus among nonrollers in the top three broods with the most rollers (*n*= number of nonrollers screened).

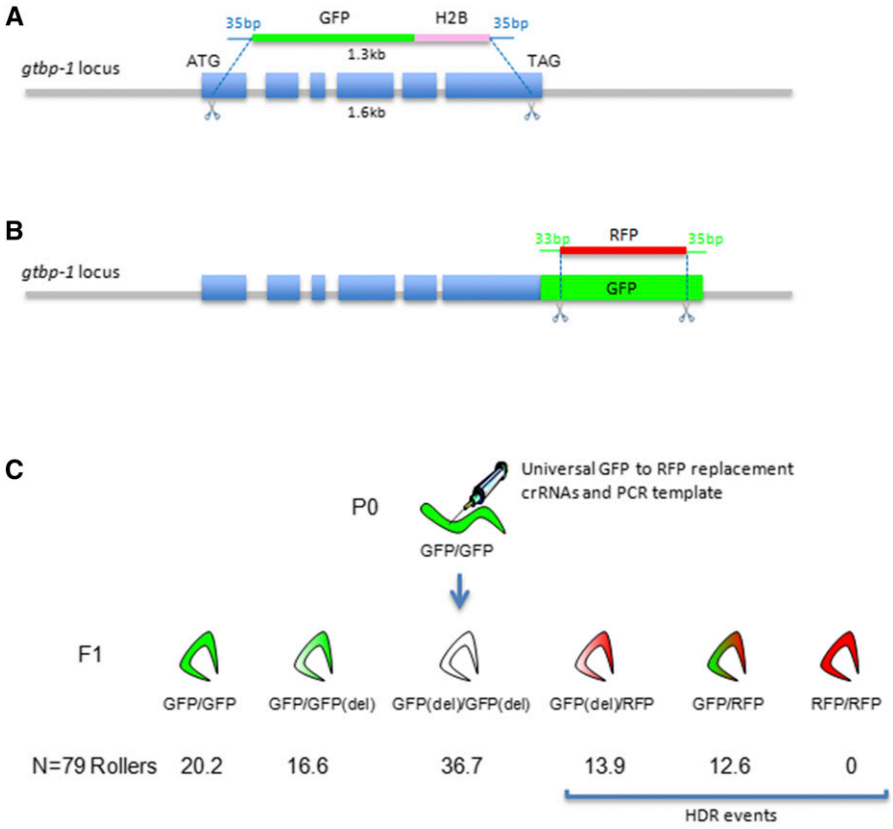

Figure 3 Gene replacement strategy. (A) Replacement of the *gtbp-1* ORF with GFP::H2B. Two cuts were made at either end of the *gtbp-1* ORF and repaired using a PCR template containing GFP::H2B flanked by 35 bases that were homologous to the 5′ and 3′ ends of *gtbp-1*. (B) Replacement of GFP with RFP. Two cuts were made in GFP and repaired using a PCR template containing RFP flanked by 33 and 35 bases that were homologous to the 5′ and 3′ ends of GFP, respectively. (C) Experimental results for replacing GFP with RFP at the *gtbp-1* locus. The percentages of each genotype among roller F_1 progeny are indicated.

(Arribere *et al.* 2014; Paix *et al.* 2014). This inefficiency can be compensated partially by increasing the template concentration in the injection mix.

Tagging two genes at once: We tested whether the new protocol could be used to recover edits at two target loci simultaneously (in addition to the marker locus *dpy-10*). We targeted *gtbp-1* and *fbf-2* for GFP and RFP insertion, respectively. We obtained 28% GFP/RFP double edits among rollers and 7% GFP/RFP double edits among nonrollers (Table 2, experiment AP103). The frequency of GFP edits overall (63%) exceeded that of RFP edits (35%) almost by a factor of 2 (Table S1), possibly as a result of higher efficiency of the sgRNA targeting *gtbp-1* compared to *fbf-2*, as also seen when these sgRNAs were used separately (Paix *et al.* 2014). We conclude that protein delivery can support robust HDR at three loci simultaneously (including *dpy-10*) even when using sgRNAs with different editing efficiencies.

ORF replacement: We showed previously that a repair ssODN could be used to insert a restriction site at the junction of a deletion generated by two cuts. To test whether a similar approach could be used to replace an entire ORF with another, we first attempted to replace the *gtbp-1* ORF with a histone H2B::GFP fusion. We recovered 58% replacements among rollers and 7.5% replacements among nonrollers (Table 2, experiment AP115). Next, we attempted to replace GFP with RFP at two loci previously tagged with GFP. We used a pair of crRNAs that cut at the 5′ and 3′ ends of GFP and an RFP PCR repair template with 35-base homology arms directly flanking the cleavage sites. We obtained the desired GFP-to-RFP swap in 26% (*gtbp-1* locus) and 21% (*deps-1* locus) of rollers. Interestingly, we also recovered a significant number of rollers that were GFP^- and RFP^-, indicating a high degree of cutting in the GFP target without HDR (Figure 3 and Table S1). Because both maternal and paternal GFP copies were affected, these observations indicate that Cas9 complexes injected in the maternal germline persist into zygotes, where they can edit the paternal genome, as also reported by Cho *et al.* (2013). Consistent with these observations, in addition to rollers, in all our experiments we also obtained dumpy worms, which result from editing both copies of the *dpy-10* locus (Arribere *et al.* 2014).

Conclusions

We report that injection in the *C. elegans* gonad of repair templates and preassembled Cas9-crRNA-tracrRNA complexes generates HDR edits with high efficiency (~50% of injected hermaphrodites generate edits). Direct delivery allows us to take full advantage of the benefits of the co-CRISPR approach of Arribere *et al.* (2014), which provides a visual marker to identify broods with edits. By optimizing the ratio of Cas9 complexes (and repair templates) that target the gene of interest compared to the *dpy-10* marker, we found that it is possible to obtain both a high yield of *dpy-10*-edited progeny and a high yield of *dpy-10* edits that are also

edited at the gene of interest (coedits, as high as 70%). Among broods that segregate the highest numbers of *dpy-10* edits (jackpot broods), edits at the gene of interest also can be found in non-*dpy-10*-edited siblings (as high as 52%). The ability to recover edits in unmarked animals is particularly useful for linked loci that cannot be separated easily from the dominant *dpy-10* marker in the next generation. Our findings also demonstrate that the co-CRISPR strategy works efficiently even when using different types of templates to repair the marker locus (ssODN to generate a point mutation) and the gene of interest (PCR amplicon to insert a fluorescent protein). The efficiency of editing, however, still remains variable from locus to locus, likely owing to differences in sgRNA efficiency and possibly differences in locus competency for HDR. We also continue to find that edits at a distance from the Cas9 cleavage site (>10 bases) are incorporated less efficiently than edits closer to the cleavage site, as reported previously (Arribere *et al.* 2014; Paix *et al.* 2014). Although protospacer adjacent motifs (PAMs) can be found within 30 bases of most edit sites, in cases where no PAMs are available, it may be preferable to use templates with longer homology arms and selection markers, as in Dickinson *et al.* (2015).

The efficiency of our direct-delivery protocol translates into several practical advantages: workload is reduced at both the injection (10–20 injected hermaphrodites *vs.* 50) and screening (<50 F_1 progeny compared to hundreds) steps, low-efficiency sgRNAs can be used, and complex edits, including gene replacements and multigene edits, are possible. Replacement of an ORF with GFP, as we report here for the *gtbp-1* locus, is an effective method to generate marked null alleles that can be maintained using the linked fluorescent marker. We have also developed universal crRNAs and rescue templates to convert GFP fusions to RFP fusions (File S1). Another advantage of direct delivery is that the entire protocol requires no cloning: tracrRNA, crRNAs, and ssODN repair templates for short edits (<50 bases) are all synthesized chemically, and longer repair templates are made by PCR using oligonucleotides to code for the short (35-base) homology arms. In principle, direct delivery of Cas9 RNP complexes could be combined with other CRISPR protocols (Dickinson *et al.* 2015; Ward 2015) and also should be advantageous for nematodes where promoters to drive expression of Cas9 and the sgRNAs are not readily available (Chiu *et al.* 2013; Witte *et al.* 2015).

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Literature Cited

Aida, T., K. Chiyo, T. Usami, H. Ishikubo, R. Imahashi *et al.*, 2015 Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. Genome Biol. 16: 87.

Arribere, J. A., R. T. Bell, B. X. Fu, K. L. Artiles, P. S. Hartman *et al.*, 2014 Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in Caenorhabditis elegans. Genetics 198: 837–846.

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

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

Deltcheva, E., K. Chylinski, C. M. Sharma, K. Gonzales, Y. Chao *et al.*, 2011 CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471: 602–607.

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

Dickinson, D. J., A. M. Pani, J. K. Heppert, C. D. Higgins, and B. Goldstein, 2015 Streamlined genome engineering with a self-excising drug selection cassette. Genetics 115.178335.

Doench, J. G., E. Hartenian, D. B. Graham, Z. Tothova, M. Hegde *et al.*, 2014 Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nat. Biotechnol. 32: 1262–1267.

Doudna, J. A., and E. Charpentier, 2014 Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346: 1258096.

Farboud, B., and B. J. Meyer, 2015 Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. Genetics 199: 959–971.

Fu, B. X., L. L. Hansen, K. L. Artiles, M. L. Nonet, and A. Z. Fire, 2014 Landscape of target:guide homology effects on Cas9-mediated cleavage. Nucleic Acids Res. 42: 13778–13787.

Hsu, P. D., E. S. Lander, and F. Zhang, 2014 Development and applications of CRISPR-Cas9 for genome engineering. Cell 157: 1262–1278.

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

Katic, I., L. Xu, and R. Ciosk, 2015 CRISPR/Cas9 genome editing in Caenorhabditis elegans: evaluation of templates for homology-mediated repair and knock-ins by homology-independent DNA repair. G3 115.019273.

Kelly, W. G., S. Xu, M. K. Montgomery, and A. Fire, 1997 Distinct requirements for somatic and germline expression of a generally expressed Caernorhabditis elegans gene. Genetics 146: 227–238.

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

Liang, X., J. Potter, S. Kumar, Y. Zou, R. Quintanilla *et al.*, 2015 Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. J. Biotechnol. 208: 44–53.

Lin, S., B. T. Staahl, R. K. Alla, and J. A. Doudna, 2014 Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. eLife 3: e04766.

Paix, A., Y. Wang, H. E. Smith, C. Y. Lee, D. Calidas *et al.*, 2014 Scalable and versatile genome editing using linear DNAs with microhomology to Cas9 Sites in Caenorhabditis elegans. Genetics 198: 1347–1356.

Shah, A. N., C. F. Davey, A. C. Whitebirch, A. C. Miller, and C. B. Moens, 2015 Rapid reverse genetic screening using CRISPR in zebrafish. Nat. Methods 12: 535–540.

van Schendel, R., S. F. Roerink, V. Portegijs, S. van den Heuvel, and M. Tijsterman, 2015 Polymerase theta is a key driver of genome evolution and of CRISPR/Cas9-mediated mutagenesis. Nat. Commun. 6: 7394.

Ward, J. D., 2015 Rapid and precise engineering of the Caenorhabditis elegans genome with lethal mutation co-conversion and inactivation of NHEJ repair. Genetics 199: 363–377.

Witte, H., E. Moreno, C. Rodelsperger, J. Kim, J. S. Kim *et al.*, 2015 Gene inactivation using the CRISPR/Cas9 system in the nematode Pristionchus pacificus. Dev. Genes Evol. 225: 55–62.

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High Efficiency, Homology-Directed Genome Editing in *Caenorhabditis elegans* Using CRISPR-Cas9 Ribonucleoprotein Complexes

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Table S1: Summary of all results

Exp	Experiment purpose	Injection Volume (ul)	Gene	Background	Editing	crRNA	Distance Cut/insertion	crRNA mutation	Repair template (type, size)	% P0 giving Roller at day2 (total nd of P0s transferred at the end of day1)	nb of Roller per total P0s at day2	Cas9 source	Cas9 (pmol/ul)	Tracer RNA (pmol/ul)	Targeted gene/*dpy-10* template ratio	template (pmol/ul)	*dpy-10* ssODN (pmol/ul)	Targeted gene/*dpy-10* crRNA ratio	*dpy-10* crRNA (pmol/ul)	Targeted gene crRNA (pmol/ul)	Buffer	day2: % edit among Roller (average 2 best P0s)	day2: % edit among non *dpy-10* edited worms (average 2 best P0s)	day2: % edit among Roller (average 3 best P0s)	day2: % edit among non *dpy-10* edited worms (average 3 best P0s)	Note
AP58	Commercial Cas9 + Increase template ratio	15	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, eGFP::3Xflag (800bp)	61.5 (8/13)	5.8 (76/13)	Commercial	4.1	56	2.1 (0.91/0.43)	0.91	0.43	4.2 (39.4/9.4)	9.4	39.4	100mM KCl, 13.3mM HEPES, 2.4mM Tris, 0.6% Sucrose, pH7.5-8	58.9% (23/39)	56.2% (18/32)	61.1% (33/54)	52.1% (24/46)	See Figure 2, P0s with non *dpy-10* edited broods do not give edit
AP60	Commercial Cas9	15	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, eGFP::3Xflag (800bp)	50 (15/30)	7.4 (223/30)	Commercial	4.1	56	0.9 (0.40/0.43)	0.40	0.43	4.2 (39.4/9.4)	9.4	39.4	100mM KCl, 13.3mM HEPES, 2.4mM Tris, 0.6% Sucrose, pH7.5-8	as best 3 P0s	as best 3 P0s	55% (49/89)	18.9% (7/37)	See Figure 2
AP78	Increase crRNA ratio	15	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, mCherry (700bp)	50 (10/20)	10.6 (212/20)	Homemade	20.6	56	1.3 (0.56/0.43)	0.56	0.43	2.5 (39.4/15.7)	15.7	39.4	166.6mM KCl, 6.6mM HEPES, 2.1mM Tris, 6.6% Glycerol, pH7.5-8	43.7% (42/96)	18.4% (21/114)	51.9% (68/131)	17.8% (31/174)	See Figure 2. With our home-made Cas9, we need to inject Cas9 protein at an higher concentration than for the commercial Cas9 in order to obtain the same efficiency (Files S1 and S2 and APS8/60/111).
AP80	Baseline	15	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, mCherry (700bp)	80 (12/15)	12.4 (186/15)	Homemade	20.6	31.8	1.3 (0.56/0.43)	0.56	0.43	1 (15.7/15.7)	15.7	15.7	166.6mM KCl, 6.6mM HEPES, 1.2mM Tris, 6.6% Glycerol, pH7.5-8	30.7% (32/104)	4.4% (4/90)	24.4% (32/131)	2.9% (4/134)	no
AP82	Ollas insertion at distance from cut	15	*gtbp-1*	*wt*	tagging at Ct	728	28	crRNA sequence mutations	ssODN, Ollas (27nt)	33.3 (4/12)	15.5 (186/12)	Homemade	20.6	56	1.1 (0.51/0.43)	0.51	0.43	2.5 (39.4/15.7)	15.7	39.4	166.6mM KCl, 6.6mM HEPES, 2.1mM Tris, 6.6% Glycerol, pH7.5-8	2.3% (1/42)	0% (0/42)	2.1% (1/47)	0% (0/47)	Some deletion (>20bp) could be detected: 4.2% (2/47) for the 3 best P0s
AP83	GFP insertion at distance from cut	15	*gtbp-1*	*wt*	tagging at Ct	728	28	crRNA sequence mutations	PCR, mTagGFP::Tev ::3Xflag (800bp)	53.3 (7/12)	9 (108/12)	Homemade	20.6	56	1.1 (0.48/0.43)	0.48	0.43	2.5 (39.4/15.7)	15.7	39.4	166.6mM KCl, 6.6mM HEPES, 2.1mM Tris, 6.6% Glycerol, pH7.5-8	7.8% (5/64)	4.1% (3/73)	6.0% (5/82)	3.9% (3/76)	no
AP92	RFP insertion with GG guide RNA	15	*gtbp-1*	*wt*	tagging near Ct	772	1	crRNA sequence disruption	PCR, mCherry (700bp)	53.3 (7/12)	8.9 (107/12)	Homemade	20.6	56	1.3 (0.56/0.43)	0.56	0.43	2.5 (39.4/15.7)	15.7	39.4	166.6mM KCl, 6.6mM HEPES, 2.1mM Tris, 6.6% Glycerol, pH7.5-8	22.4% (11/49)	17.2% (14/81)	20.2% (14/69)	15.8% (20/126)	no
AP95	Ollas insertion at distance from cut + increase ssODN	15	*gtbp-1*	*wt*	tagging at Ct	728	28	crRNA sequence mutations	ssODN, Ollas (27nt)	45.4 (5/11)	4.2 (47/11)	Homemade	20.6	56	6 (2.58/0.43)	2.58	0.43	2.5 (39.4/15.7)	15.7	39.4	166.6mM KCl, 6.6mM HEPES, 2.1mM Tris, 6.6% Glycerol, pH7.5-8	10.7% (3/28)	0% (0/28)	8.8% (3/34)	0% (0/34)	No deletion (>20bp) could be detected
AP96	Increase template ratio	20	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, mCherry (700bp)	45.4 (5/11)	9.6 (106/11)	Homemade	15.5	42	2.5 (1.13/0.44)	1.13	0.44	2.5 (29.6/11.8)	11.8	29.6	150mM KCl, 20mM HEPES, 1.6mM Tris, 5% Glycerol, pH7.5-8	69.4% (41/59)	19.4% (15/77)	70.2% (59/84)	22.1% (27/122)	no
AP103	Tagging two genes at once (GFP/RFP)	20	*gtbp-1* (ChrS) and *fbf-2* (Chr2)	*wt*	tagging near and at Ct	728 and 689	1 and 1	crRNA sequence disruption	PCR, eGFP (750bp) and tagRFP (750bp)	33.3 (3/9)	6.3 (57/9)	Homemade	15.5	52.5	0.4 (0.20/0.44) + 0.9 (0.41/0.44)	0.20+0.41= 0.61	0.44	1.8 (14.8/8.1) + 3.6 (29.6/8.1)	8.1	14.8+29.6= 44.4	150mM KCl, 20mM HEPES, 2.0mM Tris, 5% Glycerol, pH7.5-8	29% (16/55) for only GFP+/RFP+, 36.3% (20/55) for GFP+/RFP-, 7.2% (4/55) for GFP+/RFP-	8.6% (16/186) for GFP+/RFP+, 4.3% (8/186) for GFP+/RFP-, 0.5% (1/186) for GFP-/RFP+	28% (16/57) for GFP+/RFP+, 35% (20/57) for GFP+/RFP-, 7% (4/57) for GFP-/RFP+	7.1% (16/223) for GFP+/RFP+, 3.5% (8/223) for GFP+/RFP-, 0.4% (1/223) for GFP-/RFP+	For the 3 best P0s: 44.4% (16/36) of GFP+ are also RFP+ among Roller and 66.6% (16/24) among non *dpy-10* edited. The total % of GFP edits is 63.1% (36/57) among Roller and 10.7% (24/223) among non dpy-10 edited worms. The total % of RFP edits is 35% (20/57) among Roller and 7.6% (17/223) among non dpy-10 edited worms
AP105	Replacement of GFP with RFP	20	*gtbp-1*	*gtbp-1 ::eGFP::3Xflag*	eGFP replacement	719 and 720	0 and 1	crRNA sequence disruption	PCR, tagRFP (750bp)	52.9 (9/17)	7.9 (135/17)	Homemade	15.5	56.2	1.3 (0.59/0.44)	0.59	0.44	1.8 (22.2/11.8) + 1.8 (22.2/11.8)	11.8	22.2+22.2= 44.4	150mM KCl, 20mM HEPES, 2.15mM Tris, 5% Glycerol, pH7.5-8	34.4% (20/58) for GFP+/RFP+ or GFP-/RFP+, 34.4% (20/58) for GFP-/GFP-	3.1% (3/96) for GFP+/RFP+ or GFP-/RFP+, 6.2% (6/96) for GFP-/GFP-	26.5% (21/79) for GFP+/RFP+ or GFP-/RFP+, 36.7% (29/79) for GFP-/GFP-	2.7% (4/144) for GFP+/RFP+ or GFP-/RFP+, 5.5% (8/144) for GFP-/GFP-	See Figure 3
AP105D	Replacement of GFP with RFP	20	*deps-1*	*deps-1 ::GFP*	GFP replacement	719 and 720	0 and 1	crRNA sequence disruption	PCR, tagRFP (750bp)	46.1 (6/13)	4.4 (58/13)	Homemade	15.5	56.2	1.3 (0.59/0.44)	0.59	0.44	1.8 (22.2/11.8) + 1.8 (22.2/11.8)	11.8	22.2+22.2= 44.4	150mM KCl, 20mM HEPES, 2.15mM Tris, 5% Glycerol, pH7.5-8	26.3% (10/38) for GFP+/RFP+ or GFP-/RFP+	5% (4/80) for GFP+/RFP+ or GFP-/RFP+	21.1% (11/52) for GFP+/RFP+ or GFP-/RFP+	5.9% (8/135) for GFP+/RFP+ or GFP-/RFP+	Injection after AP105 with same mix/needle
AP106	RFP insertion with low efficiency guide RNA	20	*glh-1*	*wt*	tagging at Ct	723	6	crRNA sequence mutations	PCR, tagRFP (750bp)	36.3 (8/22)	6.5 (145/22)	Homemade	15.5	42	1.8 (0.83/0.44)	0.83	0.44	2.5 (29.6/11.8)	11.8	29.6	150mM KCl, 20mM HEPES, 1.6mM Tris, 5% Glycerol, pH7.5-8	36.4% (27/74)	3.7% (3/80)	47.5% (48/101)	16.6% (20/120)	Using a plasmid approach in Paix et al, 2014; no FP+ edit of *glh-1* could be recovered from a total 402 F1s screened (from 5 P0s examined from a total of 23 P0s injected).
AP108	Lower crRNA concentrations	20	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, mCherry (700bp)	10 (1/10)	1 (10/10)	Homemade	15.5	15.5	2.5 (1.13/0.44)	1.13	0.44	2.5 (11.1/4.4)	4.4	11.1	150mM KCl, 20mM HEPES, 0.7mM Tris, 5% Glycerol, pH7.5-8	60% (6/10)	10% (4/40)	as 2 best P0s	as 2 best P0s	Using a plasmid approach in Paix et al, 2014; no FP+ edit of *htp-3* could be recovered from a total 84 F1s screened (from 1 P0 examined from a total of 30 P0s injected).
AP111	Decrease Cas9 concentration	15	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, mCherry (700bp)	33.3 (6/18)	0.7 (14/18)	Homemade	4.1	56	1.3 (0.57/0.43)	0.57	0.43	2.5 (39.4/15.7)	15.7	39.4	166.6mM KCl, 6.6mM HEPES, 2.1mM Tris, 6.6% Glycerol, pH7.5-8	37.5% (3/8)	15.8% (19/120)	33.3% (4/12)	12.7% (27/212)	no
AP112	Plasmid delivery	15	*gtbp-1*	*wt*	tagging near Ct	728	1	crRNA sequence disruption	PCR, mCherry (700bp)	11.7 (2/17)	1 (18/17)	Plasmid	0.035 (175ng/ul total of plasmid)	NA	1.3 (0.56/0.43)	0.56	0.43	2.5 (0.025/0.010) (50/125 ng/ul)	0.010	0.025	H2O	83.3% (15/18)	14.1% (17/120)	as 2 best P0s	as 2 best P0s	*dpy-10* crRNA target sequence and *gtbp-1* crRNA target sequence were cloned in pDD162 (Peft-3::Cas9 + Empty sgRNA). See Table S2
AP114	GFP insertion with low efficiency guide RNA	20	*htp-3*	*wt*	tagging at Ct	699	8	crRNA sequence disruption	PCR, TEV::egfp::myc ::3Xflag (900bp)	72% (18/25)	12.8 (322/25)	Homemade	15.5	42	1.5 (0.70/0.44)	0.70	0.44	2.5 (29.6/11.8)	11.8	29.6	150mM KCl, 20mM HEPES, 1.6mM Tris, 5% Glycerol, pH7.5-8	2.2% (3/132)	0.7% (1/137)	2.0% (4/198)	0.7% (2/282)	no
AP115	Replacement of *gtbp-1* ORF with GFP::H2B	20	*gtbp-1*	*wt*	Gene replacement	691 and 728	1 and 0	crRNA sequence disruption	PCR, GFP::H2B (1300bp)	36.3% (8/22)	5.1 (113/22)	Homemade	15.5	56.2	0.9 (0.41/0.44)	0.41	0.44	1.8 (22.2/11.8) + 1.8 (22.2/11.8)	11.8	22.2+22.2= 44.4	150mM KCl, 20mM HEPES, 2.15mM Tris, 5% Glycerol, pH7.5-8	58.1% (43/74)	7.5% (9/120)	as 2 best P0s	as 2 best P0s	no

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

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::NLS$_{SV40}$::His6	see Fu *et al.* 2014

Table S3: List of crRNAs

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 (% Roller, 3 best P0s)
589	*dpy-10*	gctaccataggcaccacgag	in ORF	60	yes	no	AS	All	NA
728	*gtbp-1*	ccacgaggtggtatgcgcag	near STOP	65	yes	no	S	AP80/78/111/96/108/60/58/83/82/95/103/115	51.9% (AP78) and 70.2% (AP96)
723	*glh-1*	tccctcaagatgaagaaggc	near STOP	50	no	yes	S	AP106	47.5%
699	*htp-3*	agaggaaactgaacgatttc	at STOP	40	no	yes	S	AP114	2.0%
691	*gtbp-1*	ggccttaacccagaataaga	near ATG	45	no	no	S	AP115	58.1% (crRNAs 691 and 728 were used for gene replacement)
719	*gfp/egfp*	caaactcaagaaggaccatg	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*	ccatctaattcaacaagaat	near 5' end of gfp/egfp	30	no	no	AS	AP105/105D	
689	*fbf-2*	ggtagtcacggcgatgatta	at STOP	50	no	no	S	AP103	35.0% (was performed with *gtbp-1* co-edition)
772	*gtbp-1*	tcgggtggtgctccacgagg	near STOP	70	yes (GG)	no	S	AP92	20.2%

Table S4: List of rescue templates

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	GGCGGTTCGGGTGGTGCTCCACGAGGAGGAATGCGTAG**CGG**TTTCCAAAATGCGGGAC AAAAT---Ollas---tagaagctttccgttctcctttttccttcttgtaag
gtbp-1	728	mTagGFP::TEV::3Xflag insertion at the STOP codon	GGCGGTTCGGGTGGTGCTCCACGAGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGAC AAAAT---mTagGFP::TEV::3Xflag---tagaagctttccgttctcctttttccttcttgtaag
gtbp-1	728	mCherry insertion at cut	GGTTCGGGTGGTGCTCCACGAGGTGGTATGCGC---mCherry--- AGCGGTTTCCAAAATGCGGGACAAAATtagaag
gtbp-1	772	mCherry insertion at cut	CTTCCGCCGTGGCGGTTCGGGTGGTGCTCCACGA---mCherry--- GG**TGG**TATGCGCAGCGGTTTCCAAAATGCGGGAC
gtbp-1	728	eGFP insertion at cut	GGTTCGGGTGGTGCTCCACGAGGTGGTATGCGC---eGFP--- AGCGGTTTCCAAAATGCGGGACAAAATtagaag
fbf-2	689	tagRFP insertion at cut (STOP codon)	CAAATTTTCTTCTTCCGGTAGTCACGGCGATGAT---tagRFP--- ta**agg**tggaactttctcaccataaatctcatcc
GFP/eGFP	719 and 720	Replacement by tagRFP	GGAGAAGAACTTTTCACTGGAGTTGTC**CCA**ATT---tagRFP--- GTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTAC
glh-1	723	tagRFP insertion at the STOP codon	CCAGTGTACCAACTCAAGTCCCTCAAGACGAGGAAGGA**TGG**---tagRFP--- tagaaaaccgaccaattgatagtgtttcgcatttattaatgc
htp-3	699	TEV::eGFP::myc::3Xflag insertion at the STOP codon	CCAGTCGCCGAACATGCCAAGTAGAAGAGGAAAC---TEV::eGFP::myc::3Xflag--- tgaacgatttc**tgg**acaatcgtgtacaattatc
gtbp-1	691 and 728	*gtbp-1* replacement by GFP::H2B	aaATGCAGATAACCTCGGCCTTAACCCAGAATAAG---GFP::H2B--- taacag**cgg**tttccaaaatgcgggacaaaattagaagc

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).

Table S5: List of strains

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* and *fbf-2*	728 and 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):
AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU

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/rnai/public/analysis-tools/sgrna-design) (Doench *et al*. 2014).

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

Also order the crRNA for *dpy-10*: GCUACCAUAGGCACCACGAG + GUUUUAGAGCUAUGCUGUUUUG

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 with 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' en 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^{o}C and 25^{o}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): CCAUCUAAUUCAACAAGAAU + GUUUUAGAGCUAUGCUGUUUUG

crRNA GFP Ct (#719 in Table S3): CAAACUCAAGAAGGACCAUG + GUUUUAGAGCUAUGCUGUUUUG

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

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

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 gtbp-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): CCACGAGGUGGUAUGCGCAG + 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: cttctaattttgtcccgcattttggaaaccgctCTTGTACAGCTCGTCCATGCC

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: cttctaattttgtcccgcattttggaaaccgctCTTATACAATTCATCCATGCC

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::NLS_{SV40}::His_6:

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 OD_{600}=~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).

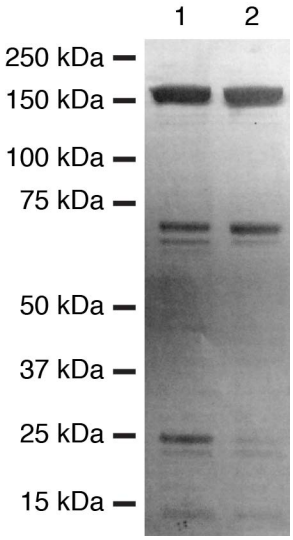
12. Flow eluent (from step 11) over Q Sepharose column. Collect flow-through and dialyze into 1L Buffer D for 5 hours at 4°C. Transfer into 1L Buffer D and dialyze overnight.

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_{SV40}::His_6 resolved by SDS-PAGE:



Recombinant Cas9::NLS_{SV40}::His_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).