

1 **Genome Editing in *Caenorhabditis briggsae* using the CRISPR/Cas9 System**

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3 Elizabeth Culp<sup>1</sup>, Cory Richman<sup>1</sup>, Devika Sharanya and Bhagwati P Gupta\*

4 Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON L8S-4K1,  
5 Canada

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8 <sup>1</sup>Co-first authors

9 \*Corresponding author. Email: [guptab@mcmaster.ca](mailto:guptab@mcmaster.ca), Phone: 905-525-9140 x26451

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12

## 1 **Author Summary**

2 The CRISPR/Cas9 system has recently emerged as a powerful tool to engineer the genome of an  
3 organism. The system is adopted from bacteria where it confers immunity against invading  
4 foreign DNA. This work reports the first successful use of the CRISPR/Cas9 system in *C.*  
5 *briggsae*, a cousin of the well-known nematode *C. elegans*. We used two plasmids, one  
6 expressing Cas9 endonuclease and the other an engineered CRISPR RNA corresponding to the  
7 DNA sequence to be cleaved. Our approach allows for the generation of loss-of-function  
8 mutations in *C. briggsae* genes thereby facilitating a comparative study of gene function between  
9 nematodes.

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11

## 12 **Abstract**

13 The CRISPR/Cas9 system is an efficient technique for generating targeted alterations in an  
14 organism's genome. Here we describe a methodology for using the CRISPR/Cas9 system to  
15 generate mutations via non-homologous end joining in the nematode *Caenorhabditis briggsae*, a  
16 sister species of *C. elegans*. Evidence for somatic mutations and off-target mutations are also  
17 reported. The use of the CRISPR/Cas9 system in *C. briggsae* will greatly facilitate comparative  
18 studies to *C. elegans*.

1 Linking genotype and phenotype is an important step in the characterization of a gene. Targeted  
2 genome editing, defined as the creation of alterations at specific sites in an organism's genome, is  
3 a powerful means to study the relationship between gene and phenotype. Genome editing  
4 techniques are based on guiding an endonuclease to a specific target in the genome in order to  
5 generate a double strand break (DSB) [1-3]. Breaks are subsequently repaired by either error  
6 prone non-homologous end joining (NHEJ) or template-directed homologous recombination  
7 (HR) [4]. While the former introduces random mutations at the point of cleavage, the latter can  
8 be used to generate specific alterations based on the presence of a donor sequence. Although  
9 several technologies currently exist for genome editing, such as zinc finger nucleases (ZFN) and  
10 transcription activator-like effector nucleases (TALEN), these techniques leave room for  
11 improvement in their ease of use, as each new sequence to be targeted requires the labor intensive  
12 process of generating a new protein construct [2].

13 Clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-  
14 associated (Cas) systems are adaptive immune mechanisms evolved by archaea and bacteria to  
15 defend against foreign plasmids and viral DNA [5]. Manipulation of the *Streptococcus pyrogenes*  
16 type II CRISPR/Cas system has been used to develop an efficient genome editing technique.  
17 First, a 20 bp sequence in a gene of interest is selected to act as a guide for the *S. pyrogenes*  
18 nuclease, Cas9. This sequence, termed the CRISPR RNA (crRNA), has the only requirement that  
19 it must precede a Protospacer Adjacent Motif (PAM) of the form 3'NGG [6]. Next, a second  
20 RNA molecule, termed the trans-activating crRNA (tracrRNA), is used for binding to Cas9 [6].  
21 For the purpose of experimental simplification, the crRNA and tracrRNA sequences can be fused  
22 into a single guide RNA (sgRNA) [7]. By expressing this sgRNA along with Cas9 in germ line  
23 cells, heritable genome mutations can be created.

24 The CRISPR/Cas9 system has been successfully established in two leading nematode  
25 models – *C. elegans* and *Pristionchus pacificus* [2, 8]. Friedland *et al.* [9] developed a simple  
26 protocol for *C. elegans* that involved injecting plasmids into the gonad of adult hermaphrodites.  
27 The authors modified Cas9 to include a SV40 NLS to ensure nuclear localization and expressed  
28 under an *eft-3* translation elongation factor promoter, chosen for its effectiveness in germ line  
29 expression. The sgRNAs were expressed under a U6 small nuclear RNA polymerase III

1 promoter, chosen for its ability to drive expression of small RNAs. As the optimal expression  
2 from this promoter requires the first base to be a purine, the sgRNA target sequence is restricted  
3 to the form (G/A)(N)<sub>19</sub>NGG [9, 10].

4         Adaptation of CRISPR/Cas9 to *C. briggsae*, a species that is closely related to *C. elegans*,  
5 would provide a powerful tool to investigate the function of any given gene. *C. briggsae* is used  
6 routinely by many laboratories in comparative evolutionary studies. The two animals diverged  
7 less than 30 million years ago yet share similar morphology [11]. A comparison of their genome  
8 sequences has revealed that roughly one-quarter of their genes lack clear orthologs including  
9 many that are highly divergent and species-specific [12]. This suggests that underlying gene  
10 networks have evolved substantially without an obvious change in phenotype [13]. Such changes  
11 are likely to have significant impacts and may confer unique advantages on animals to withstand  
12 genetic and environmental fluctuations. By generating mutations in *C. briggsae* genes and  
13 characterizing phenotypes, we can learn the functional relevance of genomic differences,  
14 including any alterations in genetic pathways and developmental mechanisms between the two  
15 species. With this goal in mind, we set out to develop a method for using this system in *C.*  
16 *briggsae*.

17         The wild type AF16 strain was used as a reference strain in all experiments. Strains  
18 generated as part of this study include DY503 *Cbr-unc-22(bh29)*, DY504 *Cbr-dpy-1(bh30)*,  
19 DY530 *Cbr-bar-1(bh31)*, DY544 *Cbr-unc-119(bh34)* and DY545 *Cbr-unc-119(bh35)*.

20         We first used the CRISPR/Cas9 system in *C. briggsae* in an attempt to generate targeted  
21 loss-of-function mutations by employing NHEJ. For this, two conserved genes were chosen  
22 based on visible phenotypes, *Cbr-dpy-1*, a cuticle protein causing a dumpy (Dpy) phenotype, and  
23 *Cbr-unc-22*, a twitchin homolog causing an uncoordinated (Unc) phenotype [14-16]. Target  
24 sgRNA sequences following the form G/A(N)<sub>19</sub>NGG were searched for in the exonic regions of  
25 these genes using the ZiFiT Targeter Version 4.2 software [17]. The sgRNA sites were screened  
26 based on predicted efficiency using empirically based scoring algorithms. Off-target sites were  
27 minimized using the sgRNACas9 software package developed by Xie *et al.* [18].

28         The plasmids containing the *C. elegans* U6 promoter and sgRNA target sequences were  
29 generated by site-directed mutagenesis. This was accomplished using either two-step overlap-

1 extension PCR on a *pU6::Cbr-unc-119\_sgRNA* template (gift from John Calarco, Addgene  
2 plasmid #46169) [9], or Q5 site-directed mutagenesis on a *pU6::Cbr-lin-10\_sgRNA* template [19]  
3 using the NEB Q5 site-directed mutagenesis kit (E0554). The target site substitution was  
4 confirmed by *AclI* digestion. See Tables S1 and S2 for sgRNA sites and primers used in this  
5 study.

6 The plasmids sgRNA and Cas9 (*Peft-3::Cas9-SV40 NLS::tbb-2 3'UTR*, also from John  
7 Calarco, Addgene #46168) were injected into the germline of young adults using standard  
8 methods [20] and F1 progeny displaying the co-injection marker, pharyngeal expression of GFP,  
9 were isolated onto separate plates. Injection mixes contained *pU6::sgRNA* (100 ng/ul), *Peft-*  
10 *3::Cas9-SV40 NLS::tbb-2 3'UTR* (100 ng/ul), and *myo-2::GFP* (10 ng/ul).

11 Following microinjection, F2 worms were screened for desired phenotypes. We  
12 successfully isolated mutants for both *Cbr-dpy-1* and *Cbr-unc-22* at comparable frequencies to  
13 those observed in *C. elegans* (Table 1) [9]. Sequencing of the alleles of each of these genes  
14 revealed insertions and deletions at the sgRNA target sites (Table 2). The phenotypes of mutant  
15 animals are indistinguishable from those in *C. elegans* corresponding to orthologous genes,  
16 demonstrating conservation of gene function. Together, these results show that the CRISPR/Cas9  
17 system works in *C. briggsae* and can utilize conserved *C. elegans* promoters to express sgRNAs  
18 and Cas9.

19 Next, we targeted six other conserved genes of the Wnt and Ras pathways (*Cbr-lin-2*,  
20 *Cbr-lin-7*, *Cbr-lin-10*, *Cbr-lin-17*, *Cbr-lin-18* and *Cbr-vit-2*). For the PCR-based assay [19] F1s  
21 were allowed to lay eggs for 24-36 hours, and then picked and lysed in pools of two. A region of  
22 the genomic DNA spanning the sgRNA site (~200 bp) was amplified and examined on a 4%  
23 high-resolution agarose gel (Invitrogen UltraPure Agarose-1000, Catalog #16550-100) for  
24 changes in band sizes (Figure S2). In some cases we recovered mutations as determined by  
25 phenotypic as well as PCR-based screening approaches but none were found to be heritable  
26 (Table 1). It is unclear to us whether it was due to sgRNAs being non-functional, less efficient or  
27 requiring much larger F1s to be screened. Similar results were previously reported in *C. elegans*  
28 [21]. In one case, *Cbr-lin-17*, we sequenced the animal that showed bi-vulva phenotype and  
29 found possible evidence for a somatic mutation (T/A transversion causing M482L substitution).

1 The bi-vulva phenotype in this line was lost in subsequent generations. Evidence of somatic  
 2 mutations has also been described in *C. elegans* [21].

3

Screening Approach	Targeted Gene	3' Target bases	Visible phenotype	Frequency of mutations	Animals screened
Phenotypic screening	<i>Cbr-bar-1</i>	GG	Egl	9.5%	22
	<i>Cbr-dpy-1</i>	GA	Dpy	2.8%	35
	<i>Cbr-lin-2</i>	UA	Vul	0	40
	<i>Cbr-lin-7</i>	GA	Vul*	0	44
	<i>Cbr-lin-10</i>	AC	Vul	0	161
	<i>Cbr-lin-17</i>	AC	Bivulva	0	63
	<i>Cbr-lin-17</i> (linear sgRNA)	AC	Bivulva <sup>#</sup>	0	3
	<i>Cbr-lin-18</i>	AG	Bivulva <sup>§</sup>	0	65
	<i>Cbr-unc-22</i>	UC	Unc	2.5%	40
	<i>Cbr-unc-119</i> (sgRNA #1)	TT	Unc	0	48
	<i>Cbr-unc-119</i> (sgRNA #2)	GG	Unc	11.1%	54
PCR-based screening	<i>Cbr-lin-7</i>	GA	Vul	0	56
	<i>Cbr-lin-10</i>	AC	Vul	0	126
	<i>Cbr-vit-2</i>	AG	WT <sup>@</sup>	1.3%	78

**Table 1.** Phenotypes of transgenic animals generated using the CRISPR/Cas9 technique. The 3' target bases are those at positions 19 and 20 in the sgRNA target sequence. \*One F2 showed Dpy phenotype. <sup>#</sup>3 bivulva worms were recovered in F3 but the phenotype was not heritable. <sup>§</sup>One F2 showed protruding vulva (Pvl) phenotype. <sup>@</sup>wild type based on the *C. elegans vit-2* mutant phenotype.

4

5 Interestingly, our screens also recovered worms with unexpected phenotypes, e.g., Dpy in  
 6 *Cbr-lin-7* screen (Table 1). Sequencing of these worms revealed no disruption in targeted genes,  
 7 raising the possibility of off-target effects of CRISPR/Cas9. Off target effects have been reported  
 8 in *C. elegans* as well as several other models including *Drosophila*, mice, zebrafish, and human  
 9 cell lines [22-25].

10 The sgRNAs with a 3'GG motif at positions 19 and 20 were recently shown to  
 11 significantly enhance the efficiency of targeted mutations in *C. elegans* [21](23). To test whether  
 12 a similar sequence structure could be effective in *C. briggsae* we selected two conserved genes

1 *Cbr-unc-119* and *Cbr-bar-1*. Mutations in *Cbr-unc-119* with Unc phenotype were recovered at a  
 2 frequency of 11.1% (Tables 1 and 2). In contrast, another sgRNA for *Cbr-unc-119* that lacked 3'  
 3 GG motif did not give rise to any mutation (Table 1). In the case of *Cbr-bar-1*, a  $\beta$ -catenin  
 4 homolog [26], the 3'GG motif sgRNA resulted in a disruption efficiency of 9.5% (Tables 1 and  
 5 2). The enhanced efficiency of the 3'GG motif sgRNA sites for these two genes suggests that  
 6 such an approach in *C. briggsae* could improve the frequency of targeted mutations in genes of  
 7 interest.

8

Strain	Sequence	Mutation
<i>Cbr-bar-1(bh31)</i>	AAGGTCAAGTTTGTGAAGATGGG <b>AGG</b> ACC ACAGAA	8bp deletion
<i>Cbr-bar-1(bh33)</i>	TTGTGAAGAACTCCTTGATGACGTTTTTC TTGGG <b>AGG</b>	21bp insertion
<i>Cbr-bar-1(bh36)</i> *	GTCAAGTTTGTGAAGA [ 147 bases ] TGGGT <b>ATC</b> GGAC	150bp insertion, 3bp deletion
<i>Cbr-dpy-1(bh30)</i>	GTGCTGATCATTGTGAATCTCAGTTCGGT GTAGGTCGTTTCGCTCCA <b>ACTGATGG</b>	31bp insertion, 1bp deletion
<i>Cbr-unc-22(bh29)</i>	GTTGAGA <b>ACTCTGTTGGATCTGATTCT</b> GGAA ATCG	5bp deletion
<i>Cbr-unc-119(bh34)</i>	CGACGGGAAGGTCGCCGAGCGACGGGAAGG TCGCCGACGGG <b>TGG</b> AATC	17bp insertion, 1bp deletion
<i>Cbr-unc-119(bh35)</i>	GCGACGGGAAGGTCGCCGAGCTTTCGGG <b>TG</b> <b>GAATC</b>	3bp insertion, 1bp deletion

**Table 2.** Alleles generated by the CRISPR/Cas9 approach. The DNA sequence includes the sgRNA target. The PAM site is bolded. Insertion and deletion sequences are underlined (dotted underline: insertion, solid underline: deletion). For clarity the 147 base pair inserted sequence in *bh36* allele has been omitted. This long sequence matches with the *E. coli* gene EF-Tu. \* The allele was recovered in a separate screen along with another allele *bh32* that has small deletion. The exact base change in *bh32* has not been determined.

9

10 In addition to the CRISPR-mediated NHEJ approach we also attempted the HR method of  
 11 gene editing in *C. briggsae*. For this donor templates were designed to either disrupt a gene (by  
 12 inserting a single-stranded oligonucleotide) or tag genes using double-stranded linear PCR

1 amplicons (or plasmids) of fluorescent reporters (GFP and dsRED). Specifically, the single strand  
 2 oligonucleotide donor templates were intended to insert a 22 bp sequence containing an *NcoI*  
 3 restriction enzyme site into *Cbr-bar-1* and *Cbr-lin-15B* (Figure S1B). Homology arms of length  
 4 75 and 49 bases were chosen directly overlapping the sgRNA site, based on previous results [19].  
 5 The double-stranded linear donor templates of *GFP* (864 bp) and *dsRED* (830 bp) containing  
 6 short microhomology arms were generated by PCR to create translational fusions with *Cbr-bar-1*  
 7 and *Cbr-vit-2*, respectively (Figure S1C). The donor vector *myo-2::dsRED::unc-54 3'UTR* was  
 8 designed to insert a *myo-2::dsRED* reporter into the *Cbr-bar-1* (Figure S1A) [27]. The vector  
 9 contained a 2 kb transgene flanked on either side by 1 kb of sequence homologous to *Cbr-bar-1*  
 10 (Gibson Assembly Cloning Kit NEB catalog #E5510). The templates were included in the  
 11 injection mix (donor plasmid 200 ng/μl, linear PCR amplicons 50 ng/μl, single-stranded  
 12 oligonucleotides 30 ng/μl) along with other DNA components as mentioned above. Although  
 13 none of these HR approaches were successful, in some cases we did observe expected genomic  
 14 changes in F1 and F2 animals (as determined by sequencing), which were not inherited in  
 15 subsequent generations (Table 3).

16

Targeted Gene	Expected phenotype	sgRNA Efficiency	HR Efficiency
<i>Cbr-bar-1</i>	Egl	25/219 (11.4%)	0/219
<i>Cbr-bar-1</i>	Egl	18/211 (8.5%)	0/211
<i>Cbr-bar-1</i>	Egl	Not Determined	0/202
<i>Cbr-lin-15B</i>	WT <sup>#</sup>	Not Determined	0/68
<i>Cbr-vit-2</i>	WT <sup>#</sup>	1/78 (1.3%)	0/78

**Table 3.** Genome editing events detected using CRISPR-mediated HR. The sgRNA efficiency shows all genome editing events, including those repaired by NHEJ and HR, based on phenotypic and PCR-based screens. HR efficiency indicates the number of HR events detected in F2 out of the total F1s screened. <sup>#</sup>Wild type based on the phenotype of *C. elegans* orthologs.

17

18 In conclusion, we have shown that the CRISPR/Cas9 system can be effectively employed  
 19 in *C. briggsae* to alter a gene of interest. Similar to *C. elegans* the 3' GG motif appears to  
 20 increase the frequency of NHEJ events. Interestingly, we observed a significant bias towards

1 insertion NHEJ events in *C. briggsae*. Of the total of 8 alleles recovered, for 4 different genes,  
2 62% had insertion of bases of varying length (range 3 to 150). Similar screens in *C. elegans* have  
3 reported 26% frequency of such events (n = 86 from 5 different studies) [9, 21, 28-30]. More  
4 work is needed to ascertain if such a bias in *C. briggsae* holds true in a larger sample size.

5 Together with the recently developed TALEN-based genome editing approach [3], the  
6 CRISPR/Cas9 approach described here provides a powerful means to investigate the functions of  
7 conserved as well as divergent genes in *C. briggsae*. This promises to accelerate comparative  
8 studies with *C. elegans* thereby leading to a greater understanding of the flexibility of genetic and  
9 molecular mechanisms during animal development.

10

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17

## 18 **Competing interests**

19 The authors declare no competing interests.

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1 **Supplementary Materials**

2 **Table S1. sgRNA target sites.**

3

Gene	sgRNA Target
<i>Cbr-bar-1</i>	GTCAAGTTTGTGAAGATGGG <b>AGG</b>
<i>Cbr-dpy-1</i>	GTGCTGATCATTGTGACTGAT <b>GG</b>
<i>Cbr-lin-2</i>	GATTAGAGACAAAGAGCATAT <b>GG</b>
<i>Cbr-lin-7</i>	GGTTCGAGAGGTTTATGAGAC <b>GG</b>
<i>Cbr-lin-10</i>	GTCCCACAGCAACAAGAAAC <b>AGG</b>
<i>Cbr-lin-15B</i>	GCCGTCAACAACACTACACCTAT <b>GG</b>
<i>Cbr-lin-17</i>	GTGTTGTCCAGTTTGACCACT <b>GG</b>
<i>Cbr-lin-18</i>	GCTCCGGAAGCAATTGCTAG <b>AGG</b>
<i>Cbr-unc-22</i>	AACTCTGTTGGATCTGATTCT <b>GG</b>
<i>Cbr-unc-119</i> #1	GGAAGTGCTAAAACGTCGTT <b>CGG</b>
<i>Cbr-unc-119</i> #2	GGGAAGGTCGCCGAGCCGGGT <b>GG</b>
<i>Cbr-vit-2</i>	AATGATGCACACCCGCC <b>AGAGG</b>
Bold indicates the PAM site.	

4

5

1 **Table S2. List of Primers.**

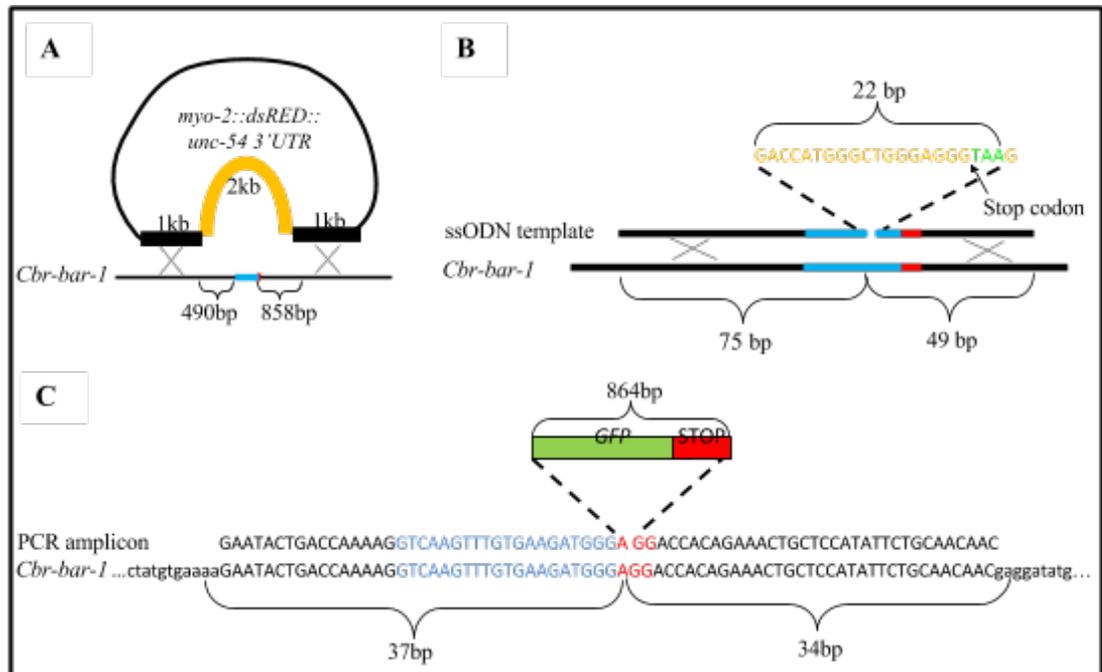
2

Gene	Purpose	Name	Direction	Sequence
<i>Cbr-bar-1</i>	sgRNA (OE)	GL964	F	GTCAAGTTTGTGAAGATGGGGTT TTAGAGCTAGAAATAGCAAGTTA
		GL965	R	CCCATCTTCACAACTTGACAAA CATTAGATTTGCAATTCAATTAT ATAG
	5' homology arm for Gibson Assembly	GL991	F	CGAGGTCGACGGTATCGATATCT GAGCAGCCACGCTAA
		GL992	R	TCTCTACTTGTCTAGAAGCTAAG ATTATGCGGTAAATAGTCTAATA ATTG
	3' homology arm for Gibson Assembly	GL1001	F	AAGTCGAAAAAATTAAGCTTTT TGAAAGACACTATATTTGGCTCG
		GL1002	R	GCTGCAGGAATTCGATATCAACC TAGTTATCAACCATGACGATAC
	Sequencing	GL1009	F	CATCTTGCTAGGCACATCACTTA TA
		GL1010	R	GGCAACAAGATGCGATCATTG
	PCR amplicon for direct HR	GL1039	F	GAATACTGACCAAAGGTCAAGT TTGTGAAGATGGGAAAAGGAGA AGAACTTTTCACTGG
		GL1040	R	GTTGTTGCAGAATATGGAGCAGT TTCTGTGGTCCCTATTTGTATAGT TCATCCATGCC
single stranded oligonucleotide donor template	GL1058	N/A	CTTCTTCTGTTATCGTCGACTTG ATCAGAGTTCTATGTGAAAAGAA TACTGACCAAAGGTCAAGTTTG TGAAGGACCATGGGCTGGGAGG GTAAGATGGGAGGACCACAGAA ACTGCTCCATATTCTGCAACAAC GAGGATATG	
PCR screening	GL1059	R	CATGGGCTGGGAGGGTAAG	
<i>Cbr-dpy-1</i>	PCR stitching	GL954	F	GTGCTGATCATTGTGACTGAGTT TTAGAGCTAGAAATAGCAAGTTA
		GL955	R	TCAGTCACAATGATCAGCACAAA CATTAGATTTGCAATTCAATTAT ATAG
	Sequencing	GL968	F	GGAGGAAGCCAACCTCACCAAG
		GL969	R	CAGCTCGATTTCAGACAATTC
<i>Cbr-lin-2</i>	sgRNA (OE)	GL974	F	GATTAGAGACAAAGAGCATAGTT TTAGAGCTAGAAATAGCAAGTT

		GL975	R	TATGCTCTTTGTCTCTAATCAAAC ATTTAGATTTGCAATTCAATTAT ATAG
<i>Cbr-lin-7</i>	sgRNA (OE)	GL976	F	GGTTCGAGAGGTTTATGAGAGTT TTAGAGCTAGAAATAGCAAGTT
		GL977	R	TCTCATAAACCTCTCGAACCAAA CATTTAGATTTGCAATTCAATTAT ATAG
	PCR-based screening and sequencing	GL1024	F	TGGGCCAATTCTATATCGATT
		GL1025	R	TTGCAGTCGAAATATGGGAT
<i>Cbr-lin-10</i>	sgRNA (OE)	GL978	F	GTCCACAGCAACAAGAAACGTT TTAGAGCTAGAAATAGCAAGTT
		GL979	R	GTTTCTTGTTGCTGTGGGACAAA CATTTAGATTTGCAATTCAATTAT ATAG
	PCR-based screening	GL1043	F	CAAGCCAATGCATAATATGCTCA ATAG
		GL1044	R	CTTCTTGATATTGTGCCGGCGAG
<i>Cbr-lin-15B</i>	sgRNA #1	GL1065	F	GCCGTCAACAACACTACACTAGTT TTAGAGCTAGAAATAGCAAG
	sgRNA #2	GL1066	F	GTTGTTGACGGCAGGACGGAGTT TTAGAGCTAGAAATAGCAAG
	PCR-based screening	GL1067	F	CGACGATCAGAAGTACCTCGTG
		GL1068	R	CGGCATCCTGTCTGAATGTATTTC
	Single stranded oligonucleotide donor template	GL1064	N/A	GTATCGAAGCGGAAACATTGCTC ACTTCCATGTGTCGTGCCCATGG GCTGGGAGGGTAAGCTAGTCAAC AACTACACCTATCGAACTGTGAA ATTCAGTAACATCGTCTGCCCA ATGAATCG
<i>Cbr-lin-17</i>	sgRNA #1 (OE)	GL960	F	GTGTTGTCCAGTTTGACCACGTTT TAGAGCTAGAAATAGCAAGTTA
		GL961	R	GTGGTCAAACACTGGACAACACAA ACATTTAGATTTGCAATTCAATT ATATAG
	sgRNA #2	GL1076	F	GGAACCTTGCTTTATTGTCCGGTTT TAGAGCTAGAAATAGCAAG
	PCR-based screening and sequencing	GL1077	F	CGGTGGGAAACCTGAATTTCGATC
GL1078		R	GTATAGTCTCACCTTGTTCTG	
<i>Cbr-lin-18</i>	sgRNA (OE)	GL962	F	GCTCCGGAAGCAATTGCTAGGTT TTAGAGCTAGAAATAGCAAGTTA
		GL963	R	CTAGCAATTGCTTCCGGAGCAAA CATTTAGATTTGCAATTCAATTAT ATAG
		GL1011	F	GCTCTTGCCACTCAGTTATCC

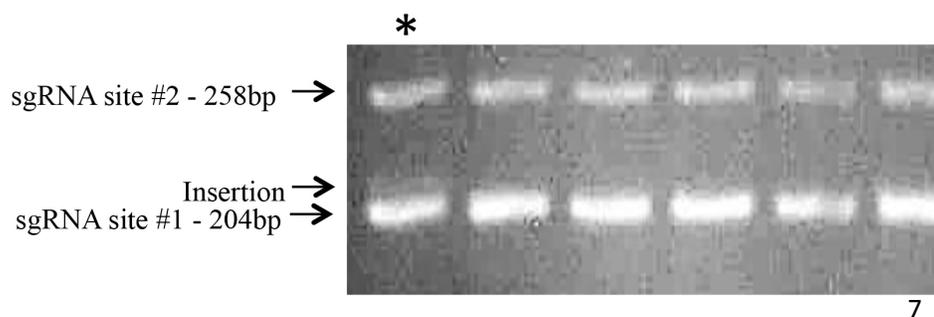
	Sequencing	GL1012	R	CACATGAGCACTCCTAGGGAC
<i>Cbr-unc-22</i>	PCR stitching	GL942	F	AACTCTGTTGGATCTGATTCGTTT TAGAGCTAGAAATAGCAAGTTA
		GL943	R	GAATCAGATCCAACAGAGTTAAA CATTTAGATTTGCAATTCAATTAT ATAG
	Sequencing	GL966	F	GGAGAAACCGTTGAGTTGAAG
		GL967	R	CCATGATCCTCCCATAGCTTC
<i>Cbr-unc-119</i>	sgRNA #1	GL1047	F	GGAAGTGCTAAAACGTCGTTGTT TTAGAGCTAGAAATAGCAAG
	sgRNA #2	GL1079	F	GGGAAGGTCGCCGAGCCGGGGT TTTAGAGCTAGAAATAGCAAG
	Sequencing	GL1099	F	GGCACCCTCTAATTACCATT
		GL1100	R	GATTCCTTGTTTCGGTGCTTG
	sgRNA (OE)	GL940	F	CGGGAATTCTCCAAGAAGCTCGT ACAAAAATGCTCT
		GL941	R	CGGAAGCTTCACAGCCGACTATG TTTGCGT
sgRNA (Q5)	GL1048	R	AAACATTTAGATTTGCAATTCAA TTATAT	
<i>Cbr-vit-2</i>	sgRNA #1 (OE)	GL1029	F	AATGATGCACACCCGCCAGGTT TTAGAGCTAGAAATAGCAAGTT
		GL1030	R	CTGGGCGGGTGTGCATCATTAAA CATTTAGATTTGCAATTCAATTAT ATAG
	sgRNA #2 (OE)	GL1033	F	GGCGGCCTCGACGGTCAAAGTT TTAGAGCTAGAAATAGCAAGTT
		GL1034	R	TTTGACCGTCGAGGCCCGCCAAA CATTTAGATTTGCAATTCAATTAT ATAG
	PCR-based screening (sgRNA #1)	GL1049	F	ACCGTCAATACGAGCCAGAA
		GL1050	R	TAGCACACTCAGTGGAACA
	PCR amplicon to direct HR	GL1053	F	GCCAGAAATCCGCATTCTTGCTC TCTGGAGAATGATGCACATGGTG CGCTCCTCCAAGAA
		GL1054	R	GAGAGACGACTTGAACGAGGAG TGGCTCCTCTGGGCGGGTGA CAGTTTAACTTACT
F indicates forward, R indicates reverse. sgRNA plasmids were either generated by overlap extension PCR (OE), or Q5 site directed mutagenesis (Q5).				

- 1 **Figure S1. Donor sequence approaches generated as templates for HR for *Cbr-bar-1*.**
- 2 Templates may take the form of a donor vector (A), ssODN (B) or PCR amplicons (C). Blue
- 3 letters represent the sgRNA target sequence while red letters represent the PAM site.



1 **Figure S2. PCR amplicons of the *Cbr-vit-2* genomic region flanking the sgRNA target site.**

2 An insertion can be seen at sgRNA site #1 in the lane marked with \*.



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