



## Supporting Online Material for

### **Targeted Genome Editing Across Species Using ZFNs and TALENs**

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#### **This PDF file includes:**

- Materials and Methods
- SOM Text
- Figs. S1 to S4
- Tables S1 to S3
- References

## SUPPORTING ONLINE MATERIAL

### Targeted Genome Editing Across Species Using ZFNs and TALENs

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#### Table of Contents

Materials and Methods.....	1
Acknowledgments.....	5
Figure S1 .....	6
β-galactosidase activity throughout both gonad arms of a <i>C. elegans</i> hermaphrodite injected with LacZ-encoding mRNA.	
Figure S2.....	7
ZFN and TALEN recognition sequences within target sites and nuclease-induced mutations.	
Figure S3.....	11
Time-course experiment for identification of ZFN-induced mutations conferring benomyl resistance.	

Figure S4.....	13
A combination of biochemical and bioinformatic analysis suggests the additional InDel in the genome of the <i>ben-1(y462)</i> mutant worm is not the result of ZFN action.	
Table S1 .....	15
ZFN target sites and designed zinc finger helices sequences.	
Table S2 .....	16
Multiple <i>ben-1</i> mutant worms originate from individual injected parents.	
Table S3 .....	17
Experimental genome-wide evaluation of ZFN action.	
Full Reference List.....	20

## Materials and Methods

### I. Procedures for nuclease design.

ZFNs were designed according to previously published principles and procedures (7-9), except that ZFNs containing tandem repeats of up to 6 zinc fingers were utilized. Amino acid sequences and DNA target sites for each ZF helix used in this study are listed in Table S1. TALENs were designed as previously described (2), except that the RVD of the C-terminal 'half repeat' was not fixed as NG. All TALENs used the +63 truncation point for fusion to the FokI cleavage domain (2). Amino acid sequences for TALENs are identical to previously described TALENs of the same length and truncation point (2) except for the identity of the RVDs indicated in Fig. 1D (or inferred from the target sites given in Fig. S2E). Full DNA sequences for the ORFs of TALENs will be provided upon request.

### II. Procedures for delivery of nucleases to the germline.

**mRNA synthesis.** Following validation in a yeast proxy system (9), the ZFN ORFs were transferred to an *SP6 in vitro* transcription plasmid backbone derived from pJK370 (a gift from T. Evans) that contains 5' and 3' UTR sequences shown previously to support germ-line translation (10). Production *in vitro* of mRNA containing 5' CAP structures and PolyA tails was performed using the Ambion mMessage Machine (#AM1340) and PolyA tailing (#AM1350) kits, and then purified over MegaClear columns (#AM1908) prior to quantification using a NanoDrop (Thermoscientific). The UTRs most favorable for germline translation of the mRNAs were the following (10).

#### 5' UTR

ATTTAGGTGACACTATAGAATACACGGAATTCTAGATGATCCCCGCGTACCGAGCTC  
AGAAAAA

#### 3'UTR

GCCTGAGCTCACGTCGACCGGGGCCCTGAGATCTGCTGCAG

**mRNA injection.** mRNA injections were performed under a Zeiss Axiovert microscope using a Narishige IM300 injector. Injection of mRNAs was performed according to standard *C. elegans* DNA injection protocols (11), with the following differences. The regulator was adjusted such that the pressure from the N<sub>2</sub> gas tank was 60 psi. The P<sub>inject</sub> and P<sub>balance</sub> measurements were adjusted to 15 psi and 2 psi, respectively. These pressure values are lower than those typically used for DNA injections to allow a more gentle release of fluid into the worm gonad. Procedures for mRNA injection into *C. briggsae* were identical to the procedures used for *C. elegans*. All ZFN mRNAs were injected at 250 ng/μl: the concentration at which β-galactosidase staining reached peak levels in worms injected with mRNAs encoding LacZ (Fig. S1). LacZ mRNAs were made from plasmid pJK370 (10). The mRNAs encoding ZFN monomers were injected as pairs; thus, the total mRNA concentration in the needle was 500 ng/μl. Injection of ZFN mRNAs at 500 ng/μl for each monomer reduced brood sizes without increasing target site mutation rates.

Non-polyadenylated ZFN mRNAs are approximately 1.2 kb in size, whereas TALEN mRNAs are approximately 3.1 kb. TALENs were initially injected at 500 ng/μl for each mRNA (1000 ng/μl total), but increasing the concentration to 1500 ng/μl for each mRNA (3000 ng/μl total) resulted in substantially more efficient mutant recovery. The higher concentration was therefore used for all subsequent TALEN experiments.

### III. Procedures for mutant isolation.

***gfp***. Strain EG4601 (a gift from E. Jorgensen and C. Frokjaer-Jensen) carries a *P<sub>pie-1</sub>::gfp::his-33* transgene integrated into chromosome II at single copy using the MosSCI approach (12). Copy number of the insert was verified by PCR amplification across the integration site (v5605F1/v5605R1) and observing a product of the expected size by agarose gel electrophoresis. Hermaphrodites homozygous for the GFP insertion were mated with wild-type males. Following mating, ZFN-encoding mRNAs made from plasmids pTY2540-pTY2543 were injected into the syncytial gonad of hermaphrodites, such that their progeny would inherit one copy of the transgene through the ZFN-exposed female germline. GFP expression in F1 animals was not visible under a regular dissecting microscope, likely because the transgene is single copy; thus F1 animals were screened under an AxioPlan 2 microscope (Zeiss) in drops of M9 containing 0.02% levamisole. Screens were performed using two different ZFN pairs. First, 20 animals were injected with mRNAs encoding ZFN-GFP-R1/L1 (Table S1), resulting in the recovery of three independent mutations (Fig. S2A). A further 36 animals were injected with mRNAs encoding a second ZFN pair (ZFN-GFP-R2/L2 –Table S1) resulting in two additional mutants (Fig. S2A). GFP fluorescence was most visible in the female germline of adult hermaphrodites (Fig. 1A) and could not be detected in male F1s; thus, ~50% of mutant F1s could not be detected in this screen. F2 progeny carrying homozygous mutant transgenes were isolated from non-green F1s by sequencing single worm PCR products spanning the target site (4601vF1/4601vR3).

***ben-1* (ZFNs)**. The *ben-1* mutant phenotype is dominant and visible in 100% of progeny under a regular dissecting microscope, and thus preferable to the *gfp* mutant phenotype for the recovery of large numbers of mutants. Benomyl screening was performed as described (13). Briefly, wild-type *C. elegans* hermaphrodites were grown on regular NGM agar plates before injection with mRNAs encoding ZFNs targeting *ben-1*, then transferred to plates containing 7  $\mu$ M benomyl. F1 self-progeny were screened as young adults by touching the anterior side of the animal. Heterozygous mutant animals respond by reversal using multiple sinusoidal-like movements, whereas wild-type animals are paralyzed and lack this ability. Non-paralyzed F1 animals were either lysed individually for PCR/CEL-1 analysis of the target site (see below), or transferred individually to fresh benomyl plates and homozygotes isolated from non-paralyzed F2 by sequencing PCR products spanning the target site (0405F2/0405R2). A pilot screen validated the efficacy of the *ben-1* ZFNs for mutagenesis (Fig. S2D). For the time course analysis, injected hermaphrodites were moved onto new benomyl plates every 4 hours and the progeny produced within each time window were analyzed independently (Fig. S3A).

To develop a strategy for mutant recovery without phenotypic selection, we initially used pre-validated ZFNs to target *ben-1* in the absence of benomyl. All 625 F1 laid between 8-16 hours post-injection were picked, as adults, individually into wells of 96 well plates containing liquid culture medium. The medium had 50  $\mu$ l S-basal medium containing OP50 *E. coli* seeded at an O.D<sub>600</sub> of 2.0, and plates were incubated at 20 °C in a humidified chamber. After four days, the wells contained hundreds of F2/F3 self-progeny. We then selected 240 wells at random for genotyping. Part of the culture volume (20  $\mu$ l) was removed from each well and mixed with 20  $\mu$ l of lysis solution (1 x PCR buffer containing 100  $\mu$ g/mL proteinase K) before flash freezing at -80 °C for 1 hour, incubation at 62 °C for 1 hour then inactivation of proteinase K at 95 °C for 15

minutes. PCR reactions were performed using 2  $\mu$ l of lysate as template in a 25  $\mu$ l reaction. Cycling conditions were as follows: 94  $^{\circ}$ C for 5 minutes, 35 cycles of (94  $^{\circ}$ C for 30 seconds, 60  $^{\circ}$ C for 30 seconds, 72  $^{\circ}$ C for 30 seconds), followed by a final elongation step of five minutes. Ten  $\mu$ l of each reaction was treated with CEL-1 Surveyor nuclease (Transgenomics #706020) according to the manufacturer's guidelines. Products were analyzed on a 2% agarose gel stained with ethidium bromide (Fig. S3D). Positive controls were obtained by placing one homozygous *ben-1* mutant and one wild-type animal together in a single liquid culture well, allowing them each to produce self-progeny and performing lysis, PCR and CEL-1 digestion as described above.

***rex-1***. Mutations in the 241 bp non-coding *rex-1* locus (Fig. 1C, Fig. S2B) were obtained without phenotypic selection essentially as described above, but one modification was implemented to the recovery protocol to increase efficiency. In a representative experiment, 1325 F1 animals laid during the peak time window (8-16 hours) were pooled in groups of four per individual well of liquid culture in four 96 well plates. Lysis, PCR (primers *rex1CF5*/*rex1CR5*), and CEL-1 analysis were performed as described above. Using this approach, 18 independent mutant lines were recovered from 338 CEL-1 reactions (1.4% of F1s, 5.4% of CEL-1 reactions).

***ben-1* (TALENs)**. To obtain mutations in *ben-1* using TALENs, we initially tested 4 pairs made up of 5 different monomers at 500 ng/ $\mu$ l for each mRNA (101318/101321, 101318/101322, 101317/101321, and 101319/101322). One pair (101318/101321) produced two mutant lines, but the other pairs did not. At a higher concentration (1500 ng/ $\mu$ l of each mRNA), three pairs (101318/101321, 101318/101322, and 101317/101321) produced one or more mutant lines, and one pair (101318/101321) produced more than 10. We focused our subsequent efforts on the most effective pair from these pilot studies. TALEN-induced mutants were recovered from 705 F1 animals laid in the peak time window without phenotypic selection as described for *rex-1*. Lysis, PCR, and CEL-1 analysis were performed as described above. In this experiment, 25 mutant lines were recovered (3.5% of F1s, 14% of CEL-1 reactions). Representative mutations are shown in Fig. 1D and Fig. S2E.

***Cbr-sdc-2***. Recovery of ZFN-induced mutations in *C. briggsae* targets was achieved without modification of the ZFN delivery and screening procedures that had previously been optimized for *C. elegans*. From the peak time window, 329 F1 animals were pooled ( $\leq$  4 F1 animals per well) into 83 wells of liquid culture. Lysis, PCR (primers 4849F1/4849R1) and CEL-1 digestions were performed as described above. From this experiment, 5 independent mutant lines were recovered (1.5% of F1s, 6.1% of CEL-1 assays). Mutant alleles are shown in Fig. S2C.

Target	Primer name	Sequence
<i>gfp</i> in Mos site ttTi5605	v5605F1	ACATGCTTCGTGCAAAACAG
<i>gfp</i> in Mos site ttTi5605	v5605R1	GTTTTTGATTGCGTGCGTTA
<i>gfp</i>	4601vF1	ATTCAGCACGGAGCACTTCT
<i>gfp</i>	4601vR3	CGTGTCTTGAGTTCCCGTCA
<i>Ce-ben-1</i>	0405F2	GCCACAAAAGTTTGAAGCTACAGT
<i>Ce-ben-1</i>	0405R2	CCCTGGCTAGTTCAAACGAA
<i>Ce-rex-1</i>	rex1CF5	CTTCTTTCCCTGCCCAAT
<i>Ce-rex-1</i>	rex1CR5	TGTGTTCCAGCAGTCTTGTTC
<i>Cbr-sdc-2</i>	4849F1	GGCAAGCGCGAGTTTTACAAT
<i>Cbr-sdc-2</i>	4849R1	CCTTCCTGGTCATTACTGTCG

#### IV. Experimental analysis of ZFN specificity.

**Probing for off-target sites.** The actual binding sites recognized by ZFNs for targets *Ce-ben-1* and *Cbr-sdc-2* were determined using a SELEX approach (Table S3A) as described previously (14), under conditions known to yield a biologically relevant consensus for ZFP binding to *C. elegans* DNA (15). The genome sequences of *C. elegans* or *C. briggsae* were then scanned for matches to the experimentally determined consensus using the approximate string matching algorithm (16). These loci (Table S3B) were deemed to be the most likely sites for off-target ZFN activity, should off-target activity exist. Genotyping the top 39 (*Ce-ben-1*) or the top 19 (*Ce-sdc-2*) off-target sites by PCR/sequencing and the CEL-1 endonuclease assay (Transgenomic, #706025) failed to identify evidence for off-target mutations in four independent lines for each target gene.

**In-depth DNA sequence analysis of the *ben-1(y462)* genome.** The homozygous *ben-1(y462)* mutation, a 1 bp deletion, was obtained by mutagenizing the wild-type *C. elegans* hermaphrodite strain N2 with enhanced high-fidelity *ben-1* ZFNs (8), which target the same site as the wild-type *ben-1* ZFNs used for establishing the optimal time course for mutagenesis (Fig. 1B, Fig. S3, and Fig. S2D). The high-fidelity FokI nuclease moiety of each ZFN enhances the specificity of ZFN mutagenesis because it carries point mutations that impose a strict heterodimerization requirement for function. Individual control N2 and *ben-1(y462)* animals that had been separated by only five generations were allowed to propagate to create a population for in-depth genome sequence analysis.

The *ben-1(y462)* genome was probed for off-target InDels using 76 bp and 101 bp Illumina paired-end sequencing runs on DNA extracted from extensively washed populations of *ben-1(y462)* and N2 nematodes. Genomic DNA (5 mg) was sheared to an average length of 250 bp with a Covaris S2 instrument and prepared for paired-end sequencing using the Illumina protocol, as modified by (17).

Paired-end sequences for both genomes were aligned to the *C. elegans* WS215 reference genome with Novoalign V2.06.09 (www.novocraft.com), which utilizes a Needleman-Wunsch gapped alignment algorithm, and each aligned data set was converted to the MAQ format using novo2maq (www.novocraft.com). The 76 bp and 101 bp datasets were merged for each genome with Maq mapmerge (18). Paired-end reads with identical outer coordinates are common PCR artifacts and were removed with the Maq rmdup. Indels were called with Maq indelpe.

Several filters were used to identify InDels present in the *ben-1(y462)* genome but not the progenitor N2 genome and to remove called InDels that likely resulted from sequencing errors or incorrect alignments. (1) InDels had to be called at least once on both strands of DNA from *ben-1(y462)* but not N2. (2) InDels were eliminated if they represented a 1 bp extension or deletion of a homopolymer greater than 8 bp, or if (3) the called InDel was in a repetitive region and present in one repeat. (4) InDels were classified as homozygous if greater than 75% of all reads at the location contained the InDel. InDels were classified as heterozygous if 25%-75% of the reads contained the InDel. (5) For InDels meeting criteria 1-4, Sanger sequencing performed on PCR-amplified fragments spanning the InDel had to reveal a sequence change in *ben-1(y462)* but not N2 DNA. Fragments were made from the same DNA used to make Illumina libraries.

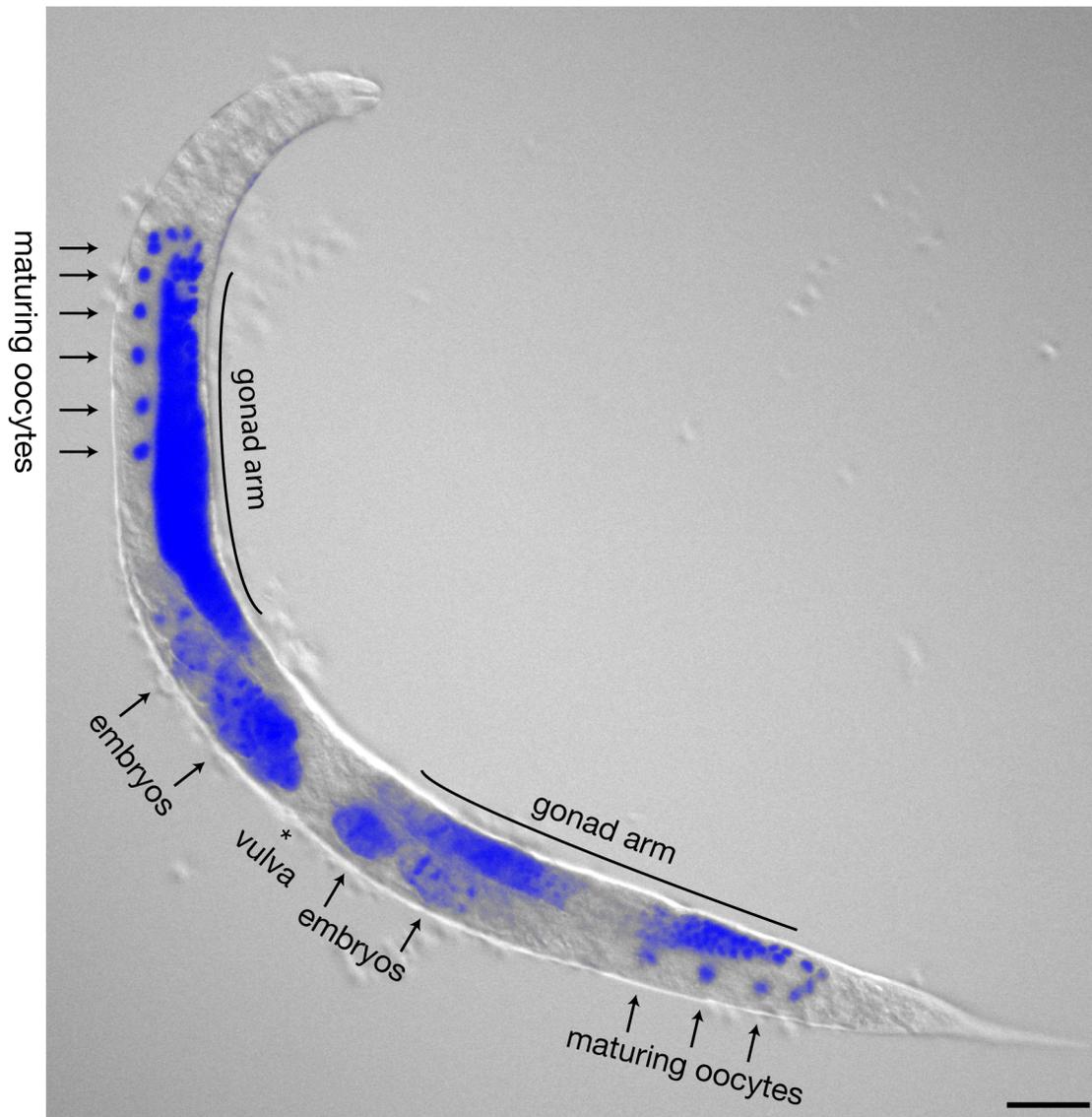
Both N2 and *ben-1(y462)* yielded  $22 \times 10^6$  aligned pairs. The average fragment sizes were 211 bp and 239 bp, respectively, and the average coverage was 65 and 64 reads per base, respectively. Greater than 97% of the N2 genome and 95% of the *ben-1(y462)* genome were covered with 4 or more reads. More than 90% of InDels called in N2 and *ben-1(y462)* (1 per 17,000 sequenced bases) were identified on only one strand and were eliminated from further consideration due to the high probability of resulting from errors in sequencing or mapping.

After filters 1-4 were applied, only two potential homozygous InDels and four potential heterozygous InDels were found in the *ben-1(y462)* genome and not the N2 genome. Sanger sequencing eliminated all but one homozygous InDel, a deletion of T at the chromosome III:3541472 position, which represented the actual *ben-1(y462)* mutation, and all but one of four heterozygous InDels, a deletion of AAC at the chromosome IV:15231117 position. This heterozygous InDel does not appear to have been caused by ZFNs, since its flanking sequence bears no homology to any predicted ZFN binding site. The InDel appears to represent either a *de novo* mutation that occurred in the 5 generations of growth that separated *ben-1(y462)* and its progenitor N2 animals or a mutation that was in the original N2 population but was lost. Small InDels arise commonly in the *C. elegans* population (19). In summary, only one non-targeted InDel arose in the *ben-1* mutant strain, and that mutation appears to have occurred spontaneously during nematode growth, rather than as a consequence of ZFN action. Mutagenesis with ZFNs is remarkably specific compared to other mutagenic regimes (20, 21).

## Acknowledgements

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**Figure S1**



**Fig. S1.  $\beta$ -galactosidase activity throughout both gonad arms of a *C. elegans* hermaphrodite injected with LacZ-encoding mRNA.**

Activity was visualized by staining with 5-bromo-4-chloro-3-indolyl- $\beta$ -D galactopyranoside. The UTR sequences for the mRNA were identical to those used for all ZFN and TALEN mRNAs in this study. LacZ contains a nuclear localization signal. Scale bar, 50 $\mu$ m.

# Figure S2

## A

*C. elegans*

**ZFN-GFP-R1**

5' GTTGTCCAATTCTTGTgaattaGATGGTgATgtTAATGG 3'  
 3' CAACAGGGTTAAGAACAActtaatCTACCACTAcAATTACC 5'

**ZFN-GFP-L1**

GAACTTTCACTGGAGTTGTCCAATTCTTGTGAATTAGATGGTATGTTAATGGGCACAAATTTCTGT (WT *gfp*)

GAACTTTCACTGGAGTTGTCCAATTCTTGTGA:TTAGATGGTATGTTAATGGGCACAAATTTCTGT (Δ1)

GAACTTTCACTGGAGTTGTCCAATTCTTGTGA:.....TGGTATGTTAATGGGCACAAATTTCTGT (Δ6)

AATCTGTAAATCTT

GAACTTTCACTGGAGTTGTCCAATTCTTGT:.....GATGGTATGTTAATGGGCACAAATTTCTGT (Δ6, +16)

**ZFN-GFP-R2**

5' GTTGTCCAATTCTTGTgaattagATGGTgATGTTAATGG 3'  
 3' CAACAGGGTTAAGAACAActtaatcTACCACtACAATTACC 5'

**ZFN-GFP-L2**

CTTGT

GAACTTTCACTGGAGTTGTCCAATTCTTGT:.....GATGGTATGTTAATGGGCACAAATTTCTGT (Δ6, +6)

GTTCTTGATTCTTGATGTTGGTTGCAATTGTGT

GAACTTTCACTGGAGTTGTCCAATTCTTGT:.....TGGTATGTTAATGGGCACAAATTTCTGT (Δ9, +34)

## B

**REX-1-ZFN-R1**

5' CAGCAGTGCCTGCGTACAAAaggaGACAAGcGCAGGgAGAACTG 3'  
 3' GTCGTACGCACGCATGTTtccctCTGTTcgCGTCCcCTTGAC 5'

**REX-1-ZFN-L1**

\*\*\*\*\*  
GCGCAGG

MEX Dup CAGCAGTGCCTGCGTACAA:.....GACAAGCGCAGGGAGAACTG (Δ5, +7)

## C

*C. briggsae*

**ZFN-SDC-2-R2**

5' CCGAGctCACATCGATgatcaaGAAAACACGGCAGCA 3'  
 3' GGCTCgaGTGTAGCTActagttCTTTTGTGCCGTCGT 5'

**ZFN-SDC-2-L2**

CGATTTGGAACACACCCGAGCTCACATCGATGATCAAGAAAACACGGCAGCAATCACCAGGAAAGGAGAAG (WT *sdc-2*)

(y466) CGATTTGGAACACACCCGAGCTCACATC:.....AAACACGGCAGCAATCACCAGGAAAGGAGAAG (Δ11)

CG

(y467) CGATTTGGAACACACCCGAGCTCACATCGAT:.....AACACGGCAGCAATCACCAGGAAAGGAGAAG (Δ9, +2)

(y468) CGATTTGGAACACACCCGAGCTCACATC:.....AAACACGGCAGCAATCACCAGGAAAGGAGAAG (Δ11)

(y469) CGATTTGGAACACACC:.....GGCAGCAATCACCAGGAAAGGAGAAG (Δ29)

GCTGCATCGAGCGAGC

(y470) CGATTTGGAACACACCCGAGCTCACATCGA:.....AAACACGGCAGCAATCACCAGGAAAGGAGAAG (Δ9, +16)



# Figure S2

## E

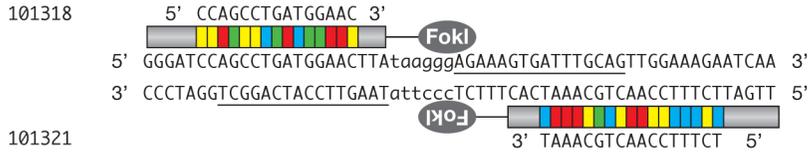
*C. elegans*

**TALE Name**

**BEN-1-TALEN-L**

**BEN-1-TALEN-R**

101317 5' GGGATCCAGCCTGAT 3'  
101319 5' CCAGCCTGATGGAAC 3'



**NT: RVD:**  
A NI  
C HD  
G NN  
T NG

101322 3' CGTCAACCTTCTTAGT 5'

GGGATCCAGCCTGATGGAACCTATAAGGGAGAAAAGTGATTTGCAGTTGGAAAGAATCAA (WT *ben-1*)

101317/101321 GGGAGAAA  
GGGATCCAGCCTGATGGAACCTATAAGGGAGAAAAGTGATTTGCAGTTGGAAAGAATCAA (+8)

101318/101322 TATAAGGAAGAAA  
GGGATCCAGCCTGATGGAACCTATAAGGAAGGAAGAAAAGTGATTTGCAGTTGGAAAGAATCAA (+13)

101318/101321 GGGATCCAGCCTGATGGAACCTATAAG:::AAAGTGATTTGCAGTTGGAAAGAATCAA ( $\Delta 4$ )

101318/101321 GGGATCCAGCCTGATGGAACCTATAAG::::::TGATTTGCAGTTGGAAAGAATCAA ( $\Delta 8$ )

101318/101321 GGGATCCAGCCTGATGGAACCT:::GATTTGCAGTTGGAAAGAATCAA ( $\Delta 14$ )

101318/101321 G  
GGGATCCAGCCTGATGGAACCT:::ATTTGCAGTTGGAAAGAATCAA ( $\Delta 15$ , +1)

101318/101321 AACITAT  
GGGATCCAGCCTGATGGAACCTATAAG:::AGTGATTTGCAGTTGGAAAGAATCAA ( $\Delta 6$ , +7)

101318/101321 TAGTGATTAT  
GGGATCCAGCCTGATGGAACCTATA:::AGTGATTTGCAGTTGGAAAGAATCAA ( $\Delta 8$ , +11)

101318/101321 TGATGGAACCTGATGGAACCTT  
GGGATCCAGCCTGATGGAACCTATA:::AAAGTGATTTGCAGTTGGAAAGAATCAA ( $\Delta 6$ , +31)

**Fig. S2. ZFN and TALEN recognition sequences within target sites and nuclease-induced mutations.**

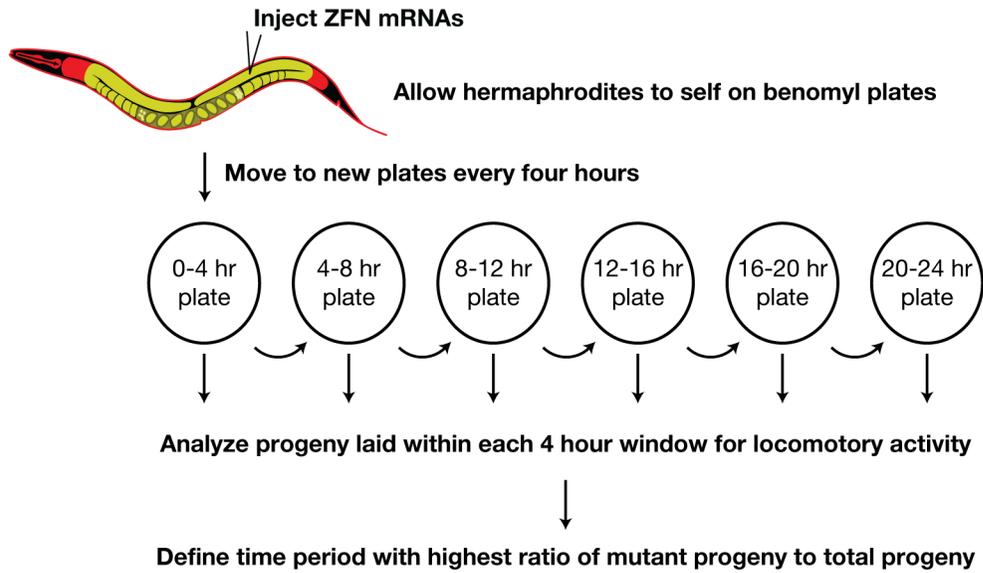
**(A-D)** ZFN recognition sequences (underlined in black) are shown within the DNA target sequence of the indicated wild-type gene. Uppercase letters depict nucleotides specifically recognized by ZF helices (Table S1). Lowercase nucleotides within a ZFN recognition site represent positions that are skipped through the use of non-canonical linkers between the fingers. The gap between the two ZFN recognition sequences is indicated by lower case nucleotides and is the target region for Fok1 endonuclease cleavage. Mutations in gene targets are shown below the wild-type sequence. Changes at target sites include short insertions (green) and deletions (red colons) that generate in-frame deletions and frame-shift mutations. Inserted sequences (green) frequently share homology (underlined in green) with sequences flanking the break site, as is typical of NHEJ-mediated repair. For *rex-1*, the MEX motif is shaded in yellow and a near-perfect duplication of this motif is indicated by asterisks. All *ben-1* mutations except *ben-1(y462)* were induced with wild-type ZFNs. *ben-1(y462)* was induced by an enhanced high fidelity ZFN.

**(E)** TALEN-induced *ben-1* mutations. TALE recognition sequences within the *ben-1* coding sequence and representative sequence changes from each of 3 TALEN pairs are shown. The nucleotide binding specificity of the lead TALEN pair (101318/101321) is indicated by colored blocks, which correspond to individual repeat variable di-residues (RVDs) within each TALE protein. The color denotes the identity of the RVD that recognizes each nucleotide (NT) of the DNA target site, as shown in the key on right. For comparison, the recognition sites for the ZFN pair used to target *ben-1* are underlined in black. Annotation for mutant sequences is the same as in (A-D).

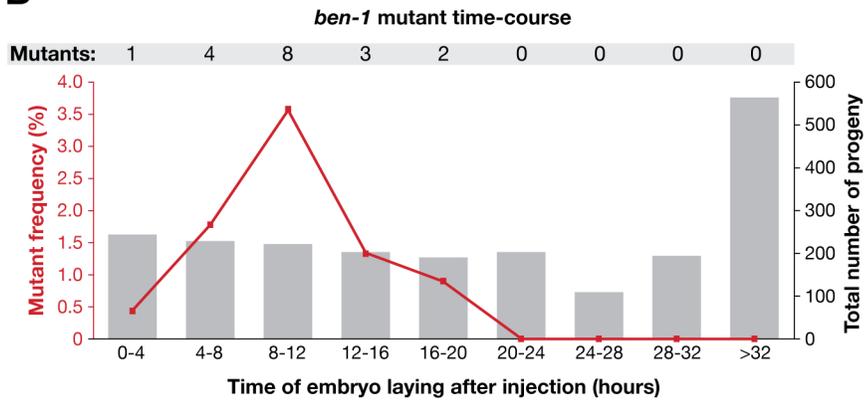
**Figure S3**

**A**

**Time course experiment**

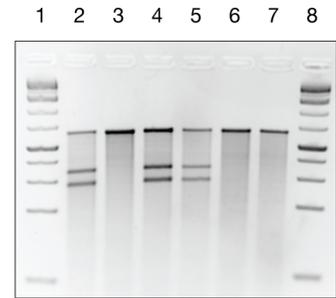


**B**



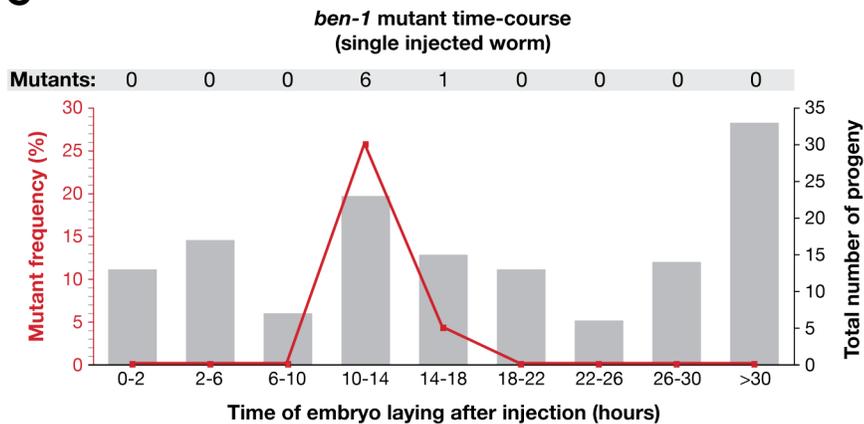
**D**

**CEL-1 assay to identify mutations**



- 1 1 kb ladder
- 2 Positive control
- 3 Negative control
- 4 *ben-1* mutant ( $\Delta 9, +12$ )
- 5 *ben-1* mutant ( $\Delta 6, +2$ )
- 6 No *ben-1* mutant
- 7 No *ben-1* mutant
- 8 1 kb ladder

**C**



**Fig. S3. Time-course experiment for identification of ZFN-induced mutations conferring benomyl resistance.**

**(A)** Diagram of a time-course experiment. F1 progeny from *C. elegans ben-1* ZFN-injected hermaphrodites were collected in defined time windows post-injection, placed on plates containing benomyl, and analyzed independently.

**(B)** Plot showing a histogram of the total number of progeny (left *y*-axis) laid by 16 hermaphrodites in each time period (*x*-axis), and a graph (red line) of the mutant frequency in each period (right *y*-axis) Total number of mutant progeny per time period is indicated above the histogram. These data represent a separate experiment from that in Fig. 1B, and the time periods have been shifted by two hours.

**(C)** Time-course for benomyl-resistant mutant progeny from an individual ZFN-treated worm. Multiple, independent *ben-1/+* mutant progeny were produced from one injected parent. Data are presented as in (B).

**(D)** CEL-1 digestion of PCR products spanning the ZFN target site in progeny of ZFN-treated hermaphrodites identifies *ben-1* mutations without use of phenotype or selection. Sequence changes of mutant lines derived from F1 progeny laid within the peak time window (8-16 hours) are shown in Fig. S2D.

## Figure S4

A

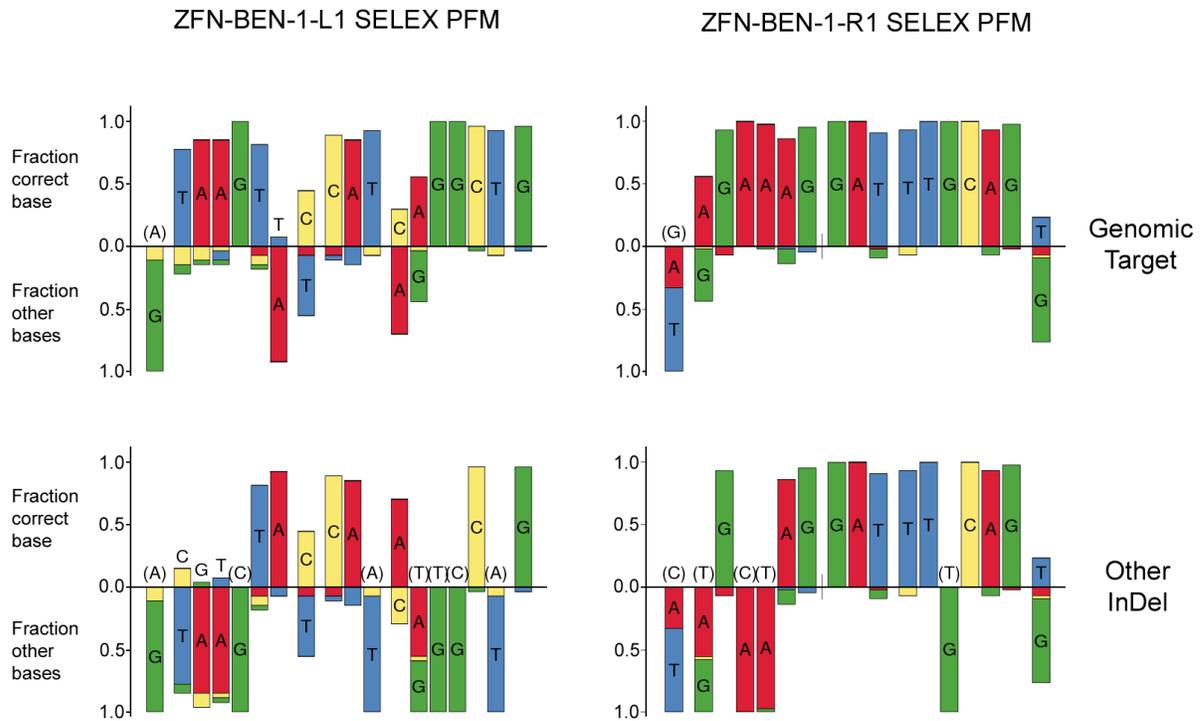
### Intended Genomic Target

5' -CAGCCTGATGGAACTTAtaagggAGAAAGTGATTTGCAGT-3' ZFN-BEN-1-R1 consensus  
 3' -GTCGACTACCTTGAATattcccTCTTTCACTAAACGTCA-5' ZFN-BEN-1-L1 consensus  
 gTCGGRATACYATGAATg

### InDel Site

5' -CTGGAATTTGGTAGACGttgtgcTGCTAGGATTTTCAGT-3' ZFN-BEN-1-R1 consensus  
 3' -GACCTTAAACCATCTGCaacacgACGATCCTAAAGTCA-5' ZFN-BEN-1-L1 consensus  
 gTCGGRATACYATGAATg

B



**Fig. S4. A combination of biochemical and bioinformatic analysis suggests the additional InDel in the genome of the *ben-1(y462)* mutant worm is not the result of ZFN action.**

**(A)** Alignment of the SELEX derived consensus for ZFN-BEN-1-L1 and ZFN-BEN-1-R1 with the intended genomic target (top half of the panel) or to the best match within a 100 bp window centered on the location of the other InDel (bottom half). The CAA highlighted in gray was deleted by the InDel. Mismatches between the ZFN consensus and the genomic sequence are shown in red. The core 15 bp consensus sequence is indicated by uppercase letters and the consensus flanking this 15 bp core is indicated by lowercase letters. W indicates A or T, R indicates A or G, and Y indicates A or C. The linker between zinc fingers 3 and 4 of both ZFN-BEN-1-L1 and ZFN-BEN-1-R1 can allow binding both with and without an intervening base pair so both possibilities were allowed. For the intended genomic target, five mismatches are observed to the full 34 bp dimer consensus. This is the best match to a valid dimer consensus in the entire *C. elegans* genome; the second best match contains eight mismatches to the dimer consensus. For the heterozygous InDel, the best match contains 16 mismatches to the consensus. It was not computationally tractable to calculate a full SELEX-derived score for every possible binding site within the *C. elegans* genome. However, an exhaustive comparison to the consensus sequences to the *C. elegans* genome indicates that there are 1,279,185 consensus matches within the *C. elegans* genome that have 16 or fewer mismatches. If this InDel was truly caused by off-target ZFN cleavage, then it is exceptionally unlikely to not observe InDels at any of the other 1,279,184 equivalent or superior sites.

**(B)** Comparison of the full SELEX-derived position frequency matrix (PFM) for ZFN-BEN-1-L1 and ZFN-BEN-1-R1 to the appropriate half sites in the genomic target or in the best match near the heterozygous InDel. All SELEX data are shown 5' to 3'; the 3' to 5' consensus sequence for ZFN-BEN-1-L1 must be reversed to align with the ZFN-BEN-1-L1 SELEX data. In the top panels, the proportion of the SELEX-derived bases at each position matching the intended genomic target are shown above the X-axis and the proportion of the SELEX-derived bases not matching the intended genomic target are shown below the X-axis. The bottom two panels show the same SELEX data compared to the best consensus match near the other InDel. This comparison indicates that the intended genomic site is a dramatically better match to the SELEX data than is the site near the other InDel. In addition, none of the 5 consensus mismatches to the intended target occur at highly specified positions (>95% of the SELEX selected sequences matching a single base) while 6 of the 16 mismatches to the best match site near the other InDel occur at highly specified positions.

**Table S1**

ZFN target sites and designed zinc finger helices sequences

Target	ZFN Binding Sequence (underlined)	ZFN	Finger 1	Finger 2	Finger 3	Finger 4	Finger 5	Finger 6
CeGFP	GTTGTCCCAATTCTTGTTgaattta <u>G</u> AIGGTGAIGtTAATGG CAACAGGGTAAAGAAACA <u>A</u> cttaatCTACCACtAcAATTACC	ZFN-GFP-R1 ZFN-GFP-L1	RSDHLSQ NSDHLTN	TSSNRIT DRANLSR	TSGNLTR RSDHLST	TSGHLSR TSSNRKT	TSSNLSR RSDALSV	- DNANRIT
CeGFP	GTTGTCCCAATTCTTGTTgaatttagAIGGTGgAIGTIAATGG CAACAGGGTAAAGAAACA <u>A</u> cttaatcTACCACtAcAATTACC	ZFN-GFP-R2 ZFN-GFP-L2	RSDHLSQ NSDHLTN	TSSNRIT DRANLSR	WRSCRSA RSDHLST	RSDALSR TSSNRKT	RSDALTQ RSDALSV	- DNANRIT
CeBEN1	AGCCTGATGgAACTTAtaagggA <u>G</u> AAAGtGATTIGCAG ICGGACTACC <u>T</u> IGAAIattcccTCTTTcAcTAAACGTC	ZFN-BEN-1-R1 ZFN-BEN-1-L1	RSDNLSQ QSSDLSR	RSFTLMQ RSDNLSR	TSSNLSR LQNLDA	RSDLSV TSGLSR	QNQHRIN QANLMMK	- -
CeREX1	CAGCAGTCCGTCGTACaagggaGACAAGcCAGGGgAACTG GTCGTCACGCACGCAIGI <u>t</u> ttccctCTGTTcgcGTCCctCTTGAC	REX-1-ZFN-R1 REX-1-ZFN-L1	RSDVLSQ RSDTLSE	QSGNLAR RRWTLVG	RSALSR QSGDLTR	QSGDLTR DKGNLTK	RSANLSV RSDTLSE	DRANLSR YRSCLSI
CbrSDC2	CCATGATGA <u>a</u> CTTGTagctgttGGIGICGATTACGAI GGTACTACT <u>e</u> GAACAICgacaaCCACAGCTAA <u>T</u> GCTA	ZFN-SDC-2-R1 ZFN-SDC-2-L1	TSGNLTR RSDHLST	NSDNRLK QSADRTK	TSGNLTR QSGDRNK	DHSARAN RSDNLSV	TSGHLSR DNANRKT	- -
CbrSDC2	CCGAGctCACATCGATgatcaaGAAAAACCGGCAGCA GGCTCGgGIGTAGCTA <u>c</u> tagttCTTTTGTGCCGTCTGT	ZFN-SDC-2-R2 ZFN-SDC-2-L2	QSGDLTR RSDNLSQ	QSGDLTR TSGSLTR	RSDLSV RSASLLR	DRSNRIT TSGNLTR	QSGNLAR DSSSRAA	- -

**Table S2****Multiple *ben-1* mutant worms originate from individual injected parents**

<b>Worm</b>	<b>No. of Progeny</b>	<b>No. of mutants</b>	<b>Frequency (%)</b>
<b><u>Time course #1</u></b>			
101	65	2	3.1
105	195	10	5.1
106	123	2	1.6
108	141	7	5.0
116	158	2	1.3
<b>Total:</b>	<b>682</b>	<b>23</b>	<b>3.3</b>
<b><u>Time course #2</u></b>			
3	118	4	3.3
8	163	11	6.7
10	181	2	1.1
16	17	1	5.9
<b>Total:</b>	<b>479</b>	<b>18</b>	<b>3.7</b>

Total number of progeny and mutant progeny from *C. elegans* hermaphrodites injected with ZFNs targeting *Ce-ben-1*. Eleven additional injected hermaphrodites gave no mutant progeny. All mutations were confirmed either by sequencing or CEL-1 endonuclease assays.

**Table S3 Experimental genome-wide evaluation of ZFN action**

**A**

**Experimentally determined binding preferences as gauged via SELEX analysis**

ZFN	Base frequency matrix																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
<b>BEN-1-L1</b>	A	0.00	0.00	0.85	0.85	0.00	0.07	0.93	0.07	0.07	0.85	0.00	0.70	0.56	0.00	0.00	0.00	0.00	0.00	
	C	0.11	0.15	0.11	0.04	0.00	0.07	0.00	0.44	0.89	0.00	0.07	0.30	0.04	0.00	0.00	0.96	0.07	0.00	
	G	0.89	0.07	0.04	0.04	1.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.41	1.00	1.00	0.04	0.00	0.96	
	T	0.00	0.78	0.00	0.07	0.00	0.81	0.07	0.48	0.04	0.15	0.93	0.00	0.00	0.00	0.00	0.00	0.93	0.04	
<b>BEN-1-R1</b>	A	0.33	0.56	0.07	1.00	0.98	0.86	0.00	0.00	1.00	0.02	0.00	0.00	0.00	0.93	0.02	0.07			
	C	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	1.00	0.00	0.00	0.02			
	G	0.00	0.42	0.93	0.00	0.02	0.12	0.95	1.00	0.00	0.07	0.00	0.00	1.00	0.00	0.07	0.98	0.67		
	T	0.67	0.00	0.00	0.00	0.00	0.02	0.05	0.00	0.00	0.91	0.93	1.00	0.00	0.00	0.00	0.00	0.23		
<b>SDC-2-L2</b>	A	0.05	0.29	0.00	0.02	0.00	0.95	0.02	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.40	0.00	0.10		
	C	0.80	0.61	0.05	0.95	0.00	0.00	0.00	0.00	0.60	0.00	0.24	0.00	0.12	0.00	0.98	0.02	0.02	0.00	
	G	0.07	0.10	0.05	0.02	1.00	0.05	0.00	1.00	0.19	0.98	0.12	1.00	0.00	0.00	0.02	0.57	0.98	0.67	
	T	0.07	0.00	0.90	0.00	0.00	0.00	0.98	0.00	0.19	0.00	0.64	0.00	0.88	1.00	0.00	0.00	0.00	0.24	
<b>SDC-2-R2</b>	A	0.09	0.02	0.96	0.76	0.24	0.96	0.00	0.89	0.04	0.00	0.00	0.18	1.00	0.00	0.00	0.14	0.00		
	C	0.24	0.00	0.02	0.00	0.58	0.00	1.00	0.04	0.44	0.00	0.00	0.82	0.00	0.00	0.98	0.02	0.12		
	G	0.36	0.98	0.02	0.24	0.11	0.04	0.00	0.02	0.02	1.00	1.00	0.00	0.00	1.00	0.02	0.00	0.09		
	T	0.30	0.00	0.00	0.00	0.07	0.00	0.00	0.04	0.49	0.00	0.00	0.00	0.00	0.00	0.00	0.84	0.79		

Each matrix provides the base preferences corresponding to the intended targets in Table S1 plus a single flanking base on either side. Matrices were derived via (i) alignment of selected targets (27-45 for each ZFP); (ii) summing of base types at each position in the alignment (using only those bases originating in the randomized region of the SELEX library); and (iii) conversion of base counts to frequencies.

**Table S3**

**B**

**Prediction and interrogation of putative off-target sites**

OT site	Score	Chromosome	Location	Site configuration ZFN / gap / ZFN	Sequence	ben-1 strains with mutations at the OTS
BEN-1.1	9.98E-15	III	10742178	BEN-1-R1 / 6 / BEN-1-L1	GCTGgAgATCGCTTTCCCTTgAgGTtCCgTcGGGCTG	0 / 5
BEN-1.2	3.99E-16	V	9655302	BEN-1-R1 / 5 / BEN-1-L1	TCTGtAAATtACTTTCTAAGTTTgAGaAAATtCGGCTG	0 / 5
BEN-1.3	3.80E-16	I	6446608	BEN-1-R1 / 6 / BEN-1-R1	ACTGCAAAaCaTTTTCTCGCGCAaAAATGGAagTGCACT	0 / 5
BEN-1.4	2.79E-16	II	13129713	BEN-1-L1 / 6 / BEN-1-R1	CAAtCCTATaATACTTtCGAGTTGAAAaCGtTTTGCAtG	0 / 5
BEN-1.5	2.05E-16	III	1675009	BEN-1-L1 / 5 / BEN-1-L1	CAGCaCaATGAAACTTtCATTtTAAGTATaATAAtGaTT	0 / 5
BEN-1.6	1.96E-16	III	13024373	BEN-1-R1 / 5 / BEN-1-R1	CCgGCAAAATaACTTTtgTGACCAaAtAGTGATTTGtgGG	0 / 5
BEN-1.7	1.72E-16	II	1825492	BEN-1-R1 / 6 / BEN-1-R1	ACTcCAAATCAgTTTCAAGAAATtGAAAGAGcTcaGCgGG	0 / 5
BEN-1.8	8.45E-17	IV	10441810	BEN-1-L1 / 5 / BEN-1-L1	CAGCaTtAaGAcAcAtTtCAGGGTtTcTATaATtGGGCTG	0 / 5
BEN-1.9	8.34E-17	III	8049590	BEN-1-L1 / 5 / BEN-1-R1	CAaCCTcAaGATAcTtGAAATAGAAAGTAttGgAGT	0 / 5
BEN-1.10	2.66E-14	III	1231193	BEN-1-L1 / 5 / BEN-1-L1	GAGCCCTtTtTACTTtCCTTGTAAGcAaATAGtTCTG	0 / 5
BEN-1.11	2.68E-15	II	14424251	BEN-1-L1 / 6 / BEN-1-R1	CAaCTgAaGaAtTtGATTTGTGGgAAGGATTTGtAGG	0 / 5
BEN-1.12	1.16E-15	III	7587740	BEN-1-L1 / 6 / BEN-1-R1	AAGgtCTATGTACTTACAATAGAAAtcATTtGtgaA	0 / 5
BEN-1.13	1.11E-15	V	1410835	BEN-1-L1 / 5 / BEN-1-R1	CAGCCTgATtagCTaACTATAGGAtAGGATTaGgAtT	0 / 5
BEN-1.14	9.81E-16	IV	12908713	BEN-1-R1 / 6 / BEN-1-L1	TtTGCAAATCCcTTcGGAAGAGTcAagtaATcAGGtTG	0 / 5
BEN-1.15	9.49E-16	III	13459570	BEN-1-R1 / 5 / BEN-1-L1	CCTGCAgATCCTcTgCAGCCcGAAAGTaaAaAaGaTG	0 / 5
BEN-1.16	5.64E-16	IV	795183	BEN-1-R1 / 6 / BEN-1-R1	CCTGCAGcaCCTcTCCATTGGAAGAAgcccAgTTGaAGT	0 / 5
BEN-1.17	5.42E-16	II	1058131	BEN-1-L1 / 5 / BEN-1-R1	CAGCtTgAaGtTtTACTAATAcAAttaATTtCcAGG	0 / 5
BEN-1.18	5.42E-16	II	1022459	BEN-1-L1 / 5 / BEN-1-R1	CAGCtTgAaGtTtTACTAATAcAAttaATTtCcAGG	0 / 5
BEN-1.19	4.93E-16	III	12198848	BEN-1-L1 / 5 / BEN-1-L1	TAGtaCTgTGTtCaTACTGTGgAAGTACATAGGggG	0 / 5
BEN-1.20	4.12E-15	I	7922056	BEN-1-R1 / 6 / BEN-1-L1	TCTGaAAATtCaTTCTCTGTTGTAAGTcTtTtGGGCTG	0 / 5
BEN-1.21	2.43E-15	V	1826797	BEN-1-R1 / 5 / BEN-1-R1	CCTGtAAATcCTTTCaAAGTAAGAAttGAgTtCaAaA	0 / 5
BEN-1.22	1.06E-15	X	14816526	BEN-1-L1 / 6 / BEN-1-R1	AAGaCCaATtGccCggACTGTTTAGAAAGGATTTGCAaA	0 / 5
BEN-1.23	9.07E-16	X	14803752	BEN-1-L1 / 6 / BEN-1-L1	CAGaCTTaaGTTACagcCCAGTCTgAGcACCAccAGGCaG	0 / 5
BEN-1.24	5.93E-16	V	5974675	BEN-1-R1 / 5 / BEN-1-R1	TCTGtAcTCCTTTCTTTCCaAAgGcGATTtCcGC	0 / 5
BEN-1.25	4.75E-16	IV	15511115	BEN-1-L1 / 6 / BEN-1-R1	CtaCCTTATGCTACcTAATTGTAAGAAAGGAggTGtAGA	0 / 5
BEN-1.26	4.38E-16	III	4921141	BEN-1-R1 / 5 / BEN-1-R1	ACTtCgAcTaCTTTCTTGATtCGAAAGaATTTtCcGG	0 / 5
BEN-1.27	3.78E-16	IV	13997007	BEN-1-L1 / 5 / BEN-1-R1	AAGCCtAaTGGTACTaACTAGTGGAgAcaAaTTGtAGT	0 / 5
BEN-1.28	3.21E-16	II	1092317	BEN-1-R1 / 6 / BEN-1-R1	AgTGgAAATtCaTctTTTGGGTGGAAGGATcTtCAGT	0 / 5
BEN-1.29	3.03E-16	V	3923153	BEN-1-L1 / 5 / BEN-1-R1	CAcCCCTATGGTgtTcACAAAAGtAAAGaATTaGaAGT	0 / 5
BEN-1.30	1.09E-15	X	3346330	BEN-1-L1 / 5 / BEN-1-R1	CAGCCTTcTGtCCTTgGTGGCcGAaAGATTGcCaG	0 / 5
BEN-1.31	1.07E-15	I	8421834	BEN-1-R1 / 5 / BEN-1-R1	ACcGaAAATtTgTTtCATTGTaAAAGTATTtCAGA	0 / 5
BEN-1.32	9.64E-16	I	14098964	BEN-1-L1 / 5 / BEN-1-R1	TAtCtGAAAatCTTACAACAAaAAAGAGATTtCAGG	0 / 5
BEN-1.33	9.50E-16	V	11292329	BEN-1-R1 / 5 / BEN-1-L1	TCTGCAAcTCTaTTTCCAATATtTtTtTtTtGGCaG	0 / 5
BEN-1.34	8.39E-16	V	7031670	BEN-1-L1 / 6 / BEN-1-R1	AAtCCagATGACTTtCTCAGCGGAAAGTGATtCcGgAcG	0 / 5
BEN-1.35	7.07E-16	V	5492075	BEN-1-R1 / 6 / BEN-1-L1	ACTGgAgATCACTTtCCAATGTcAAGaAcTtGgGGtTG	0 / 5
BEN-1.36	3.31E-16	I	9295629	BEN-1-L1 / 5 / BEN-1-L1	AAGCaTtTtTGACTTcCGGTGagAGaAaATAAGaaTG	0 / 5
BEN-1.37	2.69E-16	I	4316734	BEN-1-R1 / 5 / BEN-1-L1	CCTGCcAcTaAATTTtCTAGGAtGcACAgAAGGCTG	0 / 5
BEN-1.38	1.42E-16	II	10039952	BEN-1-R1 / 6 / BEN-1-L1	CCTGcgtATCtTaTTCTCATATGatAtTtCATAAGGCTG	0 / 5
BEN-1.39	1.28E-16	IV	12074969	BEN-1-R1 / 6 / BEN-1-L1	AaTGCAAAATCGtTTTtTCTACAGTgAGTaaAacAGGagG	0 / 5
SDC-2.1	3.50E-14	X	19375347	SDC-2-R2 / 5 / SDC-2-R2	CgGCTGCCAaGtaaTCGCTTTGAAaAcTtGaAGCTT	0 / 4
SDC-2.2	1.98E-15	V	12386556	SDC-2-R2 / 5 / SDC-2-R2	TAGgTtCCcTGtTtTtTGTCGAACACACGaCAaCTC	0 / 4
SDC-2.3	1.57E-15	I	5647186	SDC-2-R2 / 6 / SDC-2-R2	CtGCTGCCGaGtTgctCAGAGAGAACACATGGCAtaC	0 / 4
SDC-2.4	3.26E-16	V	11968850	SDC-2-R2 / 5 / SDC-2-R2	AAGCTGCCGtTtTtTCAAACtTcAaAcCtTaGcGaCTT	0 / 4
SDC-2.5	1.84E-16	unk	6672355	SDC-2-R2 / 6 / SDC-2-R2	GAGCTGgCGTGTGtaCTCGGTGGActAgtTGGAAGCaC	0 / 4
SDC-2.6	1.07E-16	V	10787253	SDC-2-R2 / 5 / SDC-2-R2	CAtCtCgGTGtTtTtCAATTGAgCACtTcGaAtCCTT	0 / 4
SDC-2.7	9.85E-17	II	10148077	SDC-2-R2 / 6 / SDC-2-R2	AAGCTtCCGTGTGggCCAGCGCAACACtaGaaAcaTG	0 / 4
SDC-2.8	9.23E-17	X	19141508	SDC-2-R2 / 5 / SDC-2-R2	CttCTgatTGTGTTCTGTCAGcAACAcGtGGCAcTCTT	0 / 4
SDC-2.9	6.39E-17	III	9278590	SDC-2-R2 / 5 / SDC-2-R2	AAGaattGcGTGTTcATGGGGAaACATtGCAGaTT	0 / 4

SDC-2.10	2.39E-13	III	6893629	SDC-2-L2 / 6 / SDC-2-R2	CaTGAACCGCATCGAGGCTCGTcAACAAaAaGaaGAGCTC	0 / 4
SDC-2.11	1.45E-15	V	14204195	SDC-2-R2 / 5 / SDC-2-L2	AAGCTttgATGctTTCAGATCaTCGATGaaGTTcttG	0 / 4
SDC-2.12	1.13E-15	V	3152553	SDC-2-R2 / 6 / SDC-2-L2	AtaCTGCCGatTtTTCaATGGCCcCGATGCcGaTgAGG	0 / 4
SDC-2.13	9.95E-16	X	19891015	SDC-2-R2 / 5 / SDC-2-L2	CtGCcGaCGTGTGcgCAATTcagCGATGCcGTTcGtG	0 / 4
SDC-2.14	7.09E-16	IV	6749771	SDC-2-L2 / 6 / SDC-2-L2	AgCGAgCctCATCGAGAACGGGctcCGATGtGGTcCtcG	0 / 4
SDC-2.15	4.76E-16	X	14045641	SDC-2-L2 / 5 / SDC-2-R2	TgTGcAtCGCcTCGctGACAGGAACAAATGGctGCTT	0 / 4
SDC-2.16	4.32E-16	I	6657292	SDC-2-R2 / 5 / SDC-2-L2	AttCatCaGTGTtTTCATATTCTctGtGGTTCCGGT	0 / 4
SDC-2.17	3.71E-16	V	15806744	SDC-2-R2 / 6 / SDC-2-R2	TgGaaGcTATGTacTCGACACTGAACACATGGaAGTTT	0 / 4
SDC-2.18	2.74E-16	V	1408569	SDC-2-R2 / 6 / SDC-2-L2	GAGCgGCaAcGTtgTCAAGAACCgaaATGgGGTTcAGG	0 / 4
SDC-2.19	2.23E-16	X	6018644	SDC-2-R2 / 6 / SDC-2-R2	AttCgGCCAcGatTgCATTCTGAgCACcCGGCAGgTT	0 / 4

The top ranked off-target sites were identified as previously described<sup>15</sup> using the SELEX-derived base frequency matrices in Table S3A. Capital letters indicate either (i) a match to the SELEX consensus or (ii) a non-targeted position within the ZFN dimer site. The genome searches for candidate off-target sites allowed for ZFN site pairings with 5 or 6 bp between individual targets to reflect the ability of our designed ZFNs to cleave equally well at these two spacings. Likewise, we also allowed an optional gap of 1 bp between the 7th and 8th positions of the BEN-1-R1 matrix in order to reflect the binding characteristics of a longer flexible linker between the third and fourth fingers of this protein which allows binding to either target type. For a similar reason (potential alternative configurations for a long, flexible linker) we also allowed optional removal of position #8 of the BEN-1-L1 matrix and position #11 the SDC-2-L2 matrix when searching for and ranking potential off-target sites.

Genomic DNA from five independent *ben-1* mutant lines was prepared from populations treated as follows. Homozygous *ben-1* mutants were isolated from among the F2 of injected hermaphrodites, and the population was expanded by selfing. All off-target mutations present in the homozygous F2 animal should therefore be represented in the population from which genomic DNA was prepared. For each site, both strands of a short amplicon centered on the OTS were directly sequenced and compared to sequence obtained from a WT control. We detected no mutations at any of the OT sites for the five *ben-1* mutant lines analyzed.

## References and Notes

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