Figure S1 Schematic representation of deletion/insertion experiments on *K08F4.2* gene

Black lines represent genomic DNA. Red lines represent inserted sequence and blue lines are homology arms on template DNA. Scissors denote the sgRNA recognition sequence (pairing site + PAM). HDR – homology-direct repair. NHEJ – non-homologous end-joining. RE: restriction enzyme site.

(A) and (B) Experiments 2 and 3 (also see Table 1). Simultaneous small deletion (24bp) and restriction enzyme site (RE) insertion.

(C) Experiment 22. The homology arms in the donor template do not extend to the cuts and as a result, NHEJ is preferred over HDR and the donor template is not used. This experiment demonstrates that separation of the homology arms from the cut sites by as little as 10-31 bp favors NHEJ. (Note that we know that at least sgRNAs APs1, 4 and 5 are functional based on Exp. 2, 3 and 6).

(D) Experiment 25. The homology arms in the donor template extend to the cuts and as a result, HDR is preferred over NHEJ, and the donor template is integrated as evidenced by insertion of the restriction enzyme site (RE).

(E) Experiment 26. Same as in Exp. 25 except that only one sgRNA (APs5) was used. No large deletions were obtained.
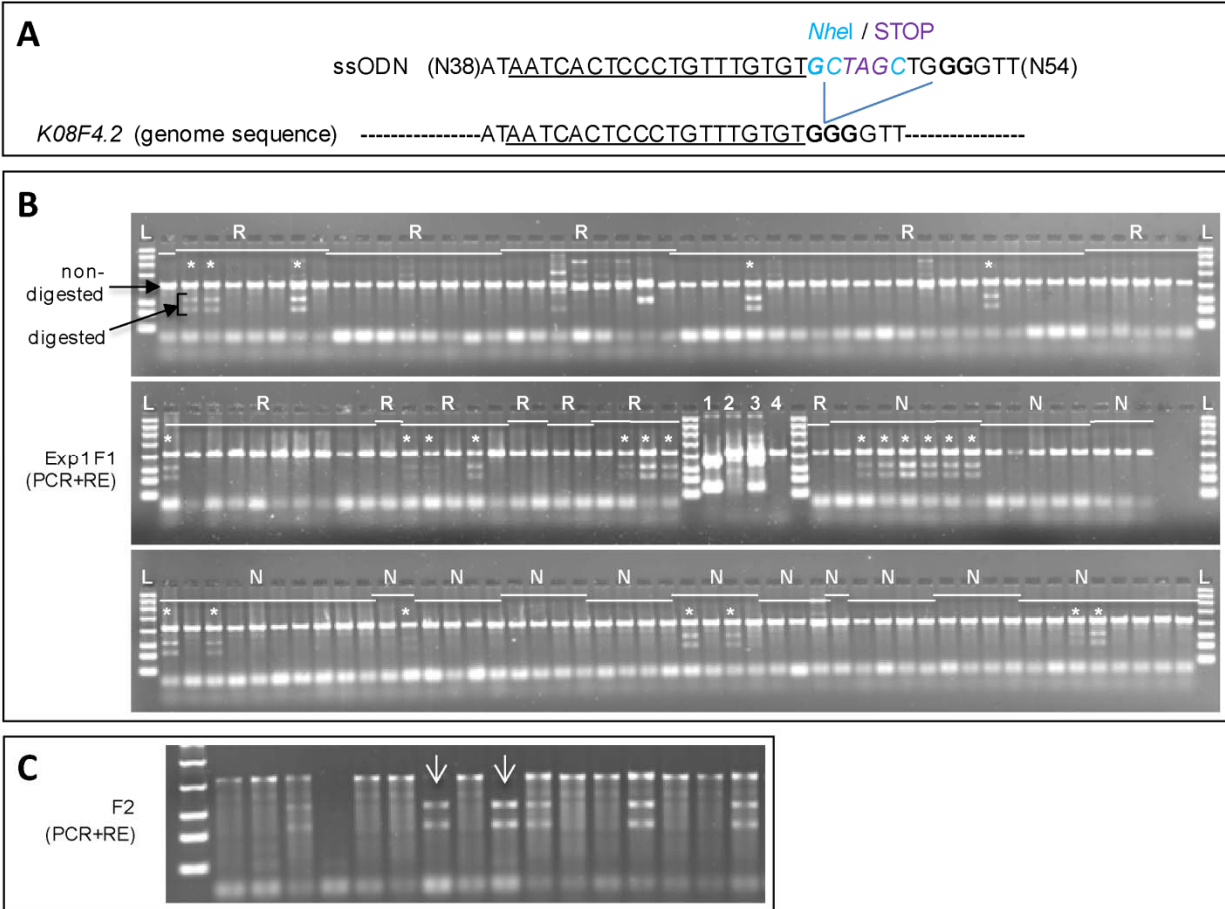
Figure S2   PCR and restriction digest analysis of F1 pools from Exp. 1

(A) Schematic representation of the ssODN1 used in Exp. 1 and the targeted locus: italic (restriction enzyme site), bold (PAM sequence), underlined (sgRNA sequence). N indicate the length of the homology arms.

(B) Subset of restriction digests used to analyze F1s from Exp. 1. White bars link F1s derived from the same injected hermaphrodite. White asterisks show F1s positive for the engineered *Nhe*I restriction site. L: DNA ladder, R: Roller F1s, N: Non-Roller F1s, Lanes labeled 1-4 are as follows: 1) Control PCR fragment containing a *Nhe*I site, 2) control PCR fragment without *Nhe*I site, 3) Mix of PCR fragments with and without *Nhe*I site, and 4) N2 lysate (wild-type control).

(C) Restriction digests used to analyse F2s from Exp. 1. Arrows point to homozygous edits.
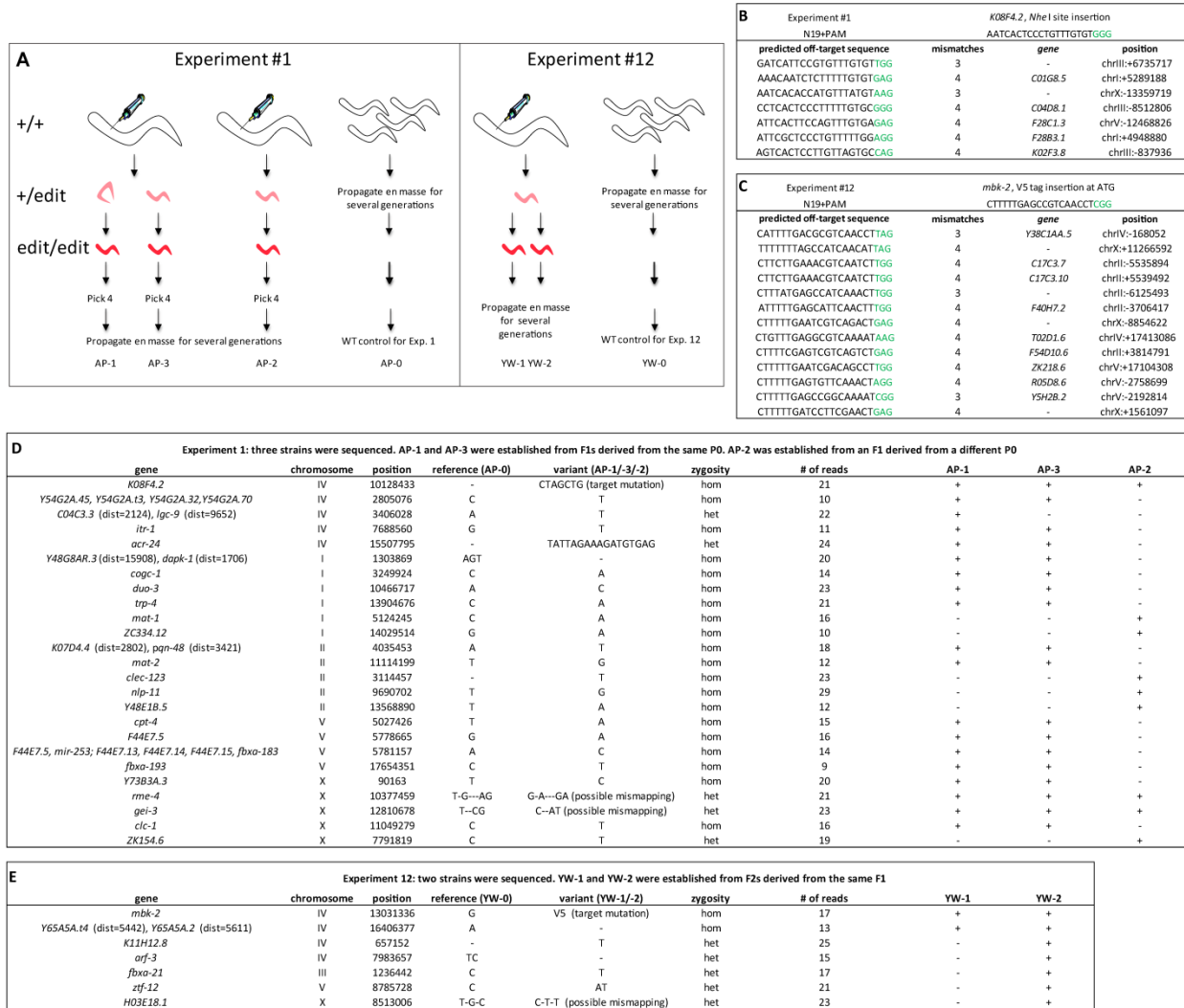
Panel A: Experiment #1 / Experiment #12 schematic diagram.

B

Experiment #1		K08F4.2, *Nhe* I site insertion	
N19+PAM		AATCACTCCCTGTTTGTGTGGG	
predicted off-target sequence	mismatches	gene	position
GATCATTCCGTGTTTGTGTTGG	3	-	chrIII:+6735717
AAACAATCTCTTTTTGTGTGAG	4	C01G8.5	chrI:+5289188
AATCACACCATGTTTATGTAAG	3	-	chrX:-13359719
CCTCACTCCCTTTTTGTGCGGG	4	C04D8.1	chrIII:-8512806
ATTCACTTCCAGTTTGTGAGAG	4	F28C1.3	chrV:-12468826
ATTCGCTCCCTGTTTTTGGAGG	4	F28B3.1	chrI:+4948880
AGTCACTCCTTGTTAGTGCCAG	4	K02F3.8	chrIII:-837936

C

Experiment #12		*mbk-2*, V5 tag insertion at ATG	
N19+PAM		CTTTTTGAGCCGTCAACCTGGG	
predicted off-target sequence	mismatches	gene	position
CATTTTGACGCGTCAACCTTAG	3	Y38C1AA.5	chrIV:-168052
TTTTTTTAGCCATCAACATTAG	4	-	chrX:+11266592
CTTCTTGAAACGTCAATCTTGG	4	C17C3.7	chrII:-5535894
CTTCTTGAAACGTCAATCTTGG	4	C17C3.10	chrII:+5539492
CTTTATGAGCCATCAAACTTGG	3	-	chrII:-6125493
ATTTTTGAGCATTCAACTTTGG	4	F40H7.2	chrII:-3706417
CTTTTTGAATCGTCAGACTGAG	4	-	chrX:-8854622
CTGTTTGAGGCGTCAAAATAAG	4	T02D1.6	chrIV:+17413086
CTTTTCGAGTCGTCAGTCTGAG	4	F54D10.6	chrII:+3814791
CTTTTTGAATCGACAGCCTTGG	4	ZK218.6	chrV:+17104308
CTTTTTGAGTGTTCAAACTAGG	4	R05D8.6	chrV:-2758699
CTTTTTGAGCCGGCAAAATCGG	3	Y5H2B.2	chrV:-2192814
CTTTTTGATCCTTCGAACTGAG	4	-	chrX:+1561097

D

Experiment 1: three strains were sequenced. AP-1 and AP-3 were established from F1s derived from the same P0. AP-2 was established from an F1 derived from a different P0								
gene	chromosome	position	reference (AP-0)	variant (AP-1/-3/-2)	zygosity	# of reads	AP-1	AP-3	AP-2
K08F4.2	IV	10128433	-	CTAGCTG (target mutation)	hom	21	+	+	+
Y54G2A.45, Y54G2A.t3, Y54G2A.32, Y54G2A.70	IV	2805076	C	T	hom	10	+	+	-
C04C3.3 (dist=2124), lgc-9 (dist=9652)	IV	3406028	A	T	het	22	+	-	-
itr-1	IV	7688560	G	T	hom	11	+	+	-
acr-24	IV	15507795	-	TATTAGAAAGATGTGAG	het	24	+	+	-
Y48G8AR.3 (dist=15908), dapk-1 (dist=1706)	I	1303869	AGT	-	hom	20	+	+	-
cogc-1	I	3249924	C	A	hom	14	+	+	-
duo-3	I	10466717	A	C	hom	23	+	+	-
trp-4	I	13904676	C	A	hom	21	+	+	-
mat-1	I	5124245	C	A	hom	16	-	-	+
ZC334.12	I	14029514	G	A	hom	10	-	-	+
K07D4.4 (dist=2802), pqn-48 (dist=3421)	II	4035453	A	T	hom	18	+	+	-
mat-2	II	11114199	T	G	hom	12	+	+	-
clec-123	II	3114457	-	T	hom	23	-	-	+
nlp-11	II	9690702	T	G	hom	29	-	-	+
Y48E1B.5	II	13568890	T	A	hom	12	-	-	+
cpt-4	V	5027426	T	A	hom	15	+	+	-
F44E7.5	V	5778665	G	A	hom	16	+	+	-
F44E7.5, mir-253; F44E7.13, F44E7.14, F44E7.15, fbxa-183	V	5781157	A	C	hom	14	+	+	-
fbxa-193	V	17654351	C	T	hom	9	+	+	-
Y73B3A.3	X	90163	T	C	hom	20	+	+	-
rme-4	X	10377459	T-G---AG	G-A---GA (possible mismapping)	het	21	+	+	+
gei-3	X	12810678	T--CG	C--AT (possible mismapping)	het	23	+	+	+
clc-1	X	11049279	C	T	hom	16	+	+	-
ZK154.6	X	7791819	C	T	het	19	-	-	+

E

Experiment 12: two strains were sequenced. YW-1 and YW-2 were established from F2s derived from the same F1								
gene	chromosome	position	reference (YW-0)	variant (YW-1/-2)	zygosity	# of reads	YW-1	YW-2
mbk-2	IV	13031336	G	V5 (target mutation)	hom	17	+	+
Y65A5A.t4 (dist=5442), Y65A5A.2 (dist=5611)	IV	16406377	A	-	hom	13	+	+
K11H12.8	IV	657152	-	T	het	25	-	+
arf-3	IV	7983657	TC	-	het	15	-	+
fbxa-21	III	1236442	C	T	het	17	-	+
ztf-12	V	8785728	C	AT	het	21	-	+
H03E18.1	X	8513006	T-G-C	C-T-T (possible mismapping)	het	23	-	+

Figure S3   Whole genome sequencing of strains obtained in Exp. 1 and 12

(A) Description of the strains used for genome sequencing.

(B) and (C) List of the possible off-targets for the sgRNAs used in Exp. 1 and 12.

(D) and (E) Lists of all variants found in edited strains obtained in Exp.1 and 12. The variants were identified by comparison with sequence obtained from wild-type populations maintained by AP and YW who conducted Exp. 1 and 12. None of the variants map to the predicted off-targets shown in B and C, and none show homology to the ssODNs used in the experiments.

Note that the edited strains were established from single hermaphrodites that underwent at least two sequential rounds of cloning to homozygoze the edits. In contrast, the wild-type reference sequences were obtained from populations that were passaged by picking multiple hermaphrodites for several generations between the time of injection and sequencing. The wild-type populations, therefore, may have lost variants present at low frequency in the population on the day of injections.

Gene: name of the gene at the indicated position in the genome. "(dist=)" indicates the genomic position of the closest gene; Position: position in the genome; Reference: sequence in the N2 reference strain; Variant: sequence variation found in the established strain; Hom: homozygous; Het: heterozygous.

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Table S1   Expanded version of Table 1

Available for download as an Excel file at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170423/-/DC1

Table S2   sgRNAs sequences

sgRNA	sense/antisense	Sequence (N19/20)	Gene	Location
APs5	s	cacgaggtggtatgcgcag	*K08F4.2*	3'end
APa4-2	a	aatcactccctgtttgtgt	*K08F4.2*	in ORF
APs1	s	gccttaacccagaataaga	*K08F4.2*	5'end
APs4	s	tattaaatgcagataacct	*K08F4.2*	5' end
APs6	s	cgcagcggtttccaaaatg	*K08F4.2*	3' end
APs12	s	gtagtcacggcgatgatta	*fbf-2*	3'end
APa13	a	taatcatcgccgtgactac	*fbf-2*	3'end
HS516	s	ctttttgagccgtcaacat	*mbk-2*	5'end
JS117	s	atggactggagtcgatggg	*mbk-2*	3'end
JS118	s	ggaggacttgccgatcatg	*mbk-2*	3'end
JS129	s	gcatcaaatagtgtctcgt	*mex-5*	5'end
JS130	s	atagtgtctcgtcggccgg	*mex-5*	5'end
SL225	a	gagtcgaagtcggttcact	*nos-2*	5'end
SL232	a	ctgattcgagagtcgaagt	*nos-2*	5'end
TL001	s	tcgtggacgtggtggttac	*pgl-1*	3'end
TL002	s	gtggtggttacgggggtcg	*pgl-1*	3'end
CSD35	a	tagccattgcaggtgataa	*swan-1*	5'end
CSD53	a	tgaagaaagttatactcga	*swan-2*	3'end
CSD54	a	acaaattgatatccaatca	*swan-2*	3'end
sg3	s	ggtctgcgaggacactatt	*mex-6*	3'end
sg4	s	ccctcaagatgaagaaggc	*glh-1*	3'end
sg5	s	gaggaaactgaacgatttc	*htp-3*	3'end
sg6	s	acaaagctcaacatcttcc	*deps-1*	3'end
sg16	a	aagattattcagaagtcat	*mes-2*	3'end
sg18	a	atagtgtcctcgcagaccg	*mex-6*	3'end
sg21	a	tatatatttaattagaccc	*deps-1*	3'end
sg23	s	ccacccagcaacgaataag	*lin-15b*	3'end
sg25	a	atttccacttattcgttgc	*lin-15b*	3'end

s: sense sgRNA / a: antisense sgRNA

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Table S3   Repair template sequences

Name	Sequence	Transcript	Description
ssODN1	GgtaaacacgacgttcttcgattttcctacaaatatatttttaagGGATACCCAACCC*CAGCTAGC*ACACAAACAGGGAGTGATTATCAATGTTATCGGAACCGTTAACCTCCGTCCATTCCTTC	*K08F4.2*	*Nhe*I site + frame shift + premature STOP
ssODN2	cgacggaaaggggaaattcaagccatttgagtttttgttcttcttgaaagATGTCTCTGGGTACT---- 15 bp deletion ----TCGACTCTCGAA*CGATCG*TAATAGTCAGACATTTTTGACGATCTTTCGTTGTATTATCTAGAGgtaaaatatcaatataaattttaaaaaataattaaattttaaaatttcagG	*ZK1127.1*	Small deletion + *Pvu*I site + premature STOP
ssODN3	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGCAGCGGTTTCCAAAATGCCGGACAAAAT----*TetraCys*----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	TetraCys Tag at STOP
ssODN4	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGACAAAAT----*Myc*----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	Myc Tag at STOP
ssODN5	GCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGACAAAAT----*3X Flag*----tagaagctttccgttctccttttttccttcttgtaagaattgcacatcc	*K08F4.2*	3X Flag Tag at STOP
ssODN6	gaaagttaaagtgagttgagagtgtaacacactcggccacacacacacaagaATG----*V5*----ACACTTTTTGAGCCGTCAACATCTGGCAATCGAATGGGGTATAGAGgtaagagagtttcaataaaacgtcactttcc	*F49E11.1a*	V5 Tag after ATG
ssODN7	ctctcttaattaattttatcgataatcaattgaatgtttcagacagagaATG----*V5*----AAAGCGGCATCAAATAGTGTGTCCTCCGCAGGAGGATCAGTGTCACCTACGACAACCCAGCCACCACTACCACCAGGGC	*W02A2.7*	V5 Tag after ATG
ssODN8	ctctcttaattaattttatcgataatcaattgaatgtttcagacagagaATG----*3X Flag*----AAAGCGGCATCAAATAGTGTGTCCTCCGCAGGAGGATCAGTGTCACCTACGACAACCCAGCCACCACTACCACCAGGGC	*W02A2.7*	3X Flag Tag after ATG
ssODN9	ctctcttaattaattttatcgataatcaattgaatgtttcagacagagaATG----*OLLAS*----AAAGCGGCATCAAATAGTGTGTCCTCCGCAGGAGGATCAGTGTCACCTACGACAACCCAGCCACCACTACCACCAGGGC	*W02A2.7*	OLLAS Tag after ATG
ssODN10	aaggggaaattcaagccatttgagtttttgttctttcttgaaagATG----*3X Flag*----TCTCTGGGTACTCCATCCGAGCCAACCAGTACCCTCGAATCAGACATTTTTGACGATCTTTCGTTGTATTATCTAGAGgta	*ZK1127.1*	3X Flag Tag after ATG
ssODN11	GAGACCGCGGAGGACGAGGTGGATACGGCGGAGATCGTTGGAAGAGGCGGCTATGGTGGGAGAGGTGGACGCGGAGGTTTC----*3X Flag*----taaactaccaactattgaatgtttaatttgtttttttaagtatacacttcttg	*ZK381.4*	3X Flag Tag at STOP
ssODN12	GTTGTGCGACTTGTTGCATAACATGAGCTTCTCCGTTCACTGCGTGACCATTGGTAGC----*V5*----CATtgcaggtgataaaggttcacaagtttctgcaagataaaacactgcgtgaggggattattagaataattggaaactagtaagcgaacgagggaaagag	*F53C11.8*	V5 Tag after ATG
ssODN13	GTTGTGCGACTTGTTGCATAACATGAGCTTCTCCGTTCACTGCGTGACCATTGGTAGC----*Myc*----CATtgcaggtgataaaggttcacaagtttctgcaagataaaacactgcgtgaggggattattagaataattggaaactagtaagcgaacgagggaaagagcgaaaaaaagt	*F53C11.8*	Myc Tag after ATG
ssODN14	cgtcatcttttacataaaccgtttttaaaattataaacaaaaataaacattttcaggtaattattaa----ORF deletion----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	ORF deletion
ssODN15	ccgtttttaaaattataaacaaaaataaacattttcaggtaattattaaATCCAGATAACCTCGGCCTTAACCCAGAATAAGA*GCTAGC*----ORF deletion-----CGGTTTCCAAAATGCGGGACAAAATtagaagctttccgttctccttttttcc	*K08F4.2*	ORF deletion + *Nhe*I site
ssODN16	ccgttttaaaattataaacaaaaataaacattttcaggtaattattaaATGCAGATAACCT*GCTAGC*T----24bp deletion----ATCCGATCAATGGTAATTTGACTTCCACCGCTCCGGTCGAGCCACTCTCATTG	*K08F4.2*	Small deletion + *Nhe*I site + premature STOP
ssODN17	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGACAAAAT----*HA*----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	HA Tag at STOP
ssODN18	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGACAAAAT----*V5*----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	V5 Tag at STOP
ssODN19	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGACAAAAT----6X His----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	6X His Tag at STOP
PCR1	GTTGGCCCTTCAAGCCGCAGAACTCCATCTGGTACTCCACAAAGCTCAACATCTTCCAGAGTC----GFP----taattaaatatatacgcatcccgtttttccccgtatttgtgtttcaaatgttctgctc	*Y65B4BL.2a*	GFP at STOP
PCR2	CGGAGCAATAAGTCTTCAGTCTCTGTCAAATTTTCTTCTTCTGGTAGTCACGGCGATGAT----GFP----taaggtggaactttctcaccataaatctcatcccacccacttatgttctgttgttttg	*F21H12.5*	GFP at STOP
PCR3	GGCAATTACGGAGCTAGTGGATTTGGGTCCAGTGTACCAACTCAAGTCCCTCAAGACGAGGAGGGATGG----GFP----TAGaaaaccgaccaattgatagtgtttcgcatttattaatgctgtcagttcccccatatttatcc	*T21G5.3*	GFP at STOP
PCR4	CCCTCCAAAGGCAATGAGGTACGGCCAGTCGCCGAACATGCCAAGTAGAAGAGGAAAC----GFP----tgaacgatttctggacaatcgtgtacaattatcAatctgttccattttttttgttgtttcttcg	*F57C9.5*	GFP at STOP
PCR5	GCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCCACGAGGTGGTATGCGC----GFP----AGCGGTTTCCAAAATGCGGGACAAAATtagaagctttccgttctccttttttccttcttg	*K08F4.2*	GFP at cut, near STOP
PCR6	GGTTTCCAACCACGCGGTGGACAATTCTTCCGCCGTGGCGGTTCGGGTGGTGCTCCGCGTGGAGGAATGCGTAGCGGTTTCCAAAATGCGGGACAAAAT----GFP----tagaagctttccgttctccttttttccttcttgtaagaattgcacatccattagattc	*K08F4.2*	GFP at Stop
PCR7	GGACGACCGCGCAAAGATGCTAATAAGCTGCCAACCCCCACACCACCCAGCAACGAA----GFP----taagtggaaatattttcatccaccgttcctgattgttgttttatatataattttttctcc	*ZK662.4*	GFP at STOP
PCR8	CCAAGGAGAGATCCGAGAAGCCCAGCAGGCCCAAAAGCCAGAAACTCTCCAAACCAATGACTTCTGAA----GFP----taatcttttttcttgacttttttttctaaatttttcccgttgatatcactttcaaatctc	*R06A4.7*	GFP at STOP
PCR9	CCAGAAGTCGTCCATCTTTCAGTACGAAATGGACATCAGTGGAGAATCTCGGTCTGCGAGGACACTAT----GFP----tagggcgtactttaccactccagattgctcactcgtgtatcatttctgtacaaaagcc	*AH6.5*	GFP at STOP

Repair templates: lower case (non coding), upper case (coding), red (silent mutations in the repair template to prevent Cas9 re-cutting), blue (insertion), green (deletion), italic (restriction enzyme), bold (PAM sequence), underlined (sgRNA sequence). GFP templates were amplified using pCM1.53 plasmid (GFP with introns). PCR6 was amplified using a synthetic DNA fragment (gBlock) containing the GFP cDNA sequence. Taq sequences are provided in the Sup Protocol, except for Exp 9 (myc: GAACAGAAACTCATCTCTGAAGAGGATCTG; V5: GGGAAAACCAATTCCAAATCCACTTCTTGGTCTCGATTCTACT); for Exp12 (V5: GGGAAAGCCAATTCCGAATCCGCTTCTCGGCTTGGATTCAACT).

Table S4   GFP edit frequencies by brood and day of egg laying

Available for download as a PDF file at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170423/-/DC1

Table S5 Strain list

Experiment	JH number	Genotype	Type of allele
1	3174	*K08F4.2(ax2027)*	Insertion
1	3175	*K08F4.2(ax2028)*	Insertion
2	3176	*K08F4.2(ax2029)*	Substitution
2	3177	*K08F4.2(ax2030)*	Substitution
4	3180	*nos-2(ax2033)*	Substitution
4	3181	*nos-2(ax2034)*	Substitution
6	3182	*K08F4.2(ax2035[K08F4.2::TetraCys])*	Insertion
6	3183	*K08F4.2(ax2036[K08F4.2::TetraCys])*	Insertion
7	3184	*K08F4.2(ax2037[K08F4.2::Myc])*	Insertion
7	3186	*K08F4.2(ax2039[K08F4.2::3xFlag])*	Insertion
8	3188	*mex-5(ax2041[3xFlag::mex-5])*	Insertion
8	3190	*mex-5(ax2043[OLLAS::mex-5])*	Insertion
9	3134	*swan-1(ax2045[V5::swan-1])*	Insertion
9	3135	*swan-1(ax2046[V5::swan-1])*	Insertion
9	3136	*swan-1(ax2047[Myc::swan-1])*	Insertion
11	3193	*nos-2(ax2049[3xFlag::nos-2])*	Insertion
11	3194	*nos-2(ax2050[3xFlag::nos-2])*	Insertion
12	3195	*mbk-2(ax2051[V5::mbk-2])*	Insertion
13	3197	*K08F4.2(ax2053[K08F4.2::gfp])*	Insertion
13	3198	*K08F4.2(ax2054[K08F4.2::gfp])*	Insertion
14	3199	*K08F4.2(ax2055[K08F4.2::gfp])*	Insertion
14	3200	*K08F4.2(ax2056[K08F4.2::gfp])*	Insertion
15	3201	*fbf-2(ax2057[fbf-2::gfp])*	Insertion
16	3203	*mes-2(ax2059[mes-2::gfp])*	Insertion
16	3204	*mes-2(ax2060[mes-2::gfp])*	Insertion
17	3205	*lin-15b(ax2061[lin-15b::gfp])*	Insertion
18	3207	*deps-1(ax2063[deps-1::gfp])*	Insertion
18	3208	*deps-1(ax2064[deps-1::gfp])*	Insertion
19	3209	*mex-6(ax2065[mex-6::gfp])*	Insertion
22	3211	*K08F4.2(ax2067)*	Deletion
22	3212	*K08F4.2(ax2068)*	Deletion
23	3213	*mbk-2(ax2069/+)*	Deletion
23	3214	*mbk-2(ax2070/+)*	Deletion
24	3158	*swan-1/2(ax2071)*	Deletion
24	3159	*swan-1/2(ax2072)*	Deletion
25	3215	*K08F4.2(ax2073)*	Deletion/Insertion of RE
25	3216	*K08F4.2(ax2074)*	Deletion/Insertion of RE

Protocol: Seamless editing of the *C. elegans* genome using CRISPR/Cas9

1. Design and cloning of the sgRNAs

-Use this website http://crispr.mit.edu/ to select sgRNAs.

Choose sgRNAs that are 1) as close as possible to the modification site and 2) have few off-target sites. If there are off-targets sites, they should have 3 or more mismatches, preferentially near the PAM. If possible, choose more than one sgRNAs near each other. Not every sgRNA works, so it's best to use more than one, until you have one that you know works.

-The website shows sgRNAs with 20 bases, but we only use the last 19 bases (skip the first base).

-Order the following primers:

Forward Q5: (N19)gttttagagctagaaatagcaagt

Reverse Q5: caagacatctcgcaatagg

Forward sequencing (for sequencing sgRNA insertion only): tatgaaatgcctacaccctctc

-Clone the sgRNAs in pDD162 using Q5 mutagenesis kit (NEB). pDD162 is the Cas9/sgRNA plasmid from Dickinson *et al*., 2013, available at Addgene.

Mix together Q5 2X master mix (12.5ul), Forward and Reverse primers 10uM (1.25ul each), pDD162 from a 1.5ml bacterial culture miniprep (0.5ul), H2O (9.5ul).

Also prepare a negative control mix without the Forward primer.

Do the PCR as follows: 30s at 98C, 10s/98C + 20s/60C + 4.30min/72C for 25 cycles, 2min at 72C, 10C forever.

Digest away pDD162: 1ul of Q5 PCR, 5ul of KLD 2X buffer, 1ul of KLD 10X enzyme, 3ul of H2O. 5min at RT

Add 5ul of the digested reaction to 50ul of kit-provided competent cells, heat shock at 42°C for 30s, add 950ul of SOC medium and shake for 1h at 37C.

Plate 25ul on Carb plate. Centrifuge the remaining 975ul for 3min at 5K and also plate the pellet.

-One half (or more) of the colonies will have the correct insertion. Pick 6 colonies to grow each in 2ml of bacterial culture. Miniprep (Qiagen kit, include the PB wash, elute in 50ul of H2O) and send to sequencing using the forward sequencing primer above. Keep several good clones. It's best to mix at least two clones for injection to avoid potential clones with mutations in Cas9.

2. Design of repair ssODNs

- The repair oligo should contain flanking bases at both the 5' and 3' ends for homologous recombination (sequences identical to genomic DNA). Ideally, flanking sequences should terminate with a C or G and contain good sequence diversity at their extremities (no hairpins). 43nt is the shortest flanking sequence we have tested and 100 is the longest. We do not know the optimal homology arm length for ssODNs. Make sure the homology arms extend as close to the sgRNA cut(s) as possible.

-The repair oligo should contain mutations that make it resistant to cutting by Cas9/sgRNA. You can mutate the PAM, insert new bases between the sgRNA sequence and the PAM, or mutate the sgRNA sequence near the PAM (we typically create 4 mismatches).

If the sgRNA targets a coding region, be careful to make only silent changes using codons that are used at similar frequency as original codon (This site for codon usage in *C. elegans* may be useful: http://www.genscript.com/cgi-bin/tools/codon_freq_table,). If possible, avoid sgRNAs that target non-coding sequences since mutations in these sequences could possibly affect regulatory (splicing, promoters) motifs.

-If possible, engineer a restriction site (RE) in your oligo to facilitate screening. Make sure that the RE site is either directly in your edit, or on the distal side of your edit relative to the cut, to ensure that both are incorporated.

-If you want to insert a premature stop, insert a *Nhe*I site between the PAM and sgRNA sequence. Use the TAG codon inside the *Nhe*I site. For maximal gene disruption, we recommend also adding a base to create a frameshift after the TAG codon.

-Suggestions for protein tags (capital letters represent bases modified to create a RE site):

V5: ggtaagcctatccctaaccctctcctcggtctAgatAGTacT (contains *Xba*I and *Sca*I sites)

HA: tacccataTgatgtCccGgattacgct (contains *Nde*I and *Nci*I sites)

TetraCys: tgctgcccaggatgctgc (contains *Bst*NI site)

3xFLAG: gactacaaagaccatgacggtgattataaagatcatgaTatcgaTtacaaggatgacgatgacaag (contains *Eco*RV and *Cla*I sites)

Myc: gaacaaaaactGatAtcagaagaggatctg (contains *Eco*RV site)

OLLAS: tccggattcgccaacGAGCTCggaccacgtctcatgggaaag (contains *Sac*I site)

-Order single stranded oligo from Operon (125nt max, 10nmol, salt free) or from IDT (200nt max, 4nM ultramer, salt free). Reconstitute oligo at 1ug/ul according to the amount provided by the manufacturer.

3. Construction of PCR donor templates for GFP insertion

Amplify the GFP plasmid pCM1.53 (available at Addgene) with primers containing the desired flanking regions (~30-60 bp), mutations in the sgRNA site(s) and GFP sequence as follows:

Fw: (flanking region/sgRNA site mutations)agtaaaggagaagaacttttcactggagttg

Rev: (flanking region/sgRNA site mutations)tttgtatagttcgtccatgccatgtgtaatccc

FYI: In one experiment where we inserted GFP right at the cut site, we obtained the highest frequencies using 33/33 bp arms. 15/19 bp arms did not work, and longer arms did not increase edit frequency (Figure 4, Paix *et al*.).

Be sure that you place GFP in frame with your ORF and that you introduce mutations to prevent recutting (as described in section for design of repair ssODNs).

PCRs are performed using Phusion taq 2X Master Mix (NEB), 45s elongation step, 30 cycles, 50ul reaction. Annealing step is done using a gradient from 60C to 72C. PCR reactions are run on agarose gel to confirm the amplification, and positive PCRs (typically three reactions) are pooled and purified using a minelute PCR purification kit (Qiagen, elution with 10ul of H2O).

Optional Nested PCR step: The nested PCR step is included to amplify your PCR further and to eliminate any long primers remaining from the first PCR. This step may be omitted if your PCR is already >500ng/ul and if you used relatively short primers (<60 bases) that are efficiently removed by the minelute PCR purification kit. Perform three nested PCR on this purification using 22-25nt Fw/Rev primers complementary to the 5'/3' ends of the template (45s elongation step, 30 cycles, 3*50ul reaction, 60C annealing). Check the PCR products on agarose gel.

Optional Dpn1 digestion step: This step may not be necessary but is included to eliminate any remaining GFP plasmid template that could form an extrachromosomal array and give you a false GFP+. Add to each 50ul PCR reactions 30ul of H2O, 10ul of Dpn1 (200 units, NEB), 10ul of 10X cutsmart buffer, and incubate at 37C for 12h and next at 80C for 20min.

Pool the reactions and purify them using one minelute PCR purification column and measure the concentration. The DNA concentration should be >500ng/ul (at this concentration, the amount of PCR oligo remaining in the mixture will be low enough

to avoid any toxicity). Oligos can significantly reduce brood size (Mello et al, 1991) and make it difficult to obtain enough F1 worms for screening.

4. Preparation of the injection mix

-We use pRF4 roller plasmid at 120ng/ul, but you can use any marker that you find convenient. The marker is included to identify successfully injected mothers. Another option to identify broods likely to give edits is to use a Co-CRISPR method (e.g. Arribere *et al.*, 2014).

Miniprep from 3ml of bacterial culture, as for the Cas9/sgRNA plasmid. Do not let cultures grow for more than 16 hours.

- Mix in a small 0.5ml eppendorf tube (Concentrations indicated are final concentrations in injection mix):

  *pRF4 (120ng/ul),

  *Repair template (30ng/ul for a 125nt ssODN, 50ng/ul for a PCR template). When using multiple oligos, we still keep each around 30ng/ul but BEWARE too much oligo can significantly reduce brood size. We use the same ssODN concentration as recommended in Zhao *et al.*, 2014.

  *Cas9/sgRNA clones (50ng/ul). When using multiple sgRNAs, we still keep each individual Cas9/sgRNA plasmid around 50ng/ul. We use the same concentration of Cas9/sgRNA as recommended in Dickinson *et al.*, 2013.

  *Add H2O to 15ul. Centrifuge at 13K for 15min on tabletop centrifuge.

- Load injection needles with the injection mix. Be careful not to touch bottom of tube with loading needle – to avoid loading precipitates that will clog your injection needle. Loading the needle under a stereomicroscope can help.

5. Preparation of worms for injection

-Bleach a large plate of worms, wash twice with M9. Plate embryos (less than 2000) on NA22 large plate. (NOT completely covered with NA22 bacteria), incubate multiple plates at different temperatures to ensure to have at least one with young adults (few embryos /one embryo row) on the day of injection. Pick hermaphrodites with a sharp pick from areas of the plate where there are no bacteria and place on injection pad.

-As a baseline: for our lab N2 strain, bleached embryos reached the correct stage for injection after 55 hours at 25°C, or at 15°C for the first night followed by 2 days at 25°C, or at 20°C for 2 days followed by one night at 25°C.

6. Injections

Inject 30-40 worms.

See Worm book protocol
http://www.wormbook.org/chapters/www_transformationmicroinjection/transformationmicroinjection.html

7. Worm recovery after injection

-About 1h after the worms have been put in recovery buffer, start adding M9. Every 5 to 10min, add 5ul of M9 (3 times), add 10ul of M9 (2 times), add 15ul of M9 (3 times), add 20ul of M9 (2 times).

- Put a drop of 20ul of M9 on a new OP50 plate, outside the bacteria layer. With a pick, transfer 5 to 10 injected worms from the recovery buffer to the M9 drop and push them away from the M9 drop towards the food. Repeat until all the worms are transferred. Even if the worms look inert at this or the next step, they are worth transferring as they may yield edited progeny.

-Leave the injected worms on OP50 plate*s* at room temperature for 5h and then transfer each worm (P0) to a new OP50 plate (1 P0 per plate).

-We recommend using fresh OP50 plates with thin bacterial lawns – easier to identify roller F1s.

8. P0 and F1 handling

-Allow the P0s to lay eggs at 20°C for 1 or 2 days. Transfer the P0s to fresh OP50 plates between the first and second day. Most of the Roller F1s are found on the first-day plates. Edits obtained using ssODNs are found on both days. Most edits made using PCR templates are found on the second day plate (see Table S4). Edits are found in both Roller and non-Roller worms but are only found among broods that contain Rollers.

 - Let the F1s grow at 20°C. When all the F1s have reached the young adult stage (4 days at 20°C), check for rollers. Expect around 20-50% of P0s to give rollers, but there may be less – in our hands this has varied greatly from experiment to experiment. Recheck plates on subsequent days if you don't see rollers on initial examination. Rollers grow more slowly than non-rollers, may not be obvious, and may be very few. Ignore P0s that don't give any rollers at all.

- GFP fusions: if you know what you are looking for, it is possible to screen directly for GFP expression in the F1 (or F2) animals. Keep in mind that, if your sgRNAs are working, 20-30% of broods will have a very high number of edits (20-60%) on the second day ("jackpot broods"). To identify jackpot broods, we recommend screening ~20 F1s from the second day plate for all the broods that segregate rollers. Once you have identified GFP+ broods, clone F1s from these to isolate homozygous edits. You can use the PCR screening to confirm the edit and to sequence it.

- PCR screening: Transfer the F1 rollers and their non-roller F1 siblings to new plates (2 to 8 F1s per plate). We typically try to screen all the F1s (roller and non roller) from P0s with roller progeny, but that may not be necessary depending on the efficiency of your sgRNA. Let the F1 to lay eggs for 24h at 20°C.

Lyse the F1s for PCR: In each 10 uL tube of lysis buffer, put 2 to 8 F1s. Up to 8 F1s may be added to each tube for lysis, if and only if you are screening for a large deletion (50% or less of the full length PCR fragment) or for a positive PCR (using internal primer for GFP insertion). Use 5ul of lysis for PCR (50ul final volume) (2ul in 25ul final volume PCR could also be used, however some multichannel pipettes are not accurate for volume under 5ul)

9. PCR Screening

We recommend testing each gene-specific PCR assay before starting the injections.

We recommend the following Taq polymerases:

If the PCR product will be processed enzymatically (restriction enzyme): Invitrogen recombinant taq (follow the manufacturer recommendation for a 50ul reaction, do 30 cycles, 1min or more by kb). Most of restriction enzymes work with the Invitrogen recombinant Taq buffer.

If the PCR product does not need processing (only looking for a size shift or a positive PCR using GFP internal primer): Promega Go Taq 2x Master mix.

For PCR products >1.5kb: NEB Phusion 2X Master mix, 30s by kb, 30 cycles. Note that, although regular Taq could amplify larger fragment, the full length DNA will compete with the edited one, and for amplification of both fragments in the same mixture, the Phusion Taq is a better choice. Most of the restriction enzymes work with the Phusion buffer.

-PCR strategies (Figure 1B):

For large insertions, use a primer specific to the insert ("internal primer"): This is best for detecting a large insert (such as GFP) in a large pool of F1s (8). Use your gene-specific forward external primer and an internal reverse primer (for GFP use the GFP reverse primer: cattaacatcaccatctaattc). Do the PCR using GoTaq, 50ul reaction, 30 cycles, 45s-1min elongation. Note that the GFP reverse primer works well with annealing temperatures from 55 to 60°C using a GoTaq.

For small insertions and modifications, design forward and reverse external primers around the edited site (but OUTSIDE the sequences in the repair template). Ideally, the PCR product should be about 500 bp centered around the edited

site. Test your primers on N2 lysis with the appropriate Taq, using a gradient from 55 to 65°C for regular Taq or 60 to 72°C for Phusion Taq

PCR size shift: use a 2.5% gel for insertion/deletion >18bp. For deletion > to 200bp, 1.25% agarose gel is the preferred. A large number of F1s can be pooled together when looking for a deletion, but we generally pool 8 F1s (makes it easier to recover edits among F2/3s).

Restriction Enzyme (RE) site insertion: This is the preferred method for a small modification/insertion. 2 F1s can be pooled. Use 5ul of the PCR product and add RE/buffer/H2O to 10ul. Do the reaction overnight. Most of the REs work with this approach (we prefer *Nhe*I because it works very well at 37°C, is available in master mix package from NEB, and contains a putative STOP codon). Run the digestion product on 2.5% gel, short run.

-Controls:

When screening F1s, we make a reaction master mix (PCR reagents and primers) that is added to each of the F1 lysis samples. We also prepare several N2 worm lysis samples with master mix only (negative control) and a few with master mix + a positive control (see below). Be sure to add this positive control PCR after all the other tubes have been closed to avoid contamination.

Positive control for RE digestion: use a PCR fragment containing the selected restriction sites. This control could be amplified from a plasmid using the same polymerase than the one used for the screen. Use 5ul of the unpurified PCR.

Positive control for GFP screen: we recommend making a synthetic template containing both your gene-specific external forward primer and the GFP internal reverse primer. Using 0.1ul of the repair GFP template, do a PCR using GoTaq, the GFP internal reverse primer, and a new forward primer containing the sequence of your forward external primer fused to 25-28nt complementary to the 5' end of your repair GFP template. Do the PCR at 60°C annealing, 50ul reaction, 30 cycles, 45s elongation. Purify the PCR product and use it as a positive control (100pg).

Another control that can be used when screening for GFP are lysed N2s that receives the PCR master mix as well as the external reverse primer (positive control).

10. F2/3 handling

-Clone the F2/3s from positive F1 plates. It is useful to let the worms crawl on a no-bacteria plate before picking to avoid accidental transfer of siblings.

If 2 F1s were pooled per plate, clone 16 F2s

If 8 F1s were pooled per plate, chunk the starved plate if necessary and clone 24 to 32 F2/F3s

-Lyse and PCR F2/3s using the same methods as for the F1s. EXCEPT: When looking for homozygous GFP worms, use primers that flank the GFP fusion.

-Use the PCR product for sequencing: Clean 25ul of the PCR reaction using Qiagen Mielute kit, elute with 10ul of H2O. Use 7ul for this elution as a template and use a primer inside the PCR product for sequencing.

If the sequence contains mismatches, use the remaining worm lysis samples to perform a second PCR with Phusion Taq (Phusion Taq has lower error rate than other Taqs).

-Once a homozygous F2/3 plate is identified, it is recommended to clone 4 worms again to new plates and to verify their genotype to ensure that the line is truly homozygous.

-Freeze the worms. We recommend freezing at least two independent lines (derived from different P0s if possible or different F1s) for each type of edit.

11. Reagents

-QIAprep Spin Miniprep Kit: Qiagen, 27104

-MinElute PCR Purification Kit: Qiagen, 28004

-Phusion High-Fidelity PCR Master Mix with HF Buffer: NEB, M0531L

-GoTaq Green Master Mix: Promega, M7122

-Taq DNA Polymerase: Invitrogen, 10342-046

-Recovery buffer: 5mm HEPES pH 7.2, 3mM CaCl2, 3mM MgCl2, 66mM NaCl, 2.4mM KCl, 4% Glucose (w/v)

-10X M9: 420mM Na2HPO4, 220mM KH2PO4, 860mM NaCl, 10mM MgSO4

-Q5 Site-Directed Mutagenesis Kit: NEB, E0554S

-Lysis buffer:50mM KCl, 10mM Tris pH8.3, 2.5mM MgCl2, 0.45% NP40, 0.45% Tween20. Before worm lysis, add proteinase K to 0.1 ug/ul.