Targeted chromosomal translocations and essential gene knockout using CRISPR/Cas9 technology in *C. elegans*

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Abstract

Many genes play essential roles in development and fertility; their disruption leads to growth arrest or sterility. Genetic balancers have been widely used to study essential genes in many organisms. However, it is technically challenging and laborious to generate and maintain the loss of function mutations of essential genes. The CRISPR/Cas9 technology has been successfully applied for gene editing and chromosome engineering. Here, we developed a method to induce chromosomal translocations and produce genetic balancers using the CRISPR/Cas9 technology, and applied this approach to edit essential genes in *C. elegans*. The co-injection of dual sgRNAs targeting genes on different chromosomes resulted in reciprocal translocation between non-homologous chromosomes. These animals with chromosomal translocations were subsequently crossed with animals that contain normal sets of chromosomes. The F1 progeny were subjected to a second round of Cas9-mediated gene editing. Through this method, we successfully produced nematode strains with specified chromosomal translocations and generated a number of loss of function alleles of two essential genes (*csr-1* and *mes-6*). Therefore, our method provides an easy and efficient approach to generate and maintain loss of function alleles of essential genes with detailed genetic background information.
Introduction

Essential genes are required for the development and fertility of organisms. Loss of function mutations of essential genes usually result in growth arrest or sterility. The production and maintenance of homozygous mutants of the essential genes are demanding and time-consuming. A series of strains with particular chromosomal rearrangements, such as duplications, translocations, and inversions, have been generated and applied to screen and grow lethal or sterile mutants. Conventional methods to elicit chromosomal rearrangements involved treating the animals with ion irradiation or chemical mutagens (JONES et al. 2011). However, the majority of existing balancer strains lack detailed sequence information. Additionally, many unintended mutations are introduced during mutagenesis and are difficult to eliminate by backcrossing with wild-type strains. Therefore, it is critical to develop a more efficient method to produce balancer strains with detailed sequence information and nominal background mutations. Furthermore, the development of more effective approaches to generate and maintain loss of function mutations of particular essential genes is required.

Recent research in targeted genome editing has made inspiring progress in genome engineering, among which is the clustered regularly interspaced short palindromic repeats (CRISPR) technology (CONG et al. 2013; JIANG et al. 2013; MALI et al. 2013; RAN et al. 2013; WANG et al. 2013; HSU et al. 2014; SHALEM et al. 2014; STERNBERG et al. 2014; WANG et al. 2014). In the CRISPR/Cas9 system, small guide RNA (sgRNA) targets its complementary genomic DNA and subsequently recruits the
Cas9 nuclease to generate double-stranded DNA breaks (DSBs). As a consequence of non-homologous end joining (NHEJ) repair, mutations are incorporated at the targeted sites.

In addition to editing a single gene, the CRISPR/Cas9 technology has also been applied to elicit chromosomal rearrangements in mammalian cell lines (Piganeau et al. 2013; Choi and Meyerson 2014; Ghezraoui et al. 2014; Torres et al. 2014; Kannan et al. 2015). A number of double-stranded DNA breaks can be induced in the presence of multiple guide RNAs. Thereafter, large genomic fragments can be reversed, deleted, or translocated to other chromosomal loci. These induced chromosomal rearrangements have been used to study the mechanism underlying cancers elicited by chromosomal inversions or translocations.

The CRISPR/Cas9 technology has been successfully applied to directing gene editing in C. elegans (Chen et al. 2013; Chiu et al. 2013; Cho et al. 2013; Dickinson et al. 2013; Friedland et al. 2013; Frokjaer-Jensen 2013; Katic and Grosshans 2013; Lo et al. 2013; Waaijers et al. 2013; Arribere et al. 2014; Chen et al. 2014; Kim et al. 2014; Paix et al. 2014; Farboud and Meyer 2015; Li et al. 2015; Ward 2015). However, there are no reports concerning Cas9-mediated genome rearrangements in C. elegans. Our previous work showed that large chromosome fragments of up to 24 kb can be eliminated through the co-injection of two sgRNAs in C. elegans (Chen et al. 2014). Here, we report the use of dual sgRNA-guided Cas9 nuclease to direct reciprocal chromosomal translocations in C. elegans. We developed a method to generate and maintain alleles of essential genes. Through the combination
of chromosomal translocation strains and the CRISPR/Cas9 technology, the loss of function mutations of essential genes can be rapidly generated, and the mutants can be easily maintained through the balancer system, therefore providing an effective approach to study these genes.

Materials and Methods

Strains. Bristol strain N2 was used as the standard wild-type strain. All strains were incubated on nematode growth medium (NGM) plates seeded with OP50 at 20°C (Brenner 1974). GR1373: eri-1(mg366), RB1441: tag-349(ok1664), YY166: ergo-1(gg098), RB1870: clec-49(ok2416), VC2575: y75b8a.11(ok3346), SHG373: dpy-13(ust040), SHG376: rde-12(ust017), CB845: unc-30(e191). uncoordinated, Unc; dumpy, Dpy; lethal, Let; sterile, Ste.

The construction of sgRNA expression plasmids. We manually searched for target sequences consisting of G(N)19NGG near the desired mutation sites (Friedland et al. 2013). The target sequences are listed in Table S1. We replaced the unc-119 target sequence in the pU6::unc-119 sgRNA expression vector (Friedland et al. 2013) with the desired target sequence using overlap extension PCR. The pU6::unc-119 sgRNA vector was diluted to 2 ng/µl and PCR amplified to generate linear products. The PCR products were digested by the DpnI restriction enzyme and transformed into Trans10 Chemically Competent Cells (Transgene Biotech, Beijing). We used the Phanta™ Super-Fidelity DNA polymerase (Vazyme Biotech, Nanjing, China, Cat. No.
P501-d1/d2/d3) in all PCR reactions. The primer sequences used for the construction of the sgRNA expression plasmids are listed in Table S2.

**Imaging.** Images were collected using Leica DM2500 and M165 FC microscopes.

**Microinjection.**

(1) Cas9-mediated chromosomal translocations. DNA mixtures were microinjected into the gonads of young adult *C. elegans*. For the chromosomal translocation experiments, we injected 50 ng/µl of the Cas9 expression vector, 50 ng/µl of the sgRNA #1 and 50 ng/µl of the sgRNA #2 expression vectors (as indicated in the figures), and 5 ng/µl of the pCFJ90 vector (a co-injection marker that expressed mCherry fluorescent protein in the pharynx). After recovering from the injection, four to five worms were placed onto individual NGM plates. Three days after the injection, F1 animals expressing mCherry were transferred to individual NGM plates and allowed to produce F2 progeny for two to three days. From an F2 plate with both wild-type and dumpy morphology animals, six to eight dumpy animals were transferred to a new NGM plate to lay F3 progeny. For an F2 plate with 100% dumpy animals, no transfer was required. Then, F2 and F3 animals were harvested and washed in M9 buffer, transferred to 50 µl lysis buffer (500 µg/ml Proteinase K, 100 mM NaCl, 50 mM Tris, and 20 mM EDTA) and screened by PCR with primers outside of the sgRNA-targeted regions. The primer sequences used for PCR screening are listed in Table S3. Mutants with chromosomal translocations were singly
transferred to NGM plates and verified by PCR amplification and DNA sequencing.

(2) The construction of balancer strains and knockout of essential genes using the CRISPR/Cas9 technology (see Figure 4 for detailed illustration). Ten males of the chromosomal translocation strain ustT2[ben-1;dpy-13](III,IV) were mated with five hermaphrodites of CB845:unc-30(e191) at 15°C. The ustT2[ben-1;dpy-13](III,IV) strain possesses the chromosomal translocation between LG III and LG IV that disrupts both ben-1 and dpy-13. Both dpy-13 and unc-30 were used as visible markers to facilitate mutant screening and maintenance. Two days later, the hermaphrodites were singly transferred to individual NGM plates to lay F1 progeny. F1 animals with wild-type morphology were selected for microinjection. We injected 50 ng/µl of the Cas9 expression plasmid, 50 ng/µl of the sgRNA #1, 50 ng/µl of the sgRNA #2, and 50 ng/µl of the sgRNA #3 expression plasmids (as indicated in the figures), and 5 ng/µl of the pSG259 (Pmyo-2::GFP::unc-54utr) plasmid. After recovering from the injection, four to five injected worms were placed onto individual NGM plates. The F1 animals produced F2 progeny with different morphological phenotypes: uncoordinated, dumpy, and wild-type. Three days after the injection, F2 animals with wild-type morphology and GFP expression in the pharynx were singly transferred to individual NGM plates and allowed to produce F3 progeny for two to three days. By analyzing the linkage between lethality and the Dpy or Unc phenotype, the alleles of essential gene X can be identified. Meanwhile, all the F2 animals were transferred to 20 µl lysis buffer and subjected to single worm PCR to directly screen for mutants.
with large chromosomal deletions.

For the *csr-1* knockout experiment, the F3 plates were examined for the linkage between sterility and morphological markers. Then, six to eight dumpy sterile F3 animals or six to eight uncoordinated sterile F3 animals were transferred to 30 µl lysis buffer and verified by PCR and DNA sequencing.

For the *mes-6* knockout experiment, six to eight F3 dumpy young adults or six to eight F3 uncoordinated young adults were transferred to individual plates to produce F4 progeny and used to assess the linkage between the sterility phenotype and morphological markers. Then, six to eight dumpy sterile F4 animals or six to eight uncoordinated sterile F4 animals were transferred to 30 µl lysis buffer and verified by PCR amplification and DNA sequencing. For plates in which all F3 animals were sterile, four to five F3 dumpy worms and four to five F3 uncoordinated animals were transferred to 30 µl of lysis buffer and examined by PCR amplification and DNA sequencing. The primer sequences used for genotyping are listed in Table S4.

**RNAi.** RNAi experiments were conducted as previously described (ZHOU et al. 2014). Synchronized embryos were grown on *unc-15* RNAi plates, and phenotypes were scored three days later. Bacteria expressing the *unc-15* dsRNA were obtained from the Ahringer RNAi library and sequenced to verify their identity (KAMATH et al. 2003).

**Benomyl assays.** The benomyl assay was conducted as previously described (DRISCOLL et al. 1989). Briefly, synchronized embryos were grown on NGM plates supplemented with 14 µM benomyl (Sigma) and maintained at 25°C for two days.
Sensitivity to benomyl was assessed by scoring the movement and body shape of the animals.

**Egg-hatching assay.** Egg hatching assays were performed as previously described (HERMAN 1977; ROSENBLUTH and BAILLIE 1981). Hermaphrodites were placed on NGM plates containing 6-10 mm diameter bacterial lawns and allowed to lay eggs for 3-4 hours, then the animals were picked off, and the eggs were counted. Three days later, the number of animals on the plates was counted again.

**Results**

**Cas9 directs chromosomal translocation between dpy-13 (LG IV) and rde-12 (LG V).** To test whether Cas9 could direct chromosomal translocation in *C. elegans*, we simultaneously injected sgRNAs targeting *dpy-13* and *rde-12* (Figure 1, A and B). *dpy-13* is a collagen gene localized on LG IV, and *dpy-13* mutant animals exhibit a dumpy morphology (ZHOU et al. 2014). The *rde-12* gene is localized on LG V and encodes an RNA helicase which engages targeted mRNA and Argonaute proteins to promote the synthesis of secondary siRNAs in *C. elegans* (SHIRAYAMA et al. 2014; YANG et al. 2014). The loss of function of *rde-12* results in resistance to exo-RNAi.

To trigger chromosomal translocation between different genomic loci by the CRISPR/Cas9 technology, these two loci needed to be cleaved concurrently and repaired through the NHEJ pathway (GHEZRAOUI et al. 2014). This process requires sgRNAs with high efficiency to engage the CRISPR/Cas9 system and trigger DNA
cleavage. Therefore, first we compared a number of sgRNAs targeting the *dpy-13* and *rde-12* loci. For *dpy-13*, three sgRNAs were designed and the gene editing efficiency of each sgRNA was assessed by phenotype analysis (Figure S1). sgRNA #2 targeting exon 2 of the *dpy-13* gene exhibited the highest efficiency and was used in the translocation experiments. For *rde-12*, the sgRNA targeting exon 2 of *rde-12* was previously reported to have a high cleavage efficiency (Chen et al. 2014) and was used in this work.

We co-injected sgRNAs targeting *rde-12* and *dpy-13* with Cas9 and mCherry expression plasmids into *eri-1(mg366)* animals (Figure S2). The mutation of *eri-1* results in an enhanced RNAi (Eri) phenotype that facilitates the analysis of exo-RNAi sensitivity (Zhou et al. 2014). The loss of function of *rde-12* results in resistance to exo-RNAi. mCherry was used as a co-injection marker. From the 150 injected animals, we obtained 369 fertile F1 animals expressing mCherry that were subsequently singly transferred to individual NGM plates to produce F2 progeny. We searched for translocation mutants by analyzing the dumpy phenotype of F2 animals and PCR screening. From the 369 F1 animals, thirty-two produced F2 progeny in which approximately 25% were dumpy, and two F1 animals produced 100% dumpy F2 progeny (Figure 1C). The dumpy animals were selected and examined by PCR to search for mutants with chromosomal translocations. We successfully identified one mutant with reciprocal chromosomal translocation between *dpy-13* and *rde-12*. This mutant exhibited both the dumpy and RNAi defective phenotypes (Figure 1D, Figure S3). Interestingly, this mutant belonged to the group of the two F1 animals that
produced 100% dumpy F2 progeny. The translocated chromosomes were further confirmed by DNA sequencing (Figure 1, E and F).

Using the CRISPR/Cas9 system to direct chromosomal translocation between ben-1 (LG III) and dpy-13 (LG IV). To further test the Cas9-directed chromosomal translocation strategy, we co-injected two sgRNAs targeting dpy-13 and ben-1 (Figure 2, A and B). The ben-1 gene is localized on LG III and encodes a β-tubulin gene that confers benomyl (an anti-microtubule drug) sensitivity. Wild-type animals exposed to benomyl at 25°C exhibit slow growth and a paralysis phenotype that is not observed in ben-1 mutants (DRISCOLL et al. 1989). An sgRNA targeting ben-1 with a high cleavage efficiency was previously reported (Figure 2B) (CHEN et al. 2013).

We co-injected the sgRNAs targeting dpy-13 and ben-1 with the Cas9 and mCherry expression plasmids into wild-type animals. From 260 injected animals, we obtained 729 fertile F1 animals expressing mCherry. These animals were singly transferred to NGM plates and then laid F2 progeny. We searched for the translocation mutants by analyzing the dumpy animals. From the 729 F1 animals, 106 produced F2 progeny in which approximately 25% were dumpy; additionally, 39 F1 animals produced 100% dumpy F2 progeny (Figure 2C). We successfully isolated two chromosomal translocation strains that exhibited the dumpy phenotype and failed to respond to benomyl (Figure 2D). Strikingly, these two mutants also belonged to the group of the 39 F1 animals that produced 100% dumpy F2 animals. The chromosomal translocation was further confirmed by DNA sequencing (Figure 2, E and F).
Interestingly, these two mutants arose from the same F1 and the sequence of one of the translocated chromosomes was identical.

**Pseudo linkage analysis and egg-hatching assay.** To further demonstrate that the two chromosomal translocation strains contain the expected chromosome fusions, we conducted genetic analysis to test the presence of pseudo linkage between the two alleles generated by chromosomal translocations (HERMAN 1977; ROSENBLUTH and BAILLIE 1981). The strain \textit{ustT1[dpy-13;rde-12](IV, V)} was generated via the cross of the strain \textit{ustT1[eri-1(mg366);dpy-13;rde-12](IV, V)} with the wild-type N2 strain, and was used in the following assays. \textit{ustT1} is short for the strain \textit{ustT1[dpy-13;rde-12](IV, V)}, and \textit{ustT2} is short for the strain \textit{ustT2[ben-1;dpy-13](III IV)} in the text.

We tested the pseudo linkage between mutant alleles of \textit{dpy-13} and \textit{rde-12} in the \textit{ustT1[dpy-13;rde-12](IV, V)} strain. \textit{ustT1} hermaphrodites were crossed with wild-type N2 strain and the F1 gravid adult animals were singled to \textit{unc-15} RNAi plates. Among more than 400 F2 animals that suppressed \textit{unc-15} RNAi (because of the mutation in \textit{rde-12}), 100% of them displayed a dumpy morphology. Meanwhile, we transferred F1 animals to NGM plates to lay F2 progeny and singled more than 200 dumpy F2 animals to \textit{unc-15} RNAi plates. All the F3 progeny generated by the dumpy F2 animals suppressed \textit{unc-15} RNAi and failed to exhibit paralysis (which is induced by \textit{unc-15} RNAi). These evidence support the presence of a pseudo linkage between \textit{dpy-13(ustT1)} and \textit{rde-12(ustT1)} and that \textit{ustT1} carries a translocation
between LG IV and LG V.

We tested the pseudo linkage between mutant alleles of *dpy-13* and *ben-1* in \( ustT2[ben-1;dpy-13](III,IV) \). \( ustT2 \) hermaphrodites were crossed with wild-type N2 strain and the F1 gravid adult animals were singled to NGM plates to lay F2 progeny. More than 200 dumpy F2 gravid adults were singled to benomyl-containing plates. All the F3 progeny exhibited resistance to the benomyl treatment and failed to display uncoordinated phenotype (which is induced by benomyl). This evidence supports the presence of a pseudo linkage between *dpy-13(ustT2)* and *ben-1(ustT2)* and that \( ustT2 \) carries a translocation between LG III and LG IV.

The chromosomal translocations were further confirmed by egg-hatching assays of the \(+/ustT1\) and \(+/ustT2\) heterozygous strains (Table 1). For eggs from the \(+/ustT1\) and \(+/ustT2\) hermaphrodites, it is expected that 37.5% of them could reach adulthood (HERMAN 1977; ROSENBLUTH and BAILIE 1981). The actual egg-surviving rates of \(+/ustT1\) and \(+/ustT2\) heterozygotes were 36.2% (n=883) and 37.2% (n=1186), respectively, which agreed well with the predicted value.

**Chromosomal translocation suppresses recombination in the non-pairing center** (non-PC) regions. Each of the six chromosomes of *C. elegans* contains a pairing center, which locates asymmetrically near one end and mediates homologous chromosome paring and segregation during meiosis (ROG and DERNBURG 2013). Chromosomes that contain homologous PC regions can pair with each other and undergo synapsis along their lengths, regardless of the non-homologous chromosomal
regions. However, chromosomes lacking these regions will fail to pair and synapse with their homologues. Chromosomal translocations have profound effects on the process of synapsis and recombination during meiosis. In heterozygotes carrying normal and translocated chromosomes, the crossover process occurs normally among the chromosomal regions from the PC end to the fusion point and is suppressed from the fusion point to the non-PC end of the chromosome (HERMAN 1977; ROSENBLUTH and BAILLIE 1981; ROG and DERNBURG 2013). We examined the recombination capability of different chromosomal regions of ustT1 and ustT2 via crossing them with a series of mutants which carry marker alleles on LG III, LG IV, and LG V, respectively (Figure 3 and Table 2). These marker alleles are all indels, which localize near the ends of each chromosome and can be easily genotyped by PCR amplification. As controls, we also crossed these marker strains with control animals that have normal chromosome sets but carry alleles in dpy-13(ust040) or rde-12(ust017). The primers used for genotyping these indels are listed in Table S5.

The eri-1 locus maps to LG IV::-26.02 in the pairing center, which is on the left arm to dpy-13 (Figure 3). We crossed eri-1(mg366) with dpy-13(ust040), selected dumpy F2 animals, and genotyped the presence of eri-1(mg366) allele by single worm PCR amplification. The recombination frequency was calculated by dividing the number of F2 dumpy animals carrying eri-1(mg366) allele by the number of total F2 animals. Crossing eri-1(mg366) with dpy-13(ust040) resulted in 12.6% recombination frequency (n=1635 total F2 animals) (Table 2). Crossing eri-1(mg366) with ustT1 and ustT2 resulted in 12.4% and 12.5% recombination, respectively. The tag-349 locus
maps to LG IV:14.18, distal to the paring center, which is on the right arm to \textit{dpy-13}. Crossing \textit{tag-349(ok1644)} with \textit{dpy-13(ust040)} generated 6.9\% recombination. However, crossing \textit{tag-349(ok1644)} with \textit{ustT1} and \textit{ustT2} did not produce any recombination (n=1680 and 2205 total F2 animals, respectively). These results suggest that the chromosomal translocations do not suppress recombination in the chromosomal region from \textit{dpy-13} to the left end of LG IV, but suppress the recombination from \textit{dpy-13} to the right end of LG IV.

\textit{ergo-1} localizes near the left end of LG V and \textit{clec-49} localizes near the right end of LG V. The chromosomal translocation in \textit{ustT1} suppressed the recombination in the region between \textit{ergo-1} and \textit{rde-12}, yet it did not suppress the recombination in the region between \textit{clec-49} and \textit{rde-12} (Table 2). \textit{y75b8a.11} localizes near the right end of LG III. We did not get any recombinants between \textit{y78b8a.11} and \textit{ben-1} from a total of 1207 F2 animals when crossing \textit{y75b8a.11(ok3346)} with \textit{ustT2}, suggesting that the recombination in the region from \textit{ben-1} to the right end of LG III is suppressed by chromosomal translocations.

We conclude, for \textit{ustT1}, recombination is suppressed from \textit{dpy-13} to the right end of LG IV and from \textit{rde-12} to the left end of LG V, yet recombination is not suppressed from the left end to \textit{dpy-13} on LG IV and from the right end to \textit{rde-12} on LG V. For \textit{ustT2}, recombination is suppressed from \textit{ben-1} to the right end of LG III, and from \textit{dpy-13} to the right end of LG IV. These results are consistent with the localization of paring centers on each chromosome (ROG and DERNBURG 2013). Recombination occurs normally in the regions containing paring centers, while is suppressed in the
The schematic of balancer construction of essential genes. Nematode strains with special chromosomal rearrangements have been used to construct genetic balancers of essential genes (Edgley et al. 2006; Jones et al. 2011). Here, we developed a new method to generate and maintain the loss of function alleles of essential genes by integrating the CRISPR/Cas9 technology with the balancer system (Figure 4). The ustT2[ben-1;dpy-13](III,IV) strain, which was generated in Figure 2 and carried a reciprocal translocation between LG III and LG IV, was used to illustrate this method.

First, we crossed ustT2[ben-1;dpy-13](III,IV) and CB845:unc-30(e191) to generate F1 heterozygous progeny. CB845 carries an unc-30(e191) allele to facilitate the screening and maintenance of mutants with the Unc morphology marker. The F1 heterozygotes were injected with the Cas9 expression plasmid, GFP expression plasmid, and the plasmids expressing sgRNAs targeting the lethal gene X. Both copies of gene X on the two chromosomes can be edited. The F1 animals generated F2 progeny with a segregation of Dpy, Unc, and the wild-type morphology (Figure S4). The F2 animals with wild-type morphology and GFP expression were singly transferred to individual NGM plates to lay F3 progeny, which exhibited Dpy, Unc, Let, or wild-type phenotype. By analyzing the linkage between lethality and the Dpy or Unc phenotype, the alleles of essential gene X can be identified. The presence of the dpy-13 or unc-30 alleles further simplified the identification and maintenance of the chromosomes on which the alleles of gene X were localized. Additionally, mutants
with large sequence deletions can be easily pinpointed by PCR-based single worm genotyping of the F2 animals.

We examined the phenotypic segregation ratio of \textit{unc-30(e191)/ustT2} heterozygote, which is expected to be 4 : 1 : 1 of wild-type : Dpy : Unc animals within the progeny (Figure S4). Among a total of 1706 progeny, we observed 1148 wild-type, 282 Dpy and 276 Unc individuals, which exhibited a ratio of 4.16 : 1.02 : 1 that agreed well with the expectation.

\textbf{Balancer construction of \textit{csr-1} using the CRISPR/Cas9 system.} CSR-1 is a germline-expressed Argonaute protein that is required for faithful chromosome segregation and embryonic viability (\textsc{Claycomb et al.} 2009). The homozygous \textit{csr-1} mutants are sterile, although they produce a very few embryos with chromosome segregation defects.

The strain \textit{ustT2[ben-1;dpy-13](III,IV)} was first crossed with \textit{CB845:unc-30(e191)}. Then, we co-injected three sgRNA expression vectors targeting exons 2 and 4 of \textit{csr-1} with the Cas9 and GFP expression plasmids into the F1 heterozygous animals (Figure 5A, Figure S5A). From the 90 injected F1 animals, we acquired 155 fertile F2 animals with wild-type morphology and GFP expression (Figure 5B). F2 animals were singly transferred to individual NGM plates to lay F3 progeny. From the 155 F2 animals, five produced F3 animals with linkage between the uncoordinated and sterile phenotypes and two F2 animals produced F3 animals with linkage between the dumpy and sterile phenotypes. The linkage between the
sterile phenotype and morphological markers indicated the chromosome on which the mutations were located (Figure S5B). Interestingly, three F2 animals with large deletions were directly screened out via single worm PCR of the F2 animals (Figure 5C). The \textit{csr-1} alleles were confirmed by PCR amplification and sequencing (Figure 5, D and E).

One of the balancer strain, \texttt{+/ustT2 [ben-1] III;csr-1(ust041) unc-30(e191) IV/ustT2 [dpy-13] IV}, which carries a \textit{csr-1(ust041)} mutation on the normal chromosome, was examined to verify its linkage with \textit{unc-30(e191)}. L4 heterozygous \texttt{+/ustT2 [ben-1] III;csr-1(ust041) unc-30(e191) IV/ustT2 [dpy-13] IV} animals were singled to NGM plates to lay eggs. Three days later, more than 800 uncoordinated larva animals were singly transferred to new NGM plates and their fertility were scored after another 4-5 days. We only observed a few dead embryos but no viable larva progeny from these 800 uncoordinated parents, which is consistent with the sterility of \textit{csr-1} homozygous mutation.

Therefore, these loss of function \textit{csr-1} alleles were balanced with the translocation of chromosomal \textit{ustT2[dpy-13]} (IV) and maintained as heterozygous states.

\textbf{Genetic balancer generation of \textit{mes-6}.} MES-6 is a member of the polycomb-like chromatin repressive complex (PRC2) (\textit{Xu et al.} 2001) that is engaged in germline development and regulation of gene expression. The loss of function of \textit{mes-6} results
in a maternal sterile phenotype in which the heterozygous mother produces 25% homozygous mes progeny that are themselves fertile but produce sterile progeny.

The strain ustT2[ben-1;dpy-13](III,IV) was first crossed with unc-30(e191). Then, we co-injected three sgRNA expression plasmids targeting exons 1 and 2 of mes-6 with the Cas9 and GFP expression plasmids into the F1 heterozygous animals (Figure 6A, Figure S6A). From 100 injected F1 animals, we obtained 165 fertile F2 animals expressing GFP with wild-type morphology (Figure 6B). F2 animals were singly transferred to NGM plates to lay F3 progeny. From each plate, six to eight dumpy or uncoordinated animals were transferred to individual NGM plates to lay F4 progeny. From the total of 165 F2 animals, seventeen produced F4 progeny with a linkage between the Dpy and maternal sterile phenotypes, twenty F2 progeny produced F4 progeny with a linkage between the uncoordinated and sterile phenotypes, and thirteen F2 animals produced sterile F3 animals with both the Dpy and Unc phenotypes. The linkage between sterility and the morphological phenotypes indicated the chromosome on which the mutations were located (Figure S6B).

Interestingly, two F2 animals with large deletions were directly screened out via single worm PCR of the F2 animals (Figure 6C). The mes-6 alleles of a subset of these mutants were verified by PCR and sequencing (Figure 6, D and E and F).

Therefore, these loss of function mes-6 alleles were balanced with the translocation of chromosomal ustT2[dpy-13] (IV) and maintained in heterozygous state.

Discussion

Recurrent chromosomal translocations in mammalian cells usually result in
chimeric fusion transcripts that lead to the expression of fusion proteins and drive oncogenesis. A series of technologies have been developed to establish cancer models by inducing chromosomal translocation in cell lines. These technologies include the rare-cutting I-SceI endonuclease (RICHARDSON and JASIN 2000), zinc finger nucleases (ZFNs), TALE nucleases (TALENs), and the CRISPR/Cas9 system, all of which are able to introduce DNA double-stranded breaks (DSBs) into distinct chromosomal loci and generate translocated chromosomes by non-homologous end-joining (NHEJ). In C. elegans, a number of strains with chromosomal rearrangements were previously generated using mutagenic chemicals or irradiation. However, these traditional methods are time-consuming, laborious, and rarely achieve specific rearrangements on purpose. Additionally, many unintended mutations can be introduced into the genome by mutagenesis. Here, we developed a method using the CRISPR/Cas9 technology to direct chromosomal translocations in C. elegans. Through the co-injection of sgRNAs targeting different chromosomes, designated chromosomal translocation strains with nominal off-target mutations were constructed rapidly and efficiently.

Targeted chromosomal translocations are beneficial for many studies, such as meiotic processes. C. elegans’ chromosomes contain specialized regions called pairing centers that localize at the end of each chromosome and mediate homologous pairing and synapsis during meiosis (MACQUEEN et al. 2005; PHILLIPS and DERNBURG 2006; PHILLIPS et al. 2009; ROG and DERNBURG 2013). However, the detailed sequence information and mechanisms involved with the paring centers
remain unclear. Using the CRISPR/Cas9 technology to generate specific chromosomal translocations will help to pinpoint these regions and illuminate the mechanisms underlying homologue pairing, synapsis, and segregation during meiosis.

Balancers are genetic constructs or chromosomal rearrangements that allow lethal or sterile mutations to be stably maintained in heterozygotes (Hackstein et al. 1992; Zheng et al. 1999; Chick et al. 2004; Hentges and Justice 2004). Balancers can be applied to a variety of tasks, including construction of strains, maintenance of mutations, and screening for new mutants. Conventional methods using existing balancer strains to screen and maintain the alleles of essential genes are very cumbersome and time-consuming. In this work, we developed a rapid and efficient method using the CRISPR/Cas9 technology to directly generate balancer strains with loss of function alleles of essential genes. Starting from the initial cross, the experiment can be finished within ten days and is able to generate many alleles of the targeted genes. Moreover, by using multiple sgRNAs in the microinjection experiment followed by genotyping of the F2s with PCR detection, we can easily isolate deletion alleles of the targeted genes.

Linking lethality to morphological markers is especially helpful for experiments because it is much easier to score for the presence of a particular morphological phenotype than to score arrested embryos or larvae. The chromosomal translocation strains generated in this work carry dpy-13, rde-12, or ben-1 mutations. We also introduced the unces-30(e191) allele in the mating step prior to the microinjection of the Cas9 expression plasmid. These markers eased the manipulation of genetic crosses
and the selection of homozygous mutants. Other markers or GFP reporters can also be included in the mating step, which will benefit the study of the balanced genes.

In summary, our work provides a novel platform with which to use the CRISPR/Cas9 technology to generate nematode strains with specified chromosomal translocations and produce and maintain loss of function alleles of essential genes via the balancer system. This method is also applicable to study essential genes in other organisms.

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LITERATURE CITED
Ghezraoui, H., M. Piganeau, B. Renouf, J. B. Renaud, A. Sallmyr et al., 2014 Chromosomal translocations in human cells are generated by canonical


Yang, H., J. Vallandingham, P. Shiu, H. Li, C. P. Hunter *et al.*, 2014 The DEAD box helicase RDE-12 promotes amplification of RNAi in cytoplasmic foci in *C.
Zhou, X., F. Xu, H. Mao, J. Ji, M. Yin et al., 2014 Nuclear RNAi contributes to the
silencing of off-target genes and repetitive sequences in Caenorhabditis
**Figure Legends**

**Figure 1.** Cas9 directs chromosomal translocation between *dpy-13* (LG IV) and *rde-12* (LG V). (A) Schematic depicting the overall strategy of generating chromosomal rearrangements. The red arrows indicate the sgRNA targets and the yellow arrows show the orientation of chromosomes. (B) Schematic of the *rde-12* and *dpy-13* genes. Positions of sgRNA-guided cleavage sites and PCR primers for genotyping are indicated. (C) Summary of the microinjection experiments. (D) The chromosomal translocation strain *ustT1[eri-1(mg366);dpy-13;rde-12](IV,V)* exhibited a dumpy phenotype. (E and F) PCR amplification (left panel) and chromatogram of DNA sequencing (right panel) of the *ustT1[eri-1(mg366);dpy-13](IV)* and *ustT1[rde-12](V)* chromosomes. Breakpoints and sgRNA targets are indicated. Scale bars, 100µm.

**Figure 2.** Cas9 directs chromosome translocation between *ben-1* (LG III) and *dpy-13* (LG IV). (A) Schematic depicting the overall strategy of generating chromosomal rearrangements. The red arrows indicate the sgRNA targets and the yellow arrows show the orientation of the chromosomes. (B) Schematic of the *ben-1* and *dpy-13* genes. Positions of sgRNA-guided cleavage sites and PCR primers for genotyping are indicated. (C) Summary of the microinjection experiments. (D) The chromosomal translocation strain *ustT2[ben-1;dpy-13](III,IV)* is benomyl-resistant. (E and F) PCR detection (left panel) and chromatogram of DNA sequencing (right panel) of the *ustT2(IV)* and *ustT2(III)* chromosomes of mutants #1 and #2. Breakpoints and sgRNA
targets are indicated. Scale bars, 100µm.

**Figure 3.** The relative position of selected alleles used to map the recombination suppression regions. The grey vertical lines indicate the pairing center (ROG and DERNBURG 2013).

**Figure 4.** Schematic of the balancer construction of the lethal gene X. The F1 heterozygous strain was generated by the mating of ustT2[ben-1;dpy-13](III,IV) with unc-30(e191), and was subjected to the microinjection of sgRNA and Cas9 expression plasmids. Both copies of the lethal gene X can be edited. The allele on wild-type chromosome was marked with red color and the allele on the translocated chromosome was marked with black color.

**Figure 5.** The gene editing and balancer construction of csr-1 using the CRISPR/Cas9 technology. (A) Schematic of the csr-1 gene. The sgRNA-targeted sites and the PCR primers for genotyping are indicated. (B) Summary of the microinjection experiments. (C) Single worm PCR detection of the examples of the balanced F2 mutants. (D and E) Sequence alignments of wild-type and mutant animals with sterile uncoordinated and sterile dumpy phenotypes. The dash indicates a deletion. The numbers in parentheses within the sequence represent the number of bases not shown. The number of deleted (-) or inserted (+) bases is indicated to the right of each indel.
Figure 6. The gene editing and balancer construction of mes-6. (A) Schematic of the mes-6 gene. The sgRNA-targeted sites and the PCR primers for genotyping are indicated. (B) Summary of the microinjection experiments. (C) Single worm PCR detection of the examples of the balanced F2 mutants. (D, E, F) Sequence alignments of wild-type and mutant animals with sterile uncoordinated and sterile dumpy phenotypes. The dash indicates a deletion. The numbers in parentheses within the sequence represent the number of bases not shown. The number of deleted (-) or inserted (+) bases is indicated to the right of each indel.

Table 1. Egg-hatching assay.

Table 2. Mapping recombination capability of different chromosomal regions of ustT1 and ustT2.
Figure 1

A

B

C

D

E

F

Figure 1
**A**

chromosomal translocation

**B**

LG III

- ben-1
- dpy-13 sgRNA

LG IV

- dF
- dpy-13
- dR

**C**

<table>
<thead>
<tr>
<th>dpy-13 sgRNA + ben-1 sgRNA</th>
<th>P0 injected</th>
<th>F1 with mCherry</th>
<th>F2 with ≤25% dumpy</th>
<th>F2 with 100% dumpy</th>
<th>translocation strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>260</td>
<td>729</td>
<td>106</td>
<td>39</td>
<td>2</td>
</tr>
</tbody>
</table>

**D**

benomyl: - +

dpy-13(ust040)

ustT2[ben-1;dpy-13](III,IV)

**E**

primers:

- dF+bR
- dR+bF

marker #2 WT #1 WT

translocation strain #1

- #1 dF+bR
- #1 dR+bF

37 bp deletion from ben-1 sgRNA site

1019 bp deletion from ben-1 sgRNA site
dpy-13 sgRNA target

dpy-13 sgRNA PAM

**F**

primers:

- dF+bR
- dR+bF

marker #2 WT #2 WT

translocation strain #2

- #2 dF+bR
- #2 dR+bF

759 bp deletion from ben-1 sgRNA site

1019 bp deletion from ben-1 sgRNA site
dpy-13 sgRNA target

dpy-13 sgRNA PAM

---

Figure 2
Figure 3
P0: \( ustT2[ben-1;dpy-13](III,IV) \) \( \text{unc-30(e191)} \)

F1: wild type

F2: single wild type F2 with GFP marker expression.

F3:

- Wild type: 4
- Dumpy: 1
- Uncoordinated: 1

Figure 4
Figure 5

A

B

C

D

E

Figure 5
A

LG IV

8,511  8,512  8,513 (kb)

mes-6

F  R

B

sgRNAs #1 + #2 + #3

wild type F1 injected 100
wild type F2 with GFP 165
F4 with sterile dpy 17
F4 with sterile unc 20
F3 with sterile dpy and sterile unc 13

C

Figure 6

mutant

wild-type

![Image]

D

F4 with sterile dpy

wild-type

ATGAGGAACAAGCGGTTGCAACTGTCGGAGGAAGCTTTGTAAGCG

mutant #6

ATGAGGAACAAGCGGTT---c-GTCGGAGGAAGCTTTGTAAGCG (+1 bp, -6 bp)

wild-type

GAACAACGGGTTGGCAAC----TGTGGGAGGAAGCTTTGTAAGCGT

mutant #8

GAACAACGGGTTGGCAACcggtTGTCGGAGGAAGCTTTGTAAGCGT (+4 bp)

E

F4 with sterile unc

wild-type

TACGCAGGAATAGATGA-------ATCAATGACATTCGTACGTGT

mutant #30

TACGCAGGAATAGATGA(383bp)ATCAATGACATTCGTACGTGT (-383bp)

wild-type

GGTTTGATCGAAGTACA-------GCTTTGTAAGCGTTATTTAGC

mutant #40

GGTTTGATCGAAGTACA(133bp)GCTTTGTAAGCGTTATTTAGC (-133bp)

F

F3 with sterile dpy and sterile unc

wild-type

TCAATACGCAGGAATAG(24bp)TCGGAGGAAGCTTTGTACTAAC

mutant #7 dpy

TCAATACGCAGGAATAG------TCGGAGGAAGCTTTGTACTAAC (-24bp)

wild-type

GATGAAGAACAAGCGGT(8bp)TCGGAGGAAGCTTTGTACTAACT

mutant #8 dpy

GATGAAGAACAAGCGGT-----TCGGAGGAAGCTTTGTACTAACT (-8bp)

wild-type

GGAACAACGGGTTGGCAAA(4bp)CGGAGGAAGCTTTGTAAGCGTTA

mutant #8 unc

GGAACAACGGGTTGGCAAA-----CGGAGGAAGCTTTGTAAGCGTTA (-4bp)

wild-type

TGAGGAACAAGCGGTTG(6bp)TCGGAGGAAGCTTTGTAAGCGTT

mutant #8 unc

TGAGGAACAAGCGGTTG-----TCGGAGGAAGCTTTGTAAGCGTT (-6bp)
Table 1: Egg-hatching assay and number of progeny

<table>
<thead>
<tr>
<th>Genotype of parental hermaphrodite</th>
<th>Percentage of eggs reaching adulthood (%)</th>
<th>Mean number of adult progeny per hermaphrodite†</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>99.7 (661)</td>
<td>298 (10)</td>
</tr>
<tr>
<td>ustT1</td>
<td>96.7 (522)</td>
<td>147 (10)</td>
</tr>
<tr>
<td>ustT2</td>
<td>99.5 (407)</td>
<td>223 (10)</td>
</tr>
<tr>
<td>+/ustT1</td>
<td>36.2 (883)</td>
<td>101 (10)</td>
</tr>
<tr>
<td>+/ustT2</td>
<td>37.2 (1186)</td>
<td>105 (10)</td>
</tr>
<tr>
<td>+/ustT2 [ben-1] III;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unc-30(e191)/ustT2 [dpy-13] IV</td>
<td>37.7 (917)</td>
<td>109 (14)</td>
</tr>
</tbody>
</table>

*Determined as described by HERMAN(1978). The total number of eggs counted is in parentheses.
†The numbers of parent hermaphrodites are in parentheses.
Table 2. Recombination capability of different chromosomal regions of *ustT1* and *ustT2*

<table>
<thead>
<tr>
<th>male*</th>
<th>hermaphrodite*</th>
<th>PCR markers (gene allele)</th>
<th>Frequencies of recombinants (total adult progeny)</th>
<th>Dpy or Rde animals with PCR markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR1373 (<em>eri-1</em>)</td>
<td>SHG373 (<em>dpy-13</em>)</td>
<td><em>mg</em>66</td>
<td>0.126 (1635)</td>
<td></td>
</tr>
<tr>
<td>GR1373 (<em>eri-1</em>)</td>
<td>SHG374 (<em>ustT1[dpy-13] IV; ustT1[rde-12] V</em>)</td>
<td><em>mg</em>66</td>
<td>0.124 (1589)</td>
<td></td>
</tr>
<tr>
<td>GR1373 (<em>eri-1</em>)</td>
<td>SHG375 (<em>ustT2[ben-1] III; ustT2[dpy-13] IV</em>)</td>
<td><em>mg</em>66</td>
<td>0.125 (2387)</td>
<td></td>
</tr>
<tr>
<td>RB1441 (tag-349)</td>
<td>SHG373 (<em>dpy-13</em>)</td>
<td><em>ok</em>1644</td>
<td>0.069 (689)</td>
<td></td>
</tr>
<tr>
<td>RB1441 (tag-349)</td>
<td>SHG374 (<em>ustT1[dpy-13] IV; ustT1[rde-12] V</em>)</td>
<td><em>ok</em>1644</td>
<td>0 (1680)</td>
<td></td>
</tr>
<tr>
<td>RB1441 (tag-349)</td>
<td>SHG375 (<em>ustT2[ben-1] III; ustT2[dpy-13] IV</em>)</td>
<td><em>ok</em>1644</td>
<td>0 (2205)</td>
<td></td>
</tr>
<tr>
<td>YY166 (<em>ergo-1</em>)</td>
<td>SHG376 (<em>rde-12</em>)</td>
<td><em>gg</em>098</td>
<td>0.125 (768) †</td>
<td></td>
</tr>
<tr>
<td>YY166 (<em>ergo-1</em>)</td>
<td>SHG374 (<em>ustT1[dpy-13] IV; ustT1[rde-12] V</em>)</td>
<td><em>gg</em>098</td>
<td>0 (1487) †</td>
<td></td>
</tr>
<tr>
<td>RB1870 (clec-49)</td>
<td>SHG376 (<em>rde-12</em>)</td>
<td><em>ok</em>2416</td>
<td>0.102 (740) †</td>
<td></td>
</tr>
<tr>
<td>RB1870 (clec-49)</td>
<td>SHG375 (<em>ustT2[ben-1] III; ustT2[dpy-13] IV</em>)</td>
<td><em>ok</em>2416</td>
<td>0.110 (556) †</td>
<td></td>
</tr>
<tr>
<td>VC2575 (<em>y75b8a.1I</em>)</td>
<td>SHG375 (<em>ustT2[ben-1] III; ustT2[dpy-13] IV</em>)</td>
<td><em>ok</em>3346</td>
<td>0 (1207)</td>
<td></td>
</tr>
</tbody>
</table>

* The genotypes of the strains are showed in parentheses.
† The F2 animals suppressing *unc-15* RNAi treatment were singled out and genotyped.