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The application of CRISPR-Cas9 genome editing in *Caenorhabditis elegans*

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Abstract

Genome editing using the Cas9 endonuclease of *Streptococcus pyogenes* has demonstrated unparalleled efficacy and facility for modifying genomes in a wide variety of organisms. *Caenorhabditis elegans* is one of the most convenient multicellular organisms for genetic analysis, and application of this novel genome editing technique to this organism promises to revolutionize analysis of gene function in the future. CRISPR-Cas9 has been successfully used to generate imprecise insertions and deletions *via* non-homologous end-joining mechanisms and to create precise mutations by homology-directed repair from donor templates. Key variables are the methods by which the Cas9 endonuclease is delivered and the efficiency of the single guide RNAs. CRISPR-Cas9 mediated editing appears to be highly specific in *C. elegans*, with no reported off-target effects. This review briefly summarizes recent progress in CRISPR-Cas9 based genome editing in *C. elegans*, highlighting technical improvements in mutagenesis and mutation detection, and discussing potential future applications of this technique.

Keywords

genome editing; CRISPR; Cas9; non-homologous end-joining (NHEJ); homology-directed repair (HDR); somatic mutation; *C. elegans*

INTRODUCTION

Recent breakthroughs in genome editing technologies that allow precise modification of the double-stranded DNA of an organism promise to open up unprecedented opportunities for identification and characterization of gene functions in biological processes. These valuable technologies rely on the finding that eukaryotic cells have endogenous mechanisms to repair DNA double-strand breaks (DSBs) (Carroll, 2011). The targeted DSBs can be repaired either *via* the error-prone non-homologous end-joining (NHEJ) repair pathway, which induces small insertion or deletion mutations (indels) that can result in a frame shift in a coding region, or *via* the homology-directed repair (HDR) pathway, which introduces

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specific point mutations or insertion/deletion of a desired sequence by homologous recombination with exogenously provided DNA templates. Recent genome editing methods also use programmable nucleases to introduce gene disruption, insertion, or substitution of genomic sequences in a controlled manner (Segal and Meckler, 2013; Maggio and Goncalves, 2015). Several customized endonucleases have been used in genome editing in the last decade, such as meganucleases, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Hsu et al., 2014; Maggio and Goncalves, 2015). They are enabling specific genome manipulations through protein-DNA interaction for targeting. All these approaches have given valuable information on genome editing; however, each has unique weaknesses that have limited their wider application.

The most recent and rapidly developing approach in genome editing is the CRISPR (clustered regularly interspaced short palindromic repeats)-associated protein 9 endonuclease (hereafter referred to as Cas9), derived from bacterial adaptive immune systems (Doudna and Charpentier, 2014). The CRISPR-Cas9 system can be used to target virtually any genomic locus through a guide RNA that recognizes the target DNA *via* Watson-Crick base pairing. The type II CRISPR-Cas9 system from *Streptococcus pyogenes* is the simplest and most widely used, and has been shown to introduce site-specific DSBs that are subsequently repaired by either NHEJ or HDR (Doudna and Charpentier, 2014). The core components of the CRISPR-Cas9 system are an endonuclease Cas9 containing two catalytic nuclease domains (RuvC and HNH), and a single guide RNA (sgRNA) chimera that combines the functions of the CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) (Jinek et al., 2012). The specific sequence requirement for chromosomal editing hinges on the 20 nt sequence at the 5' end of the sgRNA, which has to be followed by a protospacer adjacent motif (PAM) of NGG in the DNA in order for efficient cleavage (Fig. 1). Thus, the CRISPR-Cas9 mediated genome editing is programmable and can be easily targeted to most genomic locations of choice through the design of the sgRNA. Compared to other protein-guided counterparts, ZFNs and TALENs, this RNA guided genome editing tool offers several distinct advantages, such as simplicity, accessibility, affordability, and multiplexing (Cong et al., 2013; Hsu et al., 2014). Since the landmark demonstration of CRISPR-Cas9 genome editing in 2012 (Jinek et al., 2012), this system has revolutionized functional genomics in many model organisms (Bassett et al., 2013; Belhaj et al., 2013; Chang et al., 2013; Chen et al., 2013b; Cong et al., 2013; DiCarlo et al., 2013; Friedland et al., 2013; Gratz et al., 2013; Hwang et al., 2013; Li et al., 2013; Mali et al., 2013b; Wang et al., 2013b; Wei et al., 2013; Yu et al., 2013; Bassett and Liu, 2014; Ma et al., 2014).

The nematode *Caenorhabditis elegans* is a widely used genetic model organism, in which forward and reverse genetic approaches have been well developed. CRISPR-Cas9 based genome editing has been successfully applied to *C. elegans* and has been reviewed recently (Frokjaer-Jensen, 2013; Waaijers and Boxem, 2014). Here, I summarize the rapid development and recent refinement of the CRISPR-Cas9 system as a platform for obtaining custom genome modifications. I discuss recent improvements in CRISPR-Cas9 methodology and their applications in *C. elegans*.

THE DEVELOPMENT OF CRISPR-CAS9 IN *C. ELEGANS*

Although randomly induced deletion mutations are readily generated and detected by PCR based screening (Gengyo-Ando and Mitani, 2000; Edgley et al., 2002; Barstead and Moerman, 2006), *C. elegans* has long lacked an efficient homology-based reverse genetic system. Early approaches relied on the use of transposon insertions (endogenous Tc transposons or exogenous Mos1) to create site-specific DSBs (Plasterk and Groenen, 1992; Robert and Bessereau, 2007; Frokjaer-Jensen et al., 2008; Frokjaer-Jensen et al., 2010; Frokjaer-Jensen et al., 2012). The transposase is able to delete or insert desired sequence at the insertion site through HDR. This method is robust and enables precise genome editing, but is restricted by the relative rarity of the editing event and the need for a transposon insertion site (Frokjaer-Jensen et al., 2008; Frokjaer-Jensen et al., 2010).

More recently, nuclease-based genome editing approaches (ZFNs and TALENs) have also been shown to work in *C. elegans* (Wood et al., 2011; Cheng et al., 2013; Lo et al., 2013). Although such approaches may yet be useful in certain situations, it is fair to say that the efficiency and simplicity of the CRISPR-Cas9 method has taken the *C. elegans* community by storm, resulting in an explosion of research into its application and optimization.

For CRISPR-Cas9 mediated genome editing, a key requirement is the expression of Cas9 protein and sgRNA in the nucleus where the Cas9-sgRNA-DNA complex will be assembled. Germline expression of Cas9 and sgRNA is required to generate heritable changes, while somatic tissue expression can generate nonheritable somatic mutations. Several implementations of CRISPR-Cas9 have been described in *C. elegans*, each with advantages and disadvantages (Waaijers and Boxem, 2014). One important difference is how Cas9 and sgRNA are delivered to the germline nucleus (Fig. 1). Cas9 can be delivered by microinjection of *in vitro* transcribed mRNA (Chiu et al., 2013; Katic and Grosshans, 2013), pure protein (Cho et al., 2013), or plasmid DNA (Chen et al., 2013b; Dickinson et al., 2013; Friedland et al., 2013; Lo et al., 2013; Tzur et al., 2013). Similarly, the sgRNA can also be introduced by *in vitro* transcription (Chiu et al., 2013; Cho et al., 2013; Katic and Grosshans, 2013) or expressed in the germline using an RNA polymerase III promoter such as *U6* (Chen et al., 2013b; Dickinson et al., 2013; Friedland et al., 2013; Tzur et al., 2013) (Table 1). Interestingly, bacterial feeding, which can deliver gene specific double-stranded RNAs (dsRNAs), can also introduce guide RNAs into Cas9 expressing worms to achieve gene disruption (Liu et al., 2014). These foundational studies pave the way for widespread implementation of CRISPR-Cas9 technology as a genome editing tool in *C. elegans*. Despite the differences in delivery, all of the aforementioned approaches have been successful in generating mutations. Because of the ease of plasmid cloning compared to protein or RNA purification, the most popular methods currently rely on microinjection of plasmids expressing the Cas9 protein and sgRNAs into the germline, followed by phenotype-based or PCR-based screening of progeny.

Successful expression of Cas9 protein and sgRNA in the nucleus usually generates blunt-ended DSBs 3 base pairs upstream of the PAM sequence in the chromosome. Cas9 cleaves DNA using two catalytic domains: an HNH nuclease domain that cleaves the strand complementary to the sgRNA, and a RuvC nuclease domain that cleaves the non-

complementary strand (Fig. 1). Both cleavages have been shown to induce error-prone NHEJ genome disruption and HDR based precise genome modification in *C. elegans*.

Imprecise genome editing *via* Non-Homologous End-Joining

Cas9 nuclease induced DNA DSBs can be repaired through NHEJ, resulting in the efficient generation of small insertions or deletions (indels), which can disrupt the reading frame or regulatory region thus generating loss-of-function mutations (Hsu et al., 2014) (Fig. 1). The germline expression of Cas9 and sgRNA is essential for generation of this heritable mutation. In *C. elegans*, indel mutations can easily be isolated if they lead to visible phenotypes, such as Unc (uncoordinated locomotion) or Dpy (dumpy body shape). In initial experiments, the efficiency of this approach varied widely, ranging from 0.5%—88% depending on different sgRNAs (Table 1). Because of the inefficiency of mutation detection by PCR, a dual sgRNA method, which uses two sgRNAs targeting the same gene, was developed to generate larger DNA deletions that potentially results in complete molecular null mutations (Chen et al., 2014; Xu and Chisholm, 2014). Indeed, the dual sgRNA approach has been reported to delete as large as 24 kb between the sgRNAs (Chen et al., 2014).

Precise genome editing *via* Homology-Directed Repair

Compared to NHEJ events, which generate a spectrum of mutations in the target area, the HDR-mediated repair events use DNA template that contains sequence homology to the genome site to generate targeted modifications. The HDR dependent genome editing can be used to induce precise point mutation, add insertional tag (e.g. GFP, FLAG) to specific chromosomal gene, or delete the entire gene and replace it with a gene expression construct (e.g. GFP, antibiotic gene cassettes) (Fig. 1). Numerous reports have been demonstrated that Cas9 induced DSBs could be efficiently repaired by HDR in *C. elegans* (Chen et al., 2013b; Dickinson et al., 2013; Lo et al., 2013; Zhao et al., 2014; Dickinson et al., 2015).

For successful HDR, a donor DNA template is also required together with Cas9 and sgRNA in the nucleus. There are several strategies to supply the donor template. The first approach is to use plasmid DNA templates, which could be employed to engineer a wide variety of DNA alterations (Dickinson et al., 2013). However, cloning the required homology arms into a vector can take time. An attractive alternative is to use single strand oligodeoxynucleotides (ssODNs), which can be synthesized commercially (Zhao et al., 2014). Interestingly, PCR-generated DNA fragments have been successfully used as a donor template (Paix et al., 2014). One of the potential problems of HDR-mediated repair is the low efficiency (Paix et al., 2014; Zhao et al., 2014). Although it is hard to gain precise estimates of the efficiency of HDR, overall from these published works, the recombinant mutants were identified between 0.4%—26.3% of F₁ animals (Dickinson et al., 2013; Tzur et al., 2013; Paix et al., 2014; Zhao et al., 2014) (Table 1). Interestingly, disabling NHEJ activity by RNAi inactivation of the *cku-80* gene, which encodes *C. elegans* blunt-ended DNA binding protein (KU), can significantly improve HDR efficiency (Ward, 2015). Nevertheless, refined methods with more efficient HDR are a high priority for the community.

Somatic mutations

Tissue or stage-specific conditional knockout techniques are important to determine the focus of gene function in multicellular organisms. Several strategies have been used to generate conditional mutations in *C. elegans*, including the site specific recombinase-based Cre-*LoxP* (Flavell et al., 2013) and Flp-*FRT* systems (Voutev and Hubbard, 2008), or tissue and stage-specific RNAi (Hannon, 2002). CRISPR-Cas9 system can also be used to generate somatic mutations by expressing Cas9 in somatic cells under the control of an inducible (e.g., heat shock promoter, *hsp-16*) or tissue-specific (e.g., neuronal, epidermal, muscle and intestinal) promoter (Liu et al., 2014; Shen et al., 2014). Interestingly, it was reported that Cas9 activity could across over generations once the somatic Cas9 transgene is established (Shen et al., 2014), and importantly this somatic mutation can be rescued by providing GFP fusion protein (Li et al., 2015). Through CRISPR-Cas9 targeted DNA cleavage, this methodology enables the investigation of functions of essential genes in postembryonic development and provides the ability to dissect tissue specific roles of a single gene (Shen et al., 2014; Li et al., 2015; Tian et al., 2015). These studies also demonstrated that somatic CRISPR-Cas9 is rapid, versatile, and cost-effective compared to other conditional knockout techniques. However, a caveat is that the somatic CRISPR-Cas9 introduced molecular lesions are likely heterogeneous and usually not well characterized either at the level of DNA sequence or in cellular distribution. Furthermore, as heritable mutation, the efficiency of somatic mutation appears to be highly variable between genes from 10% to 90% (Shen et al., 2014; Li et al., 2015), which may depend on the sgRNA design. Negative or partial results with tissue-specific mutagenesis approaches should be at present treated with caution.

Despite the power and versatility of the CRISPR-Cas9 system, a serious concern is the potential for off-target cleavage and mutagenesis. CRISPR-Cas9 appears to cause relatively high levels of off-target mutations in human cell lines (Fu et al., 2013; Hsu et al., 2013). However, interestingly, it was demonstrated that Cas9 induced mutagenesis is highly specific in both mouse embryos (Wang et al., 2013a; Yang et al., 2013) and human iPS cells (Smith et al., 2014; Veres et al., 2014). In addition, whole genome sequencing and examination of selected off-target loci has yet to find any off-target effects in *C. elegans* (Waaijers and Boxem, 2014). Off-target mutagenesis may be less of a problem in the normal cells (Singh et al., 2015), as well as smaller and less redundant *C. elegans* genome. Despite of the low risk of off-target, the phenotype of mutant generated by CRISPR-Cas9 system should be analyzed with caution, and this target specificity can be determined follow the general rules, such as backcrossing, independent alleles generated *via* multiple sgRNAs, and synonymous mutation rescuing (Waaijers and Boxem, 2014; Li et al., 2015; Tian et al., 2015).

ENHANCED EFFICIENCY OF CRISPR-CAS9 MEDIATED GENOME EDITING

Despite several reports of highly efficient mutagenesis, it has become clear that the efficiency of CRISPR-Cas9 targeting can vary widely, from gene to gene and between different sgRNAs. Much effort is being applied to discern the causes of this variation and to boost efficiency of the reaction. Since Cas9 targeted DNA cleavage only requires two

components (Cas9 and the sgRNA), strategies to improve efficiency have focused on enhancing Cas9 activity and sgRNA binding activity.

Cas9

Cas9 generates blunt-ended DSBs close to the PAM sequence in a process mediated by two nuclease domains: HNH and RuvC, which cleave the DNA strand complementary and non-complementary to the sgRNA, respectively. Mutations of either one of the two nuclease domains (H840A in HNH and D10A in RuvC) result a nickase activity of Cas9 (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013c), which creates a single stranded break (SSB) that can be repaired *via* the high fidelity base excision repair (BER) pathway (Dianov and Hubscher, 2013). Several studies in mammalian and *Drosophila* have reported that a double-nicking approach significantly improves the on-target DSBs specificity through offset nicking (Mali et al., 2013a; Ran et al., 2013; Port et al., 2014). Moreover, in the presence of a donor template, DNA cleaved by these nickases is preferentially repaired by HDR rather than by NHEJ. Consequently, use of these Cas9 nickases significantly increased the efficiency of HDR at the expense of NHEJ (Cong et al., 2013; Mali et al., 2013c) compared to the wild-type Cas9. As yet, use of nickase Cas9 has not been reported in *C. elegans*. It would be very interesting to explore whether Cas9 nickases can increase the targeting efficiency *via* the HDR in *C. elegans*. Expression level of Cas9 also affects the frequency of mutant generation. An early *C. elegans* study demonstrated high concentration of Cas9 expression plasmid in injection significantly increases the target mutations (Friedland et al., 2013). Thus, injection of *in vitro* synthesized mRNA or protein may increase the frequency of target mutagenesis on account of the higher dose of Cas9 in the germline nucleus compared to that achieved after injection of a Cas9-encoding plasmid (Table 1) (Chiu et al., 2013; Cho et al., 2013; Katic and Grosshans, 2013).

sgRNA

The other important and critical component of the CRISPR-Cas9 system is the single guide RNA, which find and bind to the targeted genome loci. Increasing the binding activity of sgRNA may enhance the CRISPR based DNA cleavage efficiency. Recent progress in both mammalian and *C. elegans* experiments demonstrated that a modified sgRNA (E+F) (E, hairpin extension; F, A-U flip), containing an extended Cas9-binding hairpin structure and deleted for a putative Pol III terminator (4 consecutive U's) in the sgRNA stem-loop by an A-U base pair flip, displays improved activity (Chen et al., 2013a; Ward, 2015). Another provocative finding in *C. elegans* is that guide RNAs with a GG motif at the 3' end of the target-specific sequences can dramatically enhance the frequency of mutagenesis *via* both NHEJ and HDR (Farboud and Meyer, 2015). Combining these two strategies for sgRNA design may further increase the frequency of mutagenesis.

IDENTIFICATION OF MUTATION THROUGH EFFICIENT SELECTION

Even though CRISPR-Cas9 mutagenesis can be highly efficient, it is equally important to minimize the time and the labor spent in finding the mutant animals. CRISPR-Cas9 mediated genome editing can be identified by basic molecular techniques such as PCR amplification of the site surrounding the DSBs. PCR amplicons from F₁ progeny (and co-

injection marker positive) worms can then be analyzed using an endonuclease which specifically recognizes mismatched DNA, such as T7 endonuclease I or CEL I. It is also relative easy to identify heterozygotes for deletions generated by dual sgRNA directed knockout (Chen et al., 2014; Xu and Chisholm, 2014). By adding a restriction site to the donor template, HDR based genomic modification can be more easily identified by PCR followed by enzyme digestion (Dickinson et al., 2013; Paix et al., 2014). The precise mutation can then be sought by DNA sequencing. It is important to note that any mutant identified in PCR must be confirmed in subsequent generations to avoid false positives due to somatic mutations (Cho et al., 2013). In cases when mutagenesis efficiency is low, PCR screening of F₁ mutants can be labor intensive. It was recently demonstrated that detection of targeted mutants could be benefited from refinement with the combination of *C. elegans* genetics. Several promising methods have been established to exploit visible phenotypes, resistance to drug treatment or selections for markers, minimizing the hands-on labor, the time and the money spent on PCR screening (Table 2).

Visible phenotype selection

The initial reports of CRISPR-Cas9 mediated genome editing in *C. elegans* tested genes whose loss of function was well known to confer visible recessive postembryonic phenotypes, such as Unc or Dpy. Newly induced mutations could be simply identified by isolating F₁ transgenic animals (putative heterozygotes) and examining their F₂ progeny for the expected phenotype (Friedland et al., 2013). Several other papers describe similar strategies to isolate CRISPR generated mutants (Chiu et al., 2013; Cho et al., 2013). Insertion mutations could in principle also be isolated by rescue of a visible phenotype such as Unc (Dickinson et al., 2013), as successfully used in MosSCI mediated genome editing (Frokjaer-Jensen et al., 2008; Dickinson et al., 2013). Thus, visible phenotype selection, if applicable, provides a simple and powerful way to detect desired mutations.

Antibiotic-resistance selection

Selection for antibiotic- or drug-resistance is a powerful means to find dominant resistant mutants. Initially, several groups tested the efficiency of CRISPR-Cas9 targeted gene mutation using *ben-1* (*benomyl resistance-1*) (Chen et al., 2013b; Katic and Grosshans, 2013), encoding a tubulin whose loss-of-function confers dominant resistance to benomyl. Comparable selections can also be used to identify HDR events and simplify the screen strategy. Several exogenous antibiotic gene cassettes have been used in *C. elegans*, such as neomycin, puromycin, hygromycin B and blasticidin. Selection and counterselection strategies using *HygR* (hygromycin B-resistance gene) or *BSD* (blasticidin-resistance gene) have been successfully applied to select animals with a transgene inserted by Cas9 *via* HDR (Chen et al., 2013b; Kim et al., 2014). In cases where HygR/BSD expression may disrupt the function of the modified gene, the antibiotic gene cassette can be flanked with *LoxP* sites and removed by expression of Cre recombinase. The delivery of Cre can be achieved either by subsequent microinjection of plasmid (Dickinson et al., 2013), or by a heat-shock inducible promoter embedded in a self-excising drug selection cassette (SEC) that was developed recently (Dickinson et al., 2015).

Fluorescence marker selection

C. elegans is effectively transparent, allowing fluorescence imaging in live animals and providing another efficient positive selection strategy. Insertion of green fluorescent protein (GFP) to the CRISPR-Cas9 targeted site allows mutated animals to be identified by suitable fluorescence microscopy (Dickinson et al., 2013; Tzur et al., 2013; Waaijers et al., 2013). Generated mutants in any fluorescence marker background and identified morphology defects based on the fluorescence signal may provide another efficient way to isolate the mutants. This simple selection strategy by visible fluorescent marker could significantly reduce the hands-on screening work.

Co-CRISPR selection

A hallmark of the natural CRISPR-Cas9 system is its ability to cleave multiple distinct targets simultaneously and efficiently (Cong et al., 2013); within a single nucleus, the Cas9 protein can be targeted to multiple genomic loci directed by different sgRNAs. Thus, use of a previously proven sgRNA that results in an easily recognized visible phenotype may allow to identification of progeny of an animal in which Cas9 was active. The validity of such multiplexing approaches is supported by the success of co-CRISPR strategies, in which selection of animals with CRISPR-Cas9 induced NHEJ mutations at the *unc-22* locus enriched for CRISPR-Cas9 induced NHEJ events at a second unlinked locus (Kim et al., 2014). This elegant co-CRISPR strategy not only significantly improved the frequency of detecting NHEJ events (30%—80%), but also facilitated recovery of HDR events (10%—50%) (Kim et al., 2014). Such co-CRISPR strategies should be very valuable in increasing efficiency in the generation of desired mutations, and potentially without the necessity of extensive screening, or counter-selection.

Co-conversion selection

Similar to co-CRISPR, in which CRISPR-Cas9 induced NHEJ mutation at the *unc-22* locus increase the efficient recovery of other NHEJ events (Kim et al., 2014), a co-conversion strategy was also invented to efficiently recover HDR events in any gene in an otherwise unmarked genetic background (Arribere et al., 2014). This strategy is also based on the idea that multiplex CRISPR of a marker gene, where HDR yields an easily discernable phenotype, should enable identification of animals with the desired (unmarked) mutation event, since such animals must have descended from a parental germline containing active Cas9, guide RNA and donor DNA templates. Dominant mutations in morphological cuticle collagen genes such as *dpy-10(cn64)*, *sqt-1(e1350)* and *rol-6(su1006)* were induced and used to screen the F₁ animals (Arribere et al., 2014). In addition, rescue of a temperature sensitive lethal mutant *pha-1(e2123)* has also been used as an alternate co-conversion selection marker for HDR events (Ward, 2015). In contrast to co-conversion of dominant mutations, which does not depend on a specific genetic background, the *pha-1(ts)* approach has to start with a mutant animal and results in restoration of a wild-type phenotype (Ward, 2015). These co-conversion methods significantly improved the efficiency up to 80% (Arribere et al., 2014; Ward, 2015). The successes of these distinct variations of co-editing selection highlight the robustness of CRISPR-Cas9 genome editing methods to detect the

desired mutant. With this, it is potential that any mutation of a gene could be made without any constraints on the genetic background, minimizing the hands-on screening.

DESIGN CONSIDERATION AND IMPLEMENTATION

It is less than two years since CRISPR-Cas9 was first reported in *C. elegans*, and the state of the art is in rapid flux. Many variables and strategies have been tested in many laboratories, making it hard at times to fairly compare the different approaches. However, some general considerations and advice can be put forward. Based on the selection schemes summarized above, we can now design different approaches to generate desired knock-ins and knock-outs, and some potential strategies are summarized in Table 3. These approaches combine the advantages of current optimized selection methods. The main purpose of all these strategies is: (1) to increase specificity and efficiency of mutagenesis, and (2) to minimize the time and the hands-on labor needed to detect and recover mutants. As yet, no method can be absolutely guaranteed to generate the desired mutation. Possibly the most important advice is that several different sgRNAs should be tested using SUREYOR assay to compare the efficiency of the DNA cleavage, since for any given gene or site a significant fraction of sgRNAs tested fail to induce any events (Arribere et al., 2014; Kim et al., 2014). The basis of this variable effectiveness of sgRNAs remains unclear. The coupling of optimized sgRNA design [3' GG-guide RNA and sgRNA(E+F)] with co-CRISPR, co-conversion, or recently designed SEC strategies may together strongly enhance mutagenesis and facilitate mutant recovery (Dickinson et al., 2015; Farboud and Meyer, 2015).

PERSPECTIVES

The advent of CRISPR-Cas9 represents an effective next-generation method for programmable genome editing, which will have significant impacts on future advancements in genetic manipulations in *C. elegans*. It will change the way that we think of and perform the genetic and genomic analysis. Combined with the power of *C. elegans* forward genetics, existing genetic mutants and powerful developmental genetic tools that already available, the tractability of CRISPR-Cas9 offers researchers a versatile genome editing tool to modify genes of interest. The CRISPR-Cas9 system is rapidly evolving, and fresh developments are reported monthly. However, some applications of CRISPR-Cas9 have not been reported in *C. elegans*. For example, catalytically inactive Cas9 fusion proteins can be targeted to specific DNA loci to regulate gene transcription in other systems (Gilbert et al., 2013; Larson et al., 2013; Qi et al., 2013; Ji et al., 2014; Choudhary et al., 2015), but this inactive Cas9 regulated transcription has not been described yet in *C. elegans*. CRISPR-Cas9 technology should also dramatically facilitate the use in other nematodes lacking well-developed genetics, including species distantly related to *C. elegans* or parasitic nematodes. A further application is to organelle inheritance. Mitochondria contain their own mtDNA genomes and mitochondrial genetics is drastically different from the Mendelian genetics of nuclear genes. mtDNA is present in multiple copies in different stage in *C. elegans*, ranging from ~25,000 copies in the embryo and first three larvae stage to 780,000 copies in the adult stage (Lemire, 2005). Recently, mitochondrial DNA has been mutated by mitochondrial-targeted TALENs (Bacman et al., 2013; Gammage et al., 2014; Reddy et al., 2015), suggesting potential ways to edit the mitochondrial genome. Mitochondrial genome editing

by Cas9 has not so far been reported, but may be another avenue for CRISPR-Cas9 mediated targeted DNA editing not only in *C. elegans* but also in other systems. Indeed, CRISPR-Cas9 significantly reduces the technological barrier for reverse genetics in any organism, opening up a much wider arena for the genetic dissection of cellular processes.

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Fig. 1. Diagram of CRISPR-Cas9 genome editing methodology in *C. elegans*

Cas9 (dodger blue) and guided RNA (green) can be expressed in *C. elegans* germline by microinjecting the mixture of plasmids, mRNA or combination of Cas9 protein and sgRNA mRNA. Successfully expressed Cas9 and sgRNA will be assembled as a complex at its dsDNA target site. Two nuclease domains RuvC and HNH cleave the DNA close to 5' end of the PAM (yellow) sequence and generate double-strand breaks (DSBs), which typically repaired by error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ can lead to the introduction of insertion/deletion mutations (indels) (purple) of various lengths, which therefore disrupt gene function. HDR-mediated repair can introduce specific point mutation or insert/delete desired sequences (red) through recombination of the target locus with provided exogenous donor templates.

Table 1Cas9 and sgRNA delivery platforms in *C. elegans*

Cas9	sgRNA	Mutation type	Mutation frequency (%) ^a	Reference
Plasmid DNA	Plasmid DNA	NHEJ	0.5—88 ^b	Chen et al., 2013b; Friedland et al., 2013; Lo et al., 2013
		HDR	0.4—26.3	Dickinson et al., 2013; Tzur et al., 2013; Paix et al., 2014; Zhao et al., 2014
mRNA	mRNA	NHEJ	0.06—0.65 ^c	Chiu et al., 2013; Katic and Grosshans, 2013
Protein	mRNA	NHEJ	3.3—9.4 ^b	Cho et al., 2013

^aThe efficiency cannot be directly compared from different experiments;^bThe efficiency was based on F1 animal;^cThe efficiency was based on P0 animal injected.

Table 2

Selection strategies for identifying mutations by CRISPR-Cas9 system

Selection strategy	Repair event	Marker	Reference
PCR approach selection	NHEJ and HDR	Co-injection marker	Dickinson et al., 2013; Chen et al., 2014; Paix et al., 2014; Shen et al., 2014
Visible phenotype selection	NHEJ and HDR	Dpy or Unc, restored WT phenotype [<i>unc-119(+)</i>]	Chiu et al., 2013; Cho et al., 2013; Dickinson et al., 2013; Friedland et al., 2013; Zhao et al., 2014
Antibiotic resistance selection	NHEJ and HDR	Benomyl, hygromycin, ivermectin, blasticidin	Chen et al., 2013b; Katic and Grosshans, 2013; Kim et al., 2014; Zhao et al., 2014; Dickinson et al., 2015
Fluorescence selection	HDR	Fluorescence protein expression	Dickinson et al., 2013; Lo et al., 2013; Tzur et al., 2013
Co-CRISPR selection	NHEJ and HDR	Twitching (<i>unc-22</i>)	Kim et al., 2014
Co-conversion selection	HDR	Roller [<i>dpy-10(gf)</i> , <i>rol-6(gf)</i> or <i>sqt-1(gf)</i>] or restored WT phenotype [<i>pha-1(ts)</i>]	Arribere et al., 2014; Ward, 2015

Table 3CRISPR-Cas9 targeted genome editing strategies in *C. elegans*

Purpose	Potential approaches (plasmids ^a microinjection)	Marker	Reference
Gene disruption (small indels)	Cas9 + sgRNA ^b	Co-CRISPR sgRNA ^c [<i>unc-22(IV)</i> or <i>dpy-10(II)</i>]	Kim et al., 2014; Paix et al., 2014
Gene disruption (large deletion)	Cas9 + 2 sgRNAs	Co-CRISPR sgRNA [<i>unc-22(IV)</i> or <i>dpy-10(II)</i>]	Chen et al., 2014; Kim et al., 2014; Xu and Chisholm, 2014
Conditional knock-out	Cas9 expression under tissue-specific or inducible promoter + sgRNA	Co-injection marker (e.g., <i>rol-6</i>)	Shen et al., 2014; Li et al., 2015
Precise mutation (deletion, insertion or substitution)	Cas9 + sgRNA + ssODNs	Co-conversion sgRNA (<i>dpy-10</i> or <i>sqt-1</i>)	Arribere et al., 2014; Paix et al., 2014; Ward, 2015
Large gene insertion (e.g. GFP, antibiotic)	Cas9 + sgRNA + donor DNA (linearized or plasmid)	Co-conversion sgRNA (<i>dpy-10</i> or <i>sqt-1</i>) or antibiotic selection	Arribere et al., 2014; Paix et al., 2014; Ward, 2015; Dickinson et al., 2015
Single copy insertion	Cas9 + sgRNA + DNA construct	<i>unc-119(+)</i> or antibiotic as selection marker	Chen et al., 2013b; Dickinson et al., 2013; Tzur et al., 2013

^aHigh purity of plasmid DNA could help to increase the efficiency of mutagenesis (lab experience);

^bsgRNA can be designed through the online tool that was developed by Feng Zhang's group at <http://crispr.mit.edu/> (Hsu et al., 2013), which not only facilitates the selection and validation of sgRNAs but also predicts off-target loci for specificity analyses;

^cBoth *unc-22(IV)* and *dpy-10(II)* can be used as co-CRISPR markers, choose either one that is unlinked to the gene of interest.