CHAPTER TWENTY

Transgene-Free Genome Editing by Germline Injection of CRISPR/Cas RNA

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Abstract

Genome modification by CRISPR/Cas offers its users the ability to target endogenous sites in the genome for cleavage and for engineering precise genomic changes using template-directed repair, all with unprecedented ease and flexibility of targeting. As such, CRISPR/Cas is just part of a set of recently developed and rapidly improving tools that offer great potential for researchers to functionally access the genomes of organisms that have not previously been extensively used in a laboratory setting. We describe in detail protocols for using CRISPR/Cas to target genes of experimental organisms, in a manner that does not require transformation to obtain transgenic lines and that should be readily applicable to a wide range of previously little-studied species.

1. THEORY, PHILOSOPHY, AND PRACTICAL CONSIDERATIONS

1.1. Overview

CRISPR/Cas provides a method for the generation of double-strand DNA breaks at sites that the user can select with great efficiency and a high degree of freedom (Gaj, Gersbach, & Barbas, 2013; Kim & Kim, 2014; Mali, Esvelt, & Church, 2013). It should not be necessary in this context to discuss in detail the origins and nature of nucleases with engineered specificity, or of CRISPR/Cas, in particular; many others have done this in great detail, and anyone reading this chapter is likely familiar with what CRISPR/Cas can do for them. Instead, we wish to explain the particular issues and potential advantages associated with transgene-free delivery of CRISPR/Cas, and to explain in detail how this can be performed, both in general and with specific application to the nematode worm *Caenorhabditis elegans*, or indeed to other animals possessing a similarly accessible germline.

The first work toward the creation of modern standard laboratory model organisms began a little more than a century ago in T. H. Morgan's laboratory at Columbia University (Sturtevant, 2001). Since those beginnings, worldwide communities of researchers have combined their efforts to build the resources that have made it highly rewarding to perform genetic and later molecular studies in standard laboratory model organisms, including the fruit fly *Drosophila melanogaster*, the roundworm *C. elegans*, the zebrafish *Danio rerio*, the mouse *Mus musculus*, and the thale cress *Arabidopsis thaliana*. The global communities focusing on each of these organisms shared their mutant collections, developed and optimized specialized protocols for mutagenesis and for transgenesis in each species, and eventually had access to completely

sequenced genomes. Emerging technologies promise to make it feasible for researchers interested in organisms not previously well studied and lacking such a worldwide network of collaborators to establish these animals as powerful molecular genetic experimental systems: an individual researcher with limited resources can generate useable draft genome assemblies by means of high-throughput sequencing (Schatz, Delcher, & Salzberg, 2010); the use of molecular markers has made access to rich collections of visibly phenotypic mutations unnecessary for mapping and for strain construction (Rounsley & Last, 2010; Wicks, Yeh, Gish, Waterston, & Plasterk, 2001), and RNAi and now engineered nucleases make it possible to study gene function (Boutros & Ahringer, 2008; Frokjaer-Jensen, 2013; Gaj et al., 2013; Selkirk, Huang, Knox, & Britton, 2012). Application of these technologies has made it possible to engineer gene knock-outs in organisms that have only been studied sufficiently to generate on the order of 100 published papers listed in PubMed (Lo et al., 2013; Zantke, Bannister, Rajan, Raible, & Tessmar-Raible, 2014). In particular, the transgene-free delivery of CRISPR/Cas activity by direct injection of *in vitro*-synthesized RNAs (Chiu, Schwartz, Antoshechkin, & Sternberg, 2013; Katic & Grosshans, 2013; Lo et al., 2013) makes it possible to engineer the genomes of new species even without access to reliable protocols for DNA transformation in the species of interest.

1.2. When to use or not to use transgenes for delivery of CRISPR/Cas

When available protocols make the generation of transgenes straightforward and efficient, as is the case for *C. elegans*, CRISPR/Cas using transformation with DNA should be considered the strongly favored approach: it avoids the need to generate or to store reagents for injection as relatively unstable RNA, and the highest efficiencies reported using DNA transgenes to deliver CRISPR/Cas activity are better than the highest efficiencies reported using injection of *Cas9* mRNA or protein (Frokjaer-Jensen, 2013). This situation, however, results from the advantages of *C. elegans* as an established research organism; in other species there may be no reported efficient method of generating DNA transgenes, and experience suggests that some considerable effort may be required to develop transgenesis for new species, even given a similar anatomy and reproductive mechanism to that of *C. elegans* (Schlager, Wang, Braach, & Sommer, 2009). The transgene-free nature of the direct injection of CRISPR/Cas reagents is thus likely to offer a

powerful tool in organisms in which transgenesis has not been attempted, or is known to be challenging.

There have been recent developments in the application of CRISPR technology that make use of the easily programmable DNA binding of CRISPR but that use this binding to target the recruitment of transcriptional modification machinery rather than to induce double-strand DNA breaks (Gilbert et al., 2013). These methods are very exciting in their potential, but require for their use the persistent expression of the modified CRISPR reagents involved. The transient delivery of CRISPR reagents by direct injection of Cas9 mRNA is therefore unlikely to be well suited to these new technologies, transformation with DNA transgenes will be required.

1.3. Altered mutation profile from transgene-free treatment with CRISPR/Cas

Many groups have now reported on their experience using various methodologies to achieve genome modification by CRISPR/Cas in *C. elegans*; these approaches have included expressing the CRISPR/Cas reagents from a transgene, typically for a full generation; delivering the reagents as mRNA and guide RNA; or even direct injection of the guide RNA with Cas9 protein (Chiu et al., 2013; Cho, Lee, Carroll, & Kim, 2013; Dickinson, Ward, Reiner, & Goldstein, 2013; Friedland et al., 2013; Katic & Grosshans, 2013; Liu et al., 2014; Lo et al., 2013; Waaijers et al., 2013; Zhao, Zhang, Ke, Yue, & Xue, 2014). From these reports, it has become apparent that there are important differences between the use of transformation and transgene-free approaches for performing CRISPR/Cas regarding the types of mutations most frequently recovered.

Considering only those mutations recovered on the basis of a visible phenotype, rather than identified molecularly on the basis of altered sequence at the targeted site, most of the mutations induced in *C. elegans* by CRISPR/Cas cleavage using DNA transgenes were very small insertions or deletions (Friedland et al., 2013; Liu et al., 2014; Waaijers et al., 2013). By contrast, several groups have reported that the mutations induced by transient application of CRISPR/Cas reagents, whether by direct injection of guide RNAs with *Cas9* mRNA or by direct injection of Cas9 protein, showed a strong preference for very large deletions or even chromosomal rearrangements (Chiu et al., 2013; Cho et al., 2013; Lo et al., 2013); others reported mixed results or reported finding only small deletions and insertions (Katic & Grosshans, 2013; Liu et al., 2014). These large

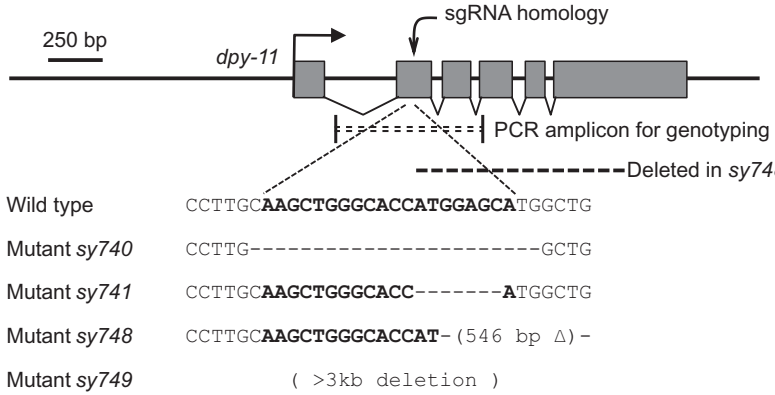

Figure 20.1 A schematic representation of the *dpy-11* locus of *C. elegans*. The position of the site selected for cleavage by targeted CRISPR/Cas activity is indicated. Note that the targeted site was within an early exon of the gene, to make it more likely that any resulting mutations would strongly disrupt gene function. Partial sequences of the wild type and of selected mutants isolated after CRISPR/Cas treatment targeting this site are shown (Chiu et al., 2013). In these sequences, the 20 nucleotides immediately prior to the **NGG** motif that were incorporated into the sgRNA used to target this locus are bolded. Although in this instance mutants were isolated on the basis of their mutant phenotype, it would have been possible to screen for molecular changes at this site, for example, by using PCR to amplify a small (600 nucleotide) region centered on the targeted site and testing for cleavage using *Nco*I (recognition sequence CCATGG). Note that this approach would only have recovered one of the four mutants shown, *sy740*; *sy748* and *sy749* mutant chromosomes would lack at least one of the primer binding sites used in the PCR amplification and would not be represented in the PCR product (compare the hypothetical PCR amplicon and the extent of the deletion *sy748* in the figure), and the seven nucleotide deletion in *sy741* destroys the endogenous *Nco*I site but the resulting sequence change generates a new *Nco*I site.

alterations have the advantage of generating mutations that are extremely likely to be molecular nulls, but these mutations are much more difficult to detect molecularly, if they cannot be recovered on the basis of a predictable viable visible phenotype. Molecular approaches to detecting changes induced by CRISPR/Cas rely on PCR amplification of the locus so that alterations can be detected using mismatch detection, by altered restriction digestion, or by a difference in amplicon size; large deletions of the sort preferentially seen with transgene-free CRISPR/Cas in *C. elegans* usually lack the primer-binding sites required for this amplification, and so such mutations are likely to be missed (Fig. 20.1). When genes are being targeted mutants of which cannot be screened for phenotypically, it may be helpful to use available template-directed repair knock-in strategies (Chen, Fenk, &

de Bono, 2013; Dickinson et al., 2013; Tzur et al., 2013; Zhao et al., 2014) in order to increase the likelihood of generating a lesion of predicable and easily detected molecular structure.

1.4. A note on specificity of CRISPR/Cas cleavage

There is considerable controversy regarding the specificity of mutations generated using CRISPR/Cas. Several studies have suggested CRISPR/Cas nuclease activity may frequently generate mutations at sites other than the one targeted for cleavage (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). Conversely, in our study we used whole-genome sequencing to assess the specificity of CRISPR/Cas activity when delivered by injection of *in vitro* synthesized RNAs in *C. elegans*, and did not observe frequent off-site changes (Chiu et al., 2013). It is conceivable that *C. elegans* for unknown reasons displays high specificity for CRISPR/Cas, or that the induction of mutations using transient delivery of CRISPR/Cas reagents inclines toward high specificity; even if so, off-target cleavage events might easily be more common in other species. If extremely high specificity is required, the researcher may wish to look into cleaving with two CRISPR/Cas complexes, each targeted very close to the other and targeting single-strand nickase activity or dimerizing Fok1 activity, such that binding at both sites is required to effect a double-strand break (Cho et al., 2014; Mali, Aach, et al., 2013; Ran et al., 2013; Tsai et al., 2014).

2. EQUIPMENT

Oligonucleotide synthesis service
DNA sequencing service
Microcentrifuge
UV spectrophotometer (e.g., NanoDrop)
Water bath
37 °C Incubator
−20 °C Freezer
−80 °C Freezer
PCR machine
Dissecting microscope
Bunsen burner
Needle puller
Inverted microscope with microinjection equipment

Agarose slab gel apparatus
Power supply
UV transilluminator

3. MATERIALS

Addgene plasmid 47911 SP6-hCas9-Ce-mRNA
Addgene plasmid 47912 SP6-sgRNA-scaffold
Restriction enzymes *Afl*II and *Kpn*I-HF, and accompanying incubation buffers
Phusion DNA polymerase (NEB)
QIAquick PCR Purification Kit (Qiagen)
QIAquick Gel Extraction Kit (Qiagen)
Gibson cloning kit (NEB)
MAXIscript SP6 Transcription Kit (Life Technologies)
mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies)
PolyA Tailing Kit (Life Technologies)
TURBO DNase (Life Technologies)
Ampicillin-sensitive transformation-competent *E. coli* (e.g., $CaCl_2$-competent DH5α)
Deoxynucleotide triphosphates (dNTPs)
RNase-free water
NGM agar
LB broth, LB agar
Agarose
Ethidium bromide (or alternative DNA visualization reagent)
Micropipettors
Micropipette tips
0.5 and 1.5-ml microcentrifuge tubes
250-μl PCR tubes
6 and 10 cm diameter Petri plates
Microcapillaries for the preparation of microinjection needles
Cover glasses for microinjection (24 × 50 mm)
20-μl micropipettes with mouth pipettor

4. IDENTIFYING A TARGET SEQUENCE

Look in your gene for suitable candidate target sites; when using the *Streptococcus pyogenes* Cas9 enzyme, these should be a 23 nucleotide sequence

ending with **NGG**. The site can be on either strand, so also seek out 23mer sequences starting with **CCN**. Cleavage will happen close to the end of the 20mer that precedes the **NGG** (after position 17). To ensure that mutations are likely to be strong loss-of-function alleles you should look within the coding sequence, ideally near to the 5′-end. The eventual sequence will be transcribed *in vitro* using SP6 RNA polymerase, which requires that the sequence start with a **G** (preferably with a **GA** or **GG**); however, this is not a consideration in identifying a target side, as if the endogenous 5′-end of the 23 nucleotide sequence is not a **G** then it can be replaced with a **G** or a **G** can be appended to the 5′-end.

Having compiled a list of candidates, use BLAST homology searches to identify target sites that do not have excessive homology to other, off-target sites in the genome. Note that it may be necessary to adjust the settings of your BLAST query for low stringency so that it will return results with appropriately weak homology; if running BLAST from the command line, include the argument "-word_size 7." Even the targeted site will only have an E-value on the order of e-05; any candidate off-target sites with an E-value less than 1 should be examined according to the criteria below. Target recognition and cleavage by CRISPR/Cas is most strongly influenced by sequences close to the 3′-end of the 20 nucleotides preceding the **NGG**, and the terminal GG sequence is required. Similar sequences elsewhere in the genome are common, with perfect matches of 15 nucleotides in a row being a common occurrence, but it should be readily feasible to identify a site for which such extended stretches of homology do not include nucleotides at or close to the 3′-end of the 20 nucleotides preceding the **NGG**, or that have high similarity but are not followed by **NGG**, indicating that these are not strong candidates for off-target cleavage by CRISPR/Cas. Note that there is another method to maximize specificity, instead of seeking sites with minimal sequence identity to other sites in the genome: as mentioned above, it is possible to modify the CRISPR/Cas cleavage protocol to replace the double-strand DNA cleavage activity with single-strand nickases or dimerizing FokI nuclease, such that two sites in close proximity must be recognized for a double-strand break to occur.

Another consideration is your ability to screen molecularly for any mutations you generate. If you can identify a cleavage site that is within or extremely close to the recognition sequence for a restriction enzyme (and one that does not also cleave again very close to the target site), PCR amplification of the target region followed by digestion with that restriction enzyme should readily detect lesions that destroy the recognition site.

5. GENERATING YOUR sgRNA CONSTRUCT

5.1. Oligonucleotide design

You will be using Gibson cloning to insert the first 20 nucleotides of your 23 nucleotide target ending in **NGG** into the vector SP6-sgRNA-scaffold. To do this, you will first order the synthesis of two oligos:

Forward oligo: Examine the first 20 nucleotides of your 23 nucleotide target site: this, or a modified version of this, will be incorporated into an oligonucleotide and cloned into an sgRNA expression vector—the final three nucleotides present in the genomic target site, the **NGG**, will not be included in this oligonucleotide or in this construct. If the first two of these 20 nucleotides are not either **GA** or **GG**, either replace these two nucleotides with **GA** or add a **G** or **GA** to the 5′-end of this 20 nucleotide sequence. Now, order the synthesis of an oligonucleotide that has this 20, 21, or 22 nucleotide sequence appended to the 3′-end of the sequence provided immediately below:

GATCCCCCGGGCTGCAGGAATTCATTTAGGTGACACTATA

Reverse oligo: Determine the reverse-complement of the sequence you appended to the 3′-end of the sequence provided above. Order the synthesis of an oligonucleotide that has this reverse-complement sequence appended to the 3′-end of the sequence immediately below:

GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

5.2. Insert generation

Mix the following in a 250-μl PCR tube:

10.0 μl of 5 × Phusion buffer
15.0 μl of 1 m*M* dNTPs
2.5 μl of 10 μ*M* forward primer
2.5 μl of 10 μ*M* reverse primer
19.5 μl H_2O
0.5 μl Phusion polymerase

Perform PCR according to the following program:

(1) 94.5 °C—3′
(2) 94.5 °C—20″
(3) 47 °C—25″
(4) 72 °C—25″
(5) Goto step 2, four times

(6) 94.5 °C—20″
(7) 50 °C—25″
(8) 72 °C—25″
(9) Goto step 6, four times
(10) 94.5 °C—20″
(11) 53 °C—25″
(12) 72 °C—25″
(13) Goto step 10, 19 times
(14) 72 °C—10′
(15) 4 °C—4″
(16) 15 °C—until stopped

Use QIAquick PCR Purification Kit to clean up the product according to manufacturer's protocol. At the end of the process, apply 10 μl ddH_2O heated to 70 °C to the column, centrifuge to collect eluate, and repeat once, collecting the eluate in the same tube. Store at −20 °C until linearized vector is available.

5.3. Preparation of linearized vector for the sgRNA construct

Mix the following in a 1.5-ml microcentrifuge tube:

4 μl of 10× NEB CutSmart buffer
5 μl of 500 ng/μl SP6-sgRNA-scaffold
29 μl H_2O
2 μl *Afl*II restriction enzyme (20 units/μl)

Incubate in 37 °C water bath for 4 h. Run product on a 0.8% agarose gel and purify using QIAquick Gel Extraction Kit according to manufacturer's protocol. At the end of the process, apply 16 μl ddH_2O heated to 70 °C to the column, centrifuge to collect eluate, and repeat once, collecting the eluate in the same tube. Store at −20 °C and use aliquots as needed.

5.4. Construction and identification of sgRNA synthesis plasmid

Mix the following in a 250 μl PCR tube:

3 μl of 2× Gibson reaction mix
1 μl Purified *Afl*II-digested SP6-sgRNA-scaffold (see Section 5.3)
2 μl Purified Phusion PCR product (see Section 5.2)

Incubate 1 h at 50 °C using a PCR machine with heated lid. It may be desirable to do a control Gibson reaction with no insert. Use at least half of the Gibson product to transform competent *E. coli* (for example, $CaCl_2$-

competent DH5α) according to standard methods (Seidman, Struhl, Sheen, & Jessen, 2001). Plate transformants on LB plates containing ampicillin or carbenicillin and grow overnight at 37 °C. Pick several (at least six) individual colonies and grow in 2–5 ml LB broth containing ampicillin or carbenicillin. Prep plasmid DNA minipreps by alkaline lysis and ethanol precipitation (Engebrecht, Brent, & Kaderbhai, 2001). Perform test digests using *Afl*II by mixing the following in a 1.5-ml microcentrifuge tube or a 250-μl PCR tube:

2.0 μl of 10× NEB CutSmart buffer
1.0 μl (~500 ng) Miniprep DNA
16.8 μl H_2O
0.2 μl *Afl*II restriction enzyme (20 units/μl)

Incubate in 37 °C water bath, heat block, or PCR machine for 2 h. Run the product on 0.8% agarose slab gel to identify colonies whose minipreps were not linearized by *Afl*II digest; these will be the colonies that have incorporated your insert and can be used to synthesize sgRNA. Errors are infrequently introduced in the amplification or cloning process; it may be desirable to confirm the DNA sequence of the clone you use prior to performing CRISPR/Cas treatment. Sequencing can be done using a T3 sequencing primer, keeping in mind that the minipreps produced by alkaline lysis were pure enough for restriction digestion but that DNA should be further purified for sequencing. Sequencing can wait until the end of Section 6.1.

6. *IN VITRO* SYNTHESIS OF sgRNA

6.1. Linearization of sgRNA template plasmid

By the end of step 5, you will have generated and miniprepped plasmids for *in vitro* transcription of your sgRNA. Before transcribing from these plasmids you should linearize them. If you have not yet confirmed the DNA sequence of your finished sgRNA template plasmid, you may wish to linearize more than one clone. You will later require linearized SP6-hCas9-Ce-mRNA plasmid, which can be prepared identically and simultaneously (see Section 7.1).

Mix the following in a 1.5-ml microcentrifuge tube:

5 μl of 10× NEB CutSmart buffer
12 μl (~6 μg) Miniprep DNA (from clone that was not linearized by *Afl*II)
31 μl H_2O
2 μl *Kpn*I-HF restriction enzyme (20 units/μl)

Incubate in 37 °C water bath for 4 h. Purify with QIAquick PCR Purification Kit or run on a 0.8% agarose slab gel and purify with QIAquick Gel Extraction Kit. At the end of the process, apply 9 μl RNase-free water heated to 70 °C to the column, centrifuge to collect eluate, and repeat once, collecting the eluate in the same tube. Product can be stored at −20 °C. Determine DNA concentration using an UV spectrophotometer. If the DNA sequence of the template plasmid has not been determined, submit part of the purified sample(s) for sequence determination prior to the next step.

6.2. *In vitro* transcription to generate sgRNA

Use MAXIscript SP6 Transcription Kit according to manufacturer's protocol; briefly, combine the following in a 1.5-ml microcentrifuge tube:

250 ng linearized sgRNA template DNA, plus RNase-free water, totaling 8 μl
2 μl of 10 × Reaction buffer
2 μl each ATP, CTP, GTP, and UTP solutions
2 μl Enzyme mix

Incubate in 37 °C water bath for 3 h. Optionally, treat reaction mix with DNase according to manufacturer's protocol. Proceed immediately to next step.

6.3. Purification of *in vitro*-transcribed sgRNA

Use MEGAclear Transcription Clean-Up Kit according to manufacturer's protocol. Elute with 30 μl RNase-free water and repeat once, collecting eluate in same tube. Determine RNA concentration using UV spectrophotometer. Store in aliquots in a −80 °C freezer.

7. *IN VITRO* SYNTHESIS OF *hCas9* mRNA

7.1. Linearization of SP6-hCas9-Ce-mRNA plasmid

If you have not already linearized the SP6-hCas9-Ce-mRNA plasmid, do so now, similarly to the procedure in Section 6.1. Mix the following in a 1.5-ml microcentrifuge tube:

5 μl of 10 × NEB CutSmart buffer
8 μl (500 ng/μl) SP6-hCas9-Ce-mRNA plasmid
35 μl H_2O
2 μl *Kpn*I-HF restriction enzyme (20 units/μl)

Incubate in a 37 °C water bath for 4 h. Purify with QIAquick PCR Purification Kit or run on an 0.8% agarose slab gel and purify with QIAquick Gel Extraction Kit. At the end of the process, apply 9 μl RNase-free water heated to 70 °C to the column, centrifuge to collect the eluate, and repeat once, collecting the eluate in the same tube. Product can be stored at -20 °C.

7.2. *In vitro* transcription of *hCas9* mRNA

Use mMESSAGE mMACHINE SP6 Transcription Kit according to the manufacturer's protocol. Briefly, mix in a 1.5-ml microcentrifuge tube:

6 μl Purified linearized SP6-hCas9-Ce-mRNA plasmid
10 μl NTP/CAP solution
2 μl of 10 × Buffer
2 μl Enzyme mix

Incubate in 37 °C water bath for 4 h. Add 1 μl TURBO DNase and incubate in a 37 °C water bath for 15 min. Immediately proceed to the next step.

7.3. Polyadenylation of *in vitro*-transcribed *hCas9* mRNA

Use the PolyA Tailing Kit according to the manufacturer's protocol. Briefly, mix in a 1.5-ml microcentrifuge tube:

20 μl DNase-treated reaction mix
36 μl RNase-free water
20 μl of 5 × *E*-PAP buffer
10 μl of 25 m*M* $MnCl_2$
10 μl of 10 m*M* ATP
4 μl *E*-PAP enzyme

Incubate in 37 °C water bath for 1 h. Proceed immediately to next step.

7.4. Purification of *in vitro*-transcribed, polyadenylated *hCas9* mRNA

Use MEGAclear Transcription Clean-Up Kit according to manufacturer's protocol. Elute with 30 μl RNase-free water and repeat once, collecting eluate in the same tube. Determine the RNA concentration using a UV spectrophotometer. Store in aliquots in a −80 °C freezer.

8. INJECTION OF sgRNA AND mRNA

You will prepare a mixture of *hCas9* mRNA and sgRNA. Note that in order to enhance your ability to molecularly identify any resulting

mutation events, it may be desirable to include a template for homology-directed repair of the double-strand breaks induced by CRISPR/Cas activity; several groups have published relevant methods (Chen et al., 2013; Dickinson et al., 2013; Tzur et al., 2013; Zhao et al., 2014).

In preparing your mixture, we recommend a ratio of sgRNA concentration to mRNA concentration of approximately 1:4 to 1:8, and have thus far found the best results when injecting the highest concentration we could readily achieve—concentrations on the order of 100 ng/μl sgRNA and 800 ng/μl mRNA. Some DNA microinjection protocols call for the use of a microinjection buffer (Mello & Fire, 1995); we did not find this to be necessary. Having prepared the mixture, spin at maximum speed in a microcentrifuge (~13,000 rcf) for at least five minutes to clear the supernatant of any particulate matter, then heat the mixture briefly to 95 °C and place it on ice. The mixture should remain on ice until it is loaded into injection needles.

For *C. elegans* and similar nematodes, follow standard microinjection procedures (Mello & Fire, 1995), injecting to flood the germline syncytia of young adults as is done for DNA transformation.

9. RECOVERY OF MUTANTS GENERATED USING CRISPR/Cas

9.1. Recovery and plating of injected animals

After being injected, animals can be floated in M9 buffer and transferred using a 20 μl micropipette and a mouth pipettor to Petri plates containing NGM that have been seeded with a bacterial food source. After the animals have visibly recovered, individual injected animals or a small number per Petri plate should be transferred to new seeded NGM Petri plates in preparation for screening their progeny. Mutations induced by injection of CRISPR/Cas mRNA and sgRNA in *C. elegans* will be found at highest frequency among the progeny produced between 8 and 16 h postinjection, and essentially none will be recovered among the progeny produced more than 24 h postinjection (Katic & Grosshans, 2013; Liu et al., 2014). It is therefore recommended that injected animals should be transferred from their recovery plate up to 8 h after being injected, and they should be discarded between 16 and 24 h postinjection, leaving behind the progeny they produced during their time on the plate.

9.2. Identification of animals carrying mutations induced by CRISPR/Cas

Induced visible mutants of *C. elegans* can be recovered by examining the F_2 progeny of injected animals for expression of the expected phenotype. If no visible phenotype can be predicted, or if homozygosity and expression of the visible phenotype is expected to be associated with an inviable phenotype such as sterility, it will be necessary to clone out individual F_1 progeny of injected animals, permit them to produce progeny, and to screen molecularly for mutations at the targeted site. This protocol will not describe in detail the methods involved in molecular detection of mutation events; briefly, the options include targeting for mutation an endogenous restriction site, such that any alteration will result in a PCR product that cannot be cleaved by the corresponding restriction enzyme (Friedland et al., 2013); mismatch detection methods such as the CEL-I Surveyor method (Colbert et al., 2001); recombinant CRISPR/Cas targeting the wild-type sequence (Kim, Kim, Kim, & Kim, 2014); or the inclusion of a template for homology-directed repair of the induced double-strand DNA break, such that repair events will produce a predictable and readily detectable sequence change. The addition of a second sgRNA previously demonstrated to reliably induce mutations in another gene that cause a visible phenotype, and screening for mutations in your targeted site only among animals displaying that visible phenotype, can enrich for the presence of mutations in the targeted site (Kim, Ishidate, et al., 2014).

Regardless of how mutations are detected, care should be taken to track all candidates back to the injected animal or small pool of injected animals that gave rise to them. If two mutants are recovered derived from the same injected animal or animals, these may not represent independent mutation events.

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