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Spotlight on CRISPR in *Strongyloides* parasitic nematodes

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Abstract

Parasitic nematodes are biomedically and economically important, but many are genetically intractable which limits our understanding of their molecular and cellular biology. Gang *et al.,* report CRISPR/Cas9 genome editing in parasites of the genus *Strongyloides*, generating both knockout and knock-ins and demonstrated heritability of the modifications, a crucial advance in the field.

Keywords

CRISPR; Cas9; Strongyloides; parasitic nematode

CRISPR in *Strongyloides*

Parasitic nematodes are a significant burden to human health and well-being. Over one billion humans are infected with parasitic nematodes, and they also infest crops and livestock, reducing food production. Despite their medical and economic importance, gaining detailed insight into their molecular and cellular biology is challenging. RNAi works well in a range of plant parasitic nematodes, but in many human and animal parasites RNAi efficacy is highly variable. Gang *et al.* [1] have recently described a major advance in parasitic nematode biology, developing CRISPR/Cas9 methods to successfully produce knockouts in *Strongyloides stercoralis* and *Strongyloides ratti*, demonstrating heritability of the modifications in *S. stercoralis*, and knocking in transgenes in *S. stercoralis* [1].

This study used the *Strongyloides* spp. *unc-22* gene as a test case (Figure 1). This gene has been successfully targeted by CRISPR/Cas9 in the free-living model nematode *Caenorhabditis elegans; unc-22* inactivation in *C. elegans* produces visible phenotypes such as impaired motility and twitching [2]. Importantly, this phenotype is dominant which allows identification of worms homozygous and heterozygous for the mutation. Gang *et al.* [1] observed the *unc-22* twitching phenotype in 16–37% of the F1 progeny from CRISPR/Cas9 edited parental *S. stercoralis* animals, indicating that they were disrupting the locus very effectively. One of the major challenges with animal and human parasitic nematodes is that

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they must be passaged through a mammalian host. Gang *et al.* [1] tested whether they could recover heritable mutations by passaging F1 *S. stercoralis* with a twitching *unc-22* phenotype through gerbil hosts (Figure 1). They recovered *unc-22* mutant animals in the F2 and F3 generation in 1.2–5% of animals examined, demonstrating that their edits were heritable. Critically, they recovered homozygous *unc-22* mutants in 2–5% of F1 progeny. This observation is important as, using traditional methodology, creating a homozygote from a mutant heterozygote is an arduous and time-consuming process that requires host passage followed by crossing of mutant males to females and passage of iL3 larvae through the host again; each additional passage would take roughly three weeks to recover next-generation larvae after introduction of iL3 (see [5 and 7] for a detailed description of how to recover and propagate homozygotes).

While the ability to create loss-of-function or reduction-of-function mutants is powerful, many of the most exciting applications of CRISPR/Cas9 editing come from knocking sequences into the genome using homologous recombination. Tagging endogenous genes with fluorescent protein cassettes, epitopes for protein purification, or knocking in specific mutations are just some of the powerful approaches enabled by CRISPR/Cas9-mediated editing in a range of organisms. Gang *et al.* [1] tested whether they could integrate transgenes by homologous recombination, knocking in a broadly expressed red fluorescent protein (*mRFPmars*) cassette into two genes (*unc-22* and *tax-4*; Figure 1). Focusing their screening on RFP positive animals (red), they obtained verified insertions in 42–75% of the screened animals. Together, these studies demonstrate the game changing feasibility of creating knock-ins and knock-outs in a parasitic nematode.

Surprises

One of the surprises from this study were observed differences in CRISPR/Cas9 editing in *Strongyloides* versus *C. elegans.* When Gang *et al.* [1] sequenced their twitching *unc-22* mutant candidates to determine the nature of the presumed mutation, they repeatedly only detected wildtype *unc-22* sequence. Reasoning that a larger deletion could remove one or both primer sites, the authors performed deep sequencing on a mixed population of wild-type and *unc-22* mutants and found deletions of more than 500 basepairs flanking the single guide RNA targeting site. While large deletions can occur in *C. elegans*, small insertion-deletions are more frequently recovered. Additionally, single-stranded oligonucleotides are very efficient repair templates to knock-in sequences using homologous recombination in *C. elegans* [3,4], while Gang *et al.* [1] could not recover any oligo-templated knock-ins in *unc-22* in *S. stercoralis.* However, it remains possible that using oligonucleotide templates may facilitate knock-ins at other loci. These data suggest that the preferred mechanisms to repair Cas9-induced DNA double-stranded breaks could differ between nematode species. Given the diversity of parasitic nematodes, an ancillary benefit to attempting CRISPR/Cas9 in a range of parasites could be insight into mechanisms of DNA repair.

Fresh challenges and exciting opportunities for study of parasitic nematodes

The efficient recovery of deletions in *unc-22* and *tax-4* creates the possibility of systematically interrogating gene function in *Strongyloides.* Moreover, *Strongyloides* can be cryopreserved so mutant lines would not need to be continuously propagated to maintain strains. If error-prone double strand break repair frequently causes large deletions in *Strongyloides*, this feature would greatly facilitate creation of nulls in a gene of interest. It will be imperative to characterize the standard features of these large deletions in order to design primers for genotyping. The authors were able to recover *unc-22* mutations on the basis of phenotype, however PCR-based genotyping is critical for identifying deletions in genes that lack a scorable mutant phenotype.

This work highlights the power of combining expertise with *C. elegans* and parasitic nematodes. Implementing transformative techniques, such as CRISPR/Cas9 genome editing, requires intimate knowledge of parasite culture and biology [5], while the *C. elegans* community offers field tested methods that have been demonstrated to be robust [6,7]. Indeed, we held a workshop at the recent International *C. elegans* meeting designed to bring *C. elegans* and parasitic nematode experts together, to drive precisely this type of research. Going forward, it will be important to develop methods to enrich for edits that do not produce scorable phenotypes. One attractive option is to use co-conversion; in *C. elegans* CRISPR/Cas9-mediated edits at a locus of interest are enriched by selecting for an unrelated edit that produces a visible phenotype. Indeed, *unc-22* was the first co-conversion marker developed in *C. elegans* [2]. However, paralyzed, twitching animals might compromise the ability of an animal to propagate in a host so other markers may need to be developed. Possibilities would include mutations that change morphology [8], ideally without compromising infectivity, or repair of a mutant allele to restore wildtype animals [3]. A variation on this theme could be to knock-in a broken mutant fluorescent reporter and use its restoration as a co-CRISPR marker to select for other knock-ins. Development of drug selection approaches would also be a powerful way to enrich for transgenics; hygromycin selection has been one of the more popular approaches for obtaining CRISPR/Cas9-mediated knock-ins in *C. elegans* [9]. Gene drive systems could be another method to increase editing efficiency and obtain homozygotes, though use comes with ethical implications [10]. Development of CRISPR/Cas9 methods in *Strongyloides* spp. is an important advance in the study of parasitic nematodes, and will hopefully provide a blueprint to establish CRISPR/Cas9 in other parasitic nematode species.

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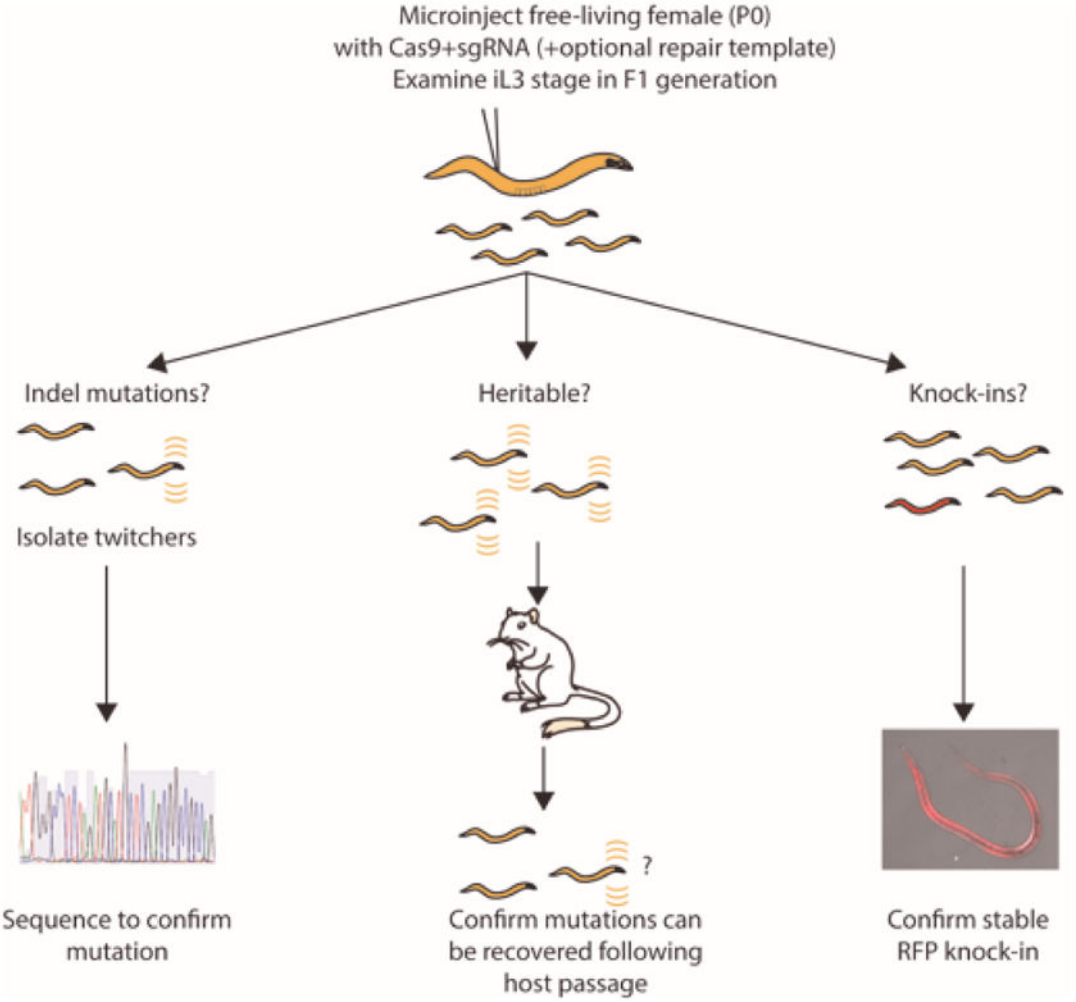

Figure 1. CRISPR/Cas9 in *Strongyloides sp.* produces knock-outs and knock-ins

Gang *et al.* [1] introduced Cas9 and single guide RNAs (sgRNA), either encoded on plasmids or as ribonucleoprotein complexes, through microinjection into *Strongyloides stercoralis* and *Strongyloides ratti* female germlines (P0). In cases where the authors wanted to knock sequences into the genome, plasmid or single-stranded oligonucleotide repair templates were included in the injection mixture. Females were injected, mated with males, and the resulting F1 progeny were allowed to develop to the infectious third larval stage (iL3). For initial proof-of-principle, the *unc-22* locus was targeted, which produces a dominant twitching phenotype. First the authors asked whether mutations could be introduced and sequenced the *unc-22* locus in twitching iL3s in *S. stercoralis* and *S. ratti*. Editing produced large deletions, confirmed by whole genome sequencing. Next the authors asked whether these edits were heritable in *S. stercoralis*, either infecting gerbils with a mixed population of wild-type and twitching iL3s or with a twitcher-enriched population of iL3s. In both cases, twitching mutants were recovered in the F2 and F3 generation, demonstrating that the initial mutations were in germ tissue and heritable. Finally, the authors asked whether exogenous DNA could be integrated by homologous recombination in *S. stercoralis*, testing both plasmids and oligonucleotides. While they were unsuccessful

with the oligonucleotide repair template, they successfully integrated a broadly expressed *mRFPmars* (RFP) transgene in to the *unc-22* locus. The gerbil image and micrograph of the *mRFPmars* knock-in were reproduced from [1] under a CC-BY 4.0 license (https://creativecommons.org/licenses/by/4.0/).