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REVIEW

Recent advances in functional genomics for parasitic nematodes of mammals

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ABSTRACT

Human-parasitic nematodes infect over a quarter of the world's population and are a major cause of morbidity in low-resource settings. Currently available treatments have not been sufficient to eliminate infections in endemic areas, and drug resistance is an increasing concern, making new treatment options a priority. The development of new treatments requires an improved understanding of the basic biology of these nematodes. Specifically, a better understanding of parasitic nematode development, reproduction and behavior may yield novel drug targets or new opportunities for intervention such as repellents or traps. Until recently, our ability to study parasitic nematode biology was limited because few tools were available for their genetic manipulation. This is now changing as a result of recent advances in the large-scale sequencing of nematode genomes and the development of new techniques for their genetic manipulation. Notably, skin-penetrating gastrointestinal nematodes in the genus *Strongyloides* are now amenable to transgenesis, RNAi and CRISPR/Cas9-mediated targeted mutagenesis, positioning the *Strongyloides* species as model parasitic nematode systems. A number of other mammalian-parasitic nematodes, including the giant roundworm *Ascaris suum* and the tissue-dwelling filarial nematode *Brugia malayi*, are also now amenable to transgenesis and/or RNAi in some contexts. Using these tools, recent studies of *Strongyloides* species have already provided insight into the molecular pathways that control the developmental decision to form infective larvae and that drive the host-seeking behaviors of infective larvae. Ultimately, a mechanistic understanding of these processes could lead to the development of new avenues for nematode control.

KEY WORDS: Parasitic nematode, Parasitic helminth, *Strongyloides*, CRISPR, Transgenesis, RNAi

Introduction

Infections with parasitic nematodes are some of the oldest known diseases of humans. Ancient Greek and Roman physicians described patients suffering from tapeworms and roundworms (Grove, 1990). Soil-transmitted intestinal parasitic nematodes such as hookworms in the genera *Necator* and *Ancylostoma*, threadworms in the genus *Strongyloides*, and giant roundworms in the genus *Ascaris* can cause intestinal distress, stunted growth and long-term cognitive impairment in children, and even death in the case of *Strongyloides stercoralis* infection (Schafer and Skopic, 2006). Moreover, vector-transmitted tissue-dwelling filarial nematodes cause very different diseases, depending on the nematode species. For example, *Onchocerca volvulus* and *Brugia malayi* cause river blindness and elephantiasis, respectively (Lustigman et al., 2012). Today, a combination of improved sanitation, education and administration of the anthelmintic (anti-parasitic-helminth) drugs ivermectin and albendazole has reduced the overall disease burden caused by these parasites (Prichard et al., 2012). Nevertheless, over one-billion people worldwide remain infected with parasitic nematodes (Lustigman et al., 2012).

Despite being familiar pathogens, many aspects of parasitic nematode biology remain mysterious. This is due in part to the complex life cycles of these parasites. Some parasitic nematodes, such as the filarial worms *B. malayi* and *Onchocerca volvulus*, require both invertebrate and vertebrate hosts and are transmitted to humans when hematophagous arthropods take a blood meal (Devaney, 2006; Nelson, 1991; Smith, 2000). Skin-penetrating nematodes such as hookworms and threadworms have both free-living stages that develop in the environment and parasitic life stages that live in mammalian hosts (Bryant and Hallem, 2018a,b; Gang and Hallem, 2016). Other parasitic nematodes such as nodular worms in the genus *Oesophagostomum* reside in the environment and infect when they are ingested (Bryant and Hallem, 2018a,b; Gang and Hallem, 2016). Once inside a host, many nematode species can survive for years as reproductive adults.

Two of the drugs commonly used to treat parasitic nematode infections include ivermectin, which targets an invertebrate-specific chloride channel, and albendazole, which targets β-tubulin (Atif et al., 2019; Prichard et al., 2012). However, these drugs do not prevent reinfection (Prichard et al., 2012). Moreover, resistance to anthelmintic drugs has already developed in parasitic nematodes that infect livestock and is likely to develop in parasitic nematodes that infect humans in the near future (Prichard, 1994). Thus, the identification of novel drug targets is a high priority. A better understanding of the development, behavior and physiology of these parasites could lead to the identification of potential drug targets and enable the development of new chemotherapeutics.

Functional genomic techniques such as transgenesis, RNA interference (RNAi) and targeted mutagenesis are necessary to delve into the biological mechanisms that enable parasitic nematodes to find and infect humans, and to develop both inside and outside their human hosts. For example, the soil-dwelling infective larvae of many parasitic nematodes must actively locate human hosts using host-emitted sensory cues, but the mechanisms that underlie these host-seeking behaviors remain poorly understood (Bryant and Hallem, 2018b; Gang and Hallem, 2016). Moreover, once they have entered a host, the parasites rely on host signals to continue development and navigate through the host body, but how this occurs is not yet understood. Understanding the sensory mechanisms used by these parasites to find and infect hosts, and to recognize and respond appropriately to the host environment, may lead to the development of traps, repellents or drugs that block these processes (Bryant and Hallem, 2018b; Gang and Hallem,

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2016). In addition, parasitic nematodes need to acquire essential metabolites, including heme and cholesterol, from their hosts (Chitwood, 1999; Luck et al., 2016). Identifying parasite-specific proteins used for nutrient uptake and metabolism could yield novel drug targets (Salinas and Risi, 2018). Thus, the ability to interfere with the function of specific genes in parasitic nematodes is necessary for mechanistic investigations into these and other aspects of their unique biology.

Functional genomic techniques, such as those listed above, are widely used in the free-living model nematode *Caenorhabditis elegans* (Ahringer, 2006; Evans, 2006; Farboud, 2017). The first requirement for adapting these techniques from *C. elegans* to parasitic nematodes is access to high-quality genome sequences. As a result of recent large-scale sequencing and annotation efforts, searchable genomes are available for over 100 nematode species through WormBase Parasite (Brindley et al., 2009; Howe et al., 2017; Hunt et al., 2016; International Helminth Genomes Consortium, 2019; Mitreva et al., 2007). Moreover, transcriptomic data are now available for many nematode species, paving the way for targeted studies of gene expression, gene function and genetic interactions (Jex et al., 2019). Thus, genomic and transcriptomic approaches are now well established and easily feasible in parasitic nematodes.

However, adapting transgenesis, RNAi and targeted mutagenesis techniques to parasitic nematodes remains challenging for a number of reasons. First, not all parasitic nematodes have free-living life stages into which exogenous RNA and DNA can be easily delivered. Many medically important parasitic nematodes, including filarial worms, exist solely inside intermediate or definitive hosts. In order to introduce transgenes into these parasites, adults or larvae must be isolated from hosts and maintained in culture for a prolonged period, and *in vitro* survival of these parasites is often low. Second, while *C. elegans* readily survives the delivery of exogenous nucleic acids, the survival of parasitic nematodes during and after delivery of exogenous nucleic acids is often low. Third, maintaining stable mutant or transgenic lines is both difficult and expensive because of the requirement for host passage. Nevertheless, in the last decade, significant progress has been made in overcoming these challenges in a number of different parasitic nematode species. This Review will describe some of the most recent advances in the development of functional genomic techniques for parasitic nematodes.

Transgenesis in parasitic nematodes

Transgenesis provides a powerful starting point for understanding many aspects of the biology of parasitic nematodes, including their development, reproduction and behavior. For example, transgenes containing reporter constructs can be used to identify genes that are expressed in sensory neurons and that are therefore likely to play a role in sensory-driven host-seeking behavior. In addition, transgenes containing functional or mutant genes can be used to study genes required for parasitic nematode development or behavior. Finally, transgenes containing exogenous constructs, including genetic silencers such as tetanus toxin (Schiavo et al., 1992) and calcium reporters such as GCaMP (Tian et al., 2009), can be used to provide some of the first insights into how the sensory nervous systems of parasitic nematodes respond to host-emitted sensory cues. In *C. elegans*, there are a number of different methods that can be used for introducing transgenes. The most commonly used method is intragonadal microinjection, in which exogenous plasmid or linear DNA is injected directly into the syncytial gonad of adult hermaphrodites and then is stably transmitted to progeny (Evans, 2006). Microparticle bombardment (also called biolistics), in which the DNA of interest is precipitated onto gold particles and then shot into worms using a 'gene gun', is also used to generate transgenic nematodes (Schweinsberg and Grant, 2013). However, whether these techniques are transferrable to parasitic nematodes depends on the biology of the parasite in question.

Transgenesis in *Strongyloides* species

The parasitic nematodes that are currently the most well-described models for genetic transformation are the human-parasitic nematode *S. stercoralis* and the closely related rat-parasitic nematode *Strongyloides ratti*. *Strongyloides stercoralis* is of particular interest because it is a parasite of humans that is estimated to infect ∼370 million people worldwide (Page et al., 2018). *Strongyloides stercoralis* and *S. ratti* are more amenable than most parasitic nematodes to genetic manipulation as a result of their life cycle, which includes a single free-living generation outside the host (Fig. 1A) (Bryant and Hallem, 2018b; Gang and Hallem, 2016). *Strongyloides* parasitic adult females are found in the host small intestine, and reproduce there by parthenogenesis. Their progeny exit the host in feces, and can then develop through one of two paths: they can either develop directly into infective third-stage larvae (iL3s), or they can develop into free-living male and female adults. All of the progeny resulting from matings between the free-living male and female adults developmentally arrest as iL3s, and must find and infect a host to continue the life cycle (Fig. 1A).

Like *C. elegans* hermaphrodites, the free-living adult females of *Strongyloides* have a syncytial gonad (Kulkarni et al., 2016; Schad, 1989). Thus, transgenics can be generated in these species by intragonadal microinjection of DNA into free-living adult females (Junio et al., 2008; Li et al., 2006, 2011; Lok, 2013, 2012; Lok and Artis, 2008; Lok et al., 2017). Expression of transgenes requires the use of endogenous regulatory regions; *C. elegans* regulatory regions generally do not function in *Strongyloides* (Junio et al., 2008; Li et al., 2006). Codon usage also differs widely across nematode species, and expression of genes from other species in *Strongyloides* often requires codon optimization of the genes for *Strongyloides* (Hunt et al., 2016; Lok et al., 2017; Massey et al., 2001; Mitreva et al., 2006; Moore et al., 1996). Intragonadal microinjection has also been used to generate transgenics in the closely related nematode *Parastrongyloides trichosuri*, a parasite of Australian brushtail possums that can cycle through multiple free-living generations (Grant et al., 2006). *Strongyloides stercoralis* free-living adult males are also amenable to genetic transformation via microinjection into the syncytial testes (Shao et al., 2017). The ability to cycle through one or more free-living generations is specific to the genera *Strongyloides* and *Parastrongyloides* (Lok, 2007). In parasitic nematodes that lack a free-living generation, the reproductive adults live exclusively within a mammalian host. As a result, gaining access to the reproductive adults for genetic manipulation requires either transiently removing them from a host or obtaining them from *in vitro* culture, and in both of these scenarios, nematode survival is generally very poor.

In *C. elegans*, injected DNA constructs are stably expressed across generations from extrachromosomal arrays, without the need for integration into the genomic DNA (Evans, 2006). However, this is not the case for the *Strongyloides* species, where extrachromosomal arrays are silenced after a single generation through an unknown mechanism (Junio et al., 2008). The generation of stably expressing transgenic lines in *Strongyloides* species requires genomic integration of the transgene, which has been achieved in both *S. stercoralis* and *S. ratti* using the piggyBac transposase system (Lok, 2013, 2012; Shao et al., 2012). The piggyBac system consists of one plasmid encoding the piggyBac transposase and a second plasmid encoding the transposon cargo to be incorporated. It has been widely used in a

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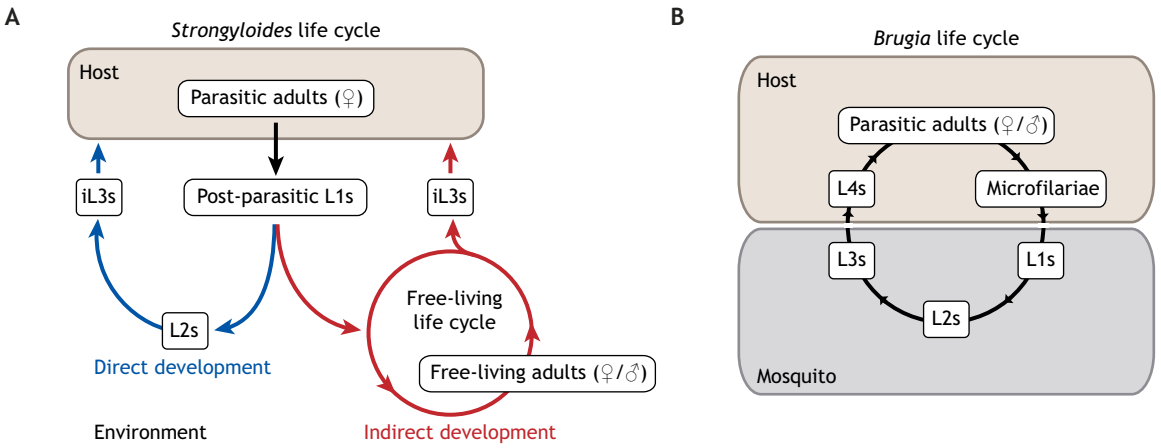

Fig. 1. The life cycles of parasitic nematode species that are currently models for genetic transformation. (A) The life cycle of the skin-penetrating gastrointestinal nematodes *Strongyloides stercoralis* and *Strongyloides ratti*. Parthenogenetically reproducing parasitic adult females reside in the host intestinal tract. Parasitic females excrete eggs (in the case of *S. ratti*) and larvae (in the case of *S. stercoralis*) into the environment in host feces. Post-parasitic L1 larvae then follow one of two distinct developmental trajectories: (1) direct development into infective third-stage larvae (iL3s) that must infect a host to continue the life cycle (blue arrows) or (2) indirect development through a single free-living generation in the environment (red arrows). Free-living male and female adults mate, and all of their progeny develop into iL3s that must infect a host. Free-living adult females of *S. stercoralis* and *S. ratti* are amenable to gonadal microinjection techniques and thus are well suited for genetic intervention. For simplicity, the indirect developmental cycle does not show post-parasitic L2–L4 larval stages or post-free-living L1–L2 larval stages. (B) The life cycle of the human-parasitic filarial nematode *Brugia malayi*. Male and female parasitic adults reside in the host lymphatic system. After mating, parasitic females release microfilariae that enter the host's bloodstream. A mosquito vector takes a bloodmeal from the infected host, resulting in ingestion of microfilariae. The microfilariae develop into L3s within the mosquito vector. When the mosquito takes another bloodmeal, the L3s are deposited into a new host, resume development, and eventually become male and female parasitic adults. Genetic transformation has been achieved in *B. malayi* female parasitic adults, microfilariae and L3s using a variety of different techniques including microinjection, microparticle bombardment and chemical transformation.

variety of different organisms for transgene integration (Kim and Pyykko, 2011; Lok, 2013). A drawback of the piggyBac system is that the genomic location at which the transgene is inserted cannot be controlled. As a result, this system has a number of disadvantages: it cannot be used to knock out specific genes, transgene expression may vary depending on the site of integration, and unintentional gene disruptions resulting from transgene integration are a possibility (Kim and Pyykko, 2011; Lok, 2013). The techniques for generating single-copy insertions at defined loci that have been used in *C. elegans*, such as MosSCI and SKI LODGE (Frøkjær-Jensen et al., 2008; Silva-García et al., 2019), have not yet been adapted to *Strongyloides*.

For *Strongyloides*, the generation of stable transgenic lines requires host passage. This is because it is not yet possible to propagate *Strongyloides* through its entire life cycle *in vitro*; as an obligate endoparasite, successive generations of the parasite can only be obtained by infecting a host and harvesting the subsequent generation of parasites from the host. The requirement for host passage presents a significant bottleneck with regard to the generation of transgenic lines of the human-parasitic nematode *S. stercoralis*, as many transgenic nematodes are required to establish a patent infection of *S. stercoralis* in either gerbils or dogs, its laboratory hosts (Lok, 2007). Nevertheless, the generation of F_2 transgenic larvae has been achieved (Lok, 2012). In this experiment, a population of 448 green fluorescent protein (GFP)-positive F_1 *S. stercoralis* infective larvae were injected into four gerbils (Lok, 2012). However, only two GFP-positive F_2 larvae were recovered, too few to infect a second round of gerbils and create a usable stable line (Lok, 2012). The generation of functional stable transgenic lines is much easier in *S. ratti* because only a few transgenic nematodes are required for establishing a patent infection in its natural host, the rat (Lok, 2012; Shao et al., 2012; Viney, 1999). Successful infection of a rat is theoretically possible with as few as one infective larva (Viney, 1999). Stable lines of transgenic *S. ratti* have been obtained starting with as few as 15 infective larvae (Shao et al., 2012). In this experiment, 19 GFP-positive transgenic infective larvae were recovered from the rat, which was enough to infect a second rat and propagate the transgenic line (Shao et al., 2012).

Transgenic *Strongyloides* have now been used in a number of different studies, primarily for analysis of gene expression patterns. The first genetic pathways studied in *Strongyloides* using transgenesis were those that control development of the infective larvae. The infective larvae of *Strongyloides* are developmentally similar to the dauer larvae of free-living nematodes such as *C. elegans*; both are developmentally arrested, non-feeding, environmentally resistant, third-larval stages (Crook, 2014; Hotez et al., 1993; Viney et al., 2005). However, in *C. elegans*, the dauer larva is an alternative larval stage that forms when environmental conditions are unfavorable; in *Strongyloides*, the infective larval stage is an obligate life stage. In *C. elegans*, the genetic mechanisms that control the formation of dauer larvae are well studied and include cGMP, insulin, transforming growth factor β (TGF-β) and dafachronic acid signaling pathways (Hu, 2007). Early studies that took advantage of the ability to generate transgenics in *Strongyloides* asked whether similar mechanisms also regulate formation of *Strongyloides* infective larvae. For example, the phosphatidylinositol-3 (PI3) kinase catalytic subunit gene *age-1* acts in a subset of head sensory neurons in *C. elegans* to regulate dauer formation as part of the insulin signaling pathway (Murphy and Hu, 2013). The *S. stercoralis age-1* gene was subsequently shown to have a similar expression pattern, raising the possibility that the function of *age-1* is at least partly conserved across species (Stoltzfus et al., 2012).

A different study focused on the role of the DAF-16 transcription factor in regulating entry into the infective larval stage in *S. stercoralis*. In *C. elegans*, the forkhead box (FOXO) transcription factor DAF-16 is a master regulator of a number of genetic pathways, including dauer entry and maintenance, where it functions as a downstream effector of the insulin pathway (Tissenbaum, 2018). The *S. stercoralis daf-16* gene was shown to be expressed in the pharynx and a number of other tissues using a *Strongyloides daf-16::GFP* reporter (Castelletto et al.,

2009). Analysis of transgenic larvae expressing GFP-tagged mutant forms of *S. stercoralis* DAF-16 identified critical residues in DAF-16, based on knowledge of DAF-16 structure and function in *C. elegans*, that regulate intracellular trafficking in *S. stercoralis*. Finally, a transgene encoding a dominant-negative form of *S. stercoralis* DAF-16, truncated to remove the activation domain but not the DNA binding domain, was used to show that loss of DAF-16 function results in pharyngeal and intestinal abnormalities (Castelletto et al., 2009). Specifically, the nematodes failed to adopt the characteristic morphology of iL3s (Castelletto et al., 2009). This study demonstrated that DAF-16 is required for the formation of *S. stercoralis* iL3s, and was one of the first *in vivo* studies of gene function in *S. stercoralis*.

More recent work investigated the host-seeking behaviors of the soil-dwelling *S. stercoralis* infective larvae. The sensory mechanisms that *C. elegans* uses to navigate its environment are well studied (Bargmann, 2006); thus, *C. elegans* provides a useful foundation for identifying candidate genetic mechanisms that may underlie sensory-driven host seeking in *S. stercoralis*. In *C. elegans*, the cGMP-gated cation channel subunit gene *tax-4* acts in a subset of head neurons to regulate chemosensory and thermosensory behaviors (Coburn and Bargmann, 1996; Komatsu et al., 1999, 1996). In *S. stercoralis*, *tax-4* was found to have a similar expression pattern, suggesting it may also play a role in sensory-driven behaviors in this species (Bryant et al., 2018). Further analysis of the *S. stercoralis tax-4* gene using CRISPR/Cas9-mediated targeted mutagenesis then confirmed a role for *tax-4* in mediating thermotaxis behavior in *S. stercoralis* (see below) (Bryant et al., 2018).

Transgenesis in other parasitic nematodes

Transgenesis has also been achieved in the human-parasitic filarial nematode *B. malayi* using several methods at different life stages (Fig. 1B). Both gonadal microinjection and microparticle bombardment have been used to introduce DNA constructs into *B. malayi* (Higazi et al., 2002; Xu et al., 2011). For example, in the initial study, a DNA construct was generated that consisted of a ubiquitously expressed *B. malayi* promoter cloned upstream of the gene encoding GFP. This construct was microinjected into adult females. As a syncytial gonad could not be identified in *B. malayi*, DNA was microinjected into the uterus of the parasitic adult females at multiple locations (Higazi et al., 2002). Expression of GFP was seen in the vicinity of the microinjection site. When this construct was bombarded into adults, GFP expression was subsequently observed in the bombarded animals under the cuticle (Higazi et al., 2002). Microparticle bombardment has also been used in *Ascaris* embryos to drive transient expression of RNA and DNA constructs (Davis et al., 1999). A method for the chemical transformation of *B. malayi* larvae using a co-precipitate of calcium phosphate and plasmid DNA has also been developed (Xu et al., 2011). *Brugia malayi* larvae were chemically transformed *in vivo* inside gerbil hosts with a luciferase reporter. Subsequently, adults and microfilariae (pre-first-stage larvae) expressing luciferase were collected from the gerbils at low efficiency (Xu et al., 2011).

Recently, *B. malayi* human-infective L3 larvae were successfully transformed with DNA plasmids containing piggyBac constructs, resulting in stable genomic integration of reporter genes encoding either luciferase or a fluorescent reporter under the control of a ubiquitiously expressed *B. malayi* promoter (Fig. 2A) (Liu et al., 2018). To improve worm survival *in vitro* during transgene delivery, a transwell culturing system was devised that included a layer of feeder bovine embryo skeletal muscle cells separated from the nematodes by a filter, incubated at 37°C in the presence of 5% CO_2. This culturing system was necessary because *B. malayi* does not have a free-living life stage and thus does not survive well outside the host; the L3 larvae are normally transmitted directly from mosquitoes to humans (Fig. 1B). Lipofectamine was added to DNA plasmids to create micelles, which were then added to the wells. Worms were induced to molt by the addition of ascorbic acid, and were subsequently collected and screened for either luciferase activity or fluorescence. Nematodes that took up plasmid DNA exhibited fluorescence throughout their body or luciferase expression (Fig. 2B). When luciferase-expressing larvae were injected into gerbils, luciferase-expressing microfilariae were subsequently obtained from the gerbils. Thus, this system can potentially be used to generate stable transgenic lines of *B. malayi* (Liu et al., 2018). However, the transgenic microfilariae were not used to infect mosquitoes, and thus it remains to be seen whether transgenic nematodes can be propagated through their entire life cycle (Liu et al., 2018). By showing successful delivery of transgenes, expression of easily visible selectable markers and stable passage to progeny, this study paves the way for functional genomic studies of *B. malayi*.

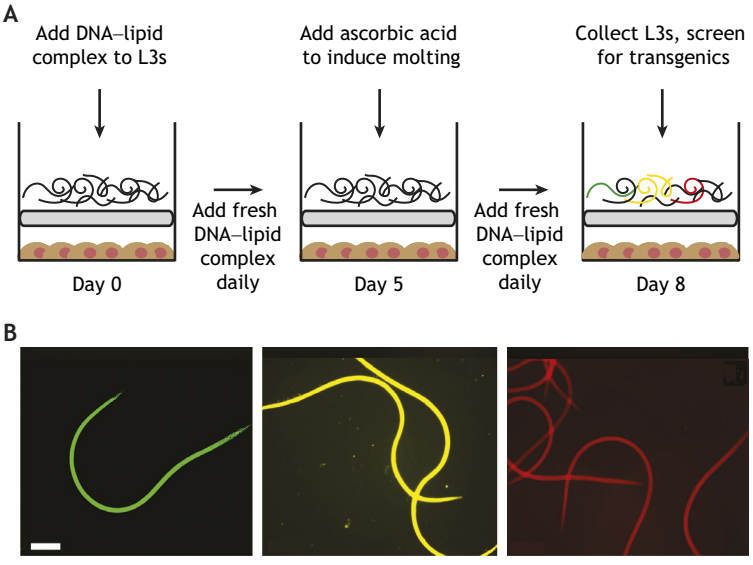

Fig. 2. Transfection of *B. malayi* using DNA–liposome complexes. (A) Schematic diagram of the transfection procedure used to make transgenic *B. malayi* L3s. Each well contained a layer of feeder bovine embryo skeletal muscle cells, a transwell insert and ~100 *B. malayi* L3s in RPMI media. Lipofectamine was added to plasmid DNA to produce DNA–lipid complexes (micelles), and the complexes were then added to the larvae in the wells. Every day for 8 days, the medium was changed and fresh DNA–lipid complex was added. On day 5, ascorbic acid was included to induce molting, as molting larvae are more likely to take up DNA. On day 8, worms were collected and screened for transgene expression. (B) Fluorescence images of *B. malayi* L3s expressing green fluorescent protein (GFP), yellow fluorescent protein (YFP) or cherry red (CHR) under the control of a ubiquitous *B. malayi* promoter (adapted from Liu et al., 2018). Scale bar: 300 nm.

A better understanding of many aspects of *B. malayi* biology, including how the larvae migrate within the mosquito, how the adults migrate within the human body, and how the adults evade the human immune system, will lead to new insights into the interactions between filarial nematodes and their hosts and may enable the identification of new targets for drug development. Furthermore, any new chemotherapeutics to arise from studies of *B. malayi* may also be effective against other filarial parasites such as *Wuchereria bancrofti*, another causative agent of elephantiasis, and *Onchocerca volvulus*, the cause of river blindness. An important next step will be the identification of endogenous promoters that drive cell-specific or cell-type-specific expression in *B. malayi* larvae and adults.

RNAi in parasitic nematodes

RNA interference has been used successfully for many years to experimentally knock down genes in *C. elegans* (Fire et al., 1998; Zhuang and Hunter, 2012). In this approach, double-stranded RNA (dsRNA) introduced into *C. elegans* knocks down gene expression by degrading the corresponding mRNA molecules (Ahringer, 2006). This technique has been widely used to investigate gene function in *C. elegans* in some contexts, including development, longevity and immune defense. However, a limitation of RNAi experiments is that gene function is reduced but not eliminated. This can make the interpretation of results from RNAi experiments difficult in some cases, particularly in the context of behavioral phenotypes.

RNAi has been applied to a number of plant-parasitic nematode and insect-parasitic nematode species (Banerjee et al., 2017; Chaudhary et al., 2019; Lilley et al., 2012; Maule et al., 2011; Morris et al., 2017; Ratnappan et al., 2016). In the case of mammalian-parasitic nematodes, RNAi has been successful in some species but not others (Maule et al., 2011). The first successful RNAi experiment in a mammalian-parasitic nematode used dsRNA to knock down acetylcholinesterase expression in the rat parasite *Nippostrongylus brasiliensis* (Hussein et al., 2002). In *C. elegans*, acetylcholinesterase activity is provided by three genes – *ace-1*, *ace-2* and *ace-3* – that act partially redundantly to regulate locomotion (Johnson et al., 1988; Kolson and Russell, 1985). Adult *N. brasiliensis* were extracted from rat intestine and soaked in a dsRNA solution. Both mRNA and protein expression were significantly reduced, but the expected movement phenotype was not observed (Hussein et al., 2002).

Since this first experiment, RNAi has been successfully applied to a number of mammalian-parasitic nematode species, including *Ascaris suum*, *B. malayi*, *Onchocerca volvulus*, the ruminant parasite *Haemonchus contortus* and the filarial parasite of livestock *Setaria digitata* (Hagen et al., 2012; Luck et al., 2016; Maule et al., 2011; McCoy et al., 2015; Misra et al., 2017; Samarasinghe et al., 2011; Somarathne et al., 2018; Verma et al., 2017). However, results from RNAi experiments are often variable in terms of both the extent of gene knockdown and the resulting mutant phenotype (Britton et al., 2016; Hagen et al., 2012; Maule et al., 2011; McCoy et al., 2015). Moreover, in some species, RNAi has been attempted without success (Lendner et al., 2008). Commonly used methods of delivery include soaking; feeding bacteria that express dsRNA constructs to worms with environmental stages; injecting dsRNA solution into the pseudocoelomic fluid of *A. suum*; and '*in squito*' delivery, where dsRNA is administered to mosquitoes infected with *B. malayi* to target nematode genes (Hagen et al., 2012; McCoy et al., 2015; Song et al., 2010).

One of the species in which RNAi has been used most extensively is *H. contortus*, although with varying degrees of success (Blanchard et al., 2018; Britton et al., 2016; He et al., 2018; Huang et al., 2017; Maule et al., 2011; Ménez et al., 2019; Samarasinghe et al., 2011; Zawadzki et al., 2012). The first successful RNAi experiment in *H. contortus* involved knocking down β-tubulin genes by soaking larvae and adults in dsRNA (Kotze and Bagnall, 2006). The β-tubulins are components of the nematode cytoskeleton (Hurd, 2018), and are of particular interest in the case of parasitic nematodes because they are targets of the anthelmintic drug albendazole (Prichard, 1994). Knockdown of β-tubulin in *H. contortus* resulted in reduced motility in infective larvae (Kotze and Bagnall, 2006). In subsequent studies, RNAi induced by soaking in dsRNA solution was found to be most reliable for genes expressed in tissues such as the intestine that are exposed to the environment (Britton et al., 2016). For example, an *H. contortus* TGF-β receptor was recently characterized (He et al., 2018). The receptor was found to be expressed in the intestine, and knockdown using dsRNA resulted in fewer L3 larvae developing into L4 stages *in vitro* (He et al., 2018). Another recent study used RNAi to demonstrate that the nuclear hormone receptor NHR-8 plays a role in resistance to the anthelmintic drug ivermectin (Ménez et al., 2019). This is thought to be due to the role of NHR-8 in regulating xenobiotic detoxification pathways, as a number of drug detoxification genes show reduced expression in *C. elegans nhr-8* mutants (Ménez et al., 2019). These and many other studies demonstrate the broad utility of RNAi for the study of gene function in *H. contortus*.

While most studies have attempted to induce RNAi using dsRNA, several recent studies have instead used small interfering RNA (siRNA) for inducing RNAi (Misra et al., 2017; Somarathne et al., 2018). In *C. elegans*, long dsRNA is processed by the dicer complex into siRNA. siRNAs act as substrates for the RNA-induced silencing complex (RISC), which then binds to the complementary mRNA to cause mRNA degradation or translational repression (Dalzell et al., 2011). In some cases, the use of siRNAs appears to improve RNAi efficacy, perhaps by bypassing the need for dsRNA processing (Dalzell et al., 2011). siRNA was used in *B. malayi* to knock down the potential drug target UCP-galactopyranose mutase, which catalyzes the production of the cell surface component galactofuranose. Galactofuranose is an essential component of cell surface glycoproteins in several nematodes, but is absent in mammals (Misra et al., 2017). UCP-galactopyranose mutase knockdown in *B. malayi* adults resulted in severe movement defects and impaired embryogenesis, and knockdown in L3 larvae resulted in severely reduced infectivity. This study validated UCP-galactopyranose mutase as a potential drug target and demonstrated that incubation in siRNA can result in robust gene knockdown in *B. malayi* (Misra et al., 2017).

Until recently, successful RNAi experiments in *Strongyloides* had not been reported (Viney and Thompson, 2008). However, a recent study demonstrated the first successful mRNA knockdown in *S. ratti* (Dulovic and Streit, 2019) via an siRNA method similar to that used in *B. malayi* (Misra et al., 2017). Soaking *S. ratti* larvae in a solution containing siRNA resulted in knockdown of three out of three tested genes. Knockdown of the nuclear hormone receptor gene *daf-12*, which in *C. elegans* acts in the dafachronic acid signaling pathway to regulate dauer formation (Hu, 2007; Wang et al., 2015), using this approach demonstrated a role for *daf-12* in regulating entry into the infective larval stage (Dulovic and Streit, 2019). Specifically, soaking first-stage larvae in an siRNA solution targeting *daf-12* resulted in a reduction in the number of worms following the direct developmental cycle to become infective larvae, and an increase in the number of worms following the indirect developmental cycle to become free-living adults (Figs 1A and 3A,B). Moreover, when the iL3s that developed from siRNA-treated L2s were injected into a rat, the rats

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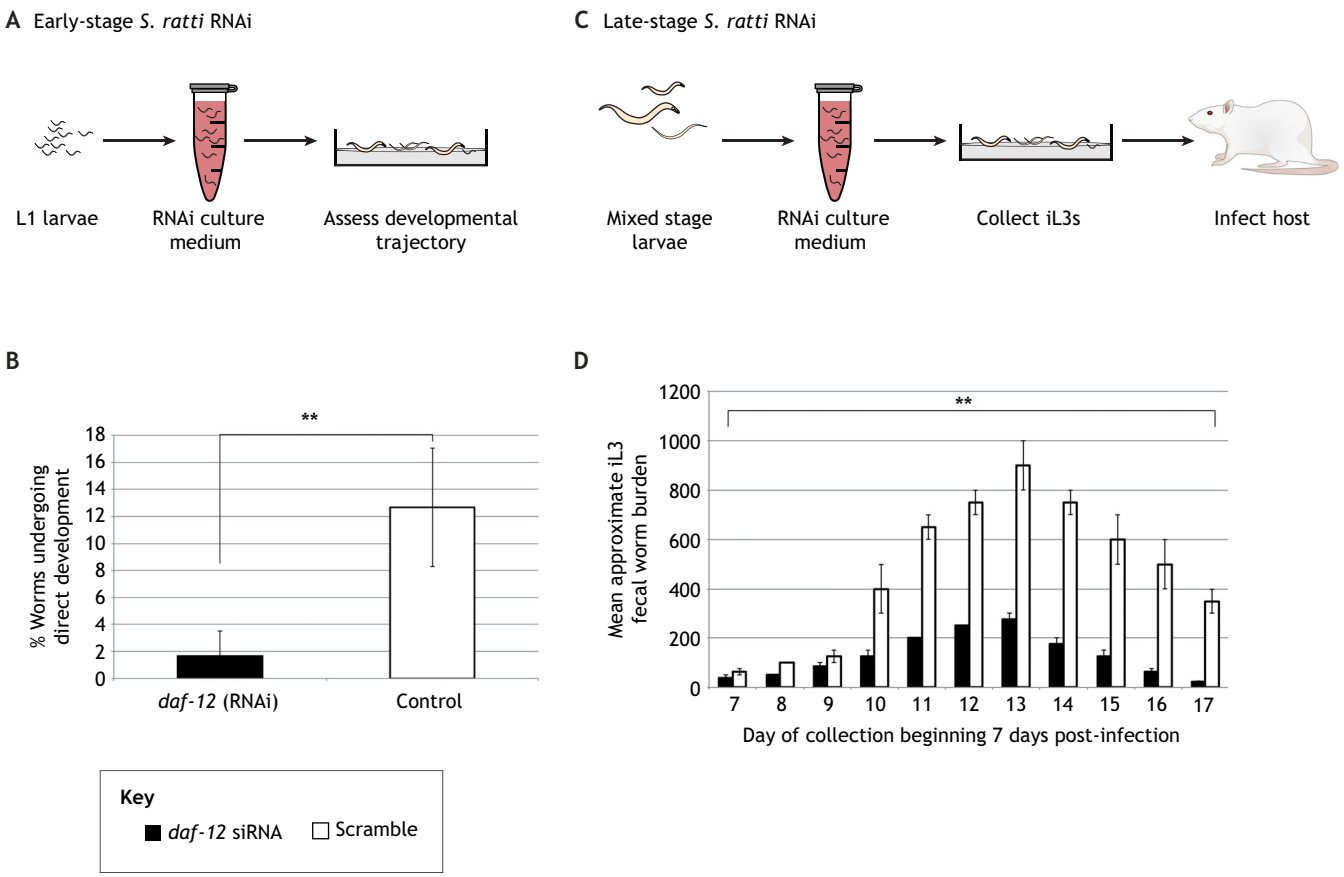

Fig. 3. RNAi-mediated knockdown of *daf-12* in *S. ratti* defines a role for *daf-12* in infective larval development. (A) Schematic diagram of early-stage RNA interference (RNAi) treatment of *S. ratti*. Post-parasitic L1 larvae were isolated and incubated in RNAi culture medium with either small interfering RNA (siRNA) targeting the *S. ratti daf-12* gene or a scramble control. Following incubation, nematodes were transferred to agar plates and their developmental trajectory was assessed. (B) Early-stage RNAi suppressing *S. ratti daf-12* reduces the proportion of nematodes that undergo direct development into iL3s. ***P*<0.001, Mann–Whitney test. (C) Schematic diagram of late-stage RNAi treatment of *S. ratti*. A mixed culture of free-living larvae and adults was incubated in RNAi culture medium with either siRNA targeting *S. ratti daf-12* or a scramble control. Following incubation, adults were transferred to agar plates supplemented with *Escherichia coli* HB101 to grow and reproduce. iL3 progeny were then collected and used to infect rat hosts. (D) Late-stage RNAi suppressing *S. ratti daf-12* results in a decreased worm burden in feces collected from infected rat hosts over the course of 7–17 days post-infection. ***P*<0.001, Student's *t*-test. (B and D adapted from Dulovic and Streit, 2019.)

had reduced worm burdens relative to rats injected with wild-type iL3s (Fig. 3C,D). These results implicate steroid hormone signaling via DAF-12 in the development and infectivity of *S. ratti* infective larvae (Dulovic and Streit, 2019). A *daf-12* homolog is also present in *S. stercoralis*; thus, these results may be valuable for understanding this human-parasitic species (Dulovic and Streit, 2019). In addition, these results make an important new addition to the *Strongyloides* genetic toolkit.

CRISPR/Cas9-mediated targeted mutagenesis in parasitic nematodes

The CRISPR/Cas9 system, which functions endogenously as a bacterial defense system, has been adapted for experimental use to modify the genomes of a wide range of both model and non-model organisms (Chen et al., 2014; Doudna and Charpentier, 2014; Tan et al., 2016). In this system, the Cas9 endonuclease complexes with a guide RNA that targets it to a specific sequence in the genome. The Cas9 enzyme then catalyzes a double-stranded break, which is repaired by the cell. In many organisms, indels are created when the non-homologous end-joining (NHEJ) pathway is used to repair the break, resulting in mutant alleles. In addition, breaks can often be repaired by homology-directed repair (HDR), in which an experimental repair template is used to incorporate transgenes of various lengths into the genome (Chen et al., 2014; Doudna and Charpentier, 2014; Tan et al., 2016). Repair events that result in large deletions or chromosomal rearrangements have also been observed in some cases (Adikusuma et al., 2018; Chiu et al., 2013; Kosicki et al., 2018; van Schendel et al., 2015).

The CRISPR/Cas9 system is widely used for genome engineering in *C. elegans* (Dickinson and Goldstein, 2016). The Cas9 endonuclease and the guide RNA can be introduced into *C. elegans* in a number of ways, including as *in vitro*-complexed ribonucleoproteins (RNPs) or as DNA plasmids (Dickinson and Goldstein, 2016). Repair templates for HDR can consist of DNA in a number of different forms, including small single-stranded oligodeoxynucleotides (ssODNs), plasmid constructs, large double-stranded linear DNA pieces, or large partially single-stranded linear DNA pieces (Dickinson and Goldstein, 2016; Dokshin et al., 2018; Paix et al., 2015). The first report of CRISPR/Cas9-mediated gene editing in a nematode other than *C. elegans* involved the creation of 'dumpy' (dpy) mutants, a class of mutants characterized by a body shape that is shorter and fatter than wild-type, in the non-parasitic nematode *Pristionchus pacificus* (Witte et al., 2015). RNP complexes targeting the *Ppa-dpy-1* gene, which encodes a collagen, were injected into adult females and their progeny were screened for mutant phenotypes. In one experiment,

multiple mutant lines with a dumpy phenotype were recovered. A few of the mutations may have been large deletions, but the majority were small indels (Witte et al., 2015).

More recently, CRISPR/Cas9-mediated targeted mutagenesis was achieved in *S. stercoralis* and *S. ratti*. In one study, a 24 base pair sequence was inserted into the *daf-16* gene, which encodes a FOXO transcription factor that controls developmental fate (Lok et al., 2017). However, a mutant phenotype was not observed (Lok et al., 2017). This study provided an initial proof-of-concept for the applicability of CRISPR/Cas9 to *Strongyloides*. In a second study, the twitchin gene *unc-22*, which encodes a large intracellular protein required for muscle function, was targeted in both *S. stercoralis* and *S. ratti* (Gang et al., 2017). The *unc-22* gene was selected because *C. elegans unc-22* mutants have an easily identifiable dominant motility ('Uncoordinated', or Unc) defect (Moerman and Baillie, 1979; Moerman et al., 1988). CRISPR components were introduced into free-living adult females by intragonadal microinjection, either as DNA plasmids or as RNP complexes. In both cases, a subset of the iL3 progeny from the microinjected females was found to have severe motility defects (Gang et al., 2017). Genotyping of *S. stercoralis unc-22* mutant iL3s revealed that in the absence of a repair template for HDR, indels resulting from NHEJ were not observed but large deletions of over 500 base pairs were observed. When a repair template was included, precise integration of the repair template occurred. When *S. stercoralis unc-22* iL3s were injected into a naive gerbil host, mutant iL3s were subsequently recovered, indicating that the CRISPR-induced mutations were heritable.

Importantly, CRISPR worked efficiently enough in *S. stercoralis* that *unc-22* homozygous mutants were generated in the F_1 generation (Gang et al., 2017). The ability to obtain homozygous mutants in the F_1 generation is critical for a number of reasons. In many cases, CRISPR/Cas9-induced DNA mutations will affect only one of the two alleles of the gene of interest, thus necessitating several cycles of breeding to generate an animal with two loss-of-function alleles. However, multiple cycles of breeding are difficult with *S. stercoralis* because this requires propagating the mutant parasites through laboratory hosts, and mutant parasites are generally not obtained in sufficient numbers to support propagation through hosts. Moreover, many of the most interesting genes to mutate will be those required for the parasites to infect a host, and parasites with loss-of-function mutations in these genes cannot be propagated through a host because of their inability to infect. Thus, obtaining homozygous knockouts in the F_1 generation bypasses the need for host passage and enables the study of genes with recessive phenotypes that are required for host infectivity. The rate at which homozygous mutants can be obtained in *S. stercoralis* varies depending on the efficiency of the Cas9 target site. Optimization of CRISPR/Cas9-induced mutagenesis in *S. stercoralis* to increase the efficiency with which homozygous mutants can be obtained is an active area of research.

This study established a CRISPR pipeline for *Strongyloides* (Fig. 4A) (Gang et al., 2017). Plasmids encoding Cas9, the guide RNA, and a repair template encoding a red fluorescent marker under the control of a strong *actin* promoter, which drives expression in body wall muscle, are microinjected into free-living adult females. The F_1 iL3 progeny of these females are screened for expression of the red fluorescent marker. The iL3s are then subjected to behavioral assays to analyze the phenotype of interest, and are PCR-genotyped *post hoc* for homozygous disruption of the gene of interest and integration of the repair template (Fig. 4A). In the case of *S. stercoralis unc-22* mutants, the behavioral assay involved exposing iL3s to a 1% nicotine solution, as nicotine is known to enhance the twitching phenotype of *C. elegans unc-22* mutants (Moerman et al., 1988). Consistent with the *C. elegans* phenotypes, *Strongyloides unc-22* iL3s twitch in the presence of nicotine, whereas wild-type iL3s paralyze (Fig. 4B). A subsequent study used the same CRISPR pipeline to interrogate the molecular basis of thermotaxis, as positive thermotaxis toward host body temperature is thought to be critical for host seeking (Bryant and Hallem, 2018a). This study found that positive thermotaxis requires the cGMP-gated cation channel subunit TAX-4 (Fig. 4C) (Bryant et al., 2018). In the absence of *tax-4*, *S. stercoralis* iL3s failed to migrate up a thermal gradient toward host body temperature (Bryant et al., 2018). Together, these studies demonstrate the utility of the *Strongyloides* CRISPR system for elucidating the molecular basis of behavior in parasitic nematodes.

Another recent study used CRISPR to target the *S. stercoralis* homolog of the *C. elegans* collagen gene *rol-6* (Adams et al., 2019). The CRISPR components were introduced by microinjection into either free-living females or iL3s. These components consisted of an RNP complex and an ssODN designed to mutate key residues in the *S. stercoralis rol-6* gene to create a dominant 'Roller' (Rol) phenotype. Roller worms have a helical twist in their body that impairs motility (Kramer and Johnson, 1993). In addition, lipofectamine was added to the microinjection mix to improve delivery of the CRISPR components across cell membranes. The Rol phenotype was then observed in a subset of the F_1 progeny of the microinjected females and a subset of the microinjected iL3s (Adams et al., 2019). These results demonstrate that mutant phenotypes can be induced directly in infective larvae through the inclusion of lipofectamine in the microinjection mix, and raise the possibility that a similar approach could be applied to other mammalian-parasitic nematodes such as hookworms that lack a free-living adult stage.

Conclusions and future directions

The past few years have seen a number of exciting new advances in transgenesis, RNAi and mutagenesis techniques for parasitic nematodes. These advances have greatly expanded the parasitic nematode genetic toolkit, and have enabled mechanistic studies of gene function in parasitic nematodes that were previously not feasible. Skin-penetrating nematodes in the genus *Strongyloides* are now amenable to transgenesis, RNAi and CRISPR/Cas9-mediated targeted mutagenesis, and are rapidly emerging as parasitic nematode genetic model systems. An important challenge in the future will be applying the tools that have been developed in *Strongyloides* to other parasitic nematodes, including those with very different life cycles and transmission modes. This will enable the identification of possible drug targets in these other species, including drug targets that may not be shared with *Strongyloides*. In addition, a comparative analysis of genetic mechanisms across species may lead to a better understanding of the genes and signaling pathways that shape the species-specific interactions of different parasitic nematodes with their hosts.

Another important direction for future research is the development of techniques for forward genetic screens in parasitic nematodes. Although chemical mutagenesis screens have been performed in *S. ratti*, mapping and propagating mutants obtained from these screens was not feasible (Guo et al., 2015; Viney et al., 2002). Finally, cell-type-specific transcriptional profiling has been performed in schistosomes (Wendt et al., 2018) but not parasitic nematodes. Extending cell-type-specific transcriptomic analysis to parasitic nematodes such as *Strongyloides* will be essential for the identification of candidate genes to target using CRISPR. For example, genes that are highly enriched in specific sensory neurons are likely to be important for the function of those neurons, and disrupting these genes will provide insight into sensory neuron

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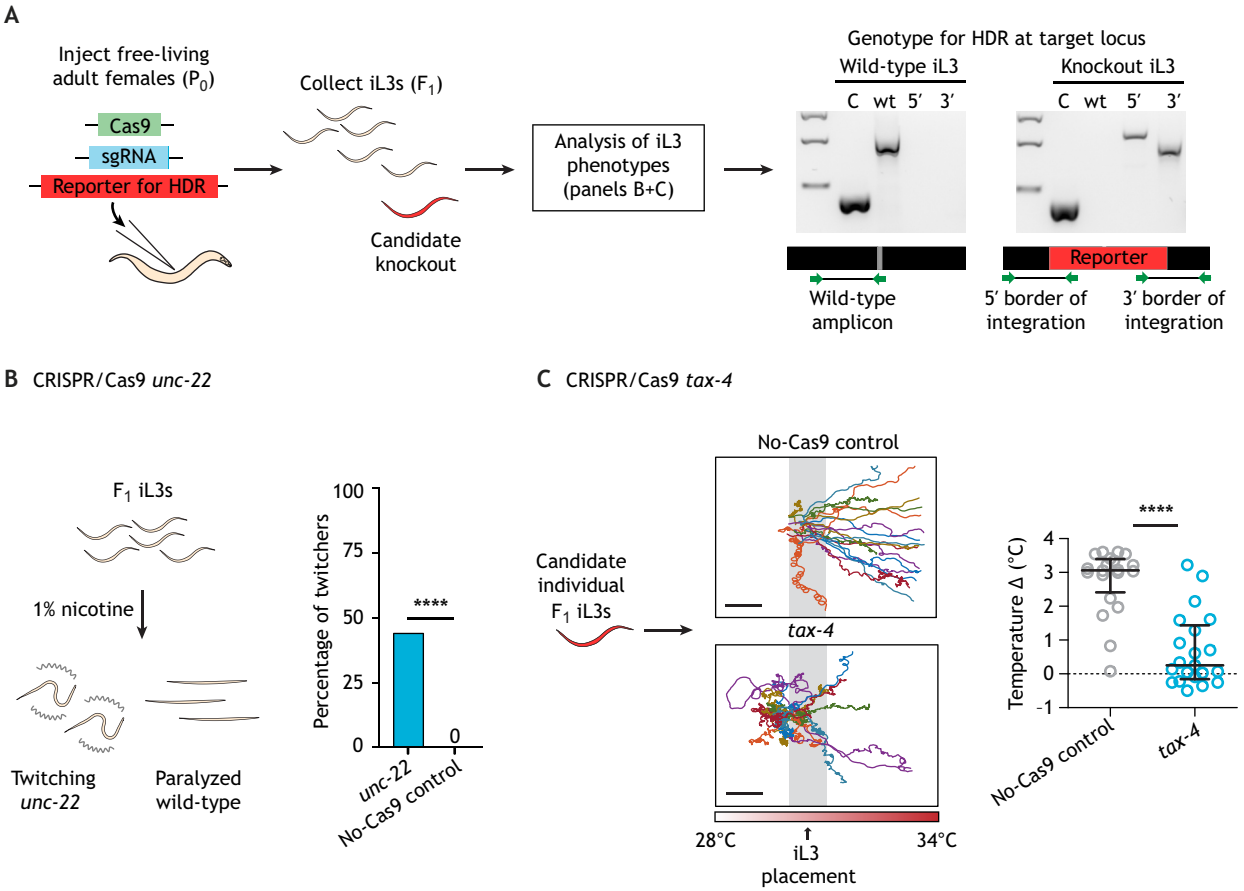

Fig. 4. CRISPR/Cas9-mediated targeted mutagenesis in *S. stercoralis*. (A) A pipeline for CRISPR/Cas9-mediated targeted mutagenesis in *S. stercoralis*. Individual plasmid vectors for Cas9, a single guide RNA (sgRNA) for the target of interest and a repair template for homology-directed repair (HDR) encoding an *mRFPmars* reporter are microinjected into *S. stercoralis* free-living adult females. iL3 progeny are screened for expression of the reporter indicating possible disruption of the target locus. Individual iL3 progeny are then tested for phenotypes. The iL3s are genotyped *post hoc* for disruption of the gene of interest by splitting the genomic DNA from each iL3 into four equal PCR reactions: C, control reaction confirming successful genomic DNA isolation; wt, reaction amplifying the wild-type locus of interest; 5′, reaction for HDR at the 5′ border of cassette integration; 3′, reaction for HDR at the 3′ border of cassette integration. 5′ and 3′ reactions only amplify if a successful HDR event has occurred at the target. Note the absence of a wt band for the knockout iL3 shown, suggesting a homozygous disruption of the gene of interest for this iL3. (B) Targeted mutagenesis of *S. stercoralis unc-22*. Free-living females were injected with CRISPR/Cas9 constructs targeting *unc-22* and F_1 iL3s were collected. F_1 iL3s were subjected to 1% nicotine treatment, which induces twitching in *unc-22* iL3s but results in paralysis in wild-type iL3s. Approximately 40% of F_1 iL3s displayed nicotine-induced twitching following CRISPR/Cas9 targeting of *unc-22*. Twitching, *mRFPmars*-expressing iL3s were collected and genotyped *post hoc* to confirm HDR at the *unc-22* target locus. As a control, the Cas9 construct was omitted from the pipeline shown in A to generate 'no-Cas9 control' iL3s; no nicotine-induced twitching was observed in these F_1 iL3s. ****P<0.0001, Fisher's exact test. (C) Targeted mutagenesis of *S. stercoralis tax-4*. Free-living adult females were injected with CRISPR/Cas9 constructs targeting *tax-4*, and *mRFPmars*-expressing F_1 iL3s were collected. Individual iL3s were then placed in a thermal gradient and allowed to crawl freely. The *tax-4* iL3s failed to migrate up the thermal gradient. By contrast, the no-Cas9 control iL3s engaged in robust positive thermotaxis. ****P<0.0001, Mann–Whitney test. (A and B adapted from Gang et al., 2017; C adapted from Bryant et al., 2018.)

function and thus sensory-driven host seeking in parasitic nematodes. In addition, cell-type-specific transcriptomic analysis may lead to the identification of genes with parasite-specific functions that could not be predicted from knowledge of *C. elegans*. Together, these approaches promise to yield new insights into the molecular basis of development, behavior and infectivity in parasitic nematodes. A better understanding of the molecular pathways that underlie parasitism in these species may lead to new avenues for nematode control.

Finally, *C. elegans* has an ever-expanding functional genomic toolkit, and an important direction for future research will be to adapt more of these new tools for use in parasitic nematodes. In particular, a number of approaches have now been used in *C. elegans* to study gene function in a temporally and spatially regulated manner. These include bipartite systems for the spatiotemporal control of transgene expression, such as the cGal4-UAS, Q-, FLP/FRT and Cre/Lox systems (Davis et al., 2008; Flavell et al., 2013; Hoier et al., 2000; Monsalve et al., 2019; Voutev and Hubbard, 2008; Wang et al., 2017); the split cGal4 system for more precise spatial or spatiotemporal control of transgene expression, including in cases where promoters that drive cell-specific expression are not available (Wang et al., 2018); the ZF1/ZIF and auxin inducible degradation (AID) systems for conditional protein degradation (Armenti et al., 2014; Zhang et al., 2015); and Cre/Lox-based systems for conditional gene disruptions (Chen et al., 2016; Kage-Nakadai et al., 2014). The adaptation of these functional genomic tools to parasitic nematodes would greatly facilitate interrogation of parasite-specific genes and behaviors, with the ultimate goal of developing novel defenses against these pathogens.

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Competing interests
The authors declare no competing or financial interests.

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