CRISPR in Parasitology: Not Exactly Cut and Dried!

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Review

CRISPR in Parasitology: Not Exactly Cut and Dried!

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CRISPR/Cas9 technology has been developing rapidly in the field of parasitology, allowing for the dissection of molecular processes with unprecedented efficiency. Optimization and implementation of a new technology like CRISPR, especially in nonmodel organisms, requires communication and collaboration throughout the field. Recently, a 'CRISPR in Parasitology' symposium was held at the Institut Pasteur Paris, bringing together scientists studying *Leishmania, Plasmodium*, *Trypanosoma*, and *Anopheles*. Here we share technological advances and challenges in using CRISPR/Cas9 in the parasite and vector systems that were discussed. As CRISPR/Cas9 continues to be applied to diverse parasite systems, the community should now focus on improvement and standardization of the technique as well as expanding the CRISPR toolkit to include Cas9 alternatives/derivatives for more advanced applications like genome-wide functional screens.

Highlights

CRISPR/Cas9 genome editing technology has greatly advanced functional studies in parasites such as *Leishmania*, *Plasmodium*, and *Trypanosoma*, and insect vectors, including *Anopheles*.

In *Plasmodium falciparum* and *Plasmodium yoelii*, alternative CRISPR-based technologies such as CRISPRi can modulate gene expression in the absence of genome editing.

In *Leishmania major, Leishmania donovani,* and *Leishmania mexicana*, a streamlined and highly efficient CRISPR/Cas9 system makes high-throughput mutant screens possible.

In *Trypanosoma brucei*, CRISPR/Cas9 makes highly efficient marker-free gene editing possible.

In *Anopheles gambiae* and *Anopheles stephensi*, CRISPR/Cas9-based gene drive systems show promise in advancing population suppression and replacement efforts.

A CRISPR/Cas9 Revolution in Parasitology

Parasitic diseases such as leishmaniasis, malaria, and trypanosomiasis remain an enormous burden on human health around the globe. While the genomes of *Leishmania*, *Plasmodium*, *Trypanosoma*, and *Anopheles* were first sequenced over a decade ago, the study of gene function has been slowed by tedious or inadequate genome-editing techniques [1–4]. Thus, the first studies using **CRISPR/Cas9** (see Glossary) gene editing in eukaryotes provided exciting new possibilities in the field of parasitology [5–7].

This simple, yet efficient system of genome editing uses the Cas9 endonuclease to generate a double-strand break (DSB) at a locus of interest in the genome (Figure 1A , Key Figure). Specificity is achieved via Cas9 binding to a **single guide RNA** (**sgRNA**), which must contain 20 nucleotides complimentary to a sequence in the genome flanking a protospacer-adjacent motif (PAM). The DSB break is then repaired with **homology-directed repair** (**HDR**) using a provided repair template or with the more error-prone **microhomology-mediated end joining** (**MMEJ**) or **nonhomologous end joining** (**NHEJ**) pathways, depending on the organism (Table 1). CRISPR/Cas9 allows for deletion, insertion, or mutation of DNA with little to no genetic scarring.

CRISPR/Cas9 technology is revolutionizing parasitology research and gene drive systems in insect vectors; and while this review focuses on *Plasmodium*, *Leishmania*, *Trypanosoma*, and *Anopheles*, CRISPR/Cas9 has been successfully adapted to many more parasite systems, including *Toxoplasma* [8], *Cryptosporidium* [9], *Strongyloides* [10], and *Trichomonas vaginalis* [11]. As CRISPR/Cas9-based technology evolves, the parasitology community is quickly discovering methods that work well, techniques that can be improved, and ongoing challenges

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that can only be solved by sharing of experiences and development of new approaches. We share here a sampling of information on the trial-and-error development of CRISPR/Cas9 in molecular parasitology that we hope might benefit those attempting this technology in the future.

Troubleshooting Traditional and Developing New CRISPR Techniques in *Plasmodium*

The development of CRISPR/Cas9 in *Plasmodium falciparum* and *Plasmodium yoelii* for genome editing has greatly advanced functional studies in these parasites by increasing precision of editing and decreasing the amount of time needed to obtain genetically modified parasites from months to weeks [12–14]. *Plasmodium* lacks the canonical NHEJ pathway and primarily uses the HDR pathway to repair DNA DSBs, resulting in fewer unintended or off-target effects [2,15–17]. Thus, the CRISPR/Cas9 system in *P. falciparum* relies on either a one- or two-plasmid system that provides the Cas9 nuclease, the sgRNA, and a DNA repair template [12,13]. The system in *P. yoelii* uses only one plasmid [14]. Despite the overwhelming success and widespread implementation of CRISPR/Cas9 in the *Plasmodium* research community (please see [18] for an excellent review of CRISPR/Cas9 success stories in apicomplexan parasites), there are several ongoing challenges that merit discussion, including difficulty with sgRNA design, unintended genomic mutations, and complications related to gene essentiality.

sgRNA and Repair Template Design in an AT-rich Genome

One challenge to CRISPR/Cas9 use in *P. falciparum* is its extremely AT-rich genome, which can make the search for a suitable PAM and the cloning of repair templates difficult (Table 2) [2]. Several parasite-specific sgRNA design tools that include various methods of scoring specificity and quality have been developed [19–21][i–iii], but anecdotal evidence within the field has provided additional guidelines for designing successful sgRNAs. First, the sgRNA binding site should be as close as possible to the desired mutation site (less than 100 bp). For large deletions, it is more effective to use two sgRNAs, one on each end of the locus to be deleted. This can be achieved by transfecting two plasmids carrying different sgRNAs simultaneously or by using the **ribozyme–guide–ribozyme** (**RGR**) system [22], recently adapted to *P. yoelii*, to generate multiple sgRNAs from the same construct or to simply lead to a more defined sgRNA length [23]. Optimization of the sgRNA structure with duplex extension and thymine mutation of the Cas9-bound loop may also help the efficacy of CRISPR/Cas9 (see loop of sgRNA in Figure 1A) [24].

For HDR template design, many laboratories have shown that the length of the homology repair regions on either side of the mutation need not exceed 400 bp. Successful genome editing has been achieved with as few as 50–100 bp homology arms [21,23]. Smaller repair templates and the ever-decreasing cost of gene synthesis will help to alleviate the difficulty of cloning AT-rich sequences.

Unintended Genomic Mutations with CRISPR/Cas9

While CRISPR/Cas9 genome editing works well in many cases, several laboratories using plasmids containing the DNA repair template have observed plasmid integration at the Cas9-edited site. It has been suggested that this problem might be helped by linearizing the DNA repair template, using two sgRNAs to generate two cut sites in the locus of interest, or including a Cas9 cut site flanking the DNA repair template on the plasmid (communicated by Marcus Lee and Michael Walker). In addition to plasmid integration, an unexpected consequence of CRISPR/Cas9 genome editing in subtelomeric regions is the loss of all DNA between the Cas9 cut site and the chromosome end, which is repaired with the addition of telomeric repeats

Glossary

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9): a genome-editing technique based on a naturally occurring antiviral defense system in bacteria. The Cas9 nuclease is targeted via an sgRNA to a specific ~20 nt genomic sequence immediately adjacent to a protospacer adjacent motif (PAM). The PAM, which is 5′-NGG-3′ (where N is any nucleobase and G is guanine) for *Streptococcus pyogenes* Cas9, is required for Cas9 binding to and cleaving of the DNA.

CRISPR interference/activation (CRISPRi/a): a genome-editing-free technique that uses a catalytically inactive Cas9 to bind to the promoter of a gene to repress or activate transcription. Fusion of epigenetic effector domains to dCas9 can enhance repressive activity or activate transcription.

Gene drive: technology in sexually reproducing organisms that aims to spread a genetic feature throughout a population via super-Mendelian genetic inheritance (i.e., higher than 50% of offspring will inherit an allele).

Homology-directed repair (HDR): an accurate pathway for repair of a DNA DSB that requires a homologous DNA sequence for repair. In general, break ends are resectioned and hybridized with the repair template, which is used to synthesize the missing sequence.

Microhomology-mediated end joining (MMEJ) and nonhomologous end joining (NHEJ): error-prone pathways for repair of a DNA DSB that do not require a homologous repair template. In MMEJ, break ends are resectioned and annealed at small homologous sequences in the single-stranded DNA. In NHEJ, break ends are processed if necessary to create compatible ends, which are then ligated.

Population suppression/replacement: a method for reducing vector-borne diseases in which vector numbers are suppressed or wild vector populations are replaced with modified organisms with reduced vector competence.

Ribonucleoprotein (RNP): a complex formed between an RNA

[25]. This phenomenon might be unavoidable if no essential genes lie between the Cas9 cut site and the chromosome end, as *P. falciparum* has been shown to be adept at repairing subtelomeric DNA damage [26]. These examples of unexpected and unintended DNA mutations make a strong case for whole-genome DNA sequencing of all edited parasites.

Using dCas9 to Manipulate Gene Transcription

With any genome-editing technique, essentiality may prevent gene knockout, and unfortunately, *Plasmodium* lacks the RNAi pathway [27]. Conditional knockout is now possible with the dimerizable Cre recombinase (DiCre) system in which 'silent' *loxP* sequences are inserted into the locus of interest and a split Cre recombinase is chemically activated to achieve recombination [28]. In addition, gene knockdown has been achieved with FKBP, EcDHFR, TetR aptamer, or the *glmS* ribozyme fusions [29–32]. However, all of these methods still require genome editing and leave a genetic scar at the endogenous locus.

Alternative tools for transcriptional manipulation that do not require genome editing are **CRISPR interference/activation (CRISPRi/a)** (Figure 1B) [33–35]. CRISPRi/a utilize an enzymatically inactive Cas9, or 'dead' Cas9 (dCas9) to bind to, but not cut, the promoter region of a gene. The binding of dCas9 alone can prevent the assembly or progression of the transcription machinery, resulting in gene knockdown. Recent studies by Baumgarten *et al.* and Walker and Lindner have demonstrated the efficacy of CRISPRi in *P. falciparum* and *P. yoelii*, respectively [23,36]. These studies suggest that gene knockdown with dCas9 requires binding at a specific 'sweet spot' that can only be found with trial and error, but which is most likely located on the nontemplate DNA strand as close to the transcriptional start site as possible where the chromatin is most open. Tiling of dCas9 across the promoter using multiple sgRNAs may increase the CRISPRi effect. Baumgarten *et al.* also provide a useful protocol for performing chromatin immunoprecipitation (ChIP) and sequencing of dCas9 to confirm sgRNA specificity, which is important in any CRISPRi/a study [36]. In addition, dCas9 ChIP can be used to test the efficacy of potential sgRNAs for CRISPR/Cas9 studies.

dCas9 can also be fused to transcriptionally repressing or activating domains to alter the local chromatin structure/composition. A recent study by Xiao *et al.* fused dCas9 with histone acetyltransferase or deacetylase domains from PfGCN5 and PfSir2a, respectively [37]. Binding of dCas9-GCN5 and dCas9-Sir2a to gene transcriptional start sites modulated local levels of histone acetylation and resulted in up- and down-regulation of the targeted genes, respectively. Although only two genes were targeted for CRISPRi in this study, the dCas9-Sir2a system may provide a stronger knockdown than using dCas9 alone.

Interestingly, CRISPRi (even with dCas9-Sir2a) achieves only a modest reduction in transcription of essential genes in *Plasmodium* [36,37]. In such experiments, parasites that emerge (normally 4–6 weeks) after transfection of the CRISPRi plasmids may have acquired a growth advantage such as lower levels of dCas9 expression. A tightly controlled, inducible dCas9 is needed to achieve immediate and maximum gene knockdown and minimize off-target effects.

CRISPR/Cas9 Transforms Genetic Manipulation in *Leishmania* and Makes High-throughput Knockout Screens Possible

Genetic engineering has always been particularly challenging and time-consuming in *Leishmania* parasites [38]. In the classical approach, deleting a gene of interest required multiple cloning steps, long flanking regions for HDR, and at least two rounds of transfection for deletion of both alleles [39,40]. Moreover, *Leishmania* parasites can adapt to environmental stress by copy number variation and aneuploidy [41–44], increasing the risk of compensatory mutations

molecule and an RNA-binding protein. A Cas9 RNP complex consists of recombinant Cas9 and sgRNA.

Ribozyme–guide–ribozyme (RGR): ribozymes are ribonucleic acid enzymes, or RNA molecules with catalytic activity. In RGR, self-cleaving ribozymes flank an sgRNA. Once transcribed, ribozyme cleavage leaves the sgRNA with a precise length and structure, which improves Cas9 binding and function. RGR allows for the use of any promoter for sgRNA transcription as well as multiple sgRNA transcription from a single promoter.

Single guide RNA (sgRNA): a hybrid RNA molecule that guides Cas9 to a specific genomic target sequence via two domains. The crispr portion is complementary to the genomic sequence of interest and the tracr portion is structured and bound by Cas9.

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Key Figure

Molecular Basis and Recent Advancements of CRISPR/Cas9 Technology in Parasites and Insect Vectors

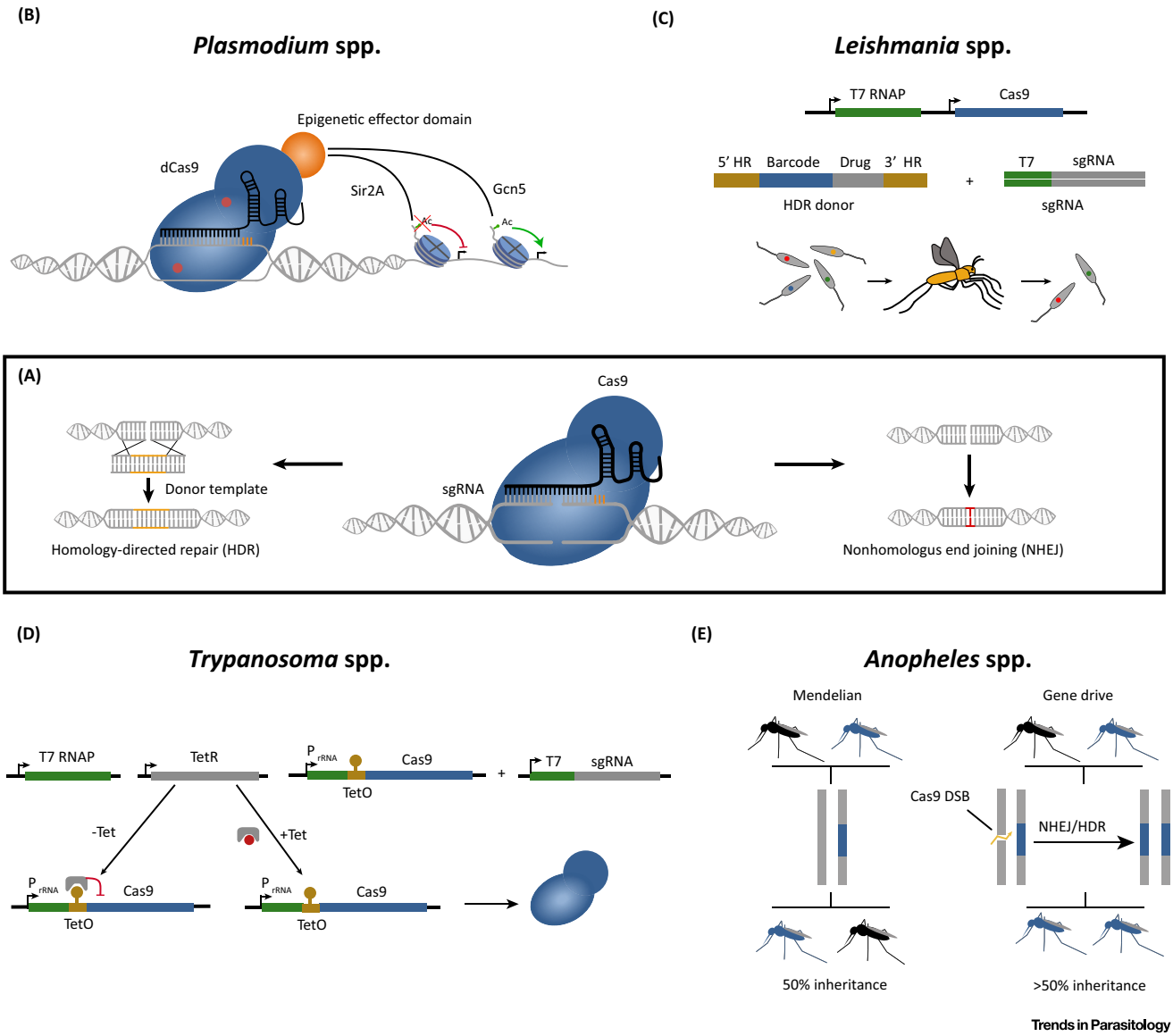

Figure 1. (A) The Cas9 protein is guided by a single guide RNA (sgRNA) to the target locus to induce a DNA double-strand break (DSB). The DSB is repaired by homology-directed repair (HDR), microhomology-mediated end joining (MMEJ), or nonhomologous end joining (NHEJ). (B) Transcriptional repression/activation via CRISPRi/a in *Plasmodium falciparum*. dCas9 (inactivating mutations shown in red) binding to gene promoters can interfere with transcriptional initiation or elongation [23,36]. Fusion of epigenetic effector domains to dCas9 can modulate histone modifications around the dCas9 binding site to activate or repress transcription via hyperacetylation by GCN5 or hypoacetylation by Sir2a, respectively [37]. (C) High-throughput gene knockout and tagging system in *Leishmania* spp. DNA repair template containing a drug-selectable marker flanked by minimal homology regions (HRs), and an optional barcode is transfected with an sgRNA template under a T7 promoter into parasites expressing Cas9 and a T7 RNA polymerase (T7 RNAP). Sandflies can be infected with mixed, barcoded knockout parasites [48,50]. (D) Inducible Cas9 expression in *Trypanosoma brucei* [53]. Transgenic *T. brucei* expresses a T7 RNAP, a Tet repressor (TetR), and Cas9 protein under a tetracycline (Tet)-

(See figure legend on the bottom of the next page.)

Table 1. Overview of CRISPR/Cas9 Systems in Parasites and Insect Vectors

	Plasmodium falciparum and *P yoelii*	*Leishmania* spp.	*Trypanosoma brucei*	*Anopheles* spp.
Application	• Deletion • Insertion • Substitution	• Deletion • Insertion • Substitution	• Deletion • Insertion • Substitution	• Deletion • Insertion
Repair mechanism	HDR	HDR, MMEJ	MMEJ, HDR	HDR, NHEJ
Repair template homology arm length	~100–400 bp	~30 bp	~25–100 bp	~1–2 kb
sgRNA promoter	• U6 snRNA [12] • T7 RNAP [13]	• T7 RNAP (*L. major, L. mexicana*) [48] • U6 snRNA (*L. major*) [45] • rRNA promoter (*L. donovani*) [47]	• T7 RNAP [48,53] • PARP [56]	U6 snRNA [64,65]
Multiplex sgRNA	• RGR [23] • Multiplasmid	Multi-sgRNA transfection [48]	Would be possible with RGR [56] and multi-sgRNA transfection [48]	Multiple sgRNA expressing construct [63]
Inducible/ conditional Cas9 expression	–	–	TetR-inducible promoter [53]	Cell-specific (i.e., *vasa*, *zpg*) [64,66]
Plasmid-free	Transfection of *Sp*Cas9 RNP complex [77]	Transfection of *Sa*Cas9 RNP complex [73]	Transfection of *Sa*Cas9 RNP complex [73]	Microinjection of embryos with Cas9 protein [64,65]
Markers for selection of transgenics	• Human dihydrofolate reductase • Blasticidin S deaminase • Neomycin phosphotransferase II (G-418) • Puromycin-*N*-acetyltransferase (puromycin) • Yeast dihydroorotate dehydrogenase	• Blasticidin S deaminase • Neomycin phosphotransferase II (G-418) • Puromycin-*N*-acetyltransferase (puromycin) • Phleomycin • Miltefosine resistance	• Hygromycin • Puromycin-*N*-acetyltransferase (puromycin dichloride) • Neomycin phosphotransferase II (G-418) • Blasticidin S deaminase • Phleomycin • Nourseothricin	Fluorescent protein
CRISPRi/a	• dCas9 [23,36] • dCas9-GCN5/Sir2A [37]	–	–	–
Time to obtain transgenics	• ~4–6 weeks	• 1 week	• 1 week	• Microinjection of embryo leads to transgenic adults

during the course of a two-step gene deletion process and masking knockout phenotypes. CRISPR/Cas9 has accelerated *Leishmania* genetic engineering substantially.

Plasmid-based CRISPR/Cas9 Systems in *Leishmania*

CRISPR/Cas9 was first adapted to *Leishmania major* by Sollelis *et al.* and was an improvement over traditional methods, allowing for the generation of knockout cell lines in a single step [45]. This system used one plasmid encoding Cas9 and a second plasmid encoding the sgRNA (under the control of a U6 promoter) and the homologous repair templates flanking a drug-

inducible rRNA promotor (P_{rRNA}). Without the addition of Tet, TetR binds to the Tet operator (TetO), inhibiting Cas9 expression. The addition of Tet leads to the removal of TetR from TetO and expression of Cas9 protein. (E) CRISPR/Cas9-based gene drive system in the *Anopheles* insect vector. Crossing a wild-type (gray) with a transgenic (blue) mosquito normally leads to a 50% Mendelian inheritance rate of the mutated locus in offspring (left). In a gene drive system (right), Cas9 is expressed by the transgene and induces a DSB in the wild-type allele, which is repaired using the transgene as a template [64,65]. This leads to super-Mendelian inheritance and spread of the transgene throughout the population.

Table 2. The Number of PAM Sequences per Kilobase in Different Genomic Regions of Parasites

	Plasmodium falciparum [2]	*Plasmodium yoelii* [82]	*Trypanosoma brucei* [61]	*Leishmania major* [4]	*Leishmania mexicana* [83]
Cas9					
Genic (promoter)	30.1 (14.3[a])	29.9 (22.2[a])	93.1	118.8	119.2
Intergenic	19.1	23.3	65.9	100.5	104.4
Cas12a					
Genic (promoter)	70.1 (76.3[a])	76.9 (80.9[a])	27.6	10.2	10.4
Intergenic	70.6	74.8	42.8	21.2	20.8

[a]A promoter is defined as the region 1000 bp upstream of a translation start site for each individual gene. Due to the polycistronic nature of transcription in kinetoplastids, this number was only calculated for *Plasmodium* spp. The latest versions of all genome sequences were obtained from EuPathDB [84].

resistance cassette. Edited parasites were successfully obtained with drug selection. The CRISPR/Cas9 system was subsequently improved by Zhang *et al.* in *Leishmania donovani* in several ways: (i) the homologous repair regions were shortened, (ii) the stronger RNA Pol I rRNA promoter was used to drive sgRNA transcription, (iii) an RGR was used for discrete and potentially multiple sgRNA production, and (iv) a single vector expressing both Cas9 and the sgRNA was generated [46,47]. Zhang *et al.* demonstrated the ability of their CRISPR/Cas9 method to delete multicopy genes, which was previously impossible with the traditional approach [47]. In this study, they cotargeted the A2 multigene family and the miltefosine transporter gene, which confers resistance to miltefosine. This method increased genome editing efficiency and eliminated the need for drug-selectable marker integration at the locus of interest. However, this approach requires repeated transfections to increase editing efficiency and selects for edited parasites with cloning, both of which increase time in culture and could lead to parasite adaptation. Both of these first systems also rely on a vector-based sgRNA, which requires plasmid cloning.

A Versatile and Cloning-free CRISPR/Cas9 Toolkit

Recently, Beneke *et al.* developed a cloning-free, PCR-based CRISPR/Cas9 method for fast and accurate genome editing that was successfully applied to *Leishmania mexicana*, *L. major*, *L. donovani,* and *Trypanosoma brucei* [48,49]. This system is provided as a toolkit with protocols for gene deletion and N terminal or C terminal tagging as well as a website[iv] for designing primers required for generating sgRNAs and DNA repair templates [48]. In this system, transgenic *Leishmania* parasites stably expressing codon-optimized *Sp*Cas9 and T7 RNA polymerase (T7 RNAP) were transfected with two PCR fragments encoding a T7-promotor-driven sgRNA and a DNA-repair template consisting of ~30 bp homology arms flanking a drug-selectable marker. After 1 week of drug selection, 100% of surviving parasites carried the intended genomic mutation. Thus, this total culture time is dramatically reduced compared with other methods. Another advantage of this method is that PCR fragments are degraded within 48 h, preventing subsequent editing.

This system has proven to be fast and reliable, and it has been successfully used to target single genes [49]. Recently, Beneke *et al.* went on to carry out the first CRISPR/Cas9-based high-throughput knockout screen in a kinetoplastid to genetically dissect the *Leishmania* flagellar proteome [50]. This study generated approximately 100 gene knockouts of flagella-associated proteins and performed an in-depth characterization of different motility phenotypes. A 17 nt barcode in the DNA repair template allowed for infection of the sandfly vector with a mixed pool of knockout parasites (Figure 1C). Thus, CRISPR/Cas9 has opened up a new era in the field of

Leishmania biology, as it is now possible to investigate entire pathways *in vitro* and *in vivo*. This is especially profound considering the fact that approximately 60% of the genes in the *Leishmania* genome are 'hypothetical' genes that have not been investigated. Deciphering the functions of these genes is crucial for understanding *Leishmania* biology and discovering novel drug targets.

Marker-free CRISPR/Cas9 Genome Editing in Trypanosomes

Genetic tool development in *T. brucei* has run ahead of that in other parasites, largely due to species-specific benefits such as relatively high transfection efficiencies, efficient homologous recombination, inducible systems such as the tetracycline repressor, RNAi, and the ability to knock out and knock in genes [51]. However, the limited number of selectable markers available has meant that generating strains with more than two simultaneous gene knockouts is challenging. While this issue has been partially overcome through the development of the DiCre system [52], CRISPR/Cas9 technology has made precise, 'scar-less', and marker-free genome editing feasible in this pathogen. Fortunately, the *T. brucei* genome has a GC content of 50.73%, and a 5′-NGG-3′ PAM sequence should be present every eight base pairs (Table 2 and [53]).

Early applications of CRISPR/Cas9 for genome editing in kinetoplastids demonstrated that Cas9 was able to form a DSB in these parasites [46,54,55]. Characterization of the resolution of these breaks revealed that repair was through MMEJ unless a donor repair template was present to facilitate HDR. Recent CRISPR/Cas9 technology development in *T. brucei* has significantly advanced the gene-editing toolbox, now allowing for rapid gene knockout, gene tagging, and precision editing in both bloodstream form and insect stage cells [48,53,56].

Establishing CRISPR/Cas9 in *T. cruzi*

Peng *et al.* established the first CRISPR/Cas9 system in a kinetoplastid by transfecting *T. cruzi* with a Cas9-encoding plasmid and *in vitro* transcribed sgRNA [55]. Gene knockout was achieved by MMEJ or, if a DNA-repair template was provided, HDR. While CRISPR/Cas9 via HDR allowed for marker-free editing, overall low frequencies of the desired mutation were observed (~ 0.1%). Lander *et al.* then streamlined the CRISPR/Cas9 system in *T. cruzi* by coexpressing sgRNA and Cas9 from a single vector [54]. Using a DNA-repair template for HDR that included a drug selection marker, the authors achieved high-efficiency knockout and tagging of endogenous genes after 5 weeks of continuous drug pressure [57]. Most recently, Costa *et al.* adapted the system developed by Beneke *et al.* (described above) to *T. cruzi*, creating a cell line stably expressing Cas9 and T7 RNA polymerase [58]. This system has made cloning-free endogenous gene tagging and gene knockout possible in *T. cruzi*.

Cloning-free CRISPR/Cas9 Systems in *T. brucei* Bloodstream Parasites

Beneke *et al.* first published CRISPR/Cas9 in bloodstream-form *T. brucei* using a system that relied on stable, integrated expression of the T7 RNAP and Cas9 [48]. Using this system, the parental strain could then be nucleofected with two PCR products: one encoding a T7 promoter-driven sgRNA and the other containing the DNA-repair template with 30 bp homology arms. The benefit of this system is that a gene knockout can be achieved with a single nucleofection with no prior plasmid cloning. While more efficient than classical knockout strategies, this system still relies on drug selection of mutants, which limits the complexity of parallel edits to the number of selectable markers.

Rico *et al.* recently developed an inducible, selection-free CRISPR/Cas9 genome editing system in *T. brucei* (Figure 1D) [53]. In this system, a Cas9-induced DSB is repaired by

HDR via a ~50 bp single-stranded oligonucleotide repair template or with MMEJ in the absence of one. Because no drug-selectable markers are used, the system was optimized in several ways. First, a *T. brucei* codon-optimized Cas9 was used under the control of an RNA polymerase I promoter to enhance expression [59,60]. Next, a tetracycline operator was inserted directly upstream of the Cas9 gene, providing for tightly inducible expression and thus reduction of potential Cas9-induced toxicity and off-target effects. Finally, this system used an sgRNA under the control of a constitutive T7 promoter, but incorporated a hepatitis delta virus (HDV) ribozyme [22] at the 3′ end to produce a discrete guide for better assembly with Cas9. This study also reported that a PAM-adjacent GC content of >50% is most optimal. In this selection-free optimized system, Rico *et al.* achieved high rates of biallelic gene editing dependent on the tightly controlled induction of Cas9 expression.

Precision CRISPR/Cas9 Editing of Multigene Families in *T. brucei* Procyclic Parasites

The *T. brucei* genome contains >190 gene families with at least five members, representing approximately 20% of the 9000 total genes [61]. To be able to edit such gene families, a repair template must be provided for a prolonged period of time. A recent marker-free CRISPR/Cas9 system developed by Vasquez *et al.* took advantage of the ability of procyclic insect-stage trypanosomes to maintain episomes without genome integration [56]. Sequentially transfected episomes – the first encoding a codon-optimized Cas9 and the second encoding an RGR and homologous repair template – are stably maintained in otherwise wild-type cells at approximately ~1 copy per cell through continuous culturing. This system uses the procyclic acidic repetitive protein (PARP) promoter for RGR transcription to obviate the need for T7 RNAP expression.

Vasquez *et al.* aimed to use their CRISPR/Cas9 system to mutate a single codon in histone H4 to achieve a lysine-to-arginine substitution at position 4, mimicking a constitutively nonacetylated state [56]. Although there are an estimated 43 copies of the histone H4 gene in the genome, 90% were edited after 5 months of culture. Interestingly, multiple copies of the H4 gene were lost completely, but no off-target effects were observed. This study demonstrates the utility of marker-free CRISPR/Cas9 systems for editing multicopy gene families.

CRISPR in *Anopheles* for Population Replacement and Suppression

The study of insect vectors is as important to the eradication of parasitic diseases as the study of the parasites themselves. While CRISPR/Cas9 technology is advancing functional studies in *Anopheles* mosquitoes, it is especially promising in the field of **gene drive**. Gene drives are genetic systems in sexually reproducing organisms that quickly spread a genetic feature throughout a population in a 'selfish' manner that defies normal Mendelian inheritance patterns (Figure 1E) [62]. In the case of *Anopheles* gene drives, the goal is to spread genetic modifications that negatively affect mosquito fertility or *Plasmodium* infection.

Using CRISPR/Cas9 for Gene Drives

CRISPR/Cas9 genome editing has been implemented successfully in *Anopheles stephensi* and *Anopheles gambiae* in studies attempting to block *Plasmodium* infection/transmission or reduce the mosquito population [63–65]. A **population suppression** approach was presented in a recent study by Hammond *et al.*, which developed a CRISPR/Cas9-based gene drive system to target haplosufficient female fertility genes in *A. gambiae* [65]. A construct flanked by regions homologous to the fertility gene and containing sgRNA, a fluorescent marker, and a *vasa2* promoter-driven Cas9 (for germline expression in either sex) was inserted into the fertility gene locus. This gene drive system achieved super-Mendelian inheritance; however, fertility was greatly reduced in heterozygous females due to leaky Cas9 expression in

somatic tissues involved in producing eggs. Follow-up studies used the *zpg* promoter to more tightly restrict Cas9 expression to the germline, which alleviated the cost to female fertility somewhat [66,67]. However, females inheriting the gene drive allele from their fathers showed reduced fertility, perhaps due to paternal deposition of Cas9 protein in the fertilized zygote. While this effect would reduce female contribution to the propagation of gene drive, strong super-Mendelian inheritance may still promote transgene propagation to an entire population and its subsequent collapse.

Another successful use of CRISPR/Cas9-based gene drive was presented by Gantz *et al.*, which sought to spread *Plasmodium* resistance factor-encoding transgenes in *A. stephensi* [64]. Larvae were microinjected with Cas9 protein, double-stranded RNAs to silence expression of the *Cas9* transgene and the Ku70 gene (involved in NHEJ), and a 21 kb plasmid containing an sgRNA, fluorescent marker, *vasa* promoter-driven Cas9, and two genes encoding single-chain antibodies against *P. falciparum* ookinete protein Chitinase 1 and the circumsporozoite protein (CSP), which were under the control of blood meal-induced promoters. All construct components were flanked by regions of homology to the eye-pigmentation gene *kh*. This method yielded a *kh* knock-in mosquito line in which significant gene drive was achieved in transgenic male-derived lineages. However, a high frequency of NHEJ DSB repair was observed in transgenic female-derived lineages, most likely due to Cas9 expression in the egg persisting through syncytial blastoderm formation.

A more recent study used CRISPR/Cas9 in *A. gambiae* to show that knockout of the fibrinogen-related protein 1 (*FREP1*) host factor suppressed *Plasmodium* infection [63]. Dong *et al.* unsuccessfully attempted Cas9/sgRNA microinjection of embryos as described above [64] and thus developed a system in which transgenic mosquitoes expressing three different sgRNAs targeting the *FREP1* gene were crossed to transgenic mosquitoes expressing Cas9 in the germline. Knockout of *FREP1* via NHEJ significantly reduced the prevalence of infection as well as the number of oocysts and sporozoites in *P. falciparum*-infected adult mosquitoes. Although *FREP1* knockout adult mosquitoes showed no difference in life span and body size from control mosquitoes, they did show a delay in development, a lower feeding propensity, and reduced fecundity and egg-hatch rate. These phenotypes may preclude the use of *FREP1* as an efficient gene drive target in **population replacement** designs. While this study showed the potential of CRISPR/Cas9 to investigate specific host factors, it highlights the possible fitness costs of such systems.

Challenges of *Anopheles* CRISPR/Cas9 Gene Drive Systems

The studies discussed here demonstrate that CRISPR/Cas9 technology has the potential to advance gene drive systems by achieving specific gene conversion at a high rate in laboratory mosquito populations. However, one drawback of using this technology in population suppression and replacement efforts is the possibly reduced fitness of transgenics, which can be related to importance of targeted genes or imprecision of Cas9 action/expression. These issues may be alleviated in *Anopheles* through the use of tightly regulated tissue-specific or blood meal-driven Cas9 or sgRNA expression.

Another challenge is the emergence of gene drive-resistant alleles due to improper target site repair of Cas9-induced DSBs, most often due to error-prone NHEJ resulting in a sequence that is refractory to future CRISPR/Cas9 editing [68,69]. Gene drive systems that utilize multiple sgRNAs, 'nicking' endonucleases that cleave a single strand, or methods that suppress the NHEJ pathway, may help to alleviate the problem of gene drive resistance [5]. However, even these systems may not be able to address the genetic diversity in potential CRISPR/Cas9

editing sites in natural populations of mosquitoes [70]. A recent study by Kyrou *et al.* avoided CRISPR-based gene drive resistance by targeting the sex-determination gene *doublesex*, which is a highly conserved, functionally constrained sequence [67]. This study highlights the importance of target choice for gene drives that will succeed in population suppression.

In addition to the technical challenges encountered, the possibility of CRISPR-Cas9-mediated gene drives in natural populations has raised concerns about ecological and ethical implications of such experiments. Although only specific species of mosquito would be targeted for population suppression, the impact of such a gene drive on the local ecosystem is unpredictable [71]. Using gene drive to prevent mosquito infection with *P. falciparum* may appear to be less risky than population suppression from an ecological perspective, but these mutations could have unforeseen effects on mosquito ecology as well. The mere presence of Cas9 in the nucleus over many generations could result in rare off-target mutations that affect the evolutionary trajectory of mosquito populations. Further optimization of these systems and long-term studies will hopefully allay these concerns.

Taking CRISPR to the Next Level

Controlling Cas9: An Ongoing Challenge in All Systems

While the TetR inducible Cas9 system works well in *T. brucei*, most CRISPR/Cas9 systems discussed here involve constitutive Cas9 expression, which can be toxic and induce genetic instability in some cases [49,53,72,73]. In *Toxoplasma gondii*, expression of a nonfunctional, 'decoy' sgRNA alleviated toxicity associated with constitutive expression of a nontargeted Cas9 [72]. A similar strategy could be applied to *Leishmania* and *Plasmodium*, but lack of inducible transcription systems in these parasites is a major obstacle in general. One potential solution is to use the DiCre system, already adapted to these parasites, to activate a *loxP*-interrupted Cas9 [74–76]. This or similar tightly controlled systems would also help to study the function of essential genes.

An alternative to an inducible system is using Cas9 **ribonucleoprotein** (**RNP**) complexes for transient transfection. Crawford *et al.* achieved genome editing in *P. falciparum* by transfecting with recombinant *Sp*Cas9, *in vitro*-transcribed sgRNA, and 200 nucleotide single-stranded oligodeoxynucleotides (for DNA repair) [77]. However, this study reported a transfection efficiency similar to plasmid-based methods, with 23% of transfections yielding correctly edited parasites. Thus, this system may be best suited for introducing mutations that confer a fitness advantage to edited parasites. Soares Medeiros *et al.* used *Sa*Cas9 RNP complexes to edit the genome in multiple life cycle stages of *T. cruzi*, procyclic forms of *L. major*, and bloodstream forms of *T. brucei* [73]. This study demonstrated that *Sa*Cas9 is more effective than *Sp*Cas9 in their system, possibly due to the smaller size and higher transfection efficiency of *Sa*Cas9. However, *Sa*Cas9 has a longer PAM sequence, which leads to fewer options for sgRNA design. Nonetheless, Cas9 RNP systems allow for serial or simultaneous genetic manipulations and obviate the need for plasmid cloning, integrated constructs for Cas9/sgRNA expression, or drug selection.

Alternative Cas Proteins: New Avenues for Genome-wide CRISPR Screens?

In a major advance for the field, Sidik *et al.* recently used CRISPR/Cas9 to perform the first genome-wide genetic screen in an apicomplexan, identifying genes that affect fitness during *T. gondii* fibroblast infection [72]. This screen relied on the presence of the NHEJ pathway in *T. gondii*, where it is not necessary to provide a DNA repair template along with the sgRNA. However, such a large-scale screen is not possible in *Plasmodium* or *Leishmania*, which primarily use HDR to repair DSBs. The streamlined, PCR-based workflow developed by

Beneke *et al.* now also allows for rapid generation of knockout constructs and genome-wide screens in kinetoplastid parasites via HDR [50]. However, for parasites requiring longer homology regions for efficient insertion/deletion (e.g., *Plasmodium*), this approach might not be as easily applied.

In contrast, and independent of DSB repair mechanisms, the recent adaptation of CRISPRi and CRISPRa to parasite systems has the potential to make large-scale knockdown and overexpression screens possible in parasites with clearly defined promoters. Since CRISPR/Cas9 was first established in various parasite systems, many variations on this gene-editing tool have been developed in other systems that could prove useful to the parasitology community. Cas12a (formerly known as Cpf1) especially has emerged as an alternative nuclease to Cas9 [78,79]. Cas12a is smaller than Cas9, requires a smaller sgRNA, and leaves a staggered DSB. Importantly, the preferred PAM sequence for Cas12a is 5′-TTTV-3′ (where 'V' is A, C, or G), a motif that occurs more frequently than 5′-NGG-3′ in the AT-rich *Plasmodium* genome (Table 2). Using Cas12a could make sgRNA design easier, especially in promoter regions, but may result in more off-target effects (Table 2). Importantly, an enzymatically inactive ('dead') version of Cas12a could be used to target the highly AT-rich promoter regions of *Plasmodium* with an sgRNA library for large-scale gene knockdown via CRISPRi (Table 2).

Other emerging alternatives to Cas9 are Cas13 nucleases. In contrast to Cas9 and Cas12a, Cas13 nucleases bind to RNA transcripts in an sgRNA-mediated manner and can be used for specific RNA degradation, base editing, or tracking [80,81]. Most importantly, Cas13 nucleases do not require a specific PAM sequence for cutting RNA as long as the secondary RNA structure allows for its binding. An additional potential option for parasite systems (e.g., *Plasmodium*, *T. cruzi*, *Leishmania*) lacking the RNAi pathway is using Cas13 nucleases in combination with a transcriptome-wide sgRNA library to perform high-throughput knockdown screens.

Concluding Remarks

While genome editing with CRISPR/Cas9 has been established in the field of parasites and insect vectors, there is an ongoing effort to improve the efficiency of this technique and apply it to new systems. Continued timely communication and sharing of advice, ideas, and reagents are required to utilize this revolutionary technology to its full potential throughout our respective fields. Making plasmids available on vector databases, disclosing information about tested sgRNA efficiency in real time, and holding regular CRISPR-specific conferences would better facilitate the widespread success of such efforts. Also important is the ongoing discussion about how the modified parasites and insect vectors we create with CRISPR/Cas9 can be used in an ethically and ecologically responsible way to stop the spread of disease (see Outstanding Questions).

Moving forward, it is crucial to use the ever-evolving CRISPR toolbox to troubleshoot traditional genome-editing applications as well as apply it to new methodologies. Cas12a has emerged as an attractive alternative to Cas9 in organisms such as *Plasmodium* with highly AT-rich genomes (see Outstanding Questions). In addition, Cas13 nucleases or dCas9/dCas12a could be used to perform gene knockdowns in organisms such as *Plasmodium* and *Leishmania* that lack the RNAi machinery and inducible expression systems (see Outstanding Questions). Finally, CRISPR/Cas9 is being used to perform larger-scale functional screens in *Toxoplasma*, *Trypanosoma*, and *Leishmania*. While these types of screens may not be easily performed with traditional CRISPR/Cas9 in *Plasmodium*, they may be possible with CRISPRi- or Cas13-mediated knockdown (see Outstanding Questions). These CRISPR-based technologies have

Outstanding Questions

Would CRISPR-based gene drive systems be effective in population suppression and replacement programs in natural populations of mosquitoes? What is the environmental impact of these systems?

Conditional or tissue-specific Cas9 expression systems are available in *Trypanosoma* and *Anopheles* mosquitoes. Will similar systems be developed for *Leishmania* and *Plasmodium*?

Can Cas12a emerge as an effective alternative to Cas9, especially for organisms like *Plasmodium* with AT-rich genomes?

Can Cas13 nucleases be adapted for post-transcriptional gene knockdown, especially in parasites that lack the RNAi pathway?

CRISPR/Cas9-based high-throughput functional screens are now possible in *Leishmania* and *Toxoplasma*. Can similar systems be applied to *Trypanosoma*? Can CRISPRi/a or Cas13 technology make these screens possible in *Plasmodium*?

the potential to finally generate knockout/knockdown libraries, a valuable resource for the parasitology community.

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Resources

[i]Protospacer Workbench: www.protospacer.com/

[ii]EuPaGDT: http://grna.ctegd.uga.edu/

[iii]Database of all protospacer sequences in the *Plasmodium falciparum* genome: http://exon.niaid.nih.gov/transcriptome/Pf26/pf-crispr4-web.xlsx

[iv]LeishGEdit: leishgedit.net

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