

New and emerging uses of CRISPR/Cas9 to genetically manipulate apicomplexan parasites

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Review Article

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Abstract

Although the application of CRISPR/Cas9 genome engineering approaches was first reported in apicomplexan parasites only 3 years ago, this technology has rapidly become an essential component of research on apicomplexan parasites. This review briefly describes the history of CRISPR/Cas9 and the principles behind its use along with documenting its implementation in apicomplexan parasites, especially *Plasmodium* spp. and *Toxoplasma gondii*. We also discuss the recent use of CRISPR/Cas9 for whole genome screening of gene knockout mutants in *T. gondii* and highlight its use for seminal genetic manipulations of *Cryptosporidium* spp. Finally, we consider new variations of CRISPR/Cas9 that have yet to be implemented in apicomplexans. Whereas CRISPR/Cas9 has already accelerated rapid interrogation of gene function in apicomplexans, the full potential of this technology is yet to be realized as new variations and innovations are integrated into the field.

Introduction

The history of the CRISPR revolution represents one of the most significant examples of basic research leading to techniques with enormous translational potential. In fact, one of the most revolutionary discoveries of the last decade took more than 25 years from the first evidence of its existence before becoming the most powerful technology for genome manipulation. CRISPR is an adaptive immune system used by several bacteria to defend themselves from infection by viruses or exogenous DNA, such as bacteriophages and plasmids, respectively. Identifying basic mechanisms underlying a viral evasion strategy may appear to be an academic interest confined to the field of microbiology. This perception, which brought frustration to many early CRISPR researchers, starting from Mojica (who first observed the presence of the CRISPR array), caused a shortage of funds, lab space and editorial rejections from leading journals (Lander, 2016). The CRISPR odyssey parallels in some ways the discovery of another technology that radically changed molecular biology research in all organisms, the polymerase chain reaction (PCR). Description of this discovery by Kary Mullis was rejected by elite journals that missed the importance of this revolutionary finding (Campanario, 2009). The lesson to the non-scientific community is that basic knowledge may lead to discoveries with virtually limitless utility such as CRISPR and PCR. The biological role of CRISPR in bacterial adaptive immunity has been comprehensively described (Karginov and Hannon, 2010; Wright *et al.* 2016; Hryhorowicz *et al.* 2017; Koonin *et al.* 2017; Patterson *et al.* 2017). The basis of CRISPR technology, its different applications, and its potential have also been well described (Hsu *et al.* 2014; Barrangou *et al.* 2015; Barrangou and Horvath, 2017; Jackson *et al.* 2017; Jiang and Doudna, 2017; Kick *et al.* 2017; Pineda *et al.* 2017; Salsman and Dellaire, 2017). This review instead aims to highlight the power of CRISPR technology in parasitology, principally concentrating on new advancements in apicomplexan parasites where considerable recent progress has been made.

The availability of genetic tools to manipulate genomes is crucial to understanding the biology of any organism. Within the phylum Apicomplexa, such tools have been most extensively applied to *Plasmodium* spp., *Toxoplasma gondii* and more recently *Babesia* spp. Research on other medically important apicomplexans such as *Cryptosporidium* spp., a leading cause of pathogen-induced diarrhea (Checkley *et al.* 2015), has been constrained due to the lack of a long-term *in vitro* culture system, animal models, and molecular genetic tools, but is now surging forward with CRISPR/Cas9 technology (Vinayak *et al.* 2015). Recent applications of CRISPR/Cas9 are creating exciting new opportunities to interrogate gene function and reveal important biological insight.

Adaptation of CRISPR in apicomplexan parasites has paralleled that of higher eukaryotes. The approach is based on initial generation of a double-strand DNA break (DSB) by the Cas9 nuclease in a site-specific manner driven by a single guide RNA (sgRNA) targeting an exact DNA sequence within the genome. The generation of a specific DSB activates DNA repair systems including non-homologous end joining (NHEJ), homologous repair (HR), or other alternative repair pathways, depending on the organism. NHEJ is active throughout the cell cycle, but is dominant in most organisms during G1-phase when HR is absent. NHEJ low fidelity repair of CRISPR/Cas9 induced DSBs causes deletions or insertions (indels), resulting in

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frame-shift mutations that typically inactivate the target gene. HR is instead an S-phase and high fidelity DNA repair pathway that can be exploited using CRISPR/Cas9 technology coupled with a 'donor DNA' that contains homologous sequences flanking the DSB. This HR donor DNA can be exploited to precisely insert mutations or sequences such as epitope tags, or for a complete gene knock out. Some parasites, such as *Plasmodium* spp. and *Cryptosporidium* spp. lack NHEJ and thus predominately use HR. In the absence of donor DNA *Plasmodium* resorts to microhomology-mediated end joining (MMEJ) where DSB is repaired using short homologous regions corresponding to as little as 4 bp flanking the lesion (Kirkman *et al.* 2014; Singer *et al.* 2015). Although it usually results in a small deletion, the low frequency of MMEJ likely renders CRISPR/Cas9-mediated gene disruption in the absence of a donor DNA template highly inefficient.

This review will focus on recent applications of CRISPR/Cas9 genetic manipulation of apicomplexans, with a particular emphasis on *Plasmodium* spp., *T. gondii* and *Cryptosporidium* spp. Other excellent recent reviews that discuss genetic manipulation of apicomplexans in general (Suarez *et al.* 2017) and CRISPR/Cas9 genome editing of protists (Lander, 2016) also illustrate various strategies for using CRISPR/Cas9.

Plasmodium: new tools advance genetic tractability

Massive efforts have been made to develop tools to study *Plasmodium* spp., and particularly *P. falciparum*, due to the impact these parasites have on society. Malaria causes severe morbidity and mortality that is sustained by the lack of an effective malaria vaccine and the ability of the parasite to develop drug resistance. *In vitro* cultivation and genetic manipulation have been available to study *P. falciparum* for decades, but these approaches have been inefficient and time-consuming. Conventional gene knockout in *P. falciparum* takes months to obtain a null mutant and relies on spontaneous single- or double-crossover recombination using plasmids containing homologous sequences to the target region. Since *P. falciparum* lacks the machinery for NHEJ, genome integration is not random and occurs mainly in the region of homology between parasite chromosomes and plasmid DNA maintained episomally. Unfortunately, integration is a stochastic event that occurs at very low frequency and thus gene disruption in *P. falciparum* requires 1–3 months of continuous culture, protracted on–off cycling of drug selection, and/or negative-selection procedures. Zinc finger nuclease technology (ZFN) has been successfully used in *P. falciparum* to improve the generation of knockout parasites (Straimer *et al.* 2012; Singer *et al.* 2015; Veiga *et al.* 2016), but is limited by its targeting capability, arduous design and implementation, and high cost. ZFN vectors are also often very large and thus not suitable for genome-wide screening.

Successful establishment of CRISPR/Cas9 technology to edit *Plasmodium* spp. genomes has provided a powerful tool to allow rapid and efficient genetic manipulation of these parasites. Several adaptations of CRISPR/Cas9 have been developed in *Plasmodium* spp. based on the use of either one- or two-vector strategies, depending on whether the donor DNA is in the same or a separate plasmid as Cas9 and the sgRNA, and if different selectable markers are used to maintain the plasmids in transformed parasites. Two seminal studies in 2014 independently reported adapting CRISPR/Cas9 technology to genetically manipulate *P. falciparum* using different approaches to express the sgRNA (Ghorbal *et al.* 2014; Wagner *et al.* 2014). In the first study, Ghorbal *et al.* developed a two-plasmid system expressing Cas9, under the HSP86 promoter, in one vector and the sgRNA, driven by the U6 promoter, along with the HR donor DNA flanking a selectable marker hDHFR (human dihydrofolate reductase) in a second vector. After co-transfecting

P. falciparum-infected erythrocytes with both vectors, drug selection for integration of hDHFR at the target locus was applied. The sgRNA/HR donor vector also carried the negative selectable marker yfcu (yeast cytosine deaminase and uridyl phosphoribosyl transferase) to subsequently eliminate parasites carrying copies of this plasmid. This study also found that transfection of linear HR donor plasmid DNA is a viable alternative to negative selection since linear DNA can mediate recombination but does not persist (Deitsch *et al.* 2001). The authors used this system to disrupt a reporter transgene (EGFP) and an endogenous gene (PfKAHRP) with hDHFR, along with introducing a point mutation in two genes (PfORC1 and PfKELCH-13) without drug selection. A possible limitation of expressing sgRNAs from the U6 promoter is that guanosine is preferred at the sgRNA 5' position for efficient RNA polymerase III transcription. Nevertheless, the authors reported that the *P. falciparum* U6 promoter was able to drive expression of gRNAs in the parasite without the functional requirement for the initial guanosine nucleotide, thus expanding in *P. falciparum* the ability of Cas9 to target any sequence with the -NGG PAM motif. We have similarly observed in *T. gondii* that expression from the U6 promoter seems to be free of this initial guanosine nucleotide restriction, having successfully used sgRNAs starting with one of the other three nucleotides, although efficiency has not been determined (Di Cristina *et al.* 2017).

In the second study, Wagner *et al.* also used a two-plasmid system, wherein Cas9 and the sgRNA were placed in the same vector along with a BSD (blasticidin-S deaminase) selection marker. In this system, expression of the sgRNA was driven by the T7 promoter, thus requiring expression of the T7 RNA polymerase from a second plasmid. This second plasmid also carried the HR donor and a NEO (neomycin) resistance cassette to maintain this vector episomally. The authors demonstrated disruption of two individual genes (PfKAHRP and PfEBA-175) without integration of a selectable marker.

These studies were followed by several other reports utilizing similar strategies, including some with new innovations. The Ghorbal approach allowed Nacer *et al.* to identify PfVAP1 (virulence-associated protein 1) as a key factor involved in the *P. falciparum* cytoadherence (Nacer *et al.* 2015). Using a similar general strategy Lu *et al.* introduced the use of a suicide vector approach (Lu *et al.* 2016). In this work, the authors designed a suicide vector encoding Cas9 nuclease, sgRNA and a drug selection marker along with a second plasmid carrying the HR donor for cotransfection. Parasites receiving only the Cas9/sgRNA suicide vector die because of inefficient repair of the DSB by MMEJ, whereas those receiving both plasmids survive *via* HR repair from the donor plasmid. This approach avoids the need for a selectable marker in the donor plasmid, thus freeing up space to introduce larger knock-in tags. The system also has the potential to efficiently mediate consecutive gene manipulations. In another adaptation of the method developed by Ghorbal and colleagues, Mogollon *et al.* (2016) generated marker-free *P. falciparum* fluorescent reporter lines by redesigning the vector carrying the sgRNA and HR donor to include a hybrid positive and negative selectable marker (hDHFR-yfcu) outside the homology arms of the HR donor. In this new approach, positive selection is applied initially to maintain the HR donor plasmid followed by negative selection to eliminate parasites containing the episomal plasmid. In very recent work, Bryant *et al.* used the Ghorbal system to functionally interrogated an important conserved genetic element of var genes, the var2csa intron, without the introduction of a drug-selectable marker (Bryant *et al.* 2017). Also, the Wagner approach based on the sgRNA expression driven by the T7 promoter was successfully used in two other studies to introduce point mutations in either PfMDR1 (Ng *et al.* 2016) or PfCARL

(LaMonte *et al.* 2016) to identify residues involved in drug resistance.

Distinct from the two-plasmid CRISPR/Cas9 systems developed for *P. falciparum*, Zhang and colleagues developed a *P. yoelii* system based on one-vector carrying all the products required for cleavage and DNA repair (Zhang *et al.* 2014). The authors used this system to generate targeted deletion, reporter knock-in, and nucleotide replacement in multiple genes with high efficiency and accuracy. In subsequent work, the same group improved this single-vector system by introducing the yfcu negative selection marker to remove the episomal vector in transfected parasites after the genomic editing was achieved (Zhang *et al.* 2017a, b). The CRISPR/Cas9 system was also employed by Knuepfer *et al.* to introduce a rapamycin-inducible DiCre recombinase for conditional disruption of target genes engineered with flanking loxP sites (Knuepfer *et al.* 2017). DiCre-mediated gene deletion was also achieved using the Ghorbal CRISPR/Cas9 adaptation by Volz *et al.* (2016) to demonstrate the essential role of Pfrh5/PfRipr/CyRPA complex during *P. falciparum* invasion of erythrocytes.

Very recently, *P. falciparum* transfection of a purified CRISPR/Cas9-guide RNA ribonucleoprotein complex and a 200-nucleotide single-stranded oligodeoxynucleotide (ssODN) repair template introduced drug resistance mutations without the use of plasmids or the need for cloning homologous recombination templates (Crawford *et al.* 2017). This approach is ideally suited for introducing mutations that confer a fitness advantage under selection conditions. The efficacy of using ssODN as a repair template in *P. falciparum* may greatly simplify future genome editing of these parasites.

Toxoplasma gondii: higher throughput and genome-wide screens

Contrary to *Plasmodium* spp., *T. gondii* is an apicomplexan parasite offering relative ease of growth in culture and a wide array of genetic tools that include chemical or insertional mutagenesis, homologous gene replacement, conditional knockdown and tagging techniques (Wang *et al.* 2016a). The availability of numerous selectable markers for generation of stable *Toxoplasma* strains (Roos *et al.* 1994, 1997; Donald and Roos, 1995, 1998; Fox *et al.* 1999, 2001; Soete *et al.* 1999; Wang *et al.* 2016a) and the rapid loss of exogenous non-integrated DNA, with no detectable exogenous DNA 7 days post-transfection (Soldati and Boothroyd, 1993; Black and Boothroyd, 1998), makes the study of the biology of *T. gondii* by genetic manipulations more manageable. Integration of foreign DNA in *T. gondii* is relatively efficient, with recombination rates of ~0.1% without restriction enzyme-mediated integration (REMI) and ~5% with REMI (Black *et al.* 1995; Roos *et al.* 1997). The presence of a very active NHEJ pathway in *T. gondii* is a limitation for generating strains with homology-directed knockout or the precise insertion of tags or mutations because of a high prevalence of random DNA integration. Due to this feature, conventional generation of knock out strains for *T. gondii* is inefficient and requires homology flanks of 2–3 kbp (Donald and Roos, 1994; Roos *et al.* 1997; Zhang *et al.* 1999; Craver and Knoll, 2007). To overcome this, *T. gondii* type I (e.g., RH) or type II (e.g., Prugniaud, Pru) strains were modified to disrupt NHEJ-mediated insertion by deleting one key component of this pathway, the gene encoding the Ku80 protein (Fox *et al.* 2009, 2011; Huynh and Carruthers, 2009). The impact of these two strains, named *RHΔku80* and *PruΔku80*, on understanding *Toxoplasma* biology has been substantial, allowing higher fidelity, rapid generation of knockout strains, and the introduction of epitope tags. Although the generation of *Δku80* strains minimized the problem of random DNA integration,

this approach, of course, limits the studies to these two strains currently. Moreover, since the Ku80 protein is involved in DNA repair, strains lacking this gene may be prone to accumulate genetic mutations after prolonged culture.

Introduction of CRISPR/Cas9 into the *Toxoplasma* field has revolutionized the capability to efficiently generate gene knock-outs in any strain. Several CRISPR/Cas9-based approaches have been developed in different laboratories to inactivate selected gene function. For example, complete or partial deletion of the target sequence was obtained through double crossover triggered by a site-directed sgRNA/Cas9-mediated DSB and subsequent DNA repair using a donor DNA comprised a drug resistance expression cassette flanked by about 1 kbp of DNA homologous to the target locus (Shen *et al.* 2014a). This approach also substantially increased the throughput of gene deletions, exemplified by individual or sequential disruption of entire gene families (Shen *et al.* 2014b). Gene inactivation was also obtained *via* 'indels' in the coding region generated by NHEJ repair of a sgRNA/Cas9-mediated DSB (Sidik *et al.* 2014; Wang *et al.* 2016b). A 'clean' knockout with complete gene deletion is desirable because it avoids the potential expression of a truncated protein and precludes homologous reinsertion of the gene or cDNA for genetic complementation. Nonetheless, template mediated complete gene deletion is less efficient in non-*Δku80* strains because homologous recombination is active only when parasites are in the S/G2 phase and thus NHEJ is the prevalent form of DNA repair in *T. gondii* when extracellular, G0-phase parasites are used for transfection. To enhance HR *vs.* NHEJ events, Behnke and colleagues (Behnke *et al.* 2015) developed a new CRISPR vector that expresses Cas9 and two sgRNAs. The two sgRNAs direct Cas9 to generate two DSBs, one at each end of the target gene or locus. This tactic not only improves efficiency, but it also permits efficient disruption of large genes or tandem gene arrays. In our hands, the two-sgRNA approach allowed successful knock out of several genes individually including those encoding TgCPL (cathepsin protease L) (Di Cristina *et al.* 2017) and TgASPI (aspartic protease 1) (Di Cristina and Carruthers, unpublished) in the *T. gondii* type II strain ME49. In summary, CRISPR/Cas9 is opening up genetic manipulation of any *T. gondii* strain and allowing for large genetic disruptions to interrogate gene function.

Although CRISPR/Cas9 is versatile for any strain, applying it in a *Δku80* strain provides the advantage of using short homology sequences, thereby permitting convenient and precise gene knockouts or knocking of tags or mutations. For example, Sidik and co-authors (Sidik *et al.* 2014) introduced tags or mutations in a *Δku80* strain using synthetic oligonucleotide repair templates with 40 bp of homology without the need for a selectable marker. This strategy is based on co-transfecting a Cas9 + sgRNA expression plasmid with a synthetic double strand oligonucleotide bearing the desired tag or mutation together with a silent mutation to eliminate the PAM site NGG beside the 20 bp sequence targeted by the gRNA. The efficiency of this approach was enhanced by fluorescence-activated cell sorting (FACS) parasites that received the Cas9/sgRNA vector, exploiting the fluorescence emitted by the GFP fused to the Cas9 protein. Only ~20–30% of parasites obtain the Cas9/sgRNA vector after electroporation, making it critical to remove the predominant fraction of non-transfected parasites to enrich the population with edited parasites. Since FACS is expensive and not available for all the laboratories, we developed a protocol that allows the enrichment of Cas9-expressing parasites with a Cas9/sgRNA vector bearing a bleomycin resistance gene (Di Cristina and Carruthers, unpublished). Parasites receiving this new plasmid, named pCas9/sgRNA/Bleo, are subjected to phleomycin treatment 24 h after transfection to eliminate parasites that have not received the

plasmid and enrich for parasites expressing Cas9 and the sgRNA. Treatment one day after transfection ensures that bleomycin resistance is transiently expressed by the 20–30% of the population that incorporate the plasmid. Stable integration is not favoured due to the Cas9 toxicity, which works as a negative selection against vector integration. In our hands, this approach results in about 30–80% efficiency of edited or tagged parasites, depending on the impact to parasite fitness of the mutation introduced, allowing easy identification of single mutant clones. Beyond this variation of the approach, the reader is referred to an excellent how-to guide for using CRISPR/Cas9 for various applications in *Toxoplasma* that was published recently (Shen *et al.* 2017).

High-throughput strategies to genetically engineer and screen large numbers of mutants or populations are powerful weapons in modern systems biology. To this end, the emergence of CRISPR/Cas9 has prompted new screening approaches using sgRNA libraries to perform genome-wide knockouts in a parasite population. Such approaches allow measuring the fitness contribution of every gene in the parasite genome. Exploiting the high rates of NHEJ in *T. gondii*, Sebastian Lourido's laboratory efficiently created frame-shift mutations and insertions at the DSBs generated by transfecting a Cas9-expressing RH strain with a library of sgRNAs containing 10 guides against each of the 8158 predicted *T. gondii* protein-coding genes (Sidik *et al.* 2016). The guide RNA library was cloned into the sgRNA expression vector and the integrated sgRNAs were exploited as barcodes to measure the contribution of each gene to parasite fitness. Generation of a Cas9-expressing RH strain was likely instrumental in obtaining high rates of gene disruption. Due to the toxicity of Cas9 expression in *T. gondii*, as observed for other microorganisms (Jiang *et al.* 2014; Peng *et al.* 2014), the authors developed a strategy to obtain strains of *T. gondii* stably expressing this nuclease by co-expressing a decoy sgRNA to prevent the detrimental effect to parasites by unintended Cas9 activity directed by endogenous RNAs. This work represents the first genome-wide functional analysis of an apicomplexan, thus providing broad-based functional information on *T. gondii* genes and their contributions to parasite fitness during infection of human fibroblasts. One initial limitation of this outstanding work is its restriction to the tachyzoite stage of *T. gondii*, the rapidly growing form responsible for the acute phase of the infection. Applying this approach to other life stages will require improvements to the transfection and integration efficiencies of strains that competently differentiate into other stages. The contribution to tachyzoite fitness of each predicted *T. gondii* protein-coding genes is now available (www.toxodb.org).

Recently, David Sibley's group developed an auxin-inducible degron (AID) tagging system for conditional protein depletion in *T. gondii* (Brown *et al.* 2017; Long *et al.* 2017). They exploited a new combination of CRISPR/Cas9-mediated gene editing and a plant-derived AID system to identify which cyclic GMP (cGMP)-dependent protein kinase G (PKG) isoforms are necessary for PKG-dependent cellular processes (Brown *et al.* 2017) and to examine the roles of three apically localized calmodulin-like proteins (Long *et al.* 2017). Adaptation of the AID system to *T. gondii* adds a powerful new tool to identify the consequences of rapidly down-regulating expression of cytosolic proteins to infer function.

CRISPR technology has been adapted to apicomplexan parasites relatively recently and thus has not been fully exploited and expanded. In mammals, evolution of this technology led to the development of tissue or time-specific promoters to restrict the genome editing to a precise cell type or developmental stage (Harrison *et al.* 2014; Ablain *et al.* 2015; Bortesi and Fischer, 2015; Wang *et al.* 2015; Yoshioka *et al.* 2015; Lee *et al.* 2016; Xu *et al.* 2017; Zhang *et al.* 2017b). In the classic CRISPR

technology, sgRNAs are usually transcribed under control of RNA polymerase III promoters to obtain transcripts devoid of both capping and poly(A) tails, thereby generating the correct 5'-end of the sgRNA and avoiding exportation of the sgRNA to the cytoplasm, respectively. Tissue/developmental-specific sgRNA expression requires using RNA polymerase II promoters active exclusively in the desired cell type or stage. To generate RNA polymerase II-driven functional sgRNAs, a strategy based on the use of ribozymes was developed by several laboratories (Yoshioka *et al.* 2015; Ng and Dean, 2017; Xu *et al.* 2017; Zhang *et al.* 2017a, b). Hammerhead or hepatitis delta virus ribozymes perform site-specific self-cleavage, resulting in mature sgRNAs with correct 5'- and 3'-ends. This ribozyme-flanked gRNA expression system can be exploited for the spatiotemporal expression of gRNA employing cell type or developmental-specific promoters. In *Toxoplasma*, this approach has not been investigated yet but may allow programmed gene inactivation in a stage-specific manner by exploiting promoters active at specific phases of the parasite life cycle. For example expressing the ribozyme-sgRNA and Cas9 under the control of a bradyzoite-specific promoter could allow stage-specific inactivation of all genes, even those that are essential for tachyzoites. This approach would allow assessing the role of essential genes during the chronic stage of *T. gondii*. Moreover, multiple sgRNAs linked with self-cleaving ribozymes could be simultaneously expressed from a single promoter to exert genome editing at different sites. Alternatively, several new approaches permit conditional expression of Cas9 via chemical or optical activation (Nihongaki *et al.* 2015; Polstein and Gersbach, 2015; Wright *et al.* 2015; Zetsche *et al.* 2015; Liu *et al.* 2016). In principle, it should also be possible to append a destabilization domain to Cas9 for ligand-dependent expression of Cas9 in any stage of the parasite.

Cryptosporidium: introducing a new genetic era

Cryptosporidium spp. causes severe diarrhoea in young children, with 10% mortality in such cases (Liu *et al.* 2012). Cryptosporidiosis also causes life-threatening chronic disease in immunocompromised individuals, including those afflicted by HIV/AIDS. Infections occur worldwide in association with oocyst contaminated water, with no vaccines available, and only a single drug (nitazoxanide) has been approved with limited benefit for malnourished children and immunocompromised patients (Amadi *et al.* 2002, 2009). Progress in understanding *Cryptosporidium* spp. biology and developing new treatments have been hindered by the limited tractability of the parasite, which includes a lack of systems for continuous culture, the absence of facile animal models, and the dearth of molecular genetic tools (Striepen, 2013; Checkley *et al.* 2015). *Cryptosporidium* spp. cultures last a few days *in vitro* since parasites undergo one or two rounds of replication at most, limiting experiments to small numbers of parasites during a fraction of the life cycle. Species that infect humans cannot be easily studied in standard model hosts such as mice. Also, since *Cryptosporidium* spp. is intrinsically refractory to antifolate drugs, selection of genetically modified parasites using these drugs, as for *Toxoplasma* and *Plasmodium* spp., is not possible. Further hindering *Cryptosporidium* spp. genomic manipulation, transient transfection is 10 000-fold less efficient than that of *T. gondii* (Vinayak *et al.* 2015). Genetic validation of potential drug targets for *Cryptosporidium* spp. is a key unmet need.

Toward this goal, CRISPR/Cas9 technology has proven again to be a powerful system even for *Cryptosporidium* spp. Boris Striepen's laboratory recently developed for the first time a protocol for transfecting *C. parvum* sporozoites in tissue culture and

isolation of stable genetically modified parasites (Vinayak *et al.* 2015). Notwithstanding a 10-fold optimization of transfection, the low efficiency still required the use of a highly sensitive nanoluciferase (nLuc) reporter. Sporozoite expression of nLuc was achieved using the strong enolase promoter, whilst the aminoglycoside antibiotic paromomycin was used as selection marker since it is effective in tissue culture and in immunocompromised mice (Theodos *et al.* 1998). After electroporation, transfected sporozoites were directly introduced into the mouse intestine by surgery due to the low oral infection efficiency of this stage. Expression of sgRNA and the Cas9 nuclease was achieved using the *C. parvum* U6 and aldolase promoters, respectively. Since *Cryptosporidium* spp. lack NHEJ, similar to *Plasmodium* species, transgene integration is likely to require homologous recombination. Thus, in a series of elegant experiments, the Striepen group restored a dead version of the nLuc carrying a stop codon that ablated luciferase activity by using short double-stranded templates for repair. This demonstrated that genome editing through homologous recombination was also possible in the recalcitrant *Cryptosporidium* spp. The authors also achieved for the first time a gene knock out in this parasite by deleting the gene encoding thymidine kinase (TK). This genetic manipulation provided evidence of the non-essentiality of TK and its role as an alternative route for thymidine monophosphate synthesis, explaining why *C. parvum* tolerates high doses of antifolate drugs. The Striepen group also recently published a how-to guide for genetic manipulation of *Cryptosporidium* spp. that will be invaluable to the field (Pawlolic *et al.* 2017). In summary, an adaptation of CRISPR/Cas9 technology to this nearly intractable parasite allowed generation of the first *Cryptosporidium* knock out strain and, at the same time, deletion of the TK gene provided a new potential selection marker for genome manipulation. Overcoming such barriers for *Cryptosporidium* spp. opens new avenues to import the RNA- or protein-based regulatory strategies developed from other apicomplexans.

Future directions

Another key aspect of CRISPR is the impact of off-target effects that may introduce breaks in genomic sites other than the specific sgRNA target. Cas9 tolerates mismatches between guide RNA and target DNA differently depending on the position of the mismatches. Mismatches are tolerated at the 5'-end of the target site, but not at the 3'-end 'seed' sequence beside the PAM (Semenova *et al.* 2011; Cho *et al.* 2013; Cong *et al.* 2013; Ma *et al.* 2014; Farboud and Meyer, 2015; Port and Bullock, 2016). Although the introduction of unwanted changes in sequences of the genome may cause unpredictable consequences for the parasite phenotype, this might not be a major concern for apicomplexans because their small genomes make off-target mutations less likely. No evidence of off-target mutations introduced by Cas9 was seen in both *P. falciparum* (Ghorbal *et al.* 2014; Wagner *et al.* 2014) and *P. yoelii* (Zhang *et al.* 2014), suggesting that this system is very specific in these parasites lacking NHEJ. Off-target effects have not been fully explored in other apicomplexan parasites, such as *T. gondii*, that have an active NHEJ system and thus may also repair DSBs in off-target positions. Regardless, the recent development of high-fidelity Cas9 variants may be useful in apicomplexans to minimize off-target effects (Kleinstiver *et al.* 2016; Slaymaker *et al.* 2016).

Recently, the potential of the CRISPR/Cas9 system has been further expanded to regulate transcription or introduce epigenetic modification in target genes. Activation (CRISPRa) or Repression (CRISPRi) of transcription of target genes has been achieved with a catalytically inactive Cas9 protein (dCas9) lacking endonuclease activity fused to activating or repressive effectors. These systems

have the advantage of controlling gene expression in an inducible and reversible manner (Qi *et al.* 2013). CRISPR/Cas9-directed epigenetic modifications were achieved by fusing the dCas9 protein to epigenetic effectors (e.g., DNA demethylase, histone acetyltransferase and others) for epigenomic engineering (Hilton *et al.* 2015; Kearns *et al.* 2015). The CRISPR/Cas9 system has also been adapted to cleave single-stranded RNA at specific target sites by providing a PAM as part of an oligonucleotide (PAMmer) that hybridizes to the target RNA. In this way, an RNA-targeting Cas9 protein (RCas9) was directed to bind and cleave target RNAs at specific sites using specially designed PAMmers, enabling specific RNA degradation (O'Connell *et al.* 2014). Since apicomplexans lack or have an incomplete system for RNA interference, this strategy might represent an alternative to RNA silencing. A further application of the CRISPR/Cas9 technology has been recently developed to study topologically associated domains (TADs), i.e. genome organization of chromatin into ordered and hierarchical topological structures in interphase nuclei (Bouwman and de Laat, 2015; Sexton and Cavalli, 2015; Bonev and Cavalli, 2016). TADs play important roles in various nuclear processes such as gene regulation since distal elements regulate their gene targets through specific chromatin-looping contacts such as long-distance enhancer-promoter interactions. CRISPR/Cas9 technology provides great opportunities to study TADs by probing spatial DNA-looping interactions and perturb higher-order chromatin organization (Huang and Wu, 2016). TADs have been poorly characterized in apicomplexan parasites and thus this new CRISPR/Cas9 approach offers fresh tools to better understand chromatin organization, opening new avenues to understanding the evolution of chromatin organization from unicellular to multicellular organisms.

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