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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.* 2017*a*, *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.* 2016*a*). 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.* 2016*a*) 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 Δ*ku80* 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 knockouts 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.* 2014*a*). This approach also substantially increased the throughput of gene deletions, exemplified by individual or sequential disruption of entire gene families (Shen *et al.* 2014*b*). 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.* 2016*b*). 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-Δ*ku80* 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 TgASP1 (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 Δ*ku80* 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 Δ*ku80* 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.* 2017*b*). 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.* 2017*a*, *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 (Pawlowic *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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References

Ablain J, Durand EM, Yang S, Zhou Y and Zon LI (2015) A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Developmental Cell* **32**, 756–764.

Amadi B, Mwiya M, Musuku J, Watuka A, Sianongo S, Ayoub A and Kelly P (2002) Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. *The Lancet* **360**, 1375–1380.

Amadi B, Mwiya M, Sianongo S, Payne L, Watuka A, Katubulushi M and Kelly P (2009) High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children: a randomised controlled trial. *BMC Infectious Diseases* **9**, 195.

Barrangou R and Horvath P (2017) A decade of discovery: CRISPR functions and applications. *Nature Microbiology* **2**, 17092.

Barrangou R, Birmingham A, Wiemann S, Beijersbergen RL, Hornung V and Smith A (2015) Advances in CRISPR-Cas9 genome engineering: lessons learned from RNA interference. *Nucleic Acids Research* **43**, 3407–3419.

Behnke MS, Khan A, Lauron EJ, Jimah JR, Wang Q, Tolia NH and Sibley LD (2015) Rhoptry proteins ROP5 and ROP18 are major murine virulence factors in genetically divergent South American strains of *Toxoplasma gondii*. *PLoS Genetics* **11**, e1005434.

Black M, Seeber F, Soldati D, Kim K and Boothroyd JC (1995) Restriction enzyme-mediated integration elevates transformation frequency and enables co-transfection of *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* **74**, 55–63.

Black MW and Boothroyd JC (1998) Development of a stable episomal shuttle vector for *Toxoplasma gondii*. *Journal of Biological Chemistry* **273**, 3972–3979.

Bonev B and Cavalli G (2016) Organization and function of the 3D genome. *Nature Reviews Genetics* **17**, 661–678.

Bortesi L and Fischer R (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances* **33**, 41–52.

Bouwman BA and de Laat W (2015) Architectural hallmarks of the pluripotent genome. *FEBS Letters* **589**, 2905–2913.

Brown KM, Long S and Sibley LD (2017) Plasma membrane association by N-acylation governs PKG function in *Toxoplasma gondii*. *mBio* **8**, e00375–17.

Bryant JM, Regnault C, Scheidig-Benatar C, Baumgarten S, Guizetti J and Scherf A (2017) CRISPR/cas9 genome editing reveals that the intron is not essential for var2csa gene activation or silencing in *Plasmodium falciparum*. *mBio* **8**, e00729–17.

Campanario J (2009) Rejecting and resisting Nobel class discoveries: accounts by Nobel Laureates. *Scientometrics* **81**, 549–565.

Checkley W, White AC Jr., Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, Fayer R, Griffiths JK, Guerrant RL, Hedstrom L, Huston CD, Kotloff KL, Kang G, Mead JR, Miller M, Petri WA Jr., Priest JW, Roos DS, Striepen B, Thompson RC, Ward HD, Van Voorhis WA, Xiao L, Zhu G and Houpt ER (2015) A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. *The Lancet Infectious Diseases* **15**, 85–94.

Cho SW, Kim S, Kim JM and Kim J (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotechnology* **31**, 230–232.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA and Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, NY)* **339**, 819–823.

Craver MP and Knoll LJ (2007) Increased efficiency of homologous recombination in *Toxoplasma gondii* dense granule protein 3 demonstrates that GRA3 is not necessary in cell culture but does contribute to virulence. *Molecular and Biochemical Parasitology* **153**, 149–157.

Crawford ED, Quan J, Horst JA, Ebert D, Wu W and DeRisi JL (2017) Plasmid-free CRISPR/Cas9 genome editing in Plasmodium falciparum confirms mutations conferring resistance to the dihydroisoquinolone clinical candidate SJ733. *PLoS ONE* **12**, e0178163.

Deitsch K, Driskill C and Wellems T (2001) Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Research* **29**, 850–853.

Di Cristina M, Dou Z, Lunghi M, Kannan G, Huynh MH, McGovern OL, Schultz TL, Schultz AJ, Miller AJ, Hayes BM, van der Linden W, Emiliani C, Bogyo M, Besteiro S, Coppens I and Carruthers VB (2017) Toxoplasma depends on lysosomal consumption of autophagosomes for persistent infection. *Nature Microbiology* **2**, 17096.

Donald RG and Roos DS (1998) Gene knock-outs and allelic replacements in *Toxoplasma gondii:* HXGPRT as a selectable marker for hit-and-run mutagenesis. *Molecular & Biochemical Parasitology* **91**, 295–305.

Donald RGK and Roos DS (1994) Homologous recombination and gene replacement at the dihydrofolate reductase-thymidylate synthase locus in *Toxoplasma gondii*. *Molecular & Biochemical Parasitology* **63**, 243–253.

Donald RGK and Roos DS (1995) Insertional mutagenesis and marker rescue in a protozoan parasite: cloning of the uracil phosphoribosyltransferase locus from *Toxoplasma gondii*. *Proceedings of the National Academy of Sciences USA* **92**, 5749–5753.

Farboud B and Meyer BJ (2015) Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. *Genetics* **199**, 959–971.

Fox BA, Belperron AA and Bzik DJ (1999) Stable transformation of *Toxoplasma gondii* based on a pyrimethamine resistant trifunctional dihydrofolate reductase-cytosine deaminase-thymidylate synthase gene that confers sensitivity to 5-fluorocytosine. *Molecular & Biochemical Parasitology* **98**, 93–103.

Fox BA, Belperron AA and Bzik DJ (2001) Negative selection of herpes simplex virus thymidine kinase in *Toxoplasma gondii*. *Molecular & Biochemical Parasitology* **116**, 85–88.

Fox BA, Ristuccia JG, Gigley JP and Bzik DJ (2009) Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end-joining. *Eukaryotic Cell* **8**, 520–529.

Fox BA, Falla A, Rommereim LM, Tomita T, Gigley JP, Mercier C, Cesbron-Delauw MF, Weiss LM and Bzik DJ (2011) Type II *Toxoplasma gondii* KU80 knockout strains enable functional analysis of genes required for cyst development and latent infection. *Eukaryotic Cell* **10**, 1193–1206.

Ghorbal M, Gorman M, Macpherson CR, Martins RM, Scherf A and Lopez-Rubio JJ (2014) Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. *Nature Biotechnology* **32**, 819–821.

Harrison MM, Jenkins BV, O'Connor-Giles KM and Wildonger J (2014) A CRISPR view of development. *Genes & Development* **28**, 1859–1872.

Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE and Gersbach CA (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature Biotechnology* **33**, 510–517.

Hryhorowicz M, Lipinski D, Zeyland J and Slomski R (2017) CRISPR/cas9 immune system as a tool for genome engineering. *Archivum Immunologiae et Therapiae Experimentalis* **65**, 233–240.

Hsu PD, Lander ES and Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262–1278.

Huang H and Wu Q (2016) CRISPR double cutting through the labyrinthine architecture of 3D genomes. *Journal of Genetics and Genomics = Yi chuan xue bao* **43**, 273–288.

Huynh MH and Carruthers VB (2009) Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryotic Cell* **8**, 530–539.

Jackson SA, McKenzie RE, Fagerlund RD, Kieper SN, Fineran PC and Brouns SJ (2017) CRISPR-Cas: adapting to change. *Science* (*New York, NY*) **356**, eaal5056. Epub 2017 Apr 6.

Jiang F and Doudna JA (2017) CRISPR-Cas9 structures and mechanisms. *Annual Review of Biophysics* **46**, 505–529.

Jiang W, Brueggeman AJ, Horken KM, Plucinak TM and Weeks DP (2014) Successful transient expression of Cas9 and single guide RNA genes in Chlamydomonas reinhardtii. *Eukaryotic Cell* **13**, 1465–1469.

Karginov FV and Hannon GJ (2010) The CRISPR system: small RNA-guided defense in bacteria and archaea. *Molecular Cell* **37**, 7–19.

Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, Garber M and Maehr R (2015) Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nature Methods* **12**, 401–403.

Kick L, Kirchner M and Schneider S (2017) CRISPR-Cas9: from a bacterial immune system to genome-edited human cells in clinical trials. *Bioengineered* **8**, 280–286.

Kirkman LA, Lawrence EA and Deitsch KW (2014) Malaria parasites utilize both homologous recombination and alternative end joining pathways to maintain genome integrity. *Nucleic Acids Research* **42**, 370–379.

Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z and Joung JK (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**, 490–495.

Knuepfer E, Napiorkowska M, van Ooij C and Holder AA (2017) Generating conditional gene knockouts in Plasmodium – a toolkit to produce stable DiCre recombinase-expressing parasite lines using CRISPR/Cas9. *Scientific Reports* **7**, 3881-017-03984-3.

Koonin EV, Makarova KS and Zhang F (2017) Diversity, classification and evolution of CRISPR-Cas systems. *Current Opinion in Microbiology* **37**, 67–78.

LaMonte G, Lim MY, Wree M, Reimer C, Nachon M, Corey V, Gedeck P, Plouffe D, Du A, Figueroa N, Yeung B, Bifani P and Winzeler EA (2016) Mutations in the Plasmodium falciparum cyclic amine resistance locus (PfCARL) confer multidrug resistance. *mBio* **7**, e00696-16.

Lander ES (2016) The heroes of CRISPR. *Cell* **164**, 18–28.

Lee RT, Ng AS and Ingham PW (2016) Ribozyme mediated gRNA generation for in vitro and in vivo CRISPR/Cas9 mutagenesis. *PLoS ONE* **11**, e0166020.

Liu KI, Ramli MN, Woo CW, Wang Y, Zhao T, Zhang X, Yim GR, Chong BY, Gowher A, Chua MZ, Jung J, Lee JH and Tan MH (2016) A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing. *Nature Chemical Biology* **12**, 980–987.

Liu L, Johnson HL, Cousens S, Perin J, Scott S, Lawn JE, Rudan I, Campbell H, Cibulskis R and Li M (2012) Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *The Lancet* **379**, 2151–2161.

Long S, Brown KM, Drewry LL, Anthony B, Phan IQH and Sibley LD (2017) Calmodulin-like proteins localized to the conoid regulate motility and cell invasion by *Toxoplasma gondii*. *PLoS Pathogens* **13**, e1006379.

Lu J, Tong Y, Pan J, Yang Y, Liu Q, Tan X, Zhao S, Qin L and Chen X (2016) A redesigned CRISPR/Cas9 system for marker-free genome editing in Plasmodium falciparum. *Parasites & Vectors* **9**, 198-016-1487-4.

Ma Y, Zhang L and Huang X (2014) Genome modification by CRISPR/Cas9. *The FEBS Journal* **281**, 5186–5193.

Mogollon CM, van Pul FJ, Imai T, Ramesar J, Chevalley-Maurel S, de Roo GM, Veld SA, Kroeze H, Franke-Fayard BM, Janse CJ and Khan SM (2016) Rapid generation of marker-free P. falciparum fluorescent reporter lines using modified CRISPR/Cas9 constructs and selection protocol. *PLoS ONE* **11**, e0168362.

Nacer A, Claes A, Roberts A, Scheidig-Benatar C, Sakamoto H, Ghorbal M, Lopez-Rubio JJ and Mattei D (2015) Discovery of a novel and conserved Plasmodium falciparum exported protein that is important for adhesion of PfEMP1 at the surface of infected erythrocytes. *Cellular Microbiology* **17**, 1205–1216.

Ng CL, Siciliano G, Lee MC, de Almeida MJ, Corey VC, Bopp SE, Bertuccini L, Wittlin S, Kasdin RG, Le Bihan A, Clozel M, Winzeler EA, Alano P and Fidock DA (2016) CRISPR-Cas9-modified pfmdr1 protects Plasmodium falciparum asexual blood stages and gametocytes against a class of piperazine-containing compounds but potentiates artemisinin-based combination therapy partner drugs. *Molecular Microbiology* **101**, 381–393.

Ng H and Dean N (2017) Dramatic improvement of CRISPR/Cas9 editing in Candida albicans by increased single guide RNA expression. *mSphere* **2**, e00385–16. eCollection 2017 Mar–Apr.

Nihongaki Y, Kawano F, Nakajima T and Sato M (2015) Photoactivatable CRISPR-Cas9 for optogenetic genome editing. *Nature Biotechnology* **33**, 755–760.

O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M and Doudna JA (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* **516**, 263–266.

Patterson AG, Yevstigneyeva MS and Fineran PC (2017) Regulation of CRISPR-Cas adaptive immune systems. *Current Opinion in Microbiology* **37**, 1–7.

Pawlowic MC, Vinayak S, Sateriale A, Brooks CF and Striepen B (2017) Generating and maintaining transgenic cryptosporidium parvum parasites. *Current Protocols in Microbiology* **46**, 20B.2.1-20B.2.32.

Peng D, Kurup SP, Yao PY, Minning TA and Tarleton RL (2014) CRISPR-Cas9-mediated single-gene and gene family disruption in Trypanosoma cruzi. *mBio* **6**, e02097–14.

Pineda M, Moghadam F, Ebrahimkhani MR and Kiani S (2017) Engineered CRISPR systems for next generation gene therapies. *ACS Synthetic Biology* **6**, 1614–1626.

Polstein LR and Gersbach CA (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. *Nature Chemical Biology* **11**, 198–200.

Port F and Bullock SL (2016) Creating heritable mutations in drosophila with CRISPR-Cas9. *Drosophila: Methods and Protocols. Methods in Molecular Biology* **1478**, 145–160.

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP and Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183.

Roos DS, Donald RG, Morrissette NS and Moulton AL (1994) Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods in Cell Biology* **45**, 27–63.

Roos DS, Sullivan WJ, Striepen B, Bohne W and Donald RG (1997) Tagging genes and trapping promoters in *Toxoplasma gondii* by insertional mutagenesis. *Methods* **13**, 112–122.

Salsman J and Dellaire G (2017) Precision genome editing in the CRISPR era. *Biochemistry and Cell Biology* (*Biochimie et biologie cellulaire*) **95**, 187–201.

Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, van der Oost J, Brouns SJ and Severinov K (2011) Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 10098–10103.

Sexton T and Cavalli G (2015) The role of chromosome domains in shaping the functional genome. *Cell* **160**, 1049–1059.

Shen B, Brown KM, Lee TD and Sibley LD (2014*a*) Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. *mBio* **5**, e01114–14.

Shen B, Buguliskis JS, Lee TD and Sibley LD (2014*b*) Functional analysis of rhomboid proteases during Toxoplasma invasion. *mBio* **5**, e01795–14.

Shen B, Brown K, Long S and Sibley LD (2017) Development of CRISPR/Cas9 for efficient genome editing in *Toxoplasma gondii*. *Methods in Molecular Biology (Clifton, NJ)* **1498**, 79–103.

Sidik SM, Hackett CG, Tran F, Westwood NJ and Lourido S (2014) Efficient genome engineering of *Toxoplasma gondii* using CRISPR/Cas9. *PLoS ONE* **9**, e100450.

Sidik SM, Huet D, Ganesan SM, Huynh MH, Wang T, Nasamu AS, Thiru P, Saeij JP, Carruthers VB, Niles JC and Lourido S (2016) A genome-wide CRISPR screen in toxoplasma identifies essential apicomplexan genes. *Cell* **166**, 1423–1435. e12.

Singer M, Marshall J, Heiss K, Mair GR, Grimm D, Mueller AK and Frischknecht F (2015) Zinc finger nuclease-based double-strand breaks attenuate malaria parasites and reveal rare microhomology-mediated end joining. *Genome Biology* **16**, 249-015-0811-1.

Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX and Zhang F (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science (New York, NY)* **351**, 84–88.

Soete M, Hettman C and Soldati D (1999) The importance of reverse genetics in determining gene function in apicomplexan parasites. *Parasitology* **118** (suppl.), S53–S61.

Soldati D and Boothroyd JC (1993) Transient transfection and expression in the obligate intracellular parasite, *Toxoplasma gondii*. *Science* **260**, 349–351.

Straimer J, Lee MC, Lee AH, Zeitler B, Williams AE, Pearl JR, Zhang L, Rebar EJ, Gregory PD, Llinas M, Urnov FD and Fidock DA (2012) Site-specific genome editing in Plasmodium falciparum using engineered zinc-finger nucleases. *Nature Methods* **9**, 993–998.

Striepen B (2013) Time to tackle cryptosporidiosis. *Nature* **503**, 189–191.

Suarez CE, Bishop RP, Alzan HF, Poole WA and Cooke BM (2017) Advances in the application of genetic manipulation methods to apicomplexan parasites. *International Journal for Parasitology* **47**, 701–710.

Theodos CM, Griffiths JK, D'Onfro J, Fairfield A and Tzipori S (1998) Efficacy of nitazoxanide against Cryptosporidium parvum in cell culture and in animal models. *Antimicrobial Agents and Chemotherapy* **42**, 1959–1965.

Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnadig N, Uhlemann AC, Martin RE, Lehane AM and Fidock DA (2016) Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. *Nature Communications* **7**, 11553.

Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ and Striepen B (2015) Genetic modification of the diarrhoeal pathogen Cryptosporidium parvum. *Nature* **523**, 477–480.

Volz JC, Yap A, Sisquella X, Thompson JK, Lim NT, Whitehead LW, Chen L, Lampe M, Tham WH, Wilson D, Nebl T, Marapana D, Triglia T, Wong W, Rogers KL and Cowman AF (2016) Essential role of the PfRh5/PfRipr/CyRPA complex during plasmodium falciparum invasion of erythrocytes. *Cell Host & Microbe* **20**, 60–71.

Wagner JC, Platt RJ, Goldfless SJ, Zhang F and Niles JC (2014) Efficient CRISPR-Cas9-mediated genome editing in Plasmodium falciparum. *Nature Methods* **11**, 915–918.

Wang J, Li X, Zhao Y, Li J, Zhou Q and Liu Z (2015) Generation of cell-type-specific gene mutations by expressing the sgRNA of the CRISPR system from the RNA polymerase II promoters. *Protein & Cell* **6**, 689–692.

Wang JL, Huang SY, Behnke MS, Chen K, Shen B and Zhu XQ (2016*a*) The past, present, and future of genetic manipulation in *Toxoplasma gondii*. *Trends in Parasitology* **32**, 542–553.

Wang JL, Huang SY, Li TT, Chen K, Ning HR and Zhu XQ (2016*b*) Evaluation of the basic functions of six calcium-dependent protein kinases in *Toxoplasma gondii* using CRISPR-Cas9 system. *Parasitology Research* **115**, 697–702.

Wright AV, Sternberg SH, Taylor DW, Staahl BT, Bardales JA, Kornfeld JE and Doudna JA (2015) Rational design of a split-Cas9 enzyme complex. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 2984–2989.

Wright AV, Nunez JK and Doudna JA (2016) Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering. *Cell* **164**, 29–44.

Xu L, Zhao L, Gao Y, Xu J and Han R (2017) Empower multiplex cell and tissue-specific CRISPR-mediated gene manipulation with self-cleaving ribozymes and tRNA. *Nucleic Acids Research* **45**, e28.

Yoshioka S, Fujii W, Ogawa T, Sugiura K and Naito K (2015) Development of a mono-promoter-driven CRISPR/Cas9 system in mammalian cells. *Scientific Reports* **5**, 18341.

Zetsche B, Volz SE and Zhang F (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nature Biotechnology* **33**, 139–142.

Zhang C, Xiao B, Jiang Y, Zhao Y, Li Z, Gao H, Ling Y, Wei J, Li S, Lu M, Su XZ, Cui H and Yuan J (2014) Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. *mBio* **5**, e01414–14.

Zhang C, Gao H, Yang Z, Jiang Y, Li Z, Wang X, Xiao B, Su XZ, Cui H and Yuan J (2017*a*) CRISPR/cas9 mediated sequential editing of genes critical for ookinete motility in *Plasmodium yoelii*. *Molecular and Biochemical Parasitology* **212**, 1–8.

Zhang T, Gao Y, Wang R and Zhao Y (2017*b*) Production of guide RNAs in vitro and in vivo for CRISPR using ribozymes and RNA polymerase II promoters. *Bio-protocol* **7**, e2148.

Zhang YW, Kim K, Ma YF, Wittner M, Tanowitz HB and Weiss LM (1999) Disruption of the *Toxoplasma gondii* bradyzoite-specific gene BAG1 decreases in vivo cyst formation. *Molecular Microbiology* **31**, 691–701.