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REVIEW ARTICLE

Genetic Manipulation Toolkits in Apicomplexan Parasites

Zhipeng Niu[1], Lilan Xue[1], Xiaoyan Yin[1], Bang Shen[1,2,3,*]

Abstract

Apicomplexan parasites are a group of intracellular pathogens of great medical and veterinary importance, including *Toxoplasma gondii* and *Plasmodium*, which cause toxoplasmosis and malaria, respectively. Efficient and accurate manipulation of their genomes is essential to dissect their complex biology and to design new interventions. Over the past several decades, scientists have continually optimized the methods for genetic engineering in these organisms, and tremendous progress has been made. Here, we review the genetic manipulation tools currently used in several apicomplexan parasites, and discuss their advantages and limitations. The widely used CRISPR/Cas9 genome editing technique has been adapted in several apicomplexans and shown promising efficiency. In contrast, conditional gene regulation is available in only a limited number of organisms, mainly *Plasmodium* and *Toxoplasma*, thus posing a research bottleneck for other parasites. Conditional gene regulation can be achieved with tools that regulate gene expression at the DNA, RNA or protein level. However, a universal tool to address all needs of conditional gene manipulation remains lacking. Understanding the scope of application is key to selecting the proper method for gene manipulation.

Keywords: Genome editing, CRISPR, Apicomplexa, conditional regulation

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The other two reviewers chose to be anonymous.

***Corresponding author:**
Email: shenbang@mail.hzau.edu.cn,
Tel: +86-27-87281810;
Fax: +86-27-87280408 (BS)

[1]State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei Province, PR China
[2]Key Laboratory of Preventive Veterinary Medicine in Hubei Province, Wuhan, Hubei Province, PR China
[3]Hubei Hongshan Laboratory, Wuhan, Hubei Province, PR China

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INTRODUCTION

The apicomplexan phylum contains a large group of parasitic protozoa with more than 6000 described species, including *Toxoplasma gondii*, *Neospora caninum*, *Plasmodium* spp., *Babesia* spp., *Eimeria* spp. and *Cryptosporidium* spp., which infect humans and diverse animals [1]. These parasites cause different diseases in their human and animal hosts and are of great medical and veterinary concern. *Toxoplasma gondii* infects one-third of the world's human population, and almost all warm-blooded animals are susceptible hosts [2]; infections can lead to miscarriage and stillbirth in pregnant women or animals. *Neospora caninum*, a cyst-forming coccidian parasite closely associated with *T. gondii*, is a major cause of abortion in cattle. Malaria, an ancient disease caused by *Plasmodium* spp., kills more than 600,000 people each year [3]. *Cryptosporidium* parasites are a leading cause of diarrhea in infants and young children [4]. Interventions for most of these pathogens are lacking or limited. Anti-folate drugs, such as pyrimethamine and sulfadiazine, are commonly used to treat toxoplasmosis [5] but are effective against only acute infections; therefore, cure of widespread chronic toxoplasmosis remains impossible. Prevention of toxoplasmosis by vaccination has not been achieved for most animal hosts, although the live vaccine Toxovax has been used in some countries to prevent abortion in ewes [6,7]. Similar problems also exist in malaria control. The emergence and

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spread of drug resistant isolates have posed enormous challenges in the treatment of human malaria [8–11]. In contrast, the RTS, S vaccine recommended by the World Health Organization has low efficacy, particularly in infants, who are highly susceptible to malaria [12]. Interventions against other apicomplexan parasites face similar (if not greater) challenges. Therefore, development of novel therapeutic and preventive strategies is urgently needed.

Full understanding of the pathobiology and pathogenesis of the apicomplexan parasites is key for designing novel interventions. In this regard, the availability of tools to manipulate parasite genomes to understand gene function is critical. Over the past several decades, transfection methods introducing exogenous DNA into several apicomplexan parasites have been developed. Different strategies, such as fluorescence activated cell sorting and drug selection makers, are available to select for transgenic parasites. More recently, genetic tools for targeted manipulation at the DNA, RNA and protein levels have been developed. Although these methods have greatly accelerated biological studies on these pathogens [13,14], no single tool is universally applicable to all genetic manipulation purposes, and each has advantages and drawbacks. In this review, we summarize common strategies currently used for targeted gene manipulation in several apicomplexan parasites, including *Toxoplasma*, *Plasmodium*, *Babesia*, *Cryptosporidium* and *Eimeria*. Comparisons among these methods will help researchers choose the proper method and be aware of potential drawbacks.

GENETIC MANIPULATION IN THE MODEL ORGANISM *TOXOPLASMA GONDII*

Toxoplasma has been used as a model organism to study the biology, pathogenesis and immunology of intracellular parasites, owing to the ease of culture *in vitro* and genetic manipulation. Culture of *Toxoplasma* tachyzoites *in vitro* was established in 1970 [15]. Then in 1993, methods for introducing and expressing exogenous genes in parasites were established [16–18]. Several drug markers for the selection and identification of transgenic parasites, including chloramphenicol acetyltransferase (CAT), pyrimethamine resistant mutant of dihydrofolate reductase (DHFR)-thymidylate synthase, hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) and bleomycin/phleomycin-binding protein, have been successfully identified and used in *Toxoplasma* [17–20]. In addition, negative selection markers, such as uracil phosphoribosyltransferase, HXGPRT and SNR1, whose inactivation leads to resistance to fluorodeoxyribose, 6-thioxanthine and sinefungin, respectively [19,21,22] were also discovered. These advances have paved the way to the development of sophisticated locus specific genome engineering. Currently, a wide variety of tools are available to manipulate target genes at different levels.

Enhanced homologous recombination with *KU80* deletion strains

A key requirement to achieve targeted genome engineering is site-specific modification. Initially, the efficiency of targeted gene editing by homologous recombination in wildtype *Toxoplasma* parasites was extremely low (less than 0.5%), thus making applications such as gene deletion very difficult [23–26]. The reason for the low efficiency of homologous recombination was the extremely high activity of nonhomologous end-joining (NHEJ), which allows for random insertion of exogenous DNA into the genome. Deletion of the *KU80* gene greatly decreases NHEJ activity and increases the homologous recombination efficiency to nearly 100% [23,27,28]. In addition, unlike traditional homologous recombination, which requires homologous regions longer than 500 base pairs, only short homology arms of approximately 50 base pairs are sufficient to allow for robust recombination in the Δ*ku80* mutants, thereby minimizing the cloning work required to generate constructs for recombination (Fig 1A) [23]. Because of their extremely high efficiency of site-specific recombination, Δ*ku80* mutants became the preferred choice for genetic modification in *Toxoplasma* soon after they were made available. One limitation of this approach is that the Δ*ku80* mutation was generated in only several strains, such as RH, PRU and ME49 [23,29]. Therefore, applications have been restricted to these strains. Nonetheless, with tools such as CRISPR/Cas9 based gene editing, as described below, construction of Δ*ku80* mutants in other isolates should be possible. In fact, even in strains such as RH and PRU, the combination of the Δ*ku80* strains and CRISPR/Cas9 techniques is commonly used for site specific gene editing.

CRISPR/Cas9, the method of choice for targeted gene editing

The CRISPR/Cas9 system is a powerful genome editing technique that has revolutionized biomedical research. In 2014, CRISPR/Cas9 technology was successfully adapted to *T. gondii* for diverse types of genetic modification with high efficiency [30,31]. One advantage was that this system can be used in all strains without genetic background restrictions, because the two elements (the Cas9 nuclease and the single guide RNA (sgRNA) that specifies the target) needed for gene targeting are expressed from a single plasmid that can be introduced into any *Toxoplasma* strains. As with the Δ*ku80* strategy, the key issue solved by CRISPR/Cas9 technology in *Toxoplasma* gene editing is increasing the frequency of the site-specific change in the targets—a challenge in wildtype parasites, because of NHEJ. CRISPR/Cas9 generates double-strand breaks (DSBs) at specific sites in the targeted gene according to the gRNA (Fig 1B). The DSBs must be fixed to avoid lethality. In the presence of a homologous template, the DSBs are fixed by homologous recombination with high efficiency. During gene editing, homologous templates are often modified to contain functional elements

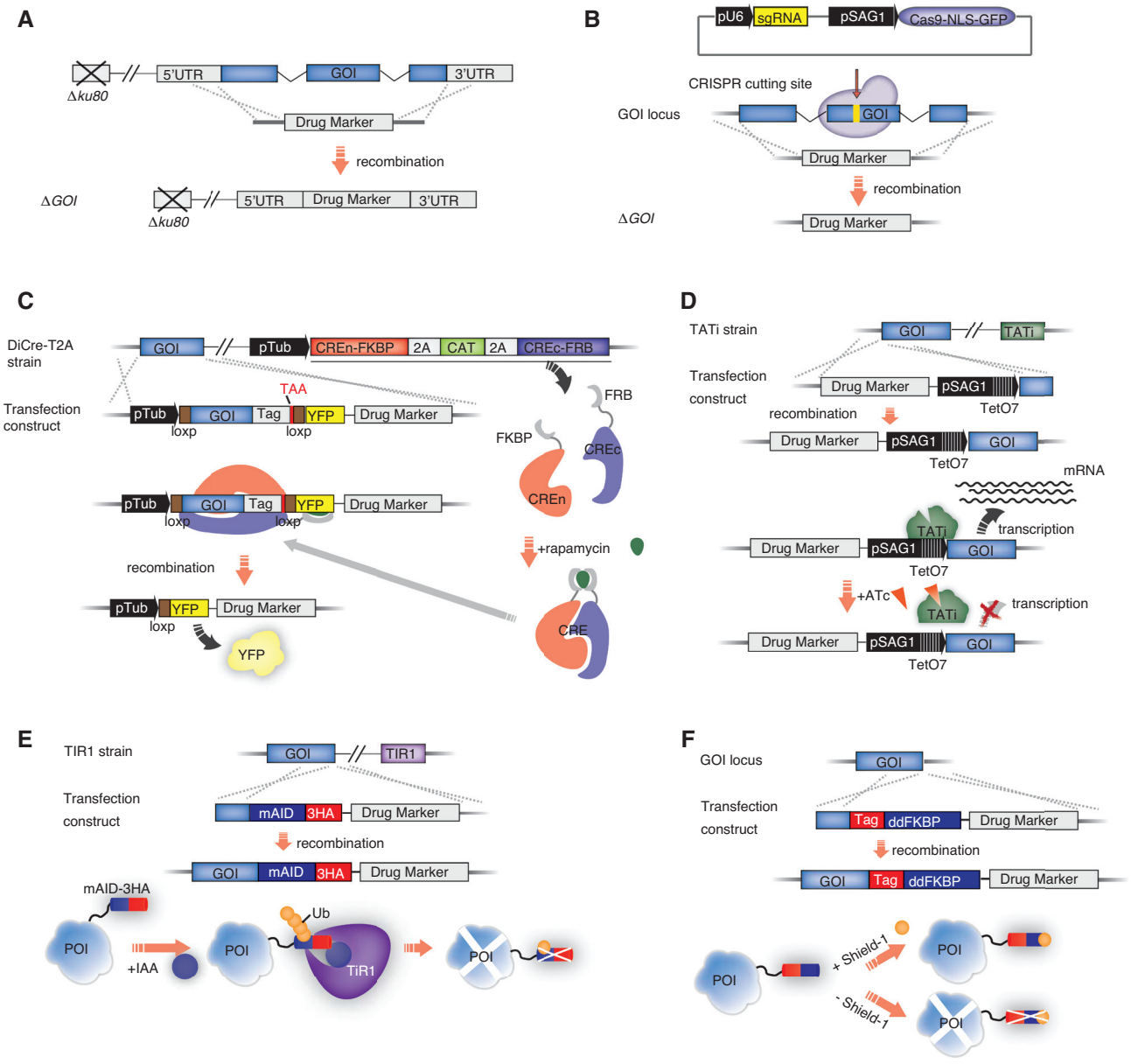

FIGURE 1 | Gene editing techniques in *Toxoplasma gondii*. (A) Inactivation of *KU80* increases homologous recombination in *Toxoplasma*, thus facilitating the replacement of a gene of interest (GOI) with drug selection markers. (B) Gene editing with the CRISPR/Cas9 system. The plasmid for expression of CAS9 and sgRNA is used to generate a target specific DSB, and the homologous repair donor containing a selection marker facilitates the repair of the DSB, thereby leading to the replacement of the GOI by the selection marker. (C) Regulatable gene excision with the DiCre-T2A strain. The Cre recombinase is split into two parts, which are fused with FKBP and FRB in the DiCre-T2A strain. Addition of rapamycin induces the dimerization of FKBP and FRB, thereby reconstituting a functional Cre recombinase. When the GOI is flanked by loxP sites, Cre mediated recombination at the loxP sites deletes the GOI, brings the YFP near the tubulin promoter (pTub) and activates its expression, thus indicating the deletion of the GOI. (D) Tunable gene transcription with the tet-off system. In the TATi strain expressing a tetracycline transactivator, the endogenous promoter of the GOI is replaced by a tetracycline regulatable promoter, such as S1O7, which consists of seven TetO sequences inserted into the *SAG1* promoter (pSAG1). The transcription of the GOI can then be turned off by addition of tetracycline or its analogs. (E) Conditional degradation of target proteins by the AID system. In a transgenic strain that expresses OsTIR1, the miniAID degron is fused to the C-terminus of the protein of interest (POI). In the presence of auxin, such as IAA, the degron tagged proteins are ubiquitinated by the SCF-TIR1 E3 ubiquitin ligase and subsequently degraded by the proteasome. (F) Conditional degradation of target proteins by the FKBP degradation system (DD). Similarly to the AID system, the destabilization domain of FKBP is fused at the C-termini of target proteins. The presence of Shield-1 maintains the stability of target proteins, and its removal induces their degradation.

(e.g., selection markers, epitope tags or regulatory elements) in addition to the homologous regions required for recombination. The DSBs may also be fixed by NHEJ if no homologous template is provided, thus leading to short insertion or deletion (indel) mutations at the targeting site and gene inactivation [32]. CRISPR/Cas9 induces site specific changes in target genes. If CRISPR/Cas9 is used in a Δ*ku80* strain, homologous recombination is the only way to repair the DSBs, because NHEJ activity is minimal. Therefore, the rate of homology-based modification

of target genes is almost 100% [27]. This method is widely used in many applications, such as epitope tagging for localization or protein stability regulation purposes. Notably, the requirement of only short homologous arms for homologous recombination in Δ*ku80* mutants makes such applications straightforward and streamlined [23]. The CRISPR/Cas9 technique has become the preferred method for targeted gene deletion or inactivation, and for the delivery of regulatory elements to specific loci to regulate the expression of target genes.

Interestingly, the *Toxoplasma* CRISPR/Cas9 plasmid for expression of Cas9 and gRNA from *Toxoplasma* gene promoters is functional in *Neospora caninum*, and can introduce site specific DSB and target gene disruption [33], probably because of the close phylogenetic relationship between these parasite species. Thus, *Toxoplasma* CRISPR plasmids can be directly used in *N. caninum* for gene editing purposes.

Inducible gene excision with the DiCre system

For genes that are essential or crucial for parasite growth, direct disruption or inactivation are not possible. However, these genes may be good targets for drug or vaccine designs. Therefore, full understanding of their physiological functions and working mechanisms is greatly needed. Fortunately in *Toxoplasma*, several tools are available to conditionally control the expression of target genes. These different tools achieve gene regulation at the DNA, messenger RNA and protein levels. At the DNA level, conditional gene manipulation tools enable the deletion of the DNA sequences of target genes under specific conditions. The most commonly used strategies use Cre recombinase, which recognizes two specific DNA sequences called loxP sites and mediates recombination between these sites [34,35]. The outcomes of Cre mediated recombination depend on the orientation of the two loxP sites [34,36]: if the sites are oriented in the same direction, the DNA sequence between them is removed [34]. Therefore, if the target gene is placed between two loxP sites with the same orientation, it is deleted in the presence of Cre recombinase. Cre recombinase was initially delivered by electroporation of a Cre expressing plasmid into parasites with a floxed target gene [34]. Nonetheless, given the relatively low efficiency of transfection, the percentage of parasites with the target gene deleted is not very high with this approach. To overcome this problem, the DiCre conditional knockout system was established in 2013 [37]. In this system, the Cre recombinase is divided into two inactive moieties (N-terminal CREn and C-terminal CREc), which are fused to the rapamycin binding proteins FKBP–rapamycin binding (FRB) and FK506 binding protein (FKBP). In a transgenic strain (called DiCre) expressing CREn-FKBP and CREc-FRB, the addition of rapamycin induces the dimerization of CREn-FKBP and CREc-FRB and reconstitutes Cre recombinase activity [37]. Therefore, if the target gene is engineered to be flanked by loxP sites in the DiCre strain, rapamycin supplementation rapidly induces excision of the target gene. The efficiency of target gene excision has been reported to exceed 90% for reporter genes. The constructs used to flox the target genes are often optimized to contain a promoterless fluorescent protein gene (such as YFP) immediately downstream of the coding sequences of the target genes (Fig 1C). With this design, rapamycin induced excision of the target genes brings the fluorescent protein gene close to the promoter that drives the expression of target genes and activates its expression. Therefore, the fluorescence serves as an indication of parasites that have the target genes deleted, and further fluorescence activated cell sorting may be used to enrich gene deletion mutants.

The DiCre system is extremely useful for conditional excision of target genes. Nonetheless, the first generation of this system had stability problems (Table 1), primarily loss of the CREn-FKBP or CREc-FRB fusions over time (probably because of the cellular toxicity of these proteins), thus decreasing gene excision efficiency. To solve this problem, a second generation DiCre system (DiCre-T2A) was generated in 2019 [38], in which CREn-FKBP and CREc-FRB are expressed along with the selection marker CAT (which provides resistance to choloramphenicol) as a single giant fusion protein with each moiety (CREn-FKBP, CREc-FRB and CAT) separated by the self-cleaving peptide T2A to produce three proteins [17,37-39] (Fig 1C). Before use, the DiCre-T2A strain can be selected with choloramphenicol to ensure that all parasites contain intact DiCre elements. The new DiCre-T2A has an excision efficiency of 98–99%. More importantly, the decrease in excision rates is minimal (less than 3%) after 2 months' passage *in vitro* [38]. The extremely high efficiency of gene excision makes DiCre-T2A an ideal system for conditional gene deletion in *Toxoplasma gondii*. In theory, this system should be able to manipulate any nuclear gene. Nonetheless, in our experience, some genes are refractory to DiCre mediated disruption, although the gene loci can be floxed. The exact reason for this resistance is currently unknown but may be due to the local chromatin structure preventing Cre recombinase from accessing the loxP sites.

Tunable gene transcription by tetracycline regulatable promoters

Tetracycline regulatable promoters regulate gene expression at the transcriptional level. Two types of tetracycline regulatable gene expression systems are used, tet-on and tet-off, which activate or suppress target gene transcription in the presence of tetracycline, respectively. Both systems require the tetracycline repressor protein (TetR) from *Escherichia coli* and the TetO (tetracycline operator) sequences to which TetR binds. Gossen *et al.* constructed the first tet-off system in 1992 for gene regulation in mammalian cells [40]. Since then, this system has been widely used and adapted to many different organisms [41-45]. Overall, the tet-off system requires a tetracycline

TABLE 1 | Comparison of conditional gene regulation approaches in apicomplexan parasites.

Approach	Method	Advantages	Limitations
Conditional gene deletion	DiCre	Complete and irreversible excision of target genes. Rapid gene deletion after rapamycin treatment. Can be used *in vivo*.	A DiCre strain is required. The original DiCre line does not appear to be stable, and the efficiency of gene excision varies. The stability issue has been solved in the DiCre-T2A system. Some genes are refractory to DiCre mediated disruption. Protein depletion may take longer than gene excision.
Transcriptional regulation	Tet-off	Reversible. Can be used *in vivo*.	Regulation efficiency varies, and complete suppression is difficult to achieve. Some genes are resistant to regulation. ATc is toxic to parasites at high levels. The *Toxoplasma* TATi line is avirulent and thus not ideal for assessing the role of genes in virulence determination. The ATc regulatable promoter may affect target gene expression even without ATc treatment. Long ATc treatment times are required to achieve depletion. ATc is toxic.
	Tet-on	Reversible. Can be used *in vivo*.	Leaky expression occurs in the absence of ATc.
Regulation of mRNA stability	Glms	Reversible. Easy to implement.	Glucosamine-6-phosphate is toxic. Knockdown efficiency varies.
Translational regulation	TetR-DOZI	Reversible. Highly efficient.	TetR-aptamer arrays are unstable. ATc is toxic.
Regulation of protein stability	FKBP-DD	Rapid protein degradation. Reversible.	Shield-1 is expensive and toxic to parasites if used for a long time. Target proteins must be accessible to the proteasome.
	DDD	Rapid protein degradation. Reversible. Inexpensive.	Trimethoprim has anti-malarial effects. Target proteins must be accessible to the proteasome.
	AID	Rapid and efficient protein degradation. Reversible. Inexpensive.	A TIR1 expressing strain is required. Target proteins must be accessible to the proteasome. Leaky degradation may occur after AID tagging. Use *in vivo* is possible but challenging.
Regulation of protein stability	Knock sideways	Rapid re-localization of target proteins.	A specialized mis-localizer strain is required. The method works only for proteins with known localization and is challenging for proteins with multiple/dynamic localization patterns. The efficiency of protein function disruption varies.

transactivator (tTA) consisting of a transcriptional activator fused to the TetR protein. *In Toxoplasma*, the transcriptional activator domain of the tTA, obtained from a genetic screen, has been used a short synthetic sequence. A transgenic line called TATi-1 expressing tTA has been constructed to enable tetracycline regulatable gene expression (Fig 1D). In practice, the endogenous promoter of the target gene is replaced by a minimal promoter containing several TetO sequences. Commonly used minimal promoters include S1O7 (SGA1 promoter with seven TetO sequences integrated) and S4O7 (SGA4 promoter with seven TetO sequences integrated) [46]. More recently, the use of the GRA2 minimal promoter has been reported to result in higher transcription of the target gene in the absence of tetracycline analogs such as anhydrotetracycline (ATc) and lower background expression when ATc is added, thus resulting in a 23-fold higher signal to noise ratio than that with the S1O7 promoter [47].

Although the tet-off system is convenient for controlling gene expression, it has several notable limitations (Table 1). First, the tetracycline analog ATc is commonly used to control gene expression. However, ATc is toxic to *Toxoplasma* parasites and should not be used for long time periods or above the concentration of 1 μM (0.5 μM is suggested for most applications). Second, leaky expression in the presence of ATc remains a concern. For some proteins, such as highly effective enzymes, residual expression may provide partial or full function. Therefore, incomplete suppression of expression causes problems in assessing the cellular functions of such proteins. Third, for unknown reasons, some genes are resistant to ATc regulation even if tetracycline regulatable promoters are placed upstream of their coding sequences. Moreover, predicting which genes are not regulatable by the tet-off system is difficult. Fourth, to achieve ATc regulated expression, the endogenous promoters of target genes must be replaced by ATc regulatable promoters, such as S1O7 or S4O7. However, on the basis of our laboratory experience, in some cases, replacing the endogenous promoter of target genes may cause severe growth defects in the parasites. If this occurs, one solution may be insertion of several TetO7 sequences in the endogenous promoter rather than replacing it. Fifth, during most applications using the tet-off system, an epitope tag is fused to the N terminus of the target protein to monitor the expression change after ATc treatment. The N terminal tagging is performed in one step along with the promoter replacement. The problem with this practice is that the N terminal tag may be removed during protein maturation for secretory proteins. Sixth, for unknown reasons, the virulence of the TATi-1

line is dramatically attenuated in mouse infection models, thus making the assessment of the roles of target genes *in vivo* or in virulence determination difficult. All these factors may affect the use of the tet-off system, and a full understanding is important for its successful application.

In contrast to the tet-off system, the tet-on system activates target gene expression in the presence of tetracycline analogs. In the tet-off system, tTA binds TetO containing promoters and activates gene expression in the absence of tetracycline analogs, whereas in the presence of tetracycline, tTA is released from the promoters, and expression of the target genes is turned off. Tet-on uses a reverse tetracycline transactivator, a mutant form of tTA that operates in an opposite manner from that of tTA [48]: it binds TetO and activates target gene expression only in the presence of tetracycline analogs. The tet-on system may be used to conditionally express target genes in parasites for diverse purposes, such as functional assessment and protein purification.

Protein depletion by auxin-induced degradation

Several approaches can be used to regulate the abundance of target proteins by controlling their stability. In *Toxoplasma*, the auxin-induced degradation system is most commonly used and relies on the plant derived auxin-inducible degron (AID) [49]. In plants, the auxin family hormones, such as indole-3-acetic acid (IAA), bind the F-box transport inhibitor response 1 (TIR1) protein and promote the degradation of proteins containing the auxin-inducible degron (a small protein motif) through ubiquitination [50]. Because TIR1 is a plant specific protein, application of the AID system in parasites requires the expression of TIR1. To construct the AID system in *Toxoplasma*, transgenic lines (RH/TIR1, ME49/TIR1, etc.) expressing *Oryza sativa* TIR1 (OsTIR1) have been constructed. To achieve auxin-induced protein degradation in these strains, the AID or the minimal degron sequence (miniAID) has been fused to the C-termini of target genes [51]. In the presence of auxins, such as IAA, the AID/miniAID tagged target proteins are recognized and ubiquitinated by the SCF-TIR1 E3 ubiquitin ligase, then degraded by the proteasome (Fig 1E) [51,52]. The AID system has been widely used in *Toxoplasma*, and depletion of target proteins can be achieved within minutes after auxin treatment.

Like other conditional gene control systems, the AID system has several drawbacks (Table 1). First, some proteins may not be efficiently degraded by the AID system even if they are AID/miniAID tagged, particularly those that localize to membrane bound organelles (such as mitochondria, apicoplasts, etc.) or are secreted outside parasites (such as to the parasite plasma membrane, parasitophorous vacuole (PV), PV membrane, host cells, etc.). This lack of degradation occurs because the proteasomes, which degrade the target proteins, are in the cytosol. Therefore, degrading proteins outside the cytosol is challenging. Nonetheless, proteins located in organelles, such as the ER and nucleus, may still be degraded by the AID system, although longer auxin treatment times may be requires. Second, incomplete depletion of target proteins may affect assessment of their biological functions. AID based degradation may be able to deplete proteins, such as highly active enzymes, to undetectable levels, but the residual amounts may still perform partial or even full function. Third, tagging of some proteins with AID/miniAID may affect their functions. In addition, for proteins with low abundance, leaky degradation (basal degradation without auxin) may prevent the tagging with AID/miniAID. To overcome the second and third problems, a second version (AID2) of AID involving the F74G mutation of OsTIR1 and the use of 5-phenyl-indole-3-acetic acid (5-Ph-IAA) has been reported. This version has no detectable leaky degradation in the absence of auxin but shows more rapid degradation than the first generation AID. Fourth, depleting target proteins during parasite infection of animal hosts is challenging with the AID system, because high levels of auxin are needed (particularly with the first generation AID), but animals typically dislike the flavor of auxins such as IAA.

In addition to AID mediated protein degradation, other degrons, such as the destabilizing-domain of the FK506 binding protein (FKBP-DD) have been developed in *Toxoplasma* [53]. FKBP-DD slightly differs from the AID system, in that it uses the small molecule Shield-1 (Shld-1) to stabilize the target protein when needed (Fig 1F) [54]. Degradation is induced by the removal of Shld-1. Therefore, the apparent drawback of the FKBP-DD system is that Shld-1, which is expensive, is continually needed to maintain the expression of target genes during daily passage of the corresponding transgenic lines (Table 1).

GENE EDITING IN *PLASMODIUM* PARASITES THAT CAUSE MALARIA

Plasmodium parasites are the most extensively studied apicomplexan parasites, not only because of the severe diseases that they cause but also because of the availability of tools developed over many years. *Plasmodium* parasites have strong host specificity, and *Plasmodium falciparum*, which infects humans and causes severe human malaria, is the focus of most *Plasmodium* research. Species that infect rodents, such as *P. berghei* and *P. yoelii*, are also commonly used. As in other parasites, genetic studies of *Plasmodium* parasites started with the establishment of methods for culture *in vitro*, introduction of exogenous DNA and selection of transgenic strains [55-57]. Blood stage *P. falciparum* can be cultured with red blood cells *in vitro*, whereas continuous culture of many other species remains challenging. Electroporation is currently the main strategy to introduce foreign DNA into *Plasmodium* parasites [57,58]. However, the efficiency is relatively low, mainly because the transfection is performed on infected erythrocytes, and the DNA molecules must travel through

the erythrocyte membrane, PV membrane and parasite membrane to enter the parasites. Several selection markers have been developed to aid in the selection of transgenic parasites [59–63]. For example, in *P. falciparum*, blasticidin S deaminase, puromycin-N-acetyl-transferase, neomycin phosphotransferase, yeast dihydroorotate dehydrogenase and human DHFR, which confer resistance to blasticidin, puromycin, G418, atovaquone or DSM1, pyrimidine or WR99210, respectively, are available. In addition, diverse techniques allowing for direct inactivation or conditional regulation of target genes have also been developed, thus markedly increasing the efficiency of gene editing in *Plasmodium*.

Genome editing with the CRISPR/Cas9 system

Unlike *Toxoplasma*, *Plasmodium* spp. lack NHEJ activity, and homologous recombination is the predominant mode of integration of foreign DNA into their genomes [63,64]. Therefore, conventional homology-based recombination had long been used for genetic manipulation in *Plasmodium*. Nonetheless, those approaches were fairly inefficient and time-consuming, because integration of foreign DNA occurs at a very low frequency without additional strategies to enhance integration. Around 2012, zinc finger nuclease technology, which generates target specific DSBs, was adapted to *P. falciparum* to improve the efficiency of homologous recombination [65,66]. This strategy was successful but had many limitations. Designing a target specific zinc finger nuclease is highly time consuming and extremely costly. Therefore, this method is not widely used. Around the same time, the CRISPR/Cas9 genome editing system was also adapted to *Plasmodium* parasites [67–70]. Because of its ease of use, CRISPR/Cas9 technology has become the most commonly used method for targeted gene editing.

The rationale for CRISPR/Cas9 assisted gene editing in *Plasmodium* is very similar to that in any other organism. This system requires a target specific CRISPR plasmid that generates a DSB and a homologous fragment to repair the DSB (Fig 2A). Several groups have designed methods to provide these elements in the parasites, by using two plasmids; one plasmid plus a linear donor construct; or one single plasmid. In addition, a centromere plasmid that can be maintained in *Plasmodium* parasites has been engineered to express Cas9 and construct a transgenic line that stably produces the nuclease for gene targeting [71]. With this line, only one plasmid providing sgRNA and a homologous template is needed, thus increasing the efficiency of gene targeting. In contrast, the Cas9-sgRNA ribonucleoprotein complex and a DSB repair template have also been transfected into *P. falciparum* to perform gene editing [72] without the use of any plasmid. Although CRISPR/Cas9 technology is often used in *Plasmodium* parasites, it is under constant optimization for various purposes. Some species, such as *P. yoelii*, lack sufficient drug markers, and the one plasmid system is preferred [73]. In other cases, multiple sgRNAs may be needed, and a ribozyme-guide-ribozyme approach has been designed for this purpose [74]. The CRISPR/Cas9 system has also been modified to control the expression of target genes. When nuclease-dead mutants of Cas9 (H840A and/or D10A) are fused with transcriptional regulators or epigenetic factors, they are delivered to target sites according to the sgRNA, and consequently activate or suppress the expression of target genes [75]. In *Toxoplasma*, CRISPR/Cas9 mediated gene targeting techniques are frequently used in combination with other gene regulation tools to achieve complex gene control. For instance, CRISPR/Cas9 may be used to flox target genes for DiCre mediated conditional gene excision [76–79] or to deliver destabilization domains to target genes for inducible protein degradation [80,81].

Control of the mRNA level of target genes in *Plasmodium* parasites

Plasmodium lacks RNA interference activity. To control mRNA levels of target genes, several approaches that control the transcription level or the stability of mRNA have been developed. Tet-off systems that suppress target gene transcription in a tetracycline (or its analogs) dependent manner are available. Two tet-off systems for *Plasmodium* parasites that differ in transactivators have been designed. In 2005, Meissner *et al.* found that artificial tTAs derived from genetic screens in *Toxoplasma* also regulate gene expression in *P. falciparum* in an anhydrotetracycline dependent manner [82]. Subsequently, a tet-off system based on the TATi-2 transactivator was developed and found to robustly shut down target gene expression in the presence of anhydrotetracycline. Because the TATi-2 based tet-off system was not able to regulate certain genes in *P. falciparum* and *P. berghei*, Pino *et al.* fused several transcriptional activation domains within apicomplexan AP2 factors with TetR and screened for tetracycline-dependent gene regulation activity [83]. Several TetR and activation domain fusions showed tTA activity and were able to knock down essential genes in *P. falciparum* and *P. berghei* (Fig 2B) [84]. Both tet-off systems have been used by the malaria research community, but difficulties continue to exist, such as those in the TATi based tet-off system in *Toxoplasma*.

Because the transcriptional control in tet-off systems does not always function as expected, other approaches have been designed to control the mRNA levels of target genes. Among them, controlling RNA stability through ribozymes has proven useful. Ribozymes, self-cleaving RNA molecules that are useful for conditional gene regulation, often show altered activity in response to certain ligands. The glmS ribozyme derived from Gram-positive bacteria has gained popularity for this purpose, because its activity is tightly regulated by glucosamine-6-phosphate. To regulate endogenous genes, the glmS ribozyme sequence is inserted into the target gene after the stop codon, so that it is transcribed along with the target gene [85]. When glucosamine-6-phosphate is added to glmS tagged strains, glmS ribozyme activity is activated,

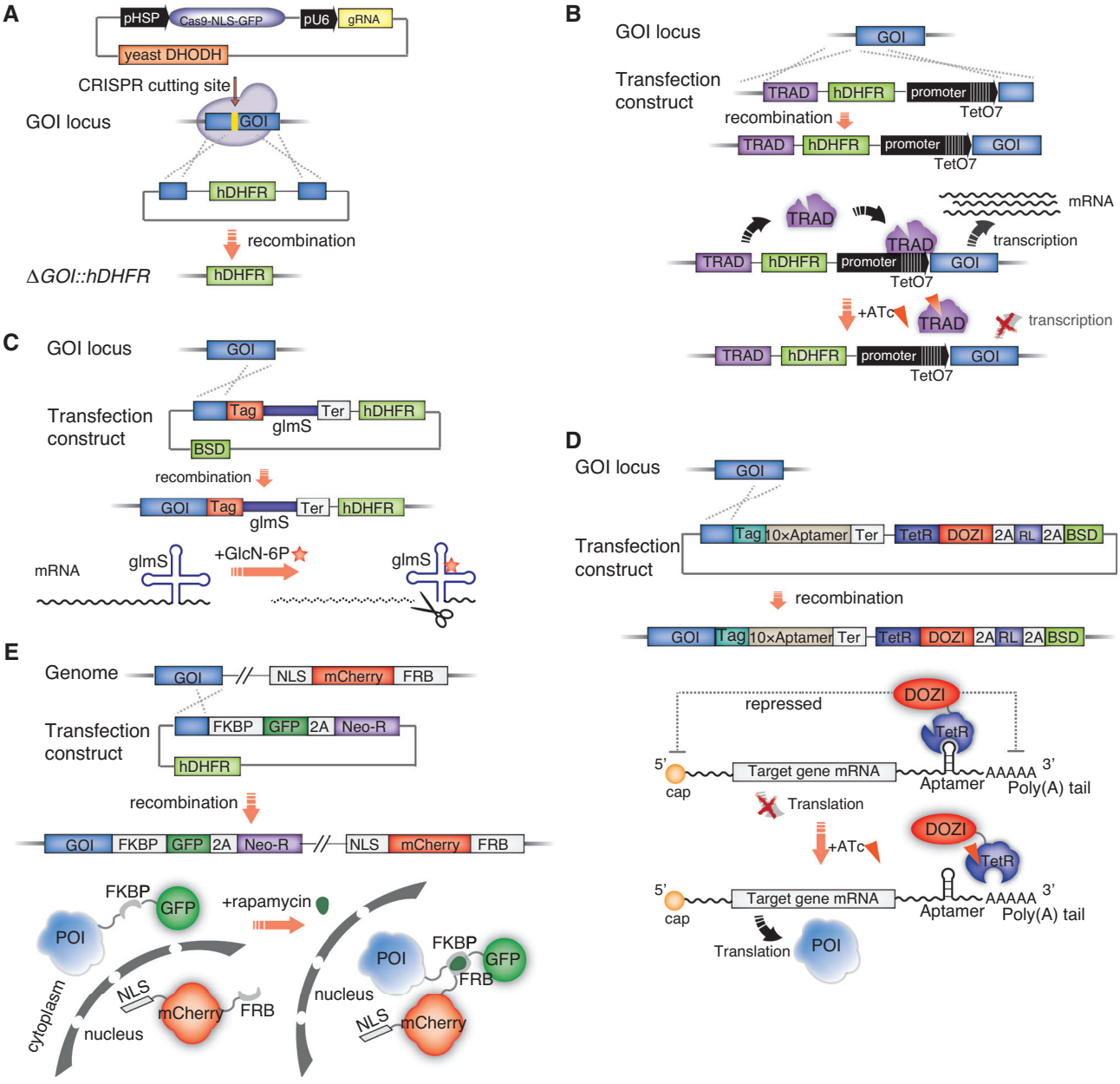

FIGURE 2 | Genetic manipulation tools in *Plasmodium* parasites. (A) Gene editing with CRISPR/Cas9 with a two-plasmid system, one providing Cas9 and sgRNA, and the other providing homology templates for target gene modification. (B) Tunable gene transcription by the TetR and activation domain fusion system. This system is very similar to the TATi system in *Toxoplasma* illustrated in Fig 1D, except that a different transactivator is used. (C) Regulation of the stability of the mRNA of target genes by glmS. The glmS ribozyme sequence is inserted into the 3′ UTR of the target gene. The target mRNA is stable in the absence of glucosamine-6-phosphate (GlcN-6P) and becomes unstable and degraded when GlcN-6P is added. (D) Control of protein translation by the TetR-DOZI system. TetR binding aptamers are inserted into the 3′ UTR of the target gene. Meanwhile, the TetR-DOZI fusion protein, which suppresses the translation of aptamer tagged genes without tetracycline (or its analogs), is expressed in the parasites. When tetracycline analog is added, TetR-DOZI is released from the aptamers, and translation is enabled. TetR-DOZI is expressed in frame with *Renilla* luciferase (RL) and the selection marker blasticidin S deaminase (BSD) to facilitate selection of transgenic parasites. (E) Inactivation of gene function by knock sideways. In this example, a cytoplasmic protein is transported to the nucleus to disrupt the normal protein function. The POI is fused with FKBP and GFP, and FRB, fused with an mCherry sequence containing a nuclear localization sequence, is also expressed in the same parasites. When rapamycin is added, FKBP binds FRB, the POI is relocated to the nucleus, and its function in the cytosol is impaired.

and degradation of the fusion mRNA is induced, thus decreasing the expression of target genes (Fig 2C). One advantage of the glmS based gene depletion system is that it is fairly easy to implement, because no additional elements are required other than the glmS ribozyme itself. However, although the glmS system has been successfully used to control diverse genes in *Plasmodium* parasites [86,87], it has several limitations (Table 1). First, glucosamine-6-phosphate is toxic to parasites when used at high concentrations. In addition, the degree of knockdown varies. In some extreme examples, knockdown of essential genes, such as ClpP in *P. falciparum*, do not cause

any growth defects, thereby indicating incomplete depletion of the target genes [88].

Regulation of target gene activity at the levels of protein translation, stability and localization

RNA aptamers that bind specific proteins in a ligand-controlled manner are commonly inserted into the untranslated regions (UTRs) of target genes to regulate protein translation. Successful gene regulation has been achieved by using the TetR-aptamers in *Plasmodium* parasites. Originally, the TetR-binding RNA aptamers were placed in the 5′-UTRs of target genes to achieve translation regulatable by tetracycline (or its analogs) [89]. In the absence of tetracycline, TetR binds aptamers and prevents the translation machinery from translating the target mRNA, thus decreasing the protein levels. In the presence of tetracycline, TetR is released from the aptamers, and translation occurs, thereby allowing normal gene expression. This system was later optimized through fusion of TetR to translational repressors, such as DOZI, which sequesters target mRNA in P-bodies and inhibits translation (Fig 2D) [90]. The TetR-DOZI fusion enables substantially better translational regulation than TetR alone, and diverse genes have been knocked down with this approach in *P. falciparum* [91-93]. Although it has gained some popularity, the TetR-DOZI system has several drawbacks. The main issue is that multiple TetR-aptamer units (each with a TetR-aptamer and a spacer) are inserted into the 3′-UTRs of target genes for regulation. In the original design, the sequence of each unit is identical. Therefore, the sequences are prone to recombination, and subsequently sequence loss and diminished efficiency of tetracycline mediated gene regulation. To solve this problem, Rajaram *et al.* have redesigned the TetR-aptamer arrays by making each spacer sequence shorter and unique [94]. The redesigned TetR-aptamer system has similar regulation efficiency but enhanced stability.

Beyond protein translation, the stability of target proteins may be conditionally regulated to control their abundance. In all these systems, a ligand controlled protein destabilization domain or degron is fused to target proteins, and the degradation of the fusion proteins is regulated by addition or removal of the corresponding ligand. Several degrons are commonly used for this purpose in *Plasmodium* parasites. The destabilization domain of the FK506-binding protein FKBP12 (FKBP-DD), which induces target protein degradation in the absence of the rapamycin derived ligand Shld-1, was among the first used [54,95]. Because of its rapid reversibility, the FKBP-DD system has been widely used to reveal protein functions in *Plasmodium* [96-101]. Nonetheless, this system has several major drawbacks (Table 1). First, it is expensive. To maintain the normal expression and function of target proteins, Shld-1 must be continually present to stabilize the target proteins but is extremely expensive. Second, Shld-1 is toxic to parasites if used for a long time. Therefore, this system may be better suited to temporal expression of exogenous genes, particularly dominant negative mutants or toxic genes in parasites, rather than the control of essential genes for functional analyses [102-104]. Third, as with other degrons, the degradation of target proteins occurs in the cytosol by the proteasome. Therefore, the degradation efficiency of non-cytosolic proteins may be low [105]. To solve some of the problems of the FKBP-DD system, other degrons have been developed. For example, the destabilization domain of *E. coli* DHFR (the DDD system) has been adapted to *P. falciparum* to decrease the cost of ligand induced degradation [106-108]. To stabilize proteins, the DDD system uses trimethoprim, a commonly used antibiotic that is much less expensive than Shld-1. Trimethoprim is anti-malarial drug that kills *Plasmodium* parasites. Therefore, use of the DDD system must be accompanied by the DHFR gene, which confers resistance to trimethoprim. To solve the problem of long-term use of Shld-1 for the stabilization of target proteins, a ligand-induced degradation technique has been designed to ensure that the target protein is stable without the ligand, and the degradation is induced by the ligand [109,110]. The ligand-induced degradation method uses the FKBP protein fused to a 19-amino-acid degron derived from a genetic screen. In the absence of the ligand Shld-1, the degron binds FKBP (thru intra-molecular binding) and is protected from degradation; consequently the fused target protein is stable. After addition of Shld-1, Shld-1 competes with the degron for binding FKBP and displaces the degron from FKBP; therefore, the degron is exposed to the proteasome, and degradation is induced. Consequently, Shld-1 is needed only when depletion of the target protein is desired. In addition to the above degrons, the AID system widely used in *Toxoplasma* is available in several *Plasmodium* species, including *P. falciparum*, *P. berghei* and *P. yoelii* [50,111-113].

Another method to disrupt the function of target proteins is to relocate them away from the normal sites where they exert biological functions, in a strategy called knock sideways (KS) [114]. In a typical design, the KS system consists of two parts. The parasite protein to be regulated is fused to the FKBP protein. In addition, the FRB protein with a mis-localization signal (a targeting signal that delivers the protein to a compartment different from the normal localization sites of the target protein) is expressed separately in the same parasite. The addition of rapamycin induces the interaction of FKBP and FRB, thus driving the protein complex to the mis-localization compartment according to the targeting signal on FRB (Fig 2E). Therefore, the target protein is relocated to a new site, and its function at the original site is disrupted. With this system, the nuclear-localized HP1 protein has been successfully relocalized to the plasma-membrane, and KS of HP1 in *P. falciparum* has been found to significantly decrease parasite growth. Since its initial development, several proteins have been relocated with the KS system. Nonetheless, this system also has several limitations. First, the exact cellular localization of target proteins must be known before the KS system is used. However, some proteins have dynamic

localization or localize to multiple sites. Second, altering the cellular localization may not always completely disrupt the function of target proteins. Third, the efficiency of re-localization is not always ensured.

GENETIC ENGINEERING IN OTHER APICOMPLEXAN PARASITES

In contrast to the availability of diverse tools in *Toxoplasma* and *Plasmodium*, gene editing techniques in other apicomplexan parasites, such as *Cryptosporidium*, *Eimeria* and *Babesia*, are less developed. The experiences gained from *Toxoplasma* and *Plasmodium* have been extremely useful in informing the design of similar tools in these other parasite species. Therefore, rapid progress has been made in the genetic manipulation of other apicomplexan parasites. One common problem in genetic studies in these parasites is the lack of continuous culture systems *in vitro*. The requirement of host animals to propagate parasites makes the collection and selection of transgenic parasites difficult. In addition, the low efficiency of transfection in many of these parasites is another hurdle. Nonetheless, several of these problems are gradually being addressed, and new solutions and techniques are emerging.

Gene editing in *Cryptosporidium*

Multiple *Cryptosporidium* species infect animals and humans, among which *C. parvum* has been the most extensively studied. Intestinal organoids can support *Cryptosporidium* propagation *in vitro* [115–118]. Later, an improved technique involving an air-liquid interface cultivation system was developed, which enables not only efficient growth of *C. parvum in vitro* but also complete parasite life cycle development [115]. Progress in *in vitro* culture techniques will greatly facilitate pathobiology studies of these parasites. Similarly to *Plasmodium* spp., *Cryptosporidium* spp. lacks NHEJ activity, and homology-based repair is predominantly used for DNA break repair, thus yielding important insights into the design of gene editing techniques. The CRISPR/Cas9 gene editing system has been adapted to *Cryptosporidium*, although the efficiency is low, mainly because of the extremely low efficiency of transfecting foreign DNA into the parasites [119,120]. Most experiments have used IFN-γ knockout mice for parasite propagation and selection, in a technically complicated process. In addition, a DDD based conditional protein depletion system has been successfully established in *C. parvum* to regulate target protein levels [121]. With the essential gene *CDPK1* as an example, this system has been demonstrated to be useful in studying the functions of essential genes.

Genetic manipulation in *Babesia*

Babesia parasites are closely related to *Plasmodium* species. Therefore, the genetic tools in *Plasmodium* are highly informative for the design of similar systems in *Babesia*. *In vitro* culture of several *Babesia* species, such as *B. bovis*, have been established or reported [122–126]. Yet these methods show high variation, and the use of different batches of sera or red blood cells may significantly affect the propagation efficiency of the parasites [127]. Nonetheless, using animal infection models or *in vitro* cultures, scientists have established transfection methods and drug resistance markers for diverse *Babesia* species, thus paving the way to molecular and genetic studies in these organisms [128–130]. *Babesia* spp. also lack NHEJ activity, as do *Plasmodium* parasites. Therefore, homologous recombination is the preferred method of integrating exogenous DNA elements. However, the efficiency in wildtype parasites, as in *Plasmodium* parasites, is low. This problem has been partially solved by adapting the CRISPR/Cas9 technology to *Babesia*. To date, the CRISPR/Cas9 system has been developed for *B. bovis*, and systems for more *Babesia* species are under development [131]. In *B. bovis*, this system has been successfully used for gene deletion and tagging [131,132]. To study the functions of genes that are critical for parasite growth, conditional gene control techniques are needed. In *Babesia*, the glmS ribozyme has been successfully used to decrease the protein level of VEAP by 82–92% after supplementation with glucosamine [85,132]. Knockdown of VEAP through this approach decreases parasite growth, thus suggesting the important role of this gene. In contrast, tagging VEAP with glmS causes an 80% decrease in VEAP in the absence of glucosamine, thus implying strong leakiness of the glmS system in this case. Although other conditional gene regulation systems are not yet available in *Babesia*, they are highly likely to be available soon, given their success in *Plasmodium* parasites and the similarity between *Babesia* and *Plasmodium*. Therefore, the DiCre, DDD, DD, AID and KS systems are worthy of testing in diverse *Babesia* parasites to assess their regulation efficiency.

Development of genetic tools in *Eimeria* parasites

Eimeria parasites include many species that are highly host specific. Those that cause coccidiosis in chicks have been the most extensively studied. No *Eimeria* parasites can currently be cultured *in vitro* over the long term, thus posing a major difficulty for molecular genetic studies in *Eimeria*. Therefore, most *Eimeria* studies have used chicks to collect *Eimeria* oocysts. With the available methods of infection and parasite collection, transient and stable transfection techniques have been established [133,134]. Transgenic lines expressing fluorescent reporters or drug selection markers have also been constructed. However, overall, genetic modifications using these old techniques have low efficiency and are time consuming. Advances in other apicomplexan parasites have led to similar studies in *Eimeria*. In 2020, two studies from the Suo group have reported the use of CRISPR-Cas9 technology for gene tagging and deletion analyses in *E. tenella* [135,136]. The first study constructed a stable line expressing Cas9, which was subsequently used for indel and homologous recombination mediated gene disruption and endogenous gene tagging

experiments. Gene targeting efficiency by CRISPR-Cas9 was high (over 29%) but the transfection efficiency appears to be low. Nonetheless, transgenic lines could be easily obtained with strong selection approaches. In the second study, the Cas9 was transiently expressed from a plasmid. The overall frequency of genetic modification using this system was not as high as that using stable Cas9 expressing lines, although it was still acceptable. Moreover, a recent study has demonstrated the transfection of *Francisella novicida* Cas12a protein along with the gene targeting crRNA into *E. tenella* for gene tagging [137]. The advantage of this approach is that it avoids introduction of CRISPR-Cas related DNA into the parasites, an aspect beneficial for downstream applications.

FUTURE PERSPECTIVES

Although *Toxoplasma* and *Plasmodium* researchers have a wide variety of genetic tools to control target genes at the DNA, RNA or protein levels and dissect their functions, each method has advantages and disadvantages, as well as specific conditions for use. A full understanding of the benefits and drawbacks of different strategies is crucial for choosing the proper method. In addition, existing tools do not always meet all needs of genetic studies, and new tools are always anticipated. For example, plasmids or artificial chromosomes that allow for rapid bacterial and yeast transformation do not yet exist for apicomplexan parasites. Circular plasmids can be maintained episomally for some time in *Plasmodium* and *Babesia* parasites. Most apicomplexan parasites have low transfection efficiencies with electroporation approaches. In mammalian cells and bacteria, viruses, such as lentivirus and lysogenic phages are widely used as vectors to introduce foreign DNA with high efficiency. Such tools are also not available in apicomplexan parasites, and future investigation is warranted. Beyond the methods for introducing foreign DNA elements into parasites, tools such as optogenetic techniques that enable rapid changes in signaling molecules are not yet widely used, although pioneering work in *Toxoplasma* using a photoactivated adenylate cyclase to control cAMP levels has demonstrated this method's utility [138]. With advances in genetic engineering in the model organisms *Toxoplasma* and *Plasmodium*, more tools are expected to be developed for other apicomplexan parasites in the near future. The availability and improvement of such tools will accelerate functional studies of parasitic genes and help identify novel targets for the design of new interventions.

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CONFLICTS OF INTEREST

No conflicts of interest are declared.

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Bang Shen is a professor at the college of veterinary medicine in Huazhong Agricultural University. He received a PhD degree in microbiology from the University of Kansas Medical Center. He is an expert in the field of microbial genetics and is the first one to develop the CRISPR/Cas9 gene editing tool for *Toxoplasma*. His current research focuses on the molecular mechanisms that govern *Toxoplasma* parasitism and life cycle development, as well as the mechanisms and regulations of carbon metabolism. He hopes to use the knowledge acquired from these studies to design novel vaccines for animal toxoplasmosis.