NEW TECHNOLOGIES: METHODS AND APPLICATIONS

Advances in molecular genetic systems in malaria

Tania F. de Koning-Ward[1], Paul R. Gilson[2,3] and Brendan S. Crabb[2,3,4]

Abstract | Robust tools for analysing gene function in *Plasmodium* parasites, which are the causative agents of malaria, are being developed at an accelerating rate. Two decades after genetic technologies for use in *Plasmodium* spp. were first described, a range of genetic tools are now available. These include conditional systems that can regulate gene expression at the genome, transcriptional or protein level, as well as more sophisticated tools for gene editing that use piggyBac transposases, integrases, zinc-finger nucleases or the CRISPR–Cas9 system. In this Review, we discuss the molecular genetic systems that are currently available for use in *Plasmodium falciparum* and *Plasmodium berghei*, and evaluate the advantages and limitations of these tools. We examine the insights that have been gained into the function of genes that are important during the blood stages of the parasites, which may help to guide the development and improvement of drug therapies and vaccines.

Artemisinin
A rapid-acting antimalarial drug isolated from the plant *Artemisia annua*. Artemisinin-based combination therapies (ACTs) are recommended by the World Health Organisation as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria.

Immunogens
Antigens that are capable of inducing an immune response.

[1]*Deakin University, Waurn Ponds, Victoria 3216, Australia.*
[2]*Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria 3004, Australia.*
[3]*Monash University, Clayton, Victoria 3800, Australia.*
[4]*The University of Melbourne, Parkville, Victoria 3010, Australia.*
e-mails: taniad@deakin.edu.au; gilson@burnet.edu.au; crabb@burnet.edu.au
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Malaria, which is caused by infection with *Plasmodium* parasites, remains one of the leading infectious causes of morbidity and mortality in humans. 40% of the world's population (3.4 billion people) are potentially exposed to infection, and 207 million clinical cases and an estimated 627,000 deaths were reported in 2012 (REF. 1). Although most such deaths are caused by *Plasmodium falciparum*, *Plasmodium vivax* also causes highly disabling disease. Many drugs are available to clear *Plasmodium* spp. infections; however, resistance has emerged to most of them. Worryingly, resistance is emerging to the last remaining front-line antimalarial drugs, the artemisinin compounds[2]. There is no antimalarial vaccine currently in clinical use, although clinical field trials of the pre-erythrocytic vaccine RTS,S show that it has partial efficacy[3]. Thus, there is an urgent need to develop new antimalarial compounds and markedly improved vaccine candidates.

An improved understanding of the biology of the malaria parasite at each stage of its life cycle (BOX 1) facilitates the identification and characterization of new targets and strategies for intervention and will increase our understanding of the molecular basis of drug resistance. The ability to genetically manipulate the malaria parasite (by knocking out or mutating genes, or introducing transgenes) to assess gene function is central to these advances. For example, such genetic techniques have provided insights into the pathways that *Plasmodium* parasites use to invade erythrocytes[4–8], which led to the identification of new vaccine approaches to block parasite invasion[8–13]. Allelic exchange has also provided a method for validating the molecular basis of resistance to particular antimalaria drugs[14–20]. Similarly, knowledge of the sexual and pre-erythrocytic stages of parasite development has been greatly advanced through reverse genetics[21,22], which led to the identification of suitable candidates for transmission-blocking vaccines[23–25] and the development of genetically attenuated parasites as potential immunogens[26–34].

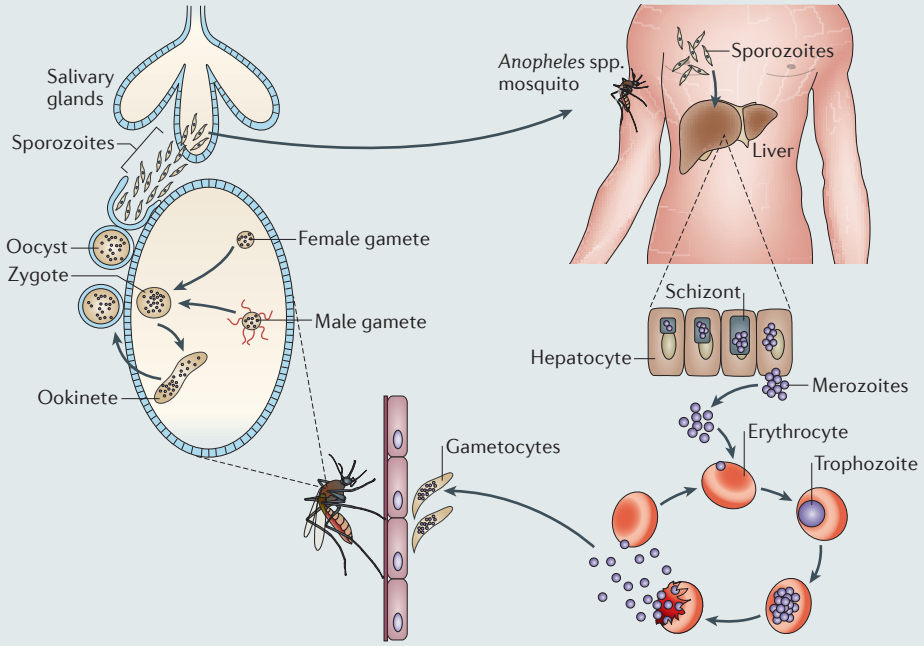
Until now, *P. falciparum* and the rodent malaria species *Plasmodium berghei* and *Plasmodium yoelii* have been the predominant species to be transfected by electroporation, and as their genomes are haploid, genetic manipulation involves the targeting of a single gene. Only the blood stages of *P. falciparum* can be cultured *in vitro* and as synchronous ring forms can be readily purified these blood stages are routinely used for transfection[35,36]. By contrast, selection of genetically manipulated *P. berghei* and *P. yoelii* must be done *in vivo*, because these species cannot be continuously propagated in cell culture. Blood that is infected with ring-form parasites is harvested from rodents and cultured overnight *in vitro* to yield mature schizonts that contain daughter merozoites, which are used instead of ring forms for transfection[37].

There has been much progress in the development of genetic technologies for *Plasmodium* spp. (FIG. 1). These include transient and stable transfection systems for expressing reporter genes, and the ability to knock out, knock down or replace genes through either conventional recombination or more directed genome-editing

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Box 1 | **The life cycle of *Plasmodium* spp.**

Malaria parasites are transmitted to a vertebrate host when *Plasmodium*-infected female *Anopheles* spp. mosquitoes take a blood meal (see the figure). In the pre-erythrocytic stages of infection, sporozoites that are injected from the salivary gland of the mosquito infect host hepatocytes and mature into schizonts that contain thousands of merozoites. When the schizonts rupture from hepatocytes, the merozoites are released into the bloodstream. The blood stages of the malaria life cycle commence when merozoites invade erythrocytes and ultimately become encased within a parasitophorous vacuole. Over the next 48 hours, *Plasmodium falciparum* parasites mature within this vacuole from ring-form trophozoites into schizonts (for *Plasmodium berghei*, this occurs over a 24 hour period), which contain 12–16 daughter merozoites as a result of asexual multiplication. Rupturing of the erythrocyte and vacuolar membrane leads to release of merozoites, enabling a new cycle of erythrocyte invasion and multiplication to begin. If the host is not treated, multiplication of parasites in the blood leads to symptomatic disease. Within erythrocytes, some parasites differentiate into male or female gametocytes, which are the sexual forms of the parasite. When a mosquito ingests the sexual stages during a blood meal, fertilization between the male and female gametocytes gives rise to a zygote. The ookinete (a motile zygote) invades through the midgut wall of the mosquito and develops into oocysts. Once the oocysts have matured, they rupture and release sporozoites, which migrate to the mosquito salivary glands, where they can infect a new host and perpetuate the malaria life cycle. With the exception of the zygote form, *Plasmodium* spp. are haploid throughout their entire life cycles. So far, only the asexual blood stages have been used for transfection, as they can be readily cultured *in vitro* or harvested from the blood of infected animals. Figure adapted from REF. 124, Nature Publishing Group.



approaches (such as zinc-finger nucleases (ZFNs) and the CRISPR–Cas9 system). However, three key issues still need to be resolved. First, it remains difficult to study genes that are crucial to parasite survival under standard blood-stage culturing conditions or during *in vivo* blood-stage growth, as disruption of such genes is lethal. Second, transfection efficiencies need to be increased to a level that will permit saturation genetic screens to identify genes that are responsible for a particular phenotype. Third, greater emphasis needs to be placed on the development of appropriate functional assays to define the phenotypes of transgenic parasites and to determine more precisely the molecular pathways in which the gene under investigation has a role. In this Review, we outline the current status of the *P. falciparum* and *P. berghei* genetic systems, the recent progress that has been made in conditional mutagenesis systems that permit the functional dissection of genes that are important to blood-stage growth, and the advances that have increased the efficiency of genome editing in *Plasmodium* spp.. In addition, we highlight recent developments that enable researchers to gain insights into the biology of *Plasmodium* spp. that are more closely related to *P. vivax*.

Plasmodium spp. molecular genetic systems

Transient transfection of the bird malaria species *Plasmodium gallinaceum* was first reported more than two decades ago[38]. Shortly after, transient and stable transfection systems were developed for *P. falciparum*[39–41] and *P. berghei*[37,42]. A history of the development of genetic tools[43] (FIG. 1) and a comparison of the transfection systems (TABLE 1) that are available for *P. falciparum*[39–41] and *P. berghei* are outlined. It should be noted that the number of *Plasmodium* species that can be genetically manipulated has expanded to include rodent malaria species *P. yoelii* and *Plasmodium chabaudi*[44–46] and the simian parasites *Plasmodium cynomolgi* and *Plasmodium knowlesi*[47–50]. Importantly, transient and stable transfection of *P. vivax* has also been described[51,52];

Transfection
The process of introducing nucleic acids into cells.

Schizonts
Mature forms of malaria parasites that are present in the liver and blood, which in blood contain ~12–16 individual merozoites.

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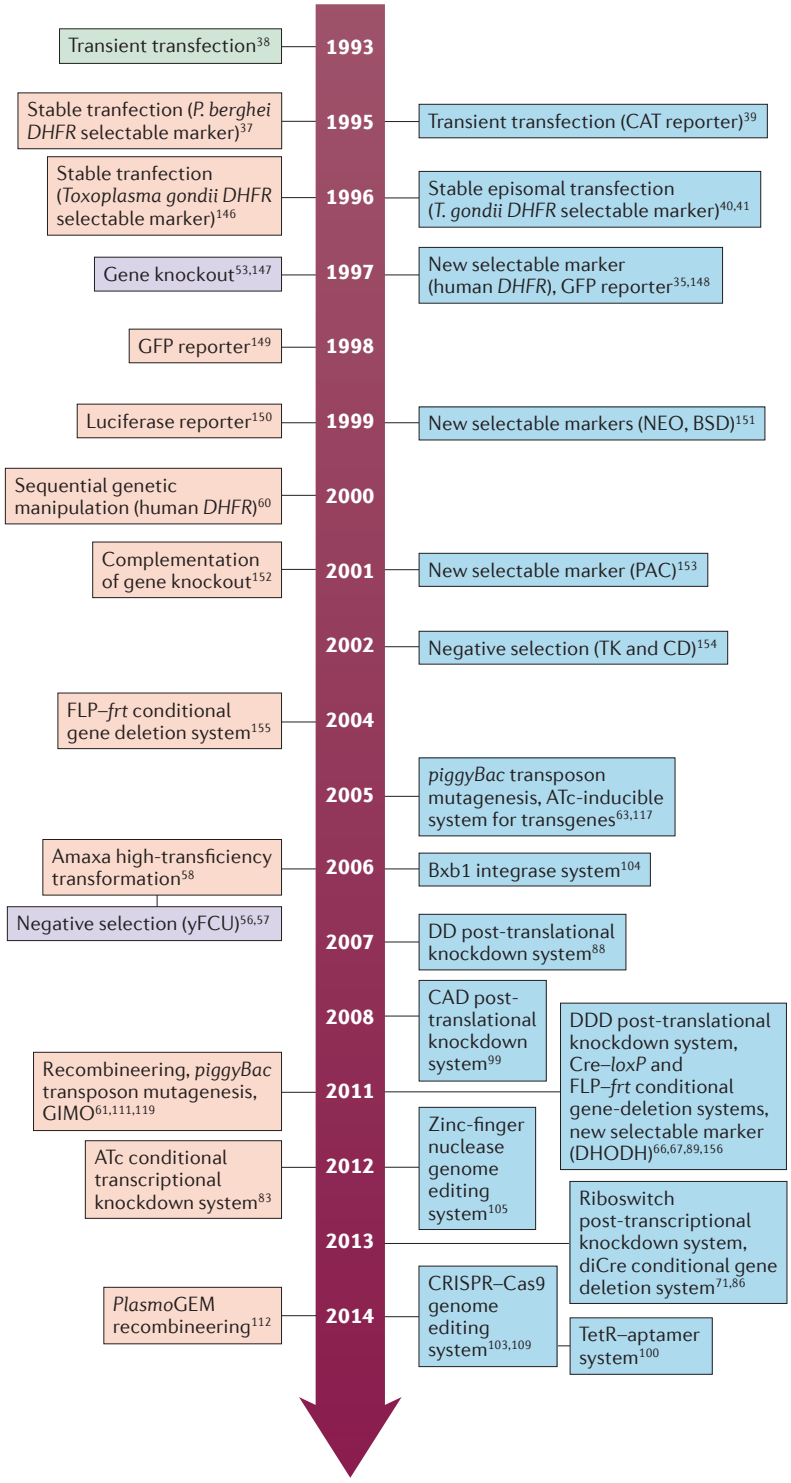


Figure 1 | **Development of transfection technologies for *Plasmodium* parasites.** Technologies that have been developed for *Plasmodium falciparum* (shown in blue boxes) and *Plasmodium berghei* (pink boxes) since transfection of a luciferase reporter was first reported in *Plasmodium gallinaceum* (green box) are shown. Technologies that were developed for both *P. falciparum* and *P. berghei* at similar times are shown in purple boxes. ATc, anhydrotetracycline; BSD, blasticidin; CAD, conditional aggregation domain; CAT, chloramphenicol acetyltransferase; CD, cytosine deaminase; DD, destabilization domain; DDD, *DHFR* degradation domain; DHFR, dihydrofolate reductase; DHODH, dihydroorotate dehydrogenase; GIMO, gene insertion/marker out; NEO, neomycin; PAC, puromycin; TetR, tetracycline repressor; TK, thymidine kinase; yFCU, yeast cytosine deaminase–uracil phosphoribosyl transferase fusion protein.

thus, when a robust *in vitro* culture system is available for this species, it will be possible to study its biology using genetic technologies.

Malaria parasites can be transfected by electroporation of plasmid DNA, which initially replicates episomally, forming large concatamers[42,53,54], and enables transgenes to be readily expressed. By contrast, to introduce, replace, mutate or knock out a particular gene, a homologous targeting sequence or sequences must be incorporated into the introduced DNA to drive integration into the genome. This is a highly inefficient process in *P. falciparum*, and parasites are generally cycled on and off positive drug-selection pressure to select for stably integrated constructs by single-crossover recombination and to remove unwanted episomes[53,55]. Alternatively, episomes can be removed by negative selection, which selects for double-crossover recombination events[56,57]. For *P. berghei*, linear DNA is used to target the genome by either single- or double-crossover recombination. This results in an integration efficiency of up to approximately four orders of magnitude higher than in *P. falciparum*[58]. The tractability of *P. berghei* is one reason that this species is so widely used to dissect gene function. Additionally, *P. berghei* can be readily maintained in the laboratory throughout the life cycle, including the mosquito and liver stages. In addition, several well-established *in vivo* and *in vitro* assays for phenotyping *P. berghei* at these stages are available; however, such assays are lacking or less efficient for *P. falciparum*. As a result, *P. berghei* gene function and phenotype can be more easily analysed at particular life cycle stages and, importantly, the *in vivo* models of infection enable the interplay between the host and parasite to be examined.

Using these approaches, meaningful progress has been made in the past 20 years in developing genetic tools to study *Plasmodium* spp., which is reflected by the steady increase in the number of malaria-related publications that report the use of these genetic tools as a core technique. However, until recently, these advances have been mostly incremental. Nevertheless, of the ~5,200 genes found in *Plasmodium* spp., we estimate (on the basis of the Rodent Malaria genetically modified Parasites Database[59] and more than 440 peer-reviewed publications that have used *Plasmodium* spp. transfection methods) that ~500 *Plasmodium* genes (predominantly those of *P. falciparum* or *P. berghei*) have been successfully targeted for gene disruption.

Several factors have limited progress in genetic manipulation. These include the paucity of robust positive selectable markers (TABLE 1), which restricts the ability to perform consecutive genetic manipulations on the same parasite. Although six selectable markers are now available for *P. falciparum*, human dihydrofolate reductase (*DHFR*) is predominantly used because of its robustness. For *P. berghei*, only three selectable markers are available: human *DHFR* and the gene encoding the DHFR–thymidylate synthase (*dhfr–ts*) from *Toxoplasma gondii* and *P. berghei*. All three of these markers confer resistance to pyrimethamine, but human *DHFR* also confers resistance to the drug WR99210 (REF. 60). Thus, for sequential genetic manipulation in *P. berghei*, either

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Table 1 | **Comparison of the transfection systems available for *Plasmodium* spp.**

	Plasmodium falciparum	*Plasmodium berghei*
Stage transfected	Ring-form trophozoites (transfection of merozoites is also possible)	Merozoites
Transient transfection	Rarely used owing to poor transfection efficiency	Used predominantly with reporter genes (for example, *gfp* and luciferase)
Stable transfection (episomal expression)	Used mainly for the expression of transgenes, reporters, epitope- and/or reporter-tagging of genes and dominant negative transgene expression	Used mainly for the expression of transgenes, reporters, epitope- and/or reporter-tagging of genes and dominant negative transgene expression
Editing the genome	• Single crossover to disrupt a gene: leads to the integration of ≥1 plasmid copy into the genome; requires a single targeting sequence on a circular plasmid and several rounds of drug cycling to select integrants • Double crossover to replace a gene: requires a circular plasmid with two targeting sequences and negative selection to identify integrants • Site-specific recombination to insert genes between *attB* and *attP* sites using the Bxb1 integrase: requires 'mother' line with integrated *attB* site; particularly useful for the expression of transgenes and gene complementation • Zinc-finger nucleases to disrupt, mutate or replace genes by double-stranded break repair • CRISPR–Cas9 to disrupt, mutate or replace genes by double-stranded break repair	• Single crossover to disrupt a gene: leads to insertion of plasmid backbone; DNA must be linearized within the targeting sequence before transfection • Double crossover to replace a gene: DNA must be linearized at 5′ and 3′ ends of respective targeting sequences • Gene insertion/marker out approach: uses double crossover to generate a mother parasite line and negative selection to identify integrants; useful for the expression of transgenes and gene complementation • *Plasmo*GEM recombineering: large targeting sequences (~9 kb) greatly increases recombination frequency; the presence of a unique barcode for each gene allows simultaneous transfection of several plasmids
Conditional systems	• Tetracycline-repressible transcriptional system: anhydrotetracycline (ATc) facilitates the rapid modulation of the transcription level of a gene of interest; used for regulating expression of transgenes only • FKBP12 destabilization domain (DD): Shield 1 stabilizes a protein containing the DD domain • *Escherichia coli* dihydrofolate reductase (DHFR) degradation domain (DDD): trimethoprim stabilizes a protein containing a DDD domain • Riboswitch: glucosamine-6-phosphate activates the *glmS* ribozyme, which cleaves mRNA, leading to mRNA degradation • Cre–*loxP*: rapamycin induces dimerization of the split Cre, which then mediates recombination between *loxP* sites	• Tetracycline-repressible transactivator system: anhydrotetracycline facilitates the rapid modulation of the transcription level of a gene of interest • *E. coli* DHFR degradation domain (DDD): trimethoprim stabilizes a protein containing the DDD domain* • FLP–*frt* system: requires cross-fertilization of parasite clones containing the *frt* sites and FLP recombinase; alternatively, insertion of *frt* sequences must be performed in a parasite line that stably expresses the FLP recombinase
Transposon shuttle mutagenesis	• *piggyBac* transposable element • Mini-Tn5-mediated shuttle transposon mutagenesis	*piggyBac* transposable element
Positive selectable markers	• *P. falciparum* or *Toxoplasma gondii dhfr*‡ (selected with pyrimethamine) • Human *DHFR*§ (selected with pyrimethamine or WR99210) • Blasticidin S deaminase (selected with blasticidin) • Neomycin phosphotransferase II (selected with G418) • Puromycin-N-acetyltransferase (selected with puromycin) • Yeast dihydroorotate dehydrogenase (selected with atovaquone or DSM1) • Fluorescent reporter genes (*gfp* and derivatives)	• *P. berghei* or *T. gondii dhfr*‡ (selected with pyrimethamine) • Human *DHFR* (selected with pyrimethamine or WR99210) • Fluorescent reporter genes (*gfp* and derivatives)
Negative selectable markers	Yeast cytosine deaminase–uracil phosphoribosyl transferase fusion protein (yFCU; selected with 5-fluorocytosine)	yFCU (selected with 5-fluorocytosine)

*Has only been used in *Plasmodium yoelii*. ‡*T. gondii dhfr* is preferable to *Plasmodium* spp. *dhfr* to prevent integration into the endogenous *dhfr* locus. §Used in preference to *Plasmodium* spp. or *T. gondii dhfr* sequences because of its smaller size and lack of homology to endogenous DHFR. FKBP, FK506-binding protein; *Plasmo*GEM, *Plasmodium* Genetic Modification Project.

T. gondii or *P. berghei dhfr–ts* must be used first, and transgenic parasites should be selected with pyrimethamine, followed by the human *DHFR* marker and selection of parasites with WR99210. Negative selection has helped to overcome the selectable marker issue, allowing recycling of the positive selectable marker as well as reducing the number of laboratory animals that are required to generate *P. berghei* transfectants[61]. Fluorescent reporter genes can also be used to directly select for parasites that carry integrated constructs using flow cytometry[62]. However, there were remaining limitations resulting from poor transfection efficiencies and the lack of a robust system to regulate the levels of gene expression. Recently, several technical advances have helped to overcome these issues (FIG. 1; see below). Although further optimization is still required, these advances are promising and are already yielding some very important insights into *Plasmodium* spp. biology. These include how malaria parasites egress from and invade erythrocytes and subsequently grow and

Single-crossover recombination
A homologous recombination event that leads to the insertion of the entire vector backbone and duplication of targeting sequences.

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replicate, and how these parasites transmit to mosquitoes and then initiate infection of another host during the pre-erythrocytic stages of the *Plasmodium* spp. life cycle.

Conditional gene expression systems

As outlined above, the study of *Plasmodium* spp. genes that are important during blood-stage growth has been hampered because the parasite is haploid, and thus conventional knockout approaches lead to parasite death or severe growth defects. To overcome this limitation, various systems for conditional mutagenesis have been developed. Several of these methodologies seemed promising, but substantial success has been sporadic.

The first of these systems was developed for *P. falciparum* nearly a decade ago[63]; however, only about a dozen attempts to regulate blood-stage genes by various techniques have been described, which highlights how difficult the technical challenges have been. As outlined below, the conditional regulatable systems that are available for *P. falciparum* and *P. berghei* differ in whether they modulate expression at the genome, transcript or protein level (FIG. 2). Each system has its advantages and disadvantages; nonetheless, there have been some genuine success stories, which provide impetus for further optimization of these technologies.

Conditional deletion of genetic loci. This system requires the expression in the parasite of a recombinase enzyme such as Cre or site-specific recombinase FLP, which triggers the non-reversible excision of genetic sequences through the recombination of two short targeting sequences (for example, *loxP* sites for Cre or *frt* sites for FLP) that flank the region that is to be excised[64,65] (FIG. 2a). However, to prevent spontaneous excision of essential blood-stage genes, which would be lethal, the recombinase must be either inducibly or stage-specifically expressed.

For *P. falciparum*, direct comparison between the Cre–*loxP* and FLP–*frt* systems found that the former was more efficient[66,67]. In the Cre–*loxP* study, conditional regulation of the Cre recombinase, and hence excision, was controlled using a tetracycline (Tet) regulatable promoter. In the presence of the tetracycline analogue anhydrotetracycline (ATc), levels of Cre are very low owing to the weak promoter, whereas removal of ATc from the culture medium leads to strong activation of the promoter and thus increased recombinase levels for excision. More recently, a split diCre protein[68–70], which can be induced to dimerize into a functional enzyme by the addition of the ligand rapamycin[71,72], has been developed and will probably become the enzyme of choice, as its levels can be controlled more efficiently than those of ATc-regulated Cre (FIG. 2a). Using the diCre excision-based approach, expression of the essential blood-stage invasion ligand apical membrane antigen 1 (AMA1) was examined in *P. falciparum*. AMA1 is expressed on the surface of invasive merozoites and strongly anchors the merozoite apex to the surface of the erythrocyte by a circumferential ring, through which the merozoite passes to enter the erythrocyte. In this case, *AMA1* was flanked with *loxP* sites and excised by diCre following addition of rapamycin, which led to a reduction in AMA1 levels by 80% in the parasite population and the arrest of proliferation of blood-stage parasites[72]. Although some merozoites failed to invade as anticipated, other merozoites (which presumably expressed very small amounts of AMA1 owing to incomplete excision) could penetrate erythrocytes but did not re-seal the host membrane, as demonstrated by their ability to reverse out of the invasion site. This led to the discovery that AMA1 has an important role, either directly or indirectly, in sealing the parasitophorous vacuole at completion of invasion[72]. A much simpler approach to positioning the *loxP* sites on either side of the entire gene of interest (GOI) — for example, as undertaken for *AMA1* (REF. 72) — is to position the *loxP* sites on either side of the 3′ UTR of the GOI. However, for some genes, *Plasmodium* parasites can overcome 3′ UTR excision-based conditional silencing by using alternative cryptic polyadenylation sites[71,73]. For example, diCre was used in *P. falciparum* to regulate expression of SERA5, which is a protease that is secreted into the parasitophorous vacuole of schizont stage parasites and is thought to have a role in breaking down the host cell prior to merozoite egress. However, excision of the 3′ UTR of *SERA5* by diCre did not decrease protein expression, probably owing to the use of cryptic polyadenylation sites[71]. Thus, it seems that excision of the entire gene rather than just the 3′ UTR may be more likely to yield a stronger phenotype for some genes.

For *P. berghei*, the FLP–*frt* system has been used to delete genetic loci; however, this system relies on parasites to be transmitted through mosquitoes to induce the recombination event[74,75], as in the currently available 'deleter' parasite clones, the gene encoding the FLP recombinase is under the control of either the thrombospondin-related anonymous protein (*TRAP*) promoter (which is expressed in the mosquito mid-gut)[74] or the *UIS4* promoter (which is expressed in salivary gland sporozoites)[75]. Thus, the sequence that is flanked by *frt* sites is deleted at the corresponding stage of parasite development. However, this system could also be designed to work in the blood stages. So far, the roles of merozoite surface protein 1 (MSP1), a cGMP-dependent protein kinase (PKG) and subtilisin 1 (SUB1) in the liver stages and AMA1 in the attachment and invasion of erythrocytes have been investigated with this system[74,76–79]. For each GOI, the *frt* sites were placed on either side of the 3′ UTR, with the exception of SUB1, for which the FLP–*frt* recombinase system was elegantly combined with recombineering technology (see below) to excise the entire *P. berghei SUB1* coding sequence. These *P. berghei* studies showed that MSP1, SUB1 and PKG have crucial roles during late liver stage development: MSP1 was shown to be important for merozoite generation within hepatocytes; SUB1 ruptures the parasitophorous vacuole membrane and facilitates egress of merozoites from the hepatocytes; and PKG helps to release extracellular merosomes.

As with most conditional mutagenesis systems, excision using the Cre–*loxP* and FLP–*frt* systems is generally not 100% efficient, owing to the level of recombinase

Negative selection
A process used to deplete parasites that express a negative-selectable marker from a population, in order to enrich parasites that contain the desired genomic integration event.

Double-crossover recombination
A homologous recombination event that results in the replacement of a DNA sequence that is flanked by two targeting sequences.

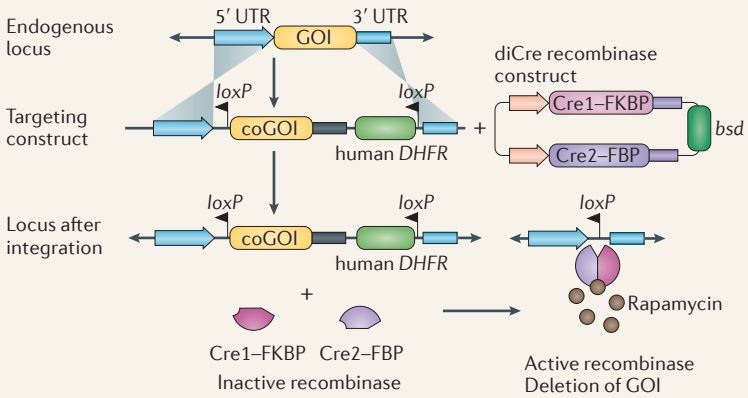
Parasitophorous vacuole
A vacuole in the host cell, in which *Plasmodium* parasites reside and develop.

Cryptic polyadenylation sites
A processing site that is not normally used for the addition of a polyadenylic acid tail to mRNA.

Merosomes
Structures containing hundreds of infectious merozoites that are surrounded by a membrane that is derived from the hepatocyte host cell.

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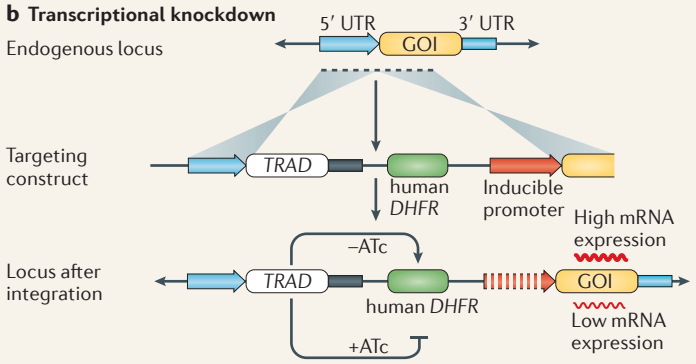
a Conditional gene deletion



Advantages
- Rapid
- Negligible leakage
- Complete ablation of gene function if gene is excised

Disadvantages
- Gene excision does not occur in 100% of parasites
- Hard to distinguish parasites in which gene excision has occurred from those parasites in which excision has not occurred
- Not reversible
- Generation of targeting constructs is difficult
- Cannot be used for very large genes (>5–10 kb)
- Costly
- Requires transfection with two plasmids

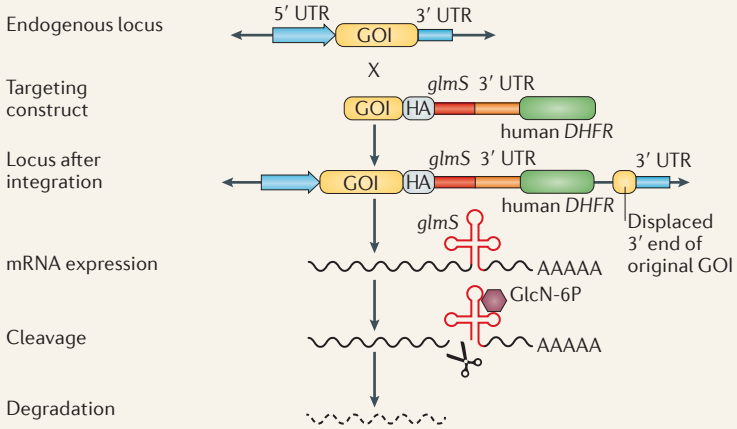
b Transcriptional knockdown



Advantages
- Reversible
- Constructs are simple to generate

Disadvantages
- Only available for *Plasmodium berghei*
- Knockdown <100%
- Difficult to tag genes N-terminally if the gene has N-terminal trafficking sequences

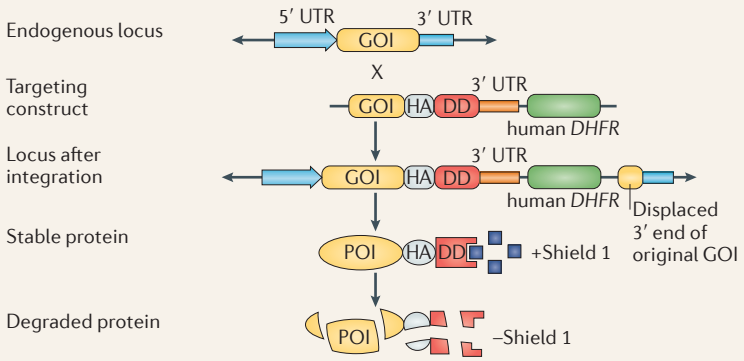
c Post-transcriptional knockdown



Advantages
- Reversible
- Construct generation is simple
- Gene can be tagged for identification

Disadvantages
- GlcN-6P toxicity
- Knockdown only 50–90%

d Post-translational knockdown



Advantages
- Reversible
- Rapid knockdown
- Gene can be tagged for identification

Disadvantages
- Requires access to the proteasome
- Knockdown <80%
- Some proteins do not tolerate DD tag

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Figure 2 | **Strategies to conditionally regulate gene expression in *Plasmodium* parasites. a** | Conditional deletion of a gene of interest (GOI) through diCre–*lox* recombination. Integration of a targeting construct by homologous recombination at the 5′ and 3′ UTR leads to replacement of the GOI with a codon-optimized version of the GOI (coGOI) and the human dihydrofolate reductase (*DHFR*) selectable marker, both of which are flanked by *loxP* sites. Parasites are then transfected with an episome that contains the blasticidin (*bsd*) selectable marker together with genes encoding two separate inactive polypeptides of Cre (Cre1 and Cre2) that are each fused to different rapamycin-binding proteins (FK506-binding protein (FKBP) and fibronectin binding protein (FBP)). Following the addition of rapamycin, the two Cre polypeptides form heterodimers (diCre), which restores recombinase activity and results in the excision of *loxP*-flanked sequences (in this case, both the coGOI and human *DHFR*). **b** | Transcriptional knockdown with the anhydrotetracycline (ATc)-inducible system. The targeting construct, which contains genes encoding a transcriptional transactivator domain (TRAD) and human DHFR, is integrated by homologous recombination at the 5′ UTR and amino terminus of the GOI. *TRAD* is placed under the transcriptional control of the promoter of the GOI, and the GOI is controlled by an inducible minimal promoter. This inducible promoter comprises seven tetracycline operator (TetO) sequences that are located immediately upstream of a minimal promoter element. In the absence of ATc, the expressed TRAD binds to the TetO sequences and induces transcription of the GOI. Addition of ATc prevents the TRAD from activating the minimal promoter and thus decreasing GOI transcription. **c** | Post-transcriptional knockdown using a ribozyme-based gene expression system. The targeting construct contains the *glmS* ribozyme, which is introduced into the genome by homologous recombination at the carboxyl terminus of the GOI; specifically, *glmS* is placed between the stop codon and the 3′ UTR downstream of the GOI. In addition, the construct contains a haemagglutinin epitope tag (HA) that is fused to the GOI and the gene encoding a selectable marker. Addition of glucosamine-6-phosphate (GlcN-6P) activates the ribozyme, which cleaves the mRNA and removes the 3′ UTR, leading to rapid degradation of the mRNA and a reduction in protein levels, as measured by HA levels. X indicates the regions where homologous recombination occurs. **d** | Post-translational knockdown. A mutant version of the human rapamycin-binding protein FKPB12, termed the destabilization domain (DD), is fused to the N terminus or C terminus of the target protein. Shield 1, which is a cell-permeable small-molecule ligand of FKPB12, binds to the DD, thereby stabilizing the protein. Removal of this ligand leads to the degradation of the protein of interest (POI). Thus, this system enables the rapid modulation of the expression level of the POI. X indicates the regions where homologous recombination occurs.

activity, which makes phenotyping problematic. This issue was addressed in a recent study[78] through the incorporation of a promoterless *gfp* coding sequence into the *SUB1* targeting construct, such that *gfp* became repositioned downstream of a promoter after excision. In this way, it was possible to link phenotypic changes directly to those parasites in which the locus had been excised, as only those parasites expressed GFP.

Conditional knockdown of mRNAs. mRNAs contain UTRs upstream and downstream of their coding regions and are appended with a 5′ cap and a 3′ poly(A) tail. Removal of the UTRs can destabilize mRNAs, leading to reduced protein expression. In many eukaryotes, the coding regions can be directly and inducibly degraded using RNA-mediated interference, but the necessary machinery is lacking in *Plasmodium* spp.[80]. For this reason, other strategies are required to regulate mRNA levels. The first conditional system that was developed for *P. falciparum*-controlled gene transcription used a 'Tet-OFF' system, whereby addition of ATc to the culture medium enables transcription of the GOI to be turned off. In this system, the promoter of the GOI is replaced with a weak promoter that contains multiple tetracycline operator (TetO) sites, and gene expression is induced when a transcriptional transactivator domain (TRAD) binds to the TetO sites and recruits transcription factors to boost promoter activity (FIG. 2b). The addition of ATc represses gene expression by preventing binding of TRADs to the TetO sites[63,81]. Although this system can be used in *P. falciparum* to express transgenes from multi-copy episomes, it does not function for the construction of conditional gene knockouts, probably because the TRAD is inefficient at recruiting transcription factors.

Attempts to improve the Tet system by using the transactivator domains of the native *P. berghei* apetala 2 family of transcription factors[82] as TRADs have yielded success in *P. berghei*[83]. Using this modified system, *P. berghei* profilin, which is involved in restructuring the actin cytoskeleton[83], N-myristoyl transferase, which helps to anchor proteins to membranes[83], and the translocon component heat shock protein 101 (HSP101)[83] were successfully downregulated, and knockdown of the genes encoding these proteins arrested parasite growth as expected. Detailed analysis of the *hsp101*-knockdown phenotype indicated that parasites with this phenotype could no longer export proteins into the host erythrocyte, thereby validating the role of the *Plasmodium* translocon of exported proteins (PTEX) complex, of which HSP101 is a component, as the protein translocator. The modified Tet system has also been used to repress the expression of an mCherry reporter protein in mouse hepatoma cultures infected with salivary gland sporozoites[83]; however, before this system can be used to regulate genes that are expressed during the mosquito stages of the malaria life cycle, delivery of ATc to infected mosquitos may need to be optimized, or alternative TRADs may be required, to yield better knockdown in mosquitoes. It is also unlikely that different blood-stage genes can be regulated to the same extent on the basis of the context of the gene locus and the strength of the endogenous promoter driving TRAD activity, and thus the development and optimization of different TRADs or inducible promoters may be required to tailor the regulation of particular genes to determine a phenotype.

Another technique to post-transcriptionally regulate mRNA levels in *P. falciparum* uses self-cleaving ribozymes, the coding sequences of which are inserted into non-coding regions (FIG. 2c). Ribozymes are RNA molecules that are capable of catalysing biochemical reactions: in this case, the *cis*-cleavage of the mRNA into which they are integrated. This leads to the removal of the UTR of the mRNA, which hastens degradation and thereby reduces protein levels. The insertion of the mRNA-cleaving *Sm1* ribozyme from *Schistosoma mansoni* into the mRNA of a luciferase reporter enzyme, and subsequent insertion of the sequence encoding these mRNAs into *P. falciparum*, greatly reduced the expression of luciferase relative to a luciferase control that did not contain the ribozyme[80]. Attempts to reverse mRNA degradation with the cleavage inhibitor compound toyocamycin were not successful, severely limiting the use of this system[85]. By contrast, placing the *glmS* ribozyme, which requires the cleavage inducer glucosamine-6-phosphate for activation[81], downstream of the stop codon of the gene encoding the *P. falciparum*

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PfDHFR–TS–GFP reporter enzyme successfully reduced expression of this mRNA 3-fold and reduced protein levels 10-fold in the presence of glucosamine-6-phosphate. The loss of the *pfdhfr–ts–gfp* selectable marker cassette, which confers resistance to the antimalarial drug pyrimethamine, also sensitized the parasites to pyrimethamine as expected. *glmS*, which is also known as the riboswitch system, has additionally been used to knock down the mRNA encoding the translocon component PTEX150 by more than 80%, which led to a protein export-defective phenotype[84], similar to observations in HSP101-deficient *P. berghei*. The riboswitch system is an attractive tool, as the *glmS* sequence can easily be targeted to the genome through a simple 3′ replacement strategy (FIG. 2c); therefore, this system is currently the method of choice to conditionally knock down mRNA levels. An alternative strategy that may promote more rapid degradation is the insertion of the *glmS* sequence into the 5′ UTR to delete the 5′ 7-methylguanosine cap, although this is technically more difficult to achieve[86]. Whether the riboswitch system also functions in *P. berghei* and whether the glucosamine levels can be adequately manipulated in mice to achieve sufficient knockdown of the transcript remain to be investigated.

Conditional knockdown of proteins. Functional protein levels can also be modified post-translationally in *P. falciparum* by promoting the premature degradation or mislocalization of the target protein in the cell. Degradation can be achieved by appending the protein of interest with either an FK506-binding protein (FKBP)-based destabilization domain (DD)[87,88] or an *Escherichia coli* DHFR destabilizing domain (DDD)[89] (FIG. 2d). These domains are structurally unstable and promote their own ubiquitylation and degradation, as well as that of the protein they are attached to, through the endoplasmic reticulum-associated degradation pathway. However, degradation can be reversed by the addition of compounds that stabilize the protein: Shield 1 for DD fusion proteins and the folate analogue trimethoprim for DDD fusion proteins. It should be noted that trimethoprim is naturally toxic to malaria parasites, and thus the parasites must contain a human *DHFR* expression cassette in their genome, which confers resistance to trimethoprim[89]. These systems are especially attractive because they are reversible, meaning that the stabilizing compounds can be added or washed out to raise or reduce levels of the protein of interest at particular time points in the cell cycle in the blood stages. The system is also suitable for regulating the expression of dominant negative transgenes that are encoded in episomes. However, these types of system have some limitations: they cannot be used to regulate proteins that are secreted by the parasite, as they are not targeted by the protein degradation machinery in the parasite cytosol or in some organelles; they also cannot be used for genes that do not tolerate fusion to the DD or DDD tag.

Systematic testing of the DD and domains that are encoded by its various alleles has shown that degradation using this system is usually about fourfold when the compounds that stabilize the DD fusion protein are removed from the culture medium, with DD24 performing most reliably for carboxy-terminal fusion[90]. So far, the DD system has been used in *P. falciparum* to C-terminally tag the endogenous sequences of the cysteine protease calpain[91], calcium-dependent protein kinase 1 (CDPK1) and CDPK5 (REFS 92,93), as well as DOC2.1, which possesses a calcium-dependent membrane-binding C2 domain[94]. The power of the DD system was elegantly demonstrated by a study in which knockdown of CDPK5 by ~60–80% showed that it has a role in parasite egress[92]. Specifically, during the blood stages, the decline in CDPK5 levels that were caused by removal of Shield 1 arrested the cell cycle at the late schizont stage when merozoites were becoming fully formed. The defect in merozoite egress could be overcome by mechanically breaking open the schizonts to release the merozoites, which suggests that distinct pathways are involved in egress and invasion in *P. falciparum*. The DD system has also been used to inducibly express a dominant negative proteolytically inactive mutant of ATP-dependent protease subunit ClpQ, which normally functions in the mitochondria of the parasites[95]. The mutant ClpQ–DD protein, when stabilized with Shield 1, disrupted transcription of the mitochondrial genome, altered organelle morphology and arrested growth, showing the importance of the mitochondrion in the asexual cell cycle. In another study, the DD system has been used to determine whether protein autoinhibitory domains can be ectopically expressed to conditionally regulate the function of the protein. For example, CDPK1 has an autoinhibitory domain that blocks kinase activity in the absence of a calcium ion signal. To conditionally regulate CDPK1, the autoinhibitory domain was expressed as a DD fusion protein, and Shield 1-mediated stabilization resulted in the arrest of parasite growth late in the cell cycle[93]. Thus, this approach could be used in the future to dissect the function of other proteins that contain autoinhibitory domains.

For the DDD system, the levels of protein knockdown that have been observed have varied widely. For example, destabilization of the *P. falciparum* proteasome lid subunit 6, which is important for proteasome assembly and function, occurred within 8 hours of trimethoprim removal, leading to the inhibition of degradation of ubiquitylated proteins and subsequent growth arrest[89]. By contrast, no evidence of actual protein degradation was observed for either *P. falciparum* HSP110–DDD[96] or HSP101–DDD[97] fusion proteins. These two unrelated heat shock proteins were studied because of their putative roles in parasite survival within the human host. It is not surprising that the DDD did not lead to the degradation of HSP101, as this protein is localized to the parasitophorous vacuole and therefore does not have access to the proteasome. Thus, it seems that the resulting lack-of-growth phenotypes that were observed for the HSP110 and HSP101 mutant parasites were a consequence of destabilization of the DHFR domain fold, which caused instability of the fusion protein rather than triggering protein degradation per se. This resulted in the dissociation of HSP110 and HSP101 from their

Ubiquitylation
A post-translational enzymatic modification involving the attachment of ubiquitin to a protein substrate.

Dominant negative transgenes
A gene that when expressed in *trans* causes an adverse effect on the normal, wild-type gene product that is expressed in the same cell.

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interacting partners, leading to an incapacity to survive heat shock[96] and a block in protein export into the host red blood cell[97], respectively.

The DDD system has only been used once in an *in vivo* setting: in the rodent malaria species *P. yoelii*. Parasites produce many different proteases that have important roles in several cellular processes such as haemoglobin digestion, egress and invasion of various host cells. Some of these proteases are regulated by endogenous inhibitory proteins. To explore the function and determine the target of the inhibitor of cysteine proteases (ICP), *P. yoelii* ICP was tagged with DDD to regulate its expression levels. Although yoelipain 2 was identified as an ICP target, a knockdown of ICP–DDD by ~50% was not sufficient to affect parasite growth or survival in infected mice[98]. Whether the DDD system facilitates adequate regulation of other proteins *in vivo* remains to be determined.

Another inducible system that is based on the conditional aggregation domain (CAD) causes self-aggregation of the fusion partner of the CAD in the endoplasmic reticulum. Aggregation is reversible following the addition of a small molecule, AP21998. Currently, this system has only been shown to function for secreted proteins: it was successfully used to control the export of *P. falciparum* proteins to the erythrocyte cytosol. Specifically, the CAD was fused to the Maurer's cleft protein skeletal-binding protein 1, or to a portion of a protein belonging to the highly variable STEVOR (subtelomeric variable open reading frame) protein family, which enabled investigation of the temporal sequence of events during export of these proteins[99]. An alternative strategy has been successfully implemented in *P. falciparum* to modulate translation without the need to modify the target protein, based on the control of a protein–RNA interaction by ATc[100]. In this system, the protein of interest is controlled by a Tet repressor (TetR)-binding aptamer (such as 5–1.17); the TetR–aptamer interaction leads to the repression of translation, whereas the addition of ATc blocks this interaction and thus results in protein expression. Translational repression is rapid, stable over prolonged periods and homogeneous (with ~80% knockdown consistently observed). This system has another advantage over the DDD and DD systems, which is that modulation of gene expression is independent of the cellular localization of the protein. Moreover, unlike the TetR–TetO system, gene expression is independent of transcriptional control. Thus, the TetR–aptamer system has some key attributes that make it very attractive, especially as there is the possibility of improving regulation even further by modifying either the TetR protein or the aptamer.

Maurer's cleft
Single-membrane-bound structure that is present in the cytoplasm of erythrocytes that are infected with *Plasmodium falciparum* and that functions in transport of proteins from the parasite to the surface of the erythrocyte.

Advances in genome editing

The suboptimal transfection efficiency of *P. falciparum* not only hinders the pace of progress as a result of the time that is required to obtain transfectants, it also makes the selection of transgenic parasites that have delayed growth extremely difficult or impossible. It has also put saturation-based genetic screens beyond reach. Accordingly, efforts have been made to tackle this issue.

For *P. berghei*, the transfection efficiency is much higher, especially with the Amaxa Nucleofection Device (Amaxa)[58], which is preferentially used over other electroporation devices for all other *Plasmodium* species in which schizont (that is, merozoite) stages are used for transfection[45,46,50,101]. The resulting increase in efficiency has reduced both the numbers of animals that are required for reverse genetic experiments and the risk of overgrowth by more robust (often wild-type) parasites in the population.

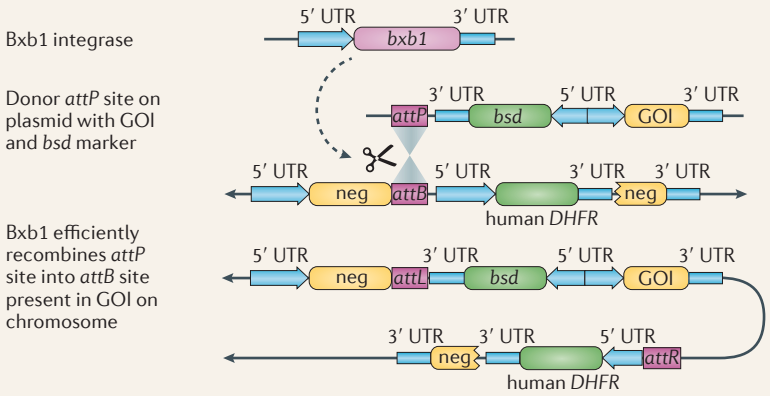
Although intracellular stages have traditionally been used for *P. falciparum* transfection, techniques have now been developed that enable large numbers of merozoites to be obtained *in vitro*. Protease inhibitors that prevent schizont rupture but still enable merozoites to develop are used during the *in vitro* culture period, and viable merozoites are obtained by mechanical rupture of schizonts[102]. A recent study that demonstrated the use of the CRISPR–Cas9 system also showed that electroporation of *P. falciparum* schizonts using Amaxa may considerably improve transfection efficiency in this species, as stably transfected parasites could be detected as early as 8 days after transfection[103]. The same study also reported *P. falciparum* nuclease-driven gene disruption using linear DNA and, although transfected parasites could not be recovered within a shorter time period than with traditional methodologies, this approach does not require negative selection to remove episomes from the population[103], which itself is an important time-saving advance.

In circumstances in which the same gene locus is to be edited on several occasions — for example, the complementation of a gene knockout or stable expression of different transgenes — the Bxb1 integrase system[104] or the gene insertion/marker out (GIMO) methodology[61] may offer a more rapid way to obtain the desired integrant. The Bxb1 integrase catalyses recombination between an *attB* site that is already present in the *P. falciparum* genome and an incoming *attP* site that is present on a plasmid containing the desired transgene (FIG. 3a). The *P. berghei* GIMO system requires transfection of a linear DNA construct in which the desired transgene is flanked by sequences targeting a modified genomic locus that already contains both a positive and a negative selectable marker. Thereby, application of negative selection selects for parasites that have incorporated the transgene into the desired locus through double homologous recombination. Both of these systems require the generation of a 'mother' line such as the *P. falciparum* Dd2*attB* and 3D7*attB* parasite lines that contain an *attB* site that is integrated at the *cg6* locus[104] and *P. berghei* and *P. yoelii* GIMO lines that allow rapid integration into the *P230p* locus[61], which are already available. The *cg6* and *P230p* loci can be readily disrupted in the blood stages without adverse effects on parasite growth.

More recently, ZFNs, which encode artificial enzymes that induce double-strand breaks in the genome and trigger homologous-directed repair of DNA double-strand breaks to restore the integrity of the genome, have been trialled in *P. falciparum* to determine whether they provide greater scope for editing the genome[105] (FIG. 3b). Rapid insertion of the targeting construct into either

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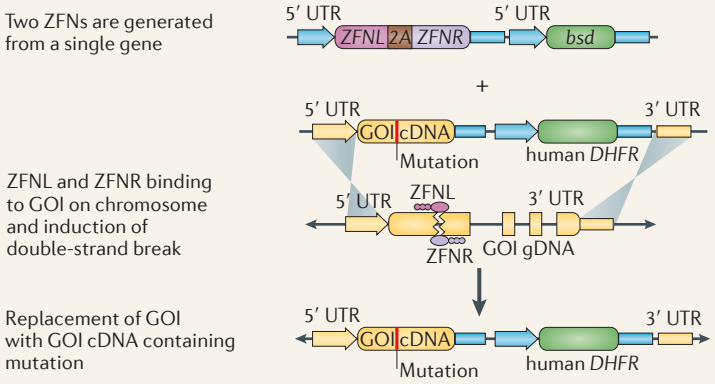
a Efficient site-specific integration



Advantages
- Rapid insertion of GOI into a chromosome without drug selection
- GOI usually inserts as a single copy which allows evenly distributed expression across the population
- Bxb1 integrase works efficiently and the plasmid does not require drug selection

Disadvantages
- Restricted to using strains modified to contain the *attB* site

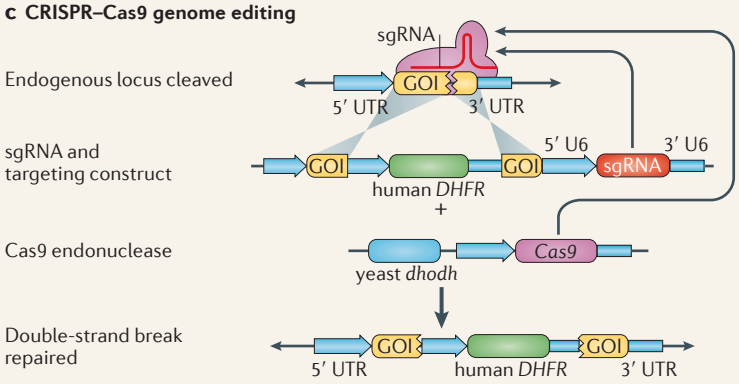
b Site-specific editing using engineered ZFNs



Advantages
- Rapid modification of GOI without drug selection

Disadvantages
- ZFN constructs are expensive to generate and require testing before being used in parasites

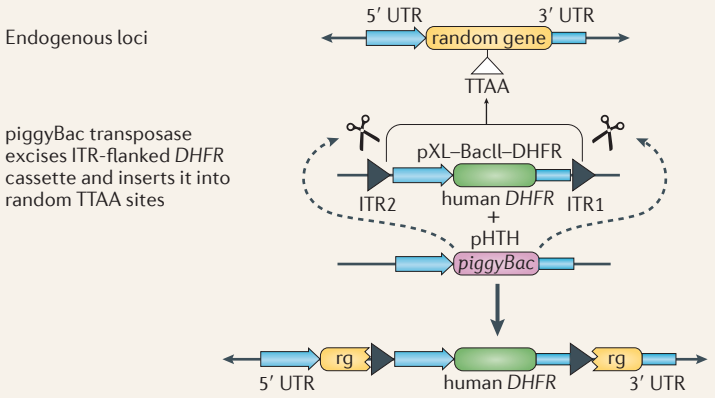
c CRISPR–Cas9 genome editing



Advantages
- Linear DNA can be used as homologous targeting template
- Very efficient
- Allows targeted gene mutations

Disadvantages
- Similar to Cre–*loxP* system in that gene modification is not reversible
- The editing of some genes will be lethal

d Random gene insertional mutagenesis



Advantages
- *Plasmodium* spp. genomes are rich in TTAA sites, allowing high coverage of random-screen libraries based on phenotype
- Identification of disrupted gene by PCR
- Recovery of knockdown gene-expression mutants with insertion into 5′ or 3′ UTRs

Disadvantages
- No recovery of gene knockout mutations in essential genes

Viral 2A ribosomal skipping peptide
A peptide derived from foot-and-mouth disease virus 2A that, when introduced as a linker between two proteins, allows autonomous intraribosomal self-processing of the resulting polyproteins that are expressed from a single polycistronic mRNA transcript.

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Figure 3 | **New strategies for editing the *Plasmodium* spp. genome. a** | Site-specific integration into the *Plasmodium* spp. genome using the Bxb1 integrase system. Two plasmids are transfected, one of which contains the gene encoding the Bxb1 integrase, which catalyses recombination between an incoming *attP* site that is present on the second plasmid containing the desired (trans)gene of interest (GOI) and a chromosomal *attB* site that has already been engineered into a gene that is not essential for blood-stage growth (neg). Recombination between the *attP* and *attB* sites produces asymmetric *attL* and *attR* sites that cannot recombine. **b** | Editing of the *Plasmodium* spp. genome using zinc-finger nucleases (ZFNs). A donor plasmid encoding a ZFN pair (ZFNL and ZFNR) that has been co-expressed from a single promoter using a viral 2A ribosomal skipping peptide is transfected into *Plasmodium* parasites, together with a plasmid containing the GOI with a specific mutation in the GOI cDNA. Following expression, dimerization of ZFNR and ZFNL results in the assembly of an artificial enzyme, which induces a double-strand break in the genome at the site homologous to sequences that are included in the ZFNs. Subsequently, the breaks are repaired by homologous-directed repair using homologous regions of the donor plasmid as the template (in this case, the 5′ and 3′ UTRs). This leads to the replacement of the GOI with a cDNA version of the GOI, which contains the desired mutation (for example, mutations that confer drug resistance). **c** | CRISPR–Cas9 genome editing requires expression of both the Cas9 endonuclease and the single-guide RNA (sgRNA). Cas9 is expressed in the parasite from an episome that contains the yeast dihydroorotate dehydrogenase (*dhodh*) drug-selectable marker. The sgRNA, which is placed under the transcriptional control of the 5′ UTR and 3′ UTR of the U6 polymerase III promoter (5′ U6 and 3′ U6, respectively), is incorporated into the targeting construct, which contains the human dihydrofolate reductase (*DHFR*) selectable marker and is flanked by targeting sequences (in this case GOI sequences) to drive homologous integration into the genome. The sgRNA must comprise 20 nucleotides that match the target DNA site, as well as a Cas9-binding domain (not shown) to guide the Cas9 endonuclease to the target DNA site, where it induces double-strand breaks. These breaks are subsequently repaired by homologous recombination. **d** | Random gene insertional mutagenesis in the *Plasmodium* spp. genome using the *piggyBac* transposon system. Two plasmids are transfected into *Plasmodium* parasites: pXL–BacII–DHFR, which contains the human *DHFR* selectable marker flanked by two inverted terminal repeats (*ITR1* and *ITR2*) of the *piggyBac* element; and pHTH, which contains a sequence encoding the piggyBac class II integrase that precisely excises the *piggyBac* element to randomly target a tetranucleotide target site (TTAA) in the genome of the parasite. Insertions can occur in a random gene (rg), as indicated in the figure, or can flank a protein-coding sequence. After a library of parasite clones has been obtained, the *piggyBac* insertions and their flanking genes can be identified by PCR. *bsd*, blasticidin selectable marker; gDNA, genomic DNA.

Ring-form parasites
The feeding stages of blood-stage parasites that show a ring-like morphology in Giemsa-stained blood smears.

Lambda Red recombination system
This tool enables targeted genetic changes to DNA in *Escherichia coli* expressing the lambda Red recombinase. This system has been used in conjunction with Gateway technology to convert *Plasmodium berghei* genomic DNA clones that have been maintained in *E. coli* into gene-targeting vectors.

Gateway cloning system
A molecular methodology that enables the transfer of DNA fragments between plasmids using *attP* recombination sequences and a mixture of commercial clonase enzymes.

an integrated copy of *gfp* or *P. falciparum* chloroquine resistance transporter (*pfCRT*) was achieved within 2 weeks[105]. For example, mutations of *pfCRT* that confer chloroquine resistance through allelic replacement of specified point mutations could be studied using this approach. Rapid insertion was possible with and without direct selection, as homologous-directed repair, which uses a homologous template to guide the repair, is the only repair mechanism that occurs in *Plasmodium* spp.. The alternative repair mechanism of stitching broken ends of DNA together by non-homologous end joining (NHEJ) does not occur in *Plasmodium* spp., as several crucial components of the NHEJ pathway are absent. ZFN-mediated gene editing has been used to confirm the role of mutations in the gene encoding phosphatidylinositol-4-OH kinase in mediating resistance to a new class of antimalarial compound: pan-active imidazopyrazines[106]. More recently, this genome editing tool has also been used to introduce or remove mutations in the gene encoding kelch protein K13 to identify their roles in conferring resistance of ring-form parasites to artemisinins *in vitro*[107]. Finally, ZFNs have been used in *P. vivax* to show that this species can be stably modified[52].

Another genome editing methodology, termed the CRISPR–Cas9 system, from *Streptococcus pyogenes*[108] has been shown to be effective in *P. falciparum*[103,109] and *P. yoelii*[110] (FIG. 3c). This system requires the heterologous expression of the nuclease Cas9 and a targeting single-guide RNA, the latter pairing with complementary DNA in the genome and thereby specifically directing the Cas9 nuclease to induce a double-strand break in the genome. In *Plasmodium* spp., this break is then repaired by homologous end joining. This system has so far been used to disrupt the knob-associated histidine-rich protein (PfHRP) and erythrocyte binding antigen-175 (EBA-175), providing proof of principle that this technology works. It has also been used to introduce single-nucleotide substitutions in origin of recognition complex 1 (*orc*) to alter the mono-allelic expression of the subtelomeric multigene *var* gene family, although this did not lead to *var* dysregulation as predicted[103,110]. Moreover, the CRISPR–Cas9 system has also been used to introduce a propeller mutation into the gene encoding the kelch protein Pf3D7_1343700 (REF. 103). This study confirmed that a particular polymorphism is associated with slow-clearing parasites in patients infected with malaria that have been treated with artemisinin. The CRISPR–Cas9 system uses a simpler approach than the ZFNs, as it does not require the engineering of specific enzymes and the guide RNA is easier to make; moreover, it is more cost-effective and possibly more efficient than ZFNs. Therefore, it is likely that this methodology will ultimately replace the conventional gene-targeting techniques to generate specific gene knockouts and to introduce single-nucleotide substitutions into genes to assess their biological function and their contribution to drug resistance.

The development of high-throughput genetic screens in *Plasmodium* spp. required further technological advances. A particular limitation for *P. berghei* is the large number of mice that are required for a high-throughput screen, as *P. berghei* transfectants can only be generated *in vivo*. However, these drawbacks have been overcome by the *Plasmodium* Genetic Modification Project (*Plasmo*GEM) at the Sanger Institute, UK[111,112], which aims to generate a production pipeline of knockout and tagging vectors for all genes in the *P. berghei* genome using the lambda Red recombination system technology and the Gateway cloning system[113,114]. These vectors are available to the malaria research community and provide the advantage that the long regions of genomic DNA homology arms that are present in the *Plasmo*GEM vectors greatly increase the recombination frequency. As each construct combines a unique 'barcode' for identification, a single mouse can be infected with a population of *P. berghei* parasites transfected with multiple knockout constructs that homologously recombine into different regions of the genome. A transgenic parasite that contains a distinct integration event can then be readily identified from the parasite population on the basis of the barcode sequence that is incorporated into its genome[111]. This should even be possible at various intervals post-transfection, paving the way for rapid functional analysis of entire pathways for the relative

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Box 2 | **Engineering malaria parasites for live cell imaging**

The ability to engineer malaria parasites has enhanced the range of biochemical and cell biological studies; for example, the dissection of genetic elements that control gene expression, the investigation of the localization of proteins within a cell, the purification of proteins at particular stages of the parasite life cycle and the identification of interacting partners have become much simpler through the appendage of reporter genes and epitope tags to the gene of interest (GOI). However, live cell imaging warrants a special mention, as this approach has only become available through the development of *Plasmodium* spp. transfection systems. Imaging has been fundamental to elucidating the cellular organization and dynamics of events within the parasite and the infected host cell, and has revealed complex parasite-trafficking pathways[125]. For example, live cell imaging of transgenic parasites expressing GFP fused to apicoplast-resident proteins was used to determine the morphology of the apicoplast and to identify motifs that direct trafficking to this organelle[126]. Advances in imaging platforms (for example, 3D structured illumination microscopy) also facilitate the detailed visualization of sub-micrometre organelles such as the Maurer's clefts that are present in the cytosol of erythrocytes infected with *Plasmodium falciparum*[127]. Additionally, with the development of newer fluorescent proteins, such as those that encode sensors for calcium, oxidative stress or pH, the ability to accurately probe particular physiological environments and responses within the parasite is now within reach.

Using intravital microscopy of transgenic parasites that express bioluminescent or fluorescent proteins, it is now also possible to trace the journey of parasites *in vivo* in tissues such as the liver, brain and spleen, which have fundamental roles in the establishment of infection, pathogenesis and clearance of infection[19,128–130]. Technological advances in microscopy, such as two-photon microscopy, are providing even greater scope for imaging living tissue at the cellular level[131,132]. Moreover, the ability to label host compartments such as immune cells with different fluorescent markers not only enables us to capture the response of the immune system to malaria infection in real time, but also to observe how this response is altered when particular *Plasmodium* spp. genes are modified[133]. Furthermore, parasite burden in tissues or live animals can be determined using bioluminescent parasites[134–136], and it is possible to detect as few as 1–5 infected hepatocytes per liver with 2D or 3D imaging[137–139], which is a much more accurate way to predict disease severity than measuring parasitaemia levels in the blood. The generation of reporter parasites has contributed to our understanding of parasite sequestration, including in experimental cerebral malaria[19,140] and liver stage immunity[141], and the ability to quantify parasites will also provide a robust means to evaluate attenuated vaccines and to assess the potency of antimalaria drugs *in vivo*[142,143].

Two-photon microscopy
A fluorescence imaging technique that absorbs two photons of infrared light to provide deeper tissue penetration, which enables living tissues to be imaged to a greater depth than conventional confocal microscopy.

Non-essential genes
Genes that can be deleted in the parasite without causing parasite death under certain conditions. These include genes that do not impart moderate or severe growth defects when mutated. However, genes that are termed 'non-essential' may in fact be essential to parasite growth when tested under different environmental conditions.

contributions of individual genes to growth and survival. Indeed, by transfecting several *Plasmo*GEM vectors that target the kinome into *P. berghei*, it was possible to show that the barcode sequencing approach could be used to phenotype mutant parasites within a pool of transfectants[115]. This screen produced results that were comparable to those obtained from a conventional *P. berghei* kinase knockout screen[116], and it was also more sensitive, in that it identified additional kinases that are amenable to gene disruption[115].

As ~50% of *Plasmodium* spp. genes show a complete lack of conservation outside the genus, there is also a need to perform high-throughput forward-genetic screens to recover mutants with particular phenotypes to determine gene function. A piggyBac transposable system has been adapted for *P. falciparum* and has been used to screen for genes that are crucial for intra-erythrocytic development of the parasite[117,118]. More recently, the system has been tailored for use in *P. berghei*[119] (FIG. 3d). Depending on the conditions that were used (such as the culture volume, the ratio of the two transfection plasmids and how soon after electroporation of these plasmids drug pressure was applied to select for transfectants), 1–10 parasite clones from a single *P. falciparum* transfection were found to contain an insertion, whereas the system developed for *P. berghei* is 16–18-fold more efficient. Nevertheless, this efficiency is still too low to generate a library of parasite clones in which the expression of all genes have been disrupted, which is required for genome-wide screening for a particular phenotype. Moreover, these systems can currently only be used to screen non-essential genes in the blood stages of parasite development[120], unless a conditionally regulatable component such as the modified Tet system described above is integrated into the system.

Conclusions

Transfection of *Plasmodium* spp., which is used to analyse gene function throughout the life cycle of the parasite and during pathogenesis, has come a long way since the technique was first described 20 years ago. Previously, biochemical, pharmacological, molecular or cell biology assays were used to assess gene function; now, gene modification tools exist to determine the role of a specific gene during the blood-stage cycle. This has been a hugely important advance, as it allows definitive functional dissection of possible drug and vaccine targets. Moreover, transfection of parasites with reporter genes has enabled the cellular organization and dynamics of events that occur within the parasite, the infected host cell or the whole infected animal to be readily studied by live cell imaging (BOX 2). Although several of these conditional mutagenesis systems have proved to be effective for such experiments, in our view, none has emerged as clearly superior to the others. Each system has its advantages and drawbacks (FIG. 2), and thus several factors need be considered when choosing a system to regulate a GOI. These factors include which *Plasmodium* species is being investigated, the cellular location of the target gene, whether the GOI is likely to tolerate modification, whether regulation needs to be reversible and at what stage of the life cycle regulation needs to occur. Even with careful consideration, one technique may work well for some genes, whereas for other genes the same approach is less effective, although the reasons for this remain unknown. Consequently, it is difficult to predict which conditional system should be preferentially used, and investigators need to have a range of approaches at their disposal.

The other main limitation in manipulating the *P. falciparum* genome has been low transfection efficiency, which has restricted the number and range of experiments and, more importantly, although 'random' transposon-based methods have been developed, large-scale forward-genetic screens are still beyond reach for *P. falciparum* and even for *P. berghei*. Recent advances will improve efficiency and facilitate the development of more robust and widely tractable tools. Innovative approaches that combine conditional mutagenesis and existing transposon systems, most notably the *piggyBac* transposon system, hold considerable promise in this regard.

It is also important to note that although our comprehension of the biology of *P. falciparum* has been enhanced by genetic tools, the lack of similar tools for

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Box 3 | **Gaining insight into *Plasmodium vivax*-like biology**

The study of *Plasmodium vivax* has been severely hampered by the lack of a continuous culture system for this species. However, the ability to derive reticulocytes that can be infected by *P. vivax* from haematopoietic stem cells *in vitro* suggests that this may one day be possible[144]. So far, the related non-human primate parasites *Plasmodium cynomolgi* and *Plasmodium knowlesi* have been used to gain insight into *P. vivax*-like biology. Although transfection techniques are still in their infancy for these species[48–50], the ability to generate transgenic *P. cynomolgi* parasites that stably express GFP has provided a means to isolate hypnozoite forms from *in vitro* cultures of infected rhesus primary hepatocytes for biological study[101,145]. For *P. knowlesi*, the transfection efficiency of the A1 strain that has been adapted to grow long-term in human erythrocytes in a continuous culture system is ~30–40%[50]. Importantly, these parasites also maintain the ability to grow in monkey erythrocytes *in vitro*[50] and can re-adapt to *in vivo* growth[49], providing an opportunity to examine the biology of parasites that closely resemble *P. vivax* in an animal setting.

Hypnozoites
Dormant forms of *Plasmodium* spp. parasites that are present in the liver.

the other main human malaria pathogen, *P. vivax*, has been a substantial impediment to understanding unique aspects of its biology, and how these lead to the formation of hypnozoites and to disease outcomes that are generally less severe than those caused by *P. falciparum* infections. The main roadblock has certainly been the lack of a continuous culture system for *P. vivax*[121], and thus insight into *P. vivax*-like parasite biology has had to be mostly gleaned from studies using the related non-human primate parasites *P. cynomolgi* and *P. knowlesi*[48–50] (BOX 3). However, it is encouraging that *P. vivax* is permissive to taking up DNA[51,52], and investigation of *P. vivax* biology using the genetic approaches that are described here for *P. falciparum* and *P. berghei* should be possible when a robust culture system has been established.

Finally, along with the expansion of the molecular toolbox for *Plasmodium* spp., there must be parallel development of robust functional assays to define the phenotypes of transgenic parasites. Moreover, as gene knockdown is unlikely to be 100% efficient, the ability to relate genotype to phenotype will aid in the functional dissection of a gene. Although assays to determine the role of a protein in parasite erythrocyte invasion pathways have considerably advanced, challenges remain: for example, defining the phenotypes of transgenic parasites at other points in the intra-erythrocytic cycle in order to identify more precisely the molecular pathways that are involved. Exposing transgenic parasites to different environmental conditions may be one strategy to reveal phenotypes, a strategy which has proven to be successful in the phenotyping of yeast mutants[122,123].

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DATABASES
Pathway Interaction Database: http://pid.nci.nih.gov
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