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Review

Functional genetics in Apicomplexa: Potentials and limits

Julien Limenitakis*, Dominique Soldati-Favre

Department of Microbiology and Molecular Medicine, CMU, University of Geneva, 1 rue Michel-Servet, 1211 Geneva 4, Switzerland

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ABSTRACT

The Apicomplexans are obligate intracellular protozoan parasites and the causative agents of severe diseases in humans and animals. Although complete genome sequences are available since many years and for several parasites, they are replete with putative genes of unassigned function. Forward and reverse genetic approaches are limited only to a few Apicomplexans that can either be propagated in vitro or in a convenient animal model. This review will compare and contrast the most recent strategies developed for the genetic manipulation of *Plasmodium falciparum*, *Plasmodium berghei* and *Toxoplasma gondii* that have taken advantage of the intrinsic features of their respective genomes. Efforts towards the improvement of the transfection efficiencies in malaria parasites, the development of approaches to study essential genes and the elaboration of high-throughput methods for the identification of gene function will be discussed.

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1. Introduction

The phylum Apicomplexa encompasses more than 5000 species of intracellular parasites, many of which are important pathogens for humans and animals. In recent years, many apicomplexan genomes have been sequenced, including those of the malaria species *Plasmodium falciparum* [1], *Plasmodium vivax* [2], *Plasmodium berghei* [3], *Plasmodium yoelii* [4], and *Plasmodium knowlesi* [5]. The genomes of two additional members of the haemosporidia, *Theileria parva* [6] and *Babesia bovis* [7], which infect the red blood cells of vertebrates during their life cycle, have been shown to be interesting sources for comparison. The group of more distantly related coccidian parasites that have been sequenced includes *Toxoplasma gondii* [8], *Neospora caninum* [8], *Eimeria tenella* (www.genedb.org), *Cryptosporidium parvum* [9] and *Cryptosporidium hominis* [10]. *P. falciparum* is responsible for the most severe form of malaria, whereas *P. vivax*, the most widespread human malaria parasite outside of sub-Saharan Africa, causes a disease prone to relapse from a dormant form called hypnozoite [11]. The closely related rodent parasites, *P. berghei* and *P. yoelii*, and the primate parasite, *P. knowlesi*, allow investigations of parasite–host cell interactions in vivo and provide access to material for studies of parasite stages from mosquitoes and from host livers. Rodent malaria parasites

constitute the model of choice for studying gene function in vivo [12]. Other Apicomplexans are important opportunistic pathogens. In patients suffering from AIDS, *T. gondii* can cause toxoplasmosis encephalitis, and *C. parvum* can lead to severe enteritis. *T. gondii* is a cyst-forming parasite found in virtually all warm blooded animals. It is chronically established in one-third of the human population, usually as a result of ingesting contaminated meat. This makes this protozoan one of the most successful parasites [13]. Some other coccidian parasites are of considerable veterinary importance, such as *E. tenella*, responsible for devastating disease in poultry, and *N. caninum*, which causes chronic infections in dogs and abortion in cattle. Other severe cattle infections are caused by *T. parva*, transmitted by a tick-bite and responsible for the so-called East Coast fever, and the mosquito-transmitted *B. bovis*, which is responsible for babesiosis in tropical and semi-tropical areas. Despite the considerable advances conferred by large-scale genome sequencing and various global transcriptomic and proteomic studies, assignment of gene functions remains limited in Apicomplexans. In the case of the well-studied organism *P. falciparum*, the frequency of identified open reading frames (ORFs) not assigned to any known function has dropped from 60% at the publication of the genome [1] to just below 50% a decade later [14].

A survey of the other apicomplexan genomes available through EupathDB [8] shows a higher frequency of 'hypothetical genes'. This finding is partly explained by the inability to propagate some apicomplexan parasites in tissue culture, which makes them less

* Corresponding author. Fax: +41 22 3795702.

E-mail address: Julien.Limenitakis@unige.ch (J. Limenitakis).

amenable to genetic manipulation. Gene annotation is also complicated by extended sequence divergence compared to other organisms. This is particularly the case for the early divergent Apicomplexan, *C. parvum*, for which putative functions were initially assigned to only 4% of the predicted ORFs [9]. Undoubtedly comparative genomics, transcriptomics and proteomics will continue to provide more information about the so-called 'hypothetical proteins', but the identification of unique parasite functions ultimately relies heavily on genetic approaches. Reverse genetics, which consists of altering or deleting a gene to unravel its biological function, harnesses information from sequenced genomes and is a favoured approach for analysing parasite gene functions. In contrast, forward genetics seeks to identify the gene(s) responsible for a particular phenotype and is often a less trivial and more time-consuming approach. To identify the gene responsible for a given phenotype, several options can be utilised. Forward genetic mapping has been successfully used to identify genes such as those responsible for natural differences in *T. gondii* virulence [15] or for resistance to drugs in the malaria parasites [16]. Genome wide quantitative trait locus (QTL) mapping was shown to be a powerful method to analyse the genetic basis of a specific trait in the progeny from genetic crosses between parental strains that differ with respect to a given phenotype. Alternatively, gene identification can be achieved by functional complementation with cDNA or even better with cosmid libraries that circumvent the inherent redundancy of cDNA libraries [17]. More recently, the power of large scale genome sequencing and analysis offers a new solution to the problem [18].

Of the approximately 5000 genes in *P. berghei*, 458 (as of February 2011) have been analysed using reverse genetics and reported in a database (www.pberghei.eu). This source of information is updated weekly with the latest data from Medline searches and with unpublished results from various laboratories working on genetically modified rodent malaria parasites [19]. This database is an invaluable tool for researchers. It contains data on genes whose knockout results in abnormal phenotypes and key negative results, such as failure to generate knockouts or knockouts that exhibited no phenotype based on classic assays. Previously, such data have been absent from the public domain, which is biased towards the reporting of knockouts with strong and clear phenotypes. Similar databases would be beneficial for *P. falciparum* and *T. gondii*, as suggested in [20].

To increase the number of genes that are assessed functionally, it is necessary to move from a gene-by-gene method to a large-scale strategy based on high-throughput approaches. Moreover, as many genes are essential for parasite survival, there is an urgent need to develop and optimise strategies for conditional gene expression controlled at the transcriptional, post-transcriptional or even post-translational levels.

Previous reviews have described in detail the tools available for genetic manipulation in apicomplexan parasites [21,22]. This review first compares the genetic features of different Apicomplexans and explains their impact on the accessibility to the panoply of genetic tools. Second, we will address recent developments that have arisen since the aforementioned reviews and have promoted gene function analysis in *P. falciparum*, *P. berghei* and *T. gondii*. Progress in developing large-scale strategies in the form of chemical and genetic screens and various approaches to controlling gene expression will be presented.

2. Parasite accessibility and limits to genetic manipulation

2.1. The malaria parasites

The specificities of parasite propagation in hosts are critical to their accessibility to genetic manipulation. The malaria parasites

exhibit a complex life cycle involving the intermediate host (human) and the definitive host (female anophelids), which also serves as the vector for transmission. The asexual stages in humans have a haploid genome, which facilitates the generation of mutants. The erythrocytic stages are used as the source for genetic manipulation. The cycle is continuously maintained in culture in the case of *P. falciparum*, whereas a single round can be passaged in vitro for *P. berghei*, which is otherwise maintained in rodents. The continuous culture of parasites remains a challenge for *P. vivax* because its restriction to reticulocytes for invasion considerably complicates its maintenance in vitro. Several groups are currently developing procedures for culturing *P. vivax* in vitro using stem cell technologies to allow production of reticulocytes and hopefully will render this parasite accessible to transfection [23].

Critical parameters for successful genetic manipulation are the ability of the exogenous DNA to access the parasite nucleus and cell survival. *P. falciparum* erythrocytic stages have been transfected while inside red blood cells due to the inability to collect free, viable merozoites. Introduction of DNA molecules into cells can be achieved by chemical-based methods or biolistic particle delivery; however, in Apicomplexans, the most robust and reproducible results have been obtained using electroporation. The poor transfection efficiencies, in the range of 10^{-6} for *P. falciparum*, are probably due to the relative inaccessibility of the parasite within the red blood cell [24]. A recent protocol has been developed for the release of viable free merozoites for invasion studies [25], and it is hoped that this methodology will enhance the efficiency of transfection. In *P. berghei*, schizonts matured in vitro are purified prior to transfection and are returned to the animal for cultivation and drug selection. Since the development of nucleofection[®] by Amaxa and its optimisation for *P. berghei*, much higher frequencies of transfection can be achieved [26]. It is thought that this method produces square wave pulses that enhance pore-formation and do not require cell division for incorporation of DNA into the nucleus. The Amaxa system also offers the advantage of requiring smaller amounts of DNA than other techniques [27].

The *Plasmodium* species exhibit a strongly biased A/T-rich genome [28]. The A/T richness ranges from 79.6% (*P. falciparum*) or 67.7% (*P. vivax*) in protein coding sequences to more than 90% in intergenic regions of the genomes [1,4]. Other non-*Plasmodium* apicomplexan genomes do not present such a bias towards A/T richness. In *P. falciparum*, the high A/T content also coincides with low complexity regions within ORFs [28]. It remains unknown why *Plasmodium* bears such low complexity regions within protein coding sequences, although these sequences often code for amino acids that are oriented towards the external surfaces of proteins after folding and do not interfere with function [29]. It has been hypothesised that these sequences could be decoys for antibodies and help immune evasion by the parasite [28,30]. Such regions are also more prone to recombination [31] and could facilitate rapid evolution of parasite populations. For researchers, such A/T richness renders *Plasmodium* DNA highly unstable in *Escherichia coli*, which hampers the generation of large plasmids for transfection [32]. If overexpressed or expressed at an inappropriate time, the products of transgenes can cause deleterious effects or artefacts affecting their localisation [33]. This phenomenon is especially relevant for the *Plasmodium* species in which transcriptome analysis has revealed waves of transcripts that are tightly regulated throughout the intra-erythrocytic cycle [34]. In this respect, the constitutive promoter for *P. berghei*, eIF1 α , is frequently used to drive expression of exogenous genes and may under some circumstances, and in conjunction with a long half-life of the expressed protein, result in inaccurate observations or even parasite lethality. More recently, a series of tightly regulated stage-specific promoters has been described and exploited to control transgene expression. The circumsporozoite protein (CS) promoter is active in oocysts and sporozoites, while the promoter

of *uis4* is active only in sporozoites from mosquito salivary glands and during development in the mammalian liver [35]. More recently, a promoter of the PB103464.00.0 gene with tight liver stage specificity has been described [36]. This promoter could be exploited to drive, in a stage-specific fashion, the expression of a FLP recombinase with the aim to achieve conditional mutagenesis leading to gene deletion as discussed below [35]. Reverse genetics in *Plasmodium* has also been hampered by the limited number of practical markers for selection of stable transformants. Emergence of natural resistance to a drug or the toxicity of the drug for the host cells or animals accounts for the need for new markers. Pyrimethamine-resistant dihydrofolate reductase-thymidylate synthase (DHFR-TS), an enzyme of folate metabolism, is routinely used in *Plasmodium* species as a selectable marker [37]. In *P. berghei*, a *T. gondii* DHFR-TS gene is mainly used for this selection. It confers resistance to pyrimethamine, which can be supplied effectively in the drinking water of mice. In *P. falciparum*, the human DHFR gene is usually used, and it confers resistance to WR99210 and pyrimethamine [38]. Although WR99210 can also be used for *P. berghei* together with the human DHFR gene, this compound has been associated with gastrointestinal intolerance [39] and hence requires to be delivered daily by subcutaneous injection in mice. Fungal blasticidin S deaminase (BSD) and bacterial neomycin phosphotransferase II (NEO) have also been adapted for *P. falciparum* [40]; however, cases of resistance to blasticidin have been reported [41]. Dihydroorotate dehydrogenase (DHOD), an enzyme of the pyrimidine biosynthesis is essential for malaria parasites due to the absence of salvage pathway for pyrimidines [42]. PfDHOD is dependent on ubiquinone regeneration by the mitochondrial electron transport chain (mtETC). The ectopic expression of *Saccharomyces cerevisiae* DHOD, which do not require ubiquinone as an electron acceptor in *P. falciparum* established that parasite survival in the absence of mtETC (in presence of atovaquone) depends only on pyrimidine biosynthesis [42]. ScDHOD has been successfully used as a new selectable marker gene for *P. falciparum* [43]. The shortage of selective marker genes can be overcome by recycling them using site-specific recombinases, as first exemplified for *T. gondii* with the Cre/LOX system [44]. This strategy has recently been adapted to malaria parasites using the FLP/FRT [45,46] and Cre/Lox [45] systems. A panoply of reporter genes is listed in Table 1. It includes fluorescent proteins that can be selected by FACS analysis and genes that have been utilised to dissect various aspects of parasite development and host–parasite interactions (reviewed in [47]).

The fate of the DNA introduced into a given parasite is a determinant of the type of reverse genetic tools that can be applied. *Plasmodium* species have the ability to maintain plasmids carrying transgenes as stable episomes, while integration into the genome occurs almost exclusively by homologous recombination. In *P. falciparum*, these plasmids or extrachromosomal replicons are maintained in a concatemeric form [48,49]. This phenomenon is not well suited for knocking out genes by homologous recombination and requires additional steps of drug cycling to eliminate the episomes. In *P. berghei*, linearised plasmids integrate into the genome by an ends-out/replacement mechanism [12] and recombination frequency is positively correlated with the length of the homology arms in the construct (Billker, pers. communication). In *P. falciparum*, negative selection based on the thymidine kinase (TK) of the herpes simplex virus (HSV) has been used successfully [50]. In this strategy, the positive selection cassette is flanked by sequences homologous to regions upstream and downstream of the target gene, while the TK gene is positioned elsewhere on the plasmid. Under ganciclovir treatment, parasites expressing TK episomally, or after single integration of the plasmid, are poisoned by competitive inhibition of dGTP incorporation into DNA during replication, whereas double homologous recombination events lead to loss of TK and survival of the parasites. Another negative selection has also been developed for *P. falciparum*, based on the conversion

of an innocuous compound, 5-fluorocytosine (5-FC) into a toxic one, 5-fluorouracil (5-FU) by *S. cerevisiae* cytosine deaminase/uracil phosphoribosyl transferase [51]. Integration can also be favoured by recombination between phage Atp sites flanking the gene of interest and an Atp site previously inserted into the genome. The recombination is mediated by a mycobacteriophage bxb1 integrase transiently expressed on a helper plasmid [52]. The tendency of transfected plasmids to persist as episomes in the malaria parasites is unfavourable for the isolation of parasites that underwent genome integration events but in counterpart, this property can be exploited to generate artificial chromosomes that serve as useful tool for gene transfer [53].

2.2. The model organism *T. gondii*

T. gondii belongs to another branch of the phylum that includes the cyst-forming coccidians. This ubiquitous parasite has emerged as a robust genetic model, easy to cultivate, efficiently transfected by electroporation (>30%) [54] and amenable to reverse genetic manipulation [55]. Attempts to optimise transfection by using other protocols have not resulted in higher efficiencies than electroporation with a BTX electroporator [27].

Similar to the *Plasmodium* species, *T. gondii* also displays a profile of transcripts that are tightly regulated during the cell cycle [56]. Therefore, the use of a constitutive promoter, such as the α -tubulin promoter, can in some circumstances be detrimental for survival and interfere with proper targeting. Epitope-tagging at an endogenous locus preserved native expression levels and proved to be the most suitable strategy to assess the level of expression of a gene and the subcellular localisation of its product. Inconveniently, stable transformation in *T. gondii* results primarily in random integration of the plasmid into the genome, as the parasite is not able to maintain episomes [54]. Ku80 is implicated in a DNA double-strand break repair mechanism called non-homologous end-joining repair (NHEJ) and is responsible for the high rate of random integration of transfected DNA. Transfection in Δ Ku80 strain of *T. gondii*, in which random integration is abolished, leads to the production of parasite mutants resulting almost exclusively from homologous recombination events [57,58]. This strategy reduces the size of the flanking sequences required to \leq 500 bp [57,59]. Importantly, Ku80 has been implicated in various additional cellular processes, including chromatin maintenance. As a result, the Δ Ku80 strain, which otherwise behaves the same as wild-type parasites, is more sensitive to double-strand DNA breaks [57] and may not be suitable for protocols that expose the parasite to stresses that can cause such breaks (e.g., chemical or UV mutagenesis). The *T. gondii* Δ Ku80 strain behaves like the *Plasmodium* parasites in which genome integration of DNA occurs mainly by homologous recombination [60]. Consistent with this behaviour, studies of DNA repair mechanisms in various protozoa showed that the Ku70 and Ku80 proteins are absent from the *Plasmodium* genomes [1,28,60]. Homologous recombination can be used not only for protein tagging by fusion with an epitope tag or a reporter gene but also for disrupting non-essential genes.

The close relationship between coccidian parasites has been explored to study the genes from parasites that are resistant to genetic techniques. *Eimeria* sequences have been expressed in *T. gondii* [61] leading to informative trans-genera complementation [62]. Inversely, there are examples of insightful expression of *T. gondii* genes in *N. caninum* [63] or in *P. berghei* [64]. A large array of selectable markers is available in *T. gondii*, including those markers originally developed to establish stable transfection, such as DHFR-TS [65], the bacterial chloramphenicol acetyl transferase (CAT) [66] and the bleomycin (BLE) selection, which confers resistance to phleomycin [67,68]. The hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene takes advantage of parasite

Table 1
Examples of reporter genes available in Apicomplexa and their use.

Reporter gene	Uses
Fluorescent reporters	
<i>gfp/egfp</i>	Pf/Pb/Tg Ex: intra vital imaging of parasites [133]
<i>rfp/Dsred/mCherry</i>	Pf/Pb/Tg Ex: visualisation of sub-cellular structures such as organelles [139]
<i>yfp</i>	Tg Ex: monitoring of protein localisation – FACS sorting [72]
Enzymatic reporters	
β -galactosidase	Pb Ex: quantification of parasite transmission [134] Tg Ex: assessment of parasite growth under drug treatment [141]
CAT	Pf Ex: assessment of transfection efficiency [135] – Tg Ex: monitoring parasite growth [66]
luciferase	Pb/Tg Ex: visualisation of parasite load and location within live animals [138,140] High-throughput screening of drugs [136,142] Analysis of promoter element activities [137]
<i>dual-luciferase</i>	Pf/Pb/Tg Renilla and Firefly luciferase co-expressed in the same parasite Ex: comparison of promoter activities [36]
Selectable markers	
Positive	Used in/conveying resistance to
HXGPRT	Tg/mycophenolic acid [69]
CAT	Tg/chloramphenicol [66]
<i>Tgdhfr-ts</i>	Tg/Pb/Pf pyrimethamine [37,65]
<i>hdhfr-ts</i>	Pb/Pf pyrimethamine, WR99210 [38]
BSD	Pf blasticidin [40]
NEO	PfG418 [40]
yDHOD	Pf atovaquone [43]
BLE	Tg phleomycin [67,68]
Negative	
CD/UPRT	Pf cytosine deaminase/uracil phosphoribosyl transferase [51]
HSV-TK	Pf/Pb ganciclovir [50]
HXGPRT	Tg 6-thiouridine [70]
YFP	Tg/FACS sorting [72]

auxotrophy for purines and exhibits a great versatility as both a positive and negative selective marker [69,70]. A dual selection can also be achieved with the salvage enzyme, uracil phosphoribosyl-transferase (UPRT) [71]. YFP can be used as a negative selectable marker gene when combined with the fluorescence activated cell sorting of the recombinant parasites [72]. Furthermore, all these selectable marker genes can be recycled by applying the Cre/LOX system [73], and the simultaneous insertion of multiple transgenes can be achieved by applying restriction enzyme-mediated insertion (REMI) [74].

3. From gene to function

Gene disruption at a specific locus can be achieved by a double homologous recombination leading to the replacement of the targeted gene by the cassette expressing the selectable marker gene. The recovery of parasites that have undergone homologous recombination events has been hindered, particularly in *T. gondii*, by the high frequency of random integration. Vectors containing large regions of homologous sequences ranging from 2 to 16 kbp have been designed to increase the frequency of homologous integration [66,75,76]. To circumvent the difficulties in generating large plasmids, the recombineering strategy [77] has been successfully applied to *T. gondii* using genomic cosmid libraries (approx. 45 kb) to generate knockouts [78]. Alternatively, the use of the *T. gondii* Δ Ku80 strain has facilitated the recovery of homologous events. Knockout mutants resulting from single crossovers in the middle of the gene of interest are easily obtained. One caveat is the reversible nature of this event, which can lead to restoration of the wild-type locus. Once the non-essentiality of a gene is established, it is recommended to generate a clean gene disruption by a double crossover, which is favoured when the 5' and 3' regions for homology are free at both ends of the linearised vector. Repeated failures to obtain an allelic replacement in the *T. gondii* Δ Ku80 strain or in

P. berghei strongly argue for the essentiality of the target gene [57,79]. In such cases, alternative approaches to identify gene function are necessary and are discussed below. Phenotype(s) assessment of a mutant obtained by a gene knockout requires validation by reintroducing a wild-type copy of the gene into the mutant with resulting rescue of the phenotype. Ideally, the complementing transgene should be driven by its own promoter. In *Plasmodium* species, complementation of gene knockouts has not been performed routinely. Instead, the isolation of two independent mutant clones exhibiting the same phenotype has been found sufficiently compelling. In view of the recent improvements in transfection technology, a request has been made to the malaria research field that complementation of knockout strains should now become a standard procedure [80].

By focusing on one-by-one gene knockout approaches, laboratories have succeeded in generating large numbers of mutants, with one study alone in *P. falciparum* producing and analysing 53 transgenic parasites implicated in one aspect of the parasite's biology [81].

However, the generation of mutants on such a scale is heroic and requires a large amount of manpower, making alternative strategies important.

4. From function to gene

Forward genetics is a powerful approach to identify a single gene or set of genes implicated in a biological process for which a given phenotype can be screened or selected. Mutants can either be natural, selected or induced chemically. N-Nitroso-N-ethylurea (ENU) is an alkylating agent used to introduce point mutations into parasite genomes. A key feature of a library is that the mutants harbour an average of one mutation per genome. If the biological process to be studied is essential for parasite survival and propagation, the mutagenesis is followed by the selection of temperature

sensitive (ts) mutants. This approach was pioneered for *T. gondii* by Elmer Pfefferkorn in the 1970s [82].

Recently, numerous essential genes implicated in the cell cycle control of *T. gondii* have been identified by the generation of a ts-library followed by functional complementation based on the cosmid genomic library [17,83]. Initially, complementation was performed using cDNA libraries [84,85]; however, this strategy proved to be limited by the bias of the cDNA library itself, in which some cDNAs were more abundant than others, and therefore complementation failed to cover all parasite genes. Complementation of a mutant with a cDNA library requires both a high transfection efficiency and a high frequency of transformation to cover the full genome. In contrast, a cosmid library offers better coverage of the genome and a reduction in the number of complemented mutants, as each cosmid contains more than one gene sequence. Alternatively, the high frequency of random integration in *T. gondii* has also been utilised to generate libraries based on insertion mutagenesis. Such an approach was developed to study the mechanism governing the conversion between tachyzoites and bradyzoites [86,87]. In this approach the gene of interest is identified by plasmid rescue.

In contrast, in organisms such as some *Plasmodium* species in which genome integration occurs almost exclusively by homologous recombination, site-specific transposable elements can be exploited. The extent of genome coverage depends on the abundance and distribution of the target sites. The first shuttle transposon mutagenesis in *P. berghei* [88] involved Tn5-derived mutagenesis. This strategy included a step in *E. coli* that has been shown to be technically cumbersome. More recently, a piggyBac transposon, which mediates insertion at TTAA sites throughout the genome [89], supplanted the previous system. In an attempt to generate a middle-scale screening based on this strategy, the authors generated approximately 180 mutants, among which 39 were inserted within gene coding sequences. Although this approach resulted in a modest yield of genes analysed, one can predict improvements concomitant with increased transfection efficiency in *P. falciparum*. Recently, this approach has been successfully adapted to *P. berghei* [90].

Additional limitations are encountered when searching for the identity of a gene responsible for a phenotype in malaria parasites. In *P. falciparum*, due to the low transfection efficiency, complementation has so far not resulted in satisfactory numbers of genes being identified in insertion mutant collections [89]. The tremendous improvement in sequencing technologies is a powerful solution to this problem. For example, genes conferring resistance to drugs have been identified by genome-wide high-density tiling microarrays, DNA sequencing, and copy number variation analysis of mutant parasites [18].

Chemical genetics has emerged in the last few years as a novel, potent approach that exploits libraries of small molecules to affect biological processes and eventually identify the targeted genes. A high-throughput microscope-based assay was developed to select compounds interfering with *T. gondii* motility and invasion [91]. More recently, a small-molecule inhibitor identified in that screen was shown to target the myosin light chain 1, a key component of the gliding machinery conserved in all Apicomplexans [92]. *P. falciparum* is also amenable to such a high-throughput approach as exemplified by a recent phenotypic forward chemical genetic screen aimed at the discovery of new anti-malarial drugs [93].

5. The headache of essential genes

When a gene is essential in the stage of the life cycle that can be manipulated, a classical gene knockout approach cannot be used to characterise it. Unravelling the function of an essential gene can be

achieved, however, by controlling its expression at several possible levels. Inducible systems control expression at the transcriptional level, whereas RNAi or ribozyme-mediated strategies influence the expression post-transcriptionally at the level of mRNA stability. Ultimately, it is also possible to modulate expression post-translationally at the level of protein stability.

5.1. Control at the transcriptional level

A commonly used methodology to control gene expression is based on the tetracycline-repressor (TetR) system of *E. coli*, which in the Apicomplexans was first adapted in *T. gondii* [94]. The bacterial TetR system can regulate gene expression by interfering with the initiation of transcription. In this scenario, the tetracycline operator (*tetO*) sequences are placed between the cis-acting element of the promoter and the site(s) of initiation of transcription. The binding of the TetR repressor physically interferes with transcription. Upon addition of tetracycline, this interference is alleviated by the release of TetR from the *tetO* sequences, resulting in gene expression. The TetR system was further improved in *T. gondii* by a fusion between a YFP gene and the bacterial TetRep, which resulted in a 6-fold increase in gene expression compared to that using TetRep alone [95]. Despite this step up, the need to maintain parasites under anhydrotetracycline (Atc) for the generation of conditional mutants was a limitation [96]. In contrast, a system based on a tetracycline-controlled transactivator (tTA) is one of the most prominent and widely accepted inducible systems so far. The Tet-off version allows an inducible expression of a gene of interest, which can be repressed in the presence of the drug. The tTA consists of a fusion between the tetR and the activating domain of Herpes simplex protein VP16. In this system, the gene of interest is placed under the control of a minimal promoter fused to several *tetO* sequences. When introduced into cells expressing the tTA, gene expression is activated and can be silenced by addition of tetracycline. Although the tetR system proved to be functional, a tTA system failed to work in *T. gondii* because the activating domain of VP16 does not interact with the parasite transcription machinery [94]. A genetic screen based on random insertion of TetR in *T. gondii* was performed to generate the transactivators Tati-1 and Tati-2 and led to the first conditional disruption of an essential gene [97]. This system was adapted to *P. falciparum* using the *Toxoplasma*-derived transactivators, and it allowed regulated expression of GFP from episomes [98,99]; however, expression was not robust enough when the transgenes were stably integrated as single copy. To improve the robustness of the system a more potent activating domain appeared to be necessary. A rudimentary transcription machinery with a very limited number of transcription factors was initially described in Apicomplexans [96,100]. The seminal discovery of a family of apicomplexan-specific DNA binding proteins, called ApiAP2 (apicomplexan APET-ALA2) [101], has considerably changed this view. Recent studies have validated several members of this family as bona fide transcription factors [102,103]. Mapping of the activating domains on some ApiAP2s allowed the generation of new transactivators fused to TetRep and called TRADs (TetRep-Activating domains). The strength and tightness of TRADs as inducible transactivators were tested in *T. gondii* and further assessed in *P. berghei* with the reporter gene integrated as single copy (Pino et al., unpublished). This new generation of tet-inducible systems has now been validated by disruption of two essential genes in *P. berghei*. This technology fills an important gap in the tools available for conditional gene expression in *P. berghei* and raises hopes for application in *P. falciparum*.

An elegant alternative strategy for conditional expression has been developed for *P. berghei* using a stage-specific expression of Flp recombinase to mediate gene deletion [35]. The Cre and Flp recombinases recognise specific short DNA sequences (the LOX

and FRT sites, respectively) and excise any DNA sequence between the two sites. The power of the Cre recombinase activity has hampered its utilisation in a controlled fashion [44]. Efforts to develop a conditional gene targeting approach have therefore focused on adapting the Flp recombinase, whose activity can be made temperature-sensitive [104]. The FRT sites have been introduced into the genome by double homologous recombination, flanking the 3' regulatory sequences of the target gene in a strain expressing the recombinase. The Flp recombinase gene expression is stage-specifically controlled by the regulatory sequences of genes, such as *Pbuis4*, which is expressed in sporozoites only. This strategy results in the loss of gene expression by removal of the FRT-flanked sequence in a stage-specific manner, such as during the liver stage in the case of *uis4* [35]. Gene essentiality for intraerythrocytic stages can be established via this strategy, however the phenotypes cannot be adequately investigated. Significantly, the impact of removing the 3' regulatory sequences on the level of gene expression is unpredictable and in some circumstances might not be sufficient to produce a phenotype due to residual expression. There is significant potential to adapt this system to *P. falciparum* using temperature shift as a trigger for the conditional expression of the recombinase [35].

5.2. Control at the post-transcriptional level

The emergence of RNA interference (RNAi) as a method of choice for targeting gene expression in various eukaryotes has motivated several laboratories to try to establish this technology in Apicomplexans. Success has been elusive, however, and whether such a technique is applicable has remained a matter of debate [105]. Furthermore, recent work has highlighted evolutionary differences between *Plasmodium* spp. and *T. gondii* [106]. While previous studies reported down regulation of gene expression by RNA degradation in *P. falciparum* [107–109] and *T. gondii* [110], compelling evidence that the effects observed were due to an RNAi-dependent mechanism was lacking. Through a rigorous experimental approach, Baum et al. ruled out the functionality of RNAi in *P. falciparum*, either introducing dsRNA directly into infected red blood cells by electroporation or by adding siRNAs to the culture medium [106]. Although the uptake of siRNA by *P. falciparum* could not be demonstrated, electroporation of *P. berghei* parasites with siRNA using the Amaxa method also failed to lead to silencing. Comparative genomics (Hidden Markov models) and phylogenetic approaches were applied to search for structurally related but potentially highly divergent homologues to RNAi machinery-related genes in *P. falciparum*. However, no homologues for Dicer and Argonaute have been found in malaria parasites although these genes are present in *T. gondii* [106]. Furthermore, components of RNA-induced silencing complexes (RISCs) have been identified in *T. gondii* by mass spectrometry as interacting with TgAGO and miRNAs [111]. The absence of retrotransposons and viruses in *P. falciparum* and *T. gondii* genomes is consistent with the role of RNAi in mediating gene silencing of transposable elements and in fighting against virus invasion [112,113]. Interestingly, no endogenous miRNAs have been detected in *P. falciparum* [114,115], while a complex set of miRNAs has been described in *T. gondii*, consistent with the presence of the RNAi enzymology [111]. Despite the presence of key players in RNAi machinery in *T. gondii*, its utilisation as a tool to silence gene expression has remained marginal. Antisense RNAs can also dramatically drop the levels of corresponding sense RNAs in an RNAi-independent fashion. Interestingly, *P. falciparum* harbours abundant levels of endogenous antisense transcripts [116]; however, the antisense approach does not appear to modulate gene expression reliably in this parasite [106].

An alternative method to regulate gene expression at the mRNA level has been reported for *T. gondii* using ribozyme-mediated

down regulation of the targeted mRNA [117]. Suppression of gene expression can be achieved by adding an 85 bp sequence coding for a hammerhead ribozyme and positioned upstream of the start codon of the endogenous locus. Gene expression can be partially rescued upon addition of toyocamycin, an adenosine analogue inhibitor of hammerhead activity [118]. The main limitation of this strategy appears to be the toxicity of toyocamycin for the parasites. Deletion of the parasite adenosine kinase activity, known in mammalian cells to participate in the toxic effect, conferred resistance to the drug; however, the regulation of the ribozyme was simultaneously lost [117].

5.3. Control at the level of protein stability

A fast response strategy known as the “destabilisation domain (DD) system” has been recently developed to control gene expression at the level of protein stability in mammalian cells [119]. When fused to the target protein, the FK506-binding protein degradation domain (ddFKBP) dramatically interferes with protein stability, resulting in rapid degradation of the protein by the proteasome. Rescue of protein stability can be achieved by adding a rapamycin-derived ligand called shield (shld-1), which specifically interacts with the ddFKBP, folds it and blocks degradation [119]. The reversible and rapid nature of this process makes it very appealing for controlling gene expression conditionally, and it has been reported to work in both *T. gondii* and *P. falciparum* [120,121]. This system allows the conditional degradation of a vital protein at a specific time and the investigation of its function. Such an allelic replacement, leading to a ddFKBP fusion with an endogenous protein, has been reported in *P. falciparum* [122,123], but has also highlighted the limitations of the system primarily due to the toxicity of the shield that needs to be permanently provided in the culture. Moreover the system has not worked satisfactorily in *P. berghei* possibly due to the poor bioavailability in vivo. More attractively, this system provides an exquisite approach to control the expression of dominant negative mutants or toxic genes. Proteins well suited for the generation of dominant negative mutants include the Rab family of GTPases and dynamins [124–127] as well as proteins acting as part of a complex or as homodimers, such as the formins [128]. Other catalytically inactive enzymes can also act as dominant negative mutants by sequestering their substrates [129].

The destabilisation domain works optimally when positioned at the N-terminus of the target protein and hence is not well suited for proteins harbouring a signal peptide and targeted to the secretory pathway. However, some polytopic proteins such as the rhomboid proteases, which exhibit an N-terminal cytosolic tail, respond well to destabilisation [129].

This approach remains suboptimal for generating conditional knockouts because shld-1 must be continuously present in the parasites. Currently, the cost of shld-1 is prohibitive and, more importantly, the compound is associated with toxicity in long-term experiments [122]. Moreover, the bioavailability of shld-1 might constitute a limitation for applications in *P. berghei*, although the ligand has been administered successfully to mice [130]. A conceptually similar approach, based on another degradation domain has recently been reported for mammalian cells [131] and adapted successfully to *P. falciparum* [29]. This method overcomes the previously mentioned drawbacks. The system is based on a mutant of the *E. coli* dihydrofolate reductase (ecDHFR) engineered to be degraded and called the DHFR degradation domain (DDD). The DDD can be stabilised using trimethoprim (TMP), an inexpensive folate analogue [131]. DDD has been combined with a fluorescent marker and an epitope-tag to generate a so-called regulatable fluorescent affinity (RFA) tag that can be studied either episomally or after tagging at an endogenous locus [29]. The rapid, reversible stabilisation

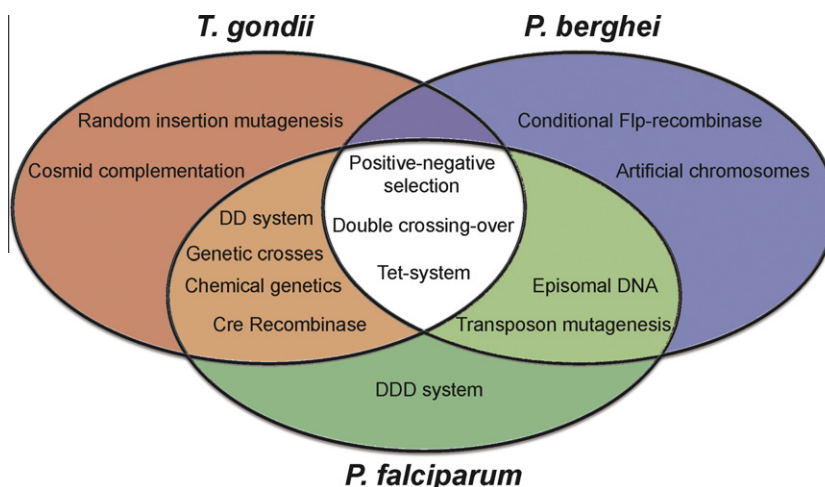


Fig. 1. Summary of the available tools for gene manipulation in Apicomplexan parasite. Scheme representing the available tools for each parasite: *T. gondii* in red, *P. falciparum* in green, *P. berghei* in blue, as well as in the intersecting sectors the tools shared between more than one parasite. The white sector in the centre contains the tools established in all three models.

of the fusion protein upon addition of low concentrations of TMP (50–100 nM) is comparable to the dynamics observed with ddFKBP fusions; however, DDD requires working with *P. falciparum* strains containing a human DHFR (hDHFR) marker to alleviate the natural toxicity of TMP for the parasite [29]. The strong inhibition by TMP of ecDHFR compared to hDHFR, in addition to the good pharmacological properties of the compound (e.g., crossing the blood–brain and placental barriers) proved to be suitable for studies on mammalian systems [131]. This raises hopes for its application to *P. berghei*. Adaptation of either the Tet-system, ddFKBP or DDD techniques to conditional knockout approaches in the vector and liver stages of *P. berghei* still represent a significant challenge in terms of accessibility of the inducers.

6. Concluding remarks

The research community has dedicated tremendous and innovative efforts to overcoming the multiple barriers encountered when analysing gene function in apicomplexan parasites. A scheme summarising the available tools for gene manipulation that have been tailored to the specific feature of each parasite is presented in Fig. 1. A combination of the technologies recently established and their adaptation to genome wide approaches can now be envisioned. Along these lines, the high random integration frequency in *T. gondii* has recently been elegantly adapted to direct insertion events by a promoter trap with a drug-selectable marker and to concomitantly substitute the promoter of the trapped gene with a conditional Tet-transactivator promoter [132].

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