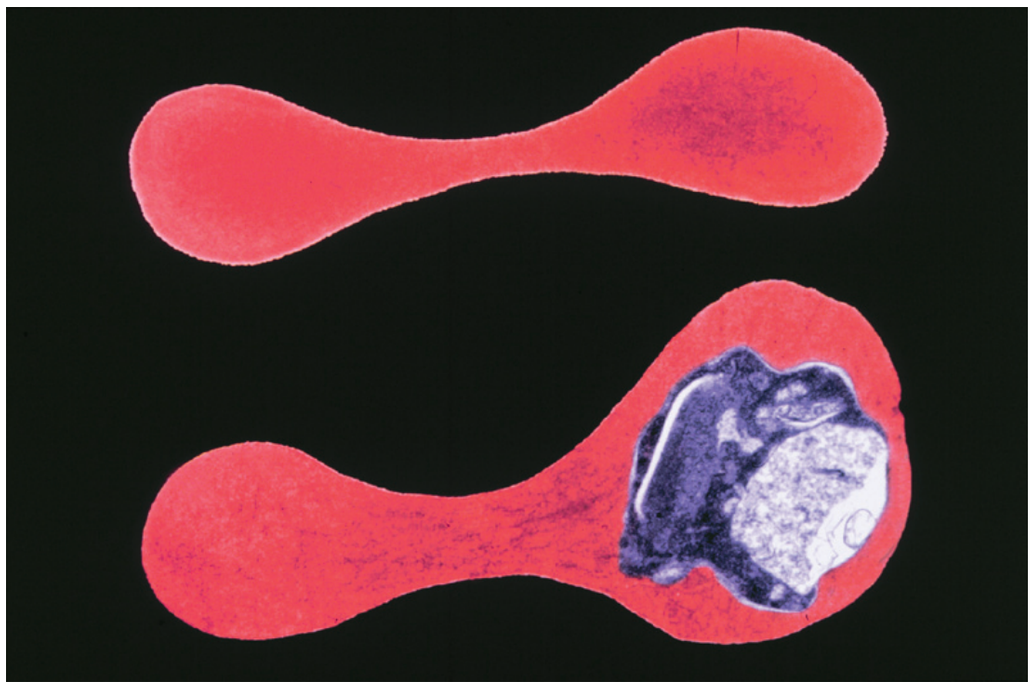
THE
JOURNAL
OF

Eukaryotic Microbiology

Volume 61 | Issue 6 | November/December 2014



Published by the
International Society
of Protistologists

WILEY
Blackwell

Journal of Eukaryotic Microbiology ISSN 1066-5234

REVIEW ARTICLE

From the Genome to the Phenome: Tools to Understand the Basic Biology of *Plasmodium falciparum*

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Keywords
Apicoplast; drug target; genetic manipulation; knockout; malaria. metabolism; phenotype.

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Received: 4 August 2014; revised 1 September 2014; accepted September 2, 2014.

doi:10.1111/jeu.12176

ABSTRACT

Malaria plagues one out of every 30 humans and contributes to almost a million deaths, and the problem could worsen. Our current therapeutic options are compromised by emerging resistance by the parasite to our front line drugs. It is thus imperative to better understand the basic biology of the parasite and develop novel drugs to stem this disease. The most facile approach to analyse a gene's function is to remove it from the genome or inhibit its activity. Although genetic manipulation of the human malaria parasite *Plasmodium falciparum* is a relatively standard procedure, there is no optimal method to perturb genes essential to the intraerythrocytic development cycle—the part of the life cycle that produces the clinical manifestation of malaria. This is a severe impediment to progress because the phenotype we wish to study is exactly the one that is so elusive. In the absence of any utilitarian way to conditionally delete essential genes, we are prevented from investigating the parasite's most vulnerable points. This review aims to focus on the development of tools identifying essential genes of *P. falciparum* and our ability to elicit phenotypic mutation.

UNDERSTANDING THE BLUEPRINT OF *PLASMODIUM FALCIPARUM* GENES

The genome of *Plasmodium falciparum* is a chimera of two eukaryotes and two prokaryotes currently housed on 14 nuclear chromosomes, a 6 kb reduced mitochondrial genome, and a 35 kb apicoplast genome (Gardner et al. 2002). Publication of the *P. falciparum* strain 3D7 genome in 2002 allowed parasitologists to accelerate the task of assigning function to the ~5,600 genes (Painter et al. 2013). Postgenomic studies indicate that only ~8% of *P. falciparum* genes are involved in metabolism compared to 17% of the ~6,000 genes in *Saccharomyces cerevisiae* (Goffeau et al. 1996). Such departures from classic eukaryotic *model* systems are hardly surprising for a parasite, where we see a high number of orphan or *hypothetical* genes that share no sequence similarity with any familiar and well understood genes (Gardner et al. 2002). In fact, at the time of initial genome publication ~60% of *P. falciparum* genes remained hypothetical, 10 yr post we are still only at ~50%.

IN VIVO/IN VITRO CONFORMATION OF IN SILICO GENE FUNCTION PREDICTIONS VIA GENETIC MANIPULATION OF PARASITES

Genomic analysis of *P. falciparum* has expanded greatly since the introduction of transfection technologies in the mid-1990s (Crabb et al. 1997; Wu et al. 1995, 1996). The ability to genetically alter the genome and express monocistronic chimeric genes has identified a great number of cellular processes essential to parasite development.

Several methodologies enable the introduction of exogenous DNA into *P. falciparum*. Parasite transfection requires the delivery of DNA across several membranes, and establishment and refinement of transfection protocols to achieve this goal has employed chemical induction (Mamoun et al. 1999b), lipid nanoparticle conjugation (Gopalakrishnan et al. 2013), and electroporation, with only the latter providing consistent reliability. Initially, electroporation was performed using high voltage/low capacitance electric pulse (Wu et al. 1995), however this practise has been replaced with the more efficient low voltage/high

capacitance electroporation of ring stage parasites (Fidock and Wellems 1997). Spontaneous uptake of exogenous DNA can also be achieved by *preloading* erythrocytes prior to infection by asexual merozoites (Deitsch et al. 2001). Direct ring stage transfection efficiency is low, 10^{-2} to 10^{-5} for transient and 10^{-6} for stable episomal expression, and penetrance can be variable within the population (Crabb et al. 1997; O'Donnell et al. 2002; VanWye and Haldar 1997). *Preloaded* transfection can produce a variable 5–180 fold increase in efficiency compared to direct electroporation (Hasenkamp et al. 2012).

Transfection is currently time and resource exhaustive, requiring large amounts of initial plasmid (~100 μg of plasmid is the current protocol for our lab) and consistent replenishment of media and O^+ human erythrocytes. Recovery timeframes are variable, ranging from 12 to 60 d, and constant visual monitoring is required to confirm resurgence of drug-resistant parasites. Vector DNA remains episomal, forming concatameric structures containing multiple copies of the transgene and resistance expression cassettes (Kadekoppala et al. 2001). The time-lag between electroporation and reemergence is related to the parasite's capacity to segregate plasmids to subsequent generations to match growth rate to drug selection. To aid plasmid segregation between sister merozoites vectors typically contain a 1.4 kbp region composed of the 21 base pair tandem repeat element, Rep20, to tether plasmids to chromosome telomeres (O'Donnell et al. 2002).

Transgenic parasites are selected via expression of a drug resistance cassette on the vector backbone, and multiple or cotransfections are achievable, yet limited by the dearth of resistance cassettes available for *P. falciparum.* Spontaneous resistance to some markers, especially when used in combination, is a further bugbear (de Koning-Ward et al. 2001; Fidock and Wellems 1997; Ganesan et al. 2011; Mamoun et al. 1999a). Transfection is typically performed in singular restricting the entire exercise to low or medium throughput. A new plate-based multi-transfection protocol, using square electro-wave pulse, has recently been established by the DeRisi lab at UCSF (Caro et al. 2012). Reemergence of parasites is monitored using *Renilla* Luciferase expression and potential users can mimic and adapt their protocol after viewing their website and Youtube video (http://derisilab.ucsf.edu/).

EXOGENOUS GENE EXPRESSION

Many initial transfection studies used genetic manipulation as a method to study temporal gene expression, and in doing so developed a minimal expression tool using deletion mapping of native *P. falciparum* genes. Wu et al. (1995) provided the first transgenic *P. falciparum* strain expressing chloramphenicol acetyltransferase (CAT) by flanking its open reading frame (ORF) between various 5′ and 3′ untranslated sequences (UTRs) copied from the parasite genome (Wu et al. 1995).

Episome housed reporter proteins have been used to localise various parasite proteins to specific sub-components including the nucleus, apicoplast, mitochondria, secretory system, invasive organelles, host cytosol and cellular membrane (for review see—Deponte et al. 2012). Many of these localisations have bioinformatic and biochemical backing via the identification of specific targeting motifs such as PTEX (Bullen et al. 2012; de Koning-Ward et al. 2009) and apicoplast N-terminal bi-partite leader sequences (Foth et al. 2003; Tonkin et al. 2008), and, combined with microscopic information (McMillan et al. 2013; Riglar et al. 2013), provide localised function and proof construction of many predicted metabolic maps (Ralph et al. 2004; van Dooren et al. 2006; Yeh et al. 2004). The wealth of cell targeting information has spurred the creation of a categorised websites such as plasmodb.org, MPMP (Ginsburg 2006), and ApiLoc (http://apiloc.biochem.unimelb.edu.au/apiloc/apiloc) to link genomic, biochemical, and literature databases. However, at the current rate of low throughput genetic manipulation, it would take until 2,050 until the localisations of all *P. falciparum* proteins were confirmed (Woodcroft et al. 2012). Reporter protein expression is also used to assess cell viability with response to drug pressure (Khan et al. 2012; Sanchez et al. 2007; Wilson et al. 2010).

CLONING *P. FALCIPARUM* GENES

Given the high A/T genomic content of *P. falciparum*, cloning and construction of expression plasmids can be difficult. A method to produce versatile expression plasmids was developed (Skinner-Adams et al. 2003; Tonkin et al. 2004) where various UTRs and ORFs flanked by bacteriophage *att* sites are constructed separately and later combined, in order, into a single expression plasmid using λ-phage LR™ Clonase® (Life Technologies, Carlsbad, CA). Transgene expression can thus be designed to match native levels through design and copy of native UTRs into donor plasmids. However, these plasmids require two expression cassettes, the transgene and resistance gene, and can be large in size (~10,000 bp), and potentially problematic for propagation within *Escherichia coli*.

To reduce plasmid size, Epp et al. (2008) used a *var* gene intron with bi-directional promoter activity to create a single expression cassette. It was shown through the sequential increase in concentration of selective drug blasticidin S (*bla*) that these plasmids, which express *Aspergillus* blasticidin S deaminase (BSD) that converts blasticidin S to a nontoxic deaminohydroxy derivative, increased in copy number and concatameric size (Epp et al. 2008). Essentially, this method exploited innate adaptive qualities of *P. falciparum* populations by selecting merozoites with a greater tolerance to blasticidin S and a consequential increase in transgene expression followed. Although a slow process, 8 wk were reported to produce a 10-fold increase in luciferase expression, regulation of expression without the need to reconstruct and retransfect parasites is possible (Epp et al. 2008).

Recently, use of the short self processing viral 2A *ribosome skip* peptide sequence enabled polycistronic expression of individual polypeptides from a single promoter

making it possible to reduce the size of the plasmid by removing the need to house two expression cassettes (Kreidenweiss et al. 2013; Straimer et al. 2012). Many of these plasmids mentioned here are available upon request from MR4.org and we support collective growth of this repository.

Transfection and transgene expression has offered a wealth of information on the biology of the parasite. However, the most informative approach is the removal/replacement of a gene or its regulatory elements.

GENE FUNCTION ABLATION IN *P. FALCIPARUM*

The methodologies employed to remove or modify gene function in *P. falciparum* rely on the same cell-intrinsic machinery for DNA repair—the dysfunction of which appears to aid in the evolution of drug resistance (Bethke et al. 2007; Castellini et al. 2011) and can also increase the chance of mutant production. The DNA rejoining/arranging process, termed homologous recombination, is initiated by the introduction of DNA double-stranded breaks and is concluded by strand exchange between two highly similar sequences of DNA (Kirkman et al. 2014), which for gene modification purposes involves a cloned sequence housed on a plasmid and a single parental genomic copy. *Plasmodium falciparum* tolerates up to ~5% difference in sequence identity between parasite genome and cloned sequence for homologous recombination and operates best when the overlapping regions are 600–1,200 bp in length.

To promote homologous recombination between the native locus and a donor plasmid, negative selective drug pressure is applied in 2–3 wk cycles and integration into the region of interest is monitored by PCR in low (Crabb et al. 1997) or high throughput conditions (Caro et al. 2012). Transfected cell cultures are a mixed populace of parasites containing wholly episomal, integrated and episomal, and wholly integrated individuals (Rug and Maier 2013; Solyakov et al. 2011). Differential growth kinetics is observed between individuals within the population and integrated parasites typically display higher growth rates compared to episomal individuals when negative selective pressure is reapplied as these parasites do not have to segregate plasmids to daughter merozoites. Genetically, identical cultures can be isolated from a mixed population using limiting dilution (Lyko et al. 2012) or by fluorescence assisted cell sorting (Miao et al. 2010). Southern blot analysis is needed to confirm correct integration into the genome. Recent advancements in speed and cost reduction in whole genome sequencing techniques may well replace this validation method in the near future (Ghorbal et al. 2014).

Gene deletion of nonessential intraerythrocytic development cycle (IDC) genes can be achieved in *P. falciparum* by replacing a native copy with a positive selective marker via double homologous crossover. Double integration events are rare and care must be taken to avoid interference of potential regulatory sequences of neighbouring genes. Successful gene replacement is selected for by the presence of a negative selective marker on the plasmid backbone. Individual parasites retaining episomal plasmids are removed from the population via their activation as only the overlapping 5′, selectable marker, and 3′ regions of the donor plasmid are incorporated into the genome (Duraisingh et al. 2002; Maier et al. 2006).

TARGETING ESSENTIAL GENES IS PROBLEMATIC

Creating a homogenous population of transfectants using homologous recombination is technically difficult requiring at least 100 d of drug cycling and cell isolation protocols, with no guarantee of integration into the desired region of the genome. Essentially, we bully the parasite into a Jack Palance-esque *pick up the gun and fight* scenario, where retention and expression of exogenic DNA is selected against progression through the IDC. When the role of the exogenic DNA is to disrupt a gene essential to the IDC the selection scenario is not achievable. Moreover, a phenotype may never develop as the parasite dies as a result of gene loss or by expressing the exogenously derived nucleic acid. If a gene cannot be removed, but can be modified, then it is inferred essential. There is no universally accepted reasonable number of attempts at this goal to imply that failure to remove a gene via homologous recombination equates to essentiality. In any case, the reliance upon inability to delete a gene is a relatively poor indicator of indispensability. As too is the presence of a single mutant parasite as the basis of phenotypic analysis.

To build information on essentiality, retention of an episomal copy and removal of the gene is a valid method, yet few studies have used complementation, and removal of episomal plasmids from the population can be problematic. To overcome these limitations, and to increase the chance of observing a mutational phenotype in the short window between gene ablation and death, the more elegant inducible mutational systems are required to target these genes in order to view their mutational phenotype.

INTEGRATION INTO THE GENOME USING *BXB1* INTEGRASE

Targeted integration enzymes evolved during the bacterial/bacteriophage arms race, and one in particular, mycobacteriophage *Bxb1* serine integrase, capable of mediating integration of bacteriophage DNA into the *Mycobacterium smegmatis groEL1* gene, is able to insert cloned genes into the *P. falciparum* genome (Nkrumah et al. 2006). Targeted recombination of short DNA regions of inverted symmetry flanking a conserved 5′ G-T dinucleotide occurs between short host *att*B and phage *att*P sites. Recombination between these sites results in the transition of DNA sequences *att*B and *att*P into asymmetric *attL* and *att*R sites, respectively, rendering them incapable of excision or reversion. *Bxb1* mediates recombination without the additional requirement of divalent cations, enzymatic cofactors, or DNA super coiling, making its use for transgenics extremely versatile.

Adaptation of the *Bxb1* integrase system as a functional *P. falciparum* genetic manipulation tool by Nkrumah and coworkers, initially relied upon the use of homologous recombination of a modified DHFR resistance cassette into the non-IDC essential 5′ region of *Pfcag6* (PF3D7_0709200) to introduce the 40 bp *att*B site into the *P. falciparum* genome (Nkrumah et al. 2006). From here, targeted integration into the genome of apicoplast localised FAS-II associated gene *P. falciparum* enoyl acyl carrier reductase (*Pfenr*—PF3D7_0615100: AKA *FAB-I*) could be achieved using their proposed *dual transfection* method involving two additional plasmids—one possessing a 50 bp 5′ *att*P *Pf*enr-*GFP* reporter expression cassette and a BSD resistance cassette, and another with the ability to express *Bxb1* integrase and a neomycin selection cassette providing resistance to drug G418. Selection using WR99210, blasticidin, and G418 resulted in a homogeneous population of *Pfenr-GFP* integrated parasites 18 d post electroporation (Nkrumah et al. 2006).

Integration of single copy *Pfenr-GFP* into the *Pfcg6* genomic locus ensured expression of a transgene using a *bona fide* native *P. falciparum* promoter with intact genomic context, resulting in correct localisation to the apicoplast (Nkrumah et al. 2006). Their selection method removed reliance on population dynamics by episomal retained resistance cassette expression. However, this method, involves the use of three resistance markers—only one of which (resistance against Neomycin) is recyclable using their published method.

The ability to select transgenic parasites in a timeframe conducive to the proposed negative effect against IDC progression or in avoidance of potential transgene toxicity has encouraged users of *Bxb1* integrase to adapt slight changes to the method to accelerate selection. In their attempt to localise the H-protein (PF3D7_1132900) of the glycine cleavage complex to mitochondria, it was shown that selection for the plasmid possessing *Bxb1* integrase using neomycin was extending the timeframe in which viable transgenic populations returned postelectroporation (Spalding et al. 2010). Furthermore, an exchange of ring stage electroporation in favour of *preloading* of erythrocytes with plasmids, and selection for the integration event itself using only *bla*, produced a homogenous population of transgenic parasites 24 d faster in comparison to using Nkrumah and coworkers' method for the same goal (Spalding et al. 2010).

The *Bxb1* integrase system was used to analyse genetic and epigenetic factors governing the absolute and temporal control of the prototypical housekeeping gene *Pfpcna* (PF3D7_1361900) during the IDC (Wong et al. 2011). The shortened time frame of genome integration, and the ability to ensure a single genomic integration, allowed for identification of several regulatory factors within the 5′ and 3′ UTRs. A signature motif (TGTGGA) within the 5′ UTR is over-represented in genes sharing a similar temporal pattern of gene expression during the IDC. A novel 3′ *cis*-acting sequence, TGAA(AT)(AT)GG, was shown to be responsible for 50% of promoter activity (Wong et al. 2011). These regions could be involved in the recruitment and stabilisation the preinitiation complex suggesting temporal control at definable regulatory sequences.

ZINC FINGER NUCLEASES

Zinc finger nucleases (ZFNs) are metalloenzymes adapted from restriction enzymes with the ability to induce DNA double-strand breaks (DSB) at a defined target sequence. Homologous recombination in *P. falciparum* occurs at undefined sites in a stochastic manner over long time frames. The action of ZFNs can induce homologous recombination at a defined DSB site if an episomally retained donor template is provided.

Tool building is a risky endeavour and many proof of concept experiments rely on changes to fluorescence as a measure of success. Integrating the target eGFP gene into the *Pfcg6* locus using *Bxb1* integrase produced a single copy target within the genome. Disruption of this single locus was measured by loss of eGFP fluorescence from parasites. Parasites exposed to erythrocytes *preloaded* with a composite ZFN-eGFP–hDHFR donor plasmid—comprising of 2A-linked eGFP-specific ZFN expression cassette as well as 5′ and 3′ eGFP homology regions that flanked the ZFN cleavage site—lost fluorescence in 14 d under WR92210 selection (Straimer et al. 2012). Selection for the homologous recombination event, as opposed to possession and retention of plasmid, by transient enrichment of ZFN expressing parasites within the population produced *Pfcg6-eGFP* disrupted progeny in less than 2 wk (Straimer et al. 2012), substantial reduction on the usual 3–6 mo previously required using standard homologous recombination.

INDUCING THE MOLECULAR BASIS OF CHLOROQUINE RESISTANCE USING ZFNS

Typically, the DNA regions encoding the functional/active sites and the targeting prerequisites of enzymes or transporters are the targets for functional studies. These DNA regions are typically cloned and a mutated copy is produced with targeted changes of specific codon(s) to produce a variant amino acid, altering the physio/chemical properties of the mature peptide. Replacement of the endogenous copy with a mutant copy can reveal the activity of the native protein either in vitro or in vivo. The ability for DNA sequence variability is also the basis for evolution of drug resistance in malaria parasites.

Since its first clinical application over 65 yr ago to the announcement of its almost complete therapeutic failure, the antimalarial drug chloroquine has placed significant pressure upon the gene *Pfcrt* of *P. falciparum*. The emergence of a clinically relevant, drug-induced resistance phenotype pushed malaria research in reverse with numerous teams chasing the basis for chloroquine resistance even though the exact mechanism of chloroquine action remained obscure. Eventually, studies of the highly polymorphic *Pfcrt* locus revealed the molecular basis of chloroquine resistance to be the result of more than 30 point mutations centred on replacement of lysine-76 with an

uncharged amino acid, either a threonine, asparagine or isoleucine (Ecker et al. 2012). Exactly why field isolates show the K76T mutation, whereas in vitro challenged parasites produce K76I mutation remains unknown. Mutational variability may highlight a difference between the evolutionary pressures of parasite–drug interaction between host infection and in vitro culture (Cooper et al. 2002).

Precise engineering of ZFNs has allowed the incorporation of site-specific pseudo-SNPs (or point mutations) into the genome of *P. falciparum* (Straimer et al. 2012). A *P. falciparum* strain (106/1) whose *Pfcrt* allele encodes six of seven CQR mutations observed in Asian and African strains while retaining the chloroquine sensitive lysine-76 codon has been employed to good effect for this work. This strain only needs one more point mutation to move from chloroquine sensitivity to chloroquine resistance (Cooper et al. 2002). Introduction of the K76I mutation via ZFN-induced homologous recombination was achieved and the chloroquine resistance phenotype selected for. Such selection of genetic mutation via phenotypic bias rather than resistance cassette expression produced chloroquine resistance parasites in 16–33 d with a 100% K76I allelic replacement record (Straimer et al. 2012). Previously, such a feat took two independent transfection events and 18 mo of continuous culture (Sidhu et al. 2002). Presumably, ZFN assisted homologous recombination reduces unintentional *side effects* of locus disruption that arise when using traditional methods of conventional allelic exchange.

As chloroquine gives ground to the more modern antimalarial drugs, validation of the drug target before global selective pressure is applied is needed. Imidazopyrazines are a new class of antimalarials identified via cell-based screening (Plouffe et al. 2008). To validate if drug target *Pf*pi(4)k (PF3D7_59800) is essential to *Plasmodium*, ZFNs were used to insert amino acid substitutions (Ser1320-Leu, His1484Tyr, and Tyr1356Phe) and a premature stop codon (Tyr1356stop) in the catalytic site of the protein (McNamara et al. 2013). Although *Δaa* parasites were raised to yield parasites with increased resistance to drug pressure, the ΔTyr1356stop mutants could not be produced without a subset of the population duplicating the copy gene to preserve function/expression (McNamara et al. 2013). Asexual parasites exposed to imidazopyrazine derivatives were shown to evolve copy number variation and point mutations in response to drug pressure (McNamara et al. 2013). Given that these mutations can evolve in the lab (in the absence of sexual selection), this should be a warning that just because expression of a full gene is essential to the IDC, the gene target is not a single static entity, and is capable of evolution towards resistance against pressure, especially on a global population scale.

CRIPSR-CAS9 SYSTEM

The ability to produce a DSB then modify a genomic locus during the repair process is also achievable using RNA-guided, modified type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR—a short sequence of nucleotides matching the target gene) and its partner, the CRISPR-associated protein (Cas9—an endonuclease that causes DSB). CRIPSR-Cas9 was developed in *P. falciparum* simultaneously by two groups (Ghorbal et al. 2014; Wagner et al. 2014), both assessing their ability of their system to alter the expression of a protein destined for the erythrocyte surface. By tracing the outcomes of their successful genomic disruption via detection of Renilla luciferase expression, Wagner et al. (2014) were able to assess the incorporation of an exogenous gene into the genome and show that the native gene transcription process was still viable. The process, which requires cotransfection with two plasmids and drug selection for episome retention, could occur over a period of ~4 to 6 wk (Wagner et al. 2014).

Ghorbal et al. (2014) used their system to first remove eGFP fluorescence then later the non-IDC essential knob-associated, histidine-rich protein coding gene with a high level of efficiency, over a period of almost 3 wk (Ghorbal et al. 2014). As an improvement, and in difference to the ZFN system, and to all other previously published *P. falciparum* genetic modifications, activation of genetic modification was achieved after transfection of linear plasmid DNA. Linear DNA is reported to degrade in *P. falciparum* infected RBCs within 4 d of electroporation (Deitsch et al. 2001), thus it is assumed that genetic manipulation occurs within the first two IDCs of the selection protocol. In addition, as the activating plasmid degrades, there is no need to use plasmid removal protocols or use negative selection (Ghorbal et al. 2014).

In silico analysis of the *P. falciparum* genome predicts 663,952 possible CRISPR-Cas9 target sites spaced at an average distance of 35 bp, meaning that virtually any *P. falciparum* locus could be targeted (Ghorbal et al. 2014). Although it remains to be shown if genes essential to IDC progression can be targeted for loss of function experiments.

Genetic polymorphisms exist in extant *Plasmodium* spp. and their identification can aid in identifying the evolutionary *push-back* necessary to survive drug pressure. Growing resistance to artemisinin derivatives is a major concern for malaria control programs and field isolates from Cambodia show a C580Y mutation in the kelch propeller domain of PF3D7_1343700 (Ariey et al. 2014). CRISPR-Cas9 SNP insertion validated this mutation, as mutants matched the field isolated resistance phenotype (Ghorbal et al. 2014). However, not all mutants created using the CRISPR-Cas9 system match previously identified and published mutational phenotypes. When CRISPR-Cas9 was used to insert the L173A mutation into the gene *Pforc1*—a mutation previously shown in vitro to cause failure of *Pf*orc1 to oligomerise, a process assumed necessary for *Pf*orc1 to associate and assist with regulation of telomeric DNA (Deshmukh et al. 2012), the genomic region associated with virulence—no such de-regulation was observed in three mutants created (Ghorbal et al. 2014). Discrepancy between the two results highlights the need to

critically assess mutational phenotypes using different genetic manipulation methods.

GLOBAL DISRUPTION OF GENE FUNCTION USING TRANSPOSABLE ELEMENTS

Transposable elements have been used to produce unbiased insertional genomic mutagenesis in organisms across kingdoms. An initial attempt to use the autonomous *Drosophila mauritania* TE *mariner* to disrupt the *P. falciparum* genome resulted in limited success (Mamoun et al. 2000). Independent of *mariner* transposase activity (suggestive of an endogenous transposase activity), the coding region of the protein kinase A gene was disrupted with no confirmed phenotype (Mamoun et al. 2000). A more successful attempt at genomic disruption of *P. falciparum* strain NF54 was achieved using the TTAA-target site-specific class II TE *piggyBac*, derived from the Cabbage Looper moth *Trichoplusia ni* (Balu et al. 2005). Late stage NF54 parasites were exposed to erythrocytes *preloaded* with two plasmids—one containing a *hsp86* driven *piggyBac* transposase, and the other a hDHFR expression cassette flanked by inverted terminal repeats (ITS) of the *piggyBac* element (Balu et al. 2005). To increase the efficiency of insertion the *hsp86* promoter was replaced with the stronger *calmodulim* promoter (Balu et al. 2009). Transposase expression inserts the drug-resistant cassette into the *Plasmodium* genome at ITS TTAA-crossover regions, leaving the plasmid backbone behind.

Within the 23 MB *P. falciparum* genome there are an estimated 311,155 TTAA sites, 124,733 (40.5%) of which are within known expressed sequence tags, which equates to ~20 targets for each gene (Balu et al. 2005). There are two typical insertions: (i) a *piggyBac* element goes into the reading frame of an exon or (ii) into a regulatory elements of the *Plasmodium* genome. The latter of these insertions may alter the spatio/temporal regulation of gene expression, altering, or compromising the efficiency of the gene product.

Forward genetic screens show that *piggyBac* preferentially inserted into predicted transcribed units of the *P. falciparum* genome, affecting 178 transcription units. Thirty-six of the insertions resulted in direct ORF disruption, three insertions were mapped to introns, 119 occurred in 5′ UTRs, and 22 were obtained in 3′ UTRs (Balu et al. 2009). The skewed distribution for the return of *piggyBac* mutants within UTRs is supported by slightly pleiotropic mutational phenotypes reported for these integration events and the potential inability to recover genes ultimately essential to the IDC. The *piggyBac* method is not useful for essential gene removal by dependence on the success of merozoite formation as a measurement of attenuation, as integration of the system takes a number of days allowing daughter clones developed by progression through the IDC every 48 h.

Disruption of noncoding genomic regions can unbalance stoichiometric or temporal gene expression, and may reduce parasite viability rather than causing cell death. Alteration of timing of expression affects the efficiency of protein function and changes to timing of gene expression can also disrupt transmission (Lindner et al. 2013). A severely affected mutant (46% reduction in viability) was produced by insertion into the coding region of *CCR4-associated factor 1* (PF3D7_0811300), a homologue of a known global gene regulator in yeast (Balu et al. 2010; Collart 2003). A similar role was suggested in *P. falciparum* as the mutant showed significant aberrant regulation of ~1,300 genes (Balu et al. 2011).

A repeating theme in the *piggyBac* screen was the retrieval of attenuated cultures with insertions within genomic regions of genes involved in nucleic acid binding, metabolism, or transcription. In particular, disruption of two putative RNA binding proteins PF3D7_0812500 and PF3D7_1360100, previously predicted as interacting partners during the IDC (Date and Stoeckert 2006), provided evidence for their predicted role in housekeeping RNA metabolism, protein synthesis, and protein trafficking. The activity of these proteins is a target for the antimalarial drug cyclosporine and its application is rationally supported by bioinformatic prediction and genetic disruption (Gavigan et al. 2003).

piggyBac insertion in the 5′ UTRs of glutaredoxin (PF3D7_0306300) and its 3′ genomic neighbour mitochondrial glycerol-3 phosphate dehydrogenase (PF3D7_0306400), resulted in increased levels of both transcripts, indicating that optimal expression of genes is essential for normal parasite growth (Balu et al. 2009). A 3′ insertion in the EPP family of ABC transporters (PF3D7_1426500), which in *Arabidopsis thaliana* is shown to transport C:16–18 fatty acids (Panikashvili et al. 2007), caused 62% reduction in IDC viability (Balu et al. 2010). Significant up-regulation of the EPP homologue occurs before transmission into the mosquito, supporting the use of lipid metabolism targeted drugs as potential transmission blocking agents.

In the hunt for *Plasmodium* drug targets affecting transmission, relation of essentiality to viability can overlook the significance of the role of the gene disrupted. Occasionally, gene replacement can only affect a subset of the asexual population, as was the case when the Nima-related kinase *Pfnek-4* was disrupted, if gene loss is more related to sexual commitment rather than IDC progression (Reininger et al. 2012). The *piggyBac* system is suitable for analysis of post-IDC stage gene identification, namely gametocytogenesis (Ikadai et al. 2013).

GENETIC ALTERATION USING SITE-SPECIFIC RECOMBINASES FLP/*FRT* OR CRE/*LOX* SYSTEMS

Precise genomic manipulation using site-specific recombinases (members of the λ integrase super-family) offers researchers the ability to remove or rearrange an almost unlimited volume of DNA. Both CRE recombinase of *E. coli* bacteriophage P1 (Causes Recombination of the *E. coli* bacteriophage P1 genome) and FLP recombinase from the 2 μm plasmid of *S. cerevisiae* (named after its ability to "FLiP" DNA sequences) catalyse single-site recombination (SSR) recombination without the need for any cofactors. Recombination occurs between sequence specific

regions—*loxP* (locus of crossover (x) in P1) and *frt* (FLP recombinase target) sites for CRE and FLP, respectively. The short target sequence length, consisting of a 13 bp palindromic sequence separated by a 8 bp core region, make them convenient loci to introduce (a process known as *flirting* a gene) into the genome with minimal sequence disruption.

The relative orientation of the target sites determines recombination activity. Linear DNA between two directly repeated target sites is excised as a circular product and reintegration of this product is kinetically unfavourable because the product is unstable and is eventually degraded. If two directly repeated target sites are present on separate DNA molecules, either separate chromosomes or one within a chromosome and the other housed upon a plasmid, recombination will result in integration or exchange of DNA sequence. If the target sites are inverted repeats, the intervening DNA becomes inverted rather than being excised. Insertion and inversion events are reversible as the *loxP*/*frt* sites are still present in the final reaction product.

Recombination of DNA is wholly dependant on FLP/CRE activity. Controlling the expression or activity of FLP/CRE would allow for control of genetic recombination. The first use of recombinase activity in Apicomplexa involved CRE in *Toxoplasma gondii* and removal of undesired sequences such as the selectable marker and native genes was achieved (Brecht et al. 1999). Attempts to conditionally control CRE expression using synthetic progesterone hormone receptor RU 486 failed and constitutive expression of CRE occurred, resulting in uncontrolled gene loss (Brecht et al. 1999). Despite this drawback, invasive *T. gondii* expressing CRE can be used to modify *flirted* host genes (Koshy et al. 2010). Recently, a system utilising rapamycin control of a split CRE (DiCRE) protein allowed for control of genetic recombination in *T. gondii* (Andenmatten et al. 2013). Adaptation in *P. falciparum* has also been recently achieved, although no native parasite gene has been silenced using this protocol (Collins et al. 2013). In a bizarre artefact of *Pfsera5* recombination, an alternate cryptic polyadenlyation site in the postrecombination *Pfsera5* 3′ UTR allowed mutants to continue expressing the gene (Collins et al. 2013). Despite this set back, the DiCRE system is a promising method with a single IDC timeframe—a much-cherished result.

Recombinase application to *Plasmodium* spp. was first achieved in *P. berghei* and involved the sexual cross of two separate transgenic strains, one possessing the FLP recombinase and the other a *flirted* target gene (Carvalho et al. 2004). Proof of concept was shown with 25% of the in vivo population genetically modified (Carvalho et al. 2004). Progeny isolation ensured a homoeogenic population of parasites containing both FLP and the target. Using stage specific promoters, FLP expression could also be compartmentalised into a life cycle stage where the action of the *flirted* gene is not essential, allowing for uninterrupted development of genetically modified parasites despite loss of the gene (Combe et al. 2009).

To disrupt sporozoite proteins during the infection process, a temperature sensitive variant of the FLP protein was introduced into blood stage parasites and the change in temperature upon transition to mosquitoes was used as an induction mechanism (Combe et al. 2009). FLP-L (FLP-L—low activity) has optimal activity at ambient temperature (20–25 °C) but minimal activity in the 37 °C environment of the mammalian host (Buchholz et al. 1996; Combe et al. 2009). By exploiting the shift in temperature experienced by the parasites upon relocation into a mosquito, the activity of the FLP-L was able to be regulated.

FLP-L expression was also maximised in mosquito stages using the TRAP promoter to drive its expression, which confers a 10,000 fold induction at the oocyst/sporozoite stage (Rosinski-Chupin et al. 2007). This approach showed no evidence of IDC stage SSR (Combe et al. 2009). Expression of FLP using stage specificity activity of UIS4 promoter during sporozoite production—a quality discovered using subtractive suppressive hybridisation (Matuschewski et al. 2002)—allowed for removal of vaccine candidate MSP1 and the subsequent production of a previously unknown mutant phenotype effecting merozoite formation in hepatocytes (Combe et al. 2009). MSP1 is a well-studied *Plasmodium* protein and the production of these tools attests to the multifactorial exploitative approach needed to further *Plasmodium* research.

Production of a genetically attenuated *P. falciparum* strain would involve multiple transfections and necessitate recycling/removal of selection markers before the introduction of a GMO into human trials. Marker excision from genetically disrupted *P. falciparum* was attempted with CRE or FLP expression using an anhydrotetracycline inducible promoter (discussed later in the review) (O'Neill et al. 2010). Using this conditional system, flirted heterologous resistance cassettes (hDHFR) were successfully removed from the haploid genome. The success of this method was somewhat restricted as the conditional expression of FLP and its SSR activity was not concurrent with genomic duplication during the IDC, which resulted in a mixed progeny of $hDHFR^{+}$ and $hDHFR^{-}$ and only 3% of the parasites undergoing marker loss (O'Neill et al. 2010). In the same study, a homogenetic population of $hDHFR^{-}$ cells was produced when with CRE/LOX recombinase. Although successful in removing hDHFR from the genome, the use of TaTi-CRE/LOX was not conditional and could not be adapted to remove essential genes (O'Neill et al. 2010).

An additional study, where culture temperature was cycled from 37 °C, where FLP activity is minimal, to 30 °C where FLP activity is optimal resulted in no significant increase in population SSR (van Schaijk et al. 2010). By integrating a *flirted* hDHFR-GFP fusion positive selective marker into the 3′ and 5′ region of nonessential genes PF3D7_0404500 and PF3D7_0404400, respectively, a modified version of FLP, termed FLPe (FLP evolved—Buchholz et al. 1998) with optimal activity at 37 °C, was ~99% efficient for SSR in *P. falciparum*.

Both the van Schaijk et al. (2010) and O'Neill et al. (2010) studies removed resistance cassettes integrated

within the parasite's genome with the goal of producing genetically attenuated parasites for future vaccination-like investigations. Importantly, these approaches restore the option of reusing a selection marker, which is of huge benefit as they are in short supply (van Schaijk et al. 2014). Regardless of the efficiency of either version of FLP or CRE, gene removal by recombinase activity can produce live parasites with missing genetic information. From here, multiple rounds of genetic alteration are possible and parasites with marker free multiple genetic disruptions have been produced (Mikolajczak et al. 2014). Attenuated parasites fail to establish clinical infection in humanised murine models (Mikolajczak et al. 2014) and in doing so allows the immune system to train its self against future challenges much the same as a champion boxer takes on a few lightweights before taking on a true title challenger. Although the parasites are genetically altered, it must be noted that none of the individual gene loss events jeopardise IDC progression and thus the method may not be useful for the identification of clinical drug targets without the ability to conditionally control the SSR event.

Conditional recombination has the potential to produce transgenic parasites poised for genetic modification. Insertion of *frt* sites into an intron and 3′ UTR of a *P. falciparum* gene by 3′ replacement should be relatively straightforward. Insertion of the 34 bp *frt* site should not affect intron/exon splicing of the gene or its downstream regulatory sequence, as they would essentially remain unchanged. Once a homogenic population of *frt*-integrated parasites is isolated, conditional expression of FLPe, which has proven *P. falciparum* activity, could remove the 3′ region of a gene, potentially making it inactive or at least producing a truncated protein. Over 50% of *P. falciparum* genes contain at least two introns, so this method is particularly attractive for conditional genetic alteration. It has also proved possible to alter the ORF of episomal plasmids, allowing for future complementation of native gene knockouts—as target gene expression is maintained on the episomal plasmid while the native gene is removed, then destroyed using FLPe (Webster 2013).

REMOVAL OR REGULATION OF GENE FUNCTION?

In the hunt for essential genes the question needs to be asked—which is better, removal of the gene or reduction in gene function to produce a phenotype? If a gene plays a multifactorial role, or is essential to a metabolic pathway, then its removal will only allow analysis of its earliest non-redundant downstream function. Many *P. falciparum* genes fit this category including proteins acting as transporters, chaperones, transcription factors, and protein recyclers. In addition, the various developmental stages of *P. falciparum* have differing requirements of different genes at different stages of the life cycle. Conditional reduction in gene function is possible by transcriptional activation/inactivation, interruption of mRNA activity, and protein stability. The following section of this review discusses the techniques available for use to alter the native gene function.

CONDITIONAL ALTERATION OF NATIVE GENE FUNCTION BY EXPRESSION ALTERATION: TRANSCRIPTIONAL REGULATION USING TETRACYCLINE RESISTANCE

Transcription is the first process of gene expression and is dependant on the recruitment of transcription factors to bind RNA polymerase II to a transcription initiation sequence. Modulation of this process allowing for conditional gene expression in eukaryotic cells has been achieved by fusing transcriptional activator or repressor domains with modified versions of tetracycline resistance (TetR) mechanisms from *E. coli* (Gossen and Bujard 1992). In its most basic implementation, the TetR system involves application or removal of an effector molecule (tetracycline) to control the interaction of a regulator (TetR) upon an operator sequence (tetO) in close proximity to the transcriptional start site to physically prevent transcription. Adaptation of this system to modulate expression using the less cytotoxic tetracycline derivative anhydrotetracycline (ATc) produced a ~10 fold reduction in myc-tagged myosin A (MyoA) expression in *T. gondii* (Meissner et al. 2001). A genetic screen of randomly integrating TetR sequences into the *T. gondii* genome revealed an artificial transcriptional activating domain TATi-1 (*T*rans-*A*ctivator *T*rap *i*dentified—derived from a noncoding sequence of the plasmid backbone) and when fused to the C-terminal of TetR, allowed for the regulation of a copied MyoA gene and the eventual disruption of the native copy to produce a noninvasive phenotype (Meissner et al. 2002).

Use of the TetR system in *P. falciparum* produced a < 50-fold induction of GFP in stable episomal lines over the IDC, with 10–20% of parasites showing high expression of GFP, and a 10–20-fold regulation was achieved with a CAT reporter over two IDCs for transient transfections (Meissner et al. 2005). Transcriptional activation/repression works best on episomal plasmids in *P. falciparum*, as this scenario most likely involved the multiple copies available on an episomal plasmid (i.e. more TetR–tetO binding interaction opportunities) and ATc induced control is reduced when TetR controlled genes are integrated into the genome (Meissner et al. 2005). Transgene expression can produce toxic impact upon culture proliferation, and the TetR system offers a chance to conditionally express transgenes tailored to elicit a phenotype, such as dominant-negative expression, once stable culture growth is observed.

To investigate the formation and behaviour of the food vacuole (FV) membrane Ehlgen et al. (2012) generated ATc inducible GFP-CQR and GFP-CQS parasites to confirm in real time previous observations of direct ER/Golgi trafficking pathway of *Pf*crt to the FV membrane during its de novo biogenesis (Ehlgen et al. 2012).

The artificial TATi1/2 domains used in apicomplexan studies contained a hydrophobic C-terminal extension unlike any other previously identified eukaryotic transcription factors, which are acidic. This feature made them nonfunctional in *HeLa* cells, highlighting a difference between Apicomplexan and *classical model* eukaryotic

gene expression (Meissner et al. 2002, 2005). To create a more efficient apicomplexan TetR system native transactivation domains (TRADs) from the ApiAp2 family of transcription factors were used (Pino et al. 2012). Due to the evolutionary distance between Apicomplexa and Opisthokonta, successful identification of TRADs using yeast hybrid assays was limited, and a *T. gondii* based assay with *A. thaliana* AP2 ANT domain as a control was used instead (Pino et al. 2012). Regulation of integrated *mCherry* expression was observed during mosquito and liver stage development and knock-downs of essential genes *profilin* and *N-myristoyltransferase* were achieved in *P. berghei* (Pino et al. 2012). Validation of N-myristoyltransferase as a drug target via regulation of its expression results in the putative inactivation of approximately 50 proteins predicted as substrates (Jones et al. 2012), and the resulting pleiotropic effect results in severe parasite impairment (Pino et al. 2012). The stage-specific expression of *N-myristoyltransferase* to the first 24 h of the IDC (Bozdech et al. 2003) required application of ATc for more than 12 h, as application below this time frame allowed for progression of later-stage parasites already past this biochemical bottleneck into functional merozoites, highlighting the need to match native, and conditional gene expression when phenotypic analysis of in vivo parasites is expected. As yet the promising Ap2-TetR system has not shown evidence of efficient regulation of genes integrated into the *P. falciparum* genome.

REGULATION OF GENE FUNCTION BY TARGETING RNA: RNA INTERFERENCE AND SIRNA IN *PLASMODIUM*

Regulation of gene function at the RNA level has shown innovative yet variable results, often due to incomplete genetic pathways or oligonucleotide toxicity in *P. falciparum*. Identification of gene function using RNA interference (RNAi) is relatively standard practise in several eukaryotes, since its Nobel Prize winning discovery in the helminth *Caenorhabditis elegans* in the late 1990s (Fire et al. 1998). Delivery systems to mediate RNAi in *C. elegans* have been varied and include microinjection, environmental saturation, and *E. coli* predation. The first report of RNAi in *P. falciparum* involved electroporation of double-stranded RNA (dsRNA) encoding a segment of the gene encoding dihydroorotate dehydrogenase (DHODH)—an enzyme in pyrimidine biosynthesis and essential for parasite growth—into parasites (McRobert and McConkey 2002). A decrease in parasite growth correlated with a decrease in DHODH mRNA level as assayed by qRT-PCR, yet no confirmation of reduced protein level was recorded. Another study added dsDNA to culture medium in an attempt to disrupt cysteine protease genes *falcipain 1* & *2* and hence inhibit the haemoglobin hydrolysis pathways (Malhotra et al. 2002). Visual quantification of IDC progression as a measure of parasite growth was replaced with [^{3}H]-hypoxanthine uptake (Chulay et al. 1983), and the resultant phenotype showed attenuated parasites with swollen FVs, argued to be due to an inability to degrade haemoglobin and form merozoites. Based on these publications, it appeared RNAi might play a major role in the progress towards understanding the basic biology of malaria parasites, and several RNA disruption studies followed (Gissot et al. 2005; Kumar et al. 2002; Tuteja et al. 2008). Later it was revealed that the *P. falciparum* genome apparently lacks the cellular machinery necessary to achieve RNAi making it hard to reconcile any effect of RNAi approaches (Baum et al. 2009). Rather, it appears that the phenotypic effect observed in previous studies was due to non-RNAi induced pathways, most likely toxic artefacts (Clark et al. 1994). Although not active in *Plasmodium* spp., RNAi can be used to identify parasite-host/vector interactions by blocking translation of host genes (Berois et al. 2012; Prudencio et al. 2008). Such factors highlight nonparasite effectors of infection and potential drug targets against which the parasite does not have the ability to evolve resistance to (Sullivan 2013).

RIBOZYMES

Ensuring correct timing of gene expression whilst attempting to limit gene function requires the use of nontrans-acting factors. One such method involves the use of inhibitory autocatalytic RNA, known as ribozymes, which have the ability to cleave mRNA to prevent translation. The application of synthetic phosphorothioated hammerhead ribozyme oligionucleotides to parasite cultures disrupted translation of carbamoyl phosphate synthetase II (PF3D7_1308200), a rate-limiting enzyme involved in pyrimidine biosynthesis, resulted in a 55% reduction in parasite viability (Flores et al. 1997). The use of synthetic ribozymes rationally designed to cleave mRNA of apicoplast targeted *Pf*gyrase-A (PF3D7_1223300) resulted in inhibition of parasite growth by 49.54% and 74.77% at concentrations of 20 and 30 μM, respectively (Ahmed and Sharma 2008). Although a background growth inhibition of 20–25% was observed within control cultures (Ahmed and Sharma 2008), neither study reported a physical phenotype other than growth inhibition. In a proof of concept study, integration of two *Sm1* hammerhead ribozyme sequences, derived from *Schistosoma mansoni,* 5′ to the ATG of the *Photinus pyralis* Luciferase reporter gene reduced its expression to levels equal to background expression in *P. falciparum* (Agop-Nersesian et al. 2008).

A strong proof of concept that ribiozymes can be used to reduce the function of an essential gene was recently reported (Prommana et al. 2013). The *glmS* ribozyme, adapted from Gram-positive bacteria, can be used to attenuate gene expression in a consistent and temporal manner in *P. falciparum* by the addition of exogenous glucosamine (GlcN). A *glmS* ribozyme was inserted into the 3′ region of a GFP tagged *P. falciparum* DHFR-TS. GlcN-induced ribozyme cleavage resulted in the knock-down of protein levels within 12 h, and recovery to original status within 12 h more. A 24 h time frame of protein reduction and recovery allowed for modulation of protein within a single IDC and resulted in *P. falciparum* parasites becoming hypersensitised to the antifolate drug pyrimethamine.

Ten genes also showed significant expression change within the integrant line after the addition of GlcN; however, cell viability appeared uncompromised within the 24 h window, yet, after 48 h, aberrant morphological changes were observed, and by 72 h a phenotype of slightly shrunken trophozoites was observed (Prommana et al. 2013).

Ribozome knock-downs are useful for targeting specific sections of larger protein complexes as knock-down of a single protein reduces the efficiency of formation of functional multicomplexes. Incorporation of the *glmS* ribozyme into the 3′ region of *PfPTEX150*, the expression of which is essential to the function of the *Plasmodium* translocon of exported proteins (PTEX) protein multicomplex, resulted in limited IDC progression (Elsworth et al. 2014). Having the ability to control protein function in a dose-dependent manner allowed Elsworth et al. (2014) to produce a transgenic line then activate the loss-of-function mutational phenotype.

APTAMERS

Some classes of antimalarial drugs, including chloroquine, interfere in the essential process of haem detoxification and polymerisation into the chemically inert pigment haemozoin. A strategy to block this process using short oligonucleotide sequences, also known as aptamers, has been achieved (Niles et al. 2009). Aptamers can be designed or chosen from large oligonucleotide libraries ($> 10^{13}$) to attach to almost any compound using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method. Binding affinities of aptamers to their targets are considered 10,000 fold greater than antibody-antigen binding, and their small size allows them to target enzymatic sites within proteins. Introduction of aptamers into erythrocytes by hypotonic lysis and resealing methods resulted in their ingestion, along with haemoglobin, into the FV (Niles et al. 2009). This delivery system, matching the parasite intrinsic pathway, resulted in aptamer-haemoglobin binding and inhibited haemozoin formation as potently as chloroquine (Niles et al. 2009). Although their experiment was not capable of removing the prospect of oligonucleotide toxicity previously observed in *Plasmodium* studies, the negative phenotype was not observed when using nonhaem specific aptamers.

Observing the natural phenotypes of *P. falciparum* during infection has provided physiological evidence for the aetiology of malarial pathogenicity. One such naturally occurring infection phenotype occurs when infected erythrocytes bind to the endothelial linings of small blood vessels (cytoadherence) or to uninfected erythrocytes (rosetting) of the host during the latter stage of the IDC to prevent clearance from the blood stream. The phenotype is directly controlled by the expression and transport of *Pf*emp1—comprised mainly of Duffy-binding ligand domains and cysteine rich inner domain regions—to the erythrocyte surface. The number of domains and size of *Pf*emp1 varies depending on which one of the ~60 *var*-genes is expressed by the parasite. The ability to produce aptamers with specific binding affinities allowed for targeting to a conserved Duffy-binding ligand amongst *Pf*emp1, and this interaction prevented resetting amongst cells in vitro (Barfod et al. 2009).

The ability to define aptamer specificity has led to the application of RNA-protein interaction to control gene expression, in particular the TetR transactivator (Belmont and Niles 2010; Hunsicker et al. 2009). The potential application of TetR-aptamer gene expression is currently being adapted to *P. falciparum*, offering inducible, sequence-specific RNA inhibition of potentially any gene within the genome (Niles, J. C., pers. commun.).

CONTROLLING PROTEIN STABILITY TO CONTROL PROTEIN FUNCTION

The activity of a protein can be disrupted if it fails to correctly fold. Organisms have a clean-up service to recycle and prevent accumulation of inactive proteins by exposing them to the proteasome. Natural degradation pathways can be exploited to target genes fused to modified version of human FKBP-12, resulting in their rapid and constitutive degradation in the absence of the small cell permeable synthetic ligand Shield^{-1} (Banaszynski et al. 2006). Proof of concept was shown by controlling protein levels of YFP appended at either the N- or C- terminus with the FKPB degradation domain (ddFKBP) in *P. falciparum* (Armstrong and Goldberg 2007) and *T. gondii* (Herm-Gotz et al. 2007). N-terminus tagging appeared more efficient, and protein stabilisation could be achieved within 3 h of Shield^{-1} application (Armstrong and Goldberg 2007). Traditional knock-out of the *P. falciparum falcipain-2* gene by homologous recombination produced a swollen FV phenotype (Sijwali and Rosenthal 2004), and phenotypic rescue was achieved using an episomally retained *hsp86* promoted (trophozite stage) ddFKBP-falcipain-2 fusion protein and the addition of Shield^{-1} (Armstrong and Goldberg 2007).

Shield^{-1}/ddFKBP control of *P. falciparum* calpain, by 3′ single-crossover recombination at the *Pfcalp* (PF3D7_1362400) locus has also been demonstrated. Calpain knock-down prevented development of parasites past the ring stage, affecting their ability to enter S-phase (Russo et al. 2009). Six independent attempts to disrupt the locus were made; none were successful. Without Shield^{-1}/ddFKBP controlled protein function, neither the egress deficient nor S-phase stalled phenotype would have been revealed, as selection for the ultimately fatal phenotype could not have been achieved.

Variants of ddFKBP with triple point mutations were shown to increase Shield^{-1} stabilisation kinetics compared to the initial ddFKBP. The best ranked of the two was used to target the ring-infected erythrocyte surface antigen at its C-terminus, proving exported dd-tagged proteins can reach the erythrocyte surface (de Azevedo et al. 2012). Exported erythrocyte surface proteins remains stable outside of the parasite's own membrane system as Shield^{-1} molecules remain attached to the protein and stabilise it; however, protein knock-down is limited and linked to protein turnover and the level of expression.

Combined use of the *Bxb1* integrase system to insert a single genomic copy of a modified (Chu et al. 2008) dd_{TM}FKBP-tagged dominant-negative calcium-dependent protein kinase-5 (*Pfcdpk-5*—PF3D7_1337800) into the genome of *P. falciparum* trapped parasites within erythrocytes until Shield^{-1} addition, revealing the role of *Pf*cdpk-5 for egress (Dvorin et al. 2010). A later study in which systematic gene-by-gene knockout via homologous recombination was attempted on *P. falciparum* kinases produced a mixed population of parasites with a modified *Pfcdpk-5* locus. However, to validate if *Pf*cdpk-5 is essential for egress a Southern blot confirming genomic disruption must be completed on isolated clones from this population (Solyakov et al. 2011).

The ddFKBP domain produces a protein of 107 residues (~10 kDa) and may potentially cause aberrant function or trafficking when fused to a protein of interest. A low level *leakiness* is also observed, and knock-down levels achieved might not be sufficient to generate a phenotype for low-level expressed proteins. In addition, tagging of mitochondrial (Balabaskaran Nina et al. 2011), apicoplast, or secreted proteins is theoretically difficult, as the FKBP-protein fusion is not exposed the proteasome, which is restricted to the cytosol. Another problem is the potential for toxicity to in vitro cultures when Shield^{-1} is applied at concentrations greater than 1 mM (de Azevedo et al. 2012). There is also the high cost of Shield^{-1} to consider, which if the goal is to integrate ddFKBP to the 3′ terminus of a target gene whilst maintaining adequate protein expression, would require constant application until integration is achieved, which, considering the Shield^{-1} induced cell cycle delay, would extend the already several month long protocol.

RFA TAGS, ASPARAGINE REPEAT GENES, AND AUXIN REGULATION OF PROTEIN LEVELS

A regulatable fluorescent strategy based upon the degradation domain of *E. coli* dihydrofolate reductase enzyme fused with a GFP was established as an alternative to ddFKBP. This dd-tag version, referred to as RFA, confers protein stability of *P. falciparum* proteins in the presence of an inexpensive cell permeable folate analogue known as trimethoprim (TMP; Muralidharan et al. 2011). Genomic 3′ integration of RFA to the asparagine repeat proteasome lid regulatory subunit 6 protein (*Pf*rpn6—PF3D7_1402300) revealed its essentiality for ubiquitinated protein degradation. Almost a quarter of the proteins of *P. falciparum* include asparagine repeat units, and their function is assumed to be involved with protein unfolding and degradation during heat stress at febrile temperatures. Expression of an asparagine repeat minus *Pf*rpn6 showed that this domain has no such role and yet its expression remains essential to the parasite, as they could not survive in the absence of TMP, suggesting that asparagine repeat elements in the proteome may occur for as yet undefined reasons (Muralidharan et al. 2011). A similar approach was applied after attempts to ablate *PfHsp110c* using double homologous recombination failed as surviving parasites showed evidence of gene duplication (Muralidharan et al. 2012). Furthermore, it was noted that TMP control of protein function is also achieved by prevention of correct protein formation or interaction, rather than degradation alone, allowing it to be used to probe the function of *Pf*HSP101-dependent translocation across the PVM and its role in the PTEX complex (Beck et al. 2014).

A recent genetic tool developed for *P. falciparum* optimised the previously developed *Thosea asigna* viral 2A "skip-peptide" (Straimer et al. 2012), this time using a sequence from the foot and mouth disease virus, and the auxin responsive plant transcription repressor to create a reduced plasmid capable of expressing regulatable transgenes (Kreidenweiss et al. 2013). As proof of concept has only just been established, this auxin-inducible degron (AID) system, allows for greater stoichiometric accuracy of expression of the target gene and its regulatable partner as the same promoter controls them (Kreidenweiss et al. 2013). In stark contrast to the FKBP and RFA systems, the AID system is always on, requiring addition of auxin to lower the protein level.

CHEMICALLY INDUCED KNOCKOUTS AND CHEMICAL RESCUE

The discovery of a plastid (apicoplast) in Apicomplexa—which arose via secondary endosymbiosis from a red algal ancestor—transformed our view of the evolutionary path these protists had marched on their road to becoming virulent intracellular parasites. A past life as photosynthetic symbionts of animals now seems most likely, and the apicoplast apparently remains as an anomalous—but potentially exploitable—evolutionary hangover from an earlier incarnation. Like all plastids, apicoplasts cannot arise de novo. Exactly why the apicoplast is essential to the infection cycle is a mystery, but these attributes certainly make them attractive targets for therapy.

Apicoplast targeted drugs include doxycycline (inhibition of apicoplast genome expression) and fosmidomycin (inhibiton of nonmevalonate isoprenoid precursor biosynthesis) and they have been shown to kill parasites. In assessing the downstream function of apicoplast metabolism, it was assumed that isoprenoid production was potentially the only nonredundant function, as knockout studies of the FAS-II pathway (Vaughan et al. 2009) and the PDH (Pei et al. 2010) have shown them to be dispensable to the IDC. Two enzymes of the linear isoprenoid pathway have already been suggested as drug targets (Crane et al. 2006; Jomaa et al. 1999). To test if this pathway was truly the only nonredundant pathway of the apicoplast, parasites were supplemented with its downstream product isopentenyl pyrophosphate (IPP). Application of 200 μM IPP to culture during doxycycline and fosmidomycin application resulted in degradation of the apicoplast genome and physical structure but not cell death. Eventually, apicoplast minus parasites proliferated and continued in the absence of compartmentalised function of ~500 apicoplast targeted genes (Yeh and DeRisi 2011).

How exactly the parasite survives without the apicoplast is itself remarkable, yet the ability to convert IPP into dimethylallyl diphosphate in the absence of a recognisable homologue of IPP isomerase, makes this finding phenomenal. It also highlights the need to find and characterise the isoprenoid transporter responsible for apicoplast to cytosol movement, as it provides a potential bottleneck in parasite metabolism.

Apicoplast minus parasites are currently being explored for their ability to produce infective sporozoite, which for *FAS-II* gene knockouts has also shown to be unsuccessful (van Schaijk et al. 2014), limiting their use as a potential attenuated live parasite vaccine. However, as an analysis tool, chemical rescue would offer protection against potential death-related phenotypic consequence to allow dissection of the finer genetic/metabolic contents of the apicoplast. Identification of what the parasite needs to survive in culture can assist in preventing death until a phenotype is observed by allowing the parasite to scavenge essential metabolites or cofactors from the environment, even if this requires presenting them in nonphysiological concentrations (Allary et al. 2007; El Bissati et al. 2008; LeRoux et al. 2009; Pillai et al. 2013). Supplementation is important for studies of drug/metabolite transporters, as physiological conditions can alter the effectiveness of drug pressure (Spillman et al. 2013), as it increases the chance of parasite survival to allow analysis.

OVERVIEW AND FUTURE RESEARCH

The recent development of elegant strategies to remove or alter malaria parasite genes that are essential in the red blood cell stage has allowed us to get our first glimpses of a phenotype and begin to understand what these genes do. Future genetic modification must allow for complementation of ablated genes to confirm their function and prevent reporting of false phenotypes or biased information about the essentially of genes. Care must also be placed upon the culture adaptation, and the reasons why some parasites isolated from patients do not undergo this transition when identifying potential drug resistance targets (Van Tyne et al. 2011). We also champion a push for the *Plasmodium* community to produce more genetic complementation experiments to confirm that reported genetic knockout phenotypes are related to gene loss of the target gene and the role of its product. The introduction of complementing genes remains a challenging but highly desirable proof of function step. It will also be desirable to explore any variation in mutant phenotypes in differing culture conditions, especially conditions that more closely match the environmental challenges the parasite faces during clinical infection as opposed to in vitro culture in a rich medium. Before death on a global scale from *Plasmodium* infection can be prevented, we must continue to further expand our understanding of the basic *Plasmodium* biology, from the genome to the phenome, or else eventually, *so it goes*, we will lose.

ACKNOWLEDGMENTS

W.A.J.W. was the recipient of a Melbourne Research Scholarship from The University of Melbourne and The School of Botany. Support from a National Health and Medical Research Council Program Grant and an Australian Research Council Discovery Grant to G.I. McF is appreciated. This review is dedicated to the life and memory of Guy A. Webster (11/11/1949-07/03/2012).

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