A Genome-Scale Vector Resource Enables High-Throughput Reverse Genetic Screening in a Malaria Parasite

Graphical Abstract

Large resource of barcoded gene KO vectors for *Plasmodium berghei*

5' homology arm

3' homology arm

5'...agggtgtgtg...3' gene-specific barcode

Cotransfection of vectors generates mixed pools of barcoded mutants

50 barcoded vectors

*P. berghei* schizonts

1 inbred mouse

Barcode counting by sequencing reveals growth phenotypes

Transfection

Drug selection

Blood sampling

Day post infection

0 1 4 5 6 7 8

Log10 Parasitaemia

Day post infection

0 5 6 7 8

Highlights

- Barcoded genetic modification vectors used to create a resource for *P. berghei*

- Parallel phenotyping of dozens of mutants can be performed using barcode sequencing

- A validation barseq screen of the kinome reveals false-positive and -negative rates

- Targetable kinase genes identified and verified with cloned mutants

Authors

Ana Rita Gomes, Ellen Bushell, ..., Julian C. Rayner, Oliver Billker

Correspondence

julian.rayner@sanger.ac.uk (J.C.R.), oliver.billker@sanger.ac.uk (O.B.)

In Brief

Gomes et al. present a large-scale resource of barcoded genetic modification vectors with high integration efficiency for *Plasmodium berghei*. The resource enables reverse genetic screening by allowing pools of barcoded mutants to be created in a single mouse and phenotyped in parallel by counting barcodes through next generation sequencing.
A Genome-Scale Vector Resource Enables High-Throughput Reverse Genetic Screening in a Malaria Parasite

Ana Rita Gomes, Ellen Bushell, Frank Schwach, Gareth Girling, Burcu Anar, Michael A. Quail, Colin Herd, Claudia Pfander, Katarzyna Modrzynska, Julian C. Rayner, and Oliver Billker

INTRODUCTION

The rate at which the genomes of intracellular malaria parasites can be modified has remained largely unchanged since methods for gene targeting by homologous recombination were developed in Plasmodium (Crabb and Cowman, 1996; van Dijk et al., 1996; Wu et al., 1995). Some notable advances have recently improved transfection efficiency in P. falciparum through the application of zinc finger nucleases (Straimer et al., 2012) and CRISPR-Cas9 (Ghorbal et al., 2014; Wagner et al., 2014). However, no currently available method is efficient enough to enable reverse genetic screens, and transposon mutagenesis in P. falciparum is at present well short of genome saturation (Balu and Adams, 2006). As a result, more than half of the protein coding genes in Plasmodium genomes still lack functional annotation.

SUMMARY

The genome-wide identification of gene functions in malaria parasites is hampered by a lack of reverse genetic screening methods. We present a large-scale resource of barcoded vectors with long homology arms for effective modification of the Plasmodium berghei genome. Cotransfected dozens of vectors into the haploid blood stages creates complex pools of barcoded mutants, whose competitive fitness can be measured during infection of a single mouse using barcode sequencing (barsseq). To validate the utility of this resource, we rescreen the P. berghei kinome, using published kinome screens for comparison. We find that several protein kinases function redundantly in asexual blood stages and confirm the targetability of kinases cdpk1, gsk3, tk3, and PBANKA_082960 by genotyping cloned mutants. Thus, parallel phenotyping of barcoded mutants unlocks the power of reverse genetic screening for a malaria parasite and will enable the systematic identification of genes essential for in vivo parasite growth and transmission.

Genome-wide collections of mutants or genetic modification vectors have greatly facilitated the discovery of gene functions in model organisms (Ni et al., 2011; Sarov et al., 2006; Skarnes et al., 2011; Winzeler et al., 1999). In malaria parasites, in contrast, efforts to scale up reverse genetics have suffered from a combination of low rates of homologous recombination and a high content of adenine and thymine (A+T) nucleotides that renders Plasmodium DNA difficult to engineer in E. coli. A malaria parasite of rodents, P. berghei, offers the most robust system for genetic manipulation with relatively high transfection efficiency (Janse et al., 2006a). In this species homologous integration can be boosted further by transfecting linear vectors with long (4–8 kb) homology arms (Pfander et al., 2011). Despite its high A+T content (>77%), P. berghei genomic DNA (gDNA) can be propagated efficiently in E. coli as large genomic inserts of up to 20 kb using a low-copy bacteriophage N15-derived linear plasmid with covalently closed hairpin telomeres (Godiska et al., 2010). In contrast to high-copy circular plasmids, an N15-based arrayed gDNA library achieved nearly complete genome coverage with sufficient insert size to represent the majority of P. berghei genes in their entirety. Clones from this library can be converted into gene targeting and tagging vectors in 96 parallel liquid cultures using robust protocols (Pfander et al., 2011), which exploit highly efficient homologous recombination mediated by the Red/ET recombinase system of lambda phage in E. coli (Zhang et al., 2000).

To accelerate the functional analysis of all P. berghei genes we here present a genome-scale community resource of long-homology genetic modification vectors that are individually quality controlled by sequencing and carry gene-specific molecular barcodes. The availability of more than 2,000 genome modification vectors raises the possibility of generating a large library of cloned and genotyped P. berghei mutants of the type that has enabled global genetic screens in yeast (Giaever et al., 2002; Winzeler et al., 1999). However, in P. berghei the lack of continuous in vitro culture of blood stages would limit the utility of such a clone collection. Signature-tagged mutagenesis, whereby thousands of mutants are simultaneously screened in a pooled approach (Hensel et al., 1995; Langridge et al., 2009; Mazurkiewicz et al., 2006), therefore offers a more attractive strategy for scaling up reverse genetics in P. berghei.

We have used the modification vector resource to enable such systematic screens for a Plasmodium parasite. We demonstrate that cotransfecting multiple gene knockout vectors in the same
electroporation reproducibly generates complex pools of bar-coded *P. berghei* mutants, and develop a barcode sequencing (barseq) approach (Smith et al., 2009) to phenotype the growth rates of all mutants within the pool over the course of an infection. To validate the approach, we compared a barseq knockout screen of protein kinases with the conventional kinome screen by Tewari et al. (2010). This comparison showed high reproducibility with previous data, but the sensitivity and robustness of the barseq approach also identified additional targetable genes. Our analysis demonstrates the power of barseq screening to robustly provide growth-rate phenotypes for dozens of mutants in single mice, and opens up the possibility for large-scale reverse genetic screens for multiple areas of *Plasmodium* biology.

**RESULTS**

**A Resource of Efficient Gene Targeting Vectors for *P. berghei***

To generate a genome-scale resource of gene knockout vectors, we used a modular pipeline for recombinase mediated engineering in *E. coli* (Pfander et al., 2011). The parasite gene of interest was first replaced in appropriately chosen gDNA clones with a marker for positive and negative selection in *E. coli* using Red/ET recombinase-mediated engineering. The bacterial markers were then exchanged under negative selection for a drug resistance cassette for *P. berghei* in a single in vitro Gateway recombinase reaction. When applied to the 2,781 *P. berghei* genes that have any level of functional annotation (57% of the genome), a first pass of the production pipeline yielded gene deletion vectors for 1,868 different protein coding genes of the core nuclear genome (Figures 1A–1C). These vectors form the foundation of the *Plasmodium* genetic modification resource, PlasmoGEM (Figure 1A), which can be viewed and requested through a searchable database at http://plasmogem.sanger.ac.uk (Schwach et al., 2015).

The vector production strategy can also be adapted to generate other modifications, including C-terminal tagging vectors, of which there are currently 278 in the resource. Tagging vectors are constructed with a C-terminal triple HA epitope tag by default. For each final vector we also make uncloned intermediate constructs available, which users can convert into targeting vectors with different functionality. A panel of Gateway entry clones are available which contain fusion tags for epitopes and different fluorescent proteins (Figure 1D). Each PlasmoGEM vector carries a gene-specific molecular barcode of 10–11 nucleotides that uniquely identifies genetically modified parasites derived from it (Figures 1C and 1D), and all final PlasmoGEM vectors contain the *hdhfr-yfcu* marker that enables positive and negative selection in *P. berghei* (Braks et al., 2006).

Manual construct design became rate limiting for vector production, and we therefore created a suite of computational tools to select gDNA library clones; to choose 50 bp homology regions for recombination in *E. coli*; to design oligonucleotides, generate sequence maps, assign barcodes, and track vectors through the production process; and to automate quality control (Schwach et al., 2015). The long homology arms of PlasmoGEM vectors...
(average sum in both arms 7.4 ± 2.9 kb SD) enhance recombination frequency in P. berghei compared to conventional designs (Pfander et al., 2011), but they also pose a risk that unintended mutations get inadvertently incorporated into the parasite genome. Homology regions and barcode modules of all targeting vectors are therefore confirmed by next-generation sequencing and pass stringent QC standards before they are used (for details, see https://plasmogem.sanger.ac.uk).

Parallel Phenotyping of Dozens of PlasmoGEM Mutants by Barcode Counting

To examine whether the improved integration efficiency of PlasmoGEM vectors would permit the reproducible generation and phenotyping of mixed pools of barcoded mutants, we selected from the resource 46 vectors targeting protein kinases that had previously been part of a systematic deletion analysis using conventional vectors (Tewari et al., 2010). Four sexual and mosquito-expressed genes known to be dispensable for asexual growth were also included to serve as reference genes to which growth rates of all other mutants could be compared. This reference set comprised of knockout vectors for the major surface proteins of the ookinetes, P25 and P28 (Tomas et al., 2001); a secreted ookinete adhesive protein, SOAP (Dessens et al., 2003); and a C-terminal tagging vector for the redundant $p_{230}p$ gene (van Dijk et al., 2010). In addition, three further control vectors were included to assess our ability to detect reduced growth rates in blood stages. One of these targeted plasmepsin IV, an aspartic protease involved in hemoglobin degradation whose deletion in P. berghei results in attenuated growth (Spaccapelo et al., 2010). We predicted parasite growth would be reduced by a deletion vector for PBANKA_110420, which encodes the E1β subunit of the mitochondrial branched chain $\alpha$-ketoadid dehydrogenase (BCKDH), given that deleting the E1α subunit of the same complex has a clear growth phenotype (Oppenheim et al., 2014). A third attenuating knockout vector targeted PBANKA_140160, a putative methyl transferase of unknown function, which emerged as a slow-growing mutant from a preliminary screen of metabolic enzymes (data not shown).

Schizonts transfected with a cocktail of 48 vectors and injected into mice (Figure 2) gave rise to drug resistant parasites 4 days later. This was indicative of an overall transfection efficiency of $\sim 10^{-4}$ and suggested that roughly 2,500 independent recombination events occurred in a transfection, enough to generate a complex mixture of mutants. Blood samples were subsequently collected exactly every 24 hr from day 4, gDNA was extracted, and vector-specific barcodes were amplified by a polymerase chain reaction (PCR) with a generic primer pair (see Figure S1A available online) and counted on a benchtop next-generation sequencer (Figure 2). In each of two replicate experiments, the same 22 barcodes from the vector pool were robustly detected and yielded nearly identical growth curves (Figure 3A). Southern hybridization of separated chromosomes showed evidence for vector integration events throughout the genome (Figure S1B). Long-range PCR products confirmed integration events for 17 of the 22 replicating barcodes (Figure S1C).

To assess the accuracy of barcode counting, we analyzed the same infected blood samples using two different methods for turning the initial PCR product after barcode amplification into sequencing libraries. The staged PCR strategy shown in Figure S1A and a conventional adaptor ligation protocol performed equally well, producing highly correlated barcode counts (Figure S1D). The PCR strategy proved faster and more economical and was therefore used in subsequent experiments. Figure S2A illustrates for a typical experiment the relative abundances of barcodes in a pool of transfected vectors and in infected mice 7 days after transfection, as determined by the PCR method.

To analyze growth curves derived from barcode counting we considered two parameters: (1) the relative abundance of each barcode within the pool, and (2) the relative fitness of each mutant, i.e., the rate at which its abundance changed each day. As expected, the four barcodes corresponding to control genes redundant for asexual development replicated rapidly. These were taken to represent wild-type growth (fitness $w = 1$). Relative abundance and growth rates were both highly reproducible for each barcode between technical and biological replicates (Figures 3B and 3C). We propose that the shape of a growth curve provides a quantitative measure for the fitness of a mutant. In contrast, the relative abundance of a mutant within a pool we consider less informative, since it may be influenced by a number of additional factors, such as the abundance of a vector in the transfection cocktail, the length of its homology arms, or any local variation in recombination rates. Plotting day 7 fitness from a ranked list (Figure 4A) showed that while the relative abundance of vectors in the transfection cocktail varied by up to one order of magnitude (Figure 4A, right axis), this was not a predictor of fitness of the resulting mutants. Fitness is therefore driven by growth rate, not by the amount of a given vector in the starting pool. The attenuating control vectors were associated with a measurable reduction in parasite fitness to between 0.60 and 0.73, as expected. The majority of protein kinase mutants either had wild-type fitness or were not detected ($w = 0$).

While all reference barcodes robustly replicated close to the average fitness of 1.0 (Figure 4B), the attenuating vectors and some kinase mutants showed statistically significant reductions in fitness that were consistently measured across different days of the infection (Figure 4C). Taken together, these data strongly
We hypothesized that in pools some slow-growing mutants may be outcompeted by faster-growing mutants before their barcodes can be detected. To eliminate this potential source of error, we performed a second-pass screening strategy by pooling only the slow or nonreplicating vectors from the previous experiments and transfecting them together with the reference set. This allowed us to measure the fitness of four additional mutants, one whose vector had a very low integration efficiency (PBANKA_040940), and three that were characterized by low growth rates during part or all of the infection (Figure S2B). These data suggest that the size and complexity of a vector pool can be increased only at the expense of losing more slow-growing mutants. However, this loss can be compensated in a second-pass screen of vectors that are not detected in the first experiment.

Barcode Counting Reveals Protein Kinase Mutants

To assess the accuracy of barseq screening, we compared the combined results from five barseq experiments with the data from our previous knockout screen, which used conventional vectors in a gene-by-gene approach with careful genotyping of cloned mutants (Tewari et al., 2010). For the majority of genes (76.1%) covered by both studies, the replication of barcodes in the mixed pool of mutants was predicted by previous data for their targetability (Table S1; Figure 5A). Two genes shown previously to be dispensable in blood stages (Tewari et al., 2010) could not be targeted in barseq screens. Failure of these vectors to integrate was not due to low amounts of vector DNA in the transfection cocktail and in the case of pK7 was reproduced when vectors were transfected individually. Absence of integration may be the result of low recombinogenicity at the target locus, or individual vector designs may interfere with the expression of essential neighboring genes. Although the fraction of technical failures was small, these observations reaffirm the need to confirm genetic essentiality by conditional methods, just as in conventional gene-by-gene studies.

Although some false negatives can be tolerated, genetic screens rely critically on the rate of false positives being very low. In barcode sequencing experiments false positives may result from episomal replication of vector DNA or from nonhomologous integration of targeting vectors. Although the former is commonly observed in P. berghei after transfecting vectors that originate from circular plasmids, we have not yet encountered spontaneous circularization of PlasmoGEM vectors, which rely on the expression of a phage telomerase to replicate in a linear form in E. coli (data not shown). The strongest candidates for false positives in our pilot screen were ten protein kinase genes for which barcode counting revealed evidence for their disruption by PlasmoGEM vectors, while previous studies had failed to delete the same genes using conventional vectors (Sebastian et al., 2012; Tewari et al., 2010). We selected six of these genes for validation by targeting them individually.

Two genes, cdpk1 and gsk3, have been considered potential drug targets in P. falciparum (Droucheau et al., 2004; Kato et al., 2008), but are shown here to be targetable in P. berghei (Figures S3A and S3B). In the case of cdpk1 our data confirm another recent study (Jebiwott et al., 2013), which also showed that the unconditional disruption of cdpk1 has no impact on blood stage growth but reproduces the ookinete phenotype we recently described for a stage-specific mutant in the same gene (Sebastian et al., 2012). Generating and genotyping individual knockout clones for gsk3, tkli, and PBANKA_082960, a putative protein kinase of unknown function, confirmed the barseq phenotype and established that these genes are indeed dispensable for blood stage growth (Figure S4).

We next turned to the rio group of protein kinases. rio1 and rio2 encode ancient atypical protein kinases that are highly conserved in most archaea and all eukaryotes, where they perform essential functions in ribosome biogenesis (LaRonde, 2014). In pooled transfections, barcodes for both genes were close to the detection threshold, and only the rio1 barcode was detected consistently at different time points. We therefore classified rio1 as targetable and rio2 as likely essential, although the latter produced a small number of replicating barcodes in some transfections. Targeted disruption of each rio gene individually yielded...
resistant parasites after a delayed prepatency period of 10–14 days. Resistant parasites grew very slowly under drug pressure and resisted dilution cloning.

Whole-genome sequencing of a putative rio1/C0 population confirmed the nearly complete absence of coverage of the target gene, consistent with the uncloned population being strongly enriched in parasites carrying a correctly integrated rio1 deletion vector (Figure 5B). Targeting of rio2, in contrast, selected for a 29.7 kb duplication on chromosome 5 that included the rio2 locus, together with three other genes (Figure 5B). Read coverage suggested these parasites carried one disrupted and one intact copy of the target gene, which was consistent with PCR evidence showing the presence of both a disrupted and a wild-type rio2 locus in the mutant population (Figure 5B). Read coverage suggested these parasites carried one disrupted and one intact copy of the target gene, which was consistent with PCR evidence showing the presence of both a disrupted and a wild-type rio2 locus in the mutant population (Figure 5B).

Finally we asked whether the interpretation of barcode counting data from parallel transfection experiments could be confounded by multiple vectors integrating into the same parasite genome. First we transfected pools of barcoded vectors into three parasite clones, each of which already carried a different barcode from the insertion of a targeting vector and subsequent recycling of the selection marker. We reasoned that if each mutant integrated one new vector, the pre-existing barcode should account for exactly half of all barcode counts after transfection and drug selection. The data were entirely consistent with this model (Figure 6A), suggesting that if multiple integration events existed, they would be too rare to be isolated by limiting dilution cloning. To detect rare double integration events, we chose three genes that are readily disrupted using PlasmoGEM vectors.

Figure 4. Fitness Measurements Obtained with PlasmoGEM Vectors Targeting Protein Kinases

(A) Distribution plot generated from a ranked list of day 7 fitness values measured in triplicate for each of 42 genes in experiment 1 (left axis). The relative abundance of a targeting vector in the electroporation cuvette at the moment of transfection (gray crosses, right axis) did not predict whether a mutant could be obtained. See Figure S2 for relative abundance data of a representative replicate experiment.

(B) Fitness of reference mutants averages 1 by definition. Error bars show standard errors (n = 6).

(C) Fitness of selected mutants. Error bars as in (B). Asterisk, different from reference mutants as determined by a two-sided t test corrected for multiple testing (p < 0.01; n = 6).
vectors. For each we transfected the final knockout vector together with a 20-fold excess of the selection marker-free intermediate vectors for the other two genes. We expected to detect replication of the marker-free vectors by PCR, but only if their genomic integration coincided with integration of the selectable construct into the same genome. These experiments failed to generate evidence for multiple integration events into the same genome (Figure 6B). The data indicate that different homologous integration events in \textit{P. berghei} are independent, and suggest that DNA uptake after electroporation is not the factor limiting transfection efficiency.

Taken together these data demonstrate that barcode counting provides a powerful tool to identify targetable \textit{P. berghei} genes and to rapidly and reliably measure the competitive fitness of dozens of mutants during asexual erythrocytic growth in a way that is neither confounded by double integration events nor likely to generate an excessive number of false-positive or false-negative results.

**DISCUSSION**

The PlasmoGEM resource has begun to facilitate conventional gene knockout and tagging experiments in \textit{P. berghei} by providing robust reagents for use in biological studies (see for instance Frénal et al., 2013). While our current emphasis is on generating and distributing the largest possible resource of knockout vectors, the same pipeline can be used to manufacture barcoded constructs that modify the 5' or 3' end of a gene. Due to the modular nature of the pipeline, the same intermediates can already be used to generate vectors for fluorescence and epitope tagging, and we anticipate that inducible systems that rely on regulatable promoters, degrons or aptamers (Armstrong and Goldberg, 2007; Goldfless et al., 2014; Pino et al., 2012) can be scaled up using the same approach. Recombinase-mediated engineering has already facilitated the generation of conditional alleles for inducible knockout approaches that rely on Flp recombinase (Suarez et al., 2013), and a similar strategy may be used for the insertion of loxP recognition sites for Cre-mediated recombination, which is becoming an important tool for generating inducible alleles in \textit{Plasmodium} (Collins et al., 2013).

As well as being highly versatile, we demonstrate here that PlasmoGEM vectors can be used to perform functional screens. Three lines of evidence support our conclusion that fitness measurements based on barcode counting generally reflect homologous integration events at the target locus: (1) a barseq screen of 46 PlasmoGEM vectors targeting protein kinases compared to the conventional kinome screen by Tewari et al. (2010); (2) barcodes of essential kinase genes did not replicate with the previous conventional knockout study by Tewari et al. (2010); (3) barcodes of essential kinase genes did not replicate with the previous conventional knockout study by Tewari et al. (2010); (3) barcodes of essential kinase genes did not replicate with the previous conventional knockout study by Tewari et al. (2010); and (4) the data indicate that DNA uptake after electroporation is not the factor limiting transfection efficiency.
in the parasite, showing that nonintegrated vectors are not maintained episomally but are effectively eliminated by day 4 of the infection; (3) unexpected mutants were in genes that we later verified as targetable by transfecting individual vectors and genotyping the resulting mutants. In one instance the inconsistent replication of a barcode raised the unexpected possibility of genetic redundancy of *rio2*, until whole-genome sequencing revealed a 29.7 kb duplication containing a second intact copy of the target gene. Whole-genome sequences of many rodent *Plasmodium* genomes are now available and contain no evidence that genome duplications commonly become fixed (Otto et al., 2014), possibly because of associated fitness costs. False positives of the *rio2* type will therefore always be rare events that can be filtered out, for instance by eliminating the least abundant 10% of mutants from the analysis, as has been recommended for barseq screens of yeast mutants (Robinson et al., 2014).

The use of vector pools to phenotype mixtures of mutants is particularly suited to *P. berghei*, where a library of cloned mutants would be of limited value, because the absence of an in vitro culture system for blood stages means that each cloned mutant has to be generated and propagated in mice. In marked contrast, the human malaria parasite *P. falciparum* has an efficient culture system for blood stages, but low recombination rates require transfection with circular vectors, and our preliminary data suggest that linear vectors of the type used here do not spontaneously integrate into the genome, even when their homology arms are each several kilobases long. The CRISPR-Cas9 system has recently been used successfully to transfect linear DNA in *P. falciparum* (Ghorbal et al., 2014). If this method increases recombination rates significantly, it raises the exciting prospect of generating large numbers of barcoded *P. falciparum* mutants that could then be pooled for genetic screens using barseq screening.

The scale of barseq screens in *P. berghei* is currently constrained by the complexity of the parasite pools that can be generated, which in turn is limited both by transfection efficiency and by the relatively large differences in recombination rates of different *PlasmoGEM* vectors. We show that as a result of such heterogeneity, screening pools of mutants poses the risk of losing genotypes that are generated in small numbers due to inefficient recombination rates at a target locus, or because they have a reduced fitness. Such mutants get outcompeted by the faster-growing genotypes in the pool, but our pilot screen suggests that in many cases their presence and growth rates can be measured in a second-pass screen that excludes most of the inefficient recombination events at a target locus, or because they have a reduced fitness. Such mutants get outcompeted by the faster-growing genotypes in the pool, but our pilot screen suggests that in many cases their presence and growth rates can be measured in a second-pass screen that excludes most of the inefficient recombination events.

The scale of barseq screens in *P. berghei* is currently constrained by the complexity of the parasite pools that can be generated, which in turn is limited both by transfection efficiency and by the relatively large differences in recombination rates of different *PlasmoGEM* vectors. We show that as a result of such heterogeneity, screening pools of mutants poses the risk of losing genotypes that are generated in small numbers due to inefficient recombination rates at a target locus, or because they have a reduced fitness. Such mutants get outcompeted by the faster-growing genotypes in the pool, but our pilot screen suggests that in many cases their presence and growth rates can be measured in a second-pass screen that excludes most of the inefficient recombination events.

The scale of barseq screens in *P. berghei* is currently constrained by the complexity of the parasite pools that can be generated, which in turn is limited both by transfection efficiency and by the relatively large differences in recombination rates of different *PlasmoGEM* vectors. We show that as a result of such heterogeneity, screening pools of mutants poses the risk of losing genotypes that are generated in small numbers due to inefficient recombination rates at a target locus, or because they have a reduced fitness. Such mutants get outcompeted by the faster-growing genotypes in the pool, but our pilot screen suggests that in many cases their presence and growth rates can be measured in a second-pass screen that excludes most of the inefficient recombination events. This will enable the creation of larger pools of mutants, which can then be combined into even more complex superpools to empower truly genome-wide screens of parasite growth and differentiation using only a fraction of the number of rodents required today to study much smaller gene sets. We anticipate that these global genetic barseq screens will make it easier to study the full genetic basis of parasite biology and drug resistance.
possible to identify systematically the parasite metabolic pathways required for replication of asexual blood stages. Dropout screens that ask which barcodes are lost from the sexual parasite stages may generate lists of candidate genes required for parasite transmission to the mosquito, and similar screens can be designed to get at genes required for virulence or for efficient replication in normocytes versus reticulocytes. In each case, vectors from the PlasmoGEM resource will be available for validation and follow-up investigations.

**EXPERIMENTAL PROCEDURES**

*Recombinase-Mediated Engineering Pipeline*

The PlasmoGEM vector resource was created by recombinase-mediated engineering in continuous liquid culture on 96-well plates largely as described (Pfander et al., 2013), but with the following modifications. To improve genome coverage, the arrayed P. berghei genomic DNA library in the pJAZZ-OK linear plasmid (Lucigen) that provides the starting point for vector production was doubled in size to ~10,000 clones. The PCR product consisting of a zeoS marker and 50 bp primer extensions homologous to the gene of interest was purified using the High Pure 96 UF Cleanup Kit (Roche) to improve lambda Red recombination efficiency in E. coli. The resulting intermediate vectors were selected in liquid culture containing 30 μg/mL kanamycin and 50 μg/mL zeocin. Kanamycin selection improved the proportion of intact intermediate vectors before selection, cultures were reinoculated into fresh selective medium and incubated for no longer than 16 hr at 37°C to favor the correct recombination product. Intermediate vector DNA was obtained using a QIAGEN Plasmid Plus 96 Miniprep kit and eluted into 65 μl Tris-EDTA buffer. DNA purity proved critical for the Gateway reaction, which was set up in 20 μl using 2 μl LR clonase (Invitrogen), 100 ng Gateway entry plasmid (e.g., pPRK-attL1-3xHA-hdhrf-ycfu-attL2), LR clonase buffer, and 300 ng purified intermediate vector. Gateway reactions were purified using the High Pure 96 UF Cleanup Kit. Electropotent E. coli TSA were transformed and plated on YEG-Cl containing kanamycin, and four colonies were picked to verify the sequence of their homology arms.

**Computational Vector Design**

A suite of software tools for automated vector design was created to work with the arrayed E. coli library of mapped P. berghei ANKA qDNA clones and has been described elsewhere (Schwach et al., 2015). In brief, the PlasmoGEM software tools select the most suitable library clone for each gene and type of modification and picks two unique 50 bp regions for lambda Red recombination, designated recUp and recDown, respectively. Together, these sites define the boundaries of the genomic region that is replaced by the selection marker cassette. A set of three PCR primers for quality-control purposes is also generated. For lambda Red recombination two highly unique 50 bp recUp and recDown regions were chosen on each selected library clone. In the case of knockout constructs these were designed to introduce the maximal deletion of the gene compatible with a homology arm length of ≥1 kb, while leaving at least 1 kb upstream and 0.8 kb downstream of neighboring genes intact. For C-terminal tagging vectors recUp and recDown were chosen to delete only the stop codon from the gene of interest. recUp and recDown sequences were included in oligonucleotides for lambda Red recombination, one of which also carried a 10–11 bp gene-specific barcode assigned automatically from a list of optimized sequences, and an 18 bp constant primer annealing site to read out the barcode after integration into the P. berghei genome. The software also automatically designed oligonucleotides for quality control during vector production (QCR1 and QCR2) and a unique oligonucleotide annealing to the P. berghei genome at least 200 bp outside of the boundary of the shorter homology arm (GT) for PCR genotyping transgenic parasites. Vector designs, primer, and barcode sequences can be viewed in a searchable database at http://plasmogem.sanger.ac.uk (Schwach et al., 2015). For quality control, up to four colonies per final vector were batch sequenced on an Illumina MiSeq instrument. Sequencing libraries were made essentially as described (Quail et al., 2012).

**New Tagging and Selection Cassettes**

Gateway entry scions were created for C-terminal tagging with fluorescent proteins by replacing the default 3xHA sequence in plasmid pR6K-attL1-3xHA-hdhrf-ycfu-attL2 (Pfander et al., 2013) with GFP-mu3 (Addgene plasmid 20410) (Franke-Fayard et al., 2004), ILoV (Addgene plasmid 26769) (Chapman et al., 2008), mEmerald (a kind gift from J. Liu), mCherry (from RmgmDB plasmid pLO046), and mVenus. Entry clones with R6K origins were maintained in E. coli PIR2 (Invitrogen).

**Parasites, Animals, Vector Preparation, and Transfection**

Transgenic P. berghei were generated either in wild-type strain ANKA 2.34 or in a selectable marker-free reporter strain of P. berghei ANKA c115c1 expressing the mu3 variant of green fluorescent protein clone Rmgm-7 (Franke-Fayard et al., 2004). Parasites were routinely propagated in 6- to 8-week-old Théiler’s original (OT) outbred mice. Schizors for barcode counting experiments were produced in female Wistar rats (200–250 g) to achieve maximal transfection efficiency. To generate pools of mutants for phenotyping by barcode counting, equal amounts of each vector were combined and the mixture digested with NotI to release the targeting vectors from the bacterial vector arms. A total of 5–8 μl of the digested vector mix, typically containing 100 ng of DNA for each vector, was used per transfection. Experiments with single vectors used 2 μg of NotI-restricted DNA per transfection. PlasmoGEM identification numbers for vectors used in this study are listed in Table S2. Transfections were done by electroporation of purified schizonts as described (Janse et al., 2006b), with the following modifications. Parasites for the schizont culture were from female Wistar rats (200–250 g) to achieve maximal transfection efficiency and were cultured for 21 hr before schizonts were isolated on a 55% Nycodenz/PBS cushion. Isolated schizonts were washed in complete media and electroporated using the 4D Nucleofector System (Lonza) in 16-well strips according to the pulse program Fi-115 (see Supplemental Information, Protocol 1, for more detail). Growth rate phenotyping of transfected parasites was done in 6- to 8-week-old Balb/c inbred mice. Rodents were from Harlan, UK. All animal research was conducted under licenses from the UK Home Office and used protocols approved by the ethics committee of the Wellcome Trust Sanger Institute.

**Growth Rate Phenotyping by Barcode Counting**

Three batches of schizonts were transfected with the same vector pool and each injected intravenously into a different Balb/c mouse. Resistant parasites were selected by pyrvinium (70 mg/L in the drinking water). Infections were monitored daily using Giemsa-stained thin blood films. A total of 30 μl of infected blood was collected from the tail at exactly the same time on days 4–8 posttransfection and diluted in 200 μl of phosphate-buffered saline. Total DNA was extracted from each sample and resuspended in water (50 μl on days 4–6 posttransfection, 100 μl for later days) using Supplemental Protocol 2. To sequence the vector-specific barcodes, 1 μl of each DNA sample served as template for a PCR reaction using Advantage 2 Taq polymerase (Clontech) with primers arg444 and arg445 (1 × 95°C/5 min denaturation, 35 × 95°C/30 s, 55°C/20 s, 68°C/8 s, 1 × 10 min at 68°C), which bind to constant annealing sites flanking each barcode. The 167 bp ampiclon was further extended in a second PCR reaction using oligonucleotides that in their 5’ extensions introduce illumina adaptors and sample-specific barcodes (Table S3) for multiplexing up to 32 samples in one run of a MiSeq instrument. For sample-specific indexing, 5 μl of the first ampiclon served as template for a further ten amplification cycles (1 × 95°C/2 min, 10 × 95°C/30 s, 68°C/15 s, 1 × 5 min, 68°C) using one generic oligonucleotide (PE1.0) and one of a set of 32 sample-specific indexing oligonucleotide. A total of 100 ng of each sequencing library was pooled and quality controlled by quantitative PCR for the presence of sequencing adaptors. Libraries were sequenced using MiSeq Reagent Kit v2 (300 cycle) from Illumina (MS-102-2002). In some experiments we compared PCR-mediated indexing to adaptor ligation libraries. Due to their low complexity, PCR amplicon libraries had to be diluted to 4 nM before loading the flow cell of a MiSeq instrument (Illumina) at low cluster density (4 × 10^5 clusters/mm²) with 30%–50% of PhIX spike-in. Sequencing of 150 bp paired-ends yielded 1.0–1.5 × 10^6 reads on average for each of the 32 samples. Using a Perl script, barcode sequences were extracted from sequencer output, counted, and the relative abundance of each barcode within the pool determined. The quantitation was considered reliable for...
barcodes accounting for at least 0.1% of all counts. Parasitaemia curves for these mutants were inferred from the relative abundance of each barcode and from the observed total parasitaemia as determined using a Giemsa stained thin blood film. The relative fitness ($\omega$) of a mutant represented by a barcode on a given day ($d$) was calculated according to Mani et al. (2008) by comparing the daily change in its relative abundance ($A$) to that of the reference genes ($A_{r,g}$) with normal growth.

$$\omega_{d} = \frac{\sum_{i=1}^{n} A_{d,i+1} - A_{d,i}}{n}$$

Statistical analyses compared each barcode against the normal-growth reference vectors using a two-tailed t test (unequal variance, p values adjusted according to the false discovery rate method). A given mutant was considered viable when consistent growth of its barcode was observed for all time points in at least two of three replicates.

**Genotyping**

To verify vector integration by diagnostic PCR on parasite genomic DNA, we designed a target gene-specific oligonucleotide to anneal to the chromosomes separated by pulsed-field gel electrophoresis (PFGE) as described previously (Pfander et al., 2011).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four tables, five figures, and two Supplemental Protocols and can be found with this article at http://dx.doi.org/10.1016/j.chom.2015.01.014.

**AUTHOR CONTRIBUTIONS**

O.B. and J.C.R. directed the research. E.B. led the PlasmoGEM resource team with additional support from K.M. The development of barcode sequencing was led by A.R.G., who also carried out the validation experiments. F.S. constructed the PlasmoGEM software tools and database and provided computational support for the project. G.G., B.A., C.H., and A.R.G. produced PlasmoGEM vectors and provided technology development. O.B., J.C.R., E.B., F.S., A.R.G., M.A.Q., and C.P. designed and analyzed experiments. O.B. and A.R.G. wrote the paper with critical input from all authors.

**ACKNOWLEDGMENTS**

We thank Tom Metcalf for technical assistance, Mandy Sanders for coordinating sequencing experiments, Thomas Dan Otto for guidance on analysing whole-genome sequence data, and the Sanger Institute sequencing teams. A plasmid encoding Emerald fluorescent proteins was a kind gift from J. Liu. A.R.G received a EViMaRaS studentship funded through the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement number 242095. Work at the Sanger Institute was funded by grants from the Wellcome Trust (098051) and the Medical Research Council (G0501670).

Received: October 20, 2014
Revised: December 1, 2014
Accepted: January 7, 2015
Published: February 26, 2015

**REFERENCES**

Armstrong, C.M., and Goldberg, D.E. (2007). An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. Nat. Methods 4, 1007–1009.

Balu, B., and Adams, J.H. (2006). Functional genomics of Plasmodium falciparum through transposon-mediated mutagenesis. Cell. Microbiol. 8, 1529–1536.

Braks, J.A., Franke-Fayard, B., Kroese, H., Janse, C.J., and Waters, A.P. (2006). Development and application of a positive-negative selectable marker system for use in reverse genetics in Plasmodium. Nucleic Acids Res. 34, e99.

Chapman, S., Faulkner, C., Kaiserli, E., Garcia-Mata, C., Savenkov, E., Roberts, A.G., Oparka, K.J., and Christie, J.M. (2008). The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. Proc. Natl. Acad. Sci. USA 105, 20038–20043.

Collins, C.R., Das, S., Wong, E.H., Andennatten, N., Stallmach, R., Hackett, F., Herman, J.P., Müller, S., Meissner, M., and Blackman, M.J. (2013). Robust inducible Cre recombinase activity in the human malaria parasite Plasmodium falciparum enables efficient gene deletion within a single asexual erythrocytic growth cycle. Mol. Microbiol. 88, 687–701.

Crabb, B.S., and Cowman, A.F. (1996). Characterization of promoters and stable transfection by homologous and nonhomologous recombination in Plasmodium falciparum. Proc. Natl. Acad. Sci. USA 93, 7289–7294.

Dessens, J.T., Siden-Kiams, I., Mendoza, J., Mahariraki, V., Khater, E., Vachou, D., Xu, X.J., Kafatos, F.C., Louis, C., Dimopoulos, G., and Sindell, R.E. (2003). SOAP, a novel malaria ookinete protein involved in mosquito midgut invasion and oocyst development. Mol. Microbiol. 49, 319–329.

Drouiche, E., Priomt, A., Thomas, V., Mattei, D., Knockaert, M., Richardson, C., Salicicandro, P., Alano, P., Jafarshad, A., Baratte, B., et al. (2004). Plasmodium falciparum glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors. Biochim. Biophys. Acta 1697, 181–196.

Franke-Fayard, B., Trauern, H., Ramesar, J., Mendoza, J., van der Keur, M., van der Linden, R., Sinder, R.E., Waters, A.P., and Janse, C.J. (2004). A Plasmodium bergheri reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol. Biochem. Parasitol. 137, 23–33.

Frênel, K., Tay, C.L., Mueller, C., Bushell, E.S., Jia, Y., Grindorge, A., Billker, O., Rayner, J.C., and Soldati-Favre, D. (2013). Global analysis of apicomplexan protein S-acyl transfersases reveals an enzyme essential for invasion. Traffic 14, 895–911.

Ghorbal, M., Gorman, M., Macpherson, C.R., Martins, R.M., Scherf, A., and Lopez-Rubi, J.J. (2014). Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. Nat. Biotechnol. 32, 819–821.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucoc-Danila, A., Anderson, K., André, B., et al. (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature 418, 387–391.

Godiska, R., Mead, D., Bhodda, V., Wu, C., Hochstein, R., Karsi, A., Uusdin, K., Entezam, A., and Ravin, N. (2010). Linear plasmid vector for cloning of repetitive or unstable sequences in Escherichia coli. Nucleic Acids Res. 38, e88.

Goldfuss, S.J., Wagner, J.C., and Niles, J.C. (2014). Versatile control of Plasmodium falciparum gene expression with an inducible protein-RNA interaction. Nat. Commun. 5, 5329.

Guler, J.L., Freeman, D.L., Ayhong, V., Patrapuvich, R., White, J., Guijar, R., Phillips, M.A., DeRisi, J., and Rathod, P.K. (2013). Asexual populations of the human malaria parasite, Plasmodium falciparum, use a two-step genomic strategy to acquire accurate, beneficial DNA amplifications. PLoS Pathog. 9, e1003375.

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995). Simultaneous identification of bacterial virulence genes by negative selection. Science 269, 400–403.

Janse, C.J., Franke-Fayard, B., Mair, G.R., Ramesar, J., Thiell, C., Englemann, S., Matuschewski, K., van Gemert, G.J., Sauerwein, R.W., and Waters, A.P. (2006a). High efficiency transfection of Plasmodium berghei facilitates novel selection procedures. Mol. Biochem. Parasitol. 145, 80–70.

Janse, C.J., Ramesar, J., and Waters, A.P. (2006b). High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. Nat. Protoc. 1, 346–356.

Jebiwott, S., Govindaswamy, K., Mbugua, A., and Bhattacharyya, P. (2013). Plasmodium berghei calcium dependent protein kinase 1 is not required for host cell invasion. PLoS ONE 8, e79171.
Kato, N., Sakata, T., Breton, G., Le Roch, K.G., Nagle, A., Andersen, C., Bursulaya, B., Henson, K., Johnson, J., Kumar, K.A., et al. (2008). Gene expression signatures and small-molecule compounds link a protein kinase to Plasmodium falciparum motility. Nat. Chem. Biol. 4, 347–356.

Langridge, G.C., Phan, M.D., Turner, D.J., Perkins, T.T., Parts, L., Haase, J., Charles, I., Maskell, D.J., Peters, S.E., Dougan, G., et al. (2009). Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. Genome Res. 19, 2308–2316.

LaRonde, N.A. (2014). The ancient microbial RIO kinases. J. Biol. Chem. 289, 9488–9492.

Mani, R., St Onge, R.P., Hartman, J.L., 4th, Giaever, G., and Roth, F.P. (2008). Defining genetic interaction. Proc. Natl. Acad. Sci. USA 105, 3461–3466.

Mazurkiewicz, P., Tang, C.M., Boone, C., and Holden, D.W. (2006). Signature-tagged mutagenesis: barcoding mutants for genome-wide screens. Nat. Rev. Genet. 7, 929–939.

Ni, J.Q., Zhou, R., Czech, B., Liu, L.P., Holderbaum, L., Yang-Zhou, D., Shim, H.S., Tao, R., Handler, D., Karpowicz, P., et al. (2011). A genome-scale shRNA resource for transgenic RNA in Drosophila. Nat. Methods 8, 405–407.

Oppenheim, R.D., Creek, D.J., Macrae, J.J., Modrzynska, K.K., Pino, P., Limentakis, J., Polonais, V., Seeber, F., Barrett, M.P., Billker, O., et al. (2014). BCKDH: the missing link in apicomplexan mitochondrial metabolism is required for full virulence of Toxoplasma gondii and Plasmodium berghei. PLoS Pathog. 10, e1004263.

Otto, T.D., Böhme, U., Jackson, A.P., Hunt, M., Franne-Fayard, B., Hoejemakers, W.A., Religa, A.A., Robertson, L., Sanders, M., Ogun, S.A., et al. (2014). A comprehensive evaluation of rodent malaria parasite genomes and gene expression. BMC Biol. 12, 86.

Pfander, C., Anar, B., Schwach, F., Otto, T.D., Brochet, M., Volkman, K., Quail, M.A., Pain, A., Rosen, B., Skarnes, W., et al. (2011). A scalable pipeline for highly effective genetic modification of a malaria parasite. Nat. Methods 8, 1078–1082.

Pfander, C., Anar, B., Brochet, M., Rayner, J.C., and Billker, O. (2013). Recombination-mediated genetic engineering of Plasmodium berghei DNA. Methods Mol. Biol. 923, 127–138.

Pino, P., Sebastian, S., Kim, E.A., Bush, E., Brochet, M., Volkman, K., Kozlowski, E., Linas, M., Billker, O., and Soldati-Favre, D. (2012). A tetracycline-repressible transactivator system to study essential genes in malaria parasites. Cell Host Microbe 12, 824–834.

Quail, M.A., Smith, M., Coupland, P., Otto, T.D., Harris, S.R., Connor, T.R., Bertoni, A., Swerdlow, H.P., and Gu, Y. (2012). A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics 13, 341.

Robinson, D.G., Chen, W., Storey, J.D., and Gresham, D. (2014). Design and analysis of Bar-seq experiments. G3 (Bethesda) 4, 11–18.

Sarav, M., Schneider, S., Pozniakowski, A., Roguev, A., Ernst, S., Zhang, Y., Hyman, A.A., and Stewart, A.F. (2006). A recombineering pipeline for functional genomics applied to Caenorhabditis elegans. Nat. Methods 3, 839–844.

Schwach, F., Bushell, E., Gomes, A.R., Anar, B., Girling, G., Herd, C., Rayner, J.C., and Billker, O. (2015). PlasmoGEM, a database supporting a community resource for large-scale experimental genetics in malaria parasites. Nucleic Acids Res. 43 (Database issue), D1176–D1182.

Sebastian, S., Brochet, M., Collins, M.O., Schwach, F., Jones, M.L., Goulding, D., Rayner, J.C., Choudhary, J.S., and Billker, O. (2012). A Plasmodium calcium-dependent protein kinase controls zygote development and transmigration by translationally activating repressed mRNAs. Cell Host Microbe 12, 9–19.

Skarnes, W.C., Rosen, B., West, A.P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A.O., Thomas, M., Harrow, J., Cox, T., et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474, 337–342.

Smith, A.M., Heisler, L.E., Meiler, J., Kaper, F., Thompson, M.J., Chee, M., Roth, F.P., Giaever, G., and Nislow, C. (2009). Quantitative phenotyping via deep barcode sequencing. Genome Res. 19, 1836–1842.

Spaccapelo, R., Janse, C.J., Caterbi, S., Franke-Fayard, B., Bonilla, J.A., Syphard, L.M., Di Cristina, M., Dottorini, T., Savarino, A., Cassone, A., et al. (2010). Plasmepsin 4-deficient Plasmodium berghei are virulence attenuated and induce protective immunity against experimental malaria. Am. J. Pathol. 176, 205–217.

Straimer, J., Lee, M.C., Lee, A.H., Zeitler, B., Williams, A.E., Pearl, J.R., Zhang, L., Rebar, E.J., Gregory, P.D., Linás, M., et al. (2012). Site-specific genome editing in Plasmodium falciparum using engineered zinc-finger nucleases. Nat. Methods 9, 993–998.

Suarez, C., Volkman, K., Gomes, A.R., Billker, O., and Blackman, M.J. (2013). The malarial serine protease SUB1 plays an essential role in parasite liver stage development. PLoS Pathog. 9, e1003811.

Tewari, R., Straschil, U., Bateman, A., Böhme, U., Chervach, L., Gong, P., Pain, A., and Billker, O. (2010). The systematic functional analysis of Plasmodium protein kinases identifies essential regulators of mosquito transmission. Cell Host Microbe 8, 377–387.

Tomas, A.M., Margos, G., Dimopoulos, G., van Lin, L.H., de Koning-Ward, T.F., Sinha, R., Beetsma, A.L., Rodriguez, M.C., Karras, M., et al. (2001). P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. EMBO J. 20, 3975–3983.

van Dijk, M.R., Janse, C.J., and Waters, A.P. (1996). Expression of a Plasmodium gene introduced into subtelomeric regions of Plasmodium berghei chromosomes. Science 271, 862–865.

van Dijk, M.R., van Schaik, B.C., Khan, S.M., van Dooren, M.W., Ramesar, J., Kaczanowski, S., van Gemert, G.J., Kroeze, H., Stunnenberg, H.G., Eling, H.W.M., et al. (2010). Three members of the 6-cys protein family of Plasmodium play a role in gamete fertility. PLoS Pathog. 6, e1000853.

Wagner, J.C., Platt, R.J., Goldfuss, S.J., Zhang, F., and Niles, J.C. (2014). Efficient CRISPR-Cas9-mediated genome editing in Plasmodium falciparum. Nat. Methods 11, 915–918.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., et al. (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285, 901–906.

Wu, Y., Sifri, C.D., Lei, H.H., Su, X.Z., and Wellems, T.E. (1995). Transfection of Plasmodium falciparum within human red blood cells. Proc. Natl. Acad. Sci. USA 92, 973–977.

Zhang, Y., Muyers, J.P., Testa, G., and Stewart, A.F. (2000). DNA cloning by homologous recombination in Escherichia coli. Nat. Biotechnol. 18, 1314–1317.

Zhang, M., Mishra, S., Sakhiviel, R., Rojas, M., Ranjan, R., Sullivan, W.J., Jr., Fontoura, B.M., Menard, R., Dever, T.E., and Nussenzweig, V. (2012). PK4, a eukaryotic initiation factor 2α(eIF2α) kinase, is essential for the development of the erythrocytic cycle of Plasmodium. Proc. Natl. Acad. Sci. USA 109, 3956–3961.