Advantages and disadvantages of conditional systems for characterization of essential genes in *Toxoplasma gondii*

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SUMMARY

The dissection of apicomplexan biology has been highly influenced by the genetic tools available for manipulation of parasite DNA. Here, we describe different techniques available for the generation of conditional mutants. Comparison of the advantages and disadvantages of the three most commonly used regulation systems: the tetracycline inducible system, the regulation of protein stability and site-specific recombination are discussed. Using some previously described examples we explore some of the pitfalls involved in gene-function analysis using these systems that can lead to wrong or over-interpretation of phenotypes. We will also mention different options to standardize the application of these techniques for the characterization of gene function in high-throughput.

Key words: apicomplexan parasites, gold-standard genetic tools, conditional mutants, tetracycline inducible system, protein destabilization, DiCre.

INTRODUCTION

The Apicomplexa phylum consists of more than 5000 species, most of which are obligate intracellular parasites and include important human and veterinary pathogens, such as *Plasmodium* spp., *Toxoplasma gondii*, *Cryptosporidium* spp., *Eimeria tenella* and *Neospora caninum*. The defining feature common to all apicomplexans is the presence of the apical complex, from which secretory organelles (e.g. rhoptries, micronemes) release their contents during cell invasion (Nichols *et al*. 1983; Carruthers *et al*. 1999). To date, no effective vaccines or treatments are available against the disease-causing apicomplexans such as *T. gondii* (Zhang *et al*. 2013) or *N. caninum* (Reichel and Ellis, 2009). Moreover, the development of drug resistance remains a serious threat against the control of malaria (Chong *et al*. 2013). In order to drive the discovery of targets for parasite disease control forward we need to invest in the development of new molecular strategies to achieve a better understanding of basic parasite biology.

Several key technologies have emerged that have allowed the molecular dissection of functions in important pathogens (Meissner *et al*. 2007; Pino, 2013). In addition, publication of several apicomplexan parasite genomes in the EuPathDB database (www.eupathdb.org) allows for fast and robust *in silico* identification of promising candidate genes through comparative genome studies (Aurrecoechea *et al*. 2010). However, not all apicomplexan parasites are amenable to genetic manipulation due to difficulties in continuous *in vitro* cultivation, transfection or isolation of intracellular developmental stages in sufficient numbers for downstream molecular applications (Oberstaller *et al*. 2013). The development of several reverse genetic tools in *T. gondii* has reinforced its role as a major model system for studying other apicomplexan parasites and also for conserved biological processes in less related organisms (Meissner *et al*. 2007). Several techniques based on the homologous recombination of exogenous DNA have allowed the generation of knockouts after the removal of the gene of interest (GOI) (reviewed in Jakot *et al*. 2013). Moreover, the efficiency of these approaches has been improved after the generation of strains deficient in the non-homologous end joining repair (NHEJ) pathway (Fox *et al*. 2009; Huynh and Carruthers 2009) allowing for repeated and efficient stable transfection in *T. gondii* (Sheiner *et al*. 2011; Andenmatten *et al*. 2013; Pieperhoff *et al*. 2013).

In a haploid organism such as *T. gondii*, the characterization of essential genes requires the employment of conditional systems that allow a tight and reliable regulation of gene activity. Current approaches for down-regulation of genes in other organisms, such as interference RNA (RNAi) and double-strand RNA (dsRNA), have been unsuccessful to date (Kolev *et al*. 2011). Here we discuss the advantages and disadvantages of the three most commonly used conditional regulation systems in

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Table 1. Summary of the technologies described in this review

	Conditional recombination (DiCre)	Tet system	ddFKBP system
Regulation efficiency	Up to 99% knockouts in population[a]	100% knockdowns with 10–50-fold regulation[b]	100% knockdowns with 1–10-fold regulation[c]
Advantages	No reversion No background expression GOI under control of endogenous promoter	Reversible Inducer (ATc) can be used *in vivo*	Rapid regulation GOI under control of endogenous promoter Single vector approach for endogenous tagging and knockdown
Disadvantage	Not reversible No clonal population	Reversion and background expression Not controlled under endogenous promoter	Reversion and background expression Only proteins accessible to the proteasome
Suggested usage	Knockouts and over-expression/ trans-dominant mutants	Reversible knockdowns	Over-expression/trans-dominant mutants

[a] Andenmatten *et al*. (2013).
[b] Meissner *et al*. (2002).
[c] Herm-Gotz *et al*. (2007).

T. gondii: Tet-inducible transcription (Meissner *et al*. 2001, 2002), rapid protein destabilization (Herm-Gotz *et al*. 2007) and conditional site-specific recombination (Andenmatten *et al*. 2013). With future phenome project development in mind, we will discuss which of these systems allow a high-throughput approach in combination with gene-editing or Recombineering technologies.

WHAT IS THE OPTIMAL SYSTEM TO GENERATE CONDITIONAL MUTANTS?

Conditional regulation systems allow the functional analysis of essential genes for the biology of an organism. When studying an essential gene, one needs to be aware of the intrinsic advantages and disadvantages of each regulation system. In our opinion, the ideal conditional knockout system should have five properties: (i) rapid regulation of the GOI; (ii) reversible and tuneable regulation of gene activity; (iii) efficient regulation of gene function without any residual activity; (iv) generation of a conditional, clonal knockout strain that can be easily analysed; and (v) prior to induction, the introduced modifications should not interfere with the GOI's function.

Unfortunately such a conditional regulation system does not yet exist for *T. gondii* and other model systems and therefore the advantages and disadvantages of each conditional system need to be carefully considered before a lengthy experimental approach is started (Table 1).

Tetracycline inducible system

The tetracycline resistance operon is recognized in genetic engineering as a useful tool for the manipulation of transcription regulation in eukaryotic cells. Two versions of tetracycline (Tet)-dependent gene regulation find widespread application, allowing either conditional repression or activation of transcription of the GOI. The repression system mimics the bacterial system, by placing Tet operator (TetO) sequences close to the transcriptional start site of a GOI, leading to a block in transcription initiation upon binding of the Tet repressor (TetR). Addition of tetracycline, or tetracycline derivatives such as doxycycline, abolishes DNA-binding and consequently allows transcription. This regulation system has been successfully employed in a variety of eukaryotes (Lovett *et al*. 2000), including protozoan parasites (Wirtz and Clayton, 1995; Hamann *et al*. 1997; Ramakrishnan *et al*. 1997; Wirtz *et al*. 1999). An analogous system was developed in *T. gondii*, but this was not sufficient for the generation of conditional mutants (Meissner *et al*. 2001). Although the expression of a bulkier version of TetR led to an increased regulation efficiency (van Poppel *et al*. 2006) this system in general suffers from a high residual activity in the repressed state. Furthermore, the necessity of keeping conditional mutants in the presence of the inducer anhydrotetracycline (ATc), less toxic for *T. gondii*, increases the risk of revertants.

In contrast, the Tet-transactivator system (Gossen and Bujard, 1992) is based on a tetracycline-responsive promoter (TRE), where TetO sequences are placed upstream of a minimal promoter. Furthermore, the fusion of TetR to a virion protein 16 (VP16) transactivating domain of the *Herpes simplex virus* generated an efficient tetracycline-dependent transactivator (tTA). Consequently, binding of tTA to the TRE activates transcription of the GOI, whereas addition of ATc abolishes binding of tTA to TetO resulting in inactivation of transcription.

While this system found widespread employment in diverse eukaryotes, a direct adaptation in apicomplexan parasites was not possible due to the inability of tTA to activate transcription from TRE (Meissner *et al*. 2001).

To overcome the lack of activity of the transcription activation domain, a screen was performed in *T. gondii* to identify *Toxoplasma* sequences that support tTA transcription (Meissner *et al*. 2002). The transactivator isolated, TATi-1 (trans-activator trap identified), showed tightly inducible regulation. This artificial transactivator has also been shown to be active in *Plasmodium falciparum* (Meissner *et al*. 2005). To date, this system has been used to functionally analyse numerous genes in *T. gondii* (Limenitakis and Soldati-Favre, 2011).

A parasite strain expressing this transactivator (RH ΔKu80 TATi-1) was created to aid the targeting of endogenous gene sequences via homologous recombination (Sheiner *et al*. 2011). Here, replacement of the endogenous GOI promoter by homologous recombination with a Tet-inducible promoter is sufficient to control the transcription of the GOI.

Recently more powerful transactivators, TRADs (trans-activating domains), derived from ApiAP2 transcription factors have been established. TRAD4 has been shown to be suitable for the study of genes essential for the erythrocytic stages of *Plasmodium berghei* development (Pino *et al*. 2012).

While the Tet-transactivator system will remain one of the workhorses for the functional characterization of essential genes there are some major pitfalls. With this technique gene expression can be reversibly regulated, although a tuneable regulation is not possible. This technology allows generation of clonal knockdown strains that can be easily analysed. However, despite the efficiency in gene regulation observed it usually takes a long time until protein levels of the GOI are undetectable depending on the relative protein stability. Moreover, every minimal promoter tested thus far shows some residual activity (Meissner *et al*. unpublished results), thus it does not allow complete down-regulation of gene function. In addition, since it does not allow expression of the GOI from its endogenous promoter, certain genes will be expressed at the wrong time and at different expression levels in the 'ON' state.

Rapid regulation of protein stability

In order to obtain de-stabilization domains based on the rapamycin-binding protein FKBP-12 (ddFKBP) random mutations were introduced in this protein. The mutated proteins were screened for Shield-1 (Shld-1)-dependent regulation. Addition of Shld-1, an analogue of rapamycin, resulted in rapid stabilization and removal of this ligand in rapid destabilization of ddFKBP (Banaszynski *et al*. 2006). Importantly, N- or C-terminal fusion of ddFKBP with a protein of interest allows efficient, rapid and reversible degradation.

This system has been successfully adapted in apicomplexan parasites (Armstrong and Goldberg, 2007; Herm-Gotz *et al*. 2007; de Azevedo *et al*. 2012) and has been used successfully in *P. falciparum* to generate knockdown mutants (Russo *et al*. 2009; Dvorin *et al*. 2010).

The rapid response kinetic of the ddFKBP-system is of particular advantage when rapid processes are to be analysed, such as components of trafficking systems or signalling cascades. A disadvantage of this technology is that proteins residing within organelles typically cannot be regulated using this destabilization domain since the fused protein needs to be accessible to the proteasome which resides in the cytosol. It has been reported that administration of Shld-1 can cause delayed parasite growth thus it is an important requirement to include appropriate controls for phenotype interpretation (de Azevedo *et al*. 2012).

Other destabilization domains have been developed, such as DHFR-based systems (DDD) (Iwamoto *et al*. 2010). This system used *Escherichia coli* DHFR as a candidate protein to engineer a second destabilizing domain with improved properties. After addition of trimethoprim (TMP), EcDDD showed faster degradation kinetics. Another important advantage of using the DDD system is that TMP ligand is commercially available, is relatively cheap, and possesses good pharmacological characteristics. This system has been demonstrated in *P. falciparum* combined with GFP and HA sequences to study the role of Rpn6 asparagine repeat (Muralidharan *et al*. 2011).

While in principle protein degradation systems should allow a rapid generation of knockdown mutants in *T. gondii* by simply tagging the endogenous GOI with this domain, our experience with ddFKBP is rather discouraging, since after successful tagging of the protein of interest no efficient destabilization could be observed (Meissner *et al*. unpublished results). One obstacle might be that during prolonged selection of parasite mutants in the presence of Shld-1 reversions occur, leading to inefficient regulation. Alternatively, it is possible that proteins that are associated in a multi-protein complex might be stabilized due to their interaction with other components of the complex. This was the case for components of the MyoA-motor complex (unpublished). In contrast, the system is particularly well suited for the regulated expression of dominant negative mutants or for the generation of over-expression phenotypes (Herm-Gotz *et al*. 2007; Agop-Nersesian *et al*. 2009, 2010; Breinich *et al*. 2009; van Dooren *et al*. 2009; Daher *et al*. 2010; Kremer *et al*. 2013).

An exciting possibility for future forward genetic screens could be the use of a cDNA library fused to ddFKBP to screen for over-expression phenotypes as recently demonstrated on a small scale for apicomplexan Rab-GTPases (Kremer *et al*. 2013).

Despite the advantages of the ddFKBP-system, major obstacles remain to be solved in order to generate conditional knockdown mutants at a larger

scale. One advantage of this system is the possibility of reversible and tuneable regulation. Moreover, it allows the generation of clonal knockdown strains that can be easily analysed. On the contrary, whereas the regulation can be efficient and rapid, the success rate for the regulation of endogenous proteins appears to be low. Inefficient regulation is frequently observed, even for over-expression and dominant negative mutants.

Site-specific recombinase systems (SSR)

Site-specific recombinase systems offer attractive possibilities for genetic manipulations and have found widespread use in different model systems to specifically remove DNA-sequences flanked by recombinase-specific recognition sites (Garcia-Otin and Guillou, 2006). A major bottleneck for the use of SSR in single-celled organisms is the necessity to tightly regulate the excision event. Different methods have been developed to control the expression of recombinases including heat-shock promoters (Combe *et al*. 2009), mutations in the loxP or FRT sites to block the reverse reaction (Arakawa *et al*. 2001), Tet-inducible systems (Plageman and Lang, 2012) and hormone-binding domain systems (Metzger *et al*. 1995; Brecht *et al*. 1999). While in *P. berghei* temporal control of the excision event is achieved by the stage-specific expression of Flp-recombinase in deletion clones (Carvalho *et al*. 2004; Lacroix *et al*. 2011), a ligand-controlled approach has been particularly successful in *T. gondii* (Andenmatten *et al*. 2013) and *P. falciparum* (Collins *et al*. 2013). This approach is known as the DiCre system, in which the Cre recombinase is expressed in the form of two separate polypeptides, each fused to a different rapamycin-binding protein (FKBP12 and FRB). The addition of rapamycin induces heterodimerization of these two components and restores recombinase activity (Jullien *et al*. 2003; Jullien *et al*. 2007).

SSR systems have a high versatility and allow multiple applications, such as removal, inversion or integration of DNA sequences (Bouabe and Okkenhaug, 2013). In our experience, the most reliable application in *T. gondii* is the GeneSwap strategy (Andenmatten *et al*. 2013). Here a vector including the cDNA of the GOI flanked by loxP sites and under control of the endogenous promoter is used. In case of intron-less GOIs it might be worth using a synthetic gene to avoid homologous recombination within the cDNA. Downstream of the second loxP site a reporter gene, YFP, was placed followed by the selectable marker and the 3′-UTR of the GOI. This cassette is introduced into the locus of the GOI by double homologous recombination. Consequently, induction of DiCre results in YFP-positive parasites that have completely lost the GOI. This approach has been successfully used to generate parasites devoid of genes, such as *myoA, mic2, act1* (Andenmatten *et al*. 2013), *ama1* (Bargieri *et al*. 2013), *dhhc7* (Frenal *et al*. 2013), *mlc1, myoA/B/C, gap40, gap45, gap50* (Egarter *et al*. 2014) and others. Also, in *P. falciparum* the induction of DiCre-recombinase results in nearly the whole parasite population excising the floxed DNA sequence (Collins *et al*. 2013).

While generation of conditional mutants with the DiCre system promises a very high success rate (>90%), there are some advantages and disadvantages that need to be considered. The introduced modifications are less likely to interfere with gene function, since the GOI will be regulated by its own endogenous promoter. Upon excision of the GOI, it is possible to obtain parasites without any residual expression. However, the regulation is irreversible and one has to keep in mind that induction results in a mixed population where 20–96% of the parasites display the desired knockout phenotype. While the GeneSwap strategy allows easy identification of knockouts due to expression of YFP, other approaches, such as proteomics, might be hindered by contamination with non-induced parasites. However, contamination with non-induced parasites could be overcome or minimized by enriching for YFP-positive parasites using sorting methods such as FACS. Despite a short induction of DiCre (<4 h) being sufficient to trigger excision of the GOI, the kinetic of down-regulation can be very slow depending on the stability of the protein, similar to the Tet-transactivation system.

DIRECT COMPARISON OF TATi, DDFKBP AND DICRE

To date the function of only a few genes have been analysed using all three technologies. Strikingly, the observed phenotypes have been different depending upon which technique was used to disrupt the same gene. This has affected the interpretation of data and conclusions, for example the work describing the role of the glideosome in host cell invasion. The glideosome is a macromolecular complex involved in gliding motility which is anchored to the plasma membrane (PM) and the inner membrane complex (IMC) (Frenal *et al*. 2010). It is described as essential machinery for host-cell invasion and egress in apicomplexan parasites of which myosin A (MyoA) was thought to play a key role.

A phenotypic characterization of MyoA was performed using the technologies described above: modification at a genomic level by removal of the gene using DiCre (Fig. 1A–C) (Andenmatten *et al*. 2013; Egarter *et al*. 2014); at a transcriptional level, using the Tet-transactivator system (Fig. 1D and E) (Meissner *et al*. 2002); and at a protein level, using the ddFKBP-system to over-express the MyoA-tail (Fig. 1F and G) (Agop-Nersesian *et al*. 2009).

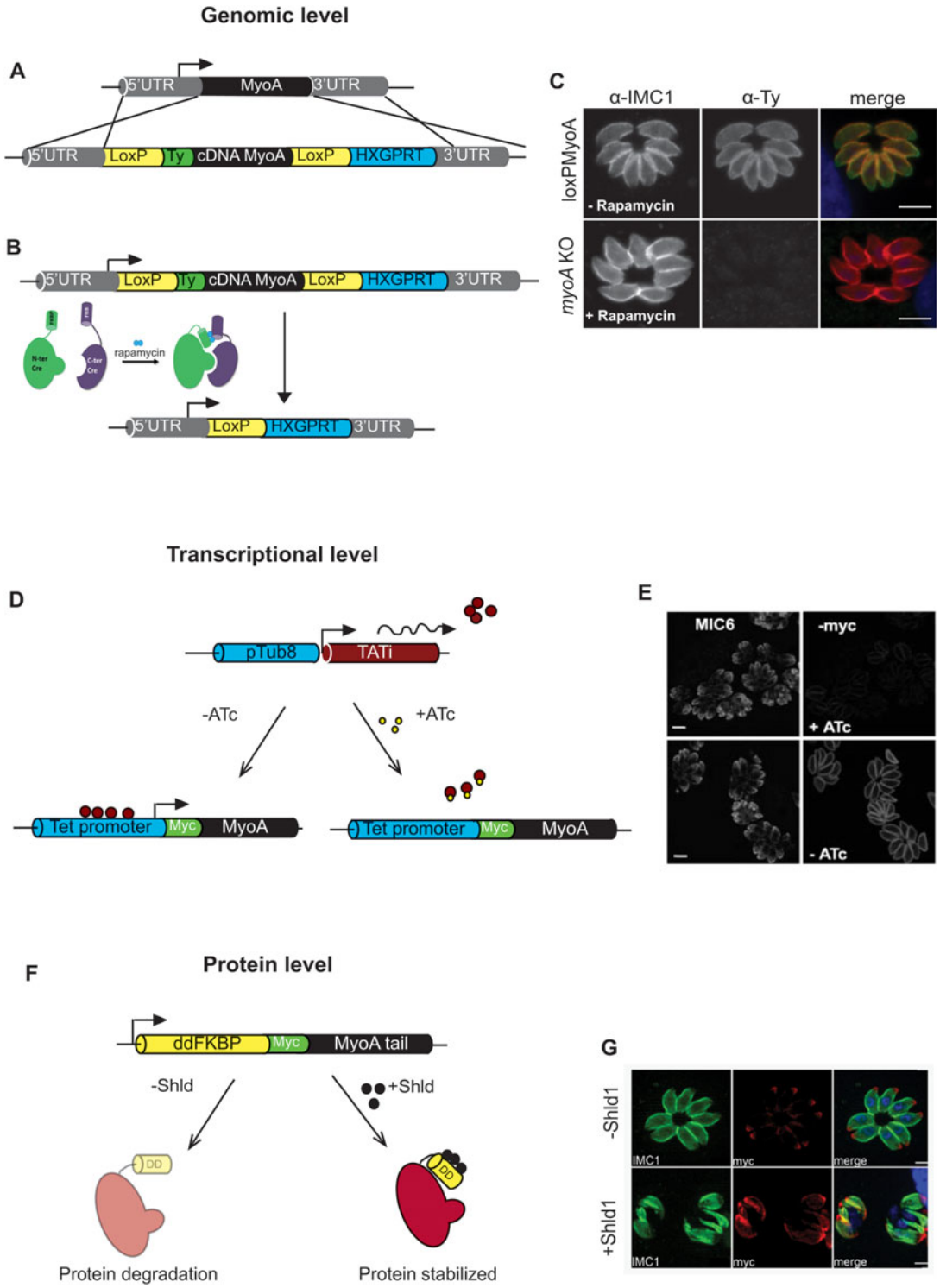

Fig. 1. Comparative analysis of inducible knockout systems and their applications for the motor protein MyoA (adapted from Meissner *et al*. 2002; Herm-Gotz *et al*. 2007; Andenmatten *et al*. 2013). Manipulation at the genomic level using the DiCre system (A)/(B). Replacement of endogenous MyoA locus with a cDNA copy (A). Upon rapamycin induction, MyoA is irreversibly removed by excision of the MyoA cDNA (B)/(C). Transcriptional manipulation using the Tet-inducible system, transcription is inhibited by addition of anhydrotetracycline (ATc) preventing transactivator binding to the promoter region and therefore silencing transcription (D). Myc tagged MyoA expression was shown to be disrupted by immunofluorescence assay (IFA) (E). Manipulation of proteins containing the DD domain, addition of Shld-1 allows stability and over-expression (F). Myc tagged MyoA degradation shown by IFA (G).

Reduction in MyoA expression levels using the Tet-transactivator system resulted in significant reduction of gliding motility and host cell invasion, leading to the conclusion that MyoA is the essential motor protein powering these processes (Meissner *et al*. 2002). Residual expression of MyoA was

interpreted as the cause for the 'minimal levels of invasion' detected with this technology.

Intriguingly, over-expression of a ddFKBP-*myoA*-tail construct not only showed reduced invasion efficiency but also a block in intracellular replication. The over-expressed DD-tagged MyoA may have interacted with critical factors required during IMC biogenesis and acted as a dominant-negative protein. This effect was interpreted as MyoA having a second function during the asexual life cycle that cannot be resolved by reduction of expression levels (Agop-Nersesian *et al*. 2009). While it will be very interesting to identify the putative interaction partners of ddFKBP-*myoA*-tail, this example demonstrates that great care has to be taken when analysing dominant negative mutants.

Therefore, while the systems provided consistent data on the function of MyoA, using the DiCre system allowed the isolation of a viable *myoA* null mutant which shed further light on its essentiality, and calls for a more detailed analysis of the mechanisms involved in host cell invasion and for IMC biogenesis (Andenmatten *et al*. 2013; Egarter *et al*. 2014).

The role of the rhomboid protease ROM4 in the cleavage of the micronemal proteins MIC2 and AMA1 was also studied using several different genetic systems. Initially down-regulation of ROM4 was established by using the Tet-transactivator system (Buguliskis *et al*. 2010) and resulted in reduced invasion. Surprisingly, expression of a dominant negative version of ROM4 using the ddFKBP system led to a block of intracellular replication. Intriguingly, simultaneous over-expression of the AMA1-tail domain (the presumed cleavage product of ROM4) fused to ddFKBP, was sufficient to release this block and it was concluded that the ROM4-AMA1 processing step is required to trigger a signal for intracellular replication of *T. gondii* (Santos *et al*. 2011).

Null mutants for *rom4, ama1* and *mic2* were recently generated using the DiCre system (Andenmatten *et al*. 2013; Bargieri *et al*. 2013; Rugarabamu *et al*. 2013), surprisingly, showing no intracellular replication defects. A possible explanation for this discrepancy is a non-specific, pleiotropic effect caused by the expression of the dominant negative forms fused to ddFKBP. This explanation is favoured by the observation that neither ROM4 nor AMA1 mutants showed replication defects. The ability to isolate viable null mutants for these genes supports the notion that host cell invasion is more complex than initially perceived.

In summary, discrepancies in phenotypic consequences can be observed especially between mutants generated by ddFKBP and Tet/DiCre. Therefore, it is our recommendation to attempt a characterization using over-expression and knockout/knockdown approaches to identify potential downstream effects or, in the worst case, artefacts.

RECOMBINATION-MEDIATED GENETIC ENGINEERING

Classical characterization of gene function using parasite lines carrying mutations in a single GOI are time consuming and only offer limited information. Improved technologies moving towards high-throughput approaches to study gene function are necessary to allow for progression in the understanding of gene regulation in apicomplexan parasites. Recombineering is an efficient method for DNA engineering using homologous recombination in *E. coli*. This technique allows modification of DNA at any chosen position based on short regions of sequence homology. Transient expression of the recombinase complex and proofreading activity of lambda red operon allows controlled Recombineering to be initiated (Wang *et al*. 2006). Recently, it was used to modify the apicoplast membrane localized phosphate translocator (APT) using COSMID libraries in *T. gondii* (Brooks *et al*. 2010). The Recombineering method has been adapted to generate *P. berghei* vectors for transfection in high throughput (Pfander *et al*. 2011). Currently, the Sanger Institute (Genome Campus, Hinxton, UK) maintain the PlasmoGem database (http://plasmogem.sanger.ac.uk) therefore gene knockout and tagged gene vectors produced using Recombineering and GateWay technologies are annotated and can be obtained for downstream functional analysis.

It will be important to establish a similar technology and distribution system for *T. gondii* large-scale genome manipulation. A combination of Recombineering with the current toolbox described above should be possible to fulfil this goal.

GENOME EDITING IN APICOMPLEXAN PARASITES

One alternative to Recombineering could be employment of genome editing tools, such as artificially developed zinc finger nucleases (ZFNs) (Kim *et al*. 1996; Nain *et al*. 2010), transcription activator-like effector (TALE) nucleases from *Xanthomonas* spp. (Boch *et al*. 2009; Kay and Bonas, 2009; Pan *et al*. 2013) or clustered regularly interspaced short palindromic repeat (CRISPR) *cas9* from *Streptococcus pyogenes* (Mali *et al*. 2013; Sampson and Weiss, 2014). Each ZF domain consists of an array of protein fingers stabilized by zinc ion that recognize a specific triplet of DNA sequence (Fig. 2A). TALE monomers directly bind to DNA via a central domain of tandem repeats (Fig. 2B). The type II CRISPR/Cas system is being used in genetic manipulation of eukaryotic cells, allowing for the introduction of double-strand breaks (DBS) after addition of a small guide RNA into target genomes (Ran *et al*. 2013)

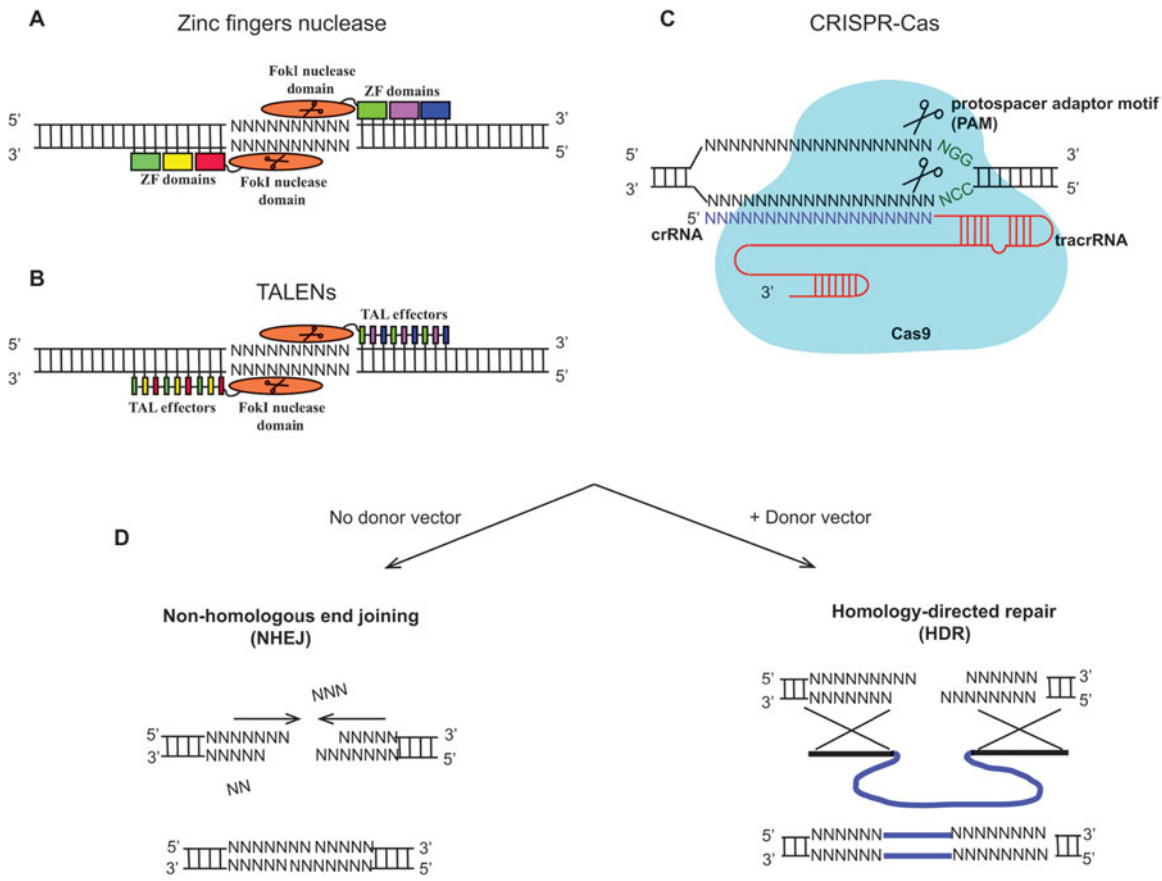

Fig. 2. Nuclease-mediated genome editing (adapted from Pan *et al*. 2013; Sampson and Weiss, 2014). Each monomer of zinc finger nuclease (ZFN) recognizes 3 bp DNA (A). Monomers of the transcription Activator-Like Effectors Nucleases (TALENs) system bind to a specific nucleotide sequence (B). Cas9 nuclease forms a complex with the chimera sgRNA, comprising a crRNA and a tracrRNA. This RNA guides the nuclease to the target locus (C). These nucleases will induce a double strand brake (DSB) in the gene that will be followed by non-homologous end joining (NHEJ) repair if no donor vector is supplied. If a plasmid with homologous regions is added the introduction of foreign DNA can be achieved (D).

(Fig. 2C). The DSB produced by any of these nuclease-based technologies can lead to non-homologous end joining (NHEJ) repair or, in case of the presence of a donor vector, to homology-directed repair (HDR) (Fig. 2D). ZF and TALE monomer construction requires specialist expertise as the probability of mismatch sequences causing off-target mutagenesis is high. The costs for generating the ZF or TALE libraries are relatively high whereas CRISPR/Cas9 technology offers a cheap alternative.

The main advantage of using nucleases for genome editing is the ability to directly engineer the genome of the organism being studied without the necessity of prior preparation of constructs with GOI DNA using recombinant DNA technology. Given the possibility for employing these new technologies for the manipulation of a wide range of organisms, adaptation of these technologies to apicomplexan gene studies would allow for greater scale of manipulation of the genome and efforts are being made to establish these new technologies into the current repertoire of molecular tools for the manipulation of *T. gondii* and *Plasmodium* spp. parasites.

OUTLOOK

The toolbox for genetic engineering discussed here demonstrates that each technology has its limitations and optimally synergistic technologies (such as knock-down and dominant negative expression) should be used to investigate gene function. However, the examples discussed above are not the only technologies employed to manipulate the genome in eukaryotic cells and several alternatives exist that we did not include in this review, such as the use of ribozymes (Agop-Nersesian *et al*. 2008; Prommana *et al*. 2013) or auxin-based degron systems (Jebiwott *et al*. 2013).

One aim for the future is the establishment of technologies that will allow the generation of mutants on a large scale. This likely will require the combination of inducible genetic tools, such as the Tet-system or DiCre with CRISPR or Recombineering for the comprehensive analysis of essential genes.

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REFERENCES

Agop-Nersesian, C., Pfahler, J., Lanzer, M. and Meissner, M. (2008). Functional expression of ribozymes in Apicomplexa: towards exogenous control of gene expression by inducible RNA-cleavage. *International Journal for Parasitology* **38**, 673–681. doi: 10.1016/j.ijpara.2007.10.015.

Agop-Nersesian, C., Naissant, B., Ben Rached, F., Rauch, M., Kretzschmar, A., Thiberge, S., Menard, R., Ferguson, D. J., Meissner, M. and Langsley, G. (2009). Rab11A-controlled assembly of the inner membrane complex is required for completion of apicomplexan cytokinesis. *PLOS Pathogen* **5**, e1000270. doi: 10.1371/journal.ppat.1000270.

Agop-Nersesian, C., Egarter, S., Langsley, G., Foth, B. J., Ferguson, D. J. and Meissner, M. (2010). Biogenesis of the inner membrane complex is dependent on vesicular transport by the alveolate specific GTPase Rab11B. *PLOS Pathogen* **6**, e1001029. doi: 10.1371/journal.ppat.1001029.

Andenmatten, N., Egarter, S., Jackson, A. J., Jullien, N., Herman, J. P. and Meissner, M. (2013). Conditional genome engineering in *Toxoplasma gondii* uncovers alternative invasion mechanisms. *Nature Methods* **10**, 125–127. doi: 10.1038/nmeth.2301.

Arakawa, H., Lodygin, D. and Buerstedde, J. M. (2001). Mutant loxP vectors for selectable marker recycle and conditional knock-outs. *BMC Biotechnology* **1**, 7.

Armstrong, C. M. and Goldberg, D. E. (2007). An FKBP destabilization domain modulates protein levels in *Plasmodium falciparum*. *Nature Methods* **4**, 1007–1009. doi: 10.1038/nmeth1132.

Aurrecoechea, C., Brestelli, J., Brunk, B. P., Fischer, S., Gajria, B., Gao, X., Gingle, A., Grant, G., Harb, O. S., Heiges, M., Innamorato, F., Iodice, J., Kissinger, J. C., Kraemer, E. T., Li, W., Miller, J. A., Nayak, V., Pennington, C., Pinney, D. F., Roos, D. S., Ross, C., Srinivasamoorthy, G., Stoeckert, C. J., Thibodeau, R., Treatman, C. and Wang, H. M. (2010). *EuPathDB*: a portal to eukaryotic pathogen databases. *Nucleic Acids Research* **38**, D415–D419. doi: 10.1093/nar/gkp941.

Banaszynski, L. A., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. and Wandless, T. J. (2006). A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **126**, 995–1004. doi: 10.1016/j.cell.2006.07.025.

Bargieri, D. Y., Andenmatten, N., Lagal, V., Thiberge, S., Whitelaw, J. A., Tardieux, I., Meissner, M. and Menard, R. (2013). Apical membrane antigen 1 mediates apicomplexan parasite attachment but is dispensable for host cell invasion. *Nature Communications* **4**, 2552. doi: 10.1038/ncomms3552.

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A. and Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-Type III effectors. *Science* **326**, 1509–1512. doi: 10.1126/science.1178811.

Bouabe, H. and Okkenhaug, K. (2013). A protocol for construction of gene targeting vectors and generation of homologous recombinant embryonic stem cells. *Methods in Molecular Biology* **1064**, 337–354. doi: 10.1007/978-1-62703-601-6_24.

Brecht, S., Erdhart, H., Soete, M. and Soldati, D. (1999). Genome engineering of *Toxoplasma gondii* using the site-specific recombinase Cre. *Gene* **234**, 239–247.

Breinich, M. S., Ferguson, D. J., Foth, B. J., van Dooren, G. G., Lebrun, M., Quon, D. V., Striepen, B., Bradley, P. J., Frischknecht, F., Carruthers, V. B. and Meissner, M. (2009). A dynamin is required for the biogenesis of secretory organelles in *Toxoplasma gondii*. *Current Biology* **19**, 277–286. doi: 10.1016/j.cub.2009.01.039.

Brooks, C. F., Johnsen, H., van Dooren, G. G., Muthalagi, M., Lin, S. S., Bohne, W., Fischer, K. and Striepen, B. (2010). The *Toxoplasma* apicoplast phosphate translocator links cytosolic and apicoplast metabolism and is essential for parasite survival. *Cell Host and Microbe* **7**, 62–73. doi: 10.1016/j.chom.2009.12.002.

Buguliskis, J. S., Brossier, F., Shuman, J. and Sibley, L. D. (2010). Rhomboid 4 (ROM4) affects the processing of surface adhesins and facilitates host cell invasion by *Toxoplasma gondii*. *PLOS Pathogens* **6**, doi: 10.1371/journal.ppat.1000858.

Carruthers, V. B., Giddings, O. K. and Sibley, L. D. (1999). Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells. *Cell Microbiology* **1**, 225–235.

Carvalho, T. G., Thiberge, S., Sakamoto, H. and Menard, R. (2004). Conditional mutagenesis using site-specific recombination in *Plasmodium berghei*. *Proceedings of the National Academy of Sciences USA* **101**, 14931–14936. doi: 10.1073/pnas.0404416101.

Chong, W.-C., Basir, R. and Fei, Y. M. (2013). Eradication of malaria through genetic engineering: the current situation. *Asian Pacific Journal of Tropical Medicine* **6**, 85–94. doi: 10.1016/s1995-7645(13)60001-2.

Collins, C. R., Das, S., Wong, E. H., Andenmatten, N., Stallmach, R., Hackett, F., Herman, J. P., Muller, 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. *Molecular Microbiology* **88**, 687–701. doi: 10.1111/mmi.12206.

Combe, A., Giovannini, D., Carvalho, T. G., Spath, S., Boisson, B., Loussert, C., Thiberge, S., Lacroix, C., Gueirard, P. and Menard, R. (2009). Clonal conditional mutagenesis in malaria parasites. *Cell Host and Microbe* **5**, 386–396. doi: 10.1016/j.chom.2009.03.008.

Daher, W., Plattner, F., Carlier, M. F. and Soldati-Favre, D. (2010). Concerted action of two formins in gliding motility and host cell invasion by *Toxoplasma gondii*. *PLOS Pathogen* **6**, e1001132. doi: 10.1371/journal.ppat.1001132.

de Azevedo, M. F., Gilson, P. R., Gabriel, H. B., Simoes, R. F., Angrisano, F., Baum, J., Crabb, B. S. and Wunderlich, G. (2012). Systematic analysis of FKBP inducible degradation domain tagging strategies for the human malaria parasite *Plasmodium falciparum*. *PLOS ONE* **7**, e40981. doi: 10.1371/journal.pone.0040981.

Dvorin, J. D., Bei, A. K., Coleman, B. I. and Duraisingh, M. T. (2010). Functional diversification between two related *Plasmodium falciparum* merozoite invasion ligands is determined by changes in the cytoplasmic domain. *Molecular Microbiology* **75**, 990–1006. doi: 10.1111/j.1365-2958.2009.07040.x.

Egarter, S., Andenmatten, N., Jackson, A. J., Whitelaw, J. A., Pall, G., Black, J. A., Ferguson, D. J. P., Tardieux, I., Mogilner, A. and Meissner, M. (2014). The *Toxoplasma* Acto-MyoA motor complex is important but not essential for gliding motility and host cell invasion. *PLOS ONE*, **9**, e91819. doi: 10.1071/journal.pone.0091819.

Fox, B. A., Ristuccia, J. G., Gigley, J. P. and Bzik, D. J. (2009). Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end joining. *Eukaryotic Cell* **8**, 520–529. doi: 10.1128/ec.00357-08.

Frenal, K., Polonais, V., Marq, J. B., Stratmann, R., Limenitakis, J. and Soldati-Favre, D. (2010). Functional dissection of the apicomplexan glideosome molecular architecture. *Cell Host and Microbe* **8**, 343–357. doi: 10.1016/j.chom.2010.09.002.

Frenal, K., Tay, C. L., Mueller, C., Bushell, E. S., Jia, Y., Graindorge, A., Billker, O., Rayner, J. C. and Soldati-Favre, D. (2013). Global analysis of apicomplexan protein S-acyl transferases reveals an enzyme essential for invasion. *Traffic* **14**, 895–911. doi: 10.1111/tra.12081.

Garcia-Otin, A. L. and Guillou, F. (2006). Mammalian genome targeting using site-specific recombinases. *Frontiers in Bioscience* **11**, 1108–1136.

Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences USA* **89**, 5547–5551.

Hamann, L., Buss, H. and Tannich, E. (1997). Tetracycline-controlled gene expression in *Entamoeba histolytica*. *Molecular and Biochemical Parasitology* **84**, 83–91.

Herm-Gotz, A., Agop-Nersesian, C., Munter, S., Grimley, J. S., Wandless, T. J., Frischknecht, F. and Meissner, M. (2007). Rapid control of protein level in the apicomplexan *Toxoplasma gondii*. *Nature Methods* **4**, 1003–1005. doi: 10.1038/nmeth1134.

Huynh, M. H. and Carruthers, V. B. (2009). Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryot Cell* **8**, 530–539. doi: 10.1128/ec.00358-08.

Iwamoto, M., Bjorklund, T., Lundberg, C., Kirik, D. and Wandless, T. J. (2010). A general chemical method to regulate protein stability in the mammalian central nervous system. *Chemistry and Biology* **17**, 981–988. doi: 10.1016/j.chembiol.2010.07.009.

Jakot, D., Meissner, M., Sheiner, L., Soldati-Fevre, D. and Striepen, B. (2013). Genetic manipulation of *Toxoplasma gondii*. In *Toxoplasma gondii*. *The Model Apicomplexan: Perspectives and Methods*, 2nd Edn (ed. Weiss, L. M. and Kim, K.), pp. 577–611. Elsevier, Amsterdam, the Netherlands.

Jebiwott, S., Govindaswamy, K., Mbugua, A. and Bhanot, P. (2013). *Plasmodium berghei* calcium dependent protein kinase 1 is not required for host cell invasion. *PLOS ONE* **8**, e79171. doi: 10.1371/journal.pone.0079171.

Jullien, N., Sampieri, F., Enjalbert, A. and Herman, J.P. (2003). Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. *Nucleic Acids Research* **31**. doi: 10.1093/nar/gng131.

Jullien, N., Goddard, I., Selmi-Ruby, S., Fina, J.L., Cremer, H. and Herman, J.P. (2007). Conditional transgenesis using dimerizable Cre (DiCre). *PLoS ONE* **2**. doi: 10.1371/journal.pone.0001355.

Kay, S. and Bonas, U. (2009). How *Xanthomonas* type III effectors manipulate the host plant. *Current Opinion in Microbiology* **12**, 37–43. doi: 10.1016/j.mib.2008.12.006.

Kim, Y.G., Cha, J. and Chandrasegaran, S. (1996). Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proceedings of the National Academy of Sciences USA* **93**, 1156–1160. doi: 10.1073/pnas.93.3.1156.

Kolev, N.G., Tschudi, C. and Ullu, E. (2011). RNA interference in protozoan parasites: achievements and challenges. *Eukaryotic Cell* **10**, 1156–1163. doi: 10.1128/ec.05114-11.

Kremer, K., Kamin, D., Rittweger, E., Wilkes, J., Flammer, H., Mahler, S., Heng, J., Tonkin, C.J., Langsley, G., Hell, S.W., Carruthers, V.B., Ferguson, D.J. and Meissner, M. (2013). An overexpression screen of *Toxoplasma gondii* Rab-GTPases reveals distinct transport routes to the micronemes. *PLOS Pathogen* **9**, e1003213. doi: 10.1371/journal.ppat.1003213.

Lacroix, C., Giovannini, D., Combe, A., Bargieri, D.Y., Spath, S., Panchal, D., Tawk, L., Thiberge, S., Carvalho, T.G., Barale, J.C., Bhanot, P. and Menard, R. (2011). FLP/FRT-mediated conditional mutagenesis in pre-erythrocytic stages of *Plasmodium berghei*. *Nature Protocols* **6**, 1412–1428. doi: 10.1038/nprot.2011.363.

Limenitakis, J. and Soldati-Favre, D. (2011). Functional genetics in *Apicomplexa*: potentials and limits. *FEBS Letters* **585**, 1579–1588. doi: 10.1016/j.febslet.2011.05.002.

Lovett, J.L., Howe, D.K. and Sibley, L.D. (2000). Molecular characterization of a thrombospondin-related anonymous protein homologue in *Neospora caninum*. *Molecular and Biochemical Parasitology* **107**, 33–43.

Mali, P., Esvelt, K.M. and Church, G.M. (2013). Cas9 as a versatile tool for engineering biology. *Nature Methods* **10**, 957–963. doi: 10.1038/nmeth.2649.

Meissner, M., Brecht, S., Bujard, H. and Soldati, D. (2001). Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. *Nucleic Acids Research* **29**, E115.

Meissner, M., Schluter, D. and Soldati, D. (2002). Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science* **298**, 837–840. doi: 10.1126/science.1074553.

Meissner, M., Krejany, E., Gilson, P.R., de Koning-Ward, T.F., Soldati, D. and Crabb, B.S. (2005). Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using *Toxoplasma gondii* transactivators. *Proceedings of the National Academy of Sciences USA* **102**, 2980–2985. doi: 10.1073/pnas.0500112102.

Meissner, M., Breinich, M.S., Gilson, P.R. and Crabb, B.S. (2007). Molecular genetic tools in *Toxoplasma* and *Plasmodium*: achievements and future needs. *Current Opinion in Microbiology* **10**, 349–356. doi: 10.1016/j.mib.2007.07.006.

Metzger, D., Clifford, J., Chiba, H. and Chambon, P. (1995). Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proceedings of the National Academy of Sciences USA* **92**, 6991–6995.

Muralidharan, V., Oksman, A., Iwamoto, M., Wandless, T.J. and Goldberg, D.E. (2011). Asparagine repeat function in a *Plasmodium falciparum* protein assessed via a regulatable fluorescent affinity tag. *Proceedings of the National Academy of Sciences USA* **108**, 4411–4416. doi: 10.1073/pnas.1018449108.

Nain, V., Sahi, S. and Verma, A. (2010). CPP-ZFN: a potential DNA-targeting anti-malarial drug. *Malaria Journal* **9**, 258. doi: 10.1186/1475-2875-9-258.

Nichols, B.A., Chiappino, M.L. and O'Connor, G.R. (1983). Secretion from the rhoptries of *Toxoplasma gondii* during host-cell invasion. *Journal of Ultrastructure Research* **83**, 85–98.

Oberstaller, J., Joseph, S.J. and Kissinger, J.C. (2013). Genome-wide upstream motif analysis of *Cryptosporidium parvum* genes clustered by expression profile. *BMC Genomics* **14**. doi: 10.1186/1471-2164-14-516.

Pan, Y.Z., Xiao, L., Li, A.S.S., Zhang, X., Sirois, P., Zhang, J. and Li, K. (2013). Biological and biomedical applications of engineered nucleases. *Molecular Biotechnology* **55**, 54–62. doi: 10.1007/s12033-012-9613-9.

Pfander, C., Anar, B., Schwach, F., Otto, T.D., Brochet, M., Volkmann, K., Quail, M.A., Pain, A., Rosen, B., Skarnes, W., Rayner, J.C. and Billker, O. (2011). A scalable pipeline for highly effective genetic modification of a malaria parasite. *Nature Methods* **8**, 1078. doi: 10.1038/nmeth.1742.

Pieperhoff, M.S., Schmitt, M., Ferguson, D.J. and Meissner, M. (2013). The role of clathrin in post-Golgi trafficking in *Toxoplasma gondii*. *PLOS ONE* **8**, e77620. doi: 10.1371/journal.pone.0077620.

Pino, P. (2013). From technology to biology: a malaria genetic toolbox for the functional dissection of essential genes. *Molecular Microbiology* **88**, 650–654. doi: 10.1111/mmi.12232.

Pino, P., Sebastian, S., Kim, E.A., Bush, E., Brochet, M., Volkmann, K., Kozlowski, E., Llinas, M., Billker, O. and Soldati-Favre, D. (2012). A tetracycline-repressible transactivator system to study essential genes in malaria parasites. *Cell Host and Microbe* **12**, 824–834. doi: 10.1016/j.chom.2012.10.016.

Plageman, T.F., Jr. and Lang, R.A. (2012). Generation of an Rx-tTA: TetOp-Cre knock-in mouse line for doxycycline regulated Cre activity in the Rx expression domain. *PLoS ONE* **7**, e50426. doi: 10.1371/journal.pone.0050426.

Prommana, P., Uthaipibull, C., Wongsombat, C., Kamchonwongpaisan, S., Yuthavong, Y., Knuepfer, E., Holder, A.A. and Shaw, P.J. (2013). Inducible knockdown of *Plasmodium* gene expression using the glmS ribozyme. *PLoS ONE* **8**, e73783. doi: 10.1371/journal.pone.0073783.

Ramakrishnan, G., Vines, R.R., Mann, B.J. and Petri, W.A., Jr. (1997). A tetracycline-inducible gene expression system in *Entamoeba histolytica*. *Molecular and Biochemical Parasitology* **84**, 93–100.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* **8**, 2281–2308. doi: 10.1038/nprot.2013.143.

Reichel, M.P. and Ellis, J.T. (2009). *Neospora caninum* – how close are we to development of an efficacious vaccine that prevents abortion in cattle? *International Journal for Parasitology* **39**, 1173–1187.

Russo, I., Oksman, A., Vaupel, B. and Goldberg, D.E. (2009). A calpain unique to alveolates is essential in *Plasmodium falciparum* and its knockdown reveals an involvement in pre-S-phase development. *Proceedings of the National Academy of Sciences USA* **106**, 1554–1559. doi: 10.1073/pnas.0806926106.

Sampson, T.R. and Weiss, D.S. (2014). Exploiting CRISPR/Cas systems for biotechnology. *Bioessays* **36**, 34–38. doi: 10.1002/bies.201300135.

Santos, T.A., Portes Jde, A., Damasceno-Sa, J.C., Caldas, L.A., Souza, W., Damatta, R.A. and Seabra, S.H. (2011). Phosphatidylserine exposure by *Toxoplasma gondii* is fundamental to balance the immune response granting survival of the parasite and of the host. *PLOS ONE* **6**, e27867. doi: 10.1371/journal.pone.0027867.

Sheiner, L., Demerly, J.L., Poulsen, N., Beatty, W.L., Lucas, O., Behnke, M.S., White, M.W. and Striepen, B. (2011). A systematic screen to discover and analyze apicoplast proteins identifies a conserved and essential protein import factor. *PLOS Pathogens* **7**. doi: 10.1371/journal.ppat.1002392.

van Dooren, G.G., Reiff, S.B., Tomova, C., Meissner, M., Humbel, B.M. and Striepen, B. (2009). A novel dynamin-related protein has been recruited for apicoplast fission in *Toxoplasma gondii*. *Current Biology* **19**, 267–276. doi: 10.1016/j.cub.2008.12.048.

van Poppel, N.F., Welagen, J., Duisters, R.F., Vermeulen, A.N. and Schaap, D. (2006). Tight control of transcription in *Toxoplasma gondii* using an alternative tet repressor. *International Journal for Parasitology* **36**, 443–452.

Wang, J., Sarov, M., Rientjes, J., Fu, J., Hollak, H., Kranz, H., Xie, W., Stewart, A.F. and Zhang, Y. (2006). An improved recombineering approach by adding RecA to lambda Red recombination. *Molecular Biotechnology* **32**, 43–53.

Wirtz, E. and Clayton, C. (1995). Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. *Science* **268**, 1179–1183.

Wirtz, E., Leal, S., Ochatt, C. and Cross, G.A. (1999). A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **99**, 89–101.

Zhang, N.Z., Chen, J., Wang, M., Petersen, E. and Zhu, X.Q. (2013). Vaccines against *Toxoplasma gondii*: new developments and perspectives. *Expert Review of Vaccines* **12**, 1287–1299.