
Copyright © 2014 Cambridge University Press

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

Content must not be changed in any way or reproduced in any format or medium without the formal permission of the copyright holder(s)

When referring to this work, full bibliographic details must be given

http://eprints.gla.ac.uk/97225/

Deposited on: 29 September 2014
Advantages and disadvantages of conditional systems for characterization of essential genes in *Toxoplasma gondii*

**ELENA JIMÉNEZ-RUIZ, ELEANOR H. WONG, GURMAN S. PALL and MARKUS MEISSNER*\**

Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

*(Received 17 January 2014; revised 11 March 2014; accepted 13 March 2014; first published online 13 June 2014)*

**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...
**Table 1. Summary of the technologies described in this review**

<table>
<thead>
<tr>
<th>Conditional recombination (DiCre)</th>
<th>Tet system</th>
<th>ddFKBP system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Regulation efficiency</strong></td>
<td>Up to 99% knockouts in population(^a)</td>
<td>100% knockdowns with 10–50-fold regulation(^b)</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>No reversion</td>
<td>Reversible</td>
</tr>
<tr>
<td></td>
<td>No background expression</td>
<td>Inducer (ATc) can be used in vivo</td>
</tr>
<tr>
<td></td>
<td>GOI under control of endogenous promoter</td>
<td></td>
</tr>
<tr>
<td><strong>Disadvantage</strong></td>
<td>Not reversible</td>
<td>Reversion and background expression</td>
</tr>
<tr>
<td></td>
<td>No clonal population</td>
<td>Not controlled under endogenous promoter</td>
</tr>
<tr>
<td><strong>Suggested usage</strong></td>
<td>Knockouts and over-expression/trans-dominant mutants</td>
<td>Reversible knockdowns</td>
</tr>
</tbody>
</table>

\(^a\) Andenmatten \textit{et al.} (2013).  
\(^b\) Meisssner \textit{et al.} (2002).  
\(^c\) Herm-Gotz \textit{et al.} (2007).

\textit{T. gondii}: Tet-inducible transcription (Meissner \textit{et al.} 2001, 2002), rapid protein destabilization (Herm-Gotz \textit{et al.} 2007) and conditional site-specific recombination (Andenmatten \textit{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 \textit{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 \textit{et al.} 2000), including protozoan parasites (Wirtz and Clayton, 1995; Hamann \textit{et al.} 1997; Ramakrishnan \textit{et al.} 1997; Wirtz \textit{et al.} 1999). An analogous system was developed in \textit{T. gondii}, but this was not sufficient for the generation of conditional mutants (Meissner \textit{et al.} 2001). Although the expression of a bulkier version of TetR led to an increased regulation efficiency (van Poppel \textit{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 \textit{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 \textit{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 \textit{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 tunable 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 Shld-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 (Murafidharian 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; Breinch 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), dhic7 (Frenal et al. 2013), mll1, 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 TAT, 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).
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

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).
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, 2010). The recently generated 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).
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 knockdown 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.

ACKNOWLEDGEMENTS
We would like to thank Dr Lilach Sheiner for helpful comments on the manuscript.
Rhomboid 4 (ROM4) a
ff
Toxoplasma gondii
Lebrun, M., Quon, D. V., Striepen, B., Bradley, P. J., metabolism and is essential for parasite survival. apicoplast phosphate translocator links cytosolic and apicoplast Toxoplasma (2010). The cub.2009.01.039.
embryonic stem cells.

REFERENCES


Frenal, K., Polonais, V., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. and Meissner, M. (2013). Conditional genome engineering in Toxoplasma gondii. The Wellcome Trust Centre for Molecular Parasitology is supported by core funding [ERC-2012-StG 309255]. The Wellcome Trust Centre for Molecular Parasitology is supported by core funding from the Wellcome Trust (0853349).

Copyright © 2013 by the American Society for Microbiology. All rights reserved.


Frenal, K., Polonais, V., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. and Meissner, M. (2013). Conditional genome engineering in Toxoplasma gondii. The Wellcome Trust Centre for Molecular Parasitology is supported by core funding [ERC-2012-StG 309255]. The Wellcome Trust Centre for Molecular Parasitology is supported by core funding from the Wellcome Trust (0853349).

Copyright © 2013 by the American Society for Microbiology. All rights reserved.


Frenal, K., Polonais, V., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. and Meissner, M. (2013). Conditional genome engineering in Toxoplasma gondii. The Wellcome Trust Centre for Molecular Parasitology is supported by core funding [ERC-2012-StG 309255]. The Wellcome Trust Centre for Molecular Parasitology is supported by core funding from the Wellcome Trust (0853349).

Copyright © 2013 by the American Society for Microbiology. All rights reserved.

Biology and biomedical applications of engineered.


