Received: 23 June 2023 | Revised: 31 August 2023 | Accepted: 5 September 2023

DOI: 10.1111/mmi.15166

REVIEW ARTICLE

WILEY

Where is the EXIT? Phenotypic screens for new egress factors in apicomplexan parasites

Elena Jimenéz-Ruiz | Wei Li | Markus Meissner

Experimental Parasitology, Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität, LMU, Munich, Germany

Correspondence
Markus Meissner, Experimental Parasitology, Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität, LMU, Munich, Germany.
Email: markus.meissner@lmu.de; markus.meissner@para.vetmed.uni-muenchen.de

Funding information
CSC fellowship, Grant/Award Number: 201806910075; Deutsche Forschungsgemeinschaft, Grant/Award Number: Project ME 2675/7-1 and SPP2225

Abstract

Apicomplexans, such as *Plasmodium* and *Toxoplasma* are obligate intracellular parasites that invade, replicate and finally EXIT their host cell. During replication within a parasitophorous vacuole (PV), the parasites establish an extensive F-actin-containing network that connects individual parasites and is required for material exchange, recycling and the final steps of daughter cell assembly. After multiple rounds of replication, the parasites exit the host cell involving multiple signalling cascades, disassembly of the network, secretion of microneme proteins and activation of the acto-myosin motor. Blocking the host cell EXIT process leads to the formation of large PVs, making the screening for genes involved in exiting the cell relatively straightforward. Given that apicomplexans are highly diverse from other eukaryotes, approximately 30% of all genes are annotated as hypothetical, some apicomplexan-specific factors are likely to be critical during EXIT. This motivated several labs to design and perform forward genetic and phenotypic screens using various approaches, such as random insertion mutagenesis, temperature-sensitive mutants and, more recently, CRISPR/Cas9-mediated targeted editing and conditional mutagenesis. Here we will provide an overview of the technological developments over recent years and the most successful stories that led to the identification of new critical factors in *Toxoplasma gondii*.

KEYWORDS
apicomplexan parasites, CRISPR/Cas9 screens, egress factors, forward genetic screens

1 | EXIT SCREENS IN APICOMPLEXANS

As with all technologies, the strategies used for a genetic screen are tied to the available methods established in a model organism at the respective time (Figure 1). There are many uncertainties, and usually high risks involved, when new technologies are developed or adapted to a model system and in general follow the so-called 'Gartner hype cycle model' that traces the evolution of technological innovations as they pass through successive stages, from awareness about the technology, peak of inflated expectations, trough of disillusionment back to a plateau of productivity (Dedehayir & Steinert, 2016). A good example is siRNA-based technologies, the former gold standard for phenotypic screening technologies, which has recently been overtaken by CRISPR/cas9. With the advancement of the CRISPR/Cas9 technology in *Toxoplasma gondii* (Sidik et al., 2016), the possibilities for forward genetic screens seem manifold, but it is worth keeping the basic strategies and pitfalls encountered in earlier screens in mind. While it is now relatively straightforward to perform genome-wide screens, results need to be validated by sometimes

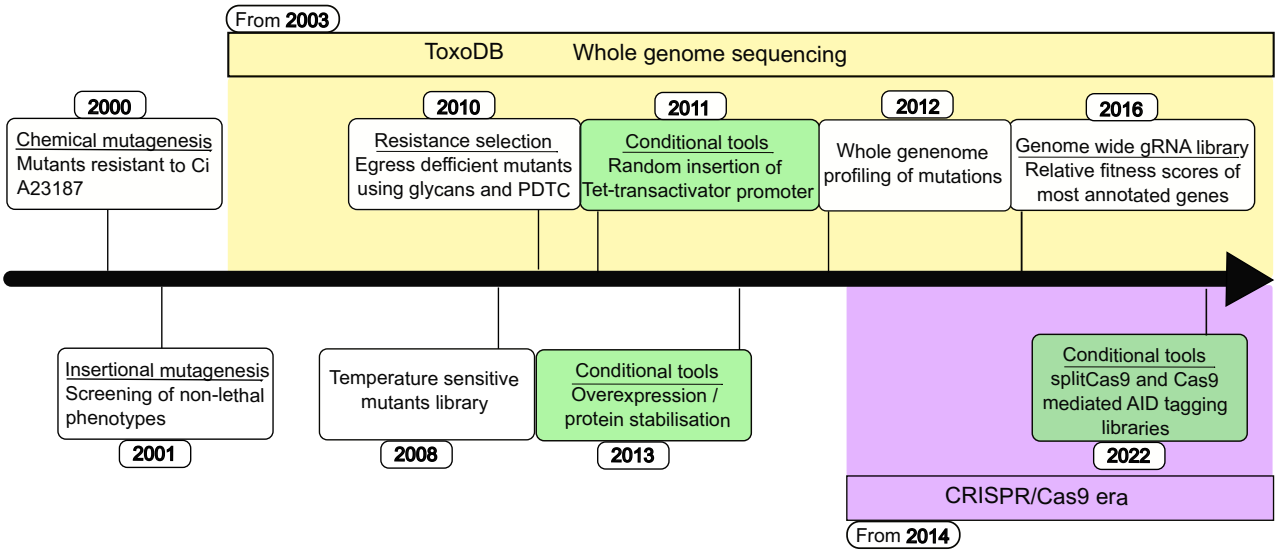

FIGURE 1 Time course of technology development for the screen of egress factors in *Toxoplasma gondii*.

time-consuming downstream assays. In fact, most screens that are currently performed are 'drop out' screens which allow the identification of fitness-conferring genes under certain conditions, meaning these mutants are lost in the resulting pool of mutants but the reason behind it needs to be investigated further.

Furthermore, it is important to consider 'older' approaches, which will still be useful for many research questions and may lead to promising and consistent results more efficient than some of the 'hyped' technologies. Therefore, the advantages and disadvantages of each technology need to be well considered (Jimenez-Ruiz et al., 2014), before embarking on sometimes time-consuming screens. Both, the available technologies for the generation of mutants, the biological question (i.e. phenotype) and the readout for the detection of the mutation need to be well-defined.

Over the years, many screens for the identification of egress factors have been performed, especially in *T. gondii*, where genetic modifications can be easily introduced and in vitro assays allow the relatively easy detection of mutants. Each screen needs to be seen in the context of our understanding of the biological question and the technology available at that time. Early attempts focused on the observation that host cell egress by *T. gondii* can be induced by the carboxylic Calcium-ionophore A23187 that rises intracellular Calcium levels (Endo et al., 1982). Exploiting this phenomenon, chemical mutagenesis was used to screen for mutants resistant to A23187 (Black et al., 2000). While at this time powerful technologies, such as genome-wide sequencing were unavailable for the identification of the corresponding mutations in the genome, three distinct classes of egress mutants were identified that behaved differently upon induction of egress and suggested that a parasite-dependent activity causes infected host cells to be permeabilised just prior to egress.

With the development of affordable, whole genome sequencing methods, whole genome profiling of chemically induced mutations became feasible (Farrell et al., 2012) and independent screens based on chemical mutagenesis allowed the identification of CDPK3 as a regulator of Calcium dependent egress (Garrison et al., 2012) and a suppressor of Ca^{2+}-dependent egress (SCE1) (McCoy et al., 2017).

With the advent of random insertional mutagenesis (Donald & Roos, 1995) a powerful technology was established allowing the relatively straightforward generation of a signature tagged mutant library for forward genetic screens (Knoll et al., 2001). Since the mutagenised locus is tagged with a known DNA sequence, identification of the mutagenised gene does not require whole genome profiling and can be achieved by genomic PCR. This strategy allowed the screening for non-lethal phenotypes, such as parasite virulence in vivo (Frankel et al., 2007), differentiation (Knoll & Boothroyd, 1998; Matrajt et al., 2002) and to some extent egress. Using the same selection scheme as for the chemical mutagenesis screen mentioned above (Black et al., 2000), random insertional mutagenesis was used to identify a previously undescribed Toxoplasma Na^+/H^+ exchanger (TgNHE1) that appears to play a role in Calcium homeostasis of the parasite and thus influences Cal-induced egress (Arrizabalaga et al., 2004).

However, while these strategies allowed the identification of some genes critically involved in (Calcium-induced) egress, given that most genes required in this critical process of the life cycle are essential, especially since egress factors are often also critical for host cell invasion, many interesting mutants cannot be isolated, since parasites will not survive the initial selection procedure (Coleman & Gubbels, 2012). Therefore, several groups attempted to use conditional systems in combination with genetic screens that allow the identification of essential genes using phenotypic/microscopy-based screening approaches.

2 | CONDITIONAL SYSTEMS ADAPTED FOR PHENOTYPIC SCREENS

Two common strategies to isolate mutants in a genetic screen based on their phenotype are replica plating and the resistance

selection method. While the latter is usually less work intense, in the case of *T. gondii* it usually only allows an enrichment of mutants with a potential phenotype. For the screening of egress mutants, besides the selection of CaI-resistant mutants, some delicate selection protocols have been established and used with some success. For example, a double selection protocol has been established that is based on the induced egress and the efficient separation of intracellular from extracellular parasites. Attachment and fast reinvasion of egressed parasites are prevented by the addition of glycans, whereas PDTC is included to specifically kill the egressed, extracellular parasites. Using this method, it is possible to enrich strong egress mutants ~1000-fold over a wild type population (Coleman & Gubbels, 2012; Eidell et al., 2010). One disadvantages of these protocols is the serious manipulation of parasites signalling cascades to trigger egress, such as activation of Ca-signalling by CaI. Therefore, factors regulating natural egress are unlikely to be identified using this strategy.

The alternative are screens that combine replica plating and automated microscopy to directly identify mutants blocked or delayed in natural egress. However, these strategies are more time consuming compared to selection-based screens and requires the use of conditional systems or generation of a temperature sensitive (ts) library upon chemical mutagenesis. In the latter case a huge effort has been undertaken to generate a library of ts mutants that allowed screening for multiple phenotypes, such as regulators of the cell cycle or egress (Gubbels et al., 2008). While in the initial study, a robust complementation strategy using a cosmid library has been used to identify several ts mutations, in a milestone study, the Gubbels lab directly used genomic profiling to identify a crucial exocytosis factor, Doc2 that is required for microneme secretion and consequently for host cell invasion and egress (Farrell et al., 2012).

The Gubbels lab also devised a different strategy based on conditional, tetracycline dependent gene activation—that interestingly has been established in *Toxoplasma* with the help of signature tagged mutagenesis itself (Meissner et al., 2002). Here random integration of the conditional Tet-transactivator promoter was predicted to integrate upstream of essential genes and thus placing them under direct control of the Tet-system (Jammallo et al., 2011). This approach resulted in the identification essential genes, such as TAF250. However, the technology appears to be prone to the generation of artefacts and has not been further developed since its first publication (Jammallo et al., 2011).

Another strategy based on conditional overexpression has been successfully employed for the characterisation and functional screening of Rab-GTPases (Kremer et al., 2013). Here cDNAs encoding for Rab-GTPases have been put under control of a strong promoter and fused to the protein degradation system (ddFKBP; Herm-Gotz et al., 2007), allowing for strong overexpression/protein stabilisation in presence of the inducer Shield-1. This focused screen resulted in the initial characterisation of the whole Rab-GTPase repertoire of *Toxoplasma* (Kremer et al., 2013) and could in principle be upgraded for overexpression screens, targeting all non-secretory proteins of the parasite, including for EXIT mutants. However, it would require the generation of a complete cDNA-library cloned under control of the inducible system, which turned out to be a cumbersome, expensive and (at that time) unrealistic approach. Furthermore, when performing overexpression screens, one needs to consider that they are prone to artefacts and the resulting, sometimes impressive phenotypes might not correspond to the respective gene function (Meissner, unpublished). The further development and optimisation of many of these screens was not followed up, since the era of CRISPR/Cas9 opened up whole new venues for forward genetic screens in this parasite.

3 | CRISPR/CAS9 SCREENS IN *TOXOPLASMA*

First described in *Caenorhabditis elegans* (Fire et al., 1998) and the protozoan parasite *Trypanosoma brucei* (Ngô et al., 1998), RNA interference (RNAi) revolutionised functional genomics screening for many eukaryotic model systems. However, in the absence of an efficient siRNA machinery, this powerful screening technology was unavailable for apicomplexans although some attempts were made to introduce siRNA-based technologies in these parasites (Hentzschel et al., 2020).

With the advent of the CRISPR revolution, several groups set out to adapt CRISPR/Cas9 also in *Toxoplasma* (Shen et al., 2014; Sidik et al., 2014). The Lourido lab subsequently spearheaded the development of forward genetic screens and performed the first genome wide CRISPR screen in an apicomplexan parasite (Sidik et al., 2016), leading to a wealth of information, including the relative fitness score for most annotated genes in the genome, which allows researchers to prioritise their candidates. Since then, several variations of the initial CRISPR screen have been described for the identification of differentiation factors (Waldman et al., 2020), identification of virulence factors in vivo (Butterworth et al., 2022; Sangaré et al., 2019; Young et al., 2019) or for drug resistance mutants (Harding et al., 2020) to name a few recent applications. Together these screens highlighted that many hypothetical genes are critical for the survival of the parasite. In these 'drop out' screens the relative abundance of gRNAs in the mutant pool is measured to identify gRNAs that are lost over time, indicating a critical function (Sidik et al., 2018). However, since the respective mutant is lost in the pool, a direct analysis of the respective phenotype is impossible, meaning that a direct information about gene function cannot be obtained. Therefore, identified candidate genes need to be analysed in a time-consuming one-by-one approach, which usually involves the generation of a conditional mutants for the respective gene of interest. Similarly, an image-based phenotypic screen is not feasible using this strategy.

Recently two strategies were used to combine the power of CRISPR screens with conditional mutagenesis (Li et al., 2022; Smith et al., 2022), allowing for both, 'drop out' screening after induction and direct phenotypic screening using automated microscopy. Smith et al. combined high-throughput CRISPR-mediated tagging

of candidate genes with the AID-system and demonstrate the efficiency of their strategy by labelling and downregulation of the *T. gondii* kinome, resulting in the characterisation of kinases, involved in diverse functions. This approach allows the direct localisation of candidate genes after cloning out individual mutants and the analysis of the resulting phenotypes upon addition of Auxin. In parallel, a pooled 'Drop-out' screen can be performed to determine which candidate is important during the asexual life cycle, analogous to standard CRISPR-screens. While this approach directly allows the generation of conditional mutants, where the gene of interest is tagged and can be localised in the parasite, it requires significant investment in the planning and generation of CRISPR-mediated tagging libraries.

Our lab followed an alternative approach to enable phenotypic screening based on conditional mutagenesis. Instead of combining CRISPR/Cas9 with an available conditional system (Jimenez-Ruiz et al., 2014), we wished to conditionally introduce indel mutations in the gene of interest by regulating cas9-activity directly. We reasoned that one main advantage of such a system is the possibility to use any previously employed gRNA-libraries. A first attempt to regulate Cas9-activity using the ddFKBP-system (Herm-Gotz et al., 2007) resulted in efficient regulation of ddFKBP-Cas9 (Serpeloni et al., 2016). However, while it was possible to keep parasites expressing a gRNA against critical genes in culture and analyse their phenotype after induction of ddFKBP-Cas9 (Serpeloni et al., 2016), the use of this system for library screens appears to be not possible, since ddFKBP-Cas9 shows a relatively high background activity. For example, it was seen that parasites expressing ddFKBP and a gRNA targeting the non-essential gene *sag1* lost SAG1 during continuous culture, without induction of ddFKBPcas9. Therefore, employment of this system would result in rapid loss of library complexity over time, hindering especially complex screens, where parasites need to be cultivated over longer periods.

Chemically induced dimerisation is a rapid and efficient technology to regulate protein activity and has been successfully used for several conditional gene expression systems in apicomplexan parasites, such as DiCre (Andenmatten et al., 2013; Collins et al., 2013) or knock sideways (Birnbaum et al., 2017). In 2015 Zetsche et al., reported on the development of a dimerisable Cas9-system, called splitCas9 (Zetsche et al., 2015). When adapted to its use in *Toxoplasma*, we developed a highly efficient and tight conditional Cas9 system that showed great potential for its application in phenotypic and drop-out screens (Li et al., 2022). However, before its use in a large-scale screen, several obstacles needed to be overcome and considered, especially when it comes to the inefficiency of NHEJ.

In conclusion, both screening strategies have their own advantages and disadvantages (Table 1) and have a huge potential to directly interrogate the phenotype caused by conditional inactivation of gene expression. As with all screens, such a project can be seen as high-risk-high-gain. In the following, a detailed discussion of the splitCas9-system that we established is provided, especially regarding pitfalls that should be considered before embarking on such a project.

4 | CONSIDERATIONS REGARDING THE SPLITCAS9 SYSTEM

In their current stage of development, CRISPR screens in *T. gondii* depend on the introduction of indel mutations upon repair of Cas9 induced DNA-double strand breaks via non-homologous-end-joining (NHEJ) (Sidik et al., 2018). During the development of the splitCas9-system, we found that induction of splitCas9 resulted in a parasite population where up to 50% of parasites showed aberrant nuclei, irrespective which gene was targeted. Interestingly, this phenotype was only seen immediately after induction (1st generation), while no such phenotype was seen after prolonged culture (2nd generation), mutant parasites could be easily isolated in case a non-essential gene was targeted. In case of essential genes, either the whole parasite population died or only non-responsive parasites with the intact candidate gene could be isolated. Whereas this demonstrated that the splitCas9-system itself is very efficient, further experiments also demonstrated that the 'aberrant nuclear' phenotype is caused by inefficient NHEJ-repair (Li et al., 2022). Moreover, it is currently unpredictable to determine the exact number of parasites that will exhibit this nonspecific phenotype. The presence of this phenotype can vary greatly depending on the specific gene being targeted, ranging

TABLE 1 Advantages and disadvantages of Cas9 based phenotypic screens.

splitCas9 screens	Cas9/AID tagging screens
Easy cloning of sgRNA library	Relative intricate cloning of sgRNA + AID libraries
Any gene can be targeted	Insertion of AID cassette might not be successful for all targeted genes
Needs extra steps to visualise the localisation of the POI	Direct tagging of targeted genes
Develop of aberrant vacuoles related to Cas9 activity (DNA damage)	Loss of library complexity due to failure in tagging of genes
Utilisation of indicator strains facilitates readouts	Usage of indicator strains possible
Possibility of scaling up to genome wide screens couple to AI tools for analysis of phenotypes	Possibility of scaling up to genome wide screens couple to AI tools for analysis of phenotypes

from its near absence to being observed in as much as 50% of the population.

While this may initially present a significant challenge to the phenotypic screening strategy, particularly when targeting genes involved in nuclear division/replication, there is a relatively simple solution to exclude this phenotype. By utilising automated imaging, it becomes feasible to accurately identify and exclude parasites displaying this non-specific phenotype, thereby ensuring that only relevant parasites are considered during the screening readout. Therefore, despite the appearance of the non-specific phenotype, splitCas9 based screens allow the identification of multiple phenotypes, especially if they are unrelated to defects in replication or nuclear morphology. Importantly, the use of indicator parasite lines will give additional specificity to the readout of the screen. In a first application the use of a parasites co-expressing CbEm and FNR-RFP to label F-actin and apicoplast respectively, we successfully identified candidates involved in apicoplast inheritance and regulation of F-actin dynamics (Li et al., 2022). Another, approach is to define replication and or nuclear phenotypes as specific, if at least 80% of the parasite population shows such a phenotype.

A promising future development overcoming the complications associated with inefficient NHEJ-repair might be the adaptation of CRISPR/Cas-derived base editing tools that bypass the NHEJ-repair machinery and instead edits suitable codons into STOP codons by using a CRISPR/Cas9 cytosine base editor (CBE) (Komor et al., 2016). This strategy has previously been used for genome-wide loss-of-function screens in human cells (Kuscu et al., 2017) and recently also successfully adapted to *Leishmania* (Engstler & Beneke, 2023). It appears feasible to adapt CBE to splitCas9 to establish a conditional gene editing system in apicomplexan parasites.

While the use of indicator strains allows for the screening of specific pathways and mechanisms, for example for genes involved in organelle biosynthesis, cytoskeletal dynamics, host cell motility or signalling (for an overview of potential indicators see Figure 2), especially screens for parasites blocked in natural

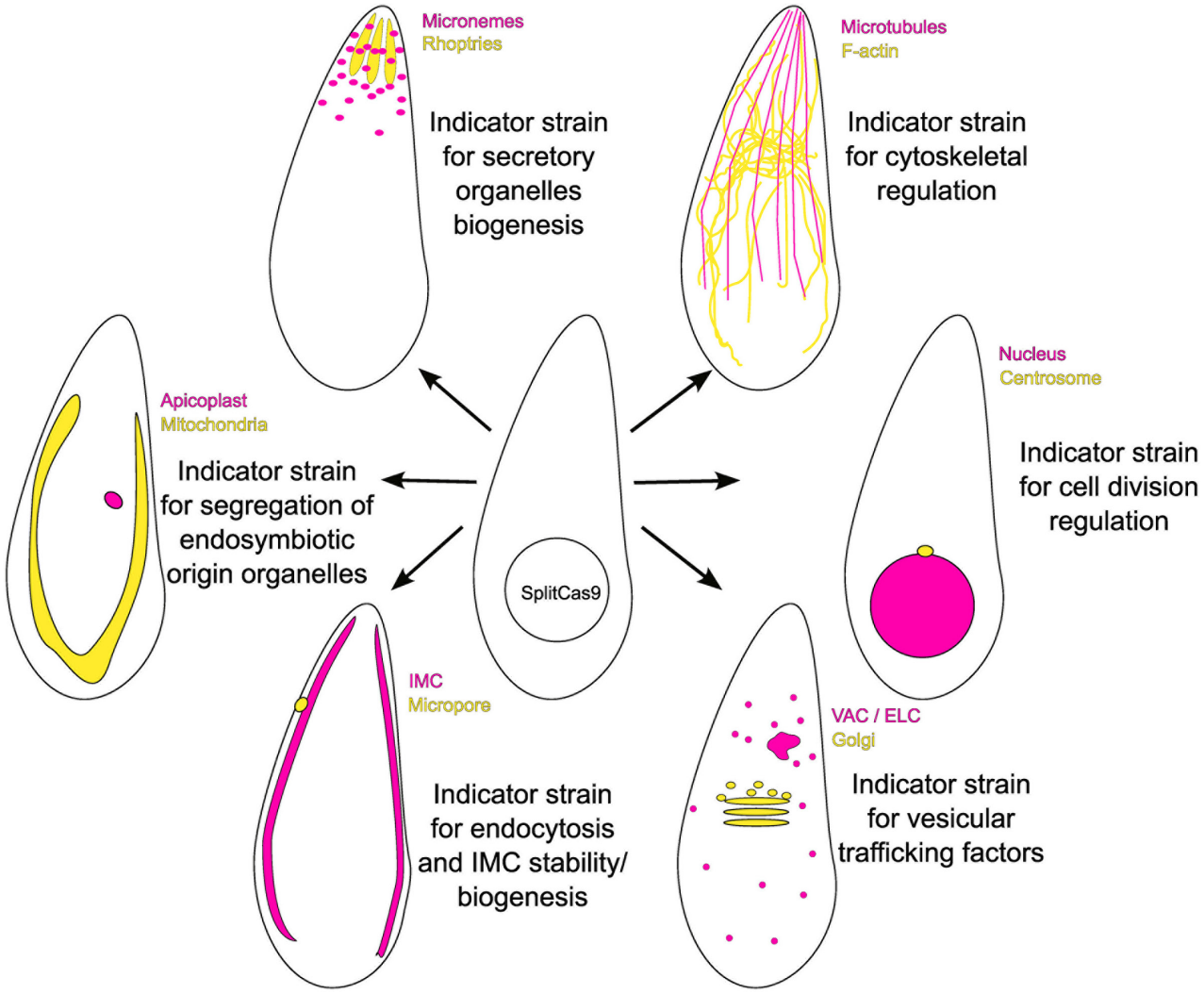

FIGURE 2 Versatility of splitCas9 screens. Creating different indicator strains would change the readout of the screen favouring the discovery of novel factors related to the organelles/structures labelled. ELC, endosomal-like compartment; IMC, inner membrane complex; VAC, vacuolar compartment.

egress and invasion turned out to be a 'low hanging fruit', since they are straightforward and reliable using the splitCas9-system (Li et al., 2022). Since significant egress and reinvasion can be observed in control parasites after 72 h of growth on an HFF-host cell monolayer, while egress mutants remain confined within the initially infected host cell, these phenotypes can be readily identified and isolated. In fact, with the availability of automated image analysis and established algorithms for automated phenotypic characterisation, it should be feasible to conduct significantly larger screens (Fisch et al., 2019).

In the first iteration of this screen, several candidates could be identified, including some invasion and egress factors, such as Tg*Nd6* that has recently been described as one of the non-discharge proteins, that is required for secretion of the rhoptries (Aquilini et al., 2021). In addition, two novel proteins were identified, CGP (Conoid Gliding Protein) and SLF (Signalling Linking Factor) that act at two independent steps during EXIT (Li et al., 2022).

5 | IDENTIFICATION OF NOVEL CONOID PROTEINS, CRITICAL FOR EXIT AND INVASION

SLF was initially discovered in a pull-down assay using Guanylate cyclase-GC, a central factor of the Signalling Platform (Bisio et al., 2019). The Signalling Platform also includes cell division control 50 related protein (CDC50.1) and unique GC organiser (UGO). Although SLF was suggested to have no significant role in signalling based on its plaque formation capability during induced Knockdown via the AID system, its critical involvement in host cell EXIT and invasion was identified through SLF knockout using the splitCas9 system. This critical role was further confirmed using the DiCre system, which allows for the complete excision of the gene flanked by loxP sites (Li et al., 2022). Depletion of SLF resulted in an early blockage of EXIT, indicating that the parasite fails to sense EXIT signals while the F-actin network remains intact. Therefore, SLF was shown to be an integral part of the Signalling Platform responsible for signal sensing. SLF's dual localisation at the residual body and the apical region is well-suited to its function, where the basal localisation of the Signalling Platform may be involved in the synchronised depolymerisation of the intravacuolar network connecting all parasites within a PV, while the apical localisation initiates signalling, leading to PVM lysis and motility initiation. Notably, treatment with Calcium Ionophore (Ci) A23187 partially rescued the phenotype, while the accumulation of cGMP triggered by the addition of BIPPO was blocked early in egress. Treatment with propranolol, which targets phosphatidic acid phosphatases, resulted in a phenotype where parasites disassembled the intravacuolar network but failed to initiate motility. This suggests that different signalling cascades can independently trigger the various events required for *T. gondii* egress. Consistent with its role in tachyzoites, SLF is essential for natural bradyzoite infection (Ye et al., 2022).

CGP is a hypothetical protein localised above the conoid marker SASL6, most likely at the preconoidal rings (Li et al., 2022). The disruption of *cgp* via splitCas9 system and deletion of CGP via the DiCre system resulted in defects in both egress and invasion. During egress induction, although the F-actin network was completely disassembled and the parasitophorous vacuole membrane dissolved, parasites were unable to EXIT since motility was not initiated. Importantly, the typical accumulation of F-actin at the posterior pole, observed during initiation of motility was not observed, leading to the speculation that CGP is involved in regulation of the acto-myosin cytoskeleton. The precise mechanism by which CGP is involved in initiating gliding motility remains unknown and further investigations are required to explore the interaction partners of CGP and their coordination with other key components involved in gliding motility, including GAC (Jacot et al., 2016), AKMT (Heaslip et al., 2011), MyoH (Graindorge et al., 2016) and FRM1 (Baum et al., 2008) among others.

The depletion of these two factors has confirmed that egress is a multi-step process involving: (1) Signalling transduction, (2) Disassembly of the F-actin network, (3) Initiation of host cell lysis and (4) Motility initiation leading to parasite egress. However, the precise interplay of various signalling cascades in this process, particularly regarding the disassembly of the intravacuolar network, remains unknown.

6 | NEW EXIT SCREENS IN *T. GONDII*

The incorporation of CRISPR/Cas9 technologies into screens aimed at discovering new key factors involved in the EXIT of *T. gondii* from the host cell opens up endless possibilities. Tailoring the readout of the screen to observe changes in signalling pathways, post-translational modifications, or secretion of key components to lyse the parasitophorous vacuole membrane (PVM) would further narrow the focus to specific steps within the egress process. Employing calcium or potassium sensors, for example, could help identify key signalling pathways involved in egress and could reveal critical steps in the molecular cascade that governs egress. Meanwhile, monitoring post-translational modifications in key proteins could uncover new targets for therapeutic intervention.

The impact of this new technology extends beyond screens aimed at identifying EXIT factors. By employing narrowed or genome-wide libraries and specific indicator parasite strains or host cells, conditional Cas9 technologies have the potential to revolutionise the field of toxoplasmosis by uncovering key factors involved in secretory pathways, cell division and host cell interaction to name a few. These new screening methods could go beyond determining the impact of gene loss-of-function during the tachyzoite phase of *T. gondii*. Advanced assays and indicator strains, such as the dual luciferase strain (DuaLuc strain), that express luciferase only during the conversion to the bradyzoite strain, may prove crucial in identifying genes responsible for bradyzoite conversion (Smith et al., 2023).

AUTHOR CONTRIBUTIONS

Markus Meissner: Conceptualization; writing – original draft; writing – review and editing. **Elena Jimenéz-Ruiz:** Conceptualization; writing – original draft; writing – review and editing. **Wei Li:** Conceptualization; writing – original draft; writing – review and editing.

ACKNOWLEDGEMENTS

We would like to thank all colleagues who contributed to research in this field and apologise if some publications were not mentioned due to space restrictions. W.L. is funded via a CSC fellowship (201806910075). This project is funded within the DFG Priority Programme SPP2225, Project ME 2675/7-1. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ETHICS STATEMENT

The work presented here did not include human or animal subjects/materials. Thus, no formal consent or approval was necessary.

ORCID

Elena Jimenéz-Ruiz https://orcid.org/0000-0003-2695-0947
Markus Meissner https://orcid.org/0000-0002-5341-5969

REFERENCES

Andenmatten, N., Egarter, S., Jackson, A.J., Jullien, N., Herman, J.P. & Meissner, M. (2013) Conditional genome engineering in *Toxoplasma gondii* uncovers alternative invasion mechanisms. *Nature Methods*, 10(2), 125–127. Available from: https://doi.org/10.1038/nmeth.2301

Aquilini, E., Cova, M.M., Mageswaran, S.K., dos Santos Pacheco, N., Sparvoli, D., Penarete-Vargas, D.M. et al. (2021) An Alveolata secretory machinery adapted to parasite host cell invasion. *Nature Microbiology*, 6(4), 425–434. Available from: https://doi.org/10.1038/s41564-020-00854-z

Arrizabalaga, G., Ruiz, F., Moreno, S. & Boothroyd, J.C. (2004) Ionophore-resistant mutant of *Toxoplasma gondii* reveals involvement of a sodium/hydrogen exchanger in calcium regulation. *The Journal of Cell Biology*, 165(5), 653–662. Available from: https://doi.org/10.1083/jcb.200309097

Baum, J., Tonkin, C.J., Paul, A.S., Rug, M., Smith, B.J., Gould, S.B. et al. (2008) A malaria parasite formin regulates actin polymerization and localizes to the parasite-erythrocyte moving junction during invasion. *Cell Host & Microbe*, 3(3), 188–198. Available from: https://doi.org/10.1016/j.chom.2008.02.006

Birnbaum, J., Flemming, S., Reichard, N., Soares, A.B., Mesén-Ramírez, P., Jonscher, E. et al. (2017) A genetic system to study *Plasmodium falciparum* protein function. *Nature Methods*, 14(4), 450–456. Available from: https://doi.org/10.1038/nmeth.4223

Bisio, H., Lunghi, M., Brochet, M. & Soldati-Favre, D. (2019) Phosphatidic acid governs natural egress in *Toxoplasma gondii* via a guanylate cyclase receptor platform. *Nature Microbiology*, 4(3), 420–428. Available from: https://doi.org/10.1038/s41564-018-0339-8

Black, M.W., Arrizabalaga, G. & Boothroyd, J.C. (2000) Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress. *Molecular and Cellular Biology*, 20(24), 9399–9408. Available from: https://doi.org/10.1128/mcb.20.24.9399-9408.2000

Butterworth, S., Torelli, F., Lockyer, E.J., Wagener, J., Song, O.-R., Broncel, M. et al. (2022) *Toxoplasma gondii* virulence factor ROP1 reduces parasite susceptibility to murine and human innate immune restriction. *PLoS Pathogens*, 18(12), e1011021. Available from: https://doi.org/10.1371/journal.ppat.1011021

Coleman, B.I. & Gubbels, M.J. (2012) A genetic screen to isolate *Toxoplasma gondii* host-cell egress mutants. *Journal of Visualized Experiments*, (60), e3807. Available from: https://doi.org/10.3791/3807

Collins, C.R., das, S., Wong, E.H., Andenmatten, N., Stallmach, R., Hackett, F. et al. (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(4), 687–701. Available from: https://doi.org/10.1111/mmi.12206

Dedehayir, O. & Steinert, M. (2016) The hype cycle model: a review and future directions. *Technological Forecasting and Social Change*, 108, 28–41. Available from: https://doi.org/10.1016/j.techfore.2016.04.005

Donald, R.G. & Roos, D.S. (1995) Insertional mutagenesis and marker rescue in a protozoan parasite: cloning of the uracil phosphoribosyltransferase locus from *Toxoplasma gondii*. *Proceedings of the National Academy of Sciences of the United States of America*, 92(12), 5749–5753. Available from: https://doi.org/10.1073/pnas.92.12.5749

Eidell, K.P., Burke, T. & Gubbels, M.J. (2010) Development of a screen to dissect *Toxoplasma gondii* egress. *Molecular and Biochemical Parasitology*, 171(2), 97–103. Available from: https://doi.org/10.1016/j.molbiopara.2010.03.004

Endo, T., Sethi, K.K. & Piekarski, G. (1982) *Toxoplasma gondii*: calcium ionophore A23187-mediated exit of trophozoites from infected murine macrophages. *Experimental Parasitology*, 53(2), 179–188. Available from: https://doi.org/10.1016/0014-4894(82)90059-5

Engstler, M. & Beneke, T. (2023) Gene editing and scalable functional genomic screening in *Leishmania* species using the CRISPR/Cas9 cytosine base editor toolbox LeishBASEedit. *eLife*, 12. Available from: https://doi.org/10.7554/eLife.85605

Farrell, A., Thirugnanam, S., Lorestani, A., Dvorin, J.D., Eidell, K.P., Ferguson, D.J. et al. (2012) A DOC2 protein identified by mutational profiling is essential for apicomplexan parasite exocytosis. *Science*, 335(6065), 218–221. Available from: https://doi.org/10.1126/science.1210829

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. & Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391(6669), 806–811. Available from: https://doi.org/10.1038/35888

Fisch, D., Yakimovich, A., Clough, B., Wright, J., Bunyan, M., Howell, M. et al. (2019) Defining host-pathogen interactions employing an artificial intelligence workflow. *eLife*, 8. Available from: https://doi.org/10.7554/eLife.40560

Frankel, M.B., Mordue, D.G. & Knoll, L.J. (2007) Discovery of parasite virulence genes reveals a unique regulator of chromosome condensation 1 ortholog critical for efficient nuclear trafficking. *Proceedings of the National Academy of Sciences of the United States of America*, 104(24), 10181–10186. Available from: https://doi.org/10.1073/pnas.0701893104

Garrison, E., Treeck, M., Ehret, E., Butz, H., Garbuz, T., Oswald, B.P. et al. (2012) A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in Toxoplasma. *PLoS Pathogens*, 8(11), e1003049. Available from: https://doi.org/10.1371/journal.ppat.1003049

Graindorge, A., Frénal, K., Jacot, D., Salamun, J., Marq, J.B. & Soldati-Favre, D. (2016) The conoid associated motor MyoH is indispensable

for *Toxoplasma gondii* entry and exit from host cells. *PLoS Pathogens*, 12(1), e1005388. Available from: https://doi.org/10.1371/journal.ppat.1005388

Gubbels, M.J., Lehmann, M., Muthalagi, M., Jerome, M.E., Brooks, C.F., Szatanek, T. et al. (2008) Forward genetic analysis of the apicomplexan cell division cycle in *Toxoplasma gondii*. *PLoS Pathogens*, 4(2), e36. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18282098

Harding, C.R., Sidik, S.M., Petrova, B., Gnädig, N.F., Okombo, J., Herneisen, A.L. et al. (2020) Genetic screens reveal a central role for heme metabolism in artemisinin susceptibility. *Nature Communications*, 11(1), 4813. Available from: https://doi.org/10.1038/s41467-020-18624-0

Heaslip, A.T., Nishi, M., Stein, B. & Hu, K. (2011) The motility of a human parasite, *Toxoplasma gondii*, is regulated by a novel lysine methyltransferase. *PLoS Pathogens*, 7(9), e1002201. Available from: https://doi.org/10.1371/journal.ppat.1002201

Hentzschel, F., Mitesser, V., Fraschka, S.A., Krzikalla, D., Carrillo, E.H., Berkhout, B. et al. (2020) Gene knockdown in malaria parasites via non-canonical RNAi. *Nucleic Acids Research*, 48(1), e2. Available from: https://doi.org/10.1093/nar/gkz927

Herm-Gotz, A., Agop-Nersesian, C., Munter, S., Grimley, J.S., Wandless, T.J., Frischknecht, F. et al. (2007) Rapid control of protein level in the apicomplexan *Toxoplasma gondii*. *Nature Methods*, 4(12), 1003–1005. Available from: https://doi.org/10.1038/nmeth1134

Jacot, D., Tosetti, N., Pires, I., Stock, J., Graindorge, A., Hung, Y.F. et al. (2016) An apicomplexan Actin-binding protein serves as a connector and lipid sensor to coordinate motility and invasion. *Cell Host & Microbe*, 20(6), 731–743. Available from: https://doi.org/10.1016/j.chom.2016.10.020

Jammallo, L., Eidell, K., Davis, P.H., Dufort, F.J., Cronin, C., Thirugnanam, S. et al. (2011) An insertional trap for conditional gene expression in *Toxoplasma gondii*: identification of TAF250 as an essential gene. *Molecular and Biochemical Parasitology*, 175(2), 133–143. Available from: https://doi.org/10.1016/j.molbiopara.2010.10.007

Jimenez-Ruiz, E., Wong, E.H., Pall, G.S. & Meissner, M. (2014) Advantages and disadvantages of conditional systems for characterization of essential genes in *Toxoplasma gondii*. *Parasitology*, 141(11), 1390–1398. Available from: https://doi.org/10.1017/s0031182014000559

Knoll, L.J. & Boothroyd, J.C. (1998) Isolation of developmentally regulated genes from *Toxoplasma gondii* by a gene trap with the positive and negative selectable marker hypoxanthine-xanthine-guanine phosphoribosyltransferase. *Molecular and Cellular Biology*, 18(2), 807–814. Available from: https://doi.org/10.1128/mcb.18.2.807

Knoll, L.J., Furie, G.L. & Boothroyd, J.C. (2001) Adaptation of signature-tagged mutagenesis for *Toxoplasma gondii*: a negative screening strategy to isolate genes that are essential in restrictive growth conditions. *Molecular and Biochemical Parasitology*, 116(1), 11–16. Available from: https://doi.org/10.1016/s0166-6851(01)00295-x

Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A. & Liu, D.R. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, 533(7603), 420–424. Available from: https://doi.org/10.1038/nature17946

Kremer, K., Kamin, D., Rittweger, E., Wilkes, J., Flammer, H., Mahler, S. et al. (2013) An overexpression screen of *Toxoplasma gondii* Rab-GTPases reveals distinct transport routes to the micronemes. *PLoS Pathogens*, 9(3), e1003213. Available from: https://doi.org/10.1371/journal.ppat.1003213

Kuscu, C., Parlak, M., Tufan, T., Yang, J., Szlachta, K., Wei, X. et al. (2017) CRISPR-STOP: gene silencing through base-editing-induced nonsense mutations. *Nature Methods*, 14(7), 710–712. Available from: https://doi.org/10.1038/nmeth.4327

Li, W., Grech, J., Stortz, J.F., Gow, M., Periz, J., Meissner, M. et al. (2022) A splitCas9 phenotypic screen in *Toxoplasma gondii* identifies proteins involved in host cell egress and invasion. *Nature Microbiology*, 7(6), 882–895. Available from: https://doi.org/10.1038/s41564-022-01114-y

Matrajt, M., Donald, R.G., Singh, U. & Roos, D.S. (2002) Identification and characterization of differentiation mutants in the protozoan parasite *Toxoplasma gondii*. *Molecular Microbiology*, 44(3), 735–747. Available from: https://doi.org/10.1046/j.1365-2958.2002.02904.x

McCoy, J.M., Stewart, R.J., Uboldi, A.D., Li, D., Schröder, J., Scott, N.E. et al. (2017) A forward genetic screen identifies a negative regulator of rapid Ca(2+)-dependent cell egress (MS1) in the intracellular parasite *Toxoplasma gondii*. *Journal of Biological Chemistry*, 292(18), 7662–7674. Available from: https://doi.org/10.1074/jbc.M117.775114

Meissner, M., Schluter, D. & Soldati, D. (2002) Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science*, 298(5594), 837–840. Available from: https://doi.org/10.1126/science.1074553

Ngô, H., Tschudi, C., Gull, K. & Ullu, E. (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences of the United States of America*, 95(25), 14687–14692. Available from: https://doi.org/10.1073/pnas.95.25.14687

Sangaré, L.O., Ólafsson, E.B., Wang, Y., Yang, N., Julien, L., Camejo, A. et al. (2019) In vivo CRISPR screen identifies TgWIP as a toxoplasma modulator of dendritic cell migration. *Cell Host & Microbe*, 26(4), 478–492.e8. Available from: https://doi.org/10.1016/j.chom.2019.09.008

Serpeloni, M., Jiménez-Ruiz, E., Vidal, N.M., Kroeber, C., Andenmatten, N., Lemgruber, L. et al. (2016) UAP56 is a conserved crucial component of a divergent mRNA export pathway in *Toxoplasma gondii*. *Molecular Microbiology*, 102(4), 672–689. Available from: https://doi.org/10.1111/mmi.13485

Shen, B., Brown, K.M., Lee, T.D. & Sibley, L.D. (2014) Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. *mBio*, 5(3), e01114-14. Available from: https://doi.org/10.1128/mBio.01114-14

Sidik, S.M., Hackett, C.G., Tran, F., Westwood, N.J. & Lourido, S. (2014) Efficient genome engineering of *Toxoplasma gondii* using CRISPR/Cas9. *PLoS One*, 9(6), e100450. Available from: https://doi.org/10.1371/journal.pone.0100450

Sidik, S.M., Huet, D., Ganesan, S.M., Huynh, M.H., Wang, T., Nasamu, A.S. et al. (2016) A genome-wide CRISPR screen in toxoplasma identifies essential apicomplexan genes. *Cell*, 166(6), 1423–1435.e12. Available from: https://doi.org/10.1016/j.cell.2016.08.019

Sidik, S.M., Huet, D. & Lourido, S. (2018) CRISPR-Cas9-based genome-wide screening of *Toxoplasma gondii*. *Nature Protocols*, 13(1), 307–323. Available from: https://doi.org/10.1038/nprot.2017.131

Smith, D., Lunghi, M., Olafsson, E.B., Hatton, O., Di Cristina, M. & Carruthers, V.B. (2023) A high-throughput amenable dual luciferase system for measuring *Toxoplasma gondii* Bradyzoite viability after drug treatment. *Analytical Chemistry*, 95(2), 668–676. Available from: https://doi.org/10.1021/acs.analchem.2c02174

Smith, T.A., Lopez-Perez, G.S., Herneisen, A.L., Shortt, E. & Lourido, S. (2022) Screening the toxoplasma kinome with high-throughput tagging identifies a regulator of invasion and egress. *Nature Microbiology*, 7(6), 868–881. Available from: https://doi.org/10.1038/s41564-022-01104-0

Waldman, B.S., Schwarz, D., Wadsworth, M.H., 2nd, Saeij, J.P., Shalek, A.K. & Lourido, S. (2020) Identification of a master regulator of differentiation in toxoplasma. *Cell*, 180(2), 359–372.e16. Available from: https://doi.org/10.1016/j.cell.2019.12.013

Ye, S., Lunghi, M. & Soldati-Favre, D. (2022) A signaling factor linked to *Toxoplasma gondii* guanylate cyclase complex controls invasion and egress during acute and chronic infection. *mBio*, 13(5), e0196522. Available from: https://doi.org/10.1128/mbio.01965-22

Young, J., Dominicus, C., Wagener, J., Butterworth, S., Ye, X., Kelly, G. et al. (2019) A CRISPR platform for targeted in vivo screens identifies *Toxoplasma gondii* virulence factors in mice. *Nature Communications*, 10(1), 3963. Available from: https://doi.org/10.1038/s41467-019-11855-w

Zetsche, B., Volz, S.E. & Zhang, F. (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nature Biotechnology*, 33(2), 139–142. Available from: https://doi.org/10.1038/nbt.3149

How to cite this article: Jimenéz-Ruiz, E., Li, W. & Meissner, M. (2023) Where is the EXIT? Phenotypic screens for new egress factors in apicomplexan parasites. *Molecular Microbiology*, 00, 1–9. Available from: https://doi.org/10.1111/mmi.15166