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Review Article

Protein kinases in *Toxoplasma gondii*

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ABSTRACT

Toxoplasma gondii is an obligatory intracellular pathogen that causes life threatening illness in immunodeficient individuals, miscarriage in pregnant woman, and blindness in newborn children. Similar to any other eukaryotic cell, protein kinases play critical and essential roles in the *Toxoplasma* life cycle. Accordingly, many studies have focused on identifying and defining the mechanism of function of these signalling proteins with a long-term goal to develop anti-*Toxoplasma* therapeutics. In this review, we briefly discuss classification and key components of the catalytic domain which are critical for functioning of kinases, with a focus on domains, families, and groups of kinases within *Toxoplasma*. More importantly, this article provides a comprehensive, current overview of research on kinase groups in *Toxoplasma* including the established eukaryotic AGC, CAMK, CK1, CMGC, STE, TKL families and the apicomplexan-specific FIKK, ROPK and WNG family of kinases. This work provides an overview and discusses current knowledge on *Toxoplasma* kinases including their localization, function, signalling network and role in acute and chronic pathogenesis, with a view towards the future in probing kinases as viable drug targets.

1. Introduction

Toxoplasma gondii is a protozoan parasite, classified under the phylum Apicomplexa, that contains obligatory intracellular pathogens. In addition to *Toxoplasma,* other important apicomplexan parasites include *Plasmodium* that causes malaria, *Cryptosporidium* that causes acute gastroenteritis, *Neospora caninum* that causes abortions in cattle, *Sarcocystis* that causes neurological disease in horses, *Babesia,* causative of babesiosis in humans and tick fever in cattle, *Eimeria* that causes coccidiosis in poultry and *Theileria* that causes east coast fever in cattle (Dubey, 1994; Striepen, 2013; Chapman, 2014; Blader et al., 2015; Suarez et al., 2019; Bishop et al., 2020).

Toxoplasma has been well-characterised at the cellular level since its identification in 1908 (Nicolle and Manceaux, 1908). *Toxoplasma* is a unicellular eukaryote (Fig. 1) that measures approximately 6 µm in length and 2 µm in width in the acute replicative form (Dubey et al., 1998; Blader et al., 2015). Similar to any other eukaryotic cell, *Toxoplasma* has standard organelles including a nucleus, endoplasmic reticulum, a single Golgi, and a mitochondrion. Most apicomplexan parasites also contain an organelle referred to as the plastid or the apicoplast that is a remnant of chloroplast in plants. However, the apicoplast does not have any photosynthetic activity but has been shown to play an essential role in lipid metabolism (Striepen et al., 2000, 2007). In addition, *Toxoplasma* contains three apicomplexan-specific sets of secretory organelles. Of these, the micronemes and rhoptries are located at the apical end and the dense granules are distributed uniformly throughout the parasite. The contents of the secretory organelles are secreted in a very organised fashion. The micronemes are the first set of organelles that are secreted by the parasite during invasion and they assist the parasite to establish a firm contact with the host-cell (Carruthers and Sibley, 1999; Alexander et al., 2005; Mital et al., 2005; Huynh and Carruthers, 2006; Carruthers and Boothroyd, 2007). Rhoptries are the second set of organelles that release their contents during host invasion and a few of the rhoptry proteins assist the parasite to penetrate the host cell together with secreted contents of micronemes (Bradley and Sibley, 2007; Boothroyd and Dubremetz, 2008; Besteiro et al., 2011). The dense granule proteins are secreted by the parasite after host invasion is complete and these have been suggested to play critical roles in

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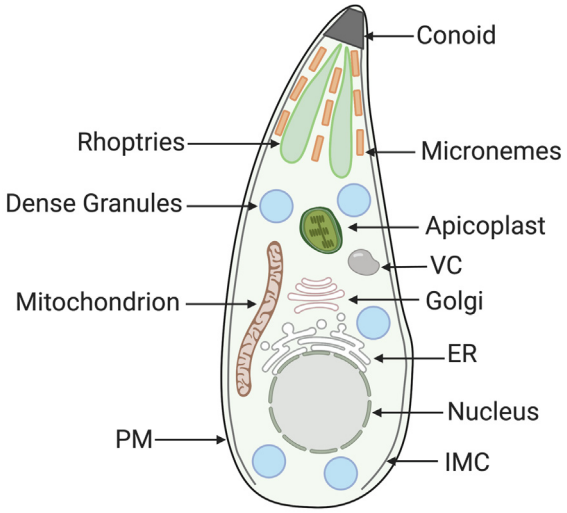

Fig. 1. Ultrastructure of *Toxoplasma gondii* tachyzoite. *Toxoplasma* is a protozoan parasite that contains organelles normally found in a eukaryotic cell. In addition, it contains apicoplast, vacuolar compartment (VC) and secretory organelles: micronemes, rhoptries and dense granules. ER, endoplasmic reticulum; PM, plasma membrane; IMC, inner membrane complex.

nutrient acquisition from the host cell (Coppens et al., 2000; Coppens, 2006, 2014, 2017).

Toxoplasma has an indirect mode of life cycle (Dubey, 1998) where cats act as the definitive host. For *Toxoplasma*, the sexual phase of the life cycle occurs in intestinal epithelial cells and the parasite is then shed as oocysts in cats' faeces. These oocysts act as a source of infection to a wide number of rodents, farm animals and birds that serve as intermediate hosts for this parasite. Humans can acquire *Toxoplasma* infection primarily by three routes: ingestion of food or water contaminated with *Toxoplasma* oocysts, ingestion of contaminated meat obtained from an infected intermediate host, or by transplacental transmission during pregnancy. Because *Toxoplasma* infection can be acquired by three different routes, the infection rate of this parasite in the human population is quite high and, in most countries, it ranges from 25% to 30% (Montoya and Liesenfeld, 2004; Robert-Gangneux and Darde, 2012). In humans infected with *Toxoplasma*, the acute form of the parasite (tachyzoites) disseminates quickly into various organs and propagates rapidly through successive cycles of invasion, replication and egress. Although the host immune response can efficiently clear tachyzoites, the parasite evades the immune system by converting into an encysted form (bradyzoites), mainly in the brain, establishing a permanent chronic infection.

Toxoplasma is an obligatory intracellular parasite. Its intracellular life begins with the parasite invading a host cell. Within the host cell, the parasite resides within a parasitophorous vacuole (PV) that is largely derived from the host cell plasma membrane (PVM). In the PV, *Toxoplasma* replicates by a process known as endodyogeny, where one parasite divides into two daughter parasites. However, endodyogeny is quite distinct from mammalian cell division because daughter cells in *Toxoplasma* do not arise by fission of the mother cell, but instead assemble within the mother cell. The process of parasite replication continues until the host cell is filled with newly formed daughter parasites. Towards the end of the replication process, through a calcium-mediated signalling event, daughter parasites egress which results in lysis of the host cell. Since the host cell is destroyed during parasite egress, the intracellular lifestyle of *Toxoplasma* tachyzoites is often referred to as the lytic cycle (Black and Boothroyd, 2000; Anderson-White et al., 2012; Francia and Striepen, 2014; Blader et al., 2015).

Although *Toxoplasma* usually does not cause any obvious clinical signs after infecting healthy adults, the parasite can cause severe pathology under certain clinical conditions (Dubey, 1994; Weiss and Dubey, 2009; Lindsay and Dubey, 2011; Dunay et al., 2018). If women are exposed to this parasite for the first time during pregnancy, it can lead to miscarriage and congenital infections in children, and can result in blindness and neurological disorders. In immunocompromised individuals and patients undergoing immunosuppressive therapy following organ transplantation, *Toxoplasma* hidden in tissue cysts can reactivate and cause fatal encephalitis. Although the currently available therapy is effective against acute toxoplasmosis, it can have toxic side effects, and importantly it does not treat the chronic form of the disease (Black and Boothroyd, 2000; Weiss and Dubey, 2009; Dunay et al., 2018). Hence, identification and characterization of unique parasite factors that play critical roles in parasite biology and pathogenesis are key in developing safe and effective therapies against toxoplasmosis. In humans, it is well established that protein kinases regulate many key events within a cell and have been validated as drug targets in many disease contexts (Cohen, 2002a; Naula et al., 2005; Johnson, 2009; Zhang et al., 2009; Harsha and Pandey, 2010; Dar and Shokat, 2011; Lucet et al., 2012; Roskoski, 2019). Similarly, many research studies have focused on the *Toxoplasma* kinome to identify kinases essential and/or important to the parasite lifecycle, to accelerate and refine their viability as drug targets (Lourido et al., 2010; Garrison et al., 2012a; Larson et al., 2012; Lourido et al., 2012, 2013b; McCoy et al., 2012; Rutaganira et al., 2017; Scheele et al., 2018).

In this review, we describe the classification and structure–function of kinases, document the current understanding of different kinase groups in *Toxoplasma* biology, and close with a short discussion of future perspectives on *Toxoplasma* kinome research.

2. Protein kinases

Kinases have been shown to play important and critical roles in diverse processes within a cell including regulation of gene transcription, translation, metabolism, cell division, motility, and responses to environmental cues (Cohen, 2002b; Brognard and Hunter, 2011). This class of enzymes acts by catalysing the transfer of a phosphoryl group from an ATP molecule onto a hydroxyl group of a substrate. Kinase substrates range from small molecules to proteins or peptides and can even self-phosphorylate (Cohen, 1999). Protein kinases phosphorylate the hydroxyl moiety of serine, threonine or tyrosine of a substrate protein (Cohen, 2002b). This post-translational modification of a substrate protein can result in its activation or inhibition, alter localization, or enable interaction with its functional partners, leading to initiation of downstream signalling (Manning et al., 2002).

In metazoans, kinases constitute a significant component of the whole genome. In humans, the kinase gene family consists of over 500 proteins and is the fifth largest gene family (1.7% of the genome) after zinc-finger proteins (3%), G-protein coupled receptors (GPCRs) (2.8%), immunoglobulins/major histocompatibility complex family (2.8%), and the protease gene family (1.9%) (Martin et al., 2009; Eswaran and Knapp, 2010). Although a large number of the protein kinases (478 kinases) in humans are true kinases, the remaining 40 are pseudo-kinases that lack one or more key residues or motifs that are essential for phosphorylation function and act in non-catalytic mechanisms such as supporting allosteric regulation (Manning et al., 2002; Zeqiraj and van Aalten, 2010; Eyers and Murphy, 2013; Taylor et al., 2013; Kung and Jura, 2016). Interestingly, several pseudokinases have also been shown to be catalytically active despite the lack of commonly conserved residues (Shi et al., 2010; Taylor and Kornev, 2010).

2.1. Classification of eukaryotic protein kinases (ePKs)

The eukaryotic protein kinases (ePKs) in humans have been classified into nine groups (Manning et al., 2002; Roskoski, 2015). These are: (1) the AGC group consists of 63 members including PKA, PKG and PKC; (2) the Ca2+/calmodulin-dependent protein kinase (CAMK) group that consists of 74 members including calcium/calmodulin dependent protein kinases (CAMK), mitogen-activated protein kinase activating protein kinases (MAPKAPKs); (3) the casein kinase 1 (CK1) group consists of 12 members including casein kinases (CKs); (4) the CMGC group consists of 61 members including cyclin-dependent protein kinases (CDK), mitogen-activated protein kinases (MAPK), glycogen synthase kinase (GSK) and cyclin dependent kinase like (CDKL) families; (5) the STE group (related to yeast non-mating or sterile genes) contains 47 members which are upstream regulators of MAPKs; (6) the tyrosine kinase (TK) group consists of 90 members including 58 receptor protein kinases (RTKs) and 32 non-receptor tyrosine kinases; (7) the tyrosine kinase like (TKL) group that consists of 43 members and proteins in this family resemble both serine/threonine kinases and tyrosine kinases; (8) the receptor guanylyl cyclase (RGC) group consists of five members; and (9) the 'other' grpoup has 83 members. In addition, 40 kinases are classified as atypical kinases, an example of which is pyruvate dehydrogenase kinase that localises to mitochondria.

The ePKs are also classified into three groups based on the phospho-acceptor residue they can modify. The major group of kinases phosphorylate serine or threonine on the substrate protein and hence are referred to as serine/threonine kinases. The second group are tyrosine kinases, and these phosphorylate tyrosine residue on the target protein. The third group includes the TKL proteins whose kinase core is a hybrid of both a serine/threonine kinase and a tyrosine kinase, and have been shown to phosphorylate all three residues (serine, threonine, and tyrosine) although in some organisms these are exclusively serine/threonine kinases.

2.2. Major domain organisation of the catalytic fold in ePKs

The protein kinase fold of ePKs is approximately 250–300 amino acids in length and is divided into 11 domains (Fig. 2A) that are mostly conserved across true kinases (Hanks et al., 1988; Roskoski, 2015). Of these, domain I is a region abundant in glycine residues that normally contains a GxGxxG motif, although variations from this signature have also been found, and provide a region rich in hydrogen bonding features. Domain II contains the Ala-Xxx-Lys motif and domain III contains a conserved glutamate residue that together forms a salt bridge that is essential in overall structural stability and important in kinase activation. Domain IV contains a conserved aliphatic residue (Leu, Ile or Val). Domain V does not contain any conserved residues and forms the αE-helix. Domain VI is further subdivided into two parts, of which VIa contains Gly-xx-Tyr/Leu motif and VIb contains the critical Y/HRD motif that possesses the catalytic aspartate. Domain VII contains the highly conserved DFG motif, retaining the essential cation-binding aspartate, that forms the start of the activation segment whereas domain VIII includes the APE sequence that marks the end of the activation segment. Domain IX contains conserved aspartate and glycine residues and domain X consists of a stretch of variable amino acids that form the αG-helix. Domain XI contains conserved hydrophobic residue and an arginine residue, the latter of which interacts with glutamate of the APE motif, forming a structural stabilising salt bridge (Huse and Kuriyan, 2002; Nolen et al., 2004; Roskoski, 2015).

2.3. Structure and key catalytic residues of the kinase fold

The first protein kinase to be crystalized was Protein Kinase A (PKA) (Knighton et al., 1991), and it has since become the model structure for the kinase domain of true kinases. The catalytic domain of PKA is designated into two lobes (Fig. 2B), the smaller N-lobe and the larger C-lobe (Taylor et al., 2012). The N-lobe is predominantly made of β-sheets, whereas the C-lobe is largely made of α-helices. The deep cleft formed between the two lobes constitutes the active site that accommodates a molecule of ATP and two divalent cations, either magnesium or manganese. The cations are critical for the phospho-transfer event as they facilitate charge stability and position of the ATP phosphates (Herberg et al., 1999; Johnson et al., 2001). Importantly, the C-lobe contains the binding motif for the substrate protein sequence (Fig. 2B).

The kinase catalytic reaction involves transfer of the phosphoryl group from the γ-phosphate of the ATP molecule onto the hydroxyl group of serine/threonine/tyrosine residue of the substrate protein (Cole et al., 1995; Skamnaki et al., 1999). A signature motif, K/E/D/D (K72, E91, D166 and D184; the residue numbers refer to human PKA, PDB ID: 4WB5) is found in active kinases and plays a structural as well as a catalytic role (Fig. 3). Of these four residues, K72 forms a salt bridge with E91 and the presence of this salt bridge is a prerequisite for formation of the active state of the kinase in which the αC helix has an inward confirmation. In addition, K72 forms salt bridges with both α- and β-phosphates of ATP. The two aspartate residues in the K/E/D/D motif are present in the large C-lobe. In the kinase core, D166 is referred to as the catalytic aspartate and during the phospho-transfer process it extracts a proton from the hydroxyl group of serine/threonine residues, thus allowing the oxygen atom to launch nucleophilic attack on the γ-phosphate of ATP (Hanks et al., 1988). The other aspartate of the K/E/D/D motif is D184 that binds one of the two essential magnesium ions (Roskoski, 2015).

3. *Toxoplasma* kinome

The *Toxoplasma* genome contains ~8000 genes, with 159 predicted to be kinases (~2% of the genome) including 108 true kinases and 51 pseudokinases based on analysis of the KEDD signature motif (Peixoto et al., 2010; Lorenzi et al., 2016). The active kinases of *Toxoplasma* include enzymes that belong to several of the established kinase groups in humans including AGC, CAMK, CK1, CMGC, STE and TKL groups of kinases (Fig. 4). Interestingly, *Toxoplasma* does not contain TK and RGC groups of kinases. Of the 51 pseudokinases that lack one or more residues of the KEDD motif, only four enzymes have been classified into the established kinase groups (two CMGCs, one CAMK and one AGC) and, importantly, most of these pseudokinases are specific to the Apicomplexa (Peixoto et al., 2010; Miranda-Saavedra et al., 2012; Lorenzi et al., 2016).

3.1. AGC group

The AGC group of kinases is widely conserved across eukaryotes and many of these are cyclic nucleotide regulated kinases (Pearce et al., 2010). A few of the prominent AGC kinases include cyclic AMP (cAMP)-dependent PKA, cGMP-dependent PKG and PKC (Manning et al., 2002; Roskoski, 2015). The activity of AGC kinases is regulated by a few separate mechanisms that involve either phosphorylation and/or change in conformation. Further, many AGC kinases contain additional domains that mediate membrane or substrate interactions (Pearce et al., 2010).

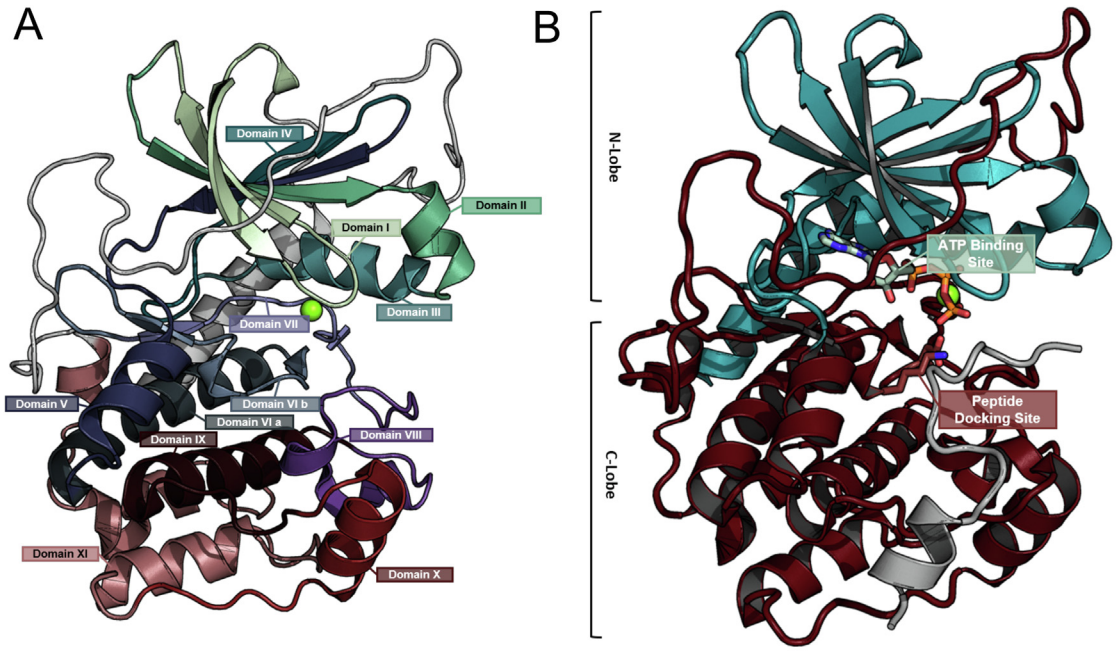

Fig. 2. Structure of the kinase fold and the key domains. (A) Structure of Protein Kinase A (PKA), shown as a cartoon and highlighting the 11 kinase domains (PDB ID: 4WB5 (Cheung et al., 2015)). Domains are colour coded and labelled by domain. The Mg^{2+} ion is shown as a green (light grey) sphere. (B) General architecture of PKA catalytic core (PDB ID: 4WB5). The N-lobe is represented in teal (dark grey) and the C-lobe is represented in maroon (black). The protein kinase inhibitor (PKI) peptide represented in grey, with ATP displayed as a stick and coloured by atom type. The Mg^{2+} ion is shown as a green (light grey) sphere.

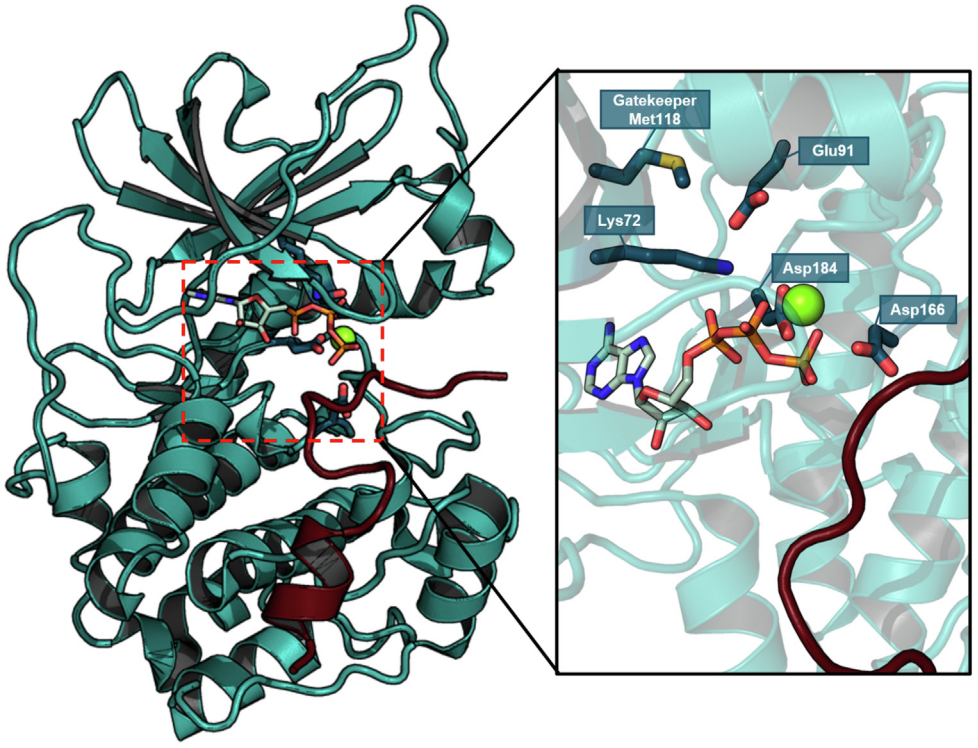

Fig. 3. Schematic representation of the protein kinase A (PKA) core structure with key catalytic residues. PKA (PDB ID: 4WB5) is represented in teal (dark grey) with protein kinase inhibitor (PKI) peptide shown in maroon (black). ATP is displayed in stick and coloured by atom type. The Mg^{2+} ion is shown as a green (light grey) sphere. Inset shows key residues and the gatekeeper residue, which are displayed as sticks, labelled, and coloured by atom type.

Normally, PKA is maintained in an inactive state in the cell as the two regulatory (R) subunits are bound to two catalytic (C) subunits that leads to the formation of a heterotetrameric complex (R_2C_2). The two regulatory subunits interact through a coiled-coil motif to form the dimer whereas within the heterotetramer a pseudo-substrate motif of each R subunit binds to the substrate binding domain of the C subunit to suppress kinase function. In higher eukaryotes, ligands such as adrenaline bind to GPCRs and

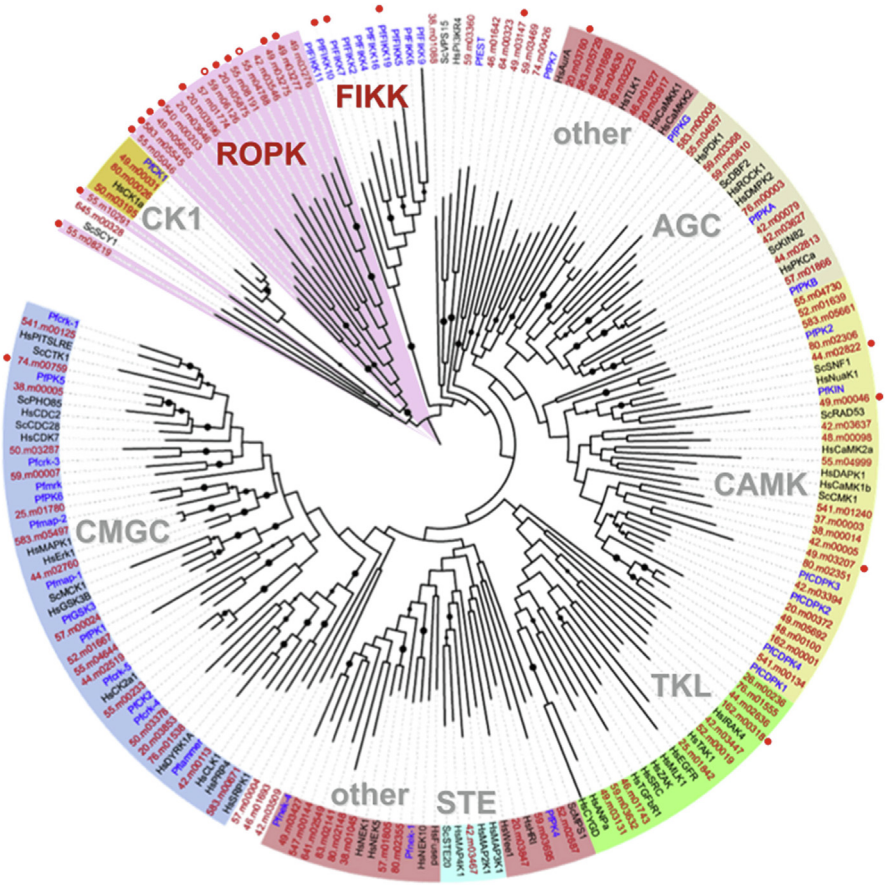

Fig. 4. The *Toxoplasma gondii* kinome. Classification of 108 active kinases predicted from the *T. gondii* genome. Black, human and yeast; blue, *Plasmodium falciparum*; red, *T. gondii*. Coloured arcs highlight major kinase groups: AGC, CMGC, CAMK, TKL, CK1 and STE. Red lettering, apicomplexan-specific groups ROPK (pink) and FIKK. Red circles, kinases with predicted secretory signal sequence or signal anchor (open, newly recognised); black dots, bootstrap support > 50%. Reprinted from: Cell Host and Microbe, Vol 8, Peixoto et al., Integrative Genomic Approaches Highlight a Family of Parasite-Specific Kinases that Regulate Host Responses, 208–218, Copyright (2010), with permission from Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this leads to the dissociation of the α-subunit from the βγ subunits of heterotrimeric G proteins. The released α-subunit binds and activates adenylate cyclase (Ac), which then hydrolyzes ATP to generate cAMP, a second messenger. cAMP then binds to the regulatory (R) subunits of the R_2C_2 complex and thus releases the catalytic subunits which undergo a conformational change to become the active kinase (Cheng et al., 1998; Nirula et al., 2006).

Toxoplasma contains a single gene that encodes the regulatory subunit, TgPKAr1, and three genes that encode catalytic subunits: TgPKAc1, TgPKAc2 and TgPKAc3 (Sugi et al., 2016; Jia et al., 2017; Uboldi et al., 2018). A CRISPR-based genome-wide study suggested that the regulatory subunit is essential for the lytic cycle in tachyzoites (Sidik et al., 2016). TgPKAc1 interacts with the regulatory subunit, TgPKAr1, and this complex localises to the inner membrane complex (IMC) as TgPKAr1 is acylated at the N-terminus. In *Toxoplasma*, TgPKAr1 plays a critical role in calcium signalling and more specifically in regulating cytoplasmic calcium levels ($[Ca^{2+}]_{cyt}$) after invasion of a host cell (Jia et al., 2017; Uboldi et al., 2018). When *Toxoplasma* is invading a host cell, a rise in cytoplasmic calcium in the tachyzoite results in secretion of micronemes to the parasite surface for engagement with host cell receptors. The gliding motility of the parasite powers the tachyzoite forward and invagination of the host cell plasma membrane around the invading parasite leads to formation of the PVM within which the intracellular parasite resides and replicates. Once the parasite completes the invasion process, $[Ca^{2+}]_{cyt}$ levels drop and the parasite becomes immotile to begin preparation for endodyogeny. However, in the absence of PKAc1, $[Ca^{2+}]_{cyt}$ levels do not decrease rapidly and this leads to microneme secretion and premature egress of the newly invaded parasite. Further studies have revealed that in *Toxoplasma,* PKA and PKG signalling pathways are interconnected (Fig. 5), as activation of PKA results in phosphorylation and activation of phospho-diesterases (PDE) that cleave cGMP (Jia et al., 2017; Brown and Sibley, 2018). Thus PDE activation results in depletion of cGMP levels and hence inhibition of PKG-mediated signalling that has been shown to play a role in parasite egress. Hence in *Toxoplasma*, TgPKAc1 is required for activation of cGMP degrading diesterases that suppresses PKG activation and blocks premature egress. In metazoans, it is well established that PKA signalling is connected to upstream GPCR signalling. However, the *Toxoplasma* genome does not appear to encode GPCRs. The signalling pathway that is responsible for activation of adenyl cyclase and the production of cAMP, the second messenger that activates PKA pathway in *Toxoplasma,* is yet to be discovered.

Transcriptomic data suggests that TgPKAc2 is not expressed in asexual stages but is expressed at high levels in the sexual stages that occur in the cat intestine (Behnke et al., 2010; Fritz et al., 2012; Sugi et al., 2016). However, when expressed under a heterologous promoter in tachyzoites, the kinase localises to the IMC region and the localization does not change in either intracellular

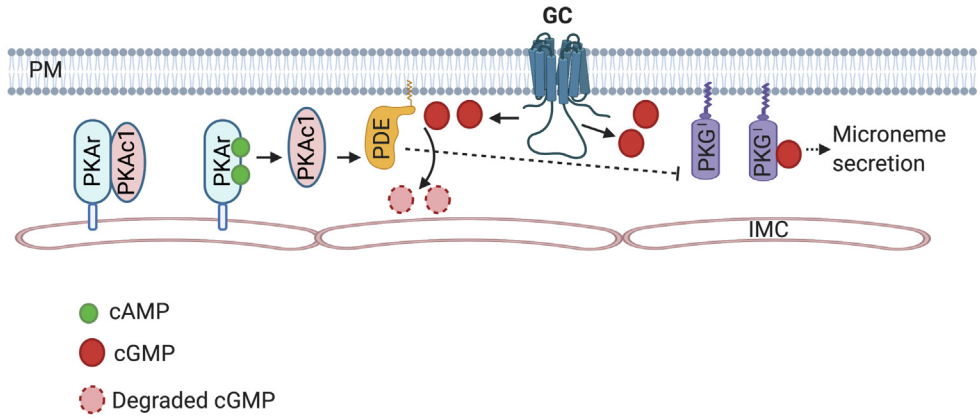

Fig. 5. Protein kinase A (PKA) and Protein kinase G (PKG) signalling in *Toxoplasma*. PKAc1 is a key enzyme that blocks premature egress and enables the parasite to initiate endodyogeny while PKG plays an essential role in parasite egress following replication. PKAr, protein kinase A regulatory subunit; PKAc, protein kinase A catalytic subunit; PDE, phosphodiesterase; IMC, inner membrane complex.

or extracellular or bradyzoite culture conditions (Sugi et al., 2016). But the precise role of this kinase in sexual developmental stages in the cat remains to be determined.

TgPKAc3 is another PKA protein kinase that is specific to coccidians and is expressed not only in tachyzoites but also in bradyzoites. Epitope tagging of TgPKAc3 revealed that the kinase localises to the cytosol in tachyzoites, whereas in bradyzoites the protein migrates to the periphery of the parasite. More importantly, ablation of TgPKAc3 results in a significant reduction in chronic cysts in the mouse brain, suggesting that this kinase plays a key role in bradyzoite differentiation and tissue cyst maintenance (Sugi et al., 2016).

PKG is another important AGC kinase and in mammalian cells there are two genes that code for three PKG proteins (Pearce et al., 2010). PKGIα and Iβ are splice variants that are both coded by the same Type I gene and localise to cytoplasm. The Type II gene, however, codes for only one protein that localises to the plasma membrane. All three PKGs have a similar architecture including an N-terminal regulatory domain (consisting of a leucine zipper domain; an autoinhibitory sequence; two tandem cyclic nucleotide-binding domains) and a C-terminal catalytic domain. Within the regulatory domain there is an autoinhibitory motif that binds to the catalytic site and hence keeps the kinase inactive. Natriuretic peptides binding to the natriuretic peptide receptor on the surface of the plasma membrane or nitric oxide (NO) binding to the NO synthase (NOS) activates particulate or soluble Guanylyl Cyclase (GC), respectively. Activated GC hydrolyzes GTP to generate cGMP that binds to the regulatory domain leading to conformation change and activation of PKG.

The *Toxoplasma* genome contains a single *pkg* gene that codes for two isoforms, PKG^{I} (residues 1–994) and PKG^{II} (residues 103–994) and both of these proteins localise to two distinct regions in the parasite, PKG^{I} (plasma membrane) and PKG^{II} (cytoplasm) (Brown et al., 2017). Although a chemical genetics approach had suggested that PKG plays a critical role in the *Toxoplasma* lytic cycle, it was not clear whether PKG^{I} and PKG^{II} are functionally distinct or redundant. A recent study (Brown et al., 2017) elegantly demonstrated that whereas PKG^{II} is dispensable in the presence of PKG^{I}, PKG^{I} however plays an essential role in in vitro growth and pathogenesis in vivo for *Toxoplasma*. More specifically, parasites deficient in PKG^{I} fail to secrete micronemes and consequently exhibit severe defects in gliding motility, host cell invasion and egress. Furthermore, PKG^{I} contains an acylation motif near the N-terminus that assists this kinase to localise to the plasma membrane and this localization is essential for proper functioning and viability of the parasite. *Toxoplasma* encodes a single guanylate cyclase, TgGC, that localises to the apical cap region of the parasite plasma membrane and loss of this gene results in phenotypes similar to the lack of PKG^{I}, suggesting that indeed TgGC and PKG^{I} function in the same signalling pathway (Brown et al., 2017; Jia et al., 2017; Brown and Sibley, 2018). The observation that loss of TgGC can be compensated if parasites are treated with a cell-permeant PDE inhibitor that suppresses cGMP cleavage (thus enhancing cGMP levels) and activates PKG^{I} further strengthens the argument that TgGC acts upstream of PKG^{I} (Brown et al., 2017; Jia et al., 2017; Brown and Sibley, 2018).

3.2. Aurora kinases

Aurora kinases are serine/threonine kinases that are conserved across eukaryotes. This kinase family has been shown to play pivotal roles in cell division including centrosome function, organisation of the mitotic spindle, chromosome segregation and cytokinesis (Roskoski, 2015).

The *Toxoplasma* genome contains three aurora kinase genes that have been named *Tgark1*, *Tgark2* and *Tgark3* (Peixoto et al., 2010; Miranda-Saavedra et al., 2012; Lorenzi et al., 2016). TgArk1 is an ortholog of aurora kinase B (ARKB) in humans that forms a chromosomal passenger complex (CPC) with three other proteins, the inner centromere protein (INCENP), Borealin and Survivin. *Tgark1* is an essential gene in *Toxoplasma* whose expression is cell cycle regulated (Behnke et al., 2010; Sidik et al., 2016). Interestingly, localization of TgArk1 is highly dynamic and varies depending on the state of the cell cycle (Suvorova et al., 2015; Berry et al., 2018). In non-dividing parasites, TgArk1 is confined to the nucleus but during endodyogeny the kinase is found throughout the parasite (both cytoplasm and nucleus). In *Toxoplasma,* TgArk1 interacts with INCENP proteins TgINCENP1 and TgINCENP2, although TgINCENP1 is dispensable for cell division. TgINCENP2, however, is essential for parasite cell division and its localization is similar to TgArk1. Importantly, dynamic localization of TgArk11 is crucial for parasite cell division and is dependent on its interaction with TgINCENP2. Further proof that these two proteins work together is evident from the observations that parasites deficient in TgArk1 or TgINCENP2 show similar phenotypes with parasites unable to properly segregate their nuclei and coordinate the formation of two daughter cells. It appears that the function of TgArk1 involves phosphorylation of cytoskeleton-associated proteins, factors involved in DNA biology, nuclear pore proteins and known cell cycle regulators (Berry et al., 2018).

TgArk2 has also been characterised in *Toxoplasma* and this protein is dispensable for parasite propagation in vitro. This kinase exhibits periodic expression and varied localization during endodyogeny. In the G1 phase, TgArk2 localises to the spindle pole prior to tubulin assembly and does not associate with the outer core of the centrosome. However, in mitosis TgArk2 associates with the mitotic spindle and disappears completely during daughter cell budding. Surprisingly, even though TgArk2 associates with key structures in the parasite, loss of this kinase does not affect the lytic cycle, suggesting that its functions are redundant (Berry et al., 2018).

TgArk3 is another member of the aurora kinase family in *Toxoplasma* that plays an essential role in parasite division (Berry et al., 2016). This kinase is not expressed in the G1 phase but is expressed in S, M and cytokinesis phases during which it shows dynamic localization. In the S phase, the protein localises to the edge of the centrosomal outer core as demonstrated by partial overlap with centrin. Interestingly, the kinase transforms into a linear structure in mitosis and cytokinesis stages, during which it appears to be associated on only one side of the IMC region in the newly forming daughter cells. Parasites deficient in this kinase show significant defects in replication and egress. More specifically, ablation of this gene results in parasites losing their ability to form rosettes within the PV. Further, TgArk3 is essential for acute toxoplasmosis in mouse as loss of this protein results in complete loss of virulence (Berry et al., 2016).

3.3. CAMK group

Calcium (Ca^{2+}) is an important, ubiquitous, and universal second messenger in any living cell. In humans and animals, calmodulin serves as the primary intracellular receptor for Ca^{2+}, and Ca^{2+} bound calmodulin binds and activates CAMK. Calmodulin is a small dumbbell-shaped protein expressed by all eukaryotic cells. This calcium binding protein has two lobes (N and C lobes) with a pair of EF hands in each lobe. Each of the EF hands can bind one Ca^{2+} ion and binding of four Ca^{2+} ions results in calmodulin adopting an open conformation. The activated calmodulin further binds and activates its target proteins including CAMKs.

The CAMK group is one of the largest kinase groups in *Toxoplasma*, which includes calcium-dependent protein kinases (CDPKs) that are restricted to plants and protozoa (Ward et al., 2004; Billker et al., 2009; Peixoto et al., 2010; Miranda-Saavedra et al., 2012). Since CDPKs are absent in humans and animals, CDPKs that play essential roles in the parasite life cycle have been proposed as drug targets. The unique feature of the CDPKs is that they contain a fusion of the kinase domain with the calmodulin domain that is referred to as CDPK-activating domain (CAD). Structural analysis of two of the CDPKs in *Toxoplasma* has revealed that the mechanism of activation of these kinases is slightly different from that of CAMKs. Binding of calcium to the CAD results in a large conformational change where the CAD is translocated to a new position opposite to the catalytic pocket, thereby allowing access to the substrate proteins (Wernimont et al., 2010).

The CDPKs in *Toxoplasma* are broadly classified into two groups: canonical and non-canonical CDPKS (Billker et al., 2009). The canonical CDPKs have an architecture that is quite similar to plant CDPKs and these typically contain a N-terminal kinase domain and a C- terminal calmodulin domain that contains four EF hands. The canonical CDPKs are further classified into two subgroups: One group has a very small N terminal extension before the kinase domain (TgCDPK1 and TgCDPK3), whereas the second group contains a long N-terminal extension (TgCDPK2, TgCDPK2A, TgCDPK2B and TgCDPK5). However, the non-canonical CDPKs contain additional features including N-terminal extensions, a variable number of EF hands, and the presence of domains such as zinc finger or pleckstrin homology (PH) domains (TgCDPK4, TgCDPK4A, TgCDPK4B, TgCDPK6, TgCDPK7, TgCDPK7A, TgCDPK8 and TgCDPK9). Further, all of the non-canonical CDPKs contain one or more incomplete EF hands.

Of the canonical group, TgCDPK1 is an essential kinase that localises to the cytoplasm and plays a key role in calcium-dependent secretion of microneme proteins for host-cell invasion, motility and parasite egress from host cells (Lourido et al., 2010) (Fig. 6). Interestingly, TgCDPK1 harbours glycine, an amino acid with no side chain as the gatekeeper residue (Fig. 3) and hence allows access to small molecules that can bind and inhibit this kinase. However, when the gatekeeper residue is changed from glycine to methionine, an amino acid with a bulky side chain, it blocks access of these small molecules to the ATP binding pocket and thus the kinase becomes insensitive to chemical inhibitors. This feature of TgCDPK1 where the drug sensitivity can be regulated by mutating the gatekeeper residue was elegantly exploited to identify substrates of this kinase (Lourido et al., 2013a). This involved use of a bulky artificial ATP analogue, N6-furfuryladenosine (kinetin)-5′-O-[3-thiotriphosphate] (KTPγS), that contains thiophosphate. When a kinase phosphorylates its substrates, γ-phosphate of the ATP molecule is added onto the serine or threonine residue of the substrate protein. As the γ-phosphate in KTPγS is a thiophosphate, this would be transferred to the substrate protein during catalysis. Subsequently, thiol modified substrates can then be identified using two approaches: immunoprecipitation using antibody specific for the thiophosphorylation modification and mass spectrometry analysis or target proteins can be trypsinized to isolate thiophosphorylated peptides followed by mass spectrometry analysis. Adoption of this approach for the kinase TgCDPK1 identified several substrates including dynamin-related protein, DrpB (Lourido et al., 2013a). However, the precise mechanism in how the phosphorylation of DrpB contributes to the downstream signalling of TgCDPK1-mediated events remains to be ascertained.

Since TgCDPK1 is essential for the *Toxoplasma* lytic cycle where it regulates microneme secretion and belongs to a group of kinases that is absent in humans and animals, many studies have focused on identifying small molecule inhibitors that could be developed as therapeutics (Johnson et al., 2012; Lourido et al., 2013b). Pyrazolopyrimidine (PP) analogues with bulky groups are one set of chemical compounds that have been explored as TgCDPK1 inhibitors and hence as anti-toxoplasmosis drugs. Indeed, many of the structurally modified PP analogues show specific inhibitory activity against TgCDPK1 in vitro. More importantly, these drugs are not only effective in reducing virulence in the acute phase of toxoplasmosis but can also substantially reduce parasite cyst burden in the brain, thus suggesting that these compounds could be promising drug candidates (Lourido et al., 2013b; Vidadala et al., 2016).

TgCDPK3 is also a canonical CDPK that localises to the inner leaflet of the plasma membrane and its localization to the plasma membrane is essential for its function. Reverse genetics studies have demonstrated that TgCDPK3 plays a key role in induced *Toxoplasma* egress (Fig. 6) from host cells (Garrison et al., 2012b; Lourido et al., 2012; McCoy et al., 2012) and is required for establishment of chronic infection in the mouse brain (Garrison et al., 2012b). It is hypothesised to be upstream of TgCDPK1 in the calcium signalling pathway and use of the proximity-based labelling approach (BioID) revealed that the interactome of this kinase includes Myosin A (TgMyoA), a key component of the parasite motor system (Gaji et al., 2015). Further phosphoproteomic analysis showed that indeed TgMyoA is less phosphorylated in TgCDPK3 mutants (Treeck et al., 2014). Based on these findings, it appears that a rise in calcium levels at the IMC region activates TgCDPK3

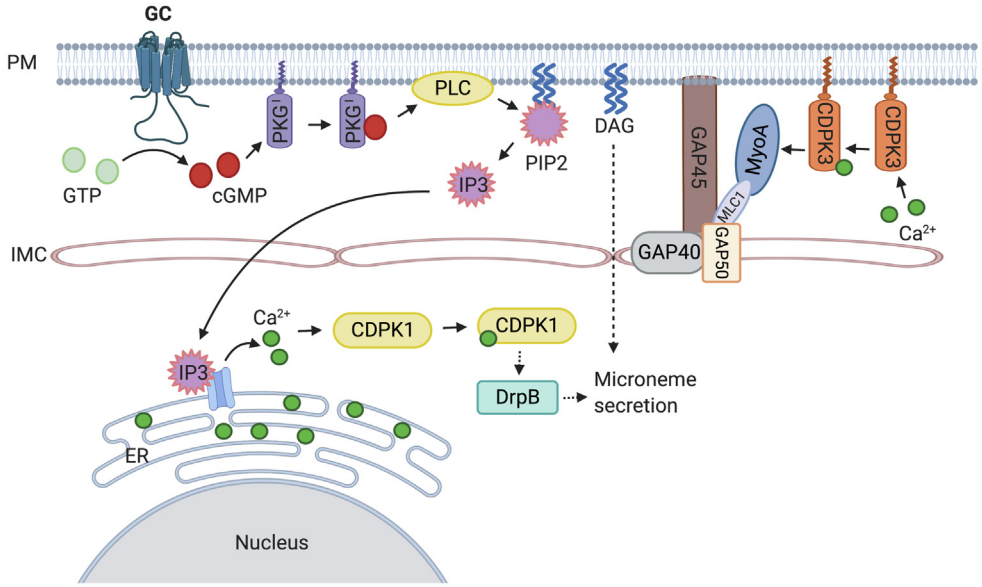

Fig. 6. Calcium dependent protein kinase 1 (CDPK1) and Calcium dependent protein kinase 3 (CDPK3) signalling in *Toxoplasma*. CDPK1 is involved in calcium mediated signalling that results in exocytosis of micronemal proteins. CDPK3 is involved in initiating parasite motility during egress. GC, gunylate cyclase; GTP, guanosine-5′-triphosphate; cGMP, cyclic guanosine monophosphate; PKG, protein kinase G; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol trisphosphate; DAG, diacylglycerol; GAP, glideosome associated protein; MyoA, myosin A; CDPK, calcium-dependent protein kinase; DrpB, dynamin related protein B; ER, endoplasmic reticulum.

to phosphorylate TgMyoA at two sites (S21 and S743). This conceivably leads to initiation of parasite motility and egress from the host-cell (Gaji et al., 2015).

The TgCDPK2 kinase is a cytoplasmic protein that contains an N-terminal carbohydrate-binding module 20 (CBM20) domain in addition to the kinase and calmodulin domains. Accordingly, reverse genetics studies have shown that TgCDPK2 plays a key role in carbohydrate metabolism and is critical for the parasite to establish chronic infection in the mouse model (Uboldi et al., 2015). In the absence of TgCDPK2, increased synthesis and decreased degradation of amylopectin leads to hyper-accumulation of amylopectin, both in tachyzoites and bradyzoites. More specifically, the accumulation is more pronounced in the bradyzoite form and results in the loss of viability and failure to establish chronic infection in the mouse brain. Quantitative phosphoproteomics combined with the thiophosphorylation technique to identify direct substrates suggested that many of the amylopectin binding proteins including pyruvate phosphate dikinase (PPDK) are targets of TgCDPK2 in *Toxoplasma* (Uboldi et al., 2015).

TgCDPK7 is a non-canonical kinase that contains two EF hands near the N-terminus and a PH domain just upstream of the kinase domain towards the C-terminus (Morlon-Guyot et al., 2014). This kinase is a cytoplasmic protein that plays an essential role in cell division of *Toxoplasma* where deletion of this gene affects partitioning and the positioning of the centrosomes, two early steps required not only for a successful co-ordination between mitosis and cytokinesis but also for the polarity of nascent IMC budding (Morlon-Guyot et al., 2014). Of the remaining seven non-canonical CDPKs, only TgCDPK6 appears to play a moderate role in *Toxoplasma* growth in vitro whereas the remaining kinases are dispensable (Long et al., 2016).

3.4. CK1 group

The CK1 group includes casein kinase 1 and close relatives that are serine/threonine kinases and have been shown to play critical roles in diverse cell functions such as vesicular trafficking, circadian rhythm, immune response and cell division events including DNA repair, cell cycle regulation, and cytokinesis (Knippschild et al., 2014). Although casein is used as a substrate in experimental studies, the milk protein is not a natural in vivo substrate. The pool of target proteins of CK1 family kinases is quite broad and includes cytoskeletal proteins, membrane receptors, membrane transporters, DNA-RNA associated proteins, ribosomal proteins, transcription and splice factors, translation factors, viral proteins, other kinases and phosphatases. These kinases show preferences for serine and threonine residues N-terminally flanked by already phosphorylated amino acid residues or acidic amino acids. The activity of CK1 kinases is predominantly regulated by either of three mechanisms: (i) phosphorylation of autoinhibitory sequence within the kinase; (ii) phosphorylation by upstream signalling kinase; (iii) interaction with functional partner proteins (Jiang, 2017).

Toxoplasma contains two CK1 isoforms that have been named TgCK1α and TgCK1β that localise to the cytosol and plasma membrane, respectively (Donald et al., 2005, 2006). Analysis of recombinant proteins of these two isoforms revealed that TgCK1α codes for an active kinase whereas TgCK1β is catalytically inactive. Further, TgCK1α can be selectively inhibited in vitro by compounds purvalanol B (half-maximal inhibitory concentration (IC_{50}) = 123 ± 23 nM) and aminopurvalanol (IC_{50} = 42 ± 7 nM) (Donald et al., 2005). Targeted disruption of the TgCK1α gene affects in vitro propagation of *Toxoplasma*, thus revealing that this kinase plays an important role in the tachyzoite lytic cycle (Wang et al., 2016). However, the precise step in the lytic cycle affected by loss of TgCK1α and its mechanism of action is yet to be determined. Interestingly, ablation of TgCK1α appears to increase parasite acute virulence in the animal model, suggesting that this kinase might be regulating the expression or function of virulence factors in *Toxoplasma*.

3.5. CMGC group

The CMGC group of kinases contains CDKs, MAPKs, GSK and CDKL families.

3.5.1. CDKs

In metazoans, cell division is regulated by a highly organised and meticulously orchestrated cell cycle machinery. The core components of this machinery include a set of temporally expressed proteins called cyclins that bind, activate and provide substrate specificity to their partner CDKs. CDKs are serine/threonine kinases that function as heterodimers, with CDK acting as the kinase unit and the cyclin acting as the regulatory unit. The CDKs are constitutively expressed whereas cyclins show periodical expression and degradation, thus ensuring tight regulation of CDK activity during cell cycle progression (Santo et al., 2015; Liu et al., 2019).

Normally a quiescent cell exists in the G_0 state before it enters the cell cycle that comprises four phases including gap 1 (G1), DNA synthesis (S), gap 2 (G2) and mitosis (M). The entry from G_0 to the G1 phase requires phosphorylation of retinoblastomal protein (pRB1) by the CDK3/cyclin C complex. Once in the G1 phase, stimulation with growth factors results in upregulation of cyclin D which, in combination with CDK4 or CDK6, ensures G1 progression. Cyclin E is the next cyclin that is upregulated, which in combination with CDK2 helps the cell to progress from G1 to the S phase. In addition to facilitating entry into the S phase, the CDK2/cyclin E complex also induces histone synthesis and centrosome duplication at the G1-S boundary. In the S phase, CDK2/cyclin E, together with CDK2/cyclin A, promotes initiation of DNA replication and progression through the S phase. During the G2 phase, sequential phosphorylation of forkhead box (FOX) transcription factors by CDK1/cyclin A and CDK1/cyclin B regulates entry into the M phase. CDK1/cyclin B holoenzyme kinase activity is required for breakdown of the nuclear envelope, chromosome condensation and spindle assembly during transition from the prophase to the metaphase in the mitotic stage. Degradation of cyclin B1 then allows the cell to progress through the remaining stages in the M phase and the cell completes cytokinesis (Santo et al., 2015; Liu et al., 2019).

Mining of the parasite genome for proteins that contain both the kinase domain and a cyclin binding sequence (C-helix) indicated that *Toxoplasma* encodes 10 such genes that have been named as CDK -related kinases (*crks*) until cyclin-dependent activation of the kinases is established (Alvarez and Suvorova, 2017). Of the 10 TgCrks, eight kinases (TgCrk1-8) are expressed in tachyzoite and/or bradyzoite stages, whereas expression of two kinases (TgCrk2-L1 and TgCrk5-L1) is restricted to merozoite and/or sporozoite stages in the life cycle. TgCrks (1–8) localise to the parasite nucleus and, interestingly, TgCrks in *Toxoplasma* show dynamic cell cycle expression unlike constitutively expressed CDKs and temporally regulated cyclins in higher eukaryotes. In addition, similar to other apicomplexans, *Toxoplasma* does not appear to have canonical cyclins (A to E types) and instead the parasite contains atypical cyclins (P, H, L and Y), most of which show constitutive expression (Alvarez and Suvorova, 2017; White and Suvorova, 2018).

The cell cycle in higher eukaryotes typically involves distinct stages including Interphase (G1, S, G2) and M (mitosis and cytokinesis) phases. In *Toxoplasma*, however, the tachyzoite cell cycle begins with a gap period (G1 phase) that is first devoted to the biosynthesis of protein and RNA components (G1a), followed by a switch to the component needs of DNA synthesis in the second half of G1 (G1b) (Fig. 7). Although *Toxoplasma* chromosomes are replicated in the S phase and segregated in the M phase, similarly to other eukaryotes, the G2 phase is either short or absent in the parasite (White and Suvorova, 2018). Recent studies using reverse genetics have revealed that cell division in *Toxoplasma* involves five Crks (Naumov et al., 2017; White and Suvorova, 2018). The kinase TgCrk2 in association P type cyclin (TgPHO80) controls the START checkpoint in G1 (G1a to G1b) whereas the DNA licencing checkpoint in the S phase is regulated by TgCrk5. During the S, M and cytokinesis phases that are interwoven in *Toxoplasma*, TgCrk4

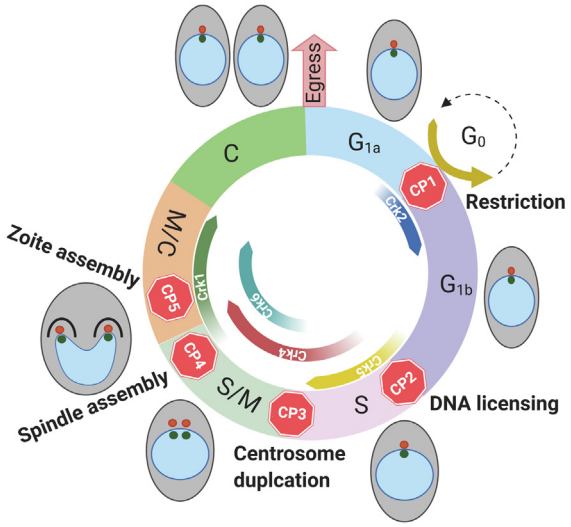

Fig. 7. TgCrks (CDK-related kinases) and cell division in *Toxoplasma*. The cell cycle in *Toxoplasma* involves five checkpoints that are tightly regulated by five Crks. Unlike a typical eukaryotic cell, *Toxoplasma* endodyogeny lacks the G2 phase of the cell cycle. The green dot indicates the centrosome outer core and the red dot indicates the centrosome inner core. CP, check point; G, gap phase; S, synthesis phase; M, mitosis; C, cytokinesis; Crk, CDK-related kinase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controls centrosome duplication, whereas TgCrk1 regulates budding of daughter cell cytoskeleton. TgCrk6 regulates the spindle cell checkpoint in *Toxoplasma* when the dividing parasite is at the transition from metaphase to anaphase. And interestingly, findings in these studies have suggested that, unlike higher eukaryotes, many of the Crks in *Toxoplasma* may function without cyclin partners (Alvarez and Suvorova, 2017; Naumov et al., 2017).

3.5.2. MAPKs

In higher eukaryotes, MAPKs are a large family of kinases that most commonly mediate signalling pathways stimulated by growth factors. Members of this family share structural and biochemical properties including their mode of activation. Dual phosphorylation of the tripeptide motif (Thr-X-Tyr) located in the kinase activation loop (T-loop) of the kinase domain of MAPKs results in their activation. Although in many of the MAPKs signalling implicates a three-tier cascade that comprises of a MAPK, a MAPK kinase (MAPKK or MAP2K), and a MAPK kinase kinase (MAPKKK or MAP3K), there are other MAPKs that do not follow this rule. In humans, MAPK family members play critical roles in converting extracellular signals to cellular responses including cell growth, migration, proliferation, differentiation and apoptosis.

The *Toxoplasma* genome encodes three MAPK enzymes, namely TgMAPK-L1 (TgMAPK1), TgMAPK2 and TgERK7 (Peixoto et al., 2010; Miranda-Saavedra et al., 2012). TgMAPK-L1 contains a MAPK-like kinase domain that is found only in coccidian parasites and localises to the centrosomal region in the parasite. Further, this kinase shows periodical expression in tachyzoites, with highest expression during the S phase and early mitosis, and is downregulated during budding and the G1 phase. Accordingly, TgMAPK-L1 plays an essential role in endodyogeny where it prevents overduplication of centrosomes and ensures preservation of the physical connection between the karyokinetic and cytokinetic centres (Brown et al., 2014; Suvorova et al., 2015).

TgMAPK2 is conserved in alveolates and localises to the cytoplasm in tachyzoites. The kinase shows temporal expression during the cell cycle with increasing transcript levels in the early

stages of cytokinesis and the G1 phase. This kinase plays an essential role during endodyogeny as loss of this gene results in arrest of the cell cycle before centrosome duplication, blockage of daughter cell budding and eventually parasite death (Hu et al., 2020).

In eukaryotes, ERK7 is quite conserved and studies have suggested that this kinase is associated with ciliogenesis both in invertebrate and vertebrate species. In *Toxoplasma*, TgERK7 localises to the apical region of the parasite, more specifically at the basal part of the conoid, and plays an essential role in conoid biogenesis (O'Shaughnessy et al., 2020). In parasites deficient in TgERK7, although the apical complex biogenesis starts, conoid maturation is completely impaired in daughter parasites. Accordingly, conoid-less TgERK7-deficient *Toxoplasma* fails to invade or egress from host cells. Hence, although TgERK7 is not a component of the conoid structure, this kinase plays a key role in regulating the maturation of the conoid in *Toxoplasma* parasites (O'Shaughnessy et al., 2020).

3.5.3. Never in mitosis-A (NIMA)-related kinases (Nek or NRK)

NIMA-related protein kinases are serine/threonine kinases that are widely conserved across eukaryotes. This family of kinases has been demonstrated to play essential roles in cell cycle regulation including chromosome condensation, mitotic entry, centrosome duplication and mitotic exit. Nek kinases are also quite abundant in organisms with ciliated cells, where their function has been associated with assembly and disassembly of cilia in coordination with the cell cycle (Miranda-Saavedra et al., 2012).

The *Toxoplasma* genome contains seven genes that code for NIMA kinases and of these *Tgnek1* is the only one that has been characterised (Chen and Gubbels, 2013). TgNEK1 shows temporal expression during the cell cycle and is seen only in intracellular parasites. This kinase localises to the proximal end of the centrosome of the parasite and plays an essential role during endodyogeny. In parasites deficient in TgNEK1, although there is duplication of the centrosome, the duplicated centrosomes fail to separate and consequently budding of two daughter cells does not occur. Unlike its orthologues in higher eukaryotes, TgNEK1 does not have a coiled-coil domain that is required for dimerization. However, TgNEK1 is catalytically active and its kinase activity is important for its correct localization and function (Chen and Gubbels, 2013).

3.5.4. TKL group

The *Toxoplasma* genome contains eight TKL kinase family members, of which six have been suggested to be important for parasite growth in vitro based on a genome-wide CRISPR screen (Peixoto et al., 2010; Sidik et al., 2016). The six TKL kinases that have been predicted to be essential or important for tachyzoite propagation and localise to distinct compartments in the parasite including the nucleus (TgTKL1 and TgTKL2), cytoplasm (TgTKL3 and TgTKL4), IMC (TgTKL5) and Golgi (TgTKL6). Of these TgTKL1 is an Enhanced Drug Resistance (EDR) family kinase that is restricted to plants and protozoa (Varberg et al., 2018). TgTKL1 localises to the parasite nucleus and appears to mediate a signalling pathway that regulates expression of invasion and virulence genes in the parasite. Accordingly, in parasites that are deficient in this plant-like kinase, many key invasion genes including TgSUB1 are down-regulated. Since TgSUB1 has been shown to play a role in post-exocytosis surface processing of microneme proteins to activate binding to host cell receptors, decreased expression of this protease impairs host cell attachment during the invasion process. In addition, TgTKL1 null mutants are completely avirulent in the mouse model, thus suggesting that the repertoire of genes that is down-regulated includes key virulence factors. Furthermore, vaccination with TgTKL1 knockout parasites generates complete protective immunity in mice, suggesting that TgTKL1 null mutants could be potentially used as a live attenuated vaccine (Varberg et al., 2018).

3.5.5. Apicomplexa-specific kinase groups (FIKK kinases and rhoptry kinases)

3.5.5.1. FIKK kinases. The FIKK kinases are a set of kinases that are specific only to apicomplexans (Ward et al., 2004). These proteins were named as FIKK kinases as they contain a four amino acid (Phe-Ile-Lys-Lys) motif in subdomain II of their kinase catalytic domain (Ward et al., 2004). Although the *Toxoplasma* genome encodes a single FIKK gene, this family of proteins is greatly expanded in the related apicomplexan parasite, *Plasmodium,* that contains 21 members (Ward et al., 2004). The single FIKK gene that is encoded by *Toxoplasma* localises to the posterior end of the tachyzoite. However, deletion of this kinase does not affect tachyzoite growth in vitro, suggesting that this kinase is dispensable for the lytic cycle of the parasite (Skariah et al., 2016).

3.5.5.2. Rhoptry kinases (ROPKs). Rhoptry kinases (ROPKs) are kinases restricted only to tissue coccidians in the Apicomplexa that includes *Toxoplasma*, *Neospora*, *Eimeria* and *Sarcocystis*. It is interesting to note that, similar to multiple members of the FIKK family in *Plasmodium*, the ROPK family is greatly expanded in tissue coccidians (Talevich et al., 2012). In *Toxoplasma*, ROPK family members vary in number across different strains with 37 genes in Type I (GT1), 55 in Type II (ME49) and 38 in Type III (VEG) (Peixoto et al., 2010; Talevich et al., 2012).

A systematic analysis based on differences in ePK-conserved residues surrounding the ATP binding pocket classified the ROPK members into three groups: active kinases, pseudokinases, and predicted kinases that may be active, but with a non-canonical catalytic mechanism. The list of catalytically active ROPKs includes 21 members (ROP11, ROP16, ROP17, ROP18, ROP19/29/38, ROP20, ROP21/27, ROP25, ROP28, ROP30, ROP31, ROP32, ROP35, ROP39, ROP41, ROP33, ROP34 and ROP46). The kinases that lack one or more conserved residues that are essential for catalytic transfer of the phosphoryl group are considered non-catalytic kinases or pseudokinases and ROPKs that are pseudokinases include a total of 17 members (ROP2/8, ROP4/7, ROP5, ROP22, ROP23, ROP26, ROP36, ROP37, ROP40, ROP42/43/44, ROP47, ROP49, ROP50). Finally, the non-canonical subgroup of ROPKs has most of the residues necessary for catalysis, but shows differences in other typically conserved residues, suggesting an unknown catalytic mechanism and it includes three members (ROP24, ROP45 and ROP48) (Talevich et al., 2012).

Structural analysis of the catalytic domain of ROP2 indeed confirmed much of the bioinformatic prediction that it is a pseudokinase (Labesse et al., 2009). The crystal structure of ROP2 revealed that although the ROP2 kinase domain adopts an open conformation, it cannot bind ATP because nucleotide entry is prevented by residues that line the ATP binding pocket. Although in catalytically active ROPKs (such as ROP11, 16, 17 and 18) the residues responsible for binding are similar to that seen in canonical kinases, in pseudokinase ROPKs (including ROP2, 4, 5, 7, and 8) the amino acids are larger with lengthy side chains that do not allow ATP binding. However, despite lacking the ability to bind ATP, the peptide binding site is well preserved in catalytically inactive ROPKs, suggesting that these kinases are able to interact with other proteins.

A few ROPK family kinases have been characterised and were shown to play key roles in *Toxoplasma* pathogenesis including ROP5, ROP16, ROP17 and ROP18. Of these ROP5, 16 and 18 were discovered by a monumental effort that combined both forward and reverse genetics tools (Saeij et al., 2006, 2007; Taylor et al., 2006). Of these three proteins, ROP16 and ROP18 are both active

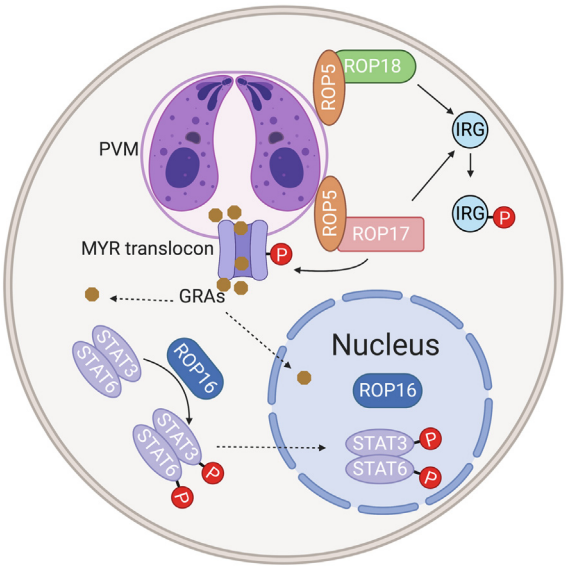

Fig. 8. Rhoptry proteins (ROP), ROP5, ROP16, ROP17 and ROP18 signalling in *Toxoplasma*. ROP proteins are injected by the parasite into the host-cell during invasion and of these ROP5, ROP17 and ROP18 accumulate on the parasitophorous vacuolar membrane (PVM) and maintain its integrity. ROP16 traffics to the host cell nucleus and alters host gene expression. IRG, immunity-related GTPase; STAT, signal transducer and activator of transcription; GRA, dense granule protein.

kinases, whereas ROP5 is catalytically inactive (Reese et al., 2011; Behnke et al., 2012).

ROP16 is a tyrosine kinase that localises to the bulb region of the rhoptry organelle and is injected into the host cell by the parasite during invasion. Within minutes after injection into the host cell, ROP16 traffics to the host cell nucleus and activates host transcription factors STAT3 and STAT6 by phosphorylating residues Tyr705 and Tyr641, respectively (Fig. 8). Activation of STAT3/6 by ROP16 attenuates the induction of IL-12, which in turn suppresses the protective TH1 cytokine response. Hence the ROP16 mediated pathway is responsible for decreased inflammation and reduced pathology, but at the same time ensures enhanced parasite survival (Saeij et al., 2006, 2007; Boothroyd, 2013).

ROP18 and ROP5 also localise to the rhoptry bulb region in the parasite, similar to ROP16. However, ROP18 is an active serine/threonine kinase, whereas ROP5 is a pseudokinase (Behnke et al., 2012). Similar to other rhoptry proteins, both ROP5 and ROP18 are also injected by the parasite into the host cell during invasion and within the host cell these kinases decorate the newly formed PVM. ROP5 and ROP18 physically interact with one another to form a complex in which ROP18 is the active kinase partner. In mice, Immunity Related GTPases (IRGs) are immune effector proteins that can disrupt the PVM and hence can eliminate *Toxoplasma* from the host cell. However, in an IFN-γ activated host cell, ROP18 in complex with ROP5 phosphorylates IRGs and inhibits recruitment of IRGs to the PVM, thus limiting damage to the PVM and ensuring parasite survival (Fig. 8) (Fentress et al., 2010; Hunter and Sibley, 2012; Behnke et al., 2016).

In addition to ROP18, ROP5 also interacts with another rhoptry kinase, ROP17, a serine/threonine kinase that appears to have multiple roles in *Toxoplasma* pathogenesis (Etheridge et al., 2014). After injection into the host cell by the parasite, ROP17 accumulates on the external face of the PVM and is in complex with ROP5. Unlike ROP18 whose kinase activity is regulated by binding to ROP5, ROP17 catalytic activity is independent of interaction with ROP5. Interestingly, deletion of ROP18 or ROP17 individually results only in partial reduction of acute virulence in the mice, whereas parasites with simultaneous deletion of these two genes are completely avirulent. Hence ROP18 and ROP17 appear to act in a synergistic manner with regard to inactivation of IRGs in an IFN-γ activated cell (Fig. 8). Further, biochemical analysis of ROP17 kinase activity revealed that phosphorylation of IRG oligomers results in their breakdown into monomers, thus suggesting that ROP17 is involved in disassembly of IRGs and thus enhancing parasite survival (Etheridge et al., 2014).

Besides its role in inactivating host immune effectors, ROP17 also is involved in regulating motility of infected immune cells of the host animal. More specifically, ROP17 is required for tissue migration of *Toxoplasma*-infected monocytes and macrophages. In infected monocytes, ROP17 acts upstream of host Rho-associated protein kinase (ROCK) signalling to promote the rapid spread of infected cells within tissues, thus promoting systemic parasite dissemination (Drewry et al., 2019).

Very surprisingly, together with roles in inactivation of IRGs and influencing tissue migration, ROP17 is also required for translocation of dense granule proteins across the PVM in the parasite-infected host cell (Fig. 8). Dense granule proteins (GRAs) are secreted by the parasite into the PV space after invasion and these proteins have three possible destinations. A few GRAs assist in formation of the intravacuolar network of the PV, others can associate with the cytoplasmic face of the PV or even integrate into the PVM. A third set of GRAs, however, translocate across the PVM to access the host cytosol or the nucleus where they can affect host signalling pathways or regulate host gene transcription. Interestingly, ROP17 kinase activity is essential for translocation of GRA proteins across the PVM through a translocon complex consisting of Myc-regulated (MYR) proteins, MYR1 and MYR3 (Panas et al., 2019).

In addition to playing important and essential roles in acute toxoplasmosis, ROPK family proteins play critical roles in establishing chronic infection. A recent study generated null mutants of 34 members of the ROPK family (ROP2/8, ROP 4/7, ROP5, ROP11, ROP16, ROP17, ROP18, ROP20, ROP21, ROP22, ROP23, ROP24, ROP25, ROP26, ROP27, ROP28, ROP30, ROP31, ROP32, ROP35, ROP36, ROP37, ROP38/29/19, ROP39, ROP40, ROP41, ROP42/43/44, and ROP45) in the Type II strain and examined cyst formation in the mouse brain. This study revealed that deletion of ROP5, ROP17, ROP18, ROP35, or ROP38/29/19 (ROP38, ROP29, and ROP19) severely reduced cyst burdens, thus suggesting that these kinases are required to establish chronic infection in the mouse model. Interestingly, in the Type II strain, deletion of ROP5 and ROP18 resulted in only approximately 10% reduction in parasite survival in IFN-γ-stimulated host cells, hinting that non-IRG functions of ROP5 and ROP18 complexes could influence establishment and maintenance of chronic infection (Fox et al., 2016).

3.5.6. With-No-Gly-loop (WNG) kinases

A set of kinases that have been recently discovered and are specific to coccidians are WNG kinases (Beraki et al., 2019). These kinases are so named because they lack the conserved glycine rich loop in the N-lobe (Fig. 2A) of canonical kinases that plays a critical role in positioning of the ATP molecule during catalysis. In *Toxoplasma*, the WNG kinases are secreted into the PV and a few of these were previously annotated as ROPK family members as they were predicted to be secreted by the parasite. Members of WNG family in *Toxoplasma* include WNG1 (previously ROP35), WNG2 (previously ROP34) and the pseudokinase TgBPK1 that was previously identified as a cyst wall protein in bradyzoites (Beraki et al., 2019). Epitope tagging and florescence microscopy revealed that WNG kinases localise to dense granules and are secreted into the PV. Crystallisation and protein structure analysis of TgBPK1 indicated that these kinases contain a modified architecture with a Gly-loop and the first β-strand that stabilises it (β1 in PKA

nomenclature) being replaced by a helical extension that packs against the top of the N-lobe of the kinase. Of the WNG family, WNG1 is an active kinase that phosphorylates at least 10 PV-resident dense granule proteins including GRA2, GRA3, GRA5, GRA6, GRA7, GRA8, GRA37, GRA47, GRA48 and GRA49. Importantly, phosphorylation of these PV-resident proteins by WNG1 assists these substrate proteins to associate with the PV membrane that is key for the formation and stability of the intravacuolar network (Beraki et al., 2019).

4. Concluding remarks

Significant progress has been made in the last two decades in understanding the role of kinases in the *Toxoplasma* life cycle. However, research on the *Toxoplasma* kinome is still in its relative infancy and many gaps remain unfilled. Ideally, the pipeline of dissecting the parasite kinome involves several steps: (i) identify genes important and/or essential for pathogenesis; (ii) determine the localization, function, and regulatory mechanism; (iii) identify the interactome, mechanism of action, and the signalling pathway within which each kinase participates; (iv) identify and/or devise specific inhibitors that could be developed as therapeutics (Solyakov et al., 2011). In recent years, techniques such as a CRISPR-based genome-wide screen have been successfully used to identify genes that are important for *Toxoplasma* propagation in vitro (Sidik et al., 2016). This identification technique helps researchers refine and probe parasite kinases that are critical for the lytic cycle. However, it is quite likely that many of the genes that are not essential for parasite propagation in vitro could prove to be crucial for pathogenesis in vivo. Therefore, it is important to identify genes that are important for acute and chronic pathology in vivo. This identification may not be as straightforward as in vitro screens because one has to take into consideration many factors including transfection efficiency and bottlenecks within infected mice. For in vivo screens, it may be ideal to use selective and focused CRISPR libraries, as recently performed in several studies (Sangaré et al., 2019; Young et al., 2019). *Toxoplasma* is a genetically tractable organism, with well-developed tools available including forward and reverse genetics. Further, in recent years the development of new conditional knockdown techniques (e.g., auxin-inducible degron or AID) to determine the roles of essential genes have greatly aided in understanding the role of kinases in the parasite life cycle (Brown et al., 2018). The structural characterization of several *Toxoplasma* kinases, some in apo or substrate/inhibitor-bound morphologies, has revealed mechanisms of activation that are quite distinct from their metazoan counterparts (Wernimont et al., 2010). It is critical for further work to be performed in structural elucidation, characterization and modelling of parasite kinases for which there is no data currently available, since several such kinases represent viable drug targets. More importantly, it will also be key to define the role of additional domains of parasite kinases and understand how they contribute to and regulate the function of the catalytic domain.

Many of the kinases in apicomplexan parasites have insertions and deletions within the catalytic domain. Subsequent studies aimed at a complete and thorough characterization of parasite kinases are necessary. Identification of kinase substrates is a challenging task given the transient nature of substrate-kinase interactions and the influence of specific parasitic life cycle stages on kinase function. Utilisation of techniques such as quantitative phosphoproteomics, thio-phosphorylation. and proximity-based labelling have yielded fruitful results and look quite promising (Lourido et al., 2013a; Gaji et al., 2015; Beraki et al., 2019). The identification of substrates of parasite kinases and signalling pathway occurrence is important for advanced targeting and combination drug strategies. Targeting multiple proteins critical for the same signalling pathway can aid in overcoming potential issues of drug resistance in the future.

The phylogenetic distance of *Toxoplasma* from humans and animals is indeed a boon, as many of the parasite kinases are divergent. This approach and thorough investigation of PKs in *Toxoplasma* can aid in developing selective and specific inhibitors of parasite kinases that could be developed as anti-*Toxoplasma* drugs. Indeed, approaches in designing small molecule inhibitors based on the knowledge of kinase structure have been adapted for a few kinases, specifically CDPKs. As *Toxoplasma* possesses close to 150 kinases, the kinome offers a great mining opportunity for developing new antiparasitic compounds. Collectively, *Toxoplasma* research in the next decade should be an exciting ride for kinase researchers.

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