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Some apicomplexan parasites have evolved distinct protein kinase families to modulate host cell structure and function. *Toxoplasma gondii* rhoptry protein kinases and pseudokinases (ROPKs) are involved in virulence and modulation of host cell signaling. The proteome of *Plasmodium falciparum* contains a family of putative kinases called FIKKs, some of which are exported to the host red blood cell and might play a role in erythrocyte remodeling. In this review we will discuss kinases known to be critical for host cell invasion, intracellular growth and egress, focusing on (i) calcium-dependent protein kinases (CDPKs) and (ii) the secreted kinases that are unique to *Toxoplasma* (ROPKs) and *Plasmodium* (FIKKs).

**Keywords**

Protein kinase, *Toxoplasma, Plasmodium*, Rhoptry, Calcium-dependent protein kinase, malaria, red blood cell
Introduction

Protein kinases mediate the transfer of phosphate groups from ATP to specific residues on their target proteins, resulting in changes in the activity, stability, interactions with ligands or localization of the phosphorylated substrates. Kinases themselves are often similarly regulated, and many of them function as signaling mediators that integrate upstream signals in the form of second messengers, post translational modifications or binding of regulatory proteins. It is therefore not surprising that many parasites have evolved distinct protein kinase families with novel domain structures and biochemical features to regulate key parasite-specific physiological processes that must be executed in a timely fashion during development or in response to external stimuli and physiological cues from the host. As signaling mediators, protein kinases are also well suited to function at the host-parasite interface, where they can perturb host signaling pathways, activate dormant mechanisms in the host cell, or disrupt the proper functioning of host proteins.

Both *Toxoplasma* and *Plasmodium* contain a family of calcium-dependent protein kinases (CDPKs), whose occurrence is restricted to plants and Alveolates (the superphylum that comprises Ciliates and Apicomplexa), although trypanosomatids also possess kinases with an EF-hand calcium binding domain that are thought to be phylogenetically distinct form plant and Alveolate CDPKs. CDPKs have a domain structure consisting of a calcium-binding domain fused to the kinase domain, such that kinase activity is stimulated upon calcium binding. Studies of apicomplexan CDPKs have revealed a conserved regulatory mechanism and highlighted the importance of calcium in regulating a number of key physiological processes including host cell attachment and invasion, gliding motility, and parasite egress.
Toxoplasma gondii, a highly prevalent obligate intracellular protozoan parasite, seems to rely for a large part on protein kinases and pseudo kinases to modify the host cell. Toxoplasma is capable of infecting all nucleated cells of most warm-blooded animals. This ability to establish a chronic infection in such a wide range of host species and cell types is likely associated with its ability to modify many aspects of the host cell’s normal physiology. It does this by secreting proteins from the rhoptry, a specialized secretory organelle that is found only in apicomplexan parasites, directly into the host cytosol where they can exert their function. Many rhoptry proteins have homology to kinases and a large proportion of them are predicted to be pseudokinases.

Malaria parasites (genus Plasmodium) also belong to the Apicomplexa, however, unlike Toxoplasma, they have a very restricted range of host cells which they can invade and in which they can replicate. After inoculation into the vertebrate host through the bite of an infected mosquito, Plasmodium sporozoites must first invade hepatocytes, where they undergo asexual proliferation (exo-erythrocytic schizogony), generating several thousand merozoites in the process. This strict dependence on hepatocytes for the pre-erythrocytic stage of infection has, however, recently been challenged by in vivo observations of P. berghei parasites developing in dermal and epidermal cells at the site of inoculation and generating infective merozoites (Gueirard et al., 2010).

The only cell type permissive for invasion by Plasmodium merozoites is the red blood cell (RBC). Invasion involves release of the contents of rhoptries and micronemes (another type of specialized secretory organelle) and formation of a parasitophorous vacuole. The parasite causes considerable modifications to its host RBC that include the establishment of a complex
trafficking system that mediates translocation of parasite-encoded proteins to the RBC membrane skeleton and surface (Cooke et al., 2004).

In the case of *P. falciparum*, the most lethal of the five species of malaria parasites that can infect humans, such proteins include components of the so-called “knob” structures, through which parasite-infected RBCs adhere to vascular endothelial cells, thereby significantly contributing to pathogenesis (Cooke et al., 2001; Cooke, B.M., N. Mohandas, and R.L. Coppel, Malaria and the red blood cell membrane. Semin Hematol, 2004. 41(2): p. 173-88; Maier, A.G., et al., Malaria parasite proteins that remodel the host erythrocyte. Nat Rev Microbiol, 2009. 7(5): p. 341-54.).

There are no orthologues of the *Toxoplasma* rhoptry kinases in malaria parasites, however, the *P. falciparum* kinome includes a family (20 members) of related kinase-like sequences called FIKKs, due to the presence of a Phe-Ile-Lys-Lys motif they share in their kinase domain (Ward et al., 2004). Most notably, 18 of the 20 fikk genes in *P. falciparum* are predicted to encode fully functional kinases, 16 of which are predicted to be exported into the host RBC (Schneider and Mercereau-Puijalon, 2005), and at least some of these enzymes have been shown to be exported to the host RBC and to be associated with kinase activity (Nunes et al., 2007; Nunes et al., 2010).

Many of the *Toxoplasma* and *Plasmodium* kinases are related to kinases with known functions in other eukaryotes and these will not be discussed in this review. Instead, we will discuss in more detail, kinases from these parasites that are known to be critical for host cell invasion, intracellular growth and parasite egress, focusing on (i) calcium-dependent protein kinases (CDPKs) and (ii) the secreted kinases that are unique to *Toxoplasma* (ROPKs) and *Plasmodium* (FIKKs).

**Calcium-dependent regulation of invasion and egress:**
Calcium levels in *T. gondii* play key roles in regulating micronemal secretion-dependent processes including host cell invasion, gliding motility and parasite egress {Nagamune, 2008 #13506}. Chelation of parasite intracellular calcium strongly inhibited both microneme release and invasion of host cells, and this effect was partially reversed by raising intracellular calcium using the ionophore A23187 (Carruthers et al., 1999). Additionally, evidence was also provided in this study for the requirement of a staurosporine-sensitive kinase activity in regulating microneme discharge and for parasite invasion of host cells.

In *Toxoplasma* parasites, calcium-dependent protein kinases (CDPKs) were shown to be involved in host cell invasion through the use of KT5926, an inhibitor of CDPKs in other systems (Kieschnick et al., 2001). KT5926 blocks the motility of *Toxoplasma* tachyzoites and their attachment to host cells. *In vivo*, the phosphorylation of only three parasite proteins was found to be blocked by KT5926 (although there may be more), and in parasite extracts, a single KT5926-sensitive protein kinase activity was detected. This activity was calcium-dependent but did not require calmodulin. In a search for CDPKs in *Toxoplasma*, two members of the class of calmodulin-like domain protein kinases (CDPKs) were detected. TgCDPK2 was only expressed at the mRNA level in tachyzoites, with no detectable protein. TgCDPK1 protein present in *Toxoplasma* tachyzoites cofractionated precisely with the peak of KT5926-sensitive protein kinase activity. TgCDPK1 kinase activity was calcium-dependent but did not require calmodulin or phospholipids. TgCDPK1 was found to be inhibited effectively by KT5926 at concentrations that block parasite attachment to host cells. *In vitro*, TgCDPK1 phosphorylated three parasite proteins that migrated identical to the three KT5926-sensitive phosphoproteins detected *in vivo*. These observations suggested a central role for TgCDPK1 in regulating *Toxoplasma* motility and host cell invasion.
Subsequent chemical genetic and conditional knockout studies have shown that TgCDPK1 is the key transducer downstream of calcium fluxes in regulating these processes. Down-regulation of TgCDPK1 expression severely impaired the secretion of micronemal proteins, including adhesins needed for gliding motility (Lourido et al., 2010). The resulting defect in gliding motility led to a significant decrease in host cell attachment and invasion. Conditional knockout of TgCDPK1 also impaired parasite egress from host cells upon induction with a calcium ionophore, with immotile parasites remaining within vacuoles. This coincided with a defect in PVM permeabilization upon calcium ionophore treatment, which likely resulted from impaired microneme secretion of the perforin-like TgPLP1 protein (Lourido et al., 2010).

The canonical CDPK domain structure consists of an N-terminal protein kinase domain, that is highly homologous to calmodulin-dependent kinases (CaMKs), and a C-terminal CDPK activation domain (CAD) with four calcium-binding EF-hands (EF1 to EF4). A recent biochemical and structural study of three apicomplexan CDPKs (TgCDPK1 and TgCDPK3 from *T. gondii*, and CpCDPK1 from *Cryptosporidium parvum*) has provided novel insights into how these CDPKs are activated upon Ca$^{2+}$ binding by the CAD (Wernimont et al., 2010). In *in vitro* assays, the kinase domain was activated by low micromolar Ca$^{2+}$ concentrations. Calcium ‘in-out’ experiments suggested a partial irreversibility of this calcium-dependent activation for TgCDPK1 and TgCDPK3, while CpCDPK1 activity was reduced to basal level upon calcium removal. Actual mapping of potential auto-phosphorylation sites may provide further insights into the reversibility of calcium-induced activation of these CDPKs.

Crystal structures have been solved for auto-inhibited (calcium-free) TgCDPK1 and TgCDPK3 and for calcium-bound TgCDPK1 and CpCDPK1 (Wernimont et al.). The kinase domain adopts the bi-lobal structure characteristic of eukaryotic protein kinases, with a smaller
N-terminal lobe (consisting mainly of β-strands) and a larger C-terminal lobe (predominantly α-helical) connected together via a single stretch of polypeptide (hinge region). A deep cleft formed between the two lobes contains the ATP binding pocket and active site (Dar, 2005). In the calcium-free state, the CAD adopts an overall dumbbell shape (resembling calmodulin in the absence of calcium) with two EF-hands at each end of the dumbbell. The CAD packs against the front face of the kinase domain, making contacts with both the N- and C-terminal lobes. The N-terminal helix of EF1 extends into a long helix (CH1) that spans the length of the CAD and packs anti-parallel along a second long helix (CH2). The N-terminus of CH1 connects directly with the C-terminus of the kinase domain and packs against the kinase C-terminal lobe to block substrate binding, analogous to the pseudo-substrate segment of CAMKII (Rosenberg et al., 2005) (Figure 1). Additional hydrophobic contacts with the N- and C-terminal lobes of the kinase domain are made by EF2 and EF3, respectively. Calcium binding to all four EF hands within the CAD causes conformational changes that expose hydrophobic surfaces and partial unwinding and bending of the CH1 and CH2 helices. This leads to a dramatic rearrangement of the CAD into a more compact structure. The calcium-bound CAD is also repositioned and binds to the back face of the kinase domain, which induces a widening of the active site cleft by altering the relative orientations of the two lobes of the kinase domain. Release of the auto-inhibitory interactions between the CAD and kinase domain also allows repositioning of the α-C helix and rearrangement of the P-loop and the activation loop regions into an active conformation as seen in other kinase structures.

Apicomplexan CDPKs have no orthologs in humans and are most closely related to CDPKs in plants. The essential functions of TgCDPK1 and the lack of orthologs in humans make it an ideal target for novel anti-Toxoplasma therapeutics. In particular, TgCDPK1 is
unique in having a glycine residue at the gatekeeper position, which is located at the rear of the adenine binding pocket. ATP-analogs with bulky substituents on the purine group can be accommodated by analog-sensitive kinases with small gatekeeper residues (with glycine being the smallest), while kinases with larger gatekeeper residues cannot accommodate these molecules (Bishop et al., 2000; Blethrow et al., 2004). Since few human kinases contain a small gatekeeper residue (no known mammalian protein kinase has glycine gatekeeper), TgCDPK1 provides a promising analog-sensitive target for bumped kinase inhibitors (BKIs) that should have relatively little off-target effects on the human kinome (Manning et al., 2002).

A number of BKIs have been developed for chemical genetic studies with engineered analog-sensitive kinase alleles in human cells. BKI analogs of 4-amino-1-tert-butylyl-3-phenylpyrazolo[3,4-d]pyrimidine, with bulky aromatic substituents at the C3 position, inhibit TgCDPK1 at sub-micromolar concentrations (Ojo et al., 2010; Sugi et al., 2010). Consistent with conditional TgCDPK1 knockout studies, BKIs greatly reduced host cell invasion by *T. gondii* and also modestly affected intracellular growth. Parasites expressing an analog-resistant TgCDPK1, in which the gatekeeper glycine is mutated to methionine (G128M) were significantly less sensitive to BKIs and thus validated TgCDPK1 as the main BKI target *in vivo*. Crystal structures of calcium-free TgCDPK1 in complex with NA-PP2 and NM-PP1 confirm the structural basis for the selectivity of these BKIs for a small gatekeeper residue and will facilitate optimization of these BKIs to further improve their affinity and specificity (Ojo et al., 2010). Of the 114 functional kinases predicted in the *T. gondii* genome, 12 are expected to be analog-sensitive based on their gatekeeper residue (Sugi et al., 2010). Thus, BKIs provide a promising approach to targeting key processes in *T. gondii* with potentially fewer side effects on the host.
That host cell invasion can be inhibited by staurosporine and involves calcium signaling has been known to also be true for malaria parasites for 2-3 decades (Scheibel et al., 1987; Ward et al., 1994). More recently, a role for a CDPK (PfCDPK1) in this process has been revealed (Green et al., 2008; Kato et al., 2008), highlighting a definite level of unity in the invasion strategies across Apicomplexa. In addition to invasion, malaria parasites exploit their repertoire of CDPKs to mediate several other developmental processes (see (Billker et al., 2009) for a comprehensive review). Briefly, these include male gametogenesis, shown in the rodent-infecting parasite *P. berghei* to be dependent on PbCDPK4 (Billker et al., 2004); gliding of ookinetes, the motile form that escapes the mosquito midgut during infection of the insect vector, a process blocked in *P. berghei* parasites lacking PbCDPK3 (Ishino et al., 2006); and switching of parasite behavior from cell traversal to cell invasion at the early stage of liver infection by *P. berghei* sporozoites, which requires PbCDPK6 (Coppi et al., 2007). There is unity across Apicomplexa not only with respect to the involvement of CDPKs in invasion, but also in the mechanism of activation of these enzymes by calcium (see above). A recent structural study of a *Plasmodium* CDPK (PfCDPK3) (Wernimont et al., 2011) revealed that the residues mediating the largest conformational change following calcium binding are the most conserved across Apicomplexa, leading the authors to propose that the mechanism described in the section above is conserved, presumably throughout the CDPK family. Another interesting observation described in this report is that although the CAD shares similar motifs and behavior with calmodulin, there are sufficient structural differences to consider the CDPK activation domain as a distinctive member of the large EF-hand family of calcium-binding proteins.

Given their key regulatory roles, malaria CDPKs also present attractive drug targets. However, in contrast to the TgCDPKs, the presence of non-glycine residues at the gatekeeper position in malaria CDPKs will preclude the use of BKIs. (maybe Christian can double check this?)
Host modification by secreted *Plasmodium* and *Toxoplasma* protein (pseudo)kinases

Unlike most other eukaryotic protein kinases, most members of the *Plasmodium* FIKK and *Toxoplasma* ROPK family have signal peptides and are predicted to be secreted into the host cell. A recent analysis of the completed genome sequence of the three most common strains of *Toxoplasma gondii* by Roos and colleagues found that the *Toxoplasma* kinome consists of 108 predicted active kinases and 51 predicted pseudokinases (Peixoto et al., 2010) (Figure 2). Compared to the human kinome, which only contains ~10% pseudokinases (Manning et al., 2002), there is a significant enrichment of pseudokinases in the *Toxoplasma* kinome. This is mainly due to an expansion of the *Toxoplasma*-specific ROPK family, consisting of ~44 genes of which about half are pseudokinases. It must be kept in mind in this context that some proteins thought to be pseudokinases on the basis of their sequence characteristics were actually shown to be competent for phosphorylation (Kannan and Taylor, 2008; Taylor and Kornev, 2010). This may be particularly relevant to sequences that are relatively distant from classical ePKs, such as the ROPKs and FIKKs (see below).

Members of both the ROPK and FIKK families are characterized by a domain structure consisting of an N-terminal signal peptide, a low complexity region, and a conserved C-terminal domain that adopts a canonical protein kinase fold. However, *Toxoplasma* ROP kinase domains constitute a phylogenetically-distinct group from other eukaryotic protein kinases, including other apicomplexan kinases such as the malaria FIKK family. Proteins of the ROPK family have roles in host cell modulation and virulence, although the molecular details of how most of these
proteins function are poorly understood. While some members of the ROPK family have been
shown to be active kinases (such as ROP16 (Yamamoto et al., 2009; Ong et al., 2010), and
ROP18 (El Hajj et al., 2007; Khan et al., 2009b)), others appear to be pseudokinases with
substitutions at key residues that abrogate catalytic activity (but see our comment above). The
inability of ROP2 to bind ATP has also been reported and may be true for other ROPK family
pseudokinases (Labesse et al., 2009).

Twelve sequence motifs or sub-domains are highly conserved in protein kinase domains
(Hanks et al., 1988). Most of these sub-domains are clustered within the active site cleft region
and are important for ATP binding and catalysis, while others are crucial for structural stability
of the protein. The crystal structures of the ROP2 (PDB accession codes 2W1Z and 3DZO) and
ROP8 (3BYV) pseudokinase domains revealed a number of structural features within their active
site region that are incompatible with catalysis and ATP binding (Labesse et al., 2009; Qiu et al.,
2009). Substitution of the VAIK motif with FEVH in both ROP2 and ROP8 results in loss of the
critical Lysine side chain that normally coordinates the α- and β-phosphates of ATP, however,
WNK is an example of a kinase that lacks this Lysine residue but is nevertheless active, through
recruiting another residue to substitute for the missing one (Xu et al., 2000). Replacement of the
DFG motif with GFE alters the magnesium coordination geometry, while substitution of the
HRD motif with HTY results in replacement of the catalytic base (Asparagine) with Tyrosine.

**Cellular destination of rhoptry kinases.**

Although it is now well established that upon invasion *Toxoplasma* secretes the contents of its
rhoptries directly into the host cytosol, it is still unclear how these secreted proteins cross the
host membrane. Patch clamp experiments have shown a short spike in conductivity at the time of
invasion, which is suggestive of a break in the host membrane followed by resealing (Suss-Toby et al., 1996). The *Toxoplasma* protein(s) that mediate this break remain unknown. Recently, two *Toxoplasma* proteins with a Membrane Attack Complex/Perforin (MACPF) domain were identified, one of which (TgPLP1) is necessary for egress, which involves rupture of the parasitophorous vacuolar membrane (PVM) (Kafsack et al., 2009). TgPLP2 could be a candidate for mediating the break in the host cell plasma membrane. Once rhoptry proteins are secreted into the host cell cytosol they traffic to distinct cellular destinations based on other signals. For example the rhoptry kinase ROP16 and the rhoptry protein phosphatase 2 C (PP2C-hn) carry a nuclear localization signal (NLS), which mediates their trafficking to the host nucleus (Gilbert et al., 2007; Saeij et al., 2007). Many ROPKs traffic back to the PVM and it was recently demonstrated that an arginine-rich amphipathic helix (RAH) domain is necessary and sufficient for targeting ROPKs to the PVM (Reese and Boothroyd, 2009). Transgenic expression of the RAH domain in host cells results in punctuate staining with some overlap with the nuclear envelope. However, after *Toxoplasma* infection of those cells, most of the staining relocated to the PVM (Reese and Boothroyd, 2009). The fact that the RAH domain only interacts weakly with a variety of host membranes but strongly with the PVM, which is derived from the host membrane, is puzzling. It has been suggested that the RAH domain has no special affinity for a particular lipid composition but rather for negative membrane curvature as the host nuclear envelope, which is negatively curved, associated somewhat with RAH domain (Reese and Boothroyd, 2009). Indeed, it is well known that *Toxoplasma* secretes proteins from its dense granules that are involved in the formation of a tubulovesicular network consisting of elongated negatively curved nanotubules of 60-90 nm in diameter, which are topologically cytosolic, and connect with the vacuole-delimiting membrane. Consistent with this, parasites without the dense
granule protein GRA2, which have an attenuated PVM tubular network, have attenuated recruitment of the RAH domain to the PVM (Reese and Boothroyd, 2009). Thus, an important function of dense granule proteins might be the formation of the extremely negatively curved tubular network, which subsequently functions to attract rhoptry proteins with a RAH domain from the cytosolic face of the PVM. Rhoptry proteins without a RAH domain or an NLS, such as Toxofilin stay in the host cell cytosol (Lodoen et al.).

**ROP16**

Although it has been known for some time that *Toxoplasma* ROPKs can be secreted into the host cytosol, their function has remained elusive. ROP2 was thought to mediate the recruitment of mitochondria to the PVM, but a recent study showed that a ROP2 knockout has no defect in mitochondrial recruitment (Pernas and Boothroyd, 2010). One of the first ROPKs for which a particular function was ascribed to was ROP16. Expression profiling of human foreskin fibroblasts (HFFs) infected with type I, II or III strain parasites demonstrated that these strains differ significantly in the modulation of host gene expression (Saeij et al., 2007). To identify the *Toxoplasma* genomic loci involved, expression profiles of HFFs infected with F1 progeny derived from crosses between type II and type III were determined. Quantitative trait locus (QTL) analysis, using host gene expression as the quantitative trait, determined that the genotype at a locus on *Toxoplasma* chromosome VIIb correlated with the strain-specific regulation of >1,000 human genes. Analyses for enrichment in functional annotation of these genes implicated the STAT signaling pathway. Indeed HFFs infected with type I or type III parasites have strong and sustained activation of STAT3 and STAT6 while the activation by type II is much weaker and only transient. A candidate gene approach identified ROP16 as the *Toxoplasma* gene
responsible for the strain-specific activation of STAT3 and STAT6. ROP16 is secreted into the
host cytosol upon infection and subsequently traffics to the host nucleus. This nuclear
translocation is dependent on an NLS in ROP16 but its nuclear localization was not important for
the activation of STAT3 and STAT6. Subsequently, these experiments were confirmed by other
groups using a type I strain where the \textit{ROP16} gene was removed by double homologous
recombination (Yamamoto et al., 2009; Ong et al., 2010). As expected, ROP16 kinase activity is
necessary for its effect on STAT3 and STAT6 (Yamamoto et al., 2009; Ong et al., 2010).
Initially it was predicted from sequence homology that ROP16 was a Serine/Threonine kinase
and was therefore unlikely to directly phosphorylate STAT3 and STAT6. However, it has
recently been shown that it can in fact directly phosphorylate these targets (Yamamoto et al.,
2009; Ong et al., 2010). One group showed that immunoprecipitated ROP16 can phosphorylate
Tyr705 of recombinant STAT3 and subsequent co-immunoprecipitation experiments determined
that the N-terminal region (amino acid 223-303) of ROP16 is required for this interaction with
STAT3. By constructing chimeras between the type I and type II ROP16 proteins, the difference
in STAT activation by these two alleles could be attributed to a single amino acid substitution; a
conversion of Serine to Leucine at position 503 made type II ROP16 also a potent activator of
STAT3.
Similarly, Boothroyd and colleagues showed that ROP16 has intrinsic tyrosine kinase activity as
determined by phosphoamino acid analysis of auto-phosphorylated ROP16. \textit{In vitro} kinase
assays with recombinant ROP16 and recombinant STAT6-GST as a substrate also showed
efficient phosphorylation of Tyr641 on STAT6 by wild-type but not K404N (kinase-dead)
recombinant ROP16. The pan-JAK inhibitor and the tyrosine kinase inhibitor K-252α severely
impaired the kinase activity of ROP16 providing further evidence that ROP16 is a tyrosine
kinase (Yamamoto et al., 2009; Ong et al., 2010). Thus, ROP16 can directly phosphorylate Tyr705 and Tyr641 from STAT3 and STAT6, respectively. This could explain why the up-regulation of the suppressor of cytokine signaling (SOCS) genes, which normally down-regulate the STAT pathway (Dalpke et al., 2008), do not down-regulate the STAT3/6 activation by ROP16 (Saeij et al., 2007). SOCS proteins bind to phosphorylated tyrosine residues on Janus kinases (JAKs) and/or cytokine receptor subunits through a central SH2 domain and subsequently mediate their degradation (Dalpke et al., 2008). Because the JAKs and cytokine receptors are bypassed by the direct phosphorylation of STAT3 and STAT6 by ROP16, the inhibitory function of SOCS proteins is abrogated. By directly phosphorylating STAT3 Toxoplasma could mimic the effects of IL-10, which also maintains constitutive activation of STAT3 because its receptor does not interact with the SOCS proteins (Yasukawa et al., 2003). IL-10 is a potent anti-inflammatory cytokine which can down-regulate the production of IFN-γ, the main mediator of resistance to Toxoplasma. The prediction would therefore be that ROP16 can enhance parasite virulence by suppressing IFN-γ production.

Indeed, ROP16 was also identified as one of the loci involved in the difference in virulence between type II and type III strains (Saeij et al., 2006). However, addition of ROP16 from type I or type III into a type II strain made it less virulent. Why type II+ROP16I/III parasites have reduced virulence is currently unknown. Although ROP16I/III can lower the level of secretion of IL-12 by macrophages infected with type II strains, which could lead to subsequent lower induction of IFN-γ secretion, one would predict that this would lead to an enhanced and not a reduced virulence phenotype.

A potential explanation for this apparent contradiction is emerging from our recent studies showing that macrophages infected with type I or type III strains are converted into an
alternatively activated or M2 macrophage (Jensen et al., 2011). These M2 macrophages are characterized by high level expression of arginase-I and several lectin receptors such as the mannose receptor and macrophage galactose specific lectin. Type II infected macrophages are converted into classically activated or M1 macrophages and secrete large amounts of proinflammatory cytokines such as IL-12p70 and IL-23. Activation of STAT6 by ROP16 is necessary to convert macrophages to the M2 phenotype, while the M1 phenotype is due to the fact that the type II strains, but not the type I and III strains, activate NFκB, a phenotype due to a new polymorphic dense granule protein GRA15 (Rosowski et al., 2011). Thus, it is possible that one of the roles of ROP16 is to limit the inflammation induced by the strong Th1 induction by *Toxoplasma* and the reduction of virulence of the II+ROP16I might be due to reduced immune pathology. Indeed, we have evidence that ROP16-mediated reduction of type II virulence is especially pronounced in Th1-prone mice such as C57/BL6 (Jensen et al., unpublished).

Alternatively, the high induction of the arginase enzyme by ROP16 could deplete L-arginine, for which *T. gondii* is auxotroph, and thereby inhibit *Toxoplasma* growth (Butcher et al., 2011).

**ROP18.**

ROP18 was identified as a rhoptry kinase involved in virulence by two different groups (Saeij et al., 2006; Taylor et al., 2006). Sibley and colleagues were interested in the genes involved in the difference in virulence between the highly virulent type I strain (LD100=1) and the avirulent type III strains (LD50 ~ 100,000). Boothroyd and colleagues used the difference in virulence between type II (LD50 ~ 500) and type III strains as the basis for their studies. Both groups used crosses between these strains to generate F1 progeny which were subsequently injected in mice to determine their virulence phenotypes. Previously a genetic map was generated for *Toxoplasma*
(Khan et al., 2005) and this was used to determine which Toxoplasma loci correlated with virulence. Interestingly, the only locus identified using the IxIII cross F1 progeny was ROP18 and indeed a type III strain expressing type I ROP18 (III+ROP18I) was as virulent as a wild-type type I strain (Taylor et al., 2006). ROP18 kinase activity was necessary to confer virulence as the type III expressing the type I kinase dead mutant was avirulent. The difference in virulence between type II and type III strains was more complicated and implicated five different loci, one of which was ROP18. Indeed a type III strain expressing type II ROP18 (III+ROP18II) became extremely virulent (LD$_{50}$~5) (Saeij et al., 2006). The expression level of ROP18 seems to be a key determinant in the strain-specific differences in virulence. Type III strains have an extremely low level of ROP18 expression probably due to an extra 2.1 kb sequence 85 bp upstream of the ATG start codon while type I and II strains express ROP18 at a high level (Saeij et al., 2006). Although both studies demonstrated that ROP18 is secreted into the host cytosol and subsequently traffics back to the PVM, its substrate(s) remained unidentified. When ROP18 is over-expressed in host cells it also traffics to the PVM of invading parasites, showing that it has a strong affinity for the PVM (El Hajj et al., 2007; Reese and Boothroyd, 2009). Like many rhoptry proteins, ROP18 is proteolytically processed. It is initially present as a 60 kDa protein but subsequently is processed to a 56 kDa protein. Indeed it contains the conserved S-Phe-X-E consensus motif (Phe represents bulky hydrophobic residues and X is any amino acid) for recognition by TgSUB2, a subtilisin-like serine proteinase (El Hajj et al., 2007). When comparing the ROP18 genes from multiple strains it was noted that it is one of most polymorphic proteins with evidence for strong diversifying selection. For example, the sequences of type I and type II ROP18 are very different, with almost no synonymous changes between the type I and type II alleles. Currently there is no evidence for a different function of the different ROP18
alleles, because quite diverse ROP18 alleles from several strains all confer virulence to type III parasites when expressed in this avirulent strain (Khan et al., 2009b). It is also unknown if over-expression of the type III ROP18 itself would also confer virulence. Comparison of the ROP18 genomic region between *Toxoplasma* and *Neospora caninum*, a close relative of *Toxoplasma gondii*, demonstrated that *Neospora* also has the extra region in the promoter of its ROP18. Thus, the most parsimonious explanation is that the ancestral ROP18 gene contained the extra sequence in its promoter resulting in low level expression and subsequently this region was deleted to give rise to the more recently derived type I and type II ROP18 alleles (Khan et al., 2009b). It was also hypothesized that strains carrying the type I ROP18 allele might be more successful because more strains have a ROP18 allele similar to the type I strain; however this could also be due to other strains being more similar to type I strains compared to type II strains.

**Proposed regulatory mechanism of ROP18.**

Protein kinases display a diverse array of regulatory mechanisms that exploit multiple ways of impeding or distorting the binding of ATP, magnesium or protein substrates to prevent catalysis. This often involves a distortion of the inter-lobe orientation, conformational change of the activation loop to block ATP and/or substrate binding, or occlusion of the active site. Activation of the kinase domain often involves phosphorylation (particularly of the activation loop), but may also involve other mechanisms such as binding or dissociation of regulatory domains or subunits (Huse and Kuriyan, 2002; Dar, 2005).

The ROP2 and ROP8 kinase domain crystal structures revealed a unique N-terminal subdomain that is conserved in other ROPK family kinase domains and contains features that preclude ATP binding in two of the ROPK pseudokinase structures determined. This ~40 residue N-terminal
extension consists of an α-helical segment (packed against the C-terminal lobe) connected via a short linker region to an α-helix and β-strand packed against the N-terminal lobe. In both 3DZO and 3BYV, an arginine side chain from the N-terminal subdomain linker region (Arg 219 in 3DZO and Arg 228 in 3BYV) forms a salt bridge interaction with the side chain of Glu 284 in 3DZO and Glu 293 in 3BYV. The glutamate and arginine side chains occupy the expected position of the ATP adenine group. Based on a homology model of the ROP18 kinase domain (with the ROP8 crystal structure as a template), a novel kinase regulatory mechanism was proposed for ROP18, in which the N-terminal subdomain was predicted to impede ATP-binding (as observed in 3DZO and 3BYV). Furthermore, phosphorylation at sites within and near the ROP18 N-terminal subdomain was proposed to regulate this auto-inhibition (Qiu et al., 2009).

Similar to other active protein kinases and in contrast to the ROP2 and ROP8 structures, the modeled active site of ROP18 was free of bulky side chains that would preclude ATP binding, with the exception of Gln 214 and Gln 216. The side chains of both residues were predicted to protrude into the ATP binding pocket from the N-terminal subdomain linker region (analogous to Arg 219 in 3DZO and Arg 228 in 3BYV). Consistent with the homology model, mutation of Gln 214 and Gln 216 to Ala resulted in a three to four fold increase in in vitro auto-phosphorylation and phosphorylation of myelin basic protein by recombinant ROP18 kinase domain. Activation was then suggested to involve a displacement or conformational change of the N-terminal subdomain to move the side chains of Gln 214 and Gln 216 out of the ATP binding pocket. ROP18 kinase domain expressed in Escherichia coli was auto-phosphorylated on Ser 221 and Thr 229 (second helix of the N-terminal subdomain), and on Thr 249 and Thr 251 (in contact with the N-terminal subdomain β-strand). Mutation of these Ser and Thr residues to Ala, reduced in vitro kinase activity by up to 90% (Qiu et al., 2009). It was therefore
proposed that phosphorylation of one or more of these Ser/Thr residues activated ROP18 by altering the interaction of the N-terminal subdomain with the N-terminal lobe to shift the linker region containing Gln 214 and Gln 216 away from the ATP binding pocket. However, several aspects of this proposed auto-inhibitory mechanism require further investigation. Comparison of the available crystal structures, suggests that loss of the salt bridge interaction between the glutamate side chain within the adenine binding pocket (Glu 284 in 3DZO and Glu 293 in 3BYV) and the arginine side chain on the N-terminal subdomain linker (Arg 219 in 3DZO and Arg 228 in 3BYV) displaces the N-terminal subdomain linker away from the active site as can be seen in 2W1Z. Since this salt bridge interaction is also not conserved in ROP18, it is unclear if the N-terminal subdomain linker in ROP18 adopts the auto-inhibitory conformation as seen in 3DZO and 3BYV. The auto-phosphorylation sites within the ROP18 N-terminal subdomain were mapped using recombinant protein, and whether these sites are phosphorylated \textit{in vivo} and even the extent to which these sites were auto-phosphorylated \textit{in vitro} were not reported. Kinase assays of recombinant ROP18 following phosphatase treatment were not presented, but may help to verify the importance of auto-phosphorylation in activating ROP18. Given the importance of the N-terminal subdomain to the proper folding of ROP18, it remains formally possible that the N-terminal Ser/Thr to Ala mutants decreased kinase activity by destabilizing ROP18 structure. Conversely, the effect of phospho-mimicking mutations of these N-terminal Ser/Thr residues to Asp or Glu on kinase activity were also not reported, but may provide a useful alternative means to test the importance of these phosphorylation sites in activating ROP18.

What are the substrates for ROP18?
The effect of ROP18\textsubscript{I} on type III virulence could be due to its effect on parasite growth; vacuoles with type III+ROP18\textsubscript{I} have on average twice as many parasites at 40hrs post-infection when compared to vacuoles with wild-type type III strain parasites (El Hajj et al., 2007). This effect of ROP18 on parasite growth seems to be vacuole autonomous as only parasites in vacuoles of type III+ROP18\textsubscript{I} have a shorter replication time even if they are next to a vacuole with a wild-type type III strain. Because this result was obtained in HFFs, possible host targets for ROP18 should be present in non-stimulated HFFs and are most likely present on the vacuole itself. Besides parasite proteins such as rhoptry proteins and dense granule proteins, several host proteins have also been described to relocalise to the PVM.

The most interesting potential substrates, at least in murine cells, would be the immunity-related GTPases (IRGs). These small GTPases are related to dynamins but are highly induced upon IFN-\(\gamma\) stimulation of cells and subsequently traffic to the PVM where they mediate dynamin-like vesiculation and subsequent rupture of the PVM and eventually the destruction of the parasite (Martens et al., 2005; Ling et al., 2006; Melzer et al., 2008). The accumulation of IRGs on the parasite PVM is also a strain-specific phenotype; Type I vacuoles seem to resist high accumulation of these IRGs and indeed type I vacuoles are resistant to destruction, while type II and III vacuoles accumulate high levels of the IRGs and are effectively destroyed by IFN-\(\gamma\) (Zhao et al., 2009a; Zhao et al., 2009b). To investigate if ROP18 had a role in this strain specific difference, the accumulation of IRGs on vacuoles of type III infected cells was compared to vacuoles of type III+ROP18\textsubscript{I} but no difference in accumulation of ROP18 was observed (Zhao et al., 2009a). Similarly, transfection of ROP18\textsubscript{I} or ROP18\textsubscript{II} into IFN-\(\gamma\)-induced L929 fibroblasts had no effect on the accumulation of IRGs on type II vacuoles (Khaminets et al., 2010).
In contrast to the above mentioned studies it was recently reported that ROP18 can bind to and
directly phosphorylate Irga6, Irgb6 and Irgb10 (Fentress et al., 2011; Steinfeldt et al., 2011),
thereby preventing their accumulation on the PVM. For Irga6 it was demonstrated that ROP18
can phosphorylate two threonine residues in the switch I loop (T102 and T108) (Steinfeldt et al.,
2011), while Irgb6 was mainly phosphorylated on a single threonine residue in its switch I loop
(Fentress et al., 2011). Because these threonine residues are at the catalytic interface that is
essential for GTP-dependent active dimer formation, it is likely that their phosphorylation
inactivates the protein. Indeed, phospho-mimetic mutants of Irga6 were inefficiently loaded onto
the PVM and could inhibit loading of wild-type Irga6 (Steinfeldt et al., 2011). Antibodies
specific for pT102 and pT108 demonstrated that type I vacuoles indeed contained significant
amounts of pT102 and pT108. Type II vacuoles did not contain any pT102 but did contain
pT108 although significantly less compared to type I strains. If the phosphorylation of T108 is
mediated by type II ROP18 remains to be determined. The phosphorylation of Irgb6 is of
particular interest as this is one of the first IRGs recruited to the vacuole and it has been proposed
to mediate the subsequent recruitment of other IRGs. Indeed knockdown of Irgb6 resulted in
significantly less killing of *Toxoplasma* by IFN-γ stimulated macrophages.

It was speculated that the difference between the studies reporting an effect of ROP18 and
studies reporting no effect (all studies were performed by the same two groups) was the IFN-γ
concentration; only at a low (<1 Unit/ml) IFN-γ concentration was an effect of ROP18t
expressed in type III on Irga6 loading on the vacuole detectable. This probably indicates that
other polymorphic parasite proteins play a role in resistance to the IRG system, because type I
strains can inhibit IRG loading even after stimulation of 200 Units/ml IFN-γ. The specific cell
type used might also be important. Sibley and colleagues demonstrated that prevention of IRG
accumulation on vacuoles of parasites expressing ROP18/I prevents the killing of Toxoplasma by GR1+ inflammatory monocytes or IFN-γ stimulated macrophages.

That ROP18 is not the only factor responsible for reduced IRG loading might also explain why type III+ROP18/I are as virulent as type III+ROP18/I, while the PVM of type II strain itself is efficiently coated by the IRGs. Possibly the type II strain lacks another polymorphic Toxoplasma protein, present in both type I and type III, that is necessary for ROP18 activity in vivo or it could contain a protein that somehow blocks ROP18 activity. Furthermore, it is unlikely that the IRGs are the only substrates for ROP18 because these IRGs are not expressed in human cells or non-stimulated cells and the effect of ROP18 on parasite growth was reported in un-stimulated HFFs.

Based on the ability of bacterially-expressed and refolded ROP18 kinase to phosphorylate 70 kD and 68 kD proteins in heat-inactivated parasite lysates, it has been suggested that ROP18 can also phosphorylate parasite-derived proteins (El Hajj et al., 2007). It is possible that these proteins are in fact ROP2, ROP8 or ROP18 itself as bacterially-expressed versions of those proteins were also shown to be phosphorylated by ROP18. However, whether these proteins are bona fide substrates in an infected cell remains unknown.

A number of unbiased strategies may be pursued for the identification of substrates or interactors of ROPK family active kinases and pseudokinases (Sopko and Andrews, 2008). Engineering a kinase of interest (by mutation of its gatekeeper residue to Gly or Ala, see below) to accept ATPγS analogues containing bulky substituents, allows chemical tagging of its direct substrates. Subsequent alkylation of the thiophosphate groups allows isolation of the tagged substrates by a thiophosphate ester-specific antibody for identification by mass spectrometry (Allen et al., 2007). Due to the cell impermeability of ATP analogues, substrates must be thiophosphorylated in lysates, where the relative spatial organization of kinases and their
substrates can be lost, potentially leading to false positives (Blethrow et al., 2004). Additionally, not all kinases are compatible with gatekeeper mutations required for analogue-sensitivity and/or with thiophosphorylation. A potential problem for the *Toxoplasma* kinome discussed earlier in the present review is the presence of a number of endogenous analog-sensitive kinases, such as TgCDPK1, that inherently have small gatekeeper residues, which may further complicate this approach (Sugi et al., 2010). An alternative method of kinase substrate identification utilizes mass spectrometry to detect changes in the abundance of phospho-sites due to the knockout of a kinase or treatment with a specific small molecule inhibitor, if available (Sopko and Andrews, 2008). Due to the effects on possible downstream kinases, substrates identified by this approach may not be direct, although it may be possible to filter out such indirect “false positives” with knowledge of the phosphorylation motif of the kinase of interest. The use of a positional-scanning peptide array (Hutti et al., 2004) to define the substrate preference of ROP18 was recently published (Fentress et al., 2011). Other approaches to identify kinase substrates that are also amenable to identifying interactors of pseudokinases include yeast 2-hybrid and co-purification of ligands / substrates with a tagged kinase or pseudokinase from cell lysates (Sopko and Andrews, 2008). The sensitivity of yeast 2-hybrid can facilitate the detection of transient kinase-substrate interactions, which may be stabilized by the use of a catalytically-inactive kinase bait.

**ROP5, a pseudokinase essential for Toxoplasma virulence.**

The analysis of virulence of the F1 progeny from IIxIII crosses identified a locus on *Toxoplasma* chromosome XII as having the largest contribution to the strain-specific differences in virulence.
Interestingly, the avirulent type III strain contributed to the enhanced virulence while the more
virulent type II strain contained the avirulent locus (Saeij et al., 2006).

It was recently demonstrated that the pseudokinase ROP5 is responsible for this difference in
virulence (Reese et al., 2011). Initially a completely avirulent F1 progeny (S22, LD_{50} > 1 \times 10^6
parasites) was complemented with a type I cosmid containing ROP5 and a 4 log increase in
virulence was noted. However, because the cosmid contained two other genes, a refined analysis
was needed. Therefore the ROP5 locus was deleted in a type I strain, and surprisingly this strain
was completely avirulent (LD_{50} > 1 \times 10^6) (Reese et al., 2011). The ROP5 locus is quite
complicated and it seems that type II strains contain 10 copies of ROP5, type I: 4 copies and type
III: 6 copies. These copies consist of at least three different alleles for each strain, with the type I
and III alleles being the most similar. Complementation of the type I knockout with one allele
increased virulence, while only complementation with two or more alleles converted the
virulence to wild-type virulence. The crystal structure of the ROP5\textsubscript{I} kinase domain showed a
canonical protein kinase domain fold and is highly similar to the crystal structures of the ROP2
and ROP8 pseudokinase domains (Reese & Boothroyd, 2011). However ROP5 was predicted to
be inactive due to the substitution of the Asp residue within the HRD motif (catalytic base) with
a basic residue (Lys or His depending on the ROP5 allele). Consistent with this, no detectable
kinase activity was reported for recombinant ROP5 protein \textit{in vitro}. The ROP5 pseudoactive site
is able to bind ATP, and the crystal structure revealed a distorted ATP binding mode, due to an
unusual positioning of magnesium coordination sites. ROP5 is also not predicted to be
processed because it lacks the subtilisin cleavage motif. Sibley and colleagues recently made a
cross between a type I and a type II strain and reported that a single QTL, containing the \textit{ROP5}
gene cluster, controls virulence difference between these strains (Behnke et al., 2011). Indeed,
through similar studies as described above they determined that ROP5 determines the differences in virulence between the type I and type II strains. The fact that ROP18 did not determine virulence differences between type I and type II indicates that indeed type I and type II ROP18 seem to be functionally equivalent. Although only a single QTL was identified this does not mean that other genes are not involved in determining virulence differences between the type I and type II strains. For example, some of the F1 progeny from the IxII cross contain both the virulent ROP5 and ROP18 alleles but are not 100% lethal indicating that other genes must be involved (Behnke et al., 2011).

The mechanism underlying ROP5-mediated virulence is currently unknown. Restoration of the catalytic base within the HRD motif by substitution of the basic residue in the ROP5AIII allele with Asp resulted in significantly reduced virulence in mice. However the equivalent substitution in the ROP5B1 allele did not restore *in vitro* catalytic activity in recombinant protein, and the basis for the reduced virulence conferred by the ROP5AIII R389D mutant is unclear (Reese & Boothroyd, 2011). To compare the contribution of ROP5 to virulence with that of ROP18, a ROP18 knockout strain was also created. Surprisingly, the type I ROP18 knockout strain was as virulent as the wild-type strain and only a slight delay till death was noted. This seems to contradict the important role of ROP18 in virulence that was previously demonstrated by complementation of the avirulent type III strain, and it also raises the question of the importance of evasion of the IRG system. Indeed, Sibley and colleagues also found that although the RHROP18 KO had a delayed time-till-death phenotype, it was still 100% lethal. It should be noted, however, that ROP18 was knocked out in the RH type I strain while the QTL analysis of virulence was performed using the GT1 type I strain. It was recently noted that these strains behave quite differently; RH has a faster duplication time, it survives much better extracellularly
and a significant number of parasite genes is differentially expressed compared to GT1 (Khan et al., 2009a). It would therefore be interesting to remove ROP18 in the GT1 background and investigate its virulence phenotype.

Although the ROPK family pseudokinases are not expected to directly phosphorylate target proteins, they may play scaffolding roles in allosteric regulation of active kinases or mediating kinase–substrate interactions (Zeqiraj and van Aalten, 2010). The crystal structure of the LKB1-STRADα-MO25α ternary complex revealed the structural basis of how the STRADα pseudokinase helps to maintain the LKB1 kinase in an active conformation. LKB1 interacts as a pseudosubstrate with regions of STRADα that correspond to the substrate binding site in active protein kinases (Zeqiraj et al., 2009). Consistent with this theme, the substrate binding loops of ROP2 and ROP8 are well ordered and structurally conserved between the 3DZO and 3BYV crystal structures. However, differences in the surface electrostatic potentials in the ROP2 (partly neutral and negative) and ROP8 (highly positively charged) pseudosubstrate binding regions suggest different ligand specificities (Qiu et al., 2009).

ROP38.

After analysing the complete Toxoplasma kinome, Roos and colleagues further characterized the ROP38 gene for the following reasons: it was predicted to be an active kinase, it was differentially expressed between strains (up >64 fold in type III; >8 fold in type II as compared to type I), it was induced upon differentiation from tachyzoites to bradyzoites, it was triplicated in both Toxoplasma and Neospora, and there was evidence for convergent evolution of Toxoplasma ROP38 and its Neospora homologue (Peixoto et al., 2010). Because its expression is very low in type I strains they decided to study its function by over-expressing HA-tagged type I
ROP38 using a tubulin promoter in a type I strain. HA-tag staining of infected cells confirmed colocalization of ROP38 with ROP2 indicating that it is indeed a rhoptry protein. ROP38 was also observed on the PVM, which contrasts with a previously published report that it does not traffic to the PVM (ROP38 corresponds to ROP2L5 in that paper) (Reese and Boothroyd, 2009). It is possible that the over-expression of ROP38 resulted in mislocalisation, and definitive localization will depend on staining with an antibody recognizing endogenous ROP38. To determine if ROP38, like ROP16, modulates host cell gene expression, host microarrays were performed after infection with type I+ROP38 or wild-type type I. Interestingly, expression of the ROP38 transgene was able to suppress a large proportion of genes that are normally regulated by type I infection. Preliminary experiments suggested that ROP38 might modulate the MAPK pathway, as the kinetics of ERK phosphorylation was different in type I-infected vs type I+ROP18 infected cells. However, this difference in the modulation of host gene expression seemed to have no consequences for virulence in mice as type I over-expressing ROP38 was as virulent as wild-type type I.

The Plasmodium kinome.

The P. falciparum kinome has been reported to comprise 85 or 99 enzymes, depending on the stringency applied for inclusion of borderline sequences (Ward et al., 2004; Anamika and Krupa, 2005). Both studies concur to present a picture of a the Plasmodium kinome as being characterized by significant divergences from the yeast or metazoan kinomes (reviewed in Doerig and Meijer, 2007; Doerig et al., 2010). Most established eukaryotic PK groups have members in Plasmodium, although TyrK (tyrosine protein kinases) and STEs (a group of PKs involved in mitogen-activated protein
kinase [MAPK] pathways) are not represented. The *Plasmodium* kinome comprises two
atypical MAP kinases, but the absence of typical MAPKKs suggests that activity of these two
enzymes is regulated in a way that differs from that found in classical eukaryotic MAPKs
pathways. These atypical MAPKs are but an example of a number of plasmodial PKs that
can be classified as belonging to established ePK families, but cannot be assigned precise
orthology with specific mammalian enzymes (for examples, see Abdi et al., 2010; Agarwal et
al., 2011; Dorin et al., 2001; Dorin et al., 1999; Reinerger et al., 2005; Fennell et al., 2009;
Reinerger et al., 2009; Halbert et al., 2010; Reinerger et al., 2011). The *Plasmodium* kinome
also includes many enzymes that do not cluster with any of the PK groups and families
established from the yeast and mammalian kinomes, such as the FIKK kinases, which are
specific to apicomplexan parasites (Schneider and Mercereau-Puijalon, 2005), and the
CDPKs (see above). Interestingly, individual PKs displaying sequence features that are
characteristic of distinct ePK groups are found in the *P. falciparum* kinome, illustrating its
evolutionary divergence from other kinomes (Dorin et al., 2001; Bracchi-Ricard et al.,
2000; Dorin et al., 2005). A notable example of such “composite kinases” is Pfnek-1, which
has a clear relatedness to the NIMA family despite the presence of a MAPKK-like putative
activation site (Dorin et al., 2001).

A systematic kinome-wide knock-out approach in *P. berghei* identified several PKs as essential
for development in the mosquito (genetic manipulations are significantly more straightforward in
the rodent malaria *P. berghei* than in *P. falciparum*, because (i) gene replacement by double
cross-over occurs at a much higher rate in the former than in the latter, and (ii) the selection of
transformed parasites occurs in the mouse and is much faster than the in vitro cultivation of *P.
falciparum* (Carvalho and Ménard, 2004)). A similar study has recently addressed essentiality
of 62 of the 65 *P. falciparum* ePKs (this study excluded the FIKKs, see above). The inability to
knock-out a given locus, together with the ability to modify the allele in a way that does not
cause loss-of-function of the gene product (e.g. tagging the C-terminal end with HA epitopes or
with GFP), is interpreted as strongly indicative of an essential role of that locus during
schizogony. 36 kinases were thus identified as playing a crucial role in erythrocytic schizogony,
while 26 are most likely dispensable for this part of the life cycle (with cloned parasite lines
having thus far been obtained for 12 of these) (Solyakov et al., Nature Communications, in
revision).

*Plasmodium exported kinases.*

*Plasmodium* expresses a set of unique kinases, the FIKKs (Ward et al., 2004), 16 of which are
predicted to be exported into the RBC because of the presence of a PEXEL motif in their N-
terminal region (Schneider and Mercereau-Puijalon, 2005). The roles played by FIKK in the
RBC are much less well understood than those of some of the ROPKs in *Toxoplasma*-infected
cells discussed above. We know that a significant subset of FIKKs are indeed exported into the
RBC shortly following parasite invasion (Nunes et al., 2007; Nunes et al., 2010) and that at least
one member of the family remains within the parasite, despite possessing a recognizable
PEXEL (Nunes et al., 2007). Most fikk genes are located in the sub-telomeric regions of 10 of the
14 chromosomes in *P. falciparum*, in the vicinity of the var genes that mediate cytoadherence
and antigenic variation. The var genes are under allelic exclusion control, with a single PfEMP1
(var-encoded) protein expressed at a time; this appears not to be the case for the fikk genes.
Interestingly, however, expression of a subset of fikk genes was modulated in parasites
undergoing a switch in their cytoadherence phenotype (Nunes et al., 2007). Knock-out studies
implicated 2 FIKKs in the remodeling of the RBC membrane skeleton, as distinct phosphorylation profiles in ghost preparations of parasite-infected RBCs were detected for each of the knock-out parasite clones (Nunes et al., 2010). In addition to FIKKs, a number of *Plasmodium* kinases have been reported to be exported into the RBC (Kun et al., 1997; Droucheau et al., 2004; Vaid et al., 2010) or even secreted to the extracellular milieu (Singh et al., 2009). Recent data demonstrate that infection with malaria parasites causes a dramatic activation of a signaling pathway involving host RBC PAK and MEK kinases, and that pharmacological interference with these enzymes using highly selective allosteric inhibitors is lethal for the parasite (Sicard et al., 2011). The parasite is also known to interfere with host cell signaling in the liver stages (reviewed in (Luder et al., 2009)), resulting in down-regulation of the NFκB pathway (Singh et al., 2007) and in prevention of apoptosis of host hepatocytes (van de Sand et al., 2005).

Thus, it appears that in both *Toxoplasma* and *Plasmodium*, two apicomplexan parasites that are phylogenetically widely divergent from each other (Kuo et al., 2008), calcium-regulated protein phosphorylation mediated by CDPKs plays central roles in regulating their entry into and egress from the host and other developmental processes, and that both parasites export proteins into their host cell to tailor it to their own needs. The secretion of virulence factors into the host is a strategy widely used not only by Apicomplexa (a striking further apicomplexan-based example of this concept is the reversible transformation of leucocytes by *Theileria* (reviewed in Shiels et al., 2006)), but also by many if not all intracellular parasites (reviewed in (Munter et al., 2006)). This not only provides a fascinating fundamental glimpse into unique signaling
mechanisms operating within parasites and between parasites and their hosts, but also suggests opportunities for novel therapeutic intervention.

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FIGURE LEGENDS

Figure 1: Comparative schematic of activation of CDPKs and CaMKs. The green lines represent the auto-inhibitory region. CaM = Calmodulin, CAD = CDPK-activating domain. See text for details. [Reproduced from “A parasite calcium switch and Achilles’ heel revealed. (Doerig and Billker, 2010)].

Figure 2: The Toxoplasma kinome.
Classification of 108 active kinases predicted from the *T. gondii* genome. Black, human and yeast; blue, *P. falciparum*; red, *T. gondii*. Colored 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 recognized); black dots, bootstrap support > 50%. [Reproduced from (Peixoto et al., 2010)]
Figure 1.

Figure 2.