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1 **_Toxoplasma_ and _Plasmodium_ protein kinases that determine how these parasites invade**
2 **and make themselves at home in their host cells.**
3

4

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10

11 **Abstract**

12 Some apicomplexan parasites have evolved distinct protein kinase families to modulate

13 host cell structure and function. *Toxoplasma gondii* rhoptry protein kinases and pseudokinases

14 (ROPKs) are involved in virulence and modulation of host cell signaling. The proteome of

15 *Plasmodium falciparum* contains a family of putative kinases called FIKKs, some of which are

16 exported to the host red blood cell and might play a role in erythrocyte remodeling. In this

17 review we will discuss kinases known to be critical for host cell invasion, intracellular growth

18 and egress, focusing on (i) calcium-dependent protein kinases (CDPKs) and (ii) the secreted

19 kinases that are unique to *Toxoplasma* (ROPKs) and *Plasmodium* (FIKKs).

20

21 **Keywords**

22 Protein kinase, *Toxoplasma*, *Plasmodium*, Rhoptry, Calcium-dependent protein kinase, malaria,

23 red blood cell

24

25

26 **Introduction**

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

39 Both *Toxoplasma* and *Plasmodium* contain a family of calcium-dependent protein kinases
40 (CDPKs), whose occurrence is restricted to plants and Alveolates (the superphylum that
41 comprises Ciliates and Apicomplexa), although trypanosomatids also possess kinases with an
42 EF-hand calcium binding domain that are thought to be phylogenetically distinct form plant and
43 Alveolate CDPKs. CDPKs have a domain structure consisting of a calcium-binding domain
44 fused to the kinase domain, such that kinase activity is stimulated upon calcium binding. Studies
45 of apicomplexan CDPKs have revealed a conserved regulatory mechanism and highlighted the
46 importance of calcium in regulating a number of key physiological processes including host cell
47 attachment and invasion, gliding motility, and parasite egress.

48 *Toxoplasma gondii*, a highly prevalent obligate intracellular protozoan parasite, seems to
49 rely for a large part on protein kinases and pseudo kinases to modify the host cell. *Toxoplasma* is
50 capable of infecting all nucleated cells of most warm-blooded animals. This ability to establish a
51 chronic infection in such a wide range of host species and cell types is likely associated with its
52 ability to modify many aspects of the host cell's normal physiology. It does this by secreting
53 proteins from the rhoptry, a specialized secretory organelle that is found only in apicomplexan
54 parasites, directly into the host cytosol where they can exert their function. Many rhoptry
55 proteins have homology to kinases and a large proportion of them are predicted to be
56 pseudokinases.
57 Malaria parasites (genus *Plasmodium*) also belong to the Apicomplexa, however, unlike
58 *Toxoplasma*, they have a very restricted range of host cells which they can invade and in which
59 they can replicate. After inoculation into the vertebrate host through the bite of an infected
60 mosquito, *Plasmodium* sporozoites must first invade hepatocytes, where they undergo asexual
61 proliferation (exo-erythrocytic schizogony), generating several thousand merozoites in the
62 process. This strict dependence on hepatocytes for the pre-erythrocytic stage of infection has,
63 however, recently been challenged by *in vivo* observations of *P. berghei* parasites developing in
64 dermal and epidermal cells at the site of inoculation and generating infective merozoites
65 (Gueirard et al., 2010).
66 The only cell type permissive for invasion by *Plasmodium* merozoites is the red blood
67 cell (RBC). Invasion involves release of the contents of rhoptries and micronemes (another type
68 of specialized secretory organelle) and formation of a parasitophorous vacuole. The parasite
69 causes considerable modifications to its host RBC that include the establishment of a complex

70 trafficking system that mediates translocation of parasite-encoded proteins to the RBC
71 membrane skeleton and surface (Cooke et al., 2004).
72 In the case of *P. falciparum*, the most lethal of the five species of malaria parasites that can infect
73 humans, such proteins include components of the so-called "knob" structures, through which
74 parasite-infected RBCs adhere to vascular endothelial cells, thereby significantly contributing to
75 pathogenesis (Cooke et al., 2001; Cooke, B.M., N. Mohandas, and R.L. Coppel, Malaria and the
76 red blood cell membrane. Semin Hematol, 2004. 41(2): p. 173-88; Maier, A.G., et al., Malaria
77 parasite proteins that remodel the host erythrocyte. Nat Rev Microbiol, 2009. 7(5): p. 341-54.).
78
79 There are no orthologues of the *Toxoplasma* rhoptry kinases in malaria parasites, however, the
80 *P. falciparum* kinome includes a family (20 members) of related kinase-like sequences called
81 FIKKs, due to the presence of a Phe-Ile-Lys-Lys motif they share in their kinase domain (Ward
82 et al., 2004). Most notably, 18 of the 20 *fikk* genes in *P. falciparum* are predicted to encode fully
83 functional kinases, 16 of which are predicted to be exported into the host RBC (Schneider and
84 Mercereau-Puijalon, 2005), and at least some of these enzymes have been shown to be exported
85 to the host RBC and to be associated with kinase activity (Nunes et al., 2007; Nunes et al., 2010).
86 Many of the *Toxoplasma* and *Plasmodium* kinases are related to kinases with known functions in
87 other eukaryotes and these will not be discussed in this review. Instead, we will discuss in more
88 detail, kinases from these parasites that are known to be critical for host cell invasion,
89 intracellular growth and parasite egress, focusing on (i) calcium-dependent protein kinases
90 (CDPKs) and (ii) the secreted kinases that are unique to *Toxoplasma* (ROPKs) and *Plasmodium*
91 (FIKKs).
92
93 **Calcium-dependent regulation of invasion and egress**:

94 Calcium levels in *T. gondii* play key roles in regulating micronemal secretion-dependent
95 processes including host cell invasion, gliding motility and parasite egress {Nagamune, 2008
96 #13506}. Chelation of parasite intracellular calcium strongly inhibited both microneme release
97 and invasion of host cells, and this effect was partially reversed by raising intracellular calcium
98 using the ionophore A23187 (Carruthers et al., 1999). Additionally, evidence was also provided
99 in this study for the requirement of a staurosporine-sensitive kinase activity in regulating
100 microneme discharge and for parasite invasion of host cells.

101 In *Toxoplasma* parasites, calcium-dependent protein kinases (CDPKs) were shown to be
102 involved in host cell invasion through the use of KT5926, an inhibitor of CDPKs in other
103 systems (Kieschnick et al., 2001). KT5926 blocks the motility of *Toxoplasma* tachyzoites and
104 their attachment to host cells. *In vivo*, the phosphorylation of only three parasite proteins was
105 found to be blocked by KT5926 (although there may be more), and in parasite extracts, a single
106 KT5926-sensitive protein kinase activity was detected. This activity was calcium-dependent but
107 did not require calmodulin. In a search for CDPKs in *Toxoplasma*, two members of the class of
108 calmodulin-like domain protein kinases (CDPKs) were detected. TgCDPK2 was only expressed
109 at the mRNA level in tachyzoites, with no detectable protein. TgCDPK1 protein present in
110 *Toxoplasma* tachyzoites cofractionated precisely with the peak of KT5926-sensitive protein
111 kinase activity. TgCDPK1 kinase activity was calcium-dependent but did not require calmodulin
112 or phospholipids. TgCDPK1 was found to be inhibited effectively by KT5926 at concentrations
113 that block parasite attachment to host cells. *In vitro*, TgCDPK1 phosphorylated three parasite
114 proteins that migrated identical to the three KT5926-sensitive phosphoproteins detected *in vivo*.
115 These observations suggested a central role for TgCDPK1 in regulating *Toxoplasma* motility and
116 host cell invasion.

117 Subsequent chemical genetic and conditional knockout studies have shown that
118 TgCDPK1 is the key transducer downstream of calcium fluxes in regulating these processes.
119 Down-regulation of TgCDPK1 expression severely impaired the secretion of micronemal
120 proteins, including adhesins needed for gliding motility (Lourido et al., 2010). The resulting
121 defect in gliding motility led to a significant decrease in host cell attachment and invasion.
122 Conditional knockout of TgCDPK1 also impaired parasite egress from host cells upon induction
123 with a calcium ionophore, with immotile parasites remaining within vacuoles. This coincided
124 with a defect in PVM permeabilization upon calcium ionophore treatment, which likely resulted
125 from impaired microneme secretion of the perforin-like TgPLP1 protein (Lourido et al., 2010).
126 The canonical CDPK domain structure consists of an N-terminal protein kinase domain,
127 that is highly homologous to calmodulin-dependent kinases (CaMKs), and a C-terminal CDPK
128 activation domain (CAD) with four calcium-binding EF-hands (EF1 to EF4). A recent
129 biochemical and structural study of three apicomplexan CDPKs (TgCDPK1 and TgCDPK3 from
130 *T. gondii*, and CpCDPK1 from *Cryptosporidium parvum*) has provided novel insights into how
131 these CDPKs are activated upon Ca^{2+} binding by the CAD (Wernimont et al., 2010). In *in vitro*
132 assays, the kinase domain was activated by low micromolar Ca^{2+} concentrations. Calcium 'in-
133 out' experiments suggested a partial irreversibility of this calcium-dependent activation for
134 TgCDPK1 and TgCDPK3, while CpCDPK1 activity was reduced to basal level upon calcium
135 removal. Actual mapping of potential auto-phosphorylation sites may provide further insights
136 into the reversibility of calcium-induced activation of these CDPKs.
137 Crystal structures have been solved for auto-inhibited (calcium-free) TgCDPK1 and
138 TgCDPK3 and for calcium-bound TgCDPK1 and CpCDPK1 (Wernimont et al.). The kinase
139 domain adopts the bi-lobal structure characteristic of eukaryotic protein kinases, with a smaller

140 N-terminal lobe (consisting mainly of β-strands) and a larger C-terminal lobe (predominantly α-
141 helical) connected together via a single stretch of polypeptide (hinge region). A deep cleft
142 formed between the two lobes contains the ATP binding pocket and active site (Dar, 2005). In
143 the calcium-free state, the CAD adopts an overall dumbbell shape (resembling calmodulin in the
144 absence of calcium) with two EF-hands at each end of the dumbbell. The CAD packs against the
145 front face of the kinase domain, making contacts with both the N- and C-terminal lobes. The N-
146 terminal helix of EF1 extends into a long helix (CH1) that spans the length of the CAD and
147 packs anti-parallel along a second long helix (CH2). The N-terminus of CH1 connects directly
148 with the C-terminus of the kinase domain and packs against the kinase C-terminal lobe to block
149 substrate binding, analogous to the pseudo-substrate segment of CAMKII (Rosenberg et al.,
150 2005) (Figure 1). Additional hydrophobic contacts with the N- and C-terminal lobes of the
151 kinase domain are made by EF2 and EF3, respectively. Calcium binding to all four EF hands
152 within the CAD causes conformational changes that expose hydrophobic surfaces and partial
153 unwinding and bending of the CH1 and CH2 helices. This leads to a dramatic rearrangement of
154 the CAD into a more compact structure. The calcium-bound CAD is also repositioned and binds
155 to the back face of the kinase domain, which induces a widening of the active site cleft by
156 altering the relative orientations of the two lobes of the kinase domain. Release of the auto-
157 inhibitory interactions between the CAD and kinase domain also allows repositioning of the α-C
158 helix and rearrangement of the P-loop and the activation loop regions into an active
159 conformation as seen in other kinase structures.
160 Apicomplexan CDPKs have no orthologs in humans and are most closely related to
161 CDPKs in plants. The essential functions of TgCDPK1 and the lack of orthologs in humans
162 make it an ideal target for novel anti-*Toxoplasma* therapeutics. In particular, TgCDPK1 is

163 unique in having a glycine residue at the gatekeeper position, which is located at the rear of the
164 adenine binding pocket. ATP-analogs with bulky substituents on the purine group can be
165 accommodated by analog-sensitive kinases with small gatekeeper residues (with glycine being
166 the smallest), while kinases with larger gatekeeper residues cannot accommodate these
167 molecules (Bishop et al., 2000; Blethrow et al., 2004). Since few human kinases contain a small
168 gatekeeper residue (no known mammalian protein kinase has glycine gatekeeper), TgCDPK1
169 provides a promising analog-sensitive target for bumped kinase inhibitors (BKIs) that should
170 have relatively little off-target effects on the human kinome (Manning et al., 2002).

171 A number of BKIs have been developed for chemical genetic studies with engineered
172 analog-sensitive kinase alleles in human cells. BKI analogs of 4-amino-1-*tert*-butyl-3-
173 phenylpyrazolo[3,4-d]pyrimidine, with bulky aromatic substituents at the C3 position, inhibit
174 TgCDPK1 at sub-micromolar concentrations (Ojo et al., 2010; Sugi et al., 2010). Consistent
175 with conditional TgCDPK1 knockout studies, BKIs greatly reduced host cell invasion by *T.*
176 *gondii* and also modestly affected intracellular growth. Parasites expressing an analog-resistant
177 TgCDPK1, in which the gatekeeper glycine is mutated to methionine (G128M) were
178 significantly less sensitive to BKIs and thus validated TgCDPK1 as the main BKI target *in vivo*.
179 Crystal structures of calcium-free TgCDPK1 in complex with NA-PP2 and NM-PP1 confirm the
180 structural basis for the selectivity of these BKIs for a small gatekeeper residue and will facilitate
181 optimization of these BKIs to further improve their affinity and specificity (Ojo et al., 2010). Of
182 the 114 functional kinases predicted in the *T. gondii* genome, 12 are expected to be analog-
183 sensitive based on their gatekeeper residue (Sugi et al., 2010) . Thus, BKIs provide a promising
184 approach to targeting key processes in *T. gondii* with potentially fewer side effects on the host.

185 That host cell invasion can be inhibited by staurosporine and involves calcium signaling has been
186 known to also be true for malaria parasites for 2-3 decades (Scheibel et al., 1987; Ward et al.,
187 1994). More recently, a role for a CDPK (PfCDPK1) in this process has been revealed (Green et
188 al., 2008; Kato et al., 2008), highlighting a definite level of unity in the invasion strategies across
189 Apicomplexa. In addition to invasion, malaria parasites exploit their repertoire of CDPKs to
190 mediate several other developmental processes (see (Billker et al., 2009) for a comprehensive
191 review). Briefly, these include male gametogenesis, shown in the rodent-infecting parasite *P.*
192 *berghei* to be dependent on PbCDPK4 (Billker et al., 2004); gliding of ookinetes, the motile form
193 that escapes the mosquito midgut during infection of the insect vector, a process blocked in *P.*
194 *berghei* parasites lacking PbCDPK3 (Ishino et al., 2006); and switching of parasite behavior
195 from cell traversal to cell invasion at the early stage of liver infection by *P. berghei* sporozoites,
196 which requires PbCDPK6 (Coppi et al., 2007). There is unity across Apicomplexa not only with
197 respect to the involvement of CDPKs in invasion, but also in the mechanism of activation of
198 these enzymes by calcium (see above). A recent structural study of a *Plasmodium* CDPK
199 (PfCDPK3) (Wernimont et al., 2011) revealed that the residues mediating the largest
200 conformational change following calcium binding are the most conserved across Apicomplexa,
201 leading the authors to propose that the mechanism described in the section above is conserved,
202 presumably throughout the CDPK family. Another interesting observation described in this
203 report is that although the CAD shares similar motifs and behavior with calmodulin, there are
204 sufficient structural differences to consider the CDPK activation domain as a distinctive member
205 of the large EF-hand family of calcium-binding proteins.

206 Given their key regulatory roles, malaria CDPKs also present attractive drug targets. However,
207 in contrast to the TgCDPKs, the presence of non-glycine residues at the gatekeeper position in
208 malaria CDPKs will preclude the use of BKIs. (maybe Christian can double check this?)

209

210 Host modification by secreted *Plasmodium* and *Toxoplasma* protein (pseudo)kinases

211 Unlike most other eukaryotic protein kinases, most members of the *Plasmodium* FIKK and
212 *Toxoplasma* ROPK family have signal peptides and are predicted to be secreted into the host
213 cell. A recent analysis of the completed genome sequence of the three most common strains of
214 *Toxoplasma gondii* by Roos and colleagues found that the *Toxoplasma* kinome consists of 108
215 predicted active kinases and 51 predicted pseudokinases (Peixoto et al., 2010) (Figure 2).
216 Compared to the human kinome, which only contains ~10% pseudokinases (Manning et al.,
217 2002), there is a significant enrichment of pseudokinases in the *Toxoplasma* kinome. This is
218 mainly due to an expansion of the *Toxoplasma*-specific ROPK family, consisting of ~44 genes of
219 which about half are pseudokinases. It must be kept in mind in this context that some proteins
220 thought to be pseudokinases on the basis of their sequence characteristics were actually shown to
221 be competent for phosphorylation (Kannan and Taylor, 2008; Taylor and Kornev, 2010). This
222 may be particularly relevant to sequences that are relatively distant from classical ePKs, such as
223 the ROPKs and FIKKs (see below).

224

225 Members of both the ROPK and FIKK families are characterized by a domain structure
226 consisting of an N-terminal signal peptide, a low complexity region, and a conserved C-terminal
227 domain that adopts a canonical protein kinase fold. However, *Toxoplasma* ROP kinase domains
228 constitute a phylogenetically-distinct group from other eukaryotic protein kinases, including
229 other apicomplexan kinases such as the malaria FIKK family. Proteins of the ROPK family have
230 roles in host cell modulation and virulence, although the molecular details of how most of these

231 proteins function are poorly understood. While some members of the ROPK family have been
232 shown to be active kinases (such as ROP16 (Yamamoto et al., 2009; Ong et al., 2010), and
233 ROP18 (El Hajj et al., 2007; Khan et al., 2009b)), others appear to be pseudokinases with
234 substitutions at key residues that abrogate catalytic activity (but see our comment above). The
235 inability of ROP2 to bind ATP has also been reported and may be true for other ROPK family
236 pseudokinases (Labesse et al., 2009).

237 Twelve sequence motifs or sub-domains are highly conserved in protein kinase domains
238 (Hanks et al., 1988). Most of these sub-domains are clustered within the active site cleft region
239 and are important for ATP binding and catalysis, while others are crucial for structural stability
240 of the protein. The crystal structures of the ROP2 (PDB accession codes 2W1Z and 3DZO) and
241 ROP8 (3BYV) pseudokinase domains revealed a number of structural features within their active
242 site region that are incompatible with catalysis and ATP binding (Labesse et al., 2009; Qiu et al.,
243 2009). Substitution of the VAIK motif with FEVH in both ROP2 and ROP8 results in loss of the
244 critical Lysine side chain that normally coordinates the α- and ß-phosphates of ATP, however,
245 WNK is an example of a kinase that lacks this Lysine residue but is nevertheless active, through
246 recruiting another residue to substitute for the missing one (Xu et al., 2000). Replacement of the
247 DFG motif with GFE alters the magnesium coordination geometry, while substitution of the
248 HRD motif with HTY results in replacement of the catalytic base (Asparagine) with Tyrosine.
249

250 **Cellular destination of rhoptry kinases**.

251 Although it is now well established that upon invasion *Toxoplasma* secretes the contents of its
252 rhoptries directly into the host cytosol, it is still unclear how these secreted proteins cross the
253 host membrane. Patch clamp experiments have shown a short spike in conductivity at the time of

254 invasion, which is suggestive of a break in the host membrane followed by resealing (Suss-Toby
255 et al., 1996). The *Toxoplasma* protein(s) that mediate this break remain unknown. Recently, two
256 *Toxoplasma* proteins with a Membrane Attack Complex/Perforin (MACPF) domain were
257 identified, one of which (TgPLP1) is necessary for egress, which involves rupture of the
258 parasitophorous vacuolar membrane (PVM) (Kafsack et al., 2009). TgPLP2 could be a candidate
259 for mediating the break in the host cell plasma membrane. Once rhoptry proteins are secreted
260 into the host cell cytosol they traffic to distinct cellular destinations based on other signals. For
261 example the rhoptry kinase ROP16 and the rhoptry protein phosphatase 2 C (PP2C-hn) carry a
262 nuclear localization signal (NLS), which mediates their trafficking to the host nucleus (Gilbert et
263 al., 2007; Saeij et al., 2007). Many ROPKs traffic back to the PVM and it was recently
264 demonstrated that an arginine-rich amphipathic helix (RAH) domain is necessary and sufficient
265 for targeting ROPKs to the PVM (Reese and Boothroyd, 2009). Transgenic expression of the
266 RAH domain in host cells results in punctuate staining with some overlap with the nuclear
267 envelope. However, after *Toxoplasma* infection of those cells, most of the staining relocated to
268 the PVM (Reese and Boothroyd, 2009). The fact that the RAH domain only interacts weakly
269 with a variety of host membranes but strongly with the PVM, which is derived from the host
270 membrane, is puzzling. It has been suggested that the RAH domain has no special affinity for a
271 particular lipid composition but rather for negative membrane curvature as the host nuclear
272 envelope, which is negatively curved, associated somewhat with RAH domain (Reese and
273 Boothroyd, 2009). Indeed, it is well known that *Toxoplasma* secretes proteins from its dense
274 granules that are involved in the formation of a tubulovesicular network consisting of elongated
275 negatively curved nanotubules of 60-90 nm in diameter, which are topologically cytosolic, and
276 connect with the vacuole-delimiting membrane. Consistent with this, parasites without the dense

277 granule protein GRA2, which have an attenuated PVM tubular network, have attenuated
278 recruitment of the RAH domain to the PVM (Reese and Boothroyd, 2009). Thus, an important
279 function of dense granule proteins might be the formation of the extremely negatively curved
280 tubular network, which subsequently functions to attract rhoptry proteins with a RAH domain
281 from the cytosolic face of the PVM. Rhoptry proteins without a RAH domain or an NLS, such as
282 Toxofilin stay in the host cell cytosol (Lodoen et al.).
283
284 **ROP16**
285 Although it has been known for some time that *Toxoplasma* ROPKs can be secreted into the host
286 cytosol, their function has remained elusive. ROP2 was thought to mediate the recruitment of
287 mitochondria to the PVM, but a recent study showed that a ROP2 knockout has no defect in
288 mitochondrial recruitment (Pernas and Boothroyd, 2010). One of the first ROPKs for which a
289 particular function was ascribed to was ROP16. Expression profiling of human foreskin
290 fibroblasts (HFFs) infected with type I, II or III strain parasites demonstrated that these strains
291 differ significantly in the modulation of host gene expression (Saeij et al., 2007). To identify the
292 *Toxoplasma* genomic loci involved, expression profiles of HFFs infected with F1 progeny
293 derived from crosses between type II and type III were determined. Quantitative trait locus
294 (QTL) analysis, using host gene expression as the quantitative trait, determined that the genotype
295 at a locus on *Toxoplasma* chromosome VIIb correlated with the strain-specific regulation of
296 >1,000 human genes. Analyses for enrichment in functional annotation of these genes implicated
297 the STAT signaling pathway. Indeed HFFs infected with type I or type III parasites have strong
298 and sustained activation of STAT3 and STAT6 while the activation by type II is much weaker
299 and only transient. A candidate gene approach identified *ROP16* as the *Toxoplasma* gene

300 responsible for the strain-specific activation of STAT3 and STAT6. ROP16 is secreted into the
301 host cytosol upon infection and subsequently traffics to the host nucleus. This nuclear
302 translocation is dependent on an NLS in ROP16 but its nuclear localization was not important for
303 the activation of STAT3 and STAT6. Subsequently, these experiments were confirmed by other
304 groups using a type I strain where the *ROP16* gene was removed by double homologous
305 recombination (Yamamoto et al., 2009; Ong et al., 2010). As expected, ROP16 kinase activity is
306 necessary for its effect on STAT3 and STAT6 (Yamamoto et al., 2009; Ong et al., 2010).
307 Initially it was predicted from sequence homology that ROP16 was a Serine/Threonine kinase
308 and was therefore unlikely to directly phosphorylate STAT3 and STAT6. However, it has
309 recently been shown that it can in fact directly phosphorylate these targets (Yamamoto et al.,
310 2009; Ong et al., 2010). One group showed that immunoprecipitated ROP16 can phosphorylate
311 Tyr705 of recombinant STAT3 and subsequent co-immunoprecipitation experiments determined
312 that the N-terminal region (amino acid 223-303) of ROP16 is required for this interaction with
313 STAT3. By constructing chimeras between the type I and type II ROP16 proteins, the difference
314 in STAT activation by these two alleles could be attributed to a single amino acid substitution; a
315 conversion of Serine to Leucine at position 503 made type II ROP16 also a potent activator of
316 STAT3.
317 Similarly, Boothroyd and colleagues showed that ROP16 has intrinsic tyrosine kinase activity as
318 determined by phosphoamino acid analysis of auto-phosphorylated ROP16. *In vitro* kinase
319 assays with recombinant ROP16 and recombinant STAT6-GST as a substrate also showed
320 efficient phosphorylation of Tyr641 on STAT6 by wild-type but not K404N (kinase-dead)
321 recombinant ROP16. The pan-JAK inhibitor and the tyrosine kinase inhibitor K-252a severely
322 impaired the kinase activity of ROP16 providing further evidence that ROP16 is a tyrosine

323 kinase (Yamamoto et al., 2009; Ong et al., 2010). Thus, ROP16 can directly phosphorylate
324 Tyr705 and Tyr641 from STAT3 and STAT6, respectively. This could explain why the up-
325 regulation of the suppressor of cytokine signaling (SOCS) genes, which normally down-regulate
326 the STAT pathway (Dalpke et al., 2008), do not down-regulate the STAT3/6 activation by
327 ROP16 (Saeij et al., 2007). SOCS proteins bind to phosphorylated tyrosine residues on Janus
328 kinases (JAKs) and/or cytokine receptor subunits through a central SH2 domain and
329 subsequently mediate their degradation (Dalpke et al., 2008). Because the JAKs and cytokine
330 receptors are bypassed by the direct phosphorylation of STAT3 and STAT6 by ROP16, the
331 inhibitory function of SOCS proteins is abrogated. By directly phosphorylating STAT3
332 *Toxoplasma* could mimic the effects of IL-10, which also maintains constitutive activation of
333 STAT3 because its receptor does not interact with the SOCS proteins (Yasukawa et al., 2003).
334 IL-10 is a potent anti-inflammatory cytokine which can down-regulate the production of IFN-γ,
335 the main mediator of resistance to *Toxoplasma*. The prediction would therefore be that ROP16
336 can enhance parasite virulence by suppressing IFN-γ production.
337 Indeed, ROP16 was also identified as one of the loci involved in the difference in virulence
338 between type II and type III strains (Saeij et al., 2006). However, addition of ROP16 from type I
339 or type III into a type II strain made it less virulent. Why type II+$ROP16_{I/III}$ parasites have
340 reduced virulence is currently unknown. Although $ROP16_{I/III}$ can lower the level of secretion of
341 IL-12 by macrophages infected with type II strains, which could lead to subsequent lower
342 induction of IFN-γ secretion, one would predict that this would lead to an enhanced and not a
343 reduced virulence phenotype.
344 A potential explanation for this apparent contradiction is emerging from our recent
345 studies showing that macrophages infected with type I or type III strains are converted into an

346 alternatively activated or M2 macrophage (Jensen et al., 2011). These M2 macrophages are
347 characterized by high level expression of arginase-I and several lectin receptors such as the
348 mannose receptor and macrophage galactose specific lectin. Type II infected macrophages are
349 converted into classically activated or M1 macrophages and secrete large amounts of
350 proinflammatory cytokines such as IL-12p70 and IL-23. Activation of STAT6 by ROP16 is
351 necessary to convert macrophages to the M2 phenotype, while the M1 phenotype is due to the
352 fact that the type II strains, but not the type I and III strains, activate NFκB, a phenotype due to a
353 new polymorphic dense granule protein GRA15 (Rosowski et al., 2011). Thus, it is possible that
354 one of the roles of ROP16 is to limit the inflammation induced by the strong Th1 induction by
355 *Toxoplasma* and the reduction of virulence of the II+$ROP16_I$ might be due to reduced immune
356 pathology. Indeed, we have evidence that ROP16-mediated reduction of type II virulence is
357 especially pronounced in Th1-prone mice such as C57/BL6 (Jensen et al., unpublished).
358 Alternatively, the high induction of the arginase enzyme by ROP16 could deplete L-arginine, for
359 which *T. gondii* is auxotroph, and thereby inhibit *Toxoplasma* growth (Butcher et al., 2011).
360

361 **ROP18.**

362 ROP18 was identified as a rhoptry kinase involved in virulence by two different groups (Saeij et
363 al., 2006; Taylor et al., 2006). Sibley and colleagues were interested in the genes involved in the
364 difference in virulence between the highly virulent type I strain (LD_{100}=1) and the avirulent type
365 III strains (LD_{50} ~ 100,000). Boothroyd and colleagues used the difference in virulence between
366 type II (LD_{50} ~ 500) and type III strains as the basis for their studies. Both groups used crosses
367 between these strains to generate F1 progeny which were subsequently injected in mice to
368 determine their virulence phenotypes. Previously a genetic map was generated for *Toxoplasma*

369 (Khan et al., 2005) and this was used to determine which *Toxoplasma* loci correlated with
370 virulence. Interestingly, the only locus identified using the IxIII cross F1 progeny was ROP18
371 and indeed a type III strain expressing type I ROP18 (III+ROP18$_{I}$) was as virulent as a wild-type
372 type I strain (Taylor et al., 2006). ROP18 kinase activity was necessary to confer virulence as the
373 type III expressing the type I kinase dead mutant was avirulent. The difference in virulence
374 between type II and type III strains was more complicated and implicated five different loci , one
375 of which was ROP18. Indeed a type III strain expressing type II ROP18 (III+ROP18$_{II}$) became
376 extremely virulent (LD$_{50}$~5) (Saeij et al., 2006). The expression level of ROP18 seems to be a
377 key determinant in the strain-specific differences in virulence. Type III strains have an extremely
378 low level of ROP18 expression probably due to an extra 2.1 kb sequence 85 bp upstream of the
379 ATG start codon while type I and II strains express ROP18 at a high level (Saeij et al., 2006).
380 Although both studies demonstrated that ROP18 is secreted into the host cytosol and
381 subsequently traffics back to the PVM, its substrate(s) remained unidentified. When ROP18 is
382 over-expressed in host cells it also traffics to the PVM of invading parasites, showing that it has
383 a strong affinity for the PVM (El Hajj et al., 2007; Reese and Boothroyd, 2009). Like many
384 rhoptry proteins, ROP18 is proteolytically processed. It is initially present as a 60 kDa protein
385 but subsequently is processed to a 56 kDa protein. Indeed it contains the conserved S-Phe-X-E
386 consensus motif (Phe represents bulky hydrophobic residues and X is any amino acid) for
387 recognition by TgSUB2, a subtilisin-like serine proteinase (El Hajj et al., 2007). When
388 comparing the ROP18 genes from multiple strains it was noted that it is one of most polymorphic
389 proteins with evidence for strong diversifying selection. For example, the sequences of type I and
390 type II *ROP18* are very different, with almost no synonymous changes between the type I and
391 type II alleles. Currently there is no evidence for a different function of the different ROP18

392 alleles, because quite diverse ROP18 alleles from several strains all confer virulence to type III
393 parasites when expressed in this avirulent strain(Khan et al., 2009b). It is also unknown if over-
394 expression of the type III ROP18 itself would also confer virulence. Comparison of the ROP18
395 genomic region between *Toxoplasma* and *Neospora caninum*, a close relative of *Toxoplasma*
396 *gondii*, demonstrated that *Neospora* also has the extra region in the promoter of its ROP18. Thus,
397 the most parsimonious explanation is that the ancestral ROP18 gene contained the extra
398 sequence in its promoter resulting in low level expression and subsequently this region was
399 deleted to give rise to the more recently derived type I and type II ROP18 alleles (Khan et al.,
400 2009b). It was also hypothesized that strains carrying the type I ROP18 allele might be more
401 successful because more strains have a ROP18 allele similar to the type I strain; however this
402 could also be due to other strains being more similar to type I strains compared to type II strains .
403
404 **Proposed regulatory mechanism of ROP18.**
405 Protein kinases display a diverse array of regulatory mechanisms that exploit multiple ways of
406 impeding or distorting the binding of ATP, magnesium or protein substrates to prevent catalysis.
407 This often involves a distortion of the inter-lobe orientation, conformational change of the
408 activation loop to block ATP and/or substrate binding, or occlusion of the active site. Activation
409 of the kinase domain often involves phosphorylation (particularly of the activation loop), but
410 may also involve other mechanisms such as binding or dissociation of regulatory domains or
411 subunits (Huse and Kuriyan, 2002; Dar, 2005).
412 The ROP2 and ROP8 kinase domain crystal structures revealed a unique N-terminal subdomain
413 that is conserved in other ROPK family kinase domains and contains features that preclude ATP
414 binding in two of the ROPK pseudokinase structures determined. This ~40 residue N-terminal

415 extension consists of an α-helical segment (packed against the C-terminal lobe) connected via a
416 short linker region to an α-helix and ß-strand packed against the N-terminal lobe. In both 3DZO
417 and 3BYV, an arginine side chain from the N-terminal subdomain linker region (Arg 219 in
418 3DZO and Arg 228 in 3BYV) forms a salt bridge interaction with the side chain of Glu 284 in
419 3DZO and Glu 293 in 3BYV. The glutamate and arginine side chains occupy the expected
420 position of the ATP adenine group. Based on a homology model of the ROP18 kinase domain
421 (with the ROP8 crystal structure as a template), a novel kinase regulatory mechanism was
422 proposed for ROP18, in which the N-terminal subdomain was predicted to impede ATP-binding
423 (as observed in 3DZO and 3BYV). Furthermore, phosphorylation at sites within and near the
424 ROP18 N-terminal subdomain was proposed to regulate this auto-inhibition (Qiu et al., 2009).
425 Similar to other active protein kinases and in contrast to the ROP2 and ROP8 structures,
426 the modeled active site of ROP18 was free of bulky side chains that would preclude ATP
427 binding, with the exception of Gln 214 and Gln 216. The side chains of both residues were
428 predicted to protrude into the ATP binding pocket from the N-terminal subdomain linker region
429 (analogous to Arg 219 in 3DZO and Arg 228 in 3BYV). Consistent with the homology model,
430 mutation of Gln 214 and Gln 216 to Ala resulted in a three to four fold increase in *in vitro* auto-
431 phosphorylation and phosphorylation of myelin basic protein by recombinant ROP18 kinase
432 domain. Activation was then suggested to involve a displacement or conformational change of
433 the N-terminal subdomain to move the side chains of Gln 214 and Gln 216 out of the ATP
434 binding pocket. ROP18 kinase domain expressed in *Escherichia coli* was auto-phosphorylated
435 on Ser 221 and Thr 229 (second helix of the N-terminal subdomain), and on Thr 249 and Thr
436 251 (in contact with the N-terminal subdomain β-strand). Mutation of these Ser and Thr residues
437 to Ala, reduced *in vitro* kinase activity by up to 90% (Qiu et al., 2009). It was therefore

438 proposed that phosphorylation of one or more of these Ser/Thr residues activated ROP18 by
439 altering the interaction of the N-terminal subdomain with the N-terminal lobe to shift the linker
440 region containing Gln 214 and Gln 216 away from the ATP binding pocket. However, several
441 aspects of this proposed auto-inhibitory mechanism require further investigation. Comparison of
442 the available crystal structures, suggests that loss of the salt bridge interaction between the
443 glutamate side chain within the adenine binding pocket (Glu 284 in 3DZO and Glu 293 in
444 3BYV) and the arginine side chain on the N-terminal subdomain linker (Arg 219 in 3DZO and
445 Arg 228 in 3BYV) displaces the N-terminal subdomain linker away from the active site as can be
446 seen in 2W1Z. Since this salt bridge interaction is also not conserved in ROP18, it is unclear if
447 the N-terminal subdomain linker in ROP18 adopts the auto-inhibitory conformation as seen in
448 3DZO and 3BYV. The auto-phosphorylation sites within the ROP18 N-terminal subdomain
449 were mapped using recombinant protein, and whether these sites are phosphorylated *in vivo* and
450 even the extent to which these sites were auto-phosphorylated *in vitro* were not reported. Kinase
451 assays of recombinant ROP18 following phosphatase treatment were not presented, but may help
452 to verify the importance of auto-phosphorylation in activating ROP18. Given the importance of
453 the N-terminal subdomain to the proper folding of ROP18, it remains formally possible that the
454 N-terminal Ser/Thr to Ala mutants decreased kinase activity by destabilizing ROP18 structure.
455 Conversely, the effect of phospho-mimicking mutations of these N-terminal Ser/Thr residues to
456 Asp or Glu on kinase activity were also not reported, but may provide a useful alternative means
457 to test the importance of these phosphorylation sites in activating ROP18.
458
459 **What are the substrates for ROP18?**

460 The effect of $ROP18_I$ on type III virulence could be due to its effect on parasite growth; vacuoles
461 with type III+$ROP18_I$ have on average twice as many parasites at 40hrs post-infection when
462 compared to vacuoles with wild-type type III strain parasites (El Hajj et al., 2007). This effect of
463 ROP18 on parasite growth seems to be vacuole autonomous as only parasites in vacuoles of type
464 III+$ROP18_I$ have a shorter replication time even if they are next to a vacuole with a wild-type
465 type III strain. Because this result was obtained in HFFs, possible host targets for ROP18 should
466 be present in non-stimulated HFFs and are most likely present on the vacuole itself. Besides
467 parasite proteins such as rhoptry proteins and dense granule proteins, several host proteins have
468 also been described to relocalise to the PVM.
469 The most interesting potential substrates, at least in murine cells, would be the immunity-related
470 GTPases (IRGs). These small GTPases are related to dynamins but are highly induced upon IFN-
471 γ stimulation of cells and subsequently traffic to the PVM where they mediate dynamin-like
472 vesiculation and subsequent rupture of the PVM and eventually the destruction of the parasite
473 (Martens et al., 2005; Ling et al., 2006; Melzer et al., 2008). The accumulation of IRGs on the
474 parasite PVM is also a strain-specific phenotype; Type I vacuoles seem to resist high
475 accumulation of these IRGs and indeed type I vacuoles are resistant to destruction, while type II
476 and III vacuoles accumulate high levels of the IRGs and are effectively destroyed by IFN-γ
477 (Zhao et al., 2009a; Zhao et al., 2009b). To investigate if ROP18 had a role in this strain specific
478 difference, the accumulation of IRGs on vacuoles of type III infected cells was compared to
479 vacuoles of type III+$ROP18_I$ but no difference in accumulation of ROP18 was observed (Zhao et
480 al., 2009a). Similarly, transfection of $ROP18_I$ or $ROP18_{II}$ into IFN-γ -induced L929 fibroblasts
481 had no effect on the accumulation of IRGs on type II vacuoles (Khaminets et al., 2010).

482 In contrast to the above mentioned studies it was recently reported that $ROP18_I$ can bind to and
483 directly phosphorylate Irga6, Irgb6 and Irgb10 (Fentress et al., 2011; Steinfeldt et al., 2011),
484 thereby preventing their accumulation on the PVM. For Irga6 it was demonstrated that ROP18
485 can phosphorylate two threonine residues in the switch I loop (T102 and T108) (Steinfeldt et al.,
486 2011), while Irgb6 was mainly phosphorylated on a single threonine residue in its switch I loop
487 (Fentress et al., 2011). Because these threonine residues are at the catalytic interface that is
488 essential for GTP-dependent active dimer formation, it is likely that their phosphorylation
489 inactivates the protein. Indeed, phospho-mimetic mutants of Irga6 were inefficiently loaded onto
490 the PVM and could inhibit loading of wild-type Irga6 (Steinfeldt et al., 2011). Antibodies
491 specific for pT102 and pT108 demonstrated that type I vacuoles indeed contained significant
492 amounts of pT102 and pT108. Type II vacuoles did not contain any pT102 but did contain
493 pT108 although significantly less compared to type I strains. If the phosphorylation of T108 is
494 mediated by type II ROP18 remains to be determined. The phosphorylation of Irgb6 is of
495 particular interest as this is one of the first IRGs recruited to the vacuole and it has been proposed
496 to mediate the subsequent recruitment of other IRGs. Indeed knockdown of Irgb6 resulted in
497 significantly less killing of *Toxoplasma* by IFN-γ stimulated macrophages.
498 It was speculated that the difference between the studies reporting an effect of ROP18 and
499 studies reporting no effect (all studies were performed by the same two groups) was the IFN-γ
500 concentration; only at a low (<1 Unit/ml) IFN-γ concentration was an effect of $ROP18_I$
501 expressed in type III on Irga6 loading on the vacuole detectable. This probably indicates that
502 other polymorphic parasite proteins play a role in resistance to the IRG system, because type I
503 strains can inhibit IRG loading even after stimulation of 200 Units/ml IFN-γ. The specific cell
504 type used might also be important. Sibley and colleagues demonstrated that prevention of IRG

505 accumulation on vacuoles of parasites expressing $ROP18_I$ prevents the killing of *Toxoplasma* by
506 $GR1^+$ inflammatory monocytes or IFN-γ stimulated macrophages.
507 That ROP18 is not the only factor responsible for reduced IRG loading might also explain why
508 type III+$ROP18_{II}$ are as virulent as type III+$ROP18_I$, while the PVM of type II strain itself is
509 efficiently coated by the IRGs. Possibly the type II strain lacks another polymorphic *Toxoplasma*
510 protein, present in both type I and type III, that is necessary for ROP18 activity *in vivo* or it could
511 contain a protein that somehow blocks ROP18 activity. Furthermore, it is unlikely that the IRGs
512 are the only substrates for ROP18 because these IRGs are not expressed in human cells or non-
513 stimulated cells and the effect of ROP18 on parasite growth was reported in un-stimulated HFFs.
514 Based on the ability of bacterially-expressed and refolded ROP18 kinase to phosphorylate 70 kD
515 and 68 kD proteins in heat-inactivated parasite lysates, it has been suggested that ROP18 can
516 also phosphorylate parasite-derived proteins (El Hajj et al., 2007). It is possible that these
517 proteins are in fact ROP2, ROP8 or ROP18 itself as bacterially-expressed versions of those
518 proteins were also shown to be phosphorylated by ROP18. However, whether these proteins are
519 *bona fide* substrates in an infected cell remains unknown.
520 A number of unbiased strategies may be pursued for the identification of substrates or
521 interactors of ROPK family active kinases and pseudokinases (Sopko and Andrews, 2008).
522 Engineering a kinase of interest (by mutation of its gatekeeper residue to Gly or Ala, see below)
523 to accept ATPγS analogues containing bulky substituents, allows chemical tagging of its direct
524 substrates. Subsequent alkylation of the thiophosphate groups allows isolation of the tagged
525 substrates by a thiophosphate ester-specific antibody for identification by mass spectrometry
526 (Allen et al., 2007). Due to the cell impermeability of ATP analogues, substrates must be
527 thiophosphorylated in lysates, where the relative spatial organization of kinases and their

528 substrates can be lost, potentially leading to false positives (Blethrow et al., 2004). Additionally,
529 not all kinases are compatible with gatekeeper mutations required for analogue-sensitivity and/or
530 with thiophosphorylation. A potential problem for the *Toxoplasma* kinome discussed earlier in
531 the present review is the presence of a number of endogenous analog-sensitive kinases, such as
532 TgCDPK1, that inherently have small gatekeeper residues, which may further complicate this
533 approach (Sugi et al., 2010). An alternative method of kinase substrate identification utilizes
534 mass spectrometry to detect changes in the abundance of phospho-sites due to the knockout of a
535 kinase or treatment with a specific small molecule inhibitor, if available (Sopko and Andrews,
536 2008). Due to the effects on possible downstream kinases, substrates identified by this approach
537 may not be direct, although it may be possible to filter out such indirect "false positives" with
538 knowledge of the phosphorylation motif of the kinase of interest. The use of a positional-
539 scanning peptide array (Hutti et al., 2004) to define the substrate preference of ROP18 was
540 recently published (Fentress et al., 2011). Other approaches to identify kinase substrates that are
541 also amenable to identifying interactors of pseudokinases include yeast 2-hybrid and co-
542 purification of ligands / substrates with a tagged kinase or pseudokinase from cell lysates (Sopko
543 and Andrews, 2008). The sensitivity of yeast 2-hybrid can facilitate the detection of transient
544 kinase-substrate interactions, which may be stabilized by the use of a catalytically-inactive
545 kinase bait.
546
547 **ROP5, a pseudokinase essential for *Toxoplasma* virulence.**
548 The analysis of virulence of the F1 progeny from IIxIII crosses identified a locus on *Toxoplasma*
549 chromosome XII as having the largest contribution to the strain-specific differences in virulence.

550 Interestingly, the avirulent type III strain contributed to the enhanced virulence while the more
551 virulent type II strain contained the avirulent locus (Saeij et al., 2006).
552 It was recently demonstrated that the pseudokinase ROP5 is responsible for this difference in
553 virulence (Reese et al., 2011). Initially a completely avirulent F1 progeny (S22, LD_{50}> $1x10^6$
554 parasites) was complemented with a type I cosmid containing ROP5 and a 4 log increase in
555 virulence was noted. However, because the cosmid contained two other genes, a refined analysis
556 was needed. Therefore the ROP5 locus was deleted in a type I strain, and surprisingly this strain
557 was completely avirulent (LD_{50} > $1x10^6$) (Reese et al., 2011). The ROP5 locus is quite
558 complicated and it seems that type II strains contain 10 copies of ROP5, type I: 4 copies and type
559 III: 6 copies. These copies consist of at least three different alleles for each strain, with the type I
560 and III alleles being the most similar. Complementation of the type I knockout with one allele
561 increased virulence, while only complementation with two or more alleles converted the
562 virulence to wild-type virulence. The crystal structure of the $ROP5B_I$ kinase domain showed a
563 canonical protein kinase domain fold and is highly similar to the crystal structures of the ROP2
564 and ROP8 pseudokinase domains (Reese & Boothroyd, 2011). However ROP5 was predicted to
565 be inactive due to the substitution of the Asp residue within the HRD motif (catalytic base) with
566 a basic residue (Lys or His depending on the ROP5 allele). Consistent with this, no detectable
567 kinase activity was reported for recombinant ROP5 protein *in vitro*. The ROP5 pseudoactive site
568 is able to bind ATP, and the crystal structure revealed a distorted ATP binding mode, due to an
569 unusual positioning of magnesium coordination sites. ROP5 is also not predicted to be
570 processed because it lacks the subtilisin cleavage motif. Sibley and colleagues recently made a
571 cross between a type I and a type II strain and reported that a single QTL, containing the *ROP5*
572 gene cluster, controls virulence difference between these strains (Behnke et al., 2011). Indeed,

573 through similar studies as described above they determined that ROP5 determines the differences
574 in virulence between the type I and type II strains. The fact that ROP18 did not determine
575 virulence differences between type I and type II indicates that indeed type I and type II ROP18
576 seem to be functionally equivalent. Although only a single QTL was identified this does not
577 mean that other genes are not involved in determining virulence differences between the type I
578 and type II strains. For example, some of the F1 progeny from the IxII cross contain both the
579 virulent ROP5 and ROP18 alleles but are not 100% lethal indicating that other genes must be
580 involved (Behnke et al., 2011).
581 The mechanism underlying ROP5-mediated virulence is currently unknown. Restoration of the
582 catalytic base within the HRD motif by substitution of the basic residue in the $ROP5A_{III}$ allele
583 with Asp resulted in significantly reduced virulence in mice. However the equivalent substitution
584 in the $ROP5B_{I}$ allele did not restore *in vitro* catalytic activity in recombinant protein, and the
585 basis for the reduced virulence conferred by the ROP5AIII R389D mutant is unclear (Reese &
586 Boothroyd, 2011). To compare the contribution of ROP5 to virulence with that of ROP18, a
587 ROP18 knockout strain was also created. Surprisingly, the type I ROP18 knockout strain was as
588 virulent as the wild-type strain and only a slight delay till death was noted. This seems to
589 contradict the important role of ROP18 in virulence that was previously demonstrated by
590 complementation of the avirulent type III strain, and it also raises the question of the importance
591 of evasion of the IRG system. Indeed, Sibley and colleagues also found that although the
592 RHROP18 KO had a delayed time-till-death phenotype, it was still 100% lethal. It should be
593 noted, however, that ROP18 was knocked out in the RH type I strain while the QTL analysis of
594 virulence was performed using the GT1 type I strain. It was recently noted that these strains
595 behave quite differently; RH has a faster duplication time, it survives much better extracellularly

596 and a significant number of parasite genes is differentially expressed compared to GT1 (Khan et
597 al., 2009a). It would therefore be interesting to remove ROP18 in the GT1 background and
598 investigate its virulence phenotype.
599 Although the ROPK family pseudokinases are not expected to directly phosphorylate target
600 proteins, they may play scaffolding roles in allosteric regulation of active kinases or mediating
601 kinase–substrate interactions (Zeqiraj and van Aalten, 2010). The crystal structure of the LKB1-
602 STRADα-MO25α ternary complex revealed the structural basis of how the STRADα
603 pseudokinase helps to maintain the LKB1 kinase in an active conformation. LKB1 interacts as a
604 pseudosubstrate with regions of STRADα that correspond to the substrate binding site in active
605 protein kinases (Zeqiraj et al., 2009). Consistent with this theme, the substrate binding loops of
606 ROP2 and ROP8 are well ordered and structurally conserved between the 3DZO and 3BYV
607 crystal structures. However, differences in the surface electrostatic potentials in the ROP2
608 (partly neutral and negative) and ROP8 (highly positively charged) pseudosubstrate binding
609 regions suggest different ligand specificities (Qiu et al., 2009).
610
611 **ROP38.**
612 After analysing the complete *Toxoplasma* kinome, Roos and colleagues further characterized the
613 *ROP38* gene for the following reasons: it was predicted to be an active kinase, it was
614 differentially expressed between strains (up >64 fold in type III; >8 fold in type II as compared
615 to type I), it was induced upon differentiation from tachyzoites to bradyzoites, it was triplicated
616 in both *Toxoplasma* and *Neospora*, and there was evidence for convergent evolution of
617 *Toxoplasma* ROP38 and its *Neospora* homologue (Peixoto et al., 2010). Because its expression is
618 very low in type I strains they decided to study its function by over-expressing HA-tagged type I

619 *ROP38* using a tubulin promoter in a type I strain. HA-tag staining of infected cells confirmed
620 colocalization of ROP38 with ROP2 indicating that it is indeed a rhoptry protein. ROP38 was
621 also observed on the PVM, which contrasts with a previously published report that it does not
622 traffic to the PVM (ROP38 corresponds to ROP2L5 in that paper) (Reese and Boothroyd, 2009).
623 It is possible that the over-expression of *ROP38* resulted in mislocalisation, anddefinitive
624 localization will depend on staining with an antibody recognizing endogenous ROP38. To
625 determine if ROP38, like ROP16, modulates host cell gene expression, host microarrays were
626 performed after infection with type I+ROP38$_I$ or wild-type type I. Interestingly, expression of the
627 ROP38 transgene was able to suppress a large proportion of genes that are normally regulated by
628 type I infection. Preliminary experiments suggested that ROP38 might modulate the MAPK
629 pathway, as the kinetics of ERK phosphorylation was different in type I-infected vs type
630 I+ROP18$_I$ infected cells. However, this difference in the modulation of host gene expression
631 seemed to have no consequences for virulence in mice as type I over-expressing ROP38 was as
632 virulent as wild-type type I.
633
634 **The *Plasmodium* kinome**.
635 The *P. falciparum* kinome has been reported to comprise 85 or 99 enzymes, depending on
636 the stringency applied for inclusion of borderline sequences (Ward et al., 2004;Anamika and
637 Krupa, 2005). Both studies concur to present a picture of a the *Plasmodium* kinome as being
638 characterized by significant divergences from the yeast or metazoan kinomes (reviewed in
639 Doerig and Meijer, 2007; Doerig et al., 2010).
640 Most established eukaryotic PK groups have members in *Plasmodium*, although TyrK
641 (tyrosine protein kinases) and STEs (a group of PKs involved in mitogen-activated protein

642 kinase [MAPK] pathways) are not represented. The *Plasmodium* kinome comprises two
643 atypical MAP kinases, but the absence of typical MAPKKs suggests that activity of these two
644 enzymes is regulated in a way that differs from that found in classical eukaryotic MAPKs
645 pathways. These atypical MAPKs are but an example of a number of plasmodial PKs that
646 can be classified as belonging to established ePK families, but cannot be assigned precise
647 orthology with specific mammalian enzymes (for examples, see Abdi et al., 2010;Agarwal et
648 al., 2011; Dorin et al., 2001;Dorin et al., 1999; Reininger et al., 2005; Fennell et al., 2009;
649 Reininger et al., 2009;Halbert et al., 2010;Reininger et al., 2011)). The *Plasmodium* kinome
650 also includes many enzymes that do not cluster with any of the PK groups and families
651 established from the yeast and mammalian kinomes, such as the FIKK kinases, which are
652 specific to apicomplexan parasites (Schneider and Mercereau-Puijalon, 2005), and the
653 CDPKs (see above) (. Interestingly, individual PKs displaying sequence features that are
654 characteristic of distinct ePK groups are found in the *P. falciparum* kinome, illustrating its
655 evolutionary divergence from other kinomes (Dorin et al., 2001;Bracchi-Ricard et al.,
656 2000;Dorin et al., 2005). A notable example of such “composite kinases” is Pfnek-1, which
657 has a clear relatedness to the NIMA family despite the presence of a MAPKK-like putative
658 activation site (Dorin et al., 2001).
659 A systematic kinome-wide knock-out approach in *P. berghei* identified several PKs as essential
660 for development in the mosquito (genetic manipulations are significantly more straightforward in
661 the rodent malaria *P. berghei* than in *P. falciparum*, because (i) gene replacement by double
662 cross-over occurs at a much higher rate in the former than in the latter, and (ii) the selection of
663 transformed parasites occurs in the mouse and is much faster than the *in vitro* cultivation of *P.*
664 *falciparum* (Carvalho and MÃ©nard, 2004)). A similar study has recently addressed essentiality

665 of 62 of the 65 *P. falciparum* ePKs (this study excluded the FIKKs, see above). The inability to
666 knock-out a given locus, together with the ability to modify the allele in a way that does not
667 cause loss-of-function of the gene product (e.g. tagging the C-terminal end with HA epitopes or
668 with GFP), is interpreted as strongly indicative of an essential role of that locus during
669 schizogony. 36 kinases were thus identified as playing a crucial role is erythrocytic schizogony,
670 while 26 are most likely dispensable for this part of the life cycle (with cloned parasite lines
671 having thus far been obtained for 12 of these) (Solyakov et al., Nature Communications, in
672 revision).
673
674 ***Plasmodium* exported kinases.**
675 *Plasmodium* expresses a set of unique kinases, the FIKKs (Ward et al., 2004), 16 of which are
676 predicted to be exported into the RBC because of the presence of a PEXEL motif in their N-
677 terminal region (Schneider and Mercereau-Puijalon, 2005). The roles played by FIKK in the
678 RBC are much less well understood than those of some of the ROPKs in *Toxoplasma*-infected
679 cells discussed above. We know that a significant subset of FIKKs are indeed exported into the
680 RBC shortly following parasite invasion (Nunes et al., 2007; Nunes et al., 2010)and that at least
681 one member of the family remains within the parasite, despite possessing a recognizable
682 PEXEL(Nunes et al., 2007). Most *fikk* genes are located in the sub-telomeric regions of 10 of the
683 14 chromosomes in *P. falciparum*, in the vicinity of the *var* genes that mediate cytoadherence
684 and antigenic variation. The *var* genes are under allelic exclusion control, with a single PfEMP1
685 (*var*-encoded) protein expressed at a time; this appears not to be the case for the *fikk* genes.
686 Interestingly, however, expression of a subset of *fikk* genes was modulated in parasites
687 undergoing a switch in their cytoadherence phenotype (Nunes et al., 2007). Knock-out studies

688 implicated 2 FIKKs in the remodeling of the RBC membrane skeleton, as distinct
689 phosphorylation profiles in ghost preparations of parasite-infected RBCs were detected for each
690 of the knock-out parasite clones (Nunes et al., 2010). In addition to FIKKs, a number of
691 *Plasmodium* kinases have been reported to be exported into the RBC (Kun et al., 1997;
692 Droucheau et al., 2004; Vaid et al., 2010) or even secreted to the extracellular milieu (Singh et
693 al., 2009). Recent data demonstrate that infection with malaria parasites causes a dramatic
694 activation of a signaling pathway involving host RBC PAK and MEK kinases, and that
695 pharmacological interference with these enzymes using highly selective allosteric inhibitors is
696 lethal for the parasite (Sicard et al., 2011). The parasite is also known to interfere with host cell
697 signaling in the liver stages (reviewed in (Luder et al., 2009)), resulting in down-regulation of the
698 NFκB pathway (Singh et al., 2007) and in prevention of apoptosis of host hepatocytes (van de
699 Sand et al., 2005).
700
701 Thus, it appears that in both *Toxoplasma* and *Plasmodium*, two apicomplexan parasites
702 that are phylogenetically widely divergent from each other (Kuo et al., 2008), calcium-regulated
703 protein phosphorylation mediated by CDPKs plays central roles in regulating their entry into and
704 egress from the host and other developmental processes, and that both parasites export proteins
705 into their host cell to tailor it to their own needs. The secretion of virulence factors into the host
706 is a strategy widely used not only by Apicomplexa (a striking further apicomplexan-based
707 example of this concept is the reversible transformation of leucocytes by *Theileria* (reviewed in
708 (Shiels et al., 2006)), but also by many if not all intracellular parasites (reviewed in (Munter et
709 al., 2006)). This not only provides a fascinating fundamental glimpse into unique signaling

710 mechanisms operating within parasites and between parasites and their hosts, but also suggests

711 opportunities for novel therapeutic intervention.

712

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1008 FIGURE LEGENDS

1009 Figure 1: Comparative schematic of activation of CDPKs and CaMKs. The green lines represent
1010 the auto-inhibitory region. CaM = Calmodulin, CAD = CDPK-activating domain. See text for
1011 details. [Reproduced from “A parasite calcium switch and Achilles’ heel revealed. (Doerig and
1012 Billker, 2010)].

1013 Figure 2: The *Toxoplasma* kinome.

1014 Classification of 108 active kinases predicted from the *T. gondii* genome. Black, human and

1015 yeast; blue, *P. falciparum*; red, *T. gondii*. Colored arcs

1016 highlight major kinase groups: AGC, CMGC, CAMK, TKL, CK1, and STE. Red lettering,

1017 apicomplexan-specific groups ROPK (pink) and FIKK. Red circles, kinases with predicted

1018 secretory signal sequence or signal anchor (open, newly recognized); black dots, bootstrap

1019 support > 50%. [Reproduced from (Peixoto et al., 2010)]

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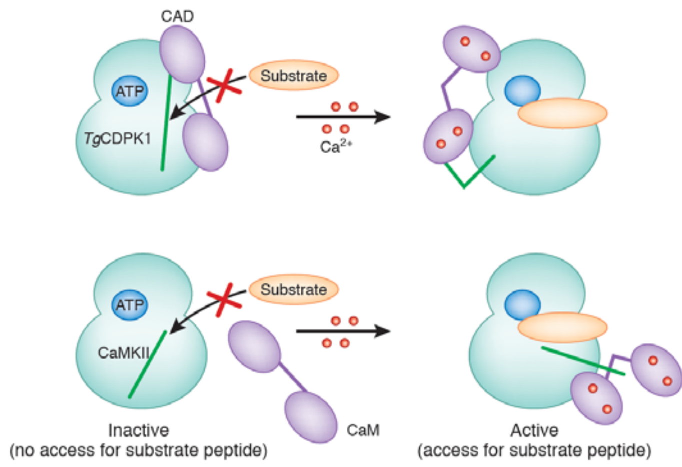
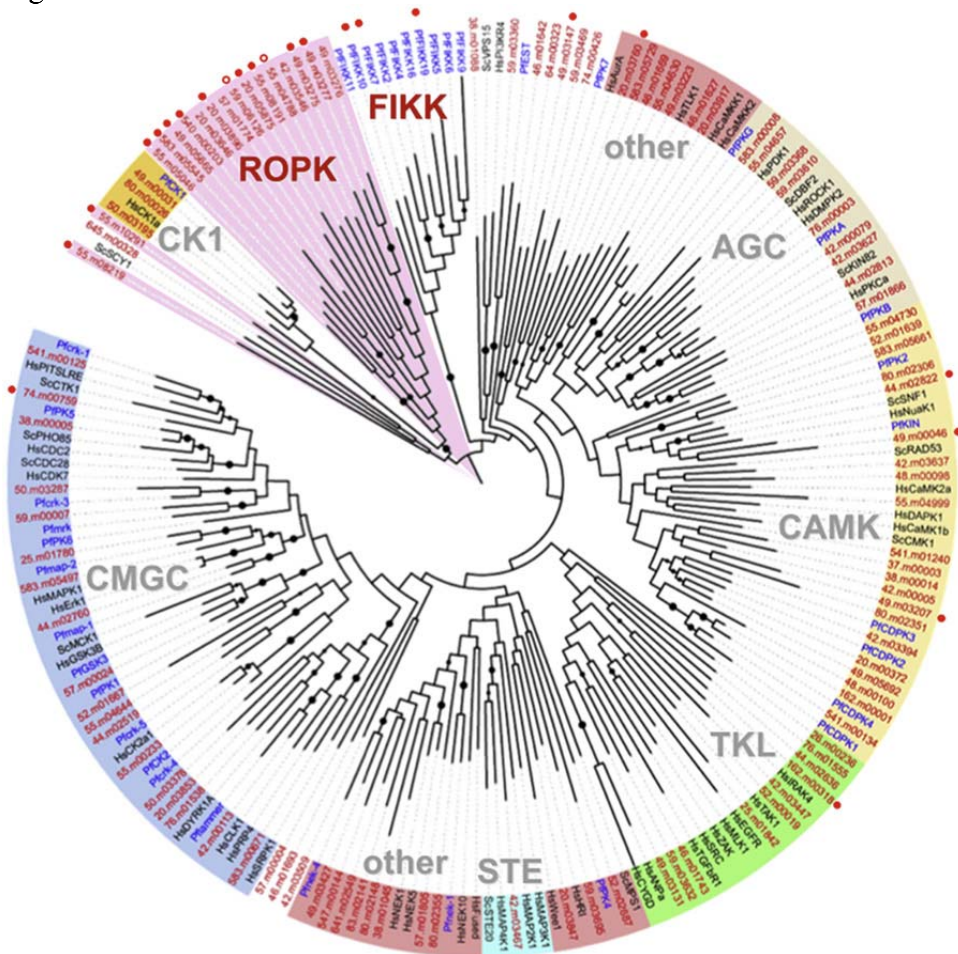
1021

1022 Figure 1.



1036

1037 Figure 2.



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