Subversion of host cellular functions by the apicomplexan parasites

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Abstract
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Subversion of host cellular functions by the apicomplexan parasites

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Abstract

Rhoptries are club-shaped secretory organelles located at the anterior pole of species belonging to the phylum of Apicomplexa. Parasites of this phylum are responsible for a huge burden of disease in humans and animals and a loss of economic productivity. Members of this elite group of obligate intracellular parasites include Plasmodium spp. that cause malaria and Cryptosporidium spp. that cause diarrhoeal disease. Although rhoptries are almost ubiquitous throughout the phylum, the relevance and role of the proteins contained within the rhoptries varies. Rhoptry contents separate into two intra-organellar compartments, the neck and the bulb. A number of rhoptry neck proteins are conserved between species and are involved in functions such as host cell invasion. The bulb proteins are less well-conserved and probably evolved for a particular lifestyle. In the majority of species studied to date, rhoptry content is involved in formation and maintenance of the parasitophorous vacuole; however some species live free within the host cytoplasm. In this review, we will summarise the knowledge available regarding rhoptry proteins. Specifically, we will discuss the role of the rhoptry kinases that are used by Toxoplasma gondii and other coccidian parasites to subvert the host cellular functions and prevent parasite death.

Introduction

Protozoan parasites belonging to the Apicomplexa phylum are ubiquitously spread across the globe. This phylum counts more than 5000 species that are obligate intracellular pathogens of specific hosts probably covering the entire Animalia kingdom. These parasites exhibit complex life cycles often involving multiple cell types and host species. Members of Apicomplexa can be split into groups based on their phylogenetic relationships and host specificities. The Haematozoans are parasites of blood cells that complete the sexual stage of their lifecycle in a vertebrate host and rely on blood-sucking invertebrates for transmission; these parasites are part of the Aconoidasida class (Adl et al., 2005). Several famous coccidian genera are known for their ability to cause severe disease in humans and animals. Eimeria causes disease in poultry (McDonald & Shirley, 2009), Neospora and Isospora are responsible for serious infections in cattle (Goodswen et al., 2012) and pigs (Worliczek et al., 2007), whereas Toxoplasma is capable of infecting a wide variety of species including humans. Cryptosporidium spp. also cause diarrhoeal disease in humans and livestock. The most distantly related subgroup of the Apicomplexa is the Gregarinia. These pathogens of invertebrates (Leander et al., 2003) will not be discussed further in this review.

Although the Apicomplexans infect a very diverse range of hosts, the mechanism of host cell invasion remains relatively conserved. Most invasive stages rely on an active form of movement called gliding motility to propel them-
selves into the host cell. This motility is powered by an actomyosin system (the ‘glideosome’) that is located underneath the plasma membrane of the parasite (Frénal et al., 2010). Essential to this process and for the subsequent survival of the parasite is the timely and sequential release of contents from secretory organelles located at the apical end of the parasite (Carruthers & Sibley, 1997). These secretory organelles are named as micronemes, rhoptries and dense granules (Fig. 1a) (Paredes-Santos et al., 2012).

Micronemes are bar-like organelles that are the first to discharge their contents in response to an increase in the intracellular calcium level within the parasite (Carruthers & Sibley, 1999). The released micronemal proteins (named MICs) are important for host cell recognition and attachment as they establish a tight and specific link between parasite and host cell receptors (Carruthers & Tomley, 2008; Friedrich et al., 2010). Several transmembrane MICs participate in gliding motility by connecting the host receptor(s) to the parasite actomyosin system via binding to parasite aldolase (Jewett & Sibley, 2003; Bosch et al., 2007; Buscaglia et al., 2007; Sheiner et al., 2010).

Rhoptries are club-shaped organelles that participate in invasion and establishment of the intracellular parasitic lifestyle adopted by most species of the phylum. The precise timing, signals and signalling pathways that trigger release of rhoptry proteins are poorly characterised. However, two recent studies suggest that the surface translocation of microneme proteins precedes release of rhoptry proteins during invasion and the functional dissection of the microneme proteins PfEBA175 and TgMIC8 connect them to the triggering event leading to rhoptry discharge in Plasmodium falciparum and Toxoplasma gondii, respectively (Kessler et al., 2008; Singh et al., 2010). In contrast to micronemes and rhoptries, dense granules appear to release their contents in a constitutive fashion, throughout the lytic cycle of T. gondii. In Theileria and Babesia species, dense granule-like organelles have been described that are probably their equivalent, but are called microspheres in Theileria and spherical bodies in Babesia (Shaw et al., 1991; Gohil et al., 2010).

Active host cell entry is initiated by release of micronemes and the invagination of the host cell plasma membrane. Subsequently, the rhoptry organelles discharge proteins and membranous materials as the parasite pulls itself into the cell, contributing to the formation of the membrane known as the parasitophorous vacuole membrane (PVM), a structure that isolates the parasite from the host cell cytosol (Sinai, 2008).

The vacuole is nonfusogenic and hence averts parasite destruction through lysosome fusion (Jones & Hirsch, 1972; Sinai & Joiner, 1997). Although invasion mechanisms are relatively well conserved across the phylum there are some exceptions. Invasion of host cells by Theileria spp., for example, occurs slightly differently. It is important to note that the zoites of this genus are non-motile and therefore they cannot invade in an active, glideosome-dependent manner. Instead, the parasites enter the host cell via a ‘zippering’ mechanism that begins with an irreversible attachment of the parasite to the host cell. Interestingly, Theileria sp. can enter the lymphocytes

![Fig. 1. (a) Schematic representation of Toxoplasma gondii tachyzoite and its secretory organelles. (b) Electron micrograph of the apical pole of a T. gondii tachyzoites showing rhoptries (marked with *). C: Indirect immunofluorescence microscopy on human foreskin fibroblasts infected with T. gondii tachyzoites. Left image shows a scheme representing a rosette of parasites. In the centre image, the rhoptries are stained in green (using anti-TgROP1 antibodies). In the right image, the neck of the rhoptries is stained in red (using anti-TgARO antibodies) and the bulbs of the rhoptries are stained in green (using anti-TgROP1 antibodies). Images courtesy of C. Mueller.](image-url)
in any orientation, whereas many other apicomplexan species must orientate their apical pole allowing the contents of the secretory organelles to be released towards the host. Micronemes are not required to secrete adhesive proteins at the apical end of the *Theileria* parasite and these organelles are consistently absent in sporozoites.

**Rhoptries: specialised secretory organelles with a dual compartment**

Rhoptries are club-shaped organelles located at the apical pole of the parasite (Fig. 1b). They generally consist of a thin electron dense neck region and a bulbous base see Lemgruber et al., 2010 for a recent study on rhoptry structure in *T. gondii*. These secretory organelles are present in most apicomplexan zoites but they vary in number and contents between different species and life stages. For example, the asexual stage zoites of *T. gondii* (tachyzoites) possess 8–12 rhoptries (Boothroyd & Dubremetz, 2008), whereas the equivalent stage in *Plasmodium* spp. (merozoites) have only two (Dubremetz, 2007). Furthermore, the slow growing cyst forms of *T. gondii* (bradyzoites) contain 1–3 rhoptries that are initially labyrinthine-like but evolve to become homogeneously electron dense in older cysts (Dubey et al., 1998). Membranous material released upon secretion of the rhoptry contents contributes to formation of the PVM and parasitophorous vacuole network (PVN). Interestingly, rhoptries are absent in *Plasmodium* ookinetes, reflecting the non-PV forming state of this stage (Tufet-Bayona et al., 2009). At least part of the rhoptry content is injected directly into the host cytosol prior to the release of membranous material. This is thought to occur via a small, transient break in the host cell membrane, which then reseals to prevent lysis of the host cell (Hakansson et al., 2001). In most species, proteins from the rhoptries and dense granules are involved in development and maintenance of the PVM and PVN. Recently, several of these secreted proteins have been implicated in subversion of host functions to sustain a niche in which the parasite is protected from the host defence mechanisms. This new class of effector molecules that includes kinases and pseudokinases will be discussed in the coming chapters.

The journey of rhoptry proteins begins in the secretory pathway. *Toxoplasma gondii* rhoptry proteins traffic through the classical secretory pathway into the premature rhoptries that emerge from the Golgi apparatus and mature concomitantly with daughter cell formation (Soldati et al., 1998). This trafficking is assisted first by a signal peptide for translocation into the endoplasmic reticulum (ER) and subsequently by pro-domains located at the N-terminus of the proteins (Binder & Kim, 2004; Bradley et al., 2005; Peixoto et al., 2010). It has been hypothesised that some prodomains could keep proteins inactive until they reach their final destination, or they may assist protein folding (Soldati et al., 1998; Turetzky et al., 2010).

A proteomic analysis of material from a purified *T. gondii* rhoptry fraction has proven to be very informative, leading to the identification of 38 nonpredictable novel rhoptry proteins (Bradley et al., 2005). The *T. gondii* rhoptry proteins characterised to date exhibit distinct localisations within the organelle, separating between the neck and bulb (Fig. 1c) (Roger et al., 1988; Bradley et al., 2005). This pattern of localisation tends to correlate with functionally distinct groups (Fig. 2).

**Rhoptry neck proteins: RONs**

The RONs refer to the proteins located in the neck of the rhoptry organelles (Bradley et al., 2005). They are the first to be secreted, responding to a triggering signal that likely comes from the discharge of micronemes (Kessler et al., 2008; Singh et al., 2010). Several RONs are involved in formation of the moving junction (Proellocks et al., 2010). This junction is the point of contact between the parasite and host cell plasma membrane that defines the zone of penetration. Some of the RONs have been shown to form a complex at the moving junction and interact with the apical membrane antigen-1 (TgAMA-1), a microneme protein implicated in invasion (Mital et al., 2005), to form a larger complex that the parasite uses as an anchor to pull itself into the host cell. In *T. gondii* this complex is composed of TgRON2, TgRON4, TgRON5, TgRON8 and TgAMA-1 (Alexander et al., 2005; Besteiro et al., 2009; Lamarque et al., 2011; Straub et al., 2011; Tyler & Boothroyd, 2011). Several members of this complex are conserved across the Apicomplexa (Table 1) and this echoes the conserved mechanism of invasion.

**Rhoptry bulb proteins: ROPs**

ROPs refer to the proteins that are located in the bulbous part of the organelle. They are largely involved in development of the parasitophorous vacuole (PV) and PVM (Boothroyd & Dubremetz, 2008). Once inside its protective niche, the parasite is secluded not only from host cell defences but also from host cell nutrients. Rhoptry and dense granule proteins contribute to modification of the PVM to access host metabolites. Importantly in this context, a molecular sieve at the PMV has been reported to allow diffusion of small molecules both in *T. gondii* and *P. falciparum* (Schwab et al., 1994; Desai & Rosenberg, 1997), however, the molecular entity responsible for this channel/pore has yet to be identified. The host ER and mitochondria are also recruited to the periphery of the PVM shortly after invasion (Sinai et al., 1997).
Among the *T. gondii* ROPs secreted into the host cell, several proteins have been identified as key virulence factors that hijack host cellular functions (Saeij *et al.*, 2006, 2007; Taylor *et al.*, 2006). Subsequently, it was shown that these proteins belong to a family of so-called rhoptry kinases (ROPks), described as a large family of coccidian specific rhoptry proteins that possess an active kinase or pseudokinase domain (Peixoto *et al.*, 2010) (Supporting Information, Table S1).

**Repetitive of rhoptry proteins across the phylum of Apicomplexa**

The Apicomplexans have very diverse host cell preferences. It is interesting to review the rhoptry protein repertoire of the Apicomplexan species for which we have information, as their similarities and differences will provide important clues about conserved processes (such as invasion) and the specific requirements for surviving in their particular niche.

**Toxoplasma**

The sequenced genomes of several *T. gondii* isolates plus the relative ease of *in vitro* culture and genetic manipulation of this parasite compared with other Apicomplexans has allowed rapid progress in identification of gene function. This holds true for the RONs described above, which were first shown in *T. gondii* to be assembled as a complex at the moving junction and implicated in invasion (Alexander *et al.*, 2005; Besteiro *et al.*, 2009; Lamarque *et al.*, 2011; Straub *et al.*, 2011; Tyler & Boothroyd, 2011).

Several rhoptry proteins, however, are still of unknown function, TgRON1 is a rhoptry neck protein whose putative homologues outside of *T. gondii* and *N. caninum* in fact share limited sequence similarity. Both the *T. gondii* and *P. falciparum* proteins, however, contain sushi domains that are typical of adhesion proteins. It is possible that although not highly conserved in sequence, these proteins may share similar functions as molecules that are required for the parasite-host interaction. Also TgRON3; this protein was initially described as a rhoptry neck protein but recent evidence has indicated that it is in fact a rhoptry bulb protein (Ito *et al.*, 2011). The function of TgRON8 has only recently been identified. It is an essential member of the moving junction complex that is found in coccidian species only (Straub *et al.*, 2009). Interestingly, it localises to the cytoplasmic face of the host cell membrane where it acts as a firm anchor, holding the remaining MJ complex in place to allow attachment and invasion (Straub *et al.*, 2011). TgRON9 and TgRON10 are newly described members of the RON group that form a high molecular mass complex (Lamarque *et al.*, 2012). They are restricted to coccidian species and do not associate with the moving junction during invasion. Disruption of either member causes degradation of the partner and so complex formation is essential for trafficking to the rhoptry. No effect was observed on rhoptry morphology or parasite growth *in vitro* and virulence *in vivo* upon disruption of the complex (Lamarque *et al.*, 2012). It is possible that this complex is responsible for interactions within a specific range of host cell types.

TgROP1 was the first gene to be knocked out in *T. gondii*. The protein is dispensable and participates in the electron lucent appearance of the rhoptries by electron microscopy (Kim *et al.*, 1993; Soldati *et al.*, 1995). Although the majority of rhoptry proteins that contain a prodomain have a conserved cleavage site, only ROP1 appears to be cleaved by a serine protease of the subtilase family TgSUB2 (Miller *et al.*, 2003).

While TgROP1 is required for rhoptry maintenance, TgROP4 is likely to be involved in PVM maintenance. TgROP4 belongs to the TgROP2 family, localises to the PVM after discharge by the rhoptries and becomes phosphorylated through the action of host or parasite kinases (Carey *et al.*, 2004).

For some time, evidence suggested that TgROP2 could be responsible for recruiting the host mitochondria to the PVM. All members of the TgROP2 family contain a hydrophobic stretch in the C-terminal region that was believed to serve as a transmembrane (TM) region, and the processed N-terminal region of the sequence appeared...
Table 1. Key rhoptry proteins of Apicomplexan parasites

<table>
<thead>
<tr>
<th>Species</th>
<th>Toxoplasma</th>
<th>Neospora</th>
<th>Eimeria</th>
<th>Cryptosporidium</th>
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All homologues for which experimental data are not available were identified using EuPathDB (Aurrecoechea et al., 2007).

1Alexander et al. (2005); 2Marugan-Hernandez et al. (2011); 3Straub et al. (2009); 4Lal et al. (2009); 5Proellocks et al. (2010); 6Cao et al. (2009); 7Bradley et al. (2005); 8Alexander et al. (2006); 9Besteiro et al. (2009); 10Straub et al. (2011); 11Curtidor et al. (2011); 12Peixoto et al. (2010); 13Reid et al. (2012); 14Leriche & Dubremetz (1991); 15Saeij et al. (2011); 16Taylor et al. (2006); 17Valentini et al. (2012); 18O’Keeffe et al. (2005); 19Srivastava et al. (2010); 20Ito et al. (2011); 21Lamarque et al. (2012); 22Cabrera et al. (2012); 23Clark et al. (1987); 24Suarez et al. (1998); 25Suarez et al. (2011); 26Proellocks et al. (2007); 27Wickramarachchi et al. (2008); 28Miller et al. (2003); 29Hajagos et al. (2012); 30Turetzky et al. (2010); 31Poupel et al. (2000); 32Que et al. (2002); 33Rieux et al. (2012); 34Aurrecoechea et al. (2007).
to mimic a mitochondrial import signal. The model suggested that TgROP2 was anchored in the PVM and recruited the host mitochondria at the vicinity of the PVM via the import signal. Recently, however, this model has been disproven. Firstly, the hydrophobic stretch was identified as a kinase domain that folded within the protein based on modelling and structural analyses (El Hajj et al., 2007a, b; Labesse et al., 2009). Secondly, deletion of the locus containing highly similar genes TgROP2a, TgROP2b and TgROP8 caused no aberrant phenotype with regards to recruitment of host organelles (Pernas & Boothroyd, 2010). Coordinated action between proteins from different secretory organelles was described for an association of TgROP2/4 with TgGRA7, although the consequence of this relationship requires further investigation (Dunn et al., 2008).

TgROP13 lacks similarity to other rhoptry proteins (Turetzky et al., 2010). This protein is soluble and released upon parasite invasion. Its function is as yet unknown and although deletion of the gene does not affect parasite virulence in mice, a growth defect is apparent in vitro. Still, it is possible that deletion of TgROP13 gene could indeed cause a virulence defect in vivo but as the AROP13 experiments were completed using a highly virulent RH strain, a virulence phenotype may have been masked.

Not all rhoptry bulb proteins are categorised as ROPs. This part of the organelle also contains various proteases such as TgSUB2 and Toxopain-1 that are implicated in rhoptry protein maturation (Que et al., 2002; Miller et al., 2003). Toxolysin-1 is a secreted parasite metalloprotease that possesses a rhoptry pro-domain at the N-terminus as well as an unusual C-terminal cleavage site. Processing at the C-terminus occurs prior to the trafficking to the rhoptries and appears to be important for proper sorting to the premature organelle (Hajagos et al., 2012). Finally, Toxofilin is an actin-binding protein located in the rhoptry bulb that is capable of sequestering actin monomers (Poupel et al., 2000). It is secreted into host cells during invasion (Lodoen et al., 2010) and has the potential to interact with host actin (Lee et al., 2007). Host cell entry by T. gondii was previously shown to depend exclusively on parasite actin (Dobrowolski & Sibley, 1996), however, recent evidence suggests a role for host actin, in conjunction with a contribution from Toxofilin, in the management of host actin disassembly and invasion kinetics (Delorme-Walker et al., 2012).

Intriguingly, a recent study has shown that T. gondii does not only secrete its rhoptry contents in the host cell to be invaded but also discharges physiologically relevant amounts of rhoptry material into neighbouring cells in vitro and in vivo (Koshy et al., 2012). Such a strategy might have important implications in situation of low parasitaemia where manipulation of multiple host cells by a single parasite could act as a decoy, directing the immune response away from the actual infected cell. It remains to be shown if those parasites that inject their contents into cells go on to invade other cells but.

Neospora

Neospora caninum is the closest relative of T. gondii and comparison of their genomes show a high degree of sequence conservation and synteny (Reid et al., 2012). Neospora and Toxoplasma differ in their intermediate host preferences and their definitive hosts are canids and felids, respectively. Furthermore, Toxoplasma infects virtually any nucleated animal cell whereas Neospora causes disease in cattle. Neospora caninum possesses the RONs involved in moving junction formation (Proellocks et al., 2010; Marugan-Hernandez et al., 2011) and the ROP kinase family is well represented in the N. caninum genome with 34 of the predicted 45 putative ROPKs identified in T. gondii having a homologue (Table S1). NcROP5 and NcROP16 are present but NcROP18 is encoded by a pseudogene (Peixoto et al., 2010). Given the distinct lifestyles of these two parasites, however, it would not be expected for all of the ROPs to be functionally orthologous. Indeed, a recent publication that compared the genomes of T. gondii and N. caninum, reported that significant differences lie within the regions coding for these secreted virulence factors and expression levels vary between the species (Reid et al., 2012).

Eimeria

Putative orthologues of all the known proteins associated with moving junction formation have been identified in Eimeria sp. via proteomics (Lal et al., 2009) and bioinformatics surveys (Proellocks et al., 2010) (Table S1). Proteome analysis of the host cells showed substantial changes in protein levels upon infection with Eimeria, with major differences found in pathways notably including stress response, apoptosis, signal transduction, immune response as well as metabolism (Lutz et al., 2011). At this point, however, no parasite effectors/modulators responsible for these changes have been identified. Recently, several hypothetical proteins that have similarity to T. gondii ROP19/ROP29/ROP38 were identified in a large-scale kinome analysis but these candidates remain to be studied experimentally (Talevich et al., 2011).

Cryptosporidium

Cryptosporidium spp. occupy an unusual niche in the host cell. They are described as residing epicellularly, at the cell
surface but surrounded by membranous, host cell derived folds (Dumenil, 2011). The vacuole is separated from the host cytoplasm by a dense band of material (Huang et al., 2004). Invasion is dependent on the host cell cytoskeleton, which ultimately encapsulates the parasite (Chen & LaRusso, 2000; Elliott et al., 2001). It is pertinent to note here that the genome of these parasites codes neither for the invasion factor AMA1 nor for the RON proteins that are required for the moving junction formation. Little information is available regarding the rhoptries in Cryptosporidium spp. A single rhoptry has been observed to fuse with the host membrane at the attachment and early internalisation process and a dense band of material then borders the host-parasite region of interaction (Huang et al., 2004). Interestingly, parasite-derived material was detected in this dense band and in the PV (Huang et al., 2004). Rhoptry proteins may be involved in triggering the internalisation process and rearrangement of host actin and may participate in the secretion of vesicles that surround the parasite prior to internalisation. The membranous content of the rhoptry is possibly required to generate the PVM and the feeder organelle that develops at the zone of contact between the PVM and host cytosol (Huang et al., 2004). Recently, a protein has been identified as a convincing marker for the rhoptries in C. parvum, however, the role of this rhoptry protein-1 (GpPRP1) is unknown (Valentini et al., 2012).

**Plasmodium**

The lifecycle of malaria parasites includes three distinct invasive stages. Merozoites infect red blood cells and are responsible for propagation of the asexual stages. Ookinetes develop after sexual reproduction in the mosquito, migrate to the gut periphery, traverse the midgut and differentiate to sporozoites in oocysts beneath the basal lamina. Sporozoites are transmitted from the vector to the vertebrate host; they migrate though the skin and reach the liver where they establish infection in hepatocytes. Rhoptry organelles are present in merozoites and sporozoites but absent in ookinetes (Tufet-Bayona et al., 2009) and the majority of information available on Plasmodium rhoptries focuses on the merozoite stage.

In *P. falciparum*, the rhoptries have recently been divided into three segments based on the sequence of protein release and concerns mainly those proteins involved in attachment and invasion (Cowman et al., 2012), however, further work is required to conclusively show this organisation.

*Plasmodium falciparum* shares few functionally homologous rhoptry proteins with other Apicomplexans, however, among them are PfRON2, PfRON4 and PfRON5 that are involved in moving junction formation (Table 1). In contrast, there is no homologue of TgRON8 outside the Coccidia (Alexander et al., 2006; Cao et al., 2009; Curtidor et al., 2011; Riglar et al., 2011). PIRON6 whose function is unknown, is remarkably conserved across all apicomplexan species studied to date (Proellocks et al., 2010). RON1 and RON3 are not as well conserved and may be genus specific. PIRON3 has been re-designated as a rhoptry bulb protein that interacts with PIRON2 and PIRON4, but not with PIAMA1 (Ito et al., 2011). This protein could perhaps act as a chaperone for these RONs.

The malaria parasites rely on alternative pathways for invasion of erythrocytes involving different host receptors and multiple RBC binding proteins. Rhoptry proteins named as reticulocyte binding-like proteins (RBLs) contribute to the alternative invasion pathways (Duraisingh et al., 2003; Stubbs et al., 2005). In *P. yoelii* a RBL multi-gene family, the Py235 family, has as many as 50 members (Gruner et al., 2004). These proteins are known as the reticulocyte-binding homologue family (PfRh) in *P. falciparum* (Kaneko, 2007; Proellocks et al., 2010). PfRh5 is a rhoptry neck protein, which localises to the moving junction during erythrocyte invasion by merozoites (Baum et al., 2009). PfRh5, has recently been shown to bind to Basigin, an erythrocyte surface protein that is an essential receptor for erythrocyte invasion (Crosnier et al., 2011). Another protein, the *P. falciparum* asparagine-rich protein (PfAARP) was shown to bind RBCs via its N-terminal region (Wickramarachchi et al., 2008).

Several rhoptry bulb proteins have been identified. A high molecular weight rhoptry complex composed of RhopH1/Clag, RhopH2 and RhopH3 has been described. Five distinct genes encode the RhopH1/Clag protein and the complex may contain any one of the members of this multigene family in combination with RhopH2 and RhopH3. Furthermore, the complexes are not mutually exclusive, multiple versions can be expressed in parallel (Kaneko, 2007). The complex is secreted from the rhoptry and associates first with the RBC membrane, then the PVM (Sam-Yellowe & Perkins, 1991). This complex might contribute to the development of the membranous network in the erythrocyte cytosol for the acquisition of lipids and other nutrients (Kaneko, 2007).

The rhoptry associated membrane antigen (RAMA) is a bulk protein that has been implicated in trafficking of other rhoptry proteins to the developing rhoptries during biogenesis (Topolska et al., 2004a, b). RAMA secreted from merozoites during invasion binds to the RBC membrane and then soon after invasion can be found associated with the PV (Topolska et al., 2004a, b).

The rhoptry-associated proteins RAP1-3 are secreted from the rhoptry bulb as a complex and they are not involved in invasion (Kats et al., 2006). It has recently been proposed that they target non-infected erythrocytes.
and erythroblasts, leading to their malformation/destruction by the immune system, and ultimately exacerbating anaemia due to reduced levels of RBCs (Awah et al., 2009).

The functions of the rhoptry bulb proteins PfRhop148 and Pf34 are not yet known, although, like RAMA, they may be involved in recruiting rhoptry contents to the organelle during their formation (Lobo et al., 2003; Proellocks et al., 2007). It has also been suggested that Pf34 can act as an adhesive at the surface of the RBC on the basis of peptide binding assays with RBCs (Arevalo-Pinzon et al., 2010). Finally, NHE, a putative orthologue of the T. gondii sodium/hydrogen exchanger (TgNHE2) has been identified and may be responsible for regulating osmotic pressure and pH within the rhoptry (Kats et al., 2006).

Most recently, a rhoptry protein anchored by acylation at the surface of the rhoptry organelles, has been described. This Armadillo Repeat Only protein PfARO, is highly conserved among all Apicomplexans and its unusual localisation suggests a role in organelle biogenesis (Cabrera et al., 2012).

Very little is known about the composition and contribution of the rhoptries in sporozoites, which traverse several hepatocytes before establishment of infection (Vanderberg et al., 1990; Mota et al., 2001). Rhoptry content is unlikely to be secreted during migration but expected to be required for formation of the parasitophorous vacuole. RON2 and RAP2/3 are conserved in sporozoites suggesting a conserved mechanism of invasion with merozoites (Lasonder et al., 2008; Tufet-Bayona et al., 2009). The sporozoite-specific thrombospondin-related sporozoite protein (TRSP) has been described as a putative rhoptry protein (Kaiser et al., 2004) with a role in sporozoite invasion (Labaied et al., 2007). A more comprehensive characterisation of the rhoptry content in sporozoites is needed to extend the extent of the role of rhoptries in the liver stage of the malaria parasites.

**Babesia**

Blood-sucking ticks transmit Babesia parasites and once in the bloodstream, the sporozoites directly invade RBCs (Yokoyama et al., 2006). This is in contrast to Plasmodium species, in which the sporozoites first invade hepatocytes and go through an initial round of replication before the merozoites enter the bloodstream and invade erythrocytes. Several rhoptry proteins have been identified in Babesia but none have so far been shown to modulate or modify the host cell.

The RAP-1 locus consists of two identical tandem genes (Suarez et al., 1998) that code for a protein, the structure of which is conserved in Babesia species (Yokoyama et al., 2006) and that is expressed at all invasive and developmental life stages (Yokoyama et al., 2002). The protein is highly immunogenic and specific antibodies block sporozoite attachment to RBCs (Mosqueda et al., 2002). Specific antibodies also reduce binding of Babesia bovis RAP-1 to RBCs in binding assays and inhibit parasite proliferation in vitro (Yokoyama et al., 2002). These data point toward an important role of BbRAP-1 in parasite attachment to host cells, however, the receptor that RAP-1 recognises still remains to be identified.

RAP-1 related antigen (BbRRA) is believed to be a functional equivalent of RAP-1 (Suarez et al., 2011). The authors hypothesise that BbRRA is produced at low levels to minimise the host-immune response against it. That way, an immune response will be mounted against the highly immunogenic BbRAP-1 as a decoy, and BbRRA will still be able to function as an invasion factor, allowing the parasites to invade successfully. Functional data for the role of both BbRRA and BbRAP-1 still require further investigations.

BbRhop68 is homologous to PfRhop148 (Lobo et al., 2003; Baravalle et al., 2010), which is suggested to play role in rhoptry biogenesis (Kats et al., 2006). This protein has two predicted transmembrane domains and is detectable only in intracellular parasites and not on free merozoites (Baravalle et al., 2010).

**Theileria**

Two of the three invasive stages of Theileria are nonmotile and as such they have a slightly different morphology when compared with other well-studied apicomplexan zoites. The sporozoites are nonmotile, exhibit a reduced microtubule network, lack a conoid, have no inner membrane complex (flattened vesicles underneath the plasma membrane) and no micronemes (Shaw, 2003). Contact with the host cell occurs by chance, attachment is irreversible, and entry occurs via ‘zippering’ of host membrane around the parasite, consequently, the PVM is completely host derived. Reorientation is not required and organelles are not discharged.

Theileria and Babesia are unusual as they escape the vacuole and resides and proliferate in the host cell cytosol. Failure to escape the PV within 30 min leads to parasite death (Shaw, 2003). For this reason, it is unsurprising that effector molecules are not released during invasion as they can be secreted directly into the host cytosol after disintegration of the PVM. Rhoptry organelle release in Theileria precedes detachment of host membrane from parasite surface and its subsequent dissolution (Shaw, 2003). It is likely that rhoptry contents are required for destruction of the vacuole membrane rather than for its construction and maintenance. At the time of
vacuolar membrane dissolution, a fuzzy coat appears on the surface of the parasite, which ultimately associates with host microtubules that accumulate around it. This material may contain rhoptry contents. A single 104 kDa rhoptry protein has been identified although its function is as yet unknown (Iams et al., 1990; Ebel et al., 1999). The publication of the sequenced genomes for two species should encourage progress in the identification of more rhoptry proteins (Gardner et al., 2005; Pain et al., 2005).

To date a diverse range of rhoptry proteins have been identified and characterised across the Apicomplexans. Some of these proteins are believed to be responsible for biogenesis of the organelle, invasion, PV formation and modulation of the host intracellular environment. These repertoires are far from being complete and many more rhoptry proteins are likely to be identified and associated with unanticipated subversive functions.

**Toxoplasma rhoptry proteins and host manipulation**

**Identification of secreted ROP kinases and their role in virulence**

In Europe and North America three major *T. gondii* lineages are predominant, types I, II and III (Howe & Sibley, 1995). The three types differ widely in a number of phenotypes in mice such as virulence, persistence, migratory capacity, attraction of different cell types and induction of cytokine expression (Saeij et al., 2005). In immunocompetent individuals, infection with *T. gondii* tends to be chronic and asymptomatic, as parasites differentiate to the slow growing bradyzoite form and encyst in tissues. Types I and II, however, are associated with congenital birth defects and abortion in first-time mothers who become infected during pregnancy. They are also linked with severe disease in immunocompromised individuals such as people with HIV/AIDS, those undergoing chemotherapy or recipients of organ transplants (Sibley & Ajioka, 2008). Type III parasites are significantly less virulent and infrequently cause disease, even in the immune compromised.

Although most likely coming from a common ancestor in North America, the South American parasite population structure is slightly different (Lehmann et al., 2006; Sibley & Ajioka, 2008). A larger diversity, probably due to increased recombination events, defines this population. In South America, a major problem from *T. gondii* comes in the form of recurrent ocular infections that appear in otherwise healthy adults (Jones et al., 2006; Khan et al., 2006). A total of 11 haplotypes exist within the *T. gondii* population (Sibley & Ajioka, 2008), this chapter will focus on the three main strains that are found in Europe and North America, Types I, II and III.

Microarray analysis of *T. gondii* infected host cells indicate changes in transcription that can be attributed to the parasite (Blader et al., 2001). This information, combined with a wealth of data gleaned from genetic crosses of the three main European *T. gondii* lineages (Saeij et al., 2006; Taylor et al., 2006; Behnke et al., 2011; Reese et al., 2011) and large scale proteomics analyses (Bradley et al., 2005), has led to the identification of several proteins important for the differences in virulence between parasite types (Table 2). These proteins are members of the ROPK family and they sit in what have been called VIR loci. Briefly, the VIR1 locus codes for a rhoptry pseudokinase (TgROP5) that is present as a tandem array of gene duplications (Saeij et al., 2006; El Hajj et al., 2007a, b), the VIR3 and VIR4 loci code for active, secreted rhoptry kinases (TgROP18 and TgROP16 respectively) and VIR2 and VIR5 have not yet been examined in detail (Saeij et al., 2006; Taylor et al., 2006). Interestingly, TgROP5, TgROP16 and TgROP18 all have a higher than average level of polymorphism across the three main *T. gondii* strains (Peixoto et al., 2010). Within the ROPK family, some genes are expressed at a higher than average level as identified by transcript abundance and there are a greater number of genes regulated in a strain-specific manner (Peixoto et al., 2010). This information is pertinent when viewed in the context of three strains with significantly different degrees of virulence and gives weight to the theory that ROPKs are important for this. Table S1 shows the conservation of the ROPKs in coccidian species *N. caninum* and *E. tenella.*

**Immune response to intracellular pathogens**

Prior to considering some of the key effector molecules shown to be critical for manipulation of the host to promote parasite survival, it is first important to understand the influence of *T. gondii* mediated changes that occur upon infection of host cells and the host response to intracellular pathogens. This is a very complex situation and micro array analyses of infected host cells highlight a large number of genes that are regulated differentially depending on the infecting *T. gondii* type and this number also varies based on the host cell type (Saeij et al., 2007; Jensen et al., 2011). However, there are some meaningful patterns and features. Many, but not all, of these gene products are

<table>
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<th>Table 2. ROPK virulence determinants in <em>T. gondii</em></th>
<th>Type I</th>
<th>Type II</th>
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<tr>
<td>ROP5</td>
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<td>ROP16</td>
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Presence of ROP5, ROP16 and ROP18 forms in the three Toxoplasma gondii lineages: -, inactive; +, minimally active; ++, highly active.
identifiers of classical or alternative macrophage differentiation (Jensen et al., 2011) including members of NFκB signalling pathways and those that lead to the activation of signal transducer and activator of transcription (STAT) factors (Saeij et al., 2007). Fittingly, one function of STATs is the regulation of genes involved in the immune response to intracellular pathogens (Quinton & Mizgerd, 2011).

Invasion into a host cell by T. gondii initially causes an increase in expression of pro-inflammatory cytokines such as IL-12. It is interesting to note that IL-12 levels vary depending on the infecting parasite strain, likely because of the combination of effector molecules produced by particular strains and their downstream effects on the host (Robben et al., 2004) (Table 2). IL-12 is mainly produced from innate immune cells such as macrophages and dendritic cells, and is involved in T helper 1 (Th1) immunity. Upon host cell infection, IL-12 stimulates activation of STAT4 and results in the differentiation of naïve T cells into Th1 cells, which along with natural killer cells, produce large amounts of IFN-γ (Murphy et al., 2000). IFN-γ in turn activates STAT1, culminating in the expression of a number of effector genes such as p47 GTPases (Leung et al., 1996; Takaoka & Yanai, 2006), which go on to aid in the destruction of the parasite (Taylor et al., 2004). Thus, the STAT4-STAT1 axis is essential for host defences against intracellular pathogens such as T. gondii. Indeed, studies observing the immune response of mice lacking Stat4 or Stat1 found they were highly susceptible to T. gondii infection (Cai et al., 2000; Gavrilescu et al., 2004; Liebermann et al., 2004). The parasite lines used in these studies were virulent types I and II, it would therefore be interesting to know if these mice also became more susceptible to the avirulent type III parasites. Intriguingly, T. gondii infection of human foreskin fibroblasts (HFFs) results in expression of interferon inducible genes (Kim et al., 2007) regardless of the fact that HFFs do not produce IFN-γ. This indicates that the parasite stimulates expression of these genes in an alternative fashion; however, for this trigger for this activation remains to be determined. One could suspect that other IFNs such as the type I interferons could trigger a similar response as IFN-γ, however, there is no significant expression of these IFNs in HFFs and this has never been shown. It would be of interest to assess if the expression of interferon inducible genes is also observed upon infection of murine fibroblasts with T. gondii. Murine fibroblasts lack Ifiβ and Ifi7, which are essential for the induction of type I interferons (Honda et al., 2006), making this a useful system to identify if type I IFNs play a role in this context. It has also been suggested that pro-inflammatory cytokines themselves could be capable of activating IFN-responsive genes (Kim et al., 2007), although this also requires further investigation.

As discussed above, invasion of T. gondii causes a pro-inflammatory response. In a normal immune setting, an anti-inflammatory reaction will also be stimulated to temper the pro-inflammatory reaction and prevent it from causing damage to the host. STAT3 is activated in innate immune cells through the action of the anti-inflammatory cytokine IL-10. In T. gondii-infected mice, IL-10 is secreted from immune cells (Murray, 2006), predominantly conventional CD4+ T cells (Jankovic et al., 2007, 2010). Meanwhile, Th2 cytokines IL-4 and IL-13 induce activation of STAT6 and eventually compete or even down-regulate the Th1 pro-inflammatory response. Together, STATs are regulated by various cytokines and can function in opposite ways to produce a balanced anti-T. gondii immune response.

STATs are fully activated through phosphorylation of a conserved tyrosine residue. Regulation of the STAT pathways occurs via a negative feedback mechanism composed of negative regulators, such as suppressors of cytokine signalling (SOCS) (Butcher et al., 2011; Tamiya et al., 2011). SOCS1 inhibits the kinase activity of JAK1 and JAK2, both of which are tyrosine kinases upstream of STAT1 and STAT2. This leads to the abrogation of STAT1 phosphorylation (Kobayashi & Yoshimura, 2005) and therefore abolition of antiparasite effector molecule stimulation.

To counteract the response against it, T. gondii induces SOCS1, which consequently suppresses the IFN-γ/STAT1-mediated cellular antiparasite response (Zimmermann et al., 2006). Another negative regulator of STATs, SOCS3, inhibits STAT3 phosphorylation by interfering with the association between JAKs and cytokine receptors (Kubo et al., 2003). SOCS3 is required for inhibition of IL-6/STAT3-mediated anti-inflammatory programs in innate immune cells. Mice lacking SOCS3 specifically in myeloid cells show reduced IL-12 production, and so reduced pro-inflammatory response, to T. gondii infection (Whitmarsh et al., 2011). This results in a failure to control acute toxoplasmosis. Thus, SOCS indirectly block phosphorylation of STATs, inhibiting their activation. For recent reviews on the innate immune response to intracellular pathogens and STAT signalling refer to (Sehgal, 2008; Rasmussen et al., 2009; Barber, 2011; Peng et al., 2011).

**TgROP16: Suppression of host Th1 immunity via STAT3 and STAT6 activation**

Strain-dependent STAT3 activation by TgROP16

TgROP16 is an active kinase that possesses a nuclear localisation signal and localises to the host cell nucleus rapidly after invasion (Fig. 3a) (Saeij et al., 2007; Ong...
et al., 2010). The TgROP16 gene of type II parasites is highly divergent when compared with types I and III and cross-type gene replacement experiments cause significant changes in virulence (Saeij et al., 2006). Type I and type III, but not type II, parasite infection induces phosphorylation of STAT3 and STAT6 (p-STAT3 and p-STAT6). Furthermore, type I parasites lacking TgROP16 fail to activate both STATs, indicating that TgROP16 is critically involved in the activation of STAT3 and STAT6 during infection. Regarding the molecular mechanism of the strain-specific STAT3 activation, over-expression of a type I TgROP16 allele (ROP16I) in a type II parasite line produces a type I profile of p-STAT3 and p-STAT6 (Saeij et al., 2007). In addition, ectopic expression of type I TgROP16, but not type II TgROP16, induces strong STAT3 activation. In vitro experiments using chimeric constructs indicate that a single amino acid change on residue 503, determines the strain-dependent difference of STAT3 activation (Yamamoto et al., 2009). In silico structural modelling of the active site of multiple TgROP16 variants showed that this residue is centrally located within the kinase domain and therefore the mutation could reduce the efficiency of binding or functionality of the active cavity (Yamamoto et al., 2009).

STAT3 and STAT6 activation by ROP16

Heterologous overexpression of parasite ROP16 alone in mammalian cells strongly induces activation of STAT3-dependent promoters and STAT3 phosphorylation in a kinase activity-dependent fashion. In addition, the N-terminal portion (220-300) of TgROP16 associates with STAT3, and the tyrosine 705 residue on STAT3 is phosphorylated by immunoprecipitates from wild-type, but not kinase-inactive, TgROP16 expressing mammalian cells (Yamamoto et al., 2009). Although this could be a direct interaction with TgROP16 phosphorylating the STATs, the possibility also exists that there is an intermediate kinase that is activated by TgROP16, one of the JAKs, for example, which in turn phosphorylates the STATs. Initial STAT3 activation upon parasite invasion appears to be TgROP16 independent as p-STAT3 is present at early time points after cells are infected with Arop16 parasites (Butcher et al., 2011). However, the maintenance of p-STAT3 does appear to require TgROP16; p-STAT3 is not detectable 90 min (Butcher et al., 2011) and 3 h (Yamamoto et al., 2009) after infection with a Arop16 strain. Intriguingly, activation of STAT3 was almost completely abrogated when JAK2 was blocked with an inhibitor (Butcher et al., 2011), suggesting that TgROP16 does target kinases upstream in the signalling pathway. However, this result must be considered with caution, as it has recently been demonstrated that in vitro TgROP16 mediated tyrosine kinase activity is also blocked >90%, by a pan-JAK inhibitor (Ong et al., 2010).

In contrast to the case for STAT3, activated STAT6 (p-STAT6) is detectable in the nuclei of infected host cells at around 1 min postinvasion of type I-WT but not type I-Arop16 parasites (Ong et al., 2010). The classical activation of STAT6 requires around 5 min, so the rapidity of STAT6 phosphorylation upon parasite invasion suggests that the normal signalling pathway leading to STAT6 activation is bypassed, and TgROP16 can do the job directly. This theory is supported by the complete inability of Arop16 parasites to phosphorylate STAT6 (Butcher et al., 2011), and the fact that blockage of kinases upstream of STAT6 does not prevent STAT6 phosphorylation upon parasite invasion (Ong et al., 2010). It must be noted that

![Fig. 3. (a) Scheme of TgROP16 protein, (b) Role of TgROP16 and TgGRA15 in the manipulation of the host-immune response.](image-url)
the JAK kinases may work redundantly in the activation of STAT6 so removing one may not abrogate function. In vitro assays using recombinant TgROP16, however, demonstrated that this kinase can recognise and phosphorylate the critical STAT6 Tyr residue, and immuno-precipitation experiments confirmed the existence of an interaction between TgROP16 and STAT6 (Yamamoto et al., 2009; Ong et al., 2010).

Infection of cells with parasites of types I and III leads to a sustained STAT activation and a suppression of pro-inflammatory cytokines such as IL-6 and IL-12 that are key to the host’s fight against T. gondii (Fig. 3b) (Hunter & Remington, 1995; Scharton-Kersten et al., 1995).

TgROP16 possesses a NLS in the N-terminus and translocates to the nucleus after the injection into host cells. Type I parasites expressing TgROP16 that lacks the NLS (ROP16ΔNLS) still induce activation of STAT3 and STAT6, and ectopic expression of recombinant ROP16ΔNLS in 293T cells also triggers p-STAT3 (Saeij et al., 2007; Yamamoto et al., 2009), suggesting that the NLS on TgROP16 is dispensable for STAT activation. Currently, the biological significance of the nuclear translocation of TgROP16 remains unclear, however, it would be interesting to study whether ectopically expressed type I TgROP16 lacking the NLS can sustain the activation of STAT3 and STAT6 for a physiologically relevant duration. Thus, TgROP16 plays an important role in activation of STAT3 and STAT6, which is dependent on the kinase activity, but appears to be independent of the NLS.

**Macrophage polarisation by TgROP16**

Recent data have begun to clarify the extent to which type I/III and type II differ in the immune response that they provoke. Closer inspection of the TgROP16-mediated host changes during infection with type I parasites revealed increased levels of arginase-1 (Arg-1) due to TgROP16-mediated STAT6 activation (Butcher et al., 2011; Jensen et al., 2011). Arg-1 consumes L-arginine to produce urea and ornithine for normal cell metabolism. L-arginine is also an essential substrate of inducible nitric oxide synthase (iNOS) that converts it instead to nitric oxide (NO), which severely inhibits parasite growth in PVs. A parasite-dependent increase in Arg-1 levels would compete L-arginine away from iNOS and reduce NO levels, thereby protecting the parasite.

The induction of arginase-1 is a result of type I/III parasites promoting alternative (M2) activation of macrophages via STAT3 and STAT6. This not only leads to increased arginase levels, but M2 polarisation of macrophages results in production of anti-inflammatory molecules that counteract Th1 immune responses and down-regulate the anti-T. gondii host defence (Martinez et al., 2009). Micro-array analyses of type II (ROP16 deficient) and type III (ROP16 sufficient) parasites confirmed an increase in a number of M2 polarisation markers in type III when compared with type II parasites (Jensen et al., 2011). Type I and III parasites therefore use TgROP16 to stimulate STAT3 and STAT6, which promotes an anti-inflammatory response via M2 activated macrophages and through an increase in host arginase-1 levels.

On the other hand, infection of macrophages by type II strains induces pro-inflammatory cytokines including IL-1β, IL-6 and IL-12 p40 (Jensen et al., 2011), largely suggesting ‘classical activation’ (or M1 polarisation) of macrophages. M1 activation is a normal cell response that occurs upon invasion of host cells by parasites through triggering of toll-like receptors (TLRs), which ultimately promote a pro-inflammatory environment [reviewed in (Pifer & Varovinsky, 2011)]. During type II infection this is also stimulated by dense granule protein TgGRA15-mediated NFκB signalling. TgGRA15 is a polymorphic dense granule protein that is responsible for the stimulation and translocation of NFκB and it is most active in type II parasites (Rosowski et al., 2011). Once in the nucleus NFκB encourages the transcription of M1 polarisation markers and increases further pro-inflammatory cytokine production. In this case, M2 polarisation is actively blocked (Jensen et al., 2011).

The M1 macrophage polarisation by type II parasites may be beneficial. This response could prevent excessive spread of the parasite that would lead to host death, and the balanced anti-inflammatory response would prevent host tissue damage, promote persistence and result in chronic infection. Thus, the presence of TgROP16 together with TgGRA15 in T. gondii is an important determinant for polarisation of the infected macrophage and determination of the immune response.

**TgROP16 and intestinal inflammation**

ROP16 plays an important role not only in macrophage polarisation in vitro but also intestinal inflammation in vivo (Jensen et al., 2011). Susceptible C57BL/6 (B6) mice infected perorally with type II strains results in severe intestinal inflammation (ileitis) and eventual death during acute infection (Liesenfeld et al., 1996). Contrary to this, almost all B6 mice infected with type II strains expressing type I or type III TgROP16 survived and had reduced intestinal inflammation with less granulocyte infiltration into the lamina propria (Jensen et al., 2011). Toxoplasma gondii ileitis can be treated by eliminating effector cytokines such as IFN-γ, IL-23 and IL-22, suggesting the role of these cytokines in pathology (Liesenfeld et al., 1996; Munoz et al., 2009). In B6 mice infected with type II strains, lymphocytes from Peyer’s Patch produced...
increased amounts of IFN-γ and IL-22, when stimulated by anti-CD3 and CD28, compared with those infected with type II strains expressing type I TgROP16 (Jensen et al., 2011). Thus, these results clearly demonstrate that TgROP16 limits intestinal inflammation by T. gondii infection in vivo.

In summary, the results gathered so far provide strong evidence to suggest that TgROP16 has a direct and important role in the modulation of host-immune responses in vitro and in vivo, via interaction with the STAT pathways. By secreting proteins that interact with STAT pathways, the parasite can manipulate the host-immune response and protect itself from destruction.

TgROP18: Suppressor of innate and acquired immunity

TgROP18 is required for in vivo virulence

TgROP18 (Fig. 4a) is an active, secreted rhoptry kinase that shares a considerable degree of homology with the TgROP2 family of S/T kinases (25% identity with ROP2) (Saeij et al., 2006; Taylor et al., 2006; El Hajj et al., 2007a, b). It is released into the PV during invasion and localises to the PVM. When parasite invasion is blocked with Cytochalasin D, an inhibitor of actin polymerisation, TgROP18 is found to be associated with whorls of rhoptry material that are secreted into the host cell and contribute to PV/PVM formation (Hakansson et al., 2001; Taylor et al., 2006; El Hajj et al., 2007a, b). Furthermore, epitope-tagged TgROP18 ectopically expressed in the cytosol of mammalian cells, redistributes to the PVM upon infection of the cells with T. gondii, indicating a high affinity of TgROP18 for the PVM (El Hajj et al., 2007a, b). As with a large number of other rhoptry proteins in T. gondii, TgROP18 is produced as a pro-protein. The N-terminal pro-region is cleaved during trafficking to the rhoptry, and cleavage is predicted to occur via the action of TgSUB2 (Miller et al., 2003). TgROP18 also contains several arginine-rich repeat regions after the cleavage site, whose basic nature is involved in PVM association (Labesse et al., 2009).

TgROP18 is highly polymorphic between types I-III; the amino acid sequence of type III TgROP18 (ROP18III) is 14% divergent when compared with the type I sequence (ROP18I) (Taylor et al., 2006) and the mRNA is expressed at a level 10,000-fold lower than that of type II (ROP18II) (Saeij et al., 2006). Sequencing of the TgROP18 gene and promoter region of the three T. gondii types revealed a large insertion (2.1 kb) 85 bp upstream of the start codon in the type III strain that is likely to be responsible for the greatly reduced levels of mRNA. The high level of polymorphism in ROP18III could also produce a protein deficient in substrate binding (Saeij et al., 2006; Taylor et al., 2006). Expression of a second copy of ROP18I (Taylor et al., 2006) or ROP18II (plus promoter) (Saeij et al., 2006) in a type III strain produces parasites that are more virulent in the mouse model when
compared with the type III parental strain. Type I parasites expressing an additional copy of ROP18I or type III parasites expressing a type I TgROP18 (type III+ROP18I) have a faster growth rate than their WT counterparts (Taylor et al., 2006; Fentress et al., 2010) in vitro and in vivo (Peng et al., 2011).

TgROP18 not only improves growth rate of parasites, it is also important for survival of tachyzoites in vivo. Firstly, it confers protection against Gr1+ monocytes that are able to kill type III parasites (TgROP18 deficient) but not type I (TgROP18 sufficient) parasites in in vitro assays. Furthermore, mice infected with Arop18I parasites survive twice as long as those infected with WT type I parasites (Fentress et al., 2010).

Kinase dead mutants of type I TgROP18 (ROP18IKD) are inactive and unable to elicit a ROP18I acute virulence phenotype upon expression in a type III strain (Taylor et al., 2006; El Hajj et al., 2007a, b) nor do they promote the fast growth rate seen with ROP18I expression (Taylor et al., 2006; El Hajj et al., 2007a, b). Thus, the kinase activity of TgROP18 is essential for parasite virulence and ROP18I is a major determinant for the virulence differences seen between strains.

Suppression of innate immunity by TgROP18

In mice, a major cellular defence mechanism against intracellular pathogens is driven by p47 immunity related GTPases (IRGs) (Taylor, 2007). Several minutes after infection with an intracellular pathogen such as *T. gondii*, IRGs accumulate on the vacuole membrane and oligomerise (Hunn et al., 2008; Khaminets et al., 2010). This leads to rupturing of the membrane, destabilisation of the PV and eventual killing of the parasite (Martens et al., 2005; Ling et al., 2006; Zhao et al., 2009a, b). One IRG, Irga6, is known to be a myristoylated protein (Martens et al., 2004) and this represents a possible mechanism by which the IRGs could associate with membranes. Interestingly, the three *T. gondii* strains vary in their susceptibility to IRG-mediated killing (Khaminets et al., 2010). Vacuoles containing type I parasites do not accumulate IRGs on the surface and parasite replication is not prohibited by IFN-γ, whereas the vacuoles of TgROP18I deficient strains are targeted by IRGs (Zhao et al., 2009a, b; Fentress et al., 2010; Steinfeldt et al., 2010).

TgROP18 was shown to directly interact with Irga6 and Irgb6 (Fentress et al., 2010; Steinfeldt et al., 2010). Furthermore, a kinase dead type I TgROP18 (ROP18IKD) binds Irgb6 more strongly than ROP18I, possibly because they get stuck together during the process of phosphate transfer that cannot be completed (Fentress et al., 2010). The phosphorylated form of Irga6 is detected in IFN-γ-stimulated MEFs infected with type I parasites, but not in uninfected cells or those infected with type II or III parasites (Steinfeldt et al., 2010). Phosphorylation sites on Irga6 and Irgb6 have been mapped to threonine residues located in the switch I loop, which is implicated in IRG oligomerisation, a process that is required for the IRG function (Fentress et al., 2010; Steinfeldt et al., 2010). Phosphorylation of IRGs by TgROP18 may block their ability to oligomerise or reduce the complex stability and preclude PVM rupture. Confirming that disruption of the threonine phosphorylation sites on Irga6 is counterproductive to its function, phosphomimetic or neutral mutations of the Irga6 threonine are detected on fewer vacuoles and at lower levels when compared with wild-type Irga6, although the interaction is not completely abolished (Steinfeldt et al., 2010). In addition, the vacuoles of less virulent Arop18I parasites are highly decorated with Irgb6 when compared with wild-type parasites (Fentress et al., 2010). RNAi of Irgb6 showed that both Arop18I and type III parasites resisted destruction when Irgb6 was not present, highlighting the important role of Irgb6 in parasite clearance. Although the fact that the Arop18I parasites are not as attenuated in virulence as type III parasites, indicates that other type I genes are contributing to parasite survival and virulence.

The N-terminal region of TgROP18 has an arginine-rich region, which folds into three helices. Mutation and deletion experiments identified that disruption of this region prevents the anchoring of TgROP18 in the PVM. Consequently, TgROP18 mislocalisation results in the accumulation of IRGs on the surface of the PVM and its subsequent destruction (Fentress et al., 2012) (Fig. 4), indicating that TgROP18 plays an essential role in the dysfunction of the IRGs and this is dependent upon correct localisation at the PVM.

The mouse genome possesses 23 IRG genes, however, the human genome contains only two and neither of these are IFN-γ inducible (Bekpen et al., 2005), so the IRGs cannot be the target of TgROP18 in human toxoplasmosis. The large guanylate-binding protein (GBP) family is an important IFN-γ inducible p65 GTPase family that is represented in both the mouse and human genomes and is important for clearance of intracellular pathogens. Mice lacking a cluster of six GBPs exhibit defects in the innate immune response to *T. gondii* infection (Yamamoto et al., 2012). Parasite burden is increased in mice lacking this cluster when infected with type II parasites. Furthermore, GBP loading on the PVM is essential for PVM targeting of IRGs, indicating an important role for GBPs in the antiparasite innate immune response. In murine cells, various GBPs are induced upon infection with *T. gondii* and localise to the PV of type II and III *T. gondii* strains (Kresse et al., 2008; Kravets et al., 2012). Similarly to the IRGs, GBP recruitment...
to type I PVs in vitro is reduced and a type III strain expressing type I TgROP18 (type III+ROP18) is able to avoid mGBP1 recruitment, but it is still not known how exactly TgROP18 blocks the accumulation (Virreira Winter et al., 2011).

Taken together, ROP18 targets two IFN-γ-inducible GTPase families, IRGs and GBPs, to evade the innate immune response in mice (Fig. 4b).

**Suppression of acquired immunity by TgROP18**

TgROP18 also appears to contribute to the efficient suppression of the host type I acquired immune response. CD4+ and CD8+ T cells from mice infected with Δrop18 parasites produce more IFN-γ than those cells from mice infected with WT parasites, indicating that the presence of TgROP18 helps to quell the IFN-γ response (Yamamoto et al., 2011). Although the mechanism underlying the TgROP18-mediated suppression of the CD4 T cell response remains unclear, disruption of ER associated degradation (ERAD) might be involved in the inhibition of CD8 T cell activation by TgROP18. Infection with live T. gondii has been shown to induce antigen cross-presentation, resulting in activation of CD8 T cell response in an ERAD-dependent fashion (Gubbels et al., 2005; Dzierszinski et al., 2007; Goldszmid et al., 2009). How could TgROP18 target the host CD8 T cell response?

In addition to IRGs, ATF6β is an ER localised transcription factor that functions as part of the unfolded protein response (UPR), and is shown to regulate transcription of genes involved in ERAD (Yoshida et al., 2001). Ectopic expression of parasite TgROP18 in mammalian cells or infection by wild-type, but not Δrop18, parasites induces proteasome-dependent degradation of ATF6β in a kinase activity-dependent fashion. In addition, ATF6β-deficient mice are highly susceptible to Δrop18 parasites, and show defective IFN-γ production from CD8+ T cells due to impaired functions of antigen presenting cells (Yamamoto et al., 2011), indicating that TgROP18 targets ATF6β in innate immune cells and indirectly suppresses the host adaptive immune response. Importantly, ATF6β is present in the human genome, and it may therefore represent a target of TgROP18 in human toxoplasmosis.

At the molecular level, the N-terminal 17 amino acids (aa) located at the 147-164 portion of TgROP18 was shown to be important for the interaction with ATF6β. Furthermore, the virulence of Δrop18 parasites expressing TgROP18 lacking the 17 aa is lower than full length TgROP18 expressing Δrop18 parasites (Yamamoto et al., 2011), suggesting that the ATF6β-dependent response plays a role in the modest resistance to Δrop18 parasites.

However, since the 17 aa N-terminal region is important for proper localisation of TgROP18 on the PV, TgROP18 lacking the 17aa is not in a position to mediate full virulence in comparison to wild-type parasites. This raises the possibility that N-terminal region(s) of TgROP18 other than the 17aa portion might be responsible for the interaction with ATF6β. The domain of ATF6β that interacts with TgROP18 is mapped to the C-terminus, which is presumably inside the lumen of the ER. Given that TgROP18 is localised on the cytoplasmic face of the PV (Reese & Boothroyd, 2009), a direct interaction is not easily foreseeable. One possibility is that during host ER-PVM fusion (Melão & de Souza, 1997; Goldszmid et al., 2009), TgROP18 may access the ER lumen, allowing the interaction with the C-terminus of ATF6β. However, it remains unclear whether direct interaction and fusion of the PV with host ER causes the stress conditions required to activate the host proteases that would cleave and activate ATF6β. Also, whether this occurs similarly in types I-III, and is a potent host response against types II and III that have a less active TgROP18 is also undetermined.

In summary, TgROP18 critically contributes to the highly pathogenic nature of type I parasites and the kinase activity is essential for its function in virulence. TgROP18 can suppress innate immunity in mice by down-regulating IFN-γ-inducible GTPases and preventing them from loading on PVM. In addition, TgROP18 also inhibits acquired immunity by targeting ATF6β in antigen
presenting cells to reduce IFN-γ production by CD8 T cells (Fig. 5).

**TgROP5: a multi-copy gene coding for pseudokinases**

TgROP5 is a pseudokinase that is delivered to the host cytosol face of the PVM (Fig. 4a) (Hanks & Hunter, 1995; El Hajj et al., 2006). Differential permeabilisation techniques and molecular modelling of TgROP5 predicted that a proposed transmembrane helix was instead buried within the protein (El Hajj et al., 2007a, b). This set a precedent for reinterpreting the structure of the other ROPs that had been thought to carry a transmembrane spanning domain (El Hajj et al., 2007a, b). It also raised questions about their actual mechanism of membrane association.

TgROP5 was identified in genetic crosses of types I-III as being highly significant for virulence and is located within the VIR1 locus (Saeij et al., 2006; Behnke et al., 2011). It is remarkable that although TgROP5 codes for a predicted pseudokinase, it is still a major virulence determinant for *T. gondii*. Recently, evidence has been presented to support the role of pseudokinases in regulating cellular functions. Pseudokinases have been implicated in activation of other kinases through receptor activity, complex formation and providing a molecular scaffold to support other active agents (Boudeau et al., 2006).

TgROP5 exists as a tandem cluster of nearly identical genes, rather than a single gene, and a different number of copies are found in types I-III (Behnke et al., 2011; Reese et al., 2011). Type I has ~6 copies, type II has ~10 and type III has ~4 TgROP5 genes (Reese et al., 2011). There are three major isoforms (A, B and C), with another two presenting only minor SNPs between their closest relatives (Reese et al., 2011). Each isoform has all the residues of a canonical kinase except for the aspartate residue of the His-Arg-Asp (HRD) domain (Behnke et al., 2011; Reese & Boothroyd, 2011). This domain helps to stabilise the interaction with the substrate (Kornev et al., 2006). In TgROP5, the aspartate residue has been replaced with either a histidine or an arginine residue (Behnke et al., 2011), both of these residues are basic in nature. These pseudokinases also have a glycine residue in place of the arginine and so the group of pseudokinases to which TgROP5 belongs are known as His-Gly-Basic (HGB) pseudokinases (Reese & Boothroyd, 2011). The majority of differences between TgROP5 isoforms are found in the C-terminal ATP binding pocket and substrate recognition domains (Behnke et al., 2011; Reese et al., 2011). There is evidence to suggest that the pseudokinase domain is undergoing a diversifying selection, which may be related to function, whereas the N-terminal region, associated with targeting to the PVM, remains unchanged (Reese et al., 2011). It is also interesting to note that none of the SNPs present in any of the isoforms occur in a region that would potentially restore catalytic activity (Reese et al., 2011). Type I TgROP5 isoforms (ROP5A, ROP5B and ROP5C) are almost identical to the type III isoforms (ROP5AIII, ROP5BIII and ROP5CIII respectively), whereas type II isoforms (ROP5AI, ROP5BI and ROP5CII) are significantly different. At least one of the type II alleles presents a frame shift, which leads to a truncated and most likely nonfunctional protein (Behnke et al., 2011; Reese et al., 2011). Interestingly, type I and type III TgROP5 isoforms are more divergent from one another than the type II alleles, and the increased number of copies within the type II cluster may act as a compensation mechanism for the loss of other functional copies (Reese et al., 2011).

Expression of a cosmids containing the TgROP5 locus in a hypovirulent parasite strain (S22) causes a >10^5 fold increase in virulence in comparison to the parental strain (Reese et al., 2011). The S22 strain is a progeny line from a type II/type III parasite cross that has nonviral TgROP5, ROP16 and ROP18 alleles, making it useful for gain of virulence studies (Saeij et al., 2006). Deletion of the entire TgROP5 locus from a type I strain, although presenting no growth phenotype, causes a drop in virulence when compared with the parental strain (Behnke et al., 2011; Reese et al., 2011). The AROP5 phenotype is complemented fully by expression of a cosmids containing the entire type I TgROP5 locus (Behnke et al., 2011). Interestingly, even expression of only one or two copies of ROP5I partially restores virulence (Reese et al., 2011), showing that each allele contributes to virulence but multiple copies are required for maximum virulence.

Furthermore, a type I AROP5 strain complemented with a mutant TgROP5 that has the canonical kinase HRD aspartate restored (AROP5 + ROP5AIH389D) is virulent but to a much lesser extent than those complemented with a WT allele. This result is fascinating as it shows that restoration of a canonical kinase residue is actually detrimental to TgROP5 function. Moreover, the minor virulence phenotype of the AROP5 + ROP5AIH389D strain is not due to any restoration of catalytic activity (Reese & Boothroyd, 2011). It is possible that even the mutant TgROP5 allele can still associate with another partner in a manner that retains some function, although it has only a minor effect on virulence.

High significance was assigned to a possible interaction between TgROP5 and TgROP18 (Reese et al., 2011) and the recent published work on pseudokinases (Boudeau et al., 2006) becomes very interesting in this context. The possibility of an interaction between TgROP5 and TgROP18 suggested that ROP5 could act as a platform or scaffold for TgROP18 at the PVM (Fig. 4).
Recently, work has been published that demonstrates the role of TgROP5 in virulence. TgROP5 appears to act as a cofactor for TgROP18 rather than a platform, as there does not seem to be a stable direct interaction between the two (Niedelman et al., 2012). TgROP5 can bind directly to IRGs and it has been shown that this alone is enough to reduce IRG burden on the PVM (Fig. 4b) (Fleckenstein et al., 2012; Niedelman et al., 2012). TgROP5 appears to bind IRGs, changing the structure to an inactive conformation and thereby preventing them from functioning to destroy the PV. TgROP18 is then able to access the essential threonine, phosphorylate it and so ensure the IRG is fully inactivated (Fig. 4b) (Fleckenstein et al., 2012). It was reported that TgROP5 binds ATP in an unusual conformation (Reese & Boothroyd, 2011), which is consistent with the new data. As TgROP5 is able to inhibit IRG activity without a highly active TgROP18 (type III parasites for example), TgROP18 cannot function without a virulent TgROP5 (as in type II) (Table 2) (Niedelman et al., 2012). Additionally, in vitro analyses confirm that TgROP18 kinase activity is enhanced in the presence of TgROP5 (Behnke et al., 2012).

In summary, TgROP5 is composed of a tandem array of genes that vary in number between types I-III. It is a secreted rhoptry pseudokinase that is essential for the virulence of type I parasites. Located on the host cytosol face of the PVM it acts as a cofactor for TgROP18 and enhances activity but may also associate with other ROPKs (Fig. 4b). TgROP5 has been shown to bind ATP but cannot complete the transfer of phosphate to a substrate even when the catalytic Asp residue is restored. This indicates that the pseudokinase status of TgROP5 was probably an evolutionary choice for a functional purpose rather than a passive drift away from active kinase ability. TgROP5 confers, independently of other ROPKs, a virulence phenotype that consists of protecting the PVM from the oligomerisation of immune-related proteins sent by the host to attack the parasite in different cell types (Behnke et al., 2012; Fleckenstein et al., 2012).

**TgROP38: impact on host genes expression**

TgROP38 was identified as a member of the ROPK family through database mining of the *T. gondii* genome (Peixoto et al., 2010). It is differentially expressed between strains with almost no expression in a virulent type I strain, but high levels of activity in avirulent type III parasites. Type I parasites alter the expression levels of around 6000 host genes during the course of infection, type III parasites affect the expression of around 650 genes. When type I parasites overexpress an additional copy of TgROP38 under the control of a tubulin promoter, the expression of ROP38 becomes similar to that of a type III strain and the number of genes differentially expressed drops from 6000 to around 400. This level of change in gene expression is more in keeping with a type III strain (Peixoto et al., 2010), suggesting that TgROP38 has an inhibitory effect on host cell transcription. Transcription factors, apoptosis-related genes and signalling molecules, which are normally upregulated in type I infection, appear to be down-regulated when TgROP38 is overexpressed.

TgROP38 not only appears to influence the expression of a large number of genes, but also is itself one of the most highly regulated genes in the genome. Furthermore, it is induced during differentiation of the parasite from one stage to another. The changes in TgROP38 levels in the type I strain expressing a type III TgROP38 do not however appear to have an impact on growth in vitro or on virulence in vivo. It will be interesting to see future reports on the characterisation of such an influential protein.

**Concluding remarks**

Rhoptry organelles can be viewed as the eukaryotic version of the different type secretion systems found in bacteria and they assist these pathogens in invasion and subversion of host cellular functions.

As rhoptries themselves are present in most apicomplexan parasites, it is now evident that the precise contents and role of these organelles differ quite substantially. The presence of the moving junction components in many species highlights the conserved mechanism of invasion shared by most members of the phylum. It is fascinating that such a group of organisms with various host cell types and lifecycles have kept a unique form of motility and invasion. RON2 is inserted into the host cell membrane and interacts with AMA-1, which is on the parasite surface. This creates a parasite-derived receptor-ligand interaction that ensures the essential components of the moving junction are present regardless of host cell type.

Differences exist in the relationship of the parasite with its host and this is clear from the diversity of proteins found within the rhoptry contents. Interestingly, *Babesia spp* and *Theileria spp* have evolved to live freely in the host cell cytoplasm. In both species, rhoptry proteins have not yet been described to be essential for modulation of the host, but are more likely to be required for initial attachment, invasion and then dissolution of the PV. It would not be surprising that in these species, host-modifying factors are not released specifically during invasion. The PVM appears exclusively host derived and dissolved shortly after entry. The PVM in other species presents another barrier to those proteins completing
their function inside the host cell, so in those species that retain a PVM it is advantageous to secrete these proteins prior to PVM formation, to avoid any unnecessary obstruction.

Toxoplasma gondii stands as a superb example of the use of specialist secretory organelles to deliver potent effector molecules that function within the host cells. The rhoptry proteins acting as virulence factors that are characterised to date probably represent only the tip of the iceberg. Several members of the ROPs are clearly dedicated to manipulate the host to ensure survival, and prevent destruction of the parasite by the host. One of these, TgROP16, interferes with transcription of host-immune response genes and actually reprograms the cell to prevent a pro-inflammatory response. The extent of their action in subverting host cellular processes is almost certainly underestimated. A comparison of the global changes in host phosphoproteome upon invasion by wild-type or mutant parasites lacking a given active secreted kinase gene should be informative in that regard.

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Subversion of host functions by apicomplexan parasites


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Apicomplexan parasites secrete kinases from apical secretory organelles to take over their host cell and manipulate their surroundings.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Conservation of ROPks in Coccidians.