Article

Subversion of host cellular functions by the apicomplexan parasites

KEMP, Louise, YAMAMOTO, Masahiro, SOLDATI-FAVRE, Dominique

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. [...]

Reference

KEMP, Louise, YAMAMOTO, Masahiro, SOLDATI-FAVRE, Dominique. Subversion of host cellular functions by the apicomplexan parasites. *FEMS Microbiology Reviews*, 2013, vol. 37, no. 4, p. 607-631

DOI : 10.1111/1574-6976.12013
PMID : 23186105

REVIEW ARTICLE

Subversion of host cellular functions by the apicomplexan parasites

Louise E. Kemp[1], Masahiro Yamamoto[2] & Dominique Soldati-Favre[1]

[1]Department of Microbiology and Molecular Medicine, Faculty of Medicine, University of Geneva, Geneva, Switzerland; and [2]Laboratory of Immunoparasitology, Department of Immunoparasitology, WPI Immunology Frontier Research Centre, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Correspondence: Louise Kemp, University of Geneva, Department of Microbiology and Molecular Medicine, CMU - rue Michel-Servet 1, 1211 Geneva 4, Switzerland. Tel.: +41 (0)22 379 5656; Fax: +41 22 379 5702; e-mail: louise.kemp@unige.ch

Received 15 April 2012; revised 21 November 2012; accepted 22 November 2012.

DOI: 10.1111/1574-6976.12013

Editor: Colin Berry

Keywords
rhoptry organelle; parasitophorous vacuole; secreted kinases; effector molecules; *Toxoplasma gondii*; immune evasion.

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) and include *Plasmodium* species that cause malaria and *Babesia* and *Theileria* species that cause severe disease in animals, most notably in cattle. The coccidian subclass composes parasites of the intestinal tract of animals that are transmitted through the faecal-oral route and are part of the Conoidasida 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-

FEMS MICROBIOLOGY REVIEWS

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 (Frènal *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 nonmotile 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

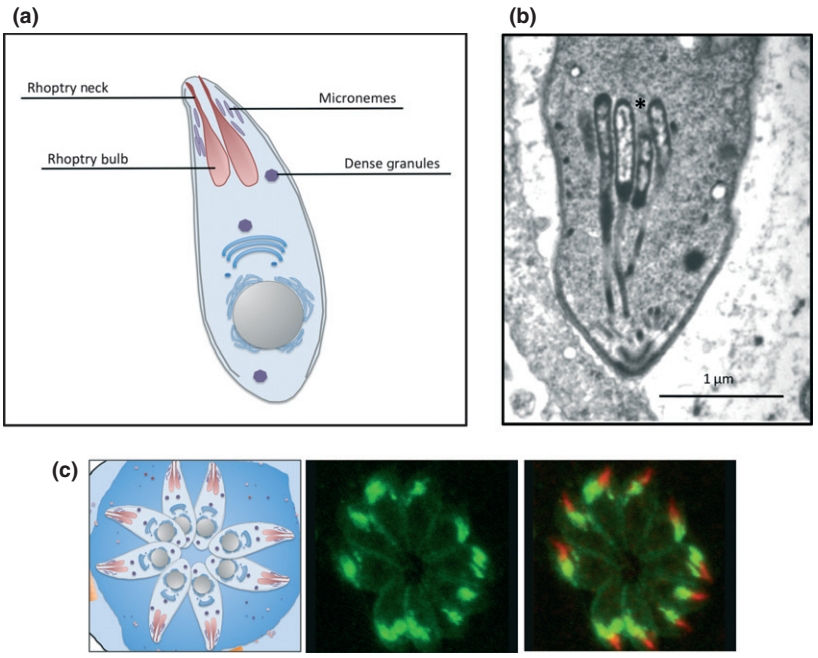

Fig. 1. (a) Schematic representation of *Toxoplasma gondii* tachyzoite and its secretory organelles. (b) Electron micrograph of the apical pole of a *T. gondii* tachyzoites showing rhoptries (marked with *). C: Indirect immunofluorescence microscopy on human foreskin fibroblasts infected with *T. gondii* 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.

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 detailing 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).

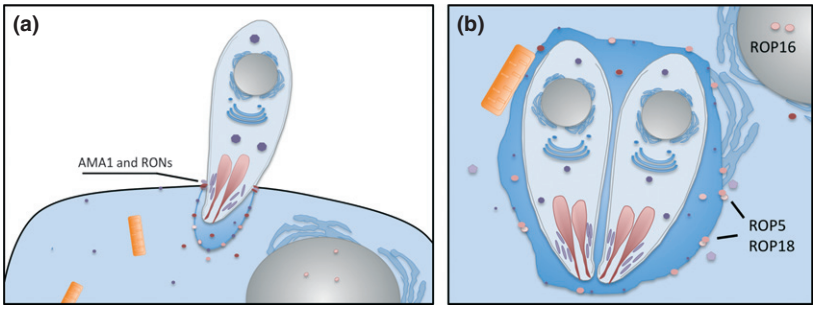

Fig. 2. (a) Distribution of secreted proteins during invasion and (b) establishment of the parasitophorous vacuole. The parasite recruits host ER and mitochondria to the viscinity of the parasitophorous vacuole membrane by an unknown mechanism.

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).

Repertoire 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

Species	*Toxoplasma*	*Neospora*	*Eimeria*	*Cryptosporidium*	*Plasmodium*	*Theileria*	*Babesia*
Lifestyle	PV	PV	PV	Extracytoplasmic PV	PV	Cytoplasm	Cytoplasm
Invasion molecules							
RON2	TGME49_100100[1]	NCLIV_064620[2,3]	ETH_00012760[4,5]	–	PF14_0495[6]	TP01_0014	BBOV_I001630
RON4	TGME49_029010[7]	NCLIV_030050[2,3]	ETH_00013525[4,5]	–	PF11_0168 8	TP02_0051	BBOV_III010920
RON5	TGME49_111470[9,10]	NCLIV_055360[3]	ETH_00015305/ETH_00015310[4,5]	–	MAL8P1.73[11]	TP01_1161	BBOV_IV011430
RON8	TGME49_106060[9,10]	NCLIV_070010[2,3]	ETH_00031645/ETH_00031650/ETH_00031655[4,5]	–	–	–	–
ROPKs[7,12,13]	45 genes	41 genes	24 genes	0 genes	Coccidian specific		
ROP5	TGME49_108080[14]	NCLIV_060730[2]	–	–	–	–	–
ROP16	TGME49_062730[15,16]	NCLIV_025120	ETH_00028765	–	–	–	–
ROP18	TGME49_005250[15]	Pseudogene	–	–	–	–	–
ROP38	TGME49_042110[12]	Triplicated	Multiple	–	–	–	–
Other rhoptry proteins							
RON1	TGME49_110010[7]	NCLIV_054120	ETH_00039940	Chro.80295[17]	PFD0295c[18,19]	–	–
RON3	TGME49_023920[7]	NCLIV_048590[2]	ETH_00001375/ETH_00030255	–	PFL2505c[20]	–	–
RON6	TGME49_097960[7]	NCLIV_006840/NCLIV_006850	ETH_00014480	Chro.10212	PFB0680w	TP01_1109	BBOV_IV012010
RON9	TGME49_108710[21]	NCLIV_053290	ETH_00015380	cgd4_2420	–	–	–
RON10	TGME49_061750[21]	NCLIV_025730	–	cgd8_2530	–	–	–
ARO	TGME49_061440[22]	NCLIV_026070	ETH_00027380	cgd2.370	PFD0720w[22]	TP03_0141	BBOV_I001940
PfRAP1 & 2	–	–	–	–	PF14_0102 PFIT_PFE0080c[23]	–	–
BbRAP1	–	–	–	–	–	–	BBOV_IV009860/BBOV_IV009870[24]
RRA	–	–	–	–	–	–	BBOV_IV010280[25]
RBLs	–	–	–	–	Multigene families	–	–
Pf34	–	–	–	–	PFD0955w[26]	–	–
PfAARP	–	–	–	–	PFD1105w[27]	–	–
Serine protease – TgSUB2	TGME49_114500[28]	NCLIV_057550	ETH_00011050[4]	cgd6_4840	–	–	–
Metallo protease – toxolysin	TGME49_069890[29]	NCLIV_036700	–	–	–	–	–
Rhoptry bulb protein ROP13	TGME49_112270[7,30]	NCLIV_055850	–	–	–	–	–
Actin–binding protein – toxofilin	TGME49_014080[7,31]	NCLIV_051340	–	–	–	–	–
Calpain–like proteinase – toxopain–1	TGME49_049670[32]	NCLIV_069550	ETH_00003570 33[4]	–	–	–	–

All homologues for which experimental data are not available were identified using EuPathDB (Aurrecoechea *et al.*, 2007).

[1]Alexander *et al.* (2005); [2]Marugan-Hernandez *et al.* (2011); [3]Straub *et al.* (2009); [4]Lal *et al.* (2009); [5]Proellocks *et al.* (2010); [6]Cao *et al.* (2009); [7]Bradley *et al.* (2005); [8]Alexander *et al.* (2006); [9]Besteiro *et al.* (2009); [10]Straub *et al.* (2011); [11]Curtidor *et al.* (2011); [12]Peixoto *et al.* (2010); [13]Reid *et al.* (2012).; [14]Leriche & Dubremetz (1991); [15]Saeij *et al.* (2006); [16]Taylor *et al.* (2006); [17]Valentini *et al.* (2012); [18]O'Keeffe *et al.* (2005); [19]Srivastava *et al.* (2010); [20]Ito *et al.* (2011); [21]Lamarque *et al.* (2012); [22]Cabrera *et al.* (2012); [23]Clark *et al.* (1987); [24]Suarez *et al.* (1998); [25]Suarez *et al.* (2011); [26]Proellocks *et al.* (2007); [27]Wickramarachchi *et al.* (2008); [28]Miller *et al.* (2003); [29]Hajagos *et al.* (2012); [30]Turetzky *et al.* (2010); [31]Poupel *et al.* (2000); [32]Que *et al.* (2002); [33]Rieux *et al.* (2012); [34]Aurrecoechea *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 *Δrop13* 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 ROPKs 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 (CpPRP1) 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). PfRON6 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. PfRON3 has been re-designated as a rhoptry bulb protein that interacts with PfRON2 and PfRON4, but not with PfAMA1 (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 multigene 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 bulb 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 adhesin 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 evaluate 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.

*Bb*Rhop68 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 2. ROPK virulence determinants in *T. gondii*

	Type I	Type II	Type III
ROP5	++	+	+
ROP16	++	−	++
ROP18	++	+	−

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 (Th-1) 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; Lieberman *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, the 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 Irf3 and Irf7, 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 (*ROP16*$_I$) 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 *Δrop16* 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 *Δrop16* 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-*Δrop16* 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 *Δrop16* 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

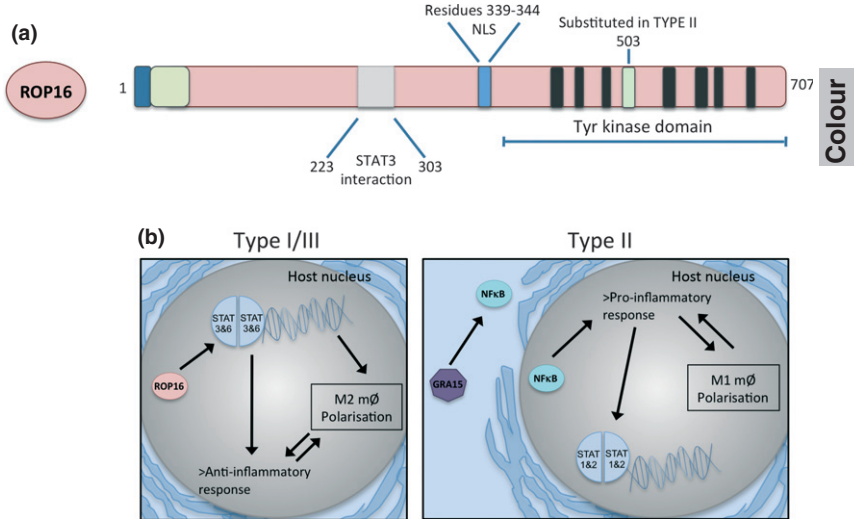

Fig. 3. (a) Scheme of TgROP16 protein, (b) Role of TgROP16 and TgGRA15 in the manipulation of the host-immune response. Upon invasion of the parasite, pro-inflammatory cytokines are produced to destroy the invading foreigner. ROP16 activates STATs to promote the alternative activation of macrophages and an anti-inflammatory response that will counteract the pro-inflammatory response of the host. GRA15 stimulates NFκB, and consequently, the classical activation of macrophages, to promote a controlled pro-inflammatory response that aids dissemination throughout the host. Nuclear localisation of TgROP16 is not essential for activation of STAT3 and STAT6.

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 immunoprecipitation 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 & Yarovinsky, 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 ($ROP18_{III}$) is 14% divergent when compared with the type I sequence ($ROP18_{I}$) (Taylor *et al.*, 2006) and the mRNA is expressed at a level 10,000-fold lower than that of type II ($ROP18_{II}$) (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 $ROP18_{III}$ could also produce a protein deficient in substrate binding (Saeij *et al.*, 2006; Taylor *et al.*, 2006). Expression of a second copy of $ROP18_{I}$ (Taylor *et al.*, 2006) or $ROP18_{II}$ (plus promoter) (Saeij *et al.*, 2006) in a type III strain produces parasites that are more virulent in the mouse model when

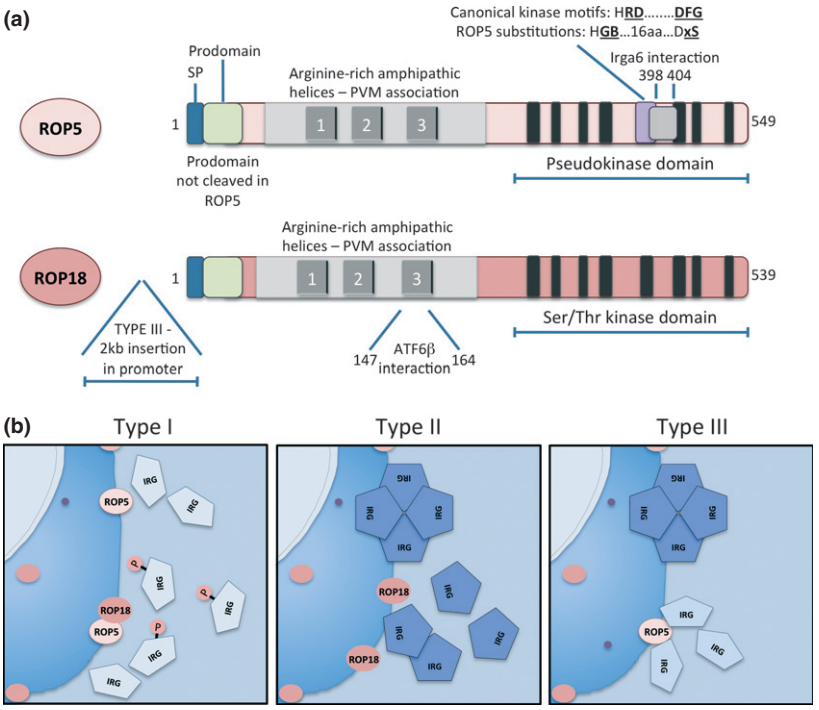

Fig. 4. (a) Scheme of TgROP5 and TgROP18 showing important features. (b) Immunity-related GTPases (IRGs) and GBPs are recruited to the PVM. Oligomerisation of IRGs and GBPs on the PVM creates pores that lead to destabilisation of the PV and ultimately parasite death. TgROP5 alters the conformation of the IRGs and prevents their oligomerisation while also making the critical threonine residue accessible for phosphorylation by TgROP18.

compared with the type III parental strain. Type I parasites expressing an additional copy of $ROP18_I$ or type III parasites expressing a type I TgROP18 (type III+$ROP18_I$) 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 *Δrop18*$_I$ parasites survive twice as long as those infected with WT type I parasites (Fentress *et al.*, 2010).

Kinase dead mutants of type I TgROP18 ($ROP18_I$KD) are inactive and unable to elicit a $ROP18_I$ 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 $ROP18_I$ expression (Taylor *et al.*, 2006; El Hajj *et al.*, 2007a, b). Thus, the kinase activity of TgROP18 is essential for parasite virulence and ROP18 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 TgROP18 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 ($ROP18_I$KD) binds Irgb6 more strongly than $ROP18_I$, 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 *Δrop18* parasites are highly decorated with Irgb6 when compared with wild-type parasites (Fentress *et al.*, 2010). RNAi of Irgb6 showed that both *Δrop18*$_I$ 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 *Δrop18*$_I$ 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$_I$) 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 has been identified as the second host factor targeted by TgROP18 (Fig. 5) (Yamamoto *et al.*, 2011). 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.

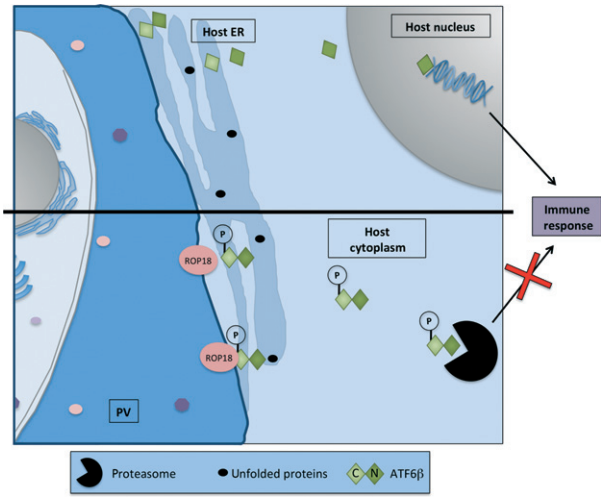

Fig. 5. Proposed mechanism of TgROP18 regulation of ATF6β and the immune system. Stress response factor ATF6β detects unfolded proteins in the ER. The N-terminal region then translocates to the nucleus where it stimulates a response to counter the stress. TgROP18 phosphorylates the C-terminal domain of ATF6β which marks the transcription factor for degradation by the proteasome.

However, since the 17 aa N-terminal region is important for proper localisation of TgROP18 on the PVM, 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 PVM (Reese & Boothroyd, 2009), a direct interaction is not easily foreseeable. One possibility is that during host ER-PVM fusion (Melo & 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 PVM 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 ($ROP5A_I$, $ROP5B_I$ and $ROP5C_I$) are almost identical to the type III isoforms ($ROP5A_{III}$, $ROP5B_{III}$ and $ROP5C_{III}$ respectively), whereas type II isoforms ($ROP5A_{II}$, $ROP5B_{II}$ and $ROP5C_{II}$) 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 cosmid 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 nonvirulent 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 *ΔROP5* phenotype is complemented fully by expression of a cosmid containing the entire type I *TgROP5* locus (Behnke *et al.*, 2011). Interestingly, even expression of only one or two copies of $ROP5_{III}$ 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 *ΔROP5* strain complemented with a mutant TgROP5 that has the canonical kinase HRD aspartate restored (*ΔROP5* + $ROP5A_{III(R389D)}$) 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 *ΔROP5* + $ROP5A_{III(R389D)}$ 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 acts 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 several 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 specialised 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 ROPKs 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.

Acknowledgements

LK was financed by FP7-funded Marie Curie Initial Training Network Project # 215281 – *InterMalTraining*. DSF is supported by the Swiss National Science Foundation (FN3100A0-116722). MY and DSF are supported by by the Japanese–Swiss Science and Technology Program. MY is supported by grants from the Ministry of Education, Culture, Sports, Science and Technology; Kato Memorial Bioscience Foundation; Mochida Memorial Foundation for Medical and Pharmaceutical Research; The Waksman Foundation of Japan INC, Senri Life Science Foundation; The Tokyo Biochemical Research Foundation; The Research Foundation for Microbial Diseases of Osaka University; The Nakajima Foundation; The Asahi Glass Foundation and The Osaka Foundation for Promotion of Clinical Immunology. We are grateful to Christina Mueller for kindly providing the immunofluorescence and electron microscopy data. Apologies to all colleagues whose data could not be included. The authors declare no conflict of interest.

References

Adl SM, Simpson AG, Farmer MA *et al.* (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J Eukaryot Microbiol* **52**: 399–451.

Alexander DL, Mital J, Ward GE, Bradley P & Boothroyd JC (2005) Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. *PLoS Pathog* **1**: e17.

Alexander DL, Arastu-Kapur S, Dubremetz JF & Boothroyd JC (2006) *Plasmodium falciparum* AMA1 binds a rhoptry neck protein homologous to TgRON4, a component of the moving junction in *Toxoplasma gondii*. *Eukaryot Cell* **5**: 1169–1173.

Arevalo-Pinzon G, Curtidor H, Vanegas M, Vizcaino C, Patarroyo MA & Patarroyo ME (2010) Conserved high activity binding peptides from the *Plasmodium falciparum* Pf34 rhoptry protein inhibit merozoites *in vitro* invasion of red blood cells. *Peptides* **31**: 1987–1994.

Aurrecoechea C, Heiges M & Wang H *et al.* (2007) ApiDB: integrated resources for the apicomplexan bioinformatics resource center. *Nucleic Acids Res* **35**: D427–D430.

Awah NW, Troye-Blomberg M, Berzins K & Gysin J (2009) Mechanisms of malarial anaemia: potential involvement of the *Plasmodium falciparum* low molecular weight rhoptry-associated proteins. *Acta Trop* **112**: 295–302.

Baravalle ME, Thompson C, de Echaide ST, Palacios C, Valentini B, Suarez CE, Christensen MF & Echaide I (2010) The novel protein BboRhop68 is expressed by intraerythrocytic stages of *Babesia bovis*. *Parasitol Int* **59**: 571–578.

Barber GN (2011) Innate immune DNA sensing pathways: STING, AIMII and the regulation of interferon production and inflammatory responses. *Curr Opin Immunol* **23**: 10–20.

Baum J, Chen L, Healer J, Lopaticki S, Boyle M, Triglia T, Ehlgen F, Ralph SA, Beeson JG & Cowman AF (2009) Reticulocyte-binding protein homologue 5 – an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int J Parasitol* **39**: 371–380.

Behnke MS, Khan A, Wootton JC, Dubey JP, Tang K & Sibley LD (2011) Virulence differences in *Toxoplasma* mediated by amplification of a family of polymorphic pseudokinases. *P Natl Acad Sci USA* **108**: 9631–9636.

Behnke MS, Fentress SJ, Mashayekhi M, Li LX, Taylor GA & Sibley LD (2012) The polymorphic pseudokinase ROP5 controls virulence in *Toxoplasma gondii* by regulating the active kinase ROP18. *PLoS Pathog* **8**: e1002992.

Bekpen C, Hunn JP, Rohde C, Parvanova I, Guethlein L, Dunn DM, Glowalla E, Leptin M & Howard JC (2005) The interferon-inducible p47 (IRG) GTPases in vertebrates: loss of the cell autonomous resistance mechanism in the human lineage. *Genome Biol* **6**: R92.

Besteiro S, Michelin A, Poncet J, Dubremetz JF & Lebrun M (2009) Export of a *Toxoplasma gondii* rhoptry neck protein complex at the host cell membrane to form the moving junction during invasion. *PLoS Pathog* **5**: e1000309.

Binder EM & Kim K (2004) Location, location, location: trafficking and function of secreted proteases of *Toxoplasma* and *Plasmodium*. *Traffic* **5**: 914–924.

Blader IJ, Manger ID & Boothroyd JC (2001) Microarray analysis reveals previously unknown changes in *Toxoplasma gondii*-infected human cells. *J Biol Chem* **276**: 24223–24231.

Boothroyd JC & Dubremetz JF (2008) Kiss and spit: the dual roles of *Toxoplasma* rhoptries. *Nat Rev Microbiol* **6**: 79–88.

Bosch J, Buscaglia CA, Krumm B, Ingason BP, Lucas R, Roach C, Cardozo T, Nussenzweig V & Hol WG (2007) Aldolase provides an unusual binding site for thrombospondin-related anonymous protein in the invasion machinery of the malaria parasite. *P Natl Acad Sci USA* **104**: 7015–7020.

Boudeau J, Miranda-Saavedra D, Barton GJ & Alessi DR (2006) Emerging roles of pseudokinases. *Trends Cell Biol* **16**: 443–452.

Bradley PJ, Ward C, Cheng SJ *et al.* (2005) Proteomic analysis of rhoptry organelles reveals many novel constituents for host-parasite interactions in *Toxoplasma gondii*. *J Biol Chem* **280**: 34245–34258.

Buscaglia CA, Hol WG, Nussenzweig V & Cardozo T (2007) Modeling the interaction between aldolase and the thrombospondin-related anonymous protein, a key connection of the malaria parasite invasion machinery. *Proteins* **66**: 528–537.

Butcher BA, Fox BA, Rommereim LM, Kim SG, Maurer KJ, Yarovinsky F, Herbert DR, Bzik DJ & Denkers EY (2011) *Toxoplasma gondii* rhoptry kinase ROP16 activates STAT3 and STAT6 resulting in cytokine inhibition and arginase-1-dependent growth control. *PLoS Pathog* **7**: e1002236.

Cabrera A, Herrmann S, Warszta D *et al.* (2012) Dissection of minimal sequence requirements for rhoptry membrane targeting in the malaria parasite. *Traffic* **13**: 1335–1350.

Cai G, Radzanowski T, Villegas EN, Kastelein R & Hunter CA (2000) Identification of STAT4-dependent and independent mechanisms of resistance to *Toxoplasma gondii*. *J Immunol* **165**: 2619–2627.

Cao J, Kaneko O, Thongkukiatkul A, Tachibana M, Otsuki H, Gao Q, Tsuboi T & Torii M (2009) Rhoptry neck protein RON2 forms a complex with microneme protein AMA1 in *Plasmodium falciparum* merozoites. *Parasitol Int* **58**: 29–35.

Carey KL, Jongco AM, Kim K & Ward GE (2004) The *Toxoplasma gondii* rhoptry protein ROP4 is secreted into the parasitophorous vacuole and becomes phosphorylated in infected cells. *Eukaryot Cell* **3**: 1320–1330.

Carruthers VB & Sibley LD (1997) Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur J Cell Biol* **73**: 114–123.

Carruthers VB & Sibley LD (1999) Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol* **31**: 421–428.

Carruthers VB & Tomley FM (2008) Microneme proteins in apicomplexans. *Subcell Biochem* **47**: 33–45.

Chen XM & LaRusso NF (2000) Mechanisms of attachment and internalization of *Cryptosporidium parvum* to biliary and intestinal epithelial cells. *Gastroenterology* **118**: 368–379.

Clark JT, Anand R, Akoglu T & McBride JS (1987) Identification and characterisation of proteins associated with the rhoptry organelles of *Plasmodium falciparum* merozoites. *Parasitol Res* **73**: 425–434.

Cowman AF, Berry D & Baum J (2012) The cell biology of disease: the cellular and molecular basis for malaria parasite invasion of the human red blood cell. *J Cell Biol* **198**: 961–971.

Crosnier C, Bustamante LY, Bartholdson SJ *et al.* (2011) Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* **480**: 534–537.

Curtidor H, Patino LC, Arevalo-Pinzon G, Patarroyo ME & Patarroyo MA (2011) Identification of the *Plasmodium falciparum* rhoptry neck protein 5 (PfRON5). *Gene* **474**: 22–28.

Delorme-Walker V, Abrivard M, Lagal V *et al.* (2012) Toxofilin upregulates the host cortical actin cytoskeleton dynamics facilitating *Toxoplasma* invasion. *J Cell Sci* **125**: 4333–4342.

Desai SA & Rosenberg RL (1997) Pore size of the malaria parasite's nutrient channel. *P Natl Acad Sci USA* **94**: 2045–2049.

Dobrowolski JM & Sibley LD (1996) *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84**: 933–939.

Dubey JP, Lindsay DS & Speer CA (1998) Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev* **11**: 267–299.

Dubremetz JF (2007) Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. *Cell Microbiol* **9**: 841–848.

Dumenil G (2011) Revisiting the extracellular lifestyle. *Cell Microbiol* **13**: 1114–1121.

Dunn JD, Ravindran S, Kim SK & Boothroyd JC (2008) The *Toxoplasma gondii* dense granule protein GRA7 is phosphorylated upon invasion and forms an unexpected association with the rhoptry proteins ROP2 and ROP4. *Infect Immun* **76**: 5853–5861.

Duraisingh MT, Triglia T, Ralph SA, Rayner JC, Barnwell JW, McFadden GI & Cowman AF (2003) Phenotypic variation of *Plasmodium falciparum* merozoite proteins directs receptor targeting for invasion of human erythrocytes. *EMBO J* **22**: 1047–1057.

Dzierszinski F, Pepper M, Stumhofer JS, LaRosa DF, Wilson EH, Turka LA, Halonen SK, Hunter CA & Roos DS (2007) Presentation of *Toxoplasma gondii* antigens via the endogenous major histocompatibility complex class I pathway in nonprofessional and professional antigen-presenting cells. *Infect Immun* **75**: 5200–5209.

Ebel T, Gerhards J, Binder BR & Lipp J (1999) *Theileria parva* 104 kDa microneme–rhoptry protein is membrane-anchored by a non-cleaved amino-terminal signal sequence for entry into the endoplasmic reticulum. *Mol Biochem Parasitol* **100**: 19–26.

El Hajj H, Demey E, Poncet J *et al.* (2006) The ROP2 family of *Toxoplasma gondii* rhoptry proteins: proteomic and genomic characterization and molecular modeling. *Proteomics* **6**: 5773–5784.

El Hajj H, Lebrun M, Fourmaux MN, Vial H & Dubremetz JF (2007a) Inverted topology of the *Toxoplasma gondii* ROP5 rhoptry protein provides new insights into the association of

the ROP2 protein family with the parasitophorous vacuole membrane. *Cell Microbiol* **9**: 54–64.

El Hajj H, Lebrun M, Arold ST, Vial H, Labesse G & Dubremetz JF, (2007b) ROP18 is a rhoptry kinase controlling the intracellular proliferation of *Toxoplasma gondii*. *PLoS Pathog* **3**: e14.

Elliott DA, Coleman DJ, Lane MA, May RC, Machesky LM & Clark DP (2001) *Cryptosporidium parvum* infection requires host cell actin polymerization. *Infect Immun* **69**: 5940–5942.

Fentress SJ, Behnke MS, Dunay IR *et al.* (2010) Phosphorylation of immunity-related GTPases by a *Toxoplasma gondii*-secreted kinase promotes macrophage survival and virulence. *Cell Host Microbe* **8**: 484–495.

Fentress SJ, Steinfeldt T, Howard JC & Sibley LD (2012) The arginine-rich N-terminal domain of ROP18 is necessary for vacuole targeting and virulence of *Toxoplasma gondii*. *Cell Microbiol* **14**: 1921–1933.

Fleckenstein MC, Reese ML, Konen-Waisman S, Boothroyd JC, Howard JC & Steinfeldt T (2012) A *Toxoplasma gondii* pseudokinase inhibits host IRG resistance proteins. *PLoS Biol* **10**: e1001358.

Frènal K, Polonais V, Marq J-B, Stratmann R, Limenitakis J & Soldati-Favre D (2010) Functional dissection of the apicomplexan glideosome molecular architecture. *Cell Host Microbe* **8**: 343–357.

Friedrich N, Matthews S & Soldati-Favre D (2010) Sialic acids: key determinants for invasion by the Apicomplexa. *Int J Parasitol* **40**: 1145–1154.

Gardner MJ, Bishop R, Shah T *et al.* (2005) Genome sequence of *Theileria parva*, a bovine pathogen that transforms lymphocytes. *Science* **309**: 134–137.

Gavrilescu LC, Butcher BA, Del Rio L, Taylor GA & Denkers EY (2004) STAT1 is essential for antimicrobial effector function but dispensable for gamma interferon production during *Toxoplasma gondii* infection. *Infect Immun* **72**: 1257–1264.

Gohil S, Kats LM, Sturm A & Cooke BM (2010) Recent insights into alteration of red blood cells by *Babesia bovis*: moovin' forward. *Trends Parasitol* **26**: 591–599.

Goldszmid RS, Coppens I, Lev A, Caspar P, Mellman I & Sher A (2009) Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *Toxoplasma gondii*-infected dendritic cells. *J Exp Med* **206**: 399–410.

Goodswen S, Kennedy P & Ellis J (2012) A review of the infection, genetics, and evolution of *Neospora caninum*: from the past to the present. *Infect Genet Evol* **15**: 133–150.

Gruner AC, Snounou G, Fuller K, Jarra W, Renia L & Preiser PR (2004) The Py235 proteins: glimpses into the versatility of a malaria multigene family. *Microbes Infect* **6**: 864–873.

Gubbels MJ, Striepen B, Shastri N, Turkoz M & Robey EA (2005) Class I major histocompatibility complex presentation of antigens that escape from the parasitophorous vacuole of *Toxoplasma gondii*. *Infect Immun* **73**: 703–711.

Hajagos BE, Turetzky JM, Peng ED, Cheng SJ, Ryan CM, Souda P, Whitelegge JP, Lebrun M, Dubremetz JF & Bradley PJ (2012) Molecular dissection of novel trafficking and processing of the *Toxoplasma gondii* rhoptry metalloprotease toxolysin-1. *Traffic* **13**: 292–304.

Hakansson S, Charron AJ & Sibley LD (2001) *Toxoplasma* evacuoles: a two-step process of secretion and fusion forms the parasitophorous vacuole. *EMBO J* **20**: 3132–3144.

Hanks SK & Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9**: 576–596.

Honda K, Takaoka A & Taniguchi T (2006) Type I interferon gene induction by the interferon regulatory factor family of transcription factors. *Immunity* **25**: 349–360.

Howe DK & Sibley LD (1995) *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis* **172**: 1561–1566.

Huang BQ, Chen XM & LaRusso NF (2004) *Cryptosporidium parvum* attachment to and internalization by human biliary epithelia *in vitro*: a morphologic study. *J Parasitol* **90**: 212–221.

Hunn JP, Koenen-Waisman S, Papic N, Schroeder N, Pawlowski N, Lange R, Kaiser F, Zerrahn J, Martens S & Howard JC (2008) Regulatory interactions between IRG resistance GTPases in the cellular response to *Toxoplasma gondii*. *EMBO J* **27**: 2495–2509.

Hunter CA & Remington JS (1995) The role of IL12 in toxoplasmosis. *Res Immunol* **146**: 546–552.

Iams KP, Young JR, Nene V, Desai J, Webster P, Ole-MoiYoi OK & Musoke AJ (1990) Characterisation of the gene encoding a 104-kilodalton microneme-rhoptry protein of *Theileria parva*. *Mol Biochem Parasitol* **39**: 47–60.

Ito D, Han ET, Takeo S, Thongkukiatkul A, Otsuki H, Torii M & Tsuboi T (2011) Plasmodial ortholog of *Toxoplasma gondii* rhoptry neck protein 3 is localized to the rhoptry body. *Parasitol Int* **60**: 132–138.

Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, Wynn TA, Kamanaka M, Flavell RA & Sher A (2007) Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med* **204**: 273–283.

Jankovic D, Kugler DG & Sher A (2010) IL-10 production by CD4 + effector T cells: a mechanism for self-regulation. *Mucosal Immunol* **3**: 239–246.

Jensen KDC, Wang Y, Wojno EDT *et al.* (2011) *Toxoplasma* polymorphic effectors determine macrophage polarization and intestinal inflammation. *Cell Host Microbe* **9**: 472–483.

Jewett TJ & Sibley LD (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. *Mol Cell* **11**: 885–894.

Jones TC & Hirsch JG (1972) The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J Exp Med* **136**: 1173–1194.

Jones JL, Muccioli C, Belfort R Jr, Holland GN, Roberts JM & Silveira C (2006) Recently acquired *Toxoplasma gondii* infection, Brazil. *Emerg Infect Dis* **12**: 582–587.

Kaiser K, Matuschewski K, Camargo N, Ross J & Kappe SH (2004) Differential transcriptome profiling identifies Plasmodium genes encoding pre-erythrocytic stage-specific proteins. *Mol Microbiol* **51**: 1221–1232.

Kaneko O (2007) Erythrocyte invasion: vocabulary and grammar of the *Plasmodium* rhoptry. *Parasitol Int* **56**: 255–262.

Kats LM, Black CG, Proellocks NI & Coppel RL (2006) *Plasmodium* rhoptries: how things went pear-shaped. *Trends Parasitol* **22**: 269–276.

Kessler H, Herm-Gotz A, Hegge S, Rauch M, Soldati-Favre D, Frischknecht F & Meissner M (2008) Microneme protein 8–a new essential invasion factor in *Toxoplasma gondii*. *J Cell Sci* **121**: 947–956.

Khaminets A, Hunn JP, Konen-Waisman S *et al.* (2010) Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell Microbiol* **12**: 939–961.

Khan A, Jordan C, Muccioli C, Vallochi AL, Rizzo LV, Belfort R Jr, Vitor RW, Silveira C & Sibley LD (2006) Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. *Emerg Infect Dis* **12**: 942–949.

Kim K, Soldati D & Boothroyd JC (1993) Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. *Science* **262**: 911–914.

Kim SK, Fouts AE & Boothroyd JC (2007) *Toxoplasma gondii* dysregulates IFN-gamma-inducible gene expression in human fibroblasts: insights from a genome-wide transcriptional profiling. *J Immunol* **178**: 5154–5165.

Kobayashi T & Yoshimura A (2005) Keeping DCs awake by putting SOCS1 to sleep. *Trends Immunol* **26**: 177–179.

Kornev AP, Haste NM, Taylor SS & Eyck LF (2006) Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *P Natl Acad Sci USA* **103**: 17783–17788.

Koshy AA, Dietrich HK, Christian DA, Melehani JH, Shastri AJ, Hunter CA & Boothroyd JC (2012) *Toxoplasma* co-opts host cells it does not invade. *PLoS Pathog* **8**: e1002825.

Kravets E, Degrandi D, Weidtkamp-Peters S *et al.* (2012) The GTPase activity of murine guanylate-binding protein 2 (mGBP2) controls the intracellular localization and recruitment to the parasitophorous vacuole of *Toxoplasma gondii*. *J Biol Chem* **287**: 27452–27466.

Kresse A, Konermann C, Degrandi D, Beuter-Gunia C, Wuerthner J, Pfeffer K & Beer S (2008) Analyses of murine GBP homology clusters based on *in silico*, *in vitro* and *in vivo* studies. *BMC Genomics* **9**: 158.

Kubo M, Hanada T & Yoshimura A (2003) Suppressors of cytokine signaling and immunity. *Nat Immunol* **4**: 1169–1176.

Labaied M, Camargo N & Kappe SH (2007) Depletion of the Plasmodium berghei thrombospondin-related sporozoite protein reveals a role in host cell entry by sporozoites. *Mol Biochem Parasitol* **153**: 158–166.

Labesse G, Gelin M, Bessin Y, Lebrun M, Papoin J, Cerdan R, Arold ST & Dubremetz JF (2009) ROP2 from *Toxoplasma gondii*: a virulence factor with a protein-kinase fold and no enzymatic activity. *Structure* **17**: 139–146.

Lal K, Bromley E, Oakes R *et al.* (2009) Proteomic comparison of four *Eimeria tenella* life-cycle stages: unsporulated oocyst, sporulated oocyst, sporozoite and second-generation merozoite. *Proteomics* **9**: 4566–4576.

Lamarque M, Besteiro S, Papoin J *et al.* (2011) The RON2-AMA1 interaction is a critical step in moving junction-dependent invasion by apicomplexan parasites. *PLoS Pathog* **7**: e1001276.

Lamarque MH, Papoin J, Finizio AL, Lentini G, Pfaff AW, Candolfi E, Dubremetz JF & Lebrun M (2012) Identification of a new rhoptry neck complex RON9/RON10 in the Apicomplexa Parasite *Toxoplasma gondii*. *PLoS ONE* **7**: e32457.

Lasonder E, Janse CJ, van Gemert GJ *et al.* (2008) Proteomic profiling of Plasmodium sporozoite maturation identifies new proteins essential for parasite development and infectivity. *PLoS Pathog* **4**: e1000195.

Leander BS, Clopton RE & Keeling PJ (2003) Phylogeny of gregarines (Apicomplexa) as inferred from small-subunit rDNA and β-tubulin. *Int J Syst Evol Micr* **53**: 345–354.

Lee SH, Hayes DB, Rebowski G, Tardieux I & Dominguez R (2007) Toxofilin from *Toxoplasma gondii* forms a ternary complex with an antiparallel actin dimer. *P Natl Acad Sci USA* **104**: 16122–16127.

Lehmann T, Marcet PL, Graham DH, Dahl ER & Dubey JP (2006) Globalization and the population structure of *Toxoplasma gondii*. *P Natl Acad Sci USA* **103**: 11423–11428.

Lemgruber L, Lupetti P, De SW & Vommaro RC (2010) New details on the fine structure of the rhoptry of *Toxoplasma gondii*. *Microsc Res Tech* **74**: 812–818.

Leriche MA & Dubremetz JF (1991) Characterization of the protein contents of rhoptries and dense granules of *Toxoplasma gondii* tachyzoites by subcellular fractionation and monoclonal antibodies. *Mol Biochem Parasitol* **45**: 249–259.

Leung S, Li X & Stark GR (1996) STATs find that hanging together can be stimulating. *Science* **273**: 750–751.

Lieberman LA, Banica M, Reiner SL & Hunter CA (2004) STAT1 plays a critical role in the regulation of antimicrobial effector mechanisms, but not in the development of Th1-type responses during toxoplasmosis. *J Immunol* **172**: 457–463.

Liesenfeld O, Kosek J, Remington JS & Suzuki Y (1996) Association of CD4 + T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with *Toxoplasma gondii*. *J Exp Med* **184**: 597–607.

Ling YM, Shaw MH, Ayala C, Coppens I, Taylor GA, Ferguson DJ & Yap GS (2006) Vacuolar and plasma membrane stripping and autophagic elimination of *Toxoplasma gondii* in primed effector macrophages. *J Exp Med* **203**: 2063–2071.

Lobo CA, Rodriguez M, Hou G, Perkins M, Oskov Y & Lustigman S (2003) Characterization of PfRhop148, a novel rhoptry protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* **128**: 59–65.

Lodoen MB, Gerke C & Boothroyd JC (2010) A highly sensitive FRET-based approach reveals secretion of the actin-binding protein toxofilin during *Toxoplasma gondii* infection. *Cell Microbiol* **12**: 55–66.

Lutz K, Schmitt S, Linder M, Hermosilla C, Zahner H & Taubert A (2011) *Eimeria bovis*-induced modulation of the host cell proteome at the meront I stage. *Mol Biochem Parasitol* **175**: 1–9.

Martens S, Sabel K, Lange R, Uthaiah R, Wolf E & Howard JC (2004) Mechanisms regulating the positioning of mouse p47 resistance GTPases LRG-47 and IIGP1 on cellular membranes: retargeting to plasma membrane induced by phagocytosis. *J Immunol* **173**: 2594–2606.

Martens S, Parvanova I, Zerrahn J, Griffiths G, Schell G, Reichmann G & Howard JC (2005) Disruption of *Toxoplasma gondii* parasitophorous vacuoles by the mouse p47-resistance GTPases. *PLoS Pathog* **1**: e24.

Martinez FO, Helming L & Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol* **27**: 451–483.

Marugan-Hernandez V, Alvarez-Garcia G, Tomley F, Hemphill A, Regidor-Cerrillo J & Ortega-Mora LM (2011) Identification of novel rhoptry proteins in *Neospora caninum* by LC/MS-MS analysis of subcellular fractions. *J Proteomics* **74**: 629–642.

McDonald V & Shirley MW (2009) Past and future: vaccination against *Eimeria*. *Parasitology* **136**: 1477–1489.

Melo EJ & de Souza W (1997) Relationship between the host cell endoplasmic reticulum and the parasitophorous vacuole containing *Toxoplasma gondii*. *Cell Struct Funct* **22**: 317–323.

Miller SA, Thathy V, Ajioka JW, Blackman MJ & Kim K (2003) TgSUB2 is a *Toxoplasma gondii* rhoptry organelle processing proteinase. *Mol Microbiol* **49**: 883–894.

Mital J, Meissner M, Soldati D & Ward GE (2005) Conditional expression of *Toxoplasma gondii* apical membrane antigen-1 (TgAMA1) demonstrates that TgAMA1 plays a critical role in host cell invasion. *Mol Biol Cell* **16**: 4341–4349.

Mosqueda J, McElwain TF, Stiller D & Palmer GH (2002) *Babesia bovis* merozoite surface antigen 1 and rhoptry-associated protein 1 are expressed in sporozoites, and specific antibodies inhibit sporozoite attachment to erythrocytes. *Infect Immun* **70**: 1599–1603.

Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V & Rodriguez A (2001) Migration of Plasmodium sporozoites through cells before infection. *Science* **291**: 141–144.

Munoz M, Heimesaat MM, Danker K *et al.* (2009) Interleukin (IL)-23 mediates *Toxoplasma gondii*-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. *J Exp Med* **206**: 3047–3059.

Murphy KM, Ouyang W, Farrar JD, Yang J, Ranganath S, Asnagli H, Afkarian M & Murphy TL (2000) Signaling and transcription in T helper development. *Annu Rev Immunol* **18**: 451–494.

Murray PJ (2006) Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. *Curr Opin Pharmacol* **6**: 379–386.

Niedelman W, Gold DA, Rosowski EE, Sprokholt JK, Lim D, Farid Arenas A, Melo MB, Spooner E, Yaffe MB & Saeij JP (2012) The rhoptry proteins ROP18 and ROP5 mediate *Toxoplasma gondii* evasion of the murine, but not the human. Interferon-gamma response. *PLoS Pathog* **8**: e1002784.

O'Keeffe AH, Green JL, Grainger M & Holder AA (2005) A novel Sushi domain-containing protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* **140**: 61–68.

Ong YC, Reese ML & Boothroyd JC (2010) *Toxoplasma* rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. *J Biol Chem* **285**: 28731–28740.

Pain A, Renauld H, Berriman M *et al.* (2005) Genome of the host-cell transforming parasite *Theileria annulata* compared with *T. parva*. *Science* **309**: 131–133.

Paredes-Santos TC, de Souza W & Attias M (2012) Dynamics and 3D organization of secretory organelles of *Toxoplasma gondii*. *J Struct Biol* **177**: 420–430.

Peixoto L, Chen F, Harb OS, Davis PH, Beiting DP, Brownback CS, Ouloguem D & Roos DS (2010) Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. *Cell Host Microbe* **8**: 208–218.

Peng H-J, Chen X-G & Lindsay DS (2011) A review: competence, compromise, and concomitance: reaction of the host cell to *Toxoplasma gondii* infection and development. *J Parasitol* **97**: 620–628.

Pernas L & Boothroyd JC (2010) Association of host mitochondria with the parasitophorous vacuole during *Toxoplasma* infection is not dependent on rhoptry proteins ROP2/8. *Int J Parasitol* **40**: 1367–1371.

Pifer R & Yarovinsky F (2011) Innate responses to *Toxoplasma gondii* in mice and humans. *Trends Parasitol* **27**: 388–393.

Poupel O, Boleti H, Axisa S, Couture-Tosi E & Tardieux I (2000) Toxofilin, a novel actin-binding protein from *Toxoplasma gondii*, sequesters actin monomers and caps actin filaments. *Mol Biol Cell* **11**: 355–368.

Proellocks NI, Kovacevic S, Ferguson DJ, Kats LM, Morahan BJ, Black CG, Waller KL & Coppel RL (2007) *Plasmodium falciparum* Pf34, a novel GPI-anchored rhoptry protein found in detergent-resistant microdomains. *Int J Parasitol* **37**: 1233–1241.

Proellocks NI, Coppel RL & Waller KL (2010) Dissecting the apicomplexan rhoptry neck proteins. *Trends Parasitol* **26**: 297–304.

Que X, Ngo H, Lawton J, Gray M, Liu Q, Engel J, Brinen L, Ghosh P, Joiner KA & Reed SL (2002) The cathepsin B of *Toxoplasma gondii*, toxopain-1, is critical for parasite

invasion and rhoptry protein processing. *J Biol Chem* **277**: 25791–25797.

Quinton LJ & Mizgerd JP (2011) NF-kappaB and STAT3 signaling hubs for lung innate immunity. *Cell Tissue Res* **343**: 153–165.

Rasmussen SB, Reinert LS & Paludan SR (2009) Innate recognition of intracellular pathogens: detection and activation of the first line of defense. *APMIS* **117**: 323–337.

Reese ML & Boothroyd JC (2009) A helical membrane-binding domain targets the *Toxoplasma* ROP2 family to the parasitophorous vacuole. *Traffic* **10**: 1458–1470.

Reese ML & Boothroyd JC (2011) A conserved noncanonical motif in the pseudoactive site of the ROP5 pseudokinase domain mediates its effect on *Toxoplasma* virulence. *J Biol Chem* **286**: 29366–29375.

Reese ML, Zeiner GM, Saeij JP, Boothroyd JC & Boyle JP (2011) Polymorphic family of injected pseudokinases is paramount in *Toxoplasma* virulence. *P Natl Acad Sci USA* **108**: 9625–9630.

Reid AJ, Vermont SJ, Cotton JA *et al.* (2012) Comparative genomics of the apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*: Coccidia differing in host range and transmission strategy. *PLoS Pathog* **8**: e1002567.

Rieux A, Gras S, Lecaille F, Niepceron A, Katrib M, Smith NC, Lalmanach G & Brossier F (2012) Eimeripain, a Cathepsin B-like cysteine protease, expressed throughout sporulation of the Apicomplexan Parasite Eimeria tenella. *PLoS ONE* **7**: e31914.

Riglar DT, Richard D, Wilson DW *et al.* (2011) Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. *Cell Host Microbe* **9**: 9–20.

Robben PM, Mordue DG, Truscott SM, Takeda K, Akira S & Sibley LD (2004) Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. *J Immunol* **172**: 3686–3694.

Roger N, Dubremetz JF, Delplace P, Fortier B, Tronchin G & Vernes A (1988) Characterization of a 225 kilodalton rhoptry protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* **27**: 135–141.

Rosowski EE, Lu D, Julien L, Rodda L, Gaiser RA, Jensen KD & Saeij JP (2011) Strain-specific activation of the NF-kappaB pathway by GRA15, a novel *Toxoplasma gondii* dense granule protein. *J Exp Med* **208**: 195–212.

Saeij JPJ, Boyle JP & Boothroyd JC (2005) Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. *Trends Parasitol* **21**: 476–481.

Saeij JP, Boyle JP, Coller S, Taylor S, Sibley LD, Brooke-Powell ET, Ajioka JW & Boothroyd JC (2006) Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* **314**: 1780–1783.

Saeij JP, Coller S, Boyle JP, Jerome ME, White MW & Boothroyd JC (2007) *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* **445**: 324–327.

Sam-Yellowe TY & Perkins ME (1991) Interaction of the 140/130/110 kDa rhoptry protein complex of *Plasmodium falciparum* with the erythrocyte membrane and liposomes. *Exp Parasitol* **73**: 161–171.

Scharton-Kersten T, Denkers EY, Gazzinelli R & Sher A (1995) Role of IL12 in induction of cell-mediated immunity to *Toxoplasma gondii*. *Res Immunol* **146**: 539–545.

Schwab JC, Beckers CJ & Joiner KA (1994) The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. *P Natl Acad Sci USA* **91**: 509–513.

Sehgal PB (2008) Paradigm shifts in the cell biology of STAT signaling. *Semin Cell Dev Biol* **19**: 329–340.

Shaw MK (2003) Cell invasion by *Theileria* sporozoites. *Trends Parasitol* **19**: 2–6.

Shaw MK, Tilney LG & Musoke AJ (1991) The entry of *Theileria parva* sporozoites into bovine lymphocytes: evidence for MHC class I involvement. *J Cell Biol* **113**: 87–101.

Sheiner L, Santos JM, Klages N, Parussini F, Jemmely N, Friedrich N, Ward GE & Soldati-Favre D (2010) *Toxoplasma gondii* transmembrane microneme proteins and their modular design. *Mol Microbiol* **77**: 912–929.

Sibley LD & Ajioka JW (2008) Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. *Annu Rev Microbiol* **62**: 329–351.

Sinai AP (2008) Biogenesis of and activities at the *Toxoplasma gondii* parasitophorous vacuole membrane. *Subcell Biochem* **47**: 155–164.

Sinai AP & Joiner KA (1997) Safe haven: the cell biology of nonfusogenic pathogen vacuoles. *Annu Rev Microbiol* **51**: 415–462.

Sinai AP, Webster P & Joiner KA (1997) Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii* parasitophorous vacuole membrane: a high affinity interaction. *J Cell Sci* **110**(Pt 17): 2117–2128.

Singh S, Alam MM, Pal-Bhowmick I, Brzostowski JA & Chitnis CE (2010) Distinct external signals trigger sequential release of apical organelles during erythrocyte invasion by malaria parasites. *PLoS Pathog* **6**: e1000746.

Soldati D, Kim K, Kampmeier J, Dubremetz JF & Boothroyd JC (1995) Complementation of a *Toxoplasma gondii* ROP1 knock-out mutant using phleomycin selection. *Mol Biochem Parasitol* **74**: 87–97.

Soldati D, Lassen A, Dubremetz JF & Boothroyd JC (1998) Processing of Toxoplasma ROP1 protein in nascent rhoptries. *Mol Biochem Parasitol* **96**: 37–48.

Srivastava A, Singh S, Dhawan S, Mahmood Alam M, Mohmmed A & Chitnis CE (2010) Localization of apical sushi protein in *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol* **174**: 66–9.

Steinfeldt T, Konen-Waisman S, Tong L, Pawlowski N, Lamkemeyer T, Sibley LD, Hunn JP & Howard JC (2010) Phosphorylation of mouse immunity-related GTPase (IRG) resistance proteins is an evasion strategy for virulent *Toxoplasma gondii*. *PLoS Biol* **8**: e1000576.

Straub KW, Cheng SJ, Sohn CS & Bradley PJ (2009) Novel components of the Apicomplexan moving junction reveal conserved and coccidia-restricted elements. *Cell Microbiol* **11**: 590–603.

Straub KW, Peng ED, Hajagos BE, Tyler JS & Bradley PJ (2011) The moving junction protein RON8 facilitates firm attachment and host cell invasion in *Toxoplasma gondii*. *PLoS Pathog* **7**: e1002007.

Stubbs J, Simpson KM, Triglia T, Plouffe D, Tonkin CJ, Duraisingh MT, Maier AG, Winzeler EA & Cowman AF (2005) Molecular mechanism for switching of *P. falciparum* invasion pathways into human erythrocytes. *Science* **309**: 1384–1387.

Suarez CE, Palmer GH, Hotzel I & McElwain TF (1998) Structure, sequence, and transcriptional analysis of the *Babesia bovis* rap-1 multigene locus. *Mol Biochem Parasitol* **93**: 215–224.

Suarez CE, Laughery JM, Bastos RG, Johnson WC, Norimine J, Asenzo G, Brown WC, Florin-Christensen M & Goff WL (2011) A novel neutralization sensitive and subdominant RAP-1-related antigen (RRA) is expressed by *Babesia bovis* merozoites. *Parasitology* **138**: 809–818.

Takaoka A & Yanai H (2006) Interferon signalling network in innate defence. *Cell Microbiol* **8**: 907–922.

Talevich E, Mirza A & Kannan N (2011) Structural and evolutionary divergence of eukaryotic protein kinases in Apicomplexa. *BMC Evol Biol* **11**: 321.

Tamiya T, Kashiwagi I, Takahashi R, Yasukawa H & Yoshimura A (2011) Suppressors of cytokine signaling (SOCS) proteins and JAK/STAT pathways: regulation of T-cell inflammation by SOCS1 and SOCS3. *Arterioscler Thromb Vasc Biol* **31**: 980–985.

Taylor GA (2007) IRG proteins: key mediators of interferon-regulated host resistance to intracellular pathogens. *Cell Microbiol* **9**: 1099–1107.

Taylor GA, Feng CG & Sher A (2004) p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol* **4**: 100–109.

Taylor S, Barragan A, Su C *et al.* (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science* **314**: 1776–1780.

Topolska AE, Richie TL, Nhan DH & Coppel RL (2004a) Associations between responses to the rhoptry-associated membrane antigen of *Plasmodium falciparum* and immunity to malaria infection. *Infect Immun* **72**: 3325–3330.

Topolska AE, Lidgett A, Truman D, Fujioka H & Coppel RL (2004b) Characterization of a membrane-associated rhoptry protein of *Plasmodium falciparum*. *J Biol Chem* **279**: 4648–4656.

Tufet-Bayona M, Janse CJ, Khan SM, Waters AP, Sinden RE & Franke-Fayard B (2009) Localisation and timing of expression of putative *Plasmodium berghei* rhoptry proteins in merozoites and sporozoites. *Mol Biochem Parasitol* **166**: 22–31.

Turetzky JM, Chu DK, Hajagos BE & Bradley PJ (2010) Processing and secretion of ROP13: a unique *Toxoplasma* effector protein. *Int J Parasitol* **40**: 1037–1044.

Tyler JS & Boothroyd JC (2011) The C-terminus of *Toxoplasma* RON2 provides the crucial link between AMA1 and the host-associated invasion complex. *PLoS Pathog* **7**: e1001282.

Valentini E, Cherchi S, Possenti A, Dubremetz JF, Pozio E & Spano F (2012) Molecular characterisation of a *Cryptosporidium parvum* rhoptry protein candidate related to the rhoptry neck proteins TgRON1 of *Toxoplasma gondii* and PfASP of *Plasmodium falciparum*. *Mol Biochem Parasitol* **183**: 94–99.

Vanderberg JP, Chew S & Stewart MJ (1990) Plasmodium sporozoite interactions with macrophages *in vitro*: a videomicroscopic analysis. *J Protozool* **37**: 528–536.

Virreira Winter S, Niedelman W, Jensen KD *et al.* (2011) Determinants of GBP recruitment to *Toxoplasma gondii* vacuoles and the parasitic factors that control it. *PLoS ONE* **6**: e24434.

Whitmarsh RJ, Gray CM, Gregg B, Christian DA, May MJ, Murray PJ & Hunter CA (2011) A critical role for SOCS3 in innate resistance to *Toxoplasma gondii*. *Cell Host Microbe* **10**: 224–236.

Wickramarachchi T, Devi YS, Mohmmed A & Chauhan VS (2008) Identification and characterization of a novel *Plasmodium falciparum* merozoite apical protein involved in erythrocyte binding and invasion. *PLoS ONE* **3**: e1732.

Worliczek HL, Buggelsheim M, Saalmüller A & Joachim A (2007) Porcine isosporosis: infection dynamics, pathophysiology and immunology of experimental infections. *Wien Klin Wochenschr* **119**: 33–39.

Yamamoto M, Standley DM, Takashima S *et al.* (2009) A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3. *J Exp Med* **206**: 2747–2760.

Yamamoto M, Ma JS, Mueller C *et al.* (2011) ATF6beta is a host cellular target of the *Toxoplasma gondii* virulence factor ROP18. *J Exp Med* **208**: 1533–1546.

Yamamoto M, Okuyama M, Ma JS *et al.* (2012) A cluster of interferon-gamma-inducible p65 GTPases plays a critical role in host defense against *Toxoplasma gondii*. *Immunity* **37**: 302–313.

Yokoyama N, Suthisak B, Hirata H, Matsuo T, Inoue N, Sugimoto C & Igarashi I (2002) Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity. *Infect Immun* **70**: 5822–5826.

Yokoyama N, Okamura M & Igarashi I (2006) Erythrocyte invasion by *Babesia* parasites: current advances in the elucidation of the molecular interactions between the protozoan ligands and host receptors in the invasion stage. *Vet Parasitol* **138**: 22–32.

Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M & Mori K (2001) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and

6beta that activates the mammalian unfolded protein response. *Mol Cell Biol* **21**: 1239–1248.

Zhao YO, Khaminets A, Hunn JP & Howard JC (2009a) Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFNgamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. *PLoS Pathog* **5**: e1000288.

Zhao YO, Rohde C, Lilue JT, Konen-Waisman S, Khaminets A, Hunn JP & Howard JC (2009b) *Toxoplasma gondii* and the immunity-related GTPase (IRG) resistance system in mice: a review. *Mem Inst Oswaldo Cruz* **104**: 234–240.

Zimmermann S, Murray PJ, Heeg K & Dalpke AH (2006) Induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii* contributes to immune evasion in macrophages by blocking IFN-gamma signaling. *J Immunol* **176**: 1840–1847.

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.