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Review

Host cell manipulation by the human pathogen *Toxoplasma gondii*

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Abstract. *Toxoplasma gondii* is an obligate intracellular parasite that can infect virtually any nucleated cell. During invasion *Toxoplasma* creates the parasitophorous vacuole, a subcellular compartment that acts as an interface between the parasite and host, and serves as a platform for modulation of host cell functions that support parasite replication and infection. Spatial reorganization of host organelles and cytoskeleton around the parasitophorous vacuole are observed following entry, and recent evidence suggests this interior redecorating promotes parasite nutrient acquisition. New findings also reveal that *Toxoplasma* manipulates host signaling pathways by deploying parasite kinases and a phosphatase, including at least two that infiltrate the host nucleus. *Toxoplasma* infection additionally controls several cellular pathways to establish an anti-apoptotic environment, and subverts immune cells as a conduit for dissemination. In this review we discuss these recent developments in understanding how *Toxoplasma* achieves widespread success as a human and animal parasite by manipulating its host.

Keywords. *Toxoplasma gondii*, host reorganization parasitophorous vacuole membrane, molecular signaling, apoptosis.

Introduction

Toxoplasma gondii is a unicellular parasite responsible for the human and animal disease toxoplasmosis. This obligate intracellular protozoan adopts several forms. The tachyzoite is a rapidly dividing haploid form of *T. gondii* that can infect a wide range of mammalian host cells including immune and non-immune cells [1]. Infection causes cellular lysis during parasite egress into the surrounding environment [2]. Tachyzoites reversibly transform into latent bradyzoites to produce intracellular tissue cysts. Encystation is triggered both by intrinsic preprogramming within the parasite and in response to immune pressure. Cysts are mainly found in muscles and the brain, appear to be largely invisible to the immune system, and are refractory to antibiotics. Healthy individuals are rarely affected by *T. gondii* infection since the immune system recognizes and eliminates rapidly any parasites that differentiate back to tachyzoites [3]. However, *T. gondii* causes severe degeneration of the central nervous system in immunocompromised patients who are unable to extinguish the resurgent infection. Congenital transmission of tachyzoites also results in grave consequences for infected fetuses and newborns [4]. Approximately 25 % of the world's human population is estimated to bear the

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chronic form of the parasite [5], placing *Toxoplasma* amongst the most successful human parasites.

Cell invasion by tachyzoites is a crucial event that shapes parasite survival, replication, and manipulation of the host cell. *T. gondii* presents three organelles involved in host cell attachment, penetration, and in the formation of the parasitophorous vacuole (PV) Micronemes, rhoptries, and dense granules fuse alternately with the parasite membrane, discharging their (protein) contents in a well-orchestrated, rapid series of deployments [6, 7]. Microneme proteins (MICs) play a central role in attachment to the host through the binding of specific receptors [8, 9]. Rhoptries, which are discharged immediately following micronemes, comprise two different substructures named the rhoptry neck and rhoptry bulb. MIC and rhoptry neck proteins (RONs) assemble on the parasite surface to form the moving junction (MJ) [10], a tight apposition of the parasite and host plasma membranes visible during cell invasion. The moving junction ensures the formation of the parasitophorous vacuole membrane (PVM) from the host cell membrane while largely excluding host membrane proteins from the forming PVM [11]. This partitioning strategy procures a non-fusogenic state of the PVM with host structures, avoiding the potential acidification and destruction of the PV contents by fusion with host cell lysosomes [12].

The PVM surrounds the intracellular parasites and provides a stable environment for parasite multiplication. Proteins from the bulbous part of the rhoptry (ROPs) are injected into the host cell coupled to evacuoles, small vesicles that subsequently fuse with the nascent PV, peppering the cytosolic face of the PVM with ROPs [13, 14]. Dense granule proteins (GRAs) are released into the PV after invasion, including some that associate with or insert into the PVM. GRAs also dictate the structure of the intravacuolar network (IVN) of tubular membranes inside the PV, which supports the characteristic rosette arrangement of parasites [15, 16]. Whereas the PVM helps protect the parasite from host cell elimination, this membrane segregates it from the abundant source of nutrients in the host cytosol. To help obviate this, the parasite elaborates pores in the PVM (and/or possibly IVN) that permit bidirectional diffusion of small molecules [17]. PVM proteins ensure nutrient acquisition and the recruitment of host organelles including mitochondria and ER. Additionally, the proximity of the host mitochondria and ER suggest a possible transfer of materials to the parasite [18].

Recent advances have shed light on how the parasite reorganizes host organelles and cytoskeletons. Also, the discovery of parasite proteins containing kinase and phosphatase domains within the host nucleus enforces the hypothesis that the parasite modulates host cell signaling and gene expression. New insight has also emerged clarifying the basis of the anti-apoptotic state imparted by the parasite in different cells. Additionally, *T. gondii* subversion of immune cell migration has been recently linked to the parasite's ability to tweak host cell intracellular signaling. This review focuses on these new aspects of host cell modulation by *T. gondii* infection that underlie the parasite's success as a ubiquitous infectious agent.

Subversion of host organelles, cytoskeleton and lysosomes

To survive and ensure its division, *T. gondii* must acquire nutrients from its host. *T. gondii* is an auxotroph for tryptophan [19–21], arginine [22, 23], polyamines [24], purines [25, 26], cholesterol [27, 28], iron [23, 29], and other essential nutrients [30]. Although parasites surrounded by the PVM are protected against acidification from the fusion of endocytic vesicles (except in activated macrophages [31, 32]), this non-fusion state deprives parasites of an abundant source of nutrients from the host's endocytic and exocytic system. The parasite instead acquires small molecules that traverse the PVM via pores in a manner independent of the temperature and energy, thereby establishing bidirectional molecular exchange with host cells. These pores allow the acquisition of small soluble metabolites ($<$1300–1900 Da) such as glucose, amino acids, nucleotides, and ions [17]. In the same manner, catabolic waste products generated by the parasite are probably discarded into the host cytosol. Although passive diffusion of some compounds can be sufficient to aliment parasites, several metabolites are found in low concentrations in the cytosol, are bound to large proteins, or are insoluble, thereby limiting acquisition by diffusion. For this reason, active transport is necessary at the plasma membrane of the parasite and at the PVM. Proteins associated with the PVM are mostly issued from the release of the bulbous part of the rhoptries during invasion and also from secretion of dense granules into the PV after entry. These PVM proteins are probably involved in several processes such as nutrient acquisition, but also the interaction with mitochondria and ER, the restructuring of the intermediate filaments and microtubules around the PV, and the modulation of certain processes of the host cell [33].

Mitochondria and ER

The association between the PV and host organelles such as mitochondria and ER is seen shortly after invasion and remains stable through the evolution of

the PV [16, 18], suggesting the involvement of molecules initially incorporated in the PVM. Intimate interaction between two organelles has been observed with the ER and mitochondria of yeast, and has been implicated in the direct transfer of phospholipids in a manner independent of vesicular transport [34, 35]. Similarly, the tight association between the PVM and host mitochondria and ER might provide a key source of new phospholipids [36]. Phospholipids could be incorporated into the PVM to ensure PV enlargement during the process of parasite division. Although *T. gondii* is capable of synthesizing certain phospholipids, it must acquire serine and choline from host cells to ensure appropriate synthesis of phosphatidylserine and phosphatidylcholine necessary for its replication. As a likely indicator of parasite siphoning, the production of choline by the host cell is increased in response to *T. gondii* invasion and replication [37]. Lipoic acid is another essential nutrient and is used as a cofactor for pyruvate dehydrogenase in the Krebs cycle. Although *T. gondii* synthesizes lipoic acid inside the apicoplast [38], intriguingly, parasite utilization of this lipid in its own mitochondrion requires scavenging of lipoic acid from the host [39, 40]. Since the largest pool of host lipoic acid is mitochondrial, it is tempting to speculate that this at least partly explains the parasite's motivation for anchoring host mitochondria. Folates and fatty acids can be acquired from the host cells or synthesised *de novo* by *T. gondii* (see above) [30, 41, 42]. These examples of a requirement for both synthesis and scavenging indicate that the genomic presence of biosynthetic genes for essential nutrients does not necessarily mean that the parasite is prototrophic for such metabolites.

ROP2, a founding member of the ROP2 subfamily, directly participates in the recruitment of host mitochondria to the PVM [36]. ROP2 subfamily proteins are often highly homologous, and in some cases even cross-reactive with monoclonal antibodies. Members of this group display a signal peptide ensuring their access into the secretory pathway and are usually synthesized as a propeptide form that is processed *en route* to the mature rhoptries. ROP2 is associated with evacuoles shortly after invasion and ultimately decorates the PVM [43]. Whereas initial experiments indicated that ROP2 is anchored to the PVM by a transmembrane domain [36], this conclusion has been reassessed recently [44, 45]. Nonetheless, there is agreement that the N-terminal portion of ROP2 is exposed to the host cytosol. Interestingly, this region of ROP2 resembles a mitochondrial matrix import signal that is exposed by cleavage of the prodomain [36]. While this element is necessary for interaction with the host mitochondrion, antibodies to the import receptor TOM-20 largely failed to block partial import of ROP2, suggesting that the protein may use an alternative import pathway [36]. Attempts to disrupt the ROP2 gene have not been successful, implying a crucial function for ROP2 in parasite invasion and development. Indeed, selectively depleting ROP2 mRNA using an antisense ribozyme resulted in less mitochondrial recruitment along with poor sterol acquisition from the host cell, defective rhoptry biogenesis, diminution of invasion, impairment of parasite division, and attenuation of virulence in mice [46]. Although the mechanism of ER recruitment is not well understood, a recent yeast two-hybrid study revealed GRA3 and GRA5 as candidate participants because of their ability to bind an ER integral membrane protein called calcium modulating ligand (CAMLG), amongst several other potential interaction partners [47]. Consistent with this hypothesis, GRA3 and GRA5 are both anchored to the PVM via a single transmembrane domain, and thereby are exposed to the cytoplasm for possible recruitment of host ER.

Cytoskeleton and lysosomes

T. gondii infection causes the reorganization of host intermediate filaments (IFs) and microtubules (MTs) around the PV (Fig. 1) [48, 49]. IF association with the PVM may provide a fortifying scaffold for this organelle and play a role in positioning the PV close to the nucleus [48]. Comprising of α- and β-tubulin, MTs stabilize the cell architecture and are responsible for the localization and the movement of intracellular organelles. Like IFs, MT encasement of the PV could support its structural integrity and juxtanuclear positioning. Remodelling of MTs around the PV is dependant on tubulin polymerisation and depolymerisation. In addition, the host microtubular organization center (MTOC) is recruited from the nuclear membrane to the PVM (Fig. 1) [49]. Although precisely how this benefits the parasite is still unclear [50], it will be interesting to see whether this reorganization of the MTOC at the PV is involved in the recently discovered ability of the parasite to induce host cell cycle arrest at the G(2)/M boundary [51, 52]. Endocytic vesicles and lysosomes are also detected in close proximity to the PV, and recent work has strongly implicated MTs as delivery conduits for parasite acquisition of endo-lysosomal components including cholesterol from the host [53].

Host cell cholesterol is crucial for *T. gondii* invasion and intracellular replication [27]. Indeed, the diminution of cholesterol at the host cell plasma membrane appears to disrupt the release of microneme and rhoptry contents during invasion by the parasite [28]. Cholesterol is mainly found in the rhoptries and in the pellicle of the parasite. It regulates the fluidity and

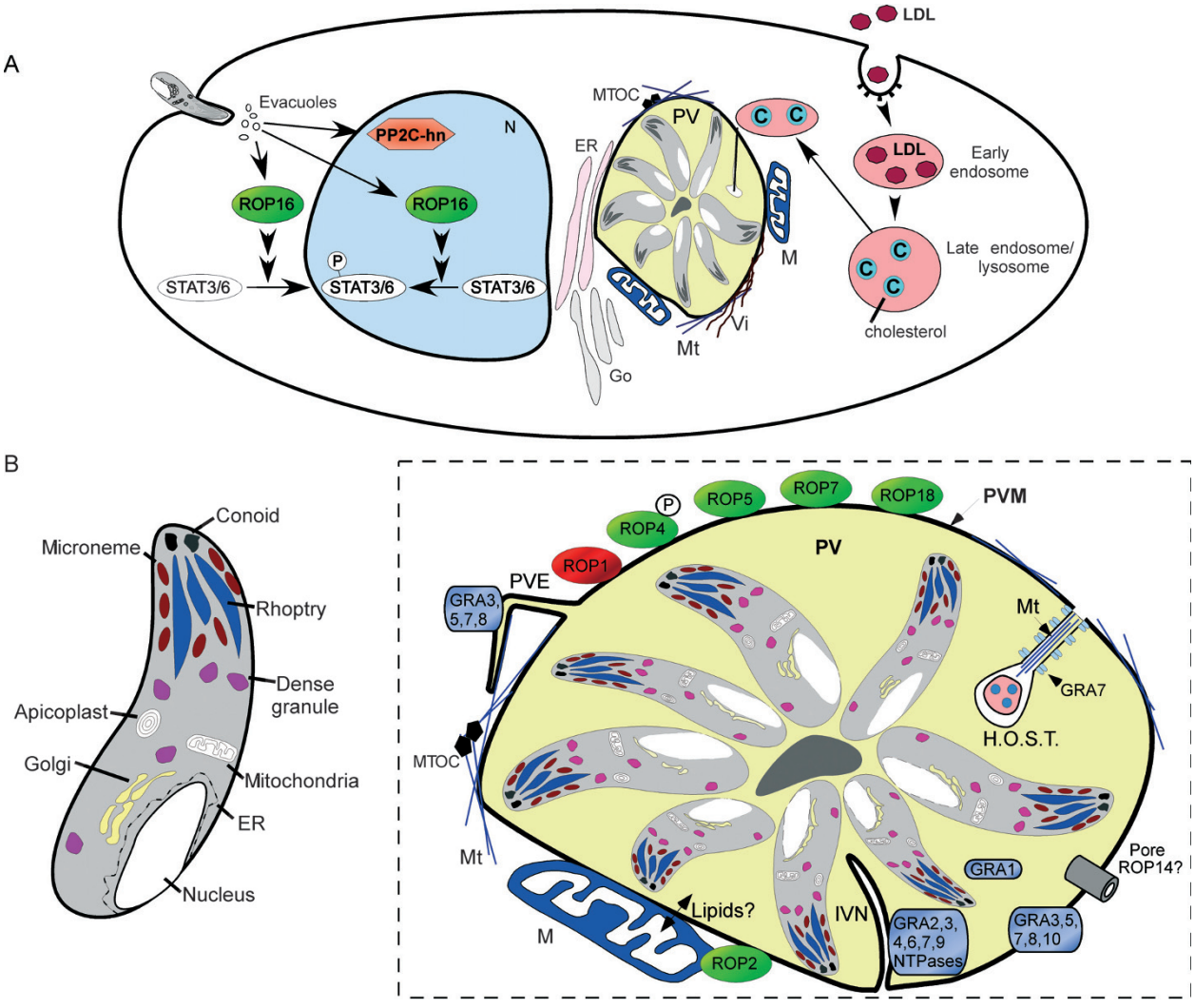

Figure 1. (*A*) Reorganization of the host mitochondria (M), endoplasmic reticulum (ER), lysosomes, microtubules (MT), microtubule organizing center (MTOC) and intermediate filaments (IF) around the parasitophorous vacuole (PV). Acquisition of cholesterol (C) via the endocytosis of low-density lipoproteins (LDL). Localization of the rhoptry proteins ROP16 and PP2C-hn in the nucleus (N) of the host cell soon after invasion. Phosphorylation of the transcription factors STAT3 and STAT6 in host cells infected with parasites of type I or III. Golgi apparatus (Go). (*B*) Proteins of the parasitophorous vacuole membrane (PVM) ROP1, 2, 4, 5, 7, 18 and GRA3, 5, 7, 8, 10. ROP2 is involved in the recruitment of mitochondria at the PVM. ROP14 is a candidate to compose the PVM pore. PVM invaginations allow encroachment of lysosomes into the PV. MT and GRA7 are involved in the rearrangement of the PVM in a structure termed H.O.S.T. GRA2, 3, 4, 6, 9 are inserted in the intravacuolar network (IVN). GRA3, 5, 7, 8 are associated with the parasitophorous vacuole extension (PVE).

rigidity of membranes, but the role of cholesterol in rhoptries is unknown. *T. gondii* does not synthesize its own cholesterol and instead scavenges it from the host. Parasite growth is enhanced by addition of free cholesterol or cholesterol bound to low-density lipoprotein (LDL) particles to the extracellular medium [27]. Also, extracellular parasites and parasites inside the purified PV can incorporate free cholesterol, suggesting the existence of cholesterol acquisition machinery at the plasma membrane and PVM [54]. Although the host cell can synthesize cholesterol within its ER, *T. gondii* does not obtain the cholesterol from this source, eliminating a role for PVM-ER association in cholesterol uptake [54]. Rather, the parasite diverts cholesterol from the host scavenging pathway involving endocytic uptake of LDL particles, which increases dramatically during *T. gondii* replication (Fig. 1A) [27]. The parasite interrupts the progression of LDL-laden endo-lysosomal vesicles to obtain host cholesterol in a manner dependent on energy and temperature. Endocytic vesicles and lysosomes normally occupy the perinuclear region in uninfected cells. However, 48 h after infection by *T. gondii*, the localization of these vesicles is observed proximal to the PV [49, 54]. The number of endocytic vesicles associated with the PVM increases with the

surface of the PV, underscoring the necessity of cholesterol and other nutrients for intracellular replication. Remarkably, MTs induce deep invaginations of the PVM into the lumen of the PV that are distinct from the IVN. These conduits deliver host endocytic vesicles to the PV interior, resulting in a double membrane structure, termed host organelle sequestering tubulo structures or H.O.S.T. (Fig. 1B). The diameter of the MT-based invaginations is estimated to be 95–115 nm and contains a network of MTs separated from each other by 15–20 nm. Coat-like material appears to bind the intrusion at regular intervals, generating a collar structure. Stabilisation of the PVM indentations is associated with the action of a protein called GRA7, which can mediate the narrowing of liposomes *in vitro*. Despite the absence of a conventional lipid-binding domain usually involved in the remodelling of membranes, GRA7 shows affinity for negatively charged lipids, and the predicted transmembrane domain of GRA7 is required for binding to such lipids. Through its ability to bind and tubulate membranes, GRA7 is proposed to constrict the PVM conduit to sequester the vesicle inside the lumen of the PV. Moreover, GRA7-deficient parasites have a growth defect that is exacerbated under serum-limiting conditions, suggesting a defect in nutrient acquisition [49]. Two hypotheses have been put forth to explain how cholesterol is transferred to the parasite (1) The outer membrane of the H.O.S.T., which corresponds to the PVM, could fuse directly with the parasite plasma membrane to liberate the endocytic vesicle inside the parasite. It should be noted, however, that this idea is inconsistent with the non-fusogenic nature of the PVM; or (2) Endocytic vesicles could transport nutrients in the intermembrane space of the H.O.S.T., which could then traverse the PVM to reach the PV lumen. In support of the latter mechanism, host cell Niemann Pick type C (NPC) cholesterol transporters are required for the parasite cholesterol acquisition, suggesting egress of cholesterol from the endo-lysosomal vesicle membrane to the PVM where additional transport activities may reside as noted above [54].

It is reasonable to predict that other essential nutrients are also obtained by the parasite via endocytosis and H.O.S.T. Iron, for example, is an essential element for all organisms, and iron uptake by pathogens is often a limiting factor for their growth. Host expression of transferrin receptor is upregulated in *T. gondii*-infected cells [23], suggesting that the parasite utilizes the host endocytic pathway for iron acquisition. In H.O.S.T. structures, it can be envisioned that host iron transporters of the Nramp family pump iron across the endo-lysosomal membrane where parasite iron transporters in the PVM could shuttle iron into the PV for uptake by the parasite. Indeed, the parasite expresses at least one Nramp-like putative metal transporter (gene ID 57.m01843; www.toxodb.org) that could function in iron uptake, but this has yet to be investigated.

Unlike IFs and MTs, actin microfilaments are not reconfigured during *T. gondii* replication [49]. However, during cell invasion the parasite must traverse the host's cortical actin cytoskeleton, presumably by either locally dismantling it or expanding a gap in its meshwork. A prime candidate for this role is a microfilament antagonist called toxofilin, which was shown recently to reside in the parasite's rhoptries [55]. This protein caps microfilaments and binds globular actin, making it unavailable for microfilament (re)polymerization [56]. The newly available crystal structure [57] showing that toxofilin uses several α-helices to embrace a host actin dimer should be invaluable for designing mutants to test its role in cortical cytoskeleton traversal.

Proteins associated with the PVM

The ROP2 subfamily members ROP4, 5, 7, 8, 16, and 18 have also been shown to associate with the PVM following invasion, suggesting a likely role in host interaction (Fig. 1B). ROP4 is highly homologous to ROP2. The carboxy-terminal portion of ROP4 displays a characteristic kinase domain. However, the lysine (subdomain II) and aspartic acid (subdomain VIb) residues typically necessary for kinase activity are absent [44]. Intriguingly, the mature form of ROP4 is phosphorylated on several serine and threonine residues following parasite invasion. Although it was shown that a kinase activity from the host cell or parasite (stimulated by host cell elements) can phosphorylate ROP4, the identity of this kinase remains unknown [14]. The function of ROP4 has not been determined at this moment, and parasites expressing a truncated ROP4 protein devoid of its kinase-like domain appear to invade and replicate normally [58]. It is possible that the function of ROP4 can be fulfilled by another ROP2 family member such as ROP7, which shares 71% identity with ROP4 and similarly lacks key residues involved in kinase activity [59]. Phosphorylation of ROP7 has not been the subject of investigation.

ROP5 shares 25% identity with ROP2. Like ROP4 and ROP7, ROP5 does not exhibit the aspartyl residues (subdomain VIb) important for phosphate transfer or the glycine motif (subdomain I) in its kinase domain. Contrary to ROP2 and ROP4, ROP5 does not undergo proteolytic maturation to remove a propeptide. Moreover, it adopts an inverted topology at the PVM compared to ROP2. These observations, coupled with the low homology between ROP2 and

ROP5, suggest specific functions for each of these proteins. Accordingly, knockouts of ROP5 and ROP2 appear to be lethal [45].

Additional proteins associated with the PVM include ROP1, which like other rhoptry proteins is initially discharged in evacuoles. Targeted deletion of the ROP1 gene does not, however, affect parasite invasion or development, although it does alter the internal ultrastructure of rhoptries [60]. ROP14 presents several predicted transmembrane domains usually found inside proteins involved in transport activity. For this reason, ROP14 is a candidate for the formation of the pore associated with the diffusion of small molecules across the PVM [44]. The dense granule proteins, GRA3, GRA5, GRA7, GRA8 and GRA10, are also localized at the PVM after the invasion of the parasite [61–64]. Future experiments may reveal their specific roles in host cell interactions via the PVM.

Rhoptry proteins manipulate host signalling pathways

ROP18 dictates parasite growth and virulence

Three main lineages of *T. gondii* are common in North America and Europe. These strains display high genetic identity, approximately 99 % overall. *T. gondii* type I strains are highly virulent in mice, and infected animals typically succumb to the acute infection [65]. Type II and III strains establish chronic infections in mice and show differences in virulence in inbred mouse lineages. As an initial survey of virulence determinants, the genomes of several progeny from a genetic cross of type I and type III parasites were analyzed and compared according to the percent mortality induced in mice [66]. A bank of primers was used to amplify specific parts of the genome for detection of restriction fragment polymorphisms (RFLPs) caused by single-nucleotide polymorphisms (SNPs) [67]. This preliminary analysis showed that a section of Chromosome VIIa is associated with lethal infection of type I strains in mice [66].

More recently, several studies have exploited the availability of genome sequences (and the associated plume of SNPs) [68] from type I, II, and III strains to identify specific genes responsible for virulence [69, 70]. With the parental type I and III strains showing clear distinctions of virulence, Taylor et al. [70] further analyzed progeny from the type I × III genetic cross. Corroborating the earlier study, genomes of the virulent progeny displayed a conserved portion of Chromosome VIIa from the type I parent. The same major virulence locus on Chromosome VIIa was also identified during analysis of progeny from a type II x III cross in which avirulent parental strains gave rise to more virulent progeny when monitored in inbred mice [69, 71]. Although the virulence locus on Chromosome VIIa contains numerous genes, one specific gene encoding ROP18 garnered particular scrutiny because it displays a kinase domain and is characterized by an exceptionally high number of SNPs. Also, expression levels of the ROP18 gene in type I and III strains are highly divergent, with ROP18-III being almost undetectable compared to the ROP18-I or ROP18-II. Analysis of the promoter region revealed an extra sequence of 2.1 kb near the start codon in the type III strain, which is absent in the genome of type I and II strains [69]. This extra sequence is probably responsible for the diminution of ROP18 expression from the type III promoter.

Although some investigators in the field had considered the idea that parasite replication rate influences virulence (for example [72]) direct evidence of it being a key virulence trait was lacking. This changed with the observation that expression of ROP18-I in the type III strain leads to a significant increase of intracellular replication [70], and that ROP18 expression levels in natural isolates directly correlates with growth rate and virulence [S. Taylor, *Toxoplasma* 2007 meeting]. Overexpression of ROP18-I in type I parasites also increases intracellular replication [73]. Moreover, mice infected with a type III strain expressing ROP18-I or ROP18-II succumb to lethal infection unlike those infected with the wild-type type III strain [70]. Although the high level of polymorphisms found between the ROP18-I and ROP18-III genes could contribute to the mortality differential, amino acids involved in the catalytic kinase activity of ROP18 are conserved in both strains. Accordingly, the virtual absence of ROP18 expression in type III strains is more likely responsible for the observed difference in mortality [70]. Although genetic disruption of ROP18 in type I parasites was unsuccessful [73], the observation that type III strains produce negligible amounts of ROP18 and yet remain viable [69] indicates that ROP18 may not be essential. Moreover, expression of ROP18-I in a type III strain is not entirely sufficient to impart the high-level mortality of a type I strain, suggesting other genes also influence virulence.

ROP18-I or ROP18-II expressed in a type III strain localizes to the rhoptries, is discharged with evacuoles, and associates with the PVM after invasion (Fig. 1B) [69, 70]. ROP18, as with several other ROP2 subfamily members, possesses stretches of arginine residues that could be involved in binding to the PVM, possibly via an ionic interaction with phospholipids. The serine/threonine kinase domain of ROP18 exhibits all essential residues to support this activity. Moreover, ROP18 kinase activity seems to be the key

to rapid growth and induction of virulence in mice since mutation of a critical amino acid (Asp) in the kinase domain failed to promote the gain of virulence observed in type III parasites expressing ROP18-I [70, 73]. With direct exposure to the host cell cytosol through its association with the PVM, ROP18 is an excellent candidate for modification of host cell signaling pathways and modulating the expression of host genes. However, *in vitro* assays failed to show evidence of host cell protein phosphorylation by ROP18. In addition, overexpression of ROP18 is required in each vacuole of the infected cell to induce the increase of parasite replication, suggesting that ROP18 acts locally [73]. Consistent with an indirect role in modulating the host, ROP18 specifically phosphorylates a 70-kDa protein in a tachyzoite protein lysate. However, the identity of the protein has not been established [73]. Also, while kinase activity has been detected at the PVM [14, 74], ROP18's contribution to this activity remains unknown. Future validation of ROP18 substrates, their associated signaling pathways, and consequential changes in host gene expression (if any) should clarify how ROP18 drives parasite growth and virulence.

ROP16 modulates host signaling and influences the course of infection

Investigating another distinction amongst type I, II and III parasites, Saeij and co-workers [69, 75] revealed conspicuous strain-dependent differences in host gene expression during *in vitro* infection. To examine this further, host cells infected with progeny from a type II × III cross were subjected to transcriptional profiling, thus confirming that the pattern of host gene expression is dependent on genes transmitted to the progeny by one of the parental strains. Chromosome VIIb was shown to contain genes involved in differential host gene expression induced by the parasite. More specifically, a locus on Chromosome VIIb for modulates genes involved in the Janus kinase (JAK)/STAT, amyloid processing, and IL-4 signaling pathways. The main transcription factors orchestrating these pathways are STAT3, STAT5b, JUN, and HIF1α. Further analysis revealed that phosphorylation of STAT3 (and STAT6) requires invasion by *T. gondii.* Although STAT3 phosphorylation is observed soon after the infection with all parasite strains, only type I and III strains maintain the phosphorylation 18 hours post infection. Remarkably, this phenotype of lingering STAT3 phosphorylation is genetically linked to another highly polymorphic rhoptry protein, ROP16 [75]. Confirming its involvement, ROP16-I or ROP16-III induce the prolonged indirect phosphorylation of STAT3 and STAT6 when expressed in a type II strain. ROP16-I accumulates in the host nucleus shortly after invasion (Fig. 1A) and it displays a conserved NLS (nuclear localization signal). Nonetheless, nuclear localization does not seem to be essential to promote STAT3/6 phosphorylation, a finding that is in agreement with STAT 3/6 usually residing in the cytosol. Contrary to other ROP2 subfamily proteins, ROP16 does not contain arginine-rich stretches potentially involved in binding to the PVM, which is consistent with its absence from this structure. Like ROP18, ROP16 contains key residues linked to kinase activity. However, again, the phosphorylation of STAT3/6 does not appear to involve a direct interaction with ROP16 since the pathway is equally stimulated by phosphorylation in all strains. Rather, ROP16-I and -III more likely activate a host factor that induces the downregulation of the STAT3/6 within 18 h of invasion. Microarray analysis of host cells infected with type II parasites versus those expressing ROP16-I confirmed the importance of ROP16 in modulating host gene expression. This modulation may be particularly important to the outcome of infection, especially during the early acute phase where infection of macrophages plays a pivotal role. Because STAT3/6 phosphorylation is not sustained in macrophages infected with type II parasites, this leads to high production of interleukin 12 (IL-12) compared to macrophages infected with type I and III strains [69, 76]. IL-12 promotes the production of interferon-γ (IFN-γ) by T cells, which suppresses parasite replication and activates natural killer cells to further limit the infection. Thus, the rapid immune response to type II strains helps ensure the survival of the host and parasite, which differentiates into encysted bradyzoites for persistence. By contrast, early suppression of IL-12 production by type I strains leads to unabated parasite replication that is ultimately fatal in mice. Why aren't type III strains similarly lethal? While the answer to this question is undoubtedly multi-factorial, the subdued replication rate of type III strains due to low expression of ROP18 is likely a major contributor. Thus, particular combinations of ROP18 and ROP16 alleles appear to dictate strain-dependent virulence by modulating parasite replication and the host immune response. Accordingly, the nearly simultaneous discovery and analysis of ROP18 and ROP16 is a leap forward in understanding the molecular basis of *Toxoplasma* virulence and manipulation of its host.

Modulation of other host transcription factors by *T. gondii*

The host transcription factor hypoxia inducible factor 1 (HIF1α) is induced following *T. gondii* infection [77]. HIF1α influences the transcription of the glycolytic enzymes, glucose transporters, transferrin recep-

tor, and vascular endothelial growth factor receptor. The activity of HIF1α is important for *T. gondii* growth at physiological oxygen levels. The enhancement of host glucose acquisition and metabolism, iron uptake, or maintenance of host cell integrity by HIF1α could explain its role in the promotion of the growth of *T. gondii* [77].

IFN-γ is crucial for controlling *T. gondii* acute infection, for inducing differentiation to bradyzoites, and for suppressing reactivation of chronic infection. Kim et al. [78] reported recently that although treatment of uninfected fibroblasts activates or represses 127 genes, none of these changes were seen in *T. gondii* infected fibroblasts after IFN-γ treatment. This unresponsiveness was attributed to two mechanisms: (1) Since approximately half of the IFN-γ-responsive genes are controlled by NF-κB and NF-κB is already activated by *T. gondii* infection, these genes cannot be further regulated by subsequent treatment with IFN-γ; and (2) The study demonstrated that *T. gondii* infection interferes with STAT1-induced expression of interferon regulatory factor 1 (IRF1), a transcription factor that regulates most of the other half of IFN-γ-responsive genes. Since STAT1 phosphorylation and translocation to the host nucleus is normal in *T. gondii*-infected fibroblasts, the parasite likely blocks STAT1 function within the nucleus, presumably via nuclear targeting of a parasite-derived effector protein. This IFN-γ-unresponsive state likely contributes to parasite persistence despite strong immune activity induced by IFN-γ.

The host nucleus: a new destination for parasite effector proteins

ROP16 abundance in the host nucleus correlates with the number of invaded parasites and decreases in a time-dependent manner after invasion [69]. Thus, nuclear-localized ROP16 most likely comes from release of the rhoptry contents during the invasion itself, rather than from deployment after entry. A similar phenomenon was also recently seen with another ROP protein initially identified by its reaction with a monoclonal antibody and its occupation of the host cell nucleus [79]. Also consistent with delivery during invasion, nuclear localization of this protein is also observed when cells are treated with cytochalasin D (CytoD), arresting the parasite at the stage between rhoptry secretion and penetration. Interestingly, this novel protein contains a protein phosphatase 2C (PP2C) domain and was accordingly named PPC2-hn, for host nucleus (Fig. 1A). Although PP2C family phosphatases are typically Mg^{2+} or Mn^{2+}-dependent, several residues implicated in metal binding are absent in PPC2-hn. Consequently, the *in vitro* phosphatase activity of PP2C-hn is low and metal-dependent. Disruption of PP2C-hn was performed to evaluate its function in the growth of the parasite. However, the absence of PP2C-hn has only a modest effect on the replication rate of intracellular parasites and no consequence on the virulence of type I parasites. Despite residing in the nucleus where it is well positioned to modulate host gene expression, no differences in host transcriptional profiles were observed comparing wild-type and PP2C-deficient parasites. Nonetheless, it remains possible that host elements are post-translationally affected by PP2C-hn [79].

Yeast two-hybrid experiments suggest that host cell partners of GRA10 are localized in the host nucleus, including the host transcription factor STAT6 [47]. As described above, phosphorylation of STAT6 following parasite invasion depends indirectly on ROP16 activity [75]. STAT6 is involved in the activation of IL-4 responses such as induction of the expression of anti-apoptotic factors [80]. In addition, GRA10 exhibits a potential NLS and is found in the host nucleus when expressed in HeLa cells [81]. Additional experiments are needed to explore the impact of GRA10 in the modulation of the molecular signaling of the host cell and its potential localization in the nucleus following invasion.

A regulatory function for catalytically deficient enzyme effectors?

The catalytic center of enzymes is typically highly conserved. The substitution of only one amino acid can lead to disruption of the enzymatic activity. Recently, proteins containing a degenerated catalytic center were shown to modulate the activity of paralogous active enzymes [82]. This regulation can take different forms through modulation of the active enzyme by direct protein-protein interactions with its inactive paralog or by substrate sequestration by the inactive enzyme [83–86]. This phenomenon is mainly observed in proteins involved in signaling pathways. For example, tyrosine phosphatase activity can be modulated by inactive enzyme paralogs [82]. Accordingly, the weak phosphatase activity of PP2C-hn in the nucleus of the host cell could interact and regulate host phosphatases. A similar phenomenon may occur for kinases. Approximately 10% of kinase proteins characterized from eukaryotes are predicted to have an inactive catalytic center [87]. Some of these pseudokinases play a role in the activation or inhibition of other kinases, or in the assembly of kinases in complex [83–87]. While ROP2 subfamily proteins contain a predicted kinase domain, sequence analysis by El Hajj et al. [44] shows that ROP2, 4, 5, 7, 8, 11, 2L3, and 2L6 proteins lack important residues usually essential for catalytic activity. A role of these proteins

in the modulation of host kinase activation remains to be tested. It should be noted, however, that enzymatic activity has been observed for some protein kinases lacking certain key residues; therefore a catalytic role for the aforementioned ROP2 family members in host modulation cannot be ruled out [87].

Interestingly, the uncharacterized proteins ROP17 and ROP2L4 also display ROP2 subfamily properties and a conserved kinase domain. The analysis of the cellular localization of these proteins, their potential substrates, and their role in virulence could also lead to interesting discoveries related to manipulation of the host signaling pathways [44].

Staying alive: new insight into anti-apoptotic mechanisms of *T. gondii*

Induction of apoptosis (also known as programmed cell death) is a common host response to infection with a variety of viruses, bacteria, and eukaryotic pathogens [88, 89]. In this manner, infected hosts reduce the replication and spread of pathogens. Not surprisingly, some microorganisms including *T. gondii* have developed tactics to hamper elimination by apoptosis (Fig. 2) [90, 91]. Blocking apoptosis helps the parasite avoid rapid clearance by macrophages, which are activated by signals emitted from apoptotic cells. Also, *T. gondii* must conserve the integrity of the host cell to obtain nutrients, since apoptotic cells undergo self-catabolism to render their macromolecules available for neighbouring cells and phagocytes. *T. gondii* impedes apoptosis of the host cell after exposure to a wide range of apoptotic inducers, including UV or gamma irradiation, growth factor scarcity, or exposure to toxins [91, 92]. These observations imply that *T. gondii* acts on common downstream apoptotic effectors or inhibits apoptotic elements of various pathways simultaneously.

Apoptosis can be induced by intracellular or extracellular stimulations. In both cases, the apoptotic process involves an activation cascade of cysteine proteases termed caspases. The successive activation of caspases results in amplification of the first signal leading to a suicide spiral [93]. Caspases are classified in two groups: initiator and executioner caspases [94]. Initiator caspases act upstream in the apoptotic pathway and are responsible for amplification of the initial signal. Executioner caspases act downstream to cleave specific proteins that result in the disassembly of the nucleus, degradation of the DNA, collapse of the cytoskeleton, and alteration of the cell surface, rendering the apoptotic cell distinguishable to surrounding cells and macrophages (Fig. 2) [95].

Role of the death receptor pathway

Initiator pro-caspases can be activated via extracellular ligation of the tumor necrosis factor receptor (TNFR) or Fas death receptors at the plasma membrane. In type I cells (e.g., SKW, H9), activation of these receptors results in assembly of components of the death-inducing signaling complex (DISC). This complex allows the recruitment and activation of the initiator caspase 8, which in turn activates the executioner caspase 3, prompting cell death [96, 97]. Although in type II cells (e.g., Jurkat, CEM) activation of Fas/CD95 leads to a weak formation of DISC, caspase 8 is still activated sufficiently to cleave the pro-apoptotic protein Bid, which induces release of cytochrome c from the mitochondria (see also below) [98, 99]. In both type I and II host cells, infection by *T. gondii* inhibits the apoptotic death receptor pathway by antagonizing caspase 8 (Fig. 2) [100]. Indeed, cells infected with *T. gondii* exhibit a lower total level of caspase 8 protein and activity, apparently via parasite-dependent proteolytic cleavage and degradation [100]. A recent study by Persson et al. [101] also shows that the ligation of death receptor or delivery of perforin/granzyme mediators leads to the egress of parasites (type I or II) from infected cells. These events initiate the caspase cascade and result in calcium release from host ER, but how this triggers parasite egress remains unclear since environmental calcium does not regulate *T. gondii* motility [102]. Nonetheless, this parasite exodus necrotizes the host cell rather than triggering apoptosis that is usually observed in uninfected cells following activation of death receptor. This immune-mediated induction of egress following death receptor ligation may aid the parasite by facilitating dissemination to adjacent cells including the very immune cells that triggered egress [101].

Induction of the intracellular apoptotic pathway

The intracellular apoptotic pathway can be induced by stress or damage to the cell and critically involves the mitochondrion. DNA damage is the most common cause of apoptosis induction via this pathway, which when activated leads to cytochrome c release into the cytosol. Cytochrome c binds and activates the apoptosome complex including the initiator caspase 9 [103]. Activated caspase 9 cleaves caspase 3, leading to disassembly of the cell. *T. gondii*-infected cells show only a small release of cytochrome c after induction of apoptosis via the mitochondrial pathway, thus dampening activation of caspases 9 in the apoptosome and subsequently precluding activation of caspase 3 (Fig. 2) [104, 105].

The permeability of the mitochondrial outer membrane is controlled by the ratio of pro-apoptotic and

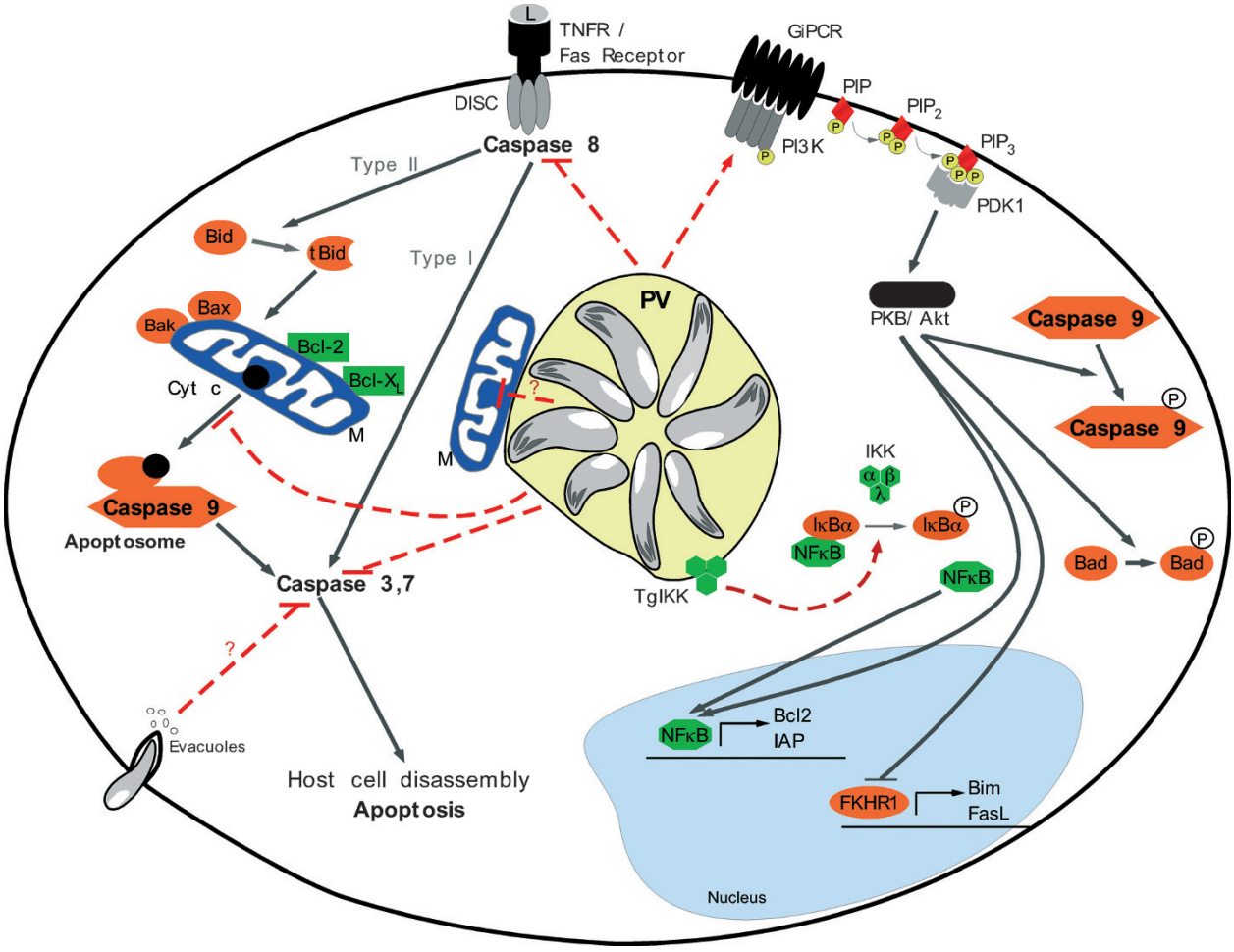

Figure 2. *T. gondii* influences the apoptotic pathways of the host cell. The inactivation of the initiator caspases 8 and 9, as well as executioner caspases 3 and 7, is seen following invasion by *T. gondii.* Induction of apoptosis in host cell types I and II, via the tumor necrosis factor receptor (TNFR) and Fas death receptors, is inhibited by *T. gondii* through degradation of caspase 8. Infection with *T. gondii* retards the release of cytochrome c from the host mitochondria intermembrane space. The role of the host mitochondria and PVM association in the blockade of apoptosis is still unclear. Proteins released from the secretory organelles during invasion could be involved in the inhibition of executioner caspases 3 and 7. In certain cells, the transcription factor NF-κB translocates to the nucleus after infection where it promotes the expression of inhibitor of apoptotic proteins (IAP) and anti-apoptotic Bcl2-family proteins. The phosphorylation of the inhibitor κBα (IκBα) by host and parasite IKK proteins is involved in the activation of NFκB. Activation of the phosphoinositol 3 kinase (PI3K) pathway in infected cells leads to inactivation of the pro-apoptotic factor Bad, the inhibition of the transcription factor forkhead transcription factor (FKHR1) and to activation of the NF-κB pathway.

anti-apoptotic Bcl-2-family proteins, which are localized at the mitochondria membrane. Indeed, infection by *T. gondii* induces the expression of anti-apoptotic members Bcl2, Bfl1, Bcl-Xl, Bcl-w, Mcl-1, and proteins involved in the degradation of the pro-apoptotic factors Bad and Bax (more on this below) [92, 104, 106]. As mentioned above, *T. gondii*-infected cells exhibit a muted release of cytochrome c following induction of the intracellular apoptotic pathway, suggesting additional parasite manipulation to restrain apoptosis.

The recruitment of mitochondria at the PVM could directly play a role in the blockage of the mitochondrial apoptosis pathway [107]. However, only a subset of the host's mitochondria is associated with the PVM, an observation that is inconsistent with global blockage of the mitochondrial pathway [104]. Also, products secreted by the parasite appear to be capable of limiting activation of the executioner caspases 3 and 7, implying that rhoptry or dense proteins may be involved [105]. Nevertheless, the abundance of caspases in the host cell versus the limited amount of proteins discharged during invasion argues against a direct stoichiometric inhibition of these enzymes (Fig. 2). Recently, proteomic analysis has identified ubiquitin ligase and de-ubiquitinating activities at the PVM (described as unpublished data in [33]). Ubiquitin ligase catalyzes the covalent attachment of the polypeptide ubiquitin to specific proteins. This modification could stimulate the target protein's degrada-

tion by the proteasome, or it may alter the target protein's activity, its cellular location, or its interactions with other proteins. Some viruses express specific ubiquitin ligases to destroy host proteins and in this way facilitate their growth [108]. Conversely, de-ubiquitinating activity results in cleavage of the ubiquitin bond. The presence of these activities associated with the PVM suggests the ability of the parasite to modulate the level of host cell proteins. For example, blockade of apoptosis could be directly linked with the elimination of pro-apoptotic elements by their specific ubiquitination by the parasite enzyme or by preservation of anti-apoptotic factors through de-ubiquitination. Future experiments aimed at confirming these activities and identifying the relevant enzymes should provide valuable insight into the role of (de-)ubiquitination in the parasite's intracellular lifestyle.

Role of the NF-κB pathway

The NF-κB transcription factor ensures the transcriptional upregulation of several cellular inhibitors of apoptosis proteins (c-IAPs) and anti-apoptotic Bcl2-family proteins that act directly on caspases and on the permeability of the mitochondria, respectively [109]. Five members of NF-κB have been identified: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel. In the cytosol, NF-κB members are retained by inhibitor κBα (IκBα) proteins. The phosphorylation of IκBα by the IκB kinase (IKK) complex results in the degradation of IκBα by the proteasome and in the translocation of NF-κB to the nucleus, where it promotes the transcription of anti-apoptotic genes, among others [110]. NF-κB members P50 (NF-κB1), p65 (RelA), p52 (NF-κB2), and RelB are found in host nuclear extracts of *T. gondii*-infected fibroblasts [106]. Additionally, infection with *T. gondii* does not protect host fibroblasts deficient in p65 in the presence of inducers of the mitochondrial or death receptor apoptotic pathways [111]. Usually, cells infected with *T. gondii* present inactive caspases 3, 8, and 9, but activities of these caspases were detected in $p65^{-/-}$ cells harbouring *T. gondii.* Consequently, the upregulated expression of Bcl-2 family proteins and c-IAPs, usually observed following infection, is not seen in p65 deficient cells [111]. This observation demonstrated the importance of NF-κB p65-component as a contributing factor in the parasite's ability to maintain infected fibroblasts in an anti-apoptotic state [106]. It should be noted, however, that movement of NF-κB to the nucleus is not observed in all types of infected cells including macrophages, which do not exhibit NF-κB translocation early after invasion [112–114]. This may be due to inherent differences between cell types or a result of examining translocation at different time points post invasion.

As mentioned above, translocation of NF-κB to the nucleus depends on phosphorylation of its inhibitor IκBα by the IKK signalosome in the cytosol. The IKK complex includes three subunits, α, β, and γ. The catalytic subunits IKKα and IKKβ are activated following stimulation by pro-inflammatory factors, while IKKγ is a regulatory subunit. Cells depleted of IKKα/β are incapable of activating NFκB. Notably, *T. gondii*-infected cells present a striking increase in phosphorylated IκBα (P-IκB) concentrated at the PVM [106]. Surprisingly, the host IKK complex does not localize to the PVM and does not seem to be involved in the phosphorylation of the IκBα observed at the PVM. Indeed, cells depleted of IKKα/β still show P-IκBα at the PVM. Intriguingly, IKK activity from a hypothetical parasite protein (TgIKK) is found at the PVM (Fig. 2)[74]. The parasite gene encoding this activity remains to be identified.

Molestina et al. [115] recently investigated the contribution of parasite TgIKK to activation of the NF-κB pathway by infection. They showed that while the host IKK signalosome is essential for translocating NF-κB to the nucleus and for the observed upregulation of NF-κB target genes early after *T. gondii* infection, maintenance of the response appears to require the contribution of parasite TgIKK during the intermediate phase of the infection at a stage where host IKK dampening is normally seen. At this point, a second peak of translocation of NF-κB to the nucleus and upregulation of its target genes is observed. Thus, in a two-phase process the host IKK complex initiates NF-κB activity and a parasite TgIKK sustains the activity.

The presence of parasite kinases targeting the host IκBα protein may extend to other apicomplexans. The closely related parasite *Neospora caninum* shares several properties with *T. gondii*, such as establishing an anti-apoptotic state in the infected host cell. However, cells infected with *N. caninum* do not show nuclear translocation of NF-κB, even though this parasite exhibits endogenous IKK activity similarly to *T. gondii* [116]. These results suggest differential roles between the IKKs of *T. gondii* and *N. caninum* in the modulation of the NF-κB pathway and by extension the inhibition of apoptosis in the infected cell. Identification of the genes coding for such factors will provide insights into their modulation of host cell processes during infection.

Role of the PI3K pathway

Maintaining an anti-apoptotic state in mammalian cells relies on activation of the phosphoinositol 3 kinase (PI3K) signaling pathway via specific ligation

of heterotrimeric G_i protein-coupled receptors (G_iPCRs), growth factor receptors, and other proteins by survival factors (certain growth factors, chemokines, and cytokines). In a series of steps, activated PI3K increases levels of phosphatidylinositol-3,4-biphosphate (PIP_2) and phosphatidylinositol-3,4,5-triphosphate (PIP_3) at the plasma membrane, leading to recruitment of the phosphoinositide-dependent kinase 1 (PKD1), which in turn phosphorylates and activates another kinase called Akt/PKB. Akt/PKB then directly phospho-inactivates caspase 9 and the pro-apoptotic protein Bad [117], and it suppresses expression of two additional pro-apoptotic factors, Bim and FasL, by inactivating the forkhead transcription factor (FKHR1) [118]. Since Akt/PKB also simultaneously triggers the NF-κB pathway [119] for expression of anti-apoptotic genes, this kinase maintains an anti-apoptotic state by inversely influencing both pro- and anti-apoptotic factors.

Kim and Denkers [120] showed that Akt/PKB phosphorylation is elevated in *T. gondii*-infected macrophages isolated from acutely infected mice. Moreover, their *in vitro* experiments revealed a PI3K- and G_iPCR-dependent increase of activated Akt/PKB in macrophages and splenic cells infected with *T. gondii* (Fig. 2). While the precise mechanism remains to be determined, it is possible that the parasite secretes an agonist or ligand of G_iPRC or it may upregulate the expression of a host survival factor to activate G_iPRC and the PI3K pathway, thus maintaining an anti-apoptotic state.

In summary, *T. gondii* induces an anti-apoptotic state by modulating several host pathways that are normally triggered during infection by extracellular and intracellular cues. However, molecular aspects of this phenomenon still remain to be elucidated. Since different *T. gondii* strains may block apoptosis in distinct ways, genetic analyses such as those employed to identify ROP18 and ROP16 may provide additional insight into the molecular basis of *T. gondii*'s ability to modulate host apoptosis.

Manipulation of host cell motility and migration

Dendritic cells (DCs) are amongst the key antigen-presenting cells of the immune system. Once DCs collect a foreign antigen in tissue, lymphatics, or the blood, they migrate to the spleen and lymphatic nodes where they efficiently activate T lymphocytes [121]. The molecular mechanism of *T. gondii* dissemination through the host is unclear. However, *T. gondii* appears to preferentially invade DCs *in vitro* rather than other blood cells [122]. Building on this observation, Lambert and colleagues recently tested the possibility that the circulating capacity of DCs is exploited by *T. gondii* as a means of promoting dissemination during acute infection. Remarkably, they found that infection of DCs by *T. gondii* tachyzoites leads to a rapid and prolonged migratory enhancement *in vitro* [123]. Compared to uninfected DCs, infected DCs migrated at higher speeds, for longer distances, and exhibited superior transmigration across endothelial monolayers *in vitro*. Moreover, inoculation of mice with *T. gondii*-infected or uninfected DCs showed that migration of DCs to the spleen is enhanced by infection with the parasite. Parasite dissemination is also accelerated in mice inoculated with infected DCs compared to inoculation with free parasites, suggesting an important role of DCs in dissemination of *T. gondii* through the infected animal. The molecular mechanism by which the parasite modulates dendritic cell motility remains to be clarified. However, a G_iPCR signaling pathway seems to play a crucial role in the migratory phenotype [123]. This observation provides a potential mechanistic link to the previous report that *T. gondii* modulation of the G_iPCR signaling pathway is involved in maintaining host cells in an anti-apoptotic state [120].

Conclusions and perspectives

While it is clear that descriptions of host manipulation by *Toxoplasma* have surged forward recently, it is also equally evident that much more is yet to be learned. Precisely what nutrients the parasite gains from its intimate association with the host mitochondrion and ER is yet to be clarified. The recent finding that *Toxoplasma* diverts cholesterol from host endosomal vesicles should motivate the design of experiments to determine other nutrients the parasite siphons from this source. The new spotlight on ROP2 subfamily members will lead to a greater understanding of their individual roles, along with determining whether catalytically deficient members serve regulatory functions. Key along these lines will be the identification of host (or parasite) substrates of the rhoptry-derived kinases and phosphatases, along with traipsing up and down the associated signaling pathways to connect molecular events to biological outcomes. Genome-wide association studies such as those used to bring ROP18 and ROP16 to the forefront will become increasingly more common and powerful, revealing the ROP18-linked genes responsible for parasite motility and possibly identifying genes involved in other critical infection-promoting strategies such as parasite regulation of apoptosis and subversion of immune cell migration.

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