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The secreted kinase ROP18 defends *Toxoplasma*'s border

Sarah J. Fentress¹ and L. David Sibley^{1,*}

¹Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave, St. Louis, MO 63130

Summary

Toxoplasma gondii is a highly successful parasite capable of infecting virtually all warm-blooded animals by actively invading nucleated host cells and forming a modified compartment where it replicates within the cytosol. The parasite-containing vacuole provides a safe haven even in professional phagocytes such as macrophages, which normally destroy foreign microbes. In effort to eliminate the parasite, the host up-regulates a family of immunity-related p47 GTPases (IRGs), which are recruited to the parasite-containing vacuole, resulting in membrane rupture and digestion of the parasite. To avoid this fate, highly virulent strains of *Toxoplasma* coat the external surface of their vacuole with a secretory serine/threonine kinase, known as ROP18. At this host-pathogen interface, ROP18 phosphorylates and inactivates IRGs, thereby protecting the parasite from killing. These findings reveal a novel molecular mechanism by which the parasite disarms host innate immunity

Introduction

Parasites are evolutionarily ancient and diverse, and as such they offer a window into adaptations for survival in a variety of niches. Because parasites often infect animals and humans with advanced immune systems, their success predicts that they have deciphered and thwarted some of our most elaborate defenses. *Toxoplasma gondii* is a single-celled, eukaryotic protozoan parasite belonging to the phylum Apicomplexa. Like other members of this phylum, *Toxoplasma* is an obligate intracellular parasite, requiring active invasion of a host cell for replication [1]. During invasion, the parasite's actin cytoskeleton and surface adhesion molecules engage, forcing the host plasma membrane to invaginate to form a vacuole around the invading parasite [1]. The invasion process is punctuated by waves of parasite secretion from three sets of specialized organelles, namely micronemes, rhoptries and dense granules, which release proteins essential to intracellular survival [2,3]. Once inside the host cell, the membrane of the parasite-containing vacuole separates the parasite from the host cytosol, providing a protective niche for the parasite to undergo cell division.

Toxoplasma has a wide geographic range as well as a broad host range, frequently infecting domestic and wild animals [4]. Transmission of the parasite typically occurs through ingestion of undercooked meat from an infected animal or through consumption of untreated contaminated water sources that may contain infectious oocysts released by infected cats [4]. *Toxoplasma* has a complex life cycle, transitioning from a sexual cycle, which only occurs in cats, to an asexual cycle, which can occur in all warm-blooded animals and birds [4]. Humans are accidental hosts for the parasite, usually presenting with mild flu-like symptoms following infection [5]. In cases of immunocompromised patients and developing fetuses, toxoplasmosis can present as clinically severe infections leading to disease of the central nervous system, eye, heart, lung and liver [5]. Although humans are by-stander hosts, other

*Corresponding author: sibley@borcim.wustl.edu, TEL: 314-362-8873, FAX: 314-286-0060.

hosts are vital to preserve the success of the parasite in the environment. Indeed, the entire life cycle of *T. gondii* can be perpetuated by the carnivorous behavior of cats on chronically infected rodents [4], making the laboratory mouse model particularly informative of the natural lifestyle of the parasite.

The population structure of *T. gondii* in North America and Europe is highly ordered, giving rise to four distinct clonal lineages [6,7]. These strains propagate largely asexually, hence isolates of a given type are highly conserved in their genomes and biological traits. Despite having less than 2% genetic diversity between strain types, large phenotypic differences, including virulence, exist between these groups [7]. Parasites belonging to the type I group are uniformly virulent in laboratory mice, causing mortality at doses as low as one parasite (Table 1) [8]. Type II, type III, and the recently described haplogroup 12 parasites [6], are much less virulent, requiring much higher doses for lethality in mice (Table 1) [8–10]. Even though laboratory mice appear to be more sensitive to toxoplasmosis than humans, this model system provides a valuable tool for investigating the molecular basis of disease.

Genetic mapping defines virulence traits

Virulence of a pathogen is often measured in terms of morbidity or mortality in a particular animal model. In the case of *Toxoplasma*, survival of acute infection in laboratory mice following challenge with needle-inoculated parasites provides a convenient means of assessing virulence. Given the large phenotypic differences, yet highly similar genotypes, between strains, one would expect that the important differences are controlled by a small subset of genes that differ. However, finding the key differences required an unbiased approach that considers the potential contribution of all possible genes. The genome of *T. gondii* is approximate 65 million base pairs, encoding an estimate 8,000–9,000 genes [11], making this a challenging task.

To identify genes involved in acute virulence in the mouse model, forward genetic analysis accompanied by genome-wide scans for quantitative trait locus mapping were employed. Two classical genetic crosses were conducted, one between a highly virulent type I strain and an avirulent type III strain [12] and one between intermediate virulent type II and avirulent type III [13]. Genes for acute virulence were identified by association of specific genomic markers with phenotypes in recombinant progeny (Table 1). An association on chromosome 7a was detected for acute virulence in both crosses [13,14]. Reverse genetic analysis identified *ROP18* as the primary contributor to the acute virulence phenotype [13,14].

ROP18 is so named because the gene encodes a protein that is targeted to the rhoptry organelle in the parasite [15,16]. *ROP18* differs between the three parental strains in both expression level and in allelic variance (Table 1) [13,14]. Type III parasites express 1000-fold lower levels of *ROP18* compared to either type I or type II parasites due to ~2 kilobase insertion in the promoter of type III *ROP18* (Table 1) [13,14]. Further analysis revealed that this difference resulted from a deletion in the type I and II lineages, associated with upregulation of expression and enhanced virulence [17]. Consequently, type III parasites provide a background on which gain-of-function phenotypes could be examined. Expression of either the type I [14] or type II [13] *ROP18* allele in the avirulent type III parasite background resulted in enhanced intracellular replication and a four log increase in virulence in the mouse model over type III parasites. Additionally, clean deletion of *ROP18* in the virulent type I background resulted in virulence attenuation [18,19]. In short, high expression levels of *ROP18* correlate with survival and growth of the parasite *in vivo* and escape from immune destruction. Although these genetic studies revealed that *ROP18* was both necessary and sufficient for acute virulence in the type I strains of *T. gondii*, they did

little to define its precise function. Understanding how ROP18 works required both an appreciation for where and when it is located, and hence, what components of the host it was likely to interact with. At the outset, the only clue to function was that ROP18 was found in a secretory organelle called the rhoptry.

What is a rhoptry anyway?

Protein secretion is key to intracellular survival of *Toxoplasma*. In particular, release of rhoptry proteins into the host cell is thought to be important in forming the protective intracellular niche within which the parasite grows and replicates. Rhoptries are unique secretory organelles that are restricted to the phylum Apicomplexa. *Toxoplasma* parasites contain a cluster of 10–12 rhoptry organelles that emanate from the anterior pole and extend ~2–3 microns in length [20]. Ultrastructure analysis demonstrates that rhoptries are club-shaped, with a large bulb extending toward the posterior and a narrow, tapering neck extending toward the anterior of the parasite. Proteins are uniquely organized in the rhoptry, with some localizing to the rhoptry neck (RONs) and some localizing to the rhoptry bulb (ROPs), suggesting that distinct sub-compartments exist within the organelle [16].

Rhoptry secretion is initiated immediately following intimate contact with a host cell, although specific triggers of this process remain elusive [3]. After rhoptries fuse with the parasite plasma membrane, RONs are delivered to the external surface of the parasite first and contribute to the formation of a novel structure called the moving junction [21,22]. The moving junction is a ring-like structure through which the parasite moves during the invasion process, providing a critical contact point between the host plasma membrane and the parasite surface [22]. Treatment of parasites with cytochalasin D inhibits the driving force behind invasion, but, interestingly, the parasite still establishes intimate contact and releases rhoptry contents [23].

Although the molecular mechanism defining how the rhoptry fuses to the parasite membrane is unknown, phospholipases are a class of enzymes that facilitate membrane fusion and fluidity. An inhibitor of phospholipase A2s called 4-bromophenacyl bromide is the only known compound capable of blocking rhoptry release while allowing for microneme secretion in *Toxoplasma* [24]. Treatment of parasites with this compound decreased the ability of *Toxoplasma* to enter host cells [24]. While the *Toxoplasma* genome encodes many genes with predicted phospholipase activity, the direct target of 4-bromophenacyl bromide responsible for blocking rhoptry release remains unknown [24]. Further study into the mechanism of rhoptry discharge will not only shed light on the biology of parasite secretion, but may lead to candidates for drug discovery.

The ROP2 family of proteins contain a kinase fold

Rhoptries are complex organelles, and their composition and biogenesis are only partly understood. However, because they discharge their contents directly into the host cell, they are particularly well suited to modify host function. Proteomic analysis of purified rhoptry fractions identified more than 30 rhoptry proteins, most of which are homologues of kinases, phosphatases, and proteases [16]. ROP18 localizes to the rhoptry bulb and is a member of the ROP2 family of predicted serine/threonine kinases (Fig. 1) [13–15]. Many members of this family lack key residues in the catalytic loop, including the catalytic aspartate, and have also lost the glycine loop responsible for stabilization of the $\alpha\beta$ -phosphate of adenosine triphosphate (ATP) [25,26]. These degenerate ROP2 family members, including ROP2, ROP4, ROP7, and ROP8, are not expected to be active kinases capable of completing phospho-transfer reactions but may function in scaffolding and/or sequestering substrates (Fig. 1). As such, they are referred to as pseudokinases. Even still, crystallographic analysis reveals ROP2 and ROP8 maintain a conserved kinase domain fold with a unique regulatory

domain N-terminal to the kinase domain [25,27]. ROP18, like distantly related ROP16 [13,28] and ROP38 [29], retains all residues required for catalytic function and is predicted to be an active kinase (Fig. 1).

To examine the role of kinase activity in acute virulence, a point mutation in the key catalytic residue (Asp³⁹⁴ Ala) was constructed in ROP18 that abrogates catalytic activity *in vitro* [14,15]. Parasites expressing this kinase dead mutation remained avirulent, whereas parasites expressing the active kinase were lethal in mice [14]. Although this demonstrated the requirement of kinase activity to confer virulence, substrates of ROP18 remained unknown until recent studies. Defining the function of ROP18, and similar parasite kinases, is hindered by the permissive nature of serine/threonine kinases *in vitro*, making identification of authentic substrates challenging.

ROP18 is anchored to the vacuolar membrane via arginine-rich amphipathic helices

Following their release into the host cell, ROP proteins are targeted to different locations, which may in turn reflect different function. Among the ROPs that have been studied, there are two known localizations in the infected cells. ROP16, a kinase that modulates STAT3/6 activity [28,30,31], and PP2C-hn, a parasite-encoded phosphatase of unknown function [32], are both targeted to the host nucleus (Fig. 1). Members of the ROP2 family, including ROP18, are delivered to the cytosolic face of the parasite-containing vacuole once the parasite has initiated invasion (Fig. 2). Membrane association is mediated via arginine-rich amphipathic helices located near the N-terminus of mature ROP2 family proteins [27,33]. Although it is presumed that targeting of ROP18 to this host-pathogen interface is critical for its virulence-enhancing activity, this remains to be directly tested. Given the flexibility of genetic studies in *T. gondii*, further elucidation of the function of this N-terminal targeting domain could be approached by testing loss-of-function phenotypes of various truncation and/or point mutants. For example, mutations that separate targeting of ROP18 to the vacuole from kinase activity, could be used to probe whether proper localization is critical to its virulence enhancing function.

Innate immune mechanisms restrict *Toxoplasma*

While the parasite sets about the task of creating a replication-permissive niche within a host cell, the host mounts defenses against the parasite. *Toxoplasma* infection induces a vigorous type I immune response in mice driven by production of interleukin 12 (IL-12) that stimulates the production of interferon- γ (IFN- γ) [34,35]. Host resistance requires IFN- γ , a cytokine essential for initiation of effector mechanisms in both hematopoietic and nonhematopoietic cells that either limit replication or directly kill the parasite [36]. The induction of parasite stasis is mediated by the production of reactive oxygen intermediates, nitric oxide, or degradation of nutrients through IFN- γ signaling [35,37]. IFN- γ facilitates parasite killing through up-regulation of two families of GTPases in the dynamin superfamily, the immunity-related GTPases (IRGs, also known as p47 GTPases) [38,39] and the guanylate binding proteins (GBPs, also known as p65 GTPases) [40].

IRGs have been studied extensively in the context of *Toxoplasma* infection and constitute a major mechanism in mice for controlling the parasite [39]. In addition, IRGs have been shown to restrict a number of other pathogens, including *Chlamydia*, *Mycobacteria*, *Leishmania*, *Listeria*, and *Salmonella* [39]. Although mostly absent in humans, the IRG family of proteins is highly expanded in the mouse, consisting of 23 members [38]. These large GTPases are known to localize to and vesiculate the vacuolar membrane of *Toxoplasma*, resulting in vacuole rupture and death of the parasite in the host cytosol (Fig.

2). In uninfected cells, IRGs normally reside on endomembranes in a GDP-bound, or inactive, form (Fig. 2). Upon infection, IRGs transition to a GTP-bound, or active, form, oligomerize on the vacuolar membrane, and, through a process that is not well understood, result in membrane blebbing and breakage (Fig. 2) [41]. The host exerts control over the IRG pathway through the autophagy protein Atg5 [42]. In the absence of Atg5, IRGs aggregate in uninfected cells in a GTP-bound form and fail to target to the parasite-containing vacuole membrane upon infection, leading to increased susceptibility *in vivo* [42]. Although the specific mechanism of Atg5 regulation remains unknown, the IRG pathway does not appear to intersect classical macro-autophagy and therefore reflects a novel function of autophagy.

Susceptibility of *Toxoplasma* to IRG killing depends on the genetic background of the parasite and correlates with virulence [43,44]. Less virulent type II and III strains of *Toxoplasma* readily accumulate IRGs on the vacuole and are killed [44]. Highly virulent type I strains largely avoid IRGs and fail to be cleared from IFN- γ -activated cells. The correlation between parasite strain type and this clearance phenotype made ROP18 a prime candidate for modulation of IRG function.

ROP18 protects the parasite from killing

Although *Toxoplasma* can infect all nucleated cells, its interactions with macrophages are especially important in determining the outcome of infection. Because macrophages are armed with many antimicrobial mechanisms, surviving in such cells is a challenge for parasites like *Toxoplasma*. In particular, in response to infection, a potent class of inflammatory monocytes is recruited to eliminate foreign invaders. Inflammatory monocytes expressing a cell surface marker called Gr1 (specifically the Ly6C isoform) are essential for clearance of *T. gondii* early after infection in the mouse [45,46]. Given the critical importance of ROP18 in acute virulence, parasite survival was examined within Gr1⁺ Ly6C⁺ monocytes that are recruited to the peritoneal cavity following interperitoneal infection [18]. Extensive parasite replication is only evident in strains expressing active ROP18 and this correlates with recruitment of inflammatory monocytes [18]. Type I parasites, which strongly express ROP18 (Table 1), grow proficiently within the peritoneal cavity, while avirulent type III parasites, which are ROP18 deficient (Table 1), are effectively controlled [18]. Transgenic type III parasites expressing an active form of type I ROP18, but not a kinase-dead form of ROP18, also readily expand *in vivo* [18]. As a direct test, isolated Gr1⁺ Ly6C⁺ monocytes are able to efficiently kill ROP18 deficient parasites *in vitro* while parasites expressing active ROP18 resist clearance (Fig.2). Parasites expressing active ROP18 largely reside within intact parasite-containing vacuoles, while those deficient in ROP18 are surrounded by highly vesiculated vacuolar membranes that eventually breach, leading to death of the parasite in the host cytosol [18].

IRGs are targeted by ROP18

The cellular features of parasite death in inflammatory monocytes have all of the hallmarks of IRG mediated killing, including vesiculated vacuolar membranes, stripping of the vacuolar membrane and rapid death of the parasite in the host cytosol [18,47]. Parasites expressing active ROP18 largely resist IRG accumulation based on localization of the protein Irgb6, while efficient Irgb6 loading occurs on vacuoles containing parasites deficient in ROP18 (Fig. 2) [18,47]. Related IRG family members, Irga6 and Irgb10, are similarly thwarted by ROP18 [18,47]. During infection with ROP18-deficient strain, Irgb6 is loaded directly on the vacuolar membrane in addition to being found on vesicles in proximity to the vacuole [18]. Collectively, these studies indicate that kinase activity of ROP18 prevents

accumulation of IRGs to the parasite-containing vacuole, thus providing an explanation for the ability of parasites expressing this secretory kinase to escape clearance.

Given that kinase activity is required to block the IRG pathway and that substrate specificity of serine/threonine kinases is often influenced by proximity to their targets, it follows that ROP18 might directly phosphorylate IRGs on the vacuolar membrane. In support of this, biochemical analysis indicates that ROP18 and IRGs interact during infection [18], and the strength of this interaction was dramatically increased using parasites expressing kinase-dead ROP18, consistent with ROP18 being trapped with its substrate when unable to complete the phospho-transfer reaction [18].

To test the function of ROP18, direct biochemical assays were used to test its ability to phosphorylate substrates *in vitro*. ROP18 was shown to phosphorylate IRGs directly using a kinase reaction containing ^{32}P -labeled ATP [18]. Given that serine/threonine kinases can be very permissive *in vitro*, this phosphorylation event needed to be verified *in vivo*. To accomplish this, cells were treated with ^{32}P -labeled orthophosphate and infected with parasites. IRGs were isolated by immunoprecipitation and checked for radiolabeling that would indicate phosphorylation [18,47]. Only strains of *Toxoplasma* that express ROP18 were capable of phosphorylating IRGs (Fig. 2) [18,47]. Together, these findings indicate that IRG phosphorylation by ROP18 prevents accumulation on the vacuole and blocks parasite clearance, hence revealing a molecular mechanism for the virulence enhancing activity of ROP18. By placing an active kinase on the surface of its vacuole, *Toxoplasma* is able to block an important defense mechanism of the host.

ROP18 inactivates IRGs through phosphorylation of the GTPase domain

In order to further understand the role of ROP18 *in vivo*, it became important to better define its substrate preference. Serine/threonine kinases add a PO_4 to the hydroxyl group of serine or threonine. Kinases have selectivity for certain substrates based on residues that surround the central phospho-acceptor site. A variety of methods exist to define substrate preferences including mass spectrometry, phospho-peptide blots and *in vitro* assays. Phospho-peptide arrays reveal that ROP18 has a strong preference for threonine over serine, and a slight preference for acidic residues at positions upstream, and bulky hydrophobic residues at positions downstream of the phosphorylation site [18]. Analysis of a subset of IRGs that have previously been implicated in resistance to *T. gondii* for potential sites matching the ROP18 preference points to a potential ROP18 phosphorylation motif occurring within switch region I (SW1) of the GTPase domain [18]. The IRG SW1 is unique in the conservation of threonine residues that match the ROP18 substrate motif [18]. Consistent with this prediction, ROP18 selectively phosphorylates conserved threonine residues within SW1 of IRGs [18,47].

SW1 is a flexible loop in the protein that coordinates GTP binding and is known to facilitate binding of adaptor proteins, with mutations in this domain often altering function. No cellular kinase has been shown to phosphorylate IRGs, so analysis of modifications in SW1 is of critical importance to understand the biochemical consequences. Mutational analysis of the conserved threonine residues in IRGs impairs activity resulting in enzymes that fail to hydrolyze GTP or oligomerize in a GTP-dependent manner [47]. Such IRG mutants are also functionally deficient in that they fail to load properly onto the vacuole [47]. Although this mutational analysis is intriguing, future efforts should be placed on determining the biochemical consequence of phosphorylation at these conserved threonine residues. Nonetheless, these studies demonstrate for the first time that ROP18 is both necessary and sufficient to avoid IRG vacuolar accumulation and promote parasite survival in the murine host [18,47].

IRGs represent a powerful defense mechanism against *Toxoplasma* present in a variety of mammals, including rodents. Survival in the mouse is beneficial to the parasite in its quest to complete its life cycle and enter the cat host for sexual reproduction. Subversion of the IRGs by ROP18, therefore, reflects an evolutionary tit-for-tat where the mouse seeks to destroy the *Toxoplasma* invader through the action of IRGs on the vacuolar membrane and the parasite defies destruction through secretion of ROP18. Although other GTPases, like the small GTPase Rac for example, also contain threonines in switch region 1 [48], the positioning of these residues differs from the conserved motif seen in IRGs. Hence, the spacing of threonine residues, and particular structural differences among GTPases, may influence their ability to be recognized by kinases such as ROP18. Additionally, few GTPases, with the notable exception of IRGs and GBPs, are present on the vacuolar membrane where ROP18 resides. This proximity of kinase and substrate is likely also crucial to the substrate selectivity of ROP18 *in vivo*.

GTPases are ubiquitous components of essential cellular machinery that pathogens have subverted in a number of ways. Host GTPase activity has been shown to be altered by pathogens by proteolytic cleavage [49], molecular mimicry [50], direct adenylation [48] and also by phosphorylation [51]. Phosphorylation of IRGs by ROP18 represents the first example of a microbe modifying these immune mediator GTPases. As other pathogens are also targeted by IRGs, it is likely that they have evolved ways to disrupt this host defense pathway as well.

Implications for human infection

Toxoplasma also infects humans, although accidentally, hence there is no similar evolutionary pressure on resistance mechanisms. As humans largely lack the IRG family, with one truncated gene and another tissue specific gene in the IRG family [52], it remains to be seen if ROP18 plays any role in modulating disease outcomes in humans. Intriguingly, similar motifs for ROP18 phosphorylation are also found in the p65 family of GBPs (Behnke and Sibley, unpublished data). GBPs are implicated in pathogen control, localize to the parasite-containing vacuole, and are conserved in humans [53], suggesting the mechanism of ROP18-mediated pathogenesis may be more widespread. Although the mechanism of GBP action has been studied in the murine system [54], little is known about how GBPs function or are regulated in humans. This family is likely to be important in human defense, however, as human GBP1 and GBP2 are able to restrict *Chlamydia* infection *in vitro* [55].

Rope-a-dope: tale of another virulence-enhancing ROP

The findings of genetic mapping studies were surprising in revealing such a strong role for just a few genes in mediating major virulence differences, and it seemed unlikely this would be a general finding. However, genetic mapping has recently revealed another member of the ROP2 family as a principal virulence factor in *Toxoplasma* (Table 1, Fig. 1) [19,56]. In one case, a previously mapped acute virulence locus in the II by III genetic cross called VIR1 was mapped to a polymorphic cluster of pseudokinases called ROP5 [19]. Somewhat surprisingly, the same locus was independently mapped as the sole determinant in a new genetic cross between type I (high virulence) and type II (intermediate virulence) [56]. Genetic knockouts of ROP5 in virulent type I parasites do not cause mortality in mice, even at extraordinarily high doses of parasites ($>10^6$). Interestingly, ROP5 has a predicted serine/threonine kinase fold but lacks critical residues required for catalytic activity, making this virulence factor a predicted pseudokinase (Fig. 1). These studies mark the first reports of a predicted pseudokinase mediating pathogen virulence.

Pseudokinases are highly expanded in *Toxoplasma*, accounting for ~30% of the total kinome and 50% of the rhoptry kinome, compared to humans, in which 10% of the kinome encodes predicted pseudokinases [29]. In humans, pseudokinases have been described to alter cellular activities by allosterically regulating active kinases [57]. One attractive hypothesis is that the virulence factor ROP5 regulates the kinase activity of the other known virulence factor ROP18. However, given that the mouse mortality phenotypes are different between the genetic knockouts of these two ROPs (Δ rop5 is much more attenuated than Δ rop18 in the type I RH strain background), it is unlikely that regulation of ROP18 is the sole function of ROP5. Thus, it is likely that ROP5 has other mechanisms of virulence enhancement, perhaps through modulation of other secreted active ROP kinases. An alternative hypothesis involves interaction with host signaling pathways through ROP5 modulation of a host-encoded kinase. *Toxoplasma* is known to alter signaling in the host cell, including apoptotic pathways [58–60], induction of hypoxia inducible factor 1 (HIF1 α) [61], and activation of phosphoinositol 3 kinase signaling pathways [62], although specific mediators of these processes remain elusive. Although the mechanism of virulence enhancement has not yet been determined for ROP5, it is also poised at the vacuolar membrane to interact and interfere with host responses to infection.

Conclusions

Toxoplasma is a highly successful parasite, a feat achieved in part by delivering secreted proteins into its host cell to modify infection outcomes. ROP18 has been shown to mediate virulence and survival of the parasite by protecting the vacuolar membrane from destruction by the host. ROP18 phosphorylates immunity-related GTPases in a conserved section on switch region 1, thereby inactivating these GTPases. With the exception of ROP16 and ROP18 (Table 1), target pathways of other ROP effectors remain unknown, although a large number of phenotypic changes have been described in host cells infected with *T. gondii*. Studies to date do not rule out the possibility that ROP16 and ROP18 have multiple target pathways and substrates within the infected cell. Defining the functions of secreted ROPs will be an area of wide interest for future studies and will undoubtedly contribute new insights to *Toxoplasma* biology.

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Abbreviations

Atg	autophagy
ATP	adenosine triphosphate
IRG	immunity related GTPase
GBP	guanylate binding protein
RON	rhoptry neck protein
ROP	rhoptry protein
IL-12	interleukin 12
IFN-γ	interferon gamma

GTPase	guanine nucleotide triphosphatase
STAT	signal transducer and activator of transcription
SW1	switch region 1

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Figure 1.

ROP kinase family allows for exploitation of intracellular survival by *Toxoplasma*. Phylogenetic analysis shows the relationship of the ROP2 family with related ROP kinases. The predicted full-length protein sequences were aligned in Clustal using a gap penalty of 15 with an elongation penalty of 0.3 [63]. The alignment was analyzed in PAUP* 4.0 [64] to generate phylogenetic trees using distance with neighbor-joining BioNJ, bootstrapped for 1000 replicates. Black circles indicate bootstrap values >90%. Many ROP2 family members display degeneracy in key residues required for ATP binding and catalysis and are of unknown function [25–27]. An exception is ROP5, a predicted pseudokinase that is necessary for acute virulence in the mouse model. ROP18, the main focus of this review, is an active serine/threonine kinase that mediates acute virulence of the parasite. ROP18 protects the parasite from killing in macrophages by phosphorylating immunity related GTPases (IRGs) and inactivating enzymatic activity (see Figure 2).

ROP18-deficient *Toxoplasma* ROP18-expressing *Toxoplasma* **Figure 2.**

Schematic model of vacuole protection by ROP18. *Toxoplasma* that do not express ROP18 are killed promptly after invasion into interferon-activated cells. Interferon up-regulates immunity related GTPases (IRGs), such as Irgb6 (green), which normally reside on endomembranes in complex with Irgm regulator proteins (blue). IRGs cycle between GDP and GTP forms, oligomerize on the vacuole membrane, and slowly hydrolyze GTP. IRG recruitment results in vesiculation and blebbing of the membrane, eventually resulting in rupture of the parasite-containing vacuole and death of the parasite. Parasites that express ROP18 are rescued from this fate. Rhoptries, red, deposit ROP18 onto the surface of the vacuolar membrane during *Toxoplasma* invasion of a host cell. ROP18 phosphorylates IRG

proteins on conserved threonines in switch region 1, thus blocking GTPase function and protecting the parasite-containing vacuole membrane.

Table 1

Role of rhostry proteins in virulence differences between *T. gondii* strains

Strain	Mouse phenotypes	ROP18		ROP16		ROP5	
		Allele	Expression	Allele	Expression	Allele/ Locus	Gene copies
I	Virulent	I	++++	I/III shared	+	I/III shared	5–6
II	Intermediate virulence	II	++++	II	+	II	9–10
III	Avirulent	III	(+)	I/III shared	+	I/III shared	5–6