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Abstract

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Reference


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The *Toxoplasma* Protein ARO Mediates the Apical Positioning of Rhoptry Organelles, a Prerequisite for Host Cell Invasion

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SUMMARY

Members of the phylum Apicomplexa actively enter host cells by a process involving the discharge of the apically localized microneme and rhoptry organelles. To unravel the processes involved in rhoptry organelle biogenesis, we focused on the *Toxoplasma gondii* armadillo repeats only protein (TgARO), a conserved acylated protein homogenously anchored to the rhoptry membrane. Conditional disruption of TgARO results in the random cytosolic dispersion of rhoptries and a severe defect in *T. gondii* invasion, with no effects on intracellular growth or host cell egress. Importantly, rhoptry displacement upon ARO depletion can be functionally complemented with wild-type TgARO but not an acylation mutant. TgARO interacts with myosin F, and inhibition of actin polymerization or myosin function also results in rhoptry dispersal, indicating that the apical positioning of rhoptries is an actomyosin-based process. Thus, TgARO mediates the apical localization of rhoptries, which is specifically required for host cell invasion.

INTRODUCTION

Protozoan pathogens of the phylum Apicomplexa, such as *Plasmodium*, *Toxoplasma*, and *Theileria*, cause a broad range of debilitating diseases in humans and animals, leading to a high socioeconomic burden in many parts of the world. Members of this phylum differ greatly in their host specificity, ecological niches, and mode of transmission, yet they are all equipped with a specific apical complex that comprises the conoid, a motile organelle in the apical end of the parasite consisting of a unique polymer of tubulin fibers and three types of secretory organelles: the micronemes, rhoptries, and dense granules (Del Carmen et al., 2009). The apical complex empowers the parasites to glide, migrate across biological barriers, actively invade host cells, and egress from infected cells (Carruthers and Boothroyd, 2007). In spite of this, the structural and functional appearance of this complex varies among the apicomplexan species and among the different life stages within a single species (Blackman and Bannister, 2001).

Invasion by *Toxoplasma gondii* tachyzoites is a multistep process that begins upon contact with the host cell and apical reorientation of the parasite. This results in a burst of micromere secretion, immediately followed by the discharge of rhoptry organelles content (Dubremetz, 2007). *T. gondii* possesses 8–12 rhoptries that cluster together at the apical pole of the parasite and occupy 10%–30% of the total cell volume. Morphologically, rhoptries appear as club-shaped organelles with a bulbous body and an elongated, narrow neck that is oriented toward the conoid. Transmission electron microscopy (TEM) of ultrathin sections showed that the neck is highly and uniformly electron-dense, whereas the bulb is amorphous and electron-lucent (Dubremetz, 2007). Data from recent stereological analysis suggest that only up to four rhoptries at a time can access the inside of the conoid, and only one organelle can discharge at a time (Paredes-Santos et al., 2012). The precise docking site for the neck of the rhoptry at the conoid has not been identified, but the neck is in close proximity to a vesicle under the apical membrane. After releasing their content, rhoptry organelles appear as empty, large electron-lucent vacuoles (Dubremetz, 2007).

The rhoptry content consists of both lipids and proteins, many of which were identified by proteomic and lipidomic analyses of purified organelles (Besteiro et al., 2008; Bradley et al., 2005). Most proteins characterized so far either localize to the bulb of the rhoptry and are referred to as ROPs or to the neck region and named RONs (Boothroyd and Dubremetz, 2008). How ROPs and RONs become segregated within the organelle is not understood. Importantly, upon rhoptry deployment, RONs are secreted before the ROPs, and a subset of RONs are found to form a complex together with a micronemal protein, apical membrane antigen 1 (AMA-1), both in *T. gondii* and *P. falciparum* (Besteiro et al., 2011). This complex participates in the assembly of the moving junction (MJ), which is a constriction produced at the point of apposition between the parasite and the host cell membranes that enables the parasite to firmly attach to the host plasma membrane and actively propel itself into the cell (Alexander et al., 2005; Besteiro et al., 2009). ROPs, in contrast, are secreted into the forming parasitophorous vacuole and implicated in the formation of the parasitophorous vacuole membrane (PVM) and in the establishment of
a successful infection by interfering with host cellular functions (Kemp et al., 2012). Moreover, a recent study showed that *T. gondii* injects ROP proteins into host cells that it does not productively invade, thereby allowing the parasite to manipulate uninfected cells (Koshy et al., 2012). Overall, the differences in function and timing of RONs/ROPs secretion might explain their subcompartmentalization within the organelle. The cellular and molecular mechanisms underlying rhoptry biogenesis and their targeting to the apical pole are not understood. Both micronemes and rhoptries are formed de novo at a late stage of tachyzoite division (Nishi et al., 2008; Ogino and Yoneda, 1966). TEM studies revealed large, globular vacuoles within the developing daughter cells. These structures, also referred to as premature rhoptries, arise from fusion of coated vesicles that derive from the trans-Golgi network and eventually develop into mature organelles (Shaw et al., 1998). A dynamin-related protein (TgDrpB) and, more recently, a sortilin-like receptor (TgSORTLR) are essential for the biogenesis of micronemes and rhoptries. Both proteins are implicated in vesicular trafficking and likely contribute by guiding proteins from the Golgi apparatus to the prerhoptries and immature micronemes (Breinich et al., 2009; Sloves et al., 2012). The majority of rhoptry proteins are synthesized as prepropolypeptides. The presequence or signal peptide is cleaved in the endoplasmic reticulum, whereas proteolytic processing of the N-terminal prodomain was shown to occur in a post-Golgi compartment, presumably in the nascent rhoptries (Soldati et al., 1998). Processing of precursor rhoptry proteins to their mature form requires an acidic environment. Shaw et al. (1998) demonstrated that prerhoptries constitute the only acidic compartments in the cell.

Proteins with armadillo (ARM) repeats, composed of a repetitive 42 amino acid long sequence motif, comprise a family that, despite little shared sequence identity, are structurally related but functionally divergent. Tewari et al. (2010) identified ten putative apicomplexan ARM repeat-containing proteins. One of them, armadillo repeat only (ARO) protein, localizes to the rhoptries, exhibits a high degree of sequence conservation throughout the Apicomplexa, and is also found in the Perkinsozoa phylum (Cabrera et al., 2012). The C terminus of ARO is comprised of two predicted ARM repeats, and the N terminus (20 first amino acids) contains acylation motifs that become covalently linked to long-chain fatty acids via N-terminal myristoylation and palmitoylation (Cabrera et al., 2012). Acylation increases the hydrophobicity of ARO and allows it, despite the absence of a transmembrane domain, to associate with the lipid bilayer that surrounds the rhoptry organelles. Myristoylation is irreversible and involves covalent attachment of myristoyl groups (C14) to the N-terminal glycine residue of a protein via an amide linkage. Palmitoylation, in contrast, is a posttranslational modification that links cysteine residues of proteins to fatty acids (most commonly palmitate [C16]) via a thioester linkage. The reversible nature of this modification allows proteins to cycle between membrane-bound and soluble states (Salaun et al., 2010). Often, palmitoylated proteins are first myristoylated, which weakly increases their membrane affinity.

In both *P. falciparum* and *T. gondii*, ARO was recently found to localize to the rhoptry membrane periphery, with the N-terminal acylation motifs being necessary and sufficient for organellar membrane attachment (Cabrera et al., 2012). Fusion of the N-terminal portion (first 20 amino acids) of ARO to GFP was enough to target the protein to the rhoptries, whereas substitutions of the glycine or the cysteine residues to alanine resulted in a cytosolic distribution of the fusion protein (Cabrera et al., 2012). We demonstrate here the importance and functional role of ARO in *T. gondii*. Based on conditional disruption of the gene, it emerged that *Toxoplasma gondii* ARO (TgARO) is critically involved in the translocation and attachment of the rhoptries to the apical pole of the parasite. Parasites depleted in TgARO display a striking abnormal rhoptry distribution and are severely impaired in host cell invasion. Three interacting partners of TgARO have been identified, among them myosin F, which offers a model for the proper positioning of rhoptries to the apical pole.

## RESULTS

**TgARO Is an Essential Gene for Parasite Survival**

Rhoptry organelles assist in host cell invasion, and thus interfering with their biogenesis or preventing their secretion is predictably detrimental to the parasite. To determine the role of this conserved acylated protein, we first confirmed the localization of the endogenous TgARO by indirect immunofluorescence assay (IFA) using rabbit polyclonal α-TgARO specific antibodies (Abs). This reagent confirmed that TgARO is localized to the bulb and to the neck of the rhoptries. In addition, α-ROP7 Abs that efficiently stain both premature and mature rhoptries indicated that TgARO is only detectable on the mature organelles (Figure 1A). To functionally characterize this gene, we generated a conditional knockout of *TgARO* by a two-step strategy using the tet-off system (Meissner et al., 2002). First, an additional inducible C-terminally epitope-tagged copy of TgARO was randomly integrated into the TATi-1 strain (AROi-Ty). Next, the endogenous copy was disrupted by double homologous recombination, thereby generating ΔAROE/AROi-Ty (Figure 1B). PCR analysis demonstrated proper gene deletion (Figure 1C; Table S1 available online) that was further confirmed by western blot and IFA analysis (Figures 1D and 1E). Expression of AROi-Ty was tightly regulated by anhydrotetracycline (ATc), with almost complete depletion observed after 48 hr of treatment. Western blot analysis confirmed the specificity of α-ARO Abs and indicated that levels of endogenous TgARO in TATi-1 and AROi-Ty in ΔAROE/AROi-Ty (controlled by 7TetO-Sag4 minimal promoter) were comparable.

Plaque assays were performed to assess the importance of TgARO for the lytic cycle of the parasite. ΔAROE/AROi-Ty parasites grown for 8 days in presence of ATc formed strikingly smaller plaques compared to parasites grown in absence of ATc or to TATi-1 (Figure 2A). Thus, parasites depleted in ARO are severely impaired in one or more steps of their lytic cycle.

**TgARO Is Essential for Invasion but Dispensable for Intracellular Growth, Egress, and Gliding**

The phenotypic consequences of TgARO depletion were investigated in each step of the lytic cycle on ΔAROE/AROi-Ty. Parasite replication within host cells was scored by counting the number of parasites per vacuole after 48 and 72 hr of ± ATc treatment. All strains examined replicated at a similar rate, which suggested that TgARO is not required for parasite growth...
and replication (Figures 2B and S1A). In contrast, invasion assays, for which we cocultured ΔAROe/AROi-Ty or TATi-1 with a red fluorescent wild-type strain (RH-mCherry) as an internal standard, revealed that parasites depleted in AROi-Ty for 48 hr with ATc were significantly impaired and reduced to only 10% compared to control strains (p value < 0.05). Parasites expressing TgARO (ΔAROe/AROi-Ty /ΔATc or ± ATc-treated TATi-1) were capable of invading host cells as efficiently as their red fluorescent internal control (Figure 2C). These data established that TgARO plays a key role in invasion and led us to dissect the entry process further by assessing the ability of

AROi-Ty-depleted parasites to attach to host cells. Committed and thus firm attachment requires the formation of the MJ and is therefore dependent on microneme and rhoptry proteins secretion. ΔAROe/AROi-Ty and TATi-1 were grown for 48 hr ± ATc, and a red/green assay was performed to allow scoring of extracellular versus intracellular parasites using α-SAG1 and α-GAP45 Abs (Figure S1B). As expected, the counts of intracellular ATc-treated ΔAROe/AROi-Ty were markedly reduced. The fact that extracellular ATc-treated ΔAROe/AROi-Ty was not increased compared to AROi-Ty-expressing ΔAROe/AROi-Ty or to TATi-1 suggested that AROi-Ty-depleted parasites are defective in host cell attachment. Indeed, when we performed the red/green assay with parasites pretreated with 1 μM cytochalasin D (CD), a drug which blocks parasite actin-based motility and invasion, we observed a significant reduction in attachment for AROi-Ty-depleted parasites (p value < 0.05) (Figure 2D). These results indicated that parasites depleted in TgARO are unable to invade host cells because they fail to firmly engage the host cell. Next, we addressed the question of whether AROi-Ty-depleted

Figure 1. Regulation of AROi-Ty-Expressing Parasites
(A) IFA and confocal microscopy of TATi-1 show colocalization of ARO with RON, ROP5, and ROP7. White arrows indicate that endogenous TgARO colocalizes with both TgRON and TgROP5 and therefore stains both the neck and the bulb of the rhoptry organelles. The α-ROP7 Abs staining shows both mature and immature (white arrowheads) rhoptries, these latter being devoid of TgARO staining. Scale bars represent 2 μm.

(B) This scheme represents the approach taken to generate a conditional knockout of the TgARO gene. FS, flanking sequence.

(C) The replacement of TgARO was confirmed by PCR analysis. Primers are depicted in (B). Briefly, primer set P1+P2 anneals to regions of the TgARO gene that were replaced by double homologous recombination with a dihydrofolate reductase (DHFR) selection cassette flanked by ARO-specific UTRs. Primer sets PS +P4 and PS +P6 anneal within the DHFR selection cassette and the adjacent ARO-specific FS.

(D) Lysates of TATi-1 and ΔAROe/AROi-Ty previously grown for 48 hr ± ATc were used for western blot analysis. Endogenous ARO is detectable in TATi-1 at 31 kDa using α-ARO and α-Ty Abs, whereas AROi-Ty is only detectable in the knockout strain in absence of ATc at 34 kDa. CAT was used as loading control.

(E) ΔAROe/AROi-Ty were grown for 48 hr ± ATc and assessed by IFA and confocal microscopy. Staining with α-Ty and α-ARO Abs confirms AROi-Ty knockdown in presence of ATc. α-SAG1 and α-GAP45 Abs were used to visualize the whole parasite. Scale bars represent 2 μm. See also Table S1.
Figure 2. Phenotypic Characterization of TgARO-Depleted Parasites

(A–F) All assays described here were performed with ΔAROeAROi-Ty and TATi-1 grown for a total of 48 hr ± ATc (except plaque assay). (A) Plaques of HFF-infected monolayers were assayed after 7 or 8 days. AROi-Ty was also included in the assay. All strains were grown ± ATc during the entire assay. (B) Intracellular growth assay was carried out after 48 hr of parasite growth ± ATc. IFA was performed with α-GAP45 Abs and the number of parasites per vacuole was counted. Values represent means ± SD from three independent experiments. (C) Invasion assay was carried out by coculturing TATi-1 and ΔAROeAROi-Ty with a red fluorescent wild-type strain (RH-mCherry) treated for a total of 48 hr ± ATc. Values represent means ± SD from at least three independent experiments. Statistical significance was determined by the Student’s t test (**p < 0.0059). (D) Attachment assay was carried out by incubating ± ATc pretreated (48 hr) TATi-1 and ΔAROeAROi-Ty in Endobuffer and normal medium containing 1 μM CD. Red/green assay was performed with α-SAG1 and α-GAP45 Abs. The number of attached parasites was assessed in at least three independent experiments, and values are represented as mean ± SD. Statistical significance was determined by the Student’s t test (***p < 0.0025). (E) Egress assay was performed by growing parasites for 48 hr ± ATc. Egress was induced upon addition of 3 μM A23187, and DMSO was used as a negative control. The data shown are mean values ± SD from three independent experiments. (F) Gliding motility was assessed after growing parasites for a total of 48 hr ± ATc. Parasites were added to Poly-L-Lysine pretreated coverslips, and trails (white arrows) were visualized using α-SAG1 Abs. See also Figure S1.
parasites were affected in their ability to egress from infected cells. For this purpose, ΔAROe/AROi-Ty and TATi-1 were grown for 48 or 72 hr in presence or absence of ATc, and egress was induced by addition of the calcium ionophore A23187. ΔAROe/AROi-Ty depleted in AROi-Ty showed no significant defect in egress, and the percentage of lysed vacuoles was almost 100%. Comparable counts were obtained for all strains treated ± ATc, except for ΔGAP45e/GAP45i-Ty, which we included as a positive control (Figures 2E and S1C; Frénal et al., 2010). The same results were obtained when AROi-Ty was stimulated with 8% ethanol (Figure S1D) or allowed to egress spontaneously (data not shown). Combined, these results clearly indicate that TgARO is not required for egress. Next, we assessed whether the observed phenotype in invasion arises from a defect in parasite gliding motility. ΔAROe/AROi-Ty and TATi-1 were grown for 48 hr ± ATc, stimulated with ionomycin, and added to poly-L-Lysine-treated coverslips. The trails were visualized by staining for TgSAG-1 deposited in the tails of gliding parasites and scored. Trails of AROi-Ty-depleted parasites were indistinguishable from trails formed by AROi-Ty-expressing ΔAROe/AROi-Ty and by TATi-1 (Figure 2F). Taken together, these results establish that TgARO depletion severely and selectively affects T. gondii host cell invasion. In contrast, other steps of the lytic cycle and most noteworthy egress from host cells are unaffected in ΔAROe/AROi-Ty ATc-treated parasites.

**TgARO Is Necessary for the Apical Positioning of Mature Rhoptyes**

To understand the link between AROi-Ty depletion and the inability of ATc-treated ΔAROe/AROi-Ty to invade host cells, we examined the nature of the rhoptry organelles and their content upon AROi-Ty knockdown by performing IFA analysis with α-RON (uncharacterized anti-RON Abs) and α-ROP markers (α-ROP1 and α-ROP2 Abs). When AROi-Ty was expressed in ΔAROe/AROi-Ty, α-RON localized to the rhoptry neck and α-ROP1 and α-ROP2 to the rhoptry body indistinguishably from their localizations in TATi-1 strain (Figures 1A and 3A). However, in absence of TgARO, neither RONs nor ROPs were localizing to the apical end but instead were found dispersed within the parasite cytosol (Figure 3A). Colocalization studies with α-ROP and α-RON Abs in ATc-treated ΔAROe/AROi-Ty revealed that the two different groups of proteins remained spatially segregated. Notably, they were found in close proximity to each other, which suggested that, even though ROPs and RONs are mislocalized within AROi-Ty-depleted parasites, they were still within the same organelle and confined to different subcompartments (Figure 3B). Based on these observations, we raised the question of whether maturation of RONs and ROPs was still taking place within the dispersed organelles. Rhoptry protein maturation involves the proteolytic cleavage of the prosequence and presumably takes place in the acidic environment of the nascent/developing rhoptry organelles (Soldati et al., 1998). To examine this, we performed western blots of lysates from ΔAROe/AROi-Ty and TATi-1 grown for 48 hr ± ATc and probed the samples with α-RON5, α-ROP1, α-ROP2, α-ROP5, α-ROP7, and α-ROP13 Abs. The results were consistent for all rhoptry proteins examined, with protein maturation occurring in presence or absence of TgARO (Figures 3C and 3D). Antibodies against TgROP2 and TgROP13 labeled both the proproteins and the processed forms (Turetzky et al., 2010). As has previously been reported, α-ROP2 Abs cross-react with TgROP3 and TgROP4 (El Hajj et al., 2006). Consistently, the overall level of rhoptry proteins in ΔAROe/AROi-Ty grown in presence of ATc was significantly lower. The levels of proROP2 and proROP13 were also reduced, which indicated to us that rhoptry protein processing was not specifically affected by TgARO depletion (Figures 3C and 3D).

Based on the distinct localization of RONs and ROPs and the evidence that processing occurred in ATc-treated parasites, we concluded that the dispersed compartments are in fact mature rhoptry organelles that have lost their proper apical distribution and thus are unable to secrete their content upon host cell invasion. To support this view, IFA analysis of ΔAROe/AROi-Ty treated with 1 μM CD showed that RON4 localization at the very tip of the parasite was lost upon AROi-Ty deletion (Figure 3E). Also, no empty vacuoles (evacuoles) were detected using α-ROP1 Abs in those parasites but in contrast were observed in about 20% of parasites expressing AROi-Ty (Figure 3E).

We also examined if AROi-Ty depletion affected other organelles. IFA analysis confirmed that micronemes, dense granules, and the apicoplast remain properly localized in AROi-Ty-depleted parasites (Figure S2A) and protein levels of TgGRA3 and TgMIC4 were comparable between lysates of ± ATc-treated ΔAROe/AROi-Ty (Figure S2B). Further, microneme secretion was not affected by AROi-Ty depletion, as we detected processed MIC2 in the supernatant of both ± ATc-treated ΔAROe/AROi-Ty (Figure S2C).

To further investigate if rhoptry organelles in AROi-Ty-depleted parasites are dispersed but fully mature, we analyzed their ultrastructure by TEM. Thin sections prepared from intracellular AROi-Ty-expressing parasites showed that their rhoptries were clustered together near the apical end as in wild-type parasites (Figures 4 and S3, left panels). In contrast, the bunch of apically localized rhoptries were undetectable from thin sections of AROi-Ty-depleted parasites. Instead, we identified single organelles, randomly dispersed in the parasite cytosol. Of note is that these dispersed rhoptries appeared as mature club-shaped organelles, and other structures, such as conoid and micronemes, were not affected (Figures 4 and S3, right panels). Occasionally, we saw mature rhoptry organelles in the residual bodies. Taken together, these results indicate that TgARO is not implicated in an early step of rhoptry biogenesis but is crucial for the translocation and attachment of the mature organelles to the apical end of the parasite. In the absence of TgARO, the rhoptries wander loosely within the parasite cytosol and fail to respond to the apical stimuli and release locally their content during the invasion process.

The virulence of ΔAROe/AROi-Ty was compared to TATi-1 parasites in BALB/c mice (n = 5) following intraperitoneal injection of ~80 parasites. Mice died within 6 days, whereas the group supplemented with ATc in the drinking water and infected with ΔAROe/AROi-Ty survived. At day 25, the serum of these mice was diluted one to five and tested by western blot to confirm seropositivity of all the mice. Importantly, when we challenged at day 26, all mice infected with 1000 wild-type parasites (RH) died within 6 days, suggesting that they had been unable to build up a protective immune response during...
Figure 3. Depletion of TgARO Impairs Rhoptry Organelle Positioning to the Apical End of the Parasite
(A) ΔAROe/AROi-Ty were grown for 48 hr ± ATc and assayed by IFA and confocal microscopy using α-ROP1, α-ROP2, and α-RON Abs. In the ATc-treated parasites, all three rhoptry proteins are dispersed in the whole parasite (white arrowheads). Staining with α-ARO Abs confirmed complete depletion of TgARO after ATc treatment.
(B) As described in (A), ΔAROe/AROi-Ty were grown for 48 hr ± ATc and stained with α-ROP1 in combination with α-RON Abs. RON and ROP proteins are adjacent to each other but do not overlap in the mature rhoptries. Arrows point to the rhoptry bulb and the arrowheads to the adjacent neck region.
(C) Western blot analysis was carried out with lysates from TATi-1 or ΔAROe/AROi-Ty previously grown for 48 hr ± ATc. A-RON5 Abs detected the two mature forms of RON5N at 110 kDa and RON5C at 30 kDa, respectively. Less RON5 protein is detectable in ATc-treated ΔAROe/AROi-Ty. CAT was used as loading control.

(legend continued on next page)
Acetylation of TgARO Is Necessary for Its Function in Apical Positioning of the Rhoptries

To confirm that TgARO is uniquely responsible for the phenotypic defect observed in AROe/AROi-Ty, we complemented this strain by stably integrating a C-terminally Myc-tagged TgARO copy controlled by the endogenous TgARO promoter (ARO-Myc; Figure 5A). ARO-Myc-expressing parasites were grown ± ATc for 72 hr and analyzed by IFA using α-Myc and α-ROP2 Abs. Expression of ARO-Myc in ATc-treated AROe/AROi-Ty fully restored the apical positioning of the rhoptries (Figure 5B). ARO-Myc was also able to restore evacuole formation (Figure 5C). Western blot analysis confirmed that ARO-Myc was constitutively expressed, while AROi-Ty remained regulatable by ATc (Figure 5E, left blot).

To determine if ARO acylation is critical for its function, we generated the construct ARO-G2A-Myc, in which the N-terminal glycine residue at amino acid position two was substituted to generate the construct ARO-G2A-Myc, in which the N-terminal glycine residue at amino acid position two was substituted to avoid the cytosolic (arrows) and occasionally in residual bodies (asterisks). Other structures, such as conoid (C) and micronemes (M), are unaffected by TgARO depletion. Scale bars represent 1 μm. See also Figure S3.

Myc was only soluble in presence of 1% Triton X-100, indicating ARO-G2A-Myc was not associated to the rhoptry membrane (Figure 5F). Plaque assays further confirmed that expression of ARO-Myc but not ARO-G2A-Myc was able to complement TgARO depletion phenotype (Figure 5G). In conclusion, these results demonstrate that anchoring of TgARO to the rhoptry membrane requires acylation and, only when properly localized, rescue of AROi-Ty depletion is ensured.

TgARO Interacts with T. gondii Myosin F

In order to establish how TgARO is promoting rhoptry organelle positioning, we searched for interacting partners. GFPTy was fused to the C terminus of TgARO (ARO-GFPTy) and expressed stably in T. gondii. As controls, we included a strain expressing the first 20 amino acids of ARO fused to GFP (20ARO-GFPTy) (Cabrera et al., 2012) and a strain expressing GFP fused to a destabilization domain (ddMycGFP) (Herm-Götz et al., 2007). We performed immunoprecipitation (IP) experiments using GFP-Trap system-based α-GFP Abs. Immune complexes were separated by SDS-PAGE, and two bands specific to ARO-GFPTy (Figure 6A) were subjected to tryptic digestion and analyzed by mass spectrometry (MS). The results obtained revealed two unique peptides that match the amino acid sequence of myosin F (TgMyoF), 25 unique peptides that match the amino acid sequence of adenylate cyclase β (TgACβ) and three unique peptides that belong to a conserved hypothetical protein, which depletes parasites. Scale bars represent 2 μm. See also Figure S2.

Figure 4. Ultrastructural Analysis of TgARO-Depleted Parasites

Thin section electron micrographs were taken from AROe/AROi-Ty that had grown for a total of 48 hr ± ATc. The left panels show untreated parasites containing clusters of rhoptry organelles (R) near the apical end. The upper left panel depicts a dividing parasite. The right panels show ATc-treated parasites. No clusters but single mature rhoptry organelles are found within the cytosol (arrows) and occasionally in residual bodies (asterisks). Other structures, such as conoid (C) and micronemes (M), are unaffected by TgARO depletion. Scale bars represent 1 μm. See also Figure S3.
Figure 5. TgARO Function Is Dependent on Protein Acylation

(A) The scheme depicts the strategy used for complementation of ΔAROe/AROi-Ty with ARO-Myc controlled by TgARO promoter. (B) ΔAROe/AROi-Ty stably expressing ARO-Myc were grown ± ATc and visualized by IFA using α-Myc Abs. The distribution of rhoptry organelles was examined using α-ROP2 Abs. Localization of the rhoptry organelles is comparable between ± ATc-treated parasites, indicating that ARO-Myc complements AROi-Ty-depleted parasites. The scale bars represent 2 μm.
hereafter is named armadillo interacting protein (TgAIP). The raw MS data are depicted in Figure S4. In order to validate TgMyoF interaction with TgARO, we transiently transfected JKU80 strain (Huynh and Carruthers, 2009) expressing the endogenous MyoF tagged with a C-terminal 3Ty-tag (MyoF-3Ty) (D.J., W. Daher, and D.S.-F., unpublished data) with either ARO-GFP Ty or 20ARO-GFP Ty. Immune complexes were pulled down using α-GFP Abs, and subsequent western blot analysis using α-Ty Abs revealed the presence of ARO-GFP Ty (58 kDa) and 20ARO-GFP Ty (34 kDa) in the eluted fractions (Figure 6B) as well as a single band at the approximate size of TgMyoF (220 kDa) that importantly was only pulled down with ARO-GFP Ty but not with 20ARO-GFP Ty. This result confirmed that TgMyoF interacts with TgARO.

In order to determine if rhoptry organelle positioning is an actomyosin-dependent event, we treated wild-type parasites overnight with either 0.2 μM CD or 20 mM butanedione monoxime (BDM). Both drugs are known to block parasite entry, while but CD is an inhibitor of actin polymerization (Shaw et al., 2000), BDM is a low-affinity inhibitor of myosin ATPase (Dobrowolski et al., 1997). IFA analysis with α-ROP2 Abs revealed that parasites treated with CD or BDM showed dispersed rhoptry organelles, similar to what we observed with AROi-Ty-depleted parasites. In contrast, micronemes (α-MIC3 Abs) localization appeared not to be affected by the drugs (Figure 6C).

TgAIP Is a TgARO Interacting Protein Localizing to the Rhoptry Neck

In order to validate that TgAIP interacts with TgARO, we epitope-tagged (3Ty-tag) the endogenous gene at its C terminus in the JKU80 strain, thereby generating TgAIP-3Ty. We transiently transfected the generated knockin strain with ARO-GFP Ty or with the control construct dMycGFP and performed colP experiments with α-GFP Abs followed by western blot analysis using α-Ty and α-Myc Abs. ARO-GFP Ty specifically pulled down TgAIP-3Ty (predicted size 89 kDa; Figure S5A), thereby confirming that this protein is an interacting partner of TgARO. Importantly, IFA analysis of TgAIP-3Ty using α-Ty, α-ARO, and α-ROP2 Abs revealed that the protein localized to the rhoptry neck (Figure S5B). The spatial overlap of TgAIP with TgARO further validated the colP data.

DISCUSSION

For a long time, rhoptry organelles have been associated with the process of invasion in Apicomplexa based on electron microcopy observations (Dubremetz, 2007). The contact between the apical end of the parasites and host cell plasma membrane was shown to trigger secretion of membrane-like materials from rhoptries, which were inserted into the host cells and contributed to the invagination and extension of the PVM. In presence of CD, parasites are blocked in motility and invasion but not attachment, which results in evacuoles that are formed by the release of rhoptry content into the host cells (Håkansson et al., 2001). Recently, further molecular insights into the contribution of rhoptry organelles to invasion were highlighted by the direct participation of the rhoptry neck proteins in the formation of the MJ (Besteiro et al., 2011).

Here we show that TgARO, an acylated rhoptry protein anchored at the surface of the organelle, plays a key role in the Toxoplasma lytic cycle. Parasites depleted in TgARO are unable to invade host cells due to a severe misplacing of the rhoptry organelles. Instead of being attached to the apical end, the rhoptries drift within the cytosol and, thus, are unable to secrete their content and assist invasion. Phenotypic analysis of AROI-Ty-depleted ΔAROe/AROi-Ty parasites revealed that host cell entry was in fact the only step in the lytic cycle to be impaired. Invasion initiates with reversible attachment of the parasite to the host cell, followed by firm, apical attachment mediated by AMA-1 and RON proteins that assemble into the MJ (Carruthers and Boothroyd, 2007). Parasites lacking TgRON8 (a component of the MJ) are severely impaired in both attachment and invasion, suggesting a role in stabilizing the MJ and promoting parasite entry (Straub et al., 2011). Tight attachment to host cells requires both secreted micronemal and rhoptry proteins. While micronemesecretion is not affected in AROI-Ty-depleted parasites, our IFA data of CD-treated AROI-Ty-depleted parasites showed that RON4 localization at the tip of the parasite is lost. Additionally, not a single evacuole was detected in those parasites, whereas evacuoles were frequently observed in parasites expressing AROI-Ty. These results establish that the absence of RONs secretion prevents the formation of the MJ and thus makes it impossible for AROI-Ty-depleted parasites to actively penetrate the host cell. These results also show that the dispersed rhoptry organelles are nonfunctional and do not secrete their content, even when stimulated with CD. Crucially, we observed no defect in spontaneous or ionophore- or ethanol-induced egress, suggesting that egress does not require rhoptry secretion and, hence, is a process mechanistically distinct from invasion. This contrasts with the assumption that egress was basically “invasion from the inside” and would require rhoptry

(C) Evacuole formation was assessed for ARO-Myc expressing ΔAROe/AROi-Ty pretreated with ATc for 48 hr. Protein secretion was stimulated by incubating parasites in Endobuffer and normal medium containing 1 μM CD. Evacuoles (arrowhead) were visualized by IFA using α-RON4 and α-ROP1 Abs. While approximately 20% of parasites expressing ARO-Myc show evacuole formation, no evacuoles were detected in the noncomplemented ΔAROe/AROi-Ty strain.

(D) ΔAROe/AROi-Ty stably expressing ARO-G2A-Myc was grown in ATc and visualized by IFA using α-Myc Abs. Distribution of rhoptry organelles was examined using α-ROP2 Abs. Rhoptry organelles remained dispersed in ΔAROe/AROi-Ty parasites, indicating that ARO-G2A-Myc is unable to complement AROI-Ty-depleted ΔAROe/AROi-Ty.

(E) Constitutive expression of integrated ARO-Myc and ARO-G2A-Myc in ΔAROe/AROi-Ty was confirmed by western blotting using α-Myc Abs. AROI-Ty remains ATc regulated (α-Ty staining). PRF was used as loading control.

(F) The solubility of ARO-Myc and ARO-G2A-Myc was studied by fractionation after protein extraction from total parasite lysates in PBS or PBS with 1% Triton X-100. Subsequent western blot analysis revealed that ARO-G2A-Myc is fully soluble in PBS, whereas ARO-Myc requires treatment with Triton X-100 due to its membrane-bound state. CAT (soluble protein) and acylated GAP45 were used as controls. S, soluble fraction; P, insoluble fraction.

(G) Plaque assays were carried out by infecting HFF monolayers with ΔAROe/AROi-Ty or ΔAROe/AROi-Ty stably expressing ARO-Myc or ARO-G2A-Myc for 7 or 8 days ± ATc. Scale bars represent 2 μm.
Figure 6. TgARO Is Interacting with Several Proteins, among which One Is a Myosin Motor
(A) The Coomassie-stained gel shows several bands that were detected after IP using α-GFP Abs. Parasite strains stably expressing ddMycGFP (treated with 1 μM Shld-1) and 20ARO-GFP-Ty were used as negative controls. Two bands that were only detected in the fraction of ARO-GFPTy were subjected to MS. MS results are listed below. Identified peptides correspond to the proteins TgMyoF (GenBank accession number DQ131541), TgAIP (GenBank accession number KC535540), and TgACβ.

(B) MyoF-3Ty knockin strain was transiently transfected with ARO-GFPTy or 20ARO-GFPTy prior to IP using α-GFP Abs and western blot analysis using α-Ty Abs. Upper and lower membranes are the same, except that the upper membrane was longer exposed. The parasite lysate of ARO-GFPTy in lane 1 was double the one of lane 2.

(C) IFA analysis was performed on RHΔHX treated o/n with either 0.2 μM CD or 20 mM BDM. Micronemes and rhoptries were visualized using α-MIC3 and α-ROP2 Abs, respectively. Scale bars represent 6 μm. See also Figure S4.
secretion and MJ formation (Alexander et al., 2005; Hoff and Carruthers, 2002). The complementation experiments performed on ΔAROe/AROi-Ty have established that acylation of TgARO is necessary for its anchoring to the rhoptry membrane and is crucial for its function. Together with the N-terminal acylation of TgGAP45 (Frénaul et al., 2010), this study stresses the crucial role played by palmitoylation on key proteins implicated in the lytic cycle of T. gondii. In this context, we have recently characterized and localized the whole repertoire of DHHC motif-containing putative palmitoyltransferases (PATs) in T. gondii and found that only one member (TGME49_052200) localizes to the rhoptry membrane. TGME49_052200 is essential and corresponds to the PAT responsible for the acylation of TgARO (K. Fre´nal, C.L. Tay, C.M., E.S. Bushell, Y. Jia, A. Graindorge, O. Billker, J.C. Rayner, and D.S.-F., unpublished data).

TgARO plays a key role in the final steps of rhoptry biogenesis, leading to apical translocation and firm positioning of these organelles to the parasite tip. Since only 2–4 rhoptries can be accommodated within the conoid (Paredes-Santos et al., 2012) and all rhoptry organelles are dispersed within the cell in absence of TgARO, it is not certain that ARO contributes to docking. The parasomes refer to permanent secretory portals at the cell plasma membrane in higher eukaryotes that allow the transient docking and fusion of secretory organelles to release intravesicular content without merging with the plasma membrane. The apical vesicle docked on the rosette of intramembrane particles located above the conoid of T. gondii tachyzoites (Porchet and Torpier, 1977) and corresponding to the site where rhoptries are exocytosed may be a functional equivalent of the parasome, although morphologically unrelated. The data collected so far on TgARO do not allow establishing a direct link with this structure.

TgARO interaction with TgMyoF strongly suggests that the repositioning of the rhoptries is an actomyosin-based process. As confirmation, drugs that interfere with actin assembly or myosin function led to dispersion of rhoptries. In a parallel study, the functional analysis of T. gondii MyoF revealed that this motor is essential and impacts on centrosomes, apicoplast, and rhoptries positioning (D.J., W. Daher, and D.S.-F., unpublished data). Thus, interaction of TgMyoF with TgARO likely occurs in a transient manner. Organellar movement involving myosin motors appears to be an evolutionary conserved mechanism. In yeast, vacuole inheritance during bud development requires the assembly of a transport complex that is composed of the class V myosin Myo2 motor and two other components Vac17 and Vac8 (Tang et al., 2003; Weisman, 2006). The latter one has been proposed as a distant homolog of TgARO, since it contains ARM repeats and is anchored to the vacuole via N-terminal acylation motifs (Cabrera et al., 2012). It is conceivable that TgAIP, which is predicted to be soluble, could act as a Vac17 homolog in linking TgMyoF to TgARO (Figure 7).

Further functional investigations on the TgARO partner TgAIP but also on TgACβ and possible connection with calcium signaling and protein kinase A activation shall shed more light on these specialized organelles that are central and unique to the biology of Apicomplexa.

**EXPERIMENTAL PROCEDURES**

**Parasite and Host Cell Culture**

* T. gondii RHΔhxgprt- parental and transgenic strains were maintained in confluent human foreskin fibroblasts (HFF) using Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen) supplemented with 5% fetal calf serum, 2 mM glutamine, and 25 μg/ml gentamicin. Parasites with tetracycline-controlled gene expression were regulated by 0.5 μg/ml ATc (Meissner et al., 2002). Parasites expressing ddGFPMye were treated with 1 μM shld-1 (Herm-Götz et al., 2007).

**Plaque Assay**

Monolayers of HFF were infected with parasites for 7 or 8 days at 37°C ± 0.5 μg/ml ATc, and cells were stained with Giemsa (Sigma-Aldrich G5500), mounted in Fluoromount G (Southern Biotech 0100-01), and visualized using Zeiss MIRAX imaging system equipped with a Plan-Apochromat 20/0.8 objective.

**Intracellular Growth Assay**

HFF were inoculated with freshly released parasites that had been pretreated or not with ATc for 24 hr or alternatively 48 hr prior to complete host cell lysis. After another 24 hr of growth ± ATc, parasites were fixed in 4% paraformaldehyde (PFA). IFA was performed using α-GAP45 Abs, and the number of parasomes per vacuole was counted from a total of 200 vacuoles per condition. The data shown are mean values and SDs from three independent experiments.

**Invasion Assay**

The assay was performed after a protocol previously described (Sheiner et al., 2010). Briefly, HFF were infected with a mixture (2:1 ratio) of TAT1-1 or ΔAROe/AROi-Ty and RHΔHx-mCherry. Before mixing the two strains, both had been cultured individually for 12 hr ± ATc prior to egress, and ± ATc treatment was continued in the mixed cultures for 36 hr. After complete host cell lysis, the ratio of mCherry to non-mCherry vacuoles were counted in at least 100 vacuoles per slide. This assay was performed at least three times independently. P value was calculated using Student’s t test, assuming equal variance, unpaired samples, and two-tailed distribution.

**Red/Green Assay and Evacuole Formation**

The red/green assay was performed as previously described (Straub et al., 2011). Briefly, parasites were grown for 48 hr ± ATc, and freshly egressed tachyzoites were resuspended in Endobuffer ± 1 μM CD and incubated for 10 min at room temperature. Equal number of parasites were used to infect...
new HFF cells and incubated for 15 min at 37°C. Endotherm was replaced by normal medium ± 1 μM CD and incubated for 20 min at 37°C prior to fixation using PFA/glutaraldehyde (GA). IFA was performed using monoclonal α-SAG1 Abs and polyclonal α-GAP45 Abs. Parasites stained with both α-SAG1 and α-GAP45 were scored as attached but noninvaded, whereas parasites only stained with α-GAP45 were denoted as internalized. Per experiment, 60 fields of each strain ± ATc were randomly counted. The data shown are mean values from three independent biological experiments. P value was calculated using Student’s t test, assuming equal variance, unpaired samples, and two-tailed distribution.

For detection of RON4 localization and evacuole formation, the same procedure was followed, except that PFA was used as a fixative and cells were stained with polyclonal α-RON4 and monoclonal Abs α-ROP1 and α-ACT.

### Induced Egress Assay
Parasites were grown for 18 hr or alternatively 48 hr ± ATc. Freshly egressed tachyzoites were added to a new monolayer of HFF and grown for 30 hr ± ATc. The infected HFF were then incubated for 5 min at 37°C with DMEM containing either 3 μM of the Ca²⁺ ionophore A23187 (from Streptomyces chartreusensis, Calbiochem) or 8% ethanol or DMSO. Host cells were fixed with PFA/GA, and IFA using α-SAG1 Abs was performed. Two hundred vacuoles were counted per strain and per condition, and the number of lysed vacuoles, which are morphologically distinct from intact ones, was scored. The data shown are mean values from three independent experiments.

### Induced Gliding Assay
Parasites were grown for 48 hr ± ATc. Freshly egressed parasites were pelleted and resuspended in a calcium-saline solution containing 1 μM ionomycin. The suspension was added to Poly-L-Lysine-coated coverslips and incubated for 15 min at 37°C prior to fixation with PFA/GA. Trails were visualized by IFA using α-SAG1 Abs.

### Cellular Fractionation
Freshly released ARO-Ty- and ddMycARO- (grown in presence of shld-1 for 24 hr prior to egress) expressing parasites were harvested and their pellets resuspended in PBS or PBS + 1% Triton X-100. Following incubation on ice for 15 min, pellet and soluble fractions were separated by centrifugation at 14,000 rpm, 45 min, and 4°C. The supernatant was subjected to IP using α-GFP lama Abs (GFP-Trap Agarose Beads from ChromoTek).

### Immunoprecipitation
Freshly released tachyzoites were harvested, washed in PBS, and lysed in coIP buffer (PBS containing 0.5% [v/v] Triton X-100, 150 mM NaCl, protease inhibitor cocktail [Roche]) and incubated on ice for 20 min. After centrifugation at 14,000 rpm, 45 min, and 4°C, the supernatant was subjected to IP using α-GFP lama Abs (GFP-Trap Agarose Beads from ChromoTek).

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