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Structural and functional dissection of *Toxoplasma gondii* armadillo repeats only protein (TgARO)

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ABSTRACT

Rhoptries are club-shaped, regulated secretory organelles that cluster at the apical pole of apicomplexan parasites. Their discharge is essential for invasion and the establishment of an intracellular lifestyle. Little is known about rhoptry biogenesis and recycling during parasite division. In *Toxoplasma gondii*, positioning of rhoptries involves the armadillo repeats only protein (TgARO) and myosin F (TgMyoF). Here, we show that two TgARO partners, ARO interacting protein (TgAIP) and adenylate cyclase β (TgAC β) localize to a rhoptry subcompartment. In absence of TgAIP, TgAC β disappears from the rhoptries. By assessing the contribution of each TgARO armadillo (ARM) repeat, we provide evidence that TgARO is multifunctional, participating not only in positioning but also in clustering of rhoptries. Structural analyses show that TgARO resembles the myosin-binding domain of the myosin chaperone UNC-45. A conserved patch of aromatic and acidic residues denotes the putative TgMyoF-binding site, and the overall arrangement of the ARM repeats explains the dramatic consequences of deleting each of them. Lastly, *Plasmodium falciparum* ARO functionally complements TgARO depletion and interacts with the same partners, highlighting the conservation of rhoptry biogenesis in Apicomplexa.

INTRODUCTION

Unicellular pathogens belonging to the phylum Apicomplexa represent a major threat to both human and animal health. The most notorious member of this phylum is the causative agent of human malaria, *Plasmodium falciparum*. Other members of considerable medical and veterinary importance include *Toxoplasma*, *Neospora*, *Cryptosporidium*, *Babesia*, *Eimeria* and *Sarcocystis*. These organisms are unified by phylum-specific cytoskeletal structures and sets of specialized secretory organelles termed micronemes, rhoptries and dense granules, discharge of which is necessary for the establishment of an obligate intracellular life cycle (reviewed in (Carruthers and Sibley, 1997; Sibley, 2010)). This cycle is initiated by host cell penetration; an active process that leads to the formation of a replication-permissive niche inside a specialised membranous sac termed the parasitophorous vacuole membrane (PVM). Rhoptries are positioned at the apical end of the parasite and adopt an elongated, club-shaped morphology with a bulbous body and a narrow neck (Dubey et al., 1998). These organelles play a key role not only during invasion, but also contribute to the formation of the PVM as well as subsequent evasion and subversion of the host cell defence mechanisms (reviewed in (Kemp et al., 2012)). During parasite entry, only one of the 10-12 rhoptries injects its contents, including the rhoptry neck proteins (RONs), rhoptry bulb proteins (ROPs) and some membranous materials, into the host cell (Boothroyd and Dubremetz, 2008). Several RONs form a complex with apical membrane antigen 1 (AMA-1), a protein secreted from the micronemes, resulting in the formation of the so-called moving junction (MJ) (Besteiro et al., 2011; Straub et al., 2009). The MJ is a tight apposition between the parasite and the host cell plasma membrane and ensures efficient propulsion of the parasite into the host cell, ultimately leading to the formation of the PVM (Besteiro et al., 2011; Straub et al., 2009). Several ROPs migrate either to the lumen of the nascent parasitophorous vacuole (PV), the PVM, or into the cytosol or nucleus of the infected host cell where they modulate cellular functions (Butcher et al., 2011; El Hajj et al., 2007a; Etheridge et al., 2014; Fleckenstein et al., 2012; Yamamoto et al., 2009). The signalling events leading to rhoptry secretion are poorly understood. However, it is known that rhoptry discharge occurs shortly after microneme discharge, and the microneme protein TgMIC8 plays a role in triggering rhoptry exocytosis (Kessler et al., 2008).

During parasite division, rhoptries are formed *de novo*, first appearing as globular vesicles in close proximity to the Golgi apparatus, and then subsequently maturing

into club-shaped organelles localizing to the parasite apex (Fig. 1B) (Nishi et al., 2008; Shaw et al., 1998). The cellular and molecular mechanisms underlying rhoptry biogenesis, including their targeting to the apical pole, are currently not well understood. Previous studies have, however, shown that perturbation of proteins involved in vesicular trafficking, such as the dynamin-related protein B (TgDrpB), sortilin-like receptor (TgSORTLR), clathrin heavy chain 1 (TgCHC1) or the Rab-GTPases Rab5A and Rab5C resulted either in a block of both rhoptry and microneme biogenesis and/or in mistargeting of the proteins to either of the two organelles (Breinich et al., 2009; Kremer et al., 2013; Pieperhoff et al., 2013; Sloves et al., 2012). TgARO is key factor ensuring the apical distribution of rhoptries (Beck et al., 2013; Mueller et al., 2013). Anchoring of TgARO to the cytosolic face of the rhoptry membrane is dependent upon acylation (Cabrera et al., 2012). Of the 18 members of the protein acyltransferases (PATs) identified in *T. gondii*, only one, TgDHHC7, was localized to the rhoptries and found to be responsible for palmitoylating TgARO (Beck et al., 2013; Frenal et al., 2013). It is currently unclear at which stage during rhoptry biogenesis this palmitoylation event takes place. Upon conditional depletion of TgARO, rhoptries are randomly dispersed within the parasite cytosol, ablating rhoptry secretion and hampering host cell invasion (Beck et al., 2013; Mueller et al., 2013). Co-immunoprecipitation (co-IP) experiments and subsequent mass spectrometry analyses revealed that TgARO interacts with myosin F (TgMyoF). This myosin belongs to the alveolate specific class XXII and participates in apicoplast inheritance and centrosome positioning (Jacot et al., 2013). The functional disruption of TgMyoF and the use of cytochalasin D, an inhibitor of actin polymerization, interfere with the positioning of rhoptries, supporting the view that this is an actomyosin-based process (Jacot et al., 2013; Mueller et al., 2013).

In analogy to the well-studied actomyosin-dependent movement of organelles in yeast and melanocytes, we anticipated additional accessory proteins to assist TgMyoF function (Hume and Seabra, 2011; Weisman, 2006). In this context, two proteins were identified co-immunoprecipitating with TgARO; a hypothetical protein with unknown function named TgARO interacting protein (TgAIP), and adenylate cyclase β (TgAC β). Although not highly conserved, AIP is present in several apicomplexan genomes. Two distinct ACs (AC α and AC β) have been described in *P. falciparum* and are present and conserved across the Apicomplexa phylum (Baker, 2004). *Plasmodium berghei* AC α was reported to mediate apically-regulated exocytosis in

Plasmodium sporozoites via cAMP signalling, whilst the function of the apparently essential AC β is unknown (Ono et al., 2008).

Here we show that TgAC β is recruited to the rhoptry surface via interactions with TgARO and that, together with TgAIP, they represent the first identified markers of a third sub-compartment separating the rhoptry bulb and neck (Lemgruber et al., 2010). TgAIP is prerequisite for the targeting of TgAC β to the rhoptry.

Depletion of TgARO prevents the apical translocation of rhoptries and hence hampers further assessment of its potential contribution to anchoring the organelles to the tip of the parasite, or in rhoptry discharge. To circumvent this limitation, we have assessed the importance of each predicted armadillo (ARM) repeat of TgARO by exploiting the functional complementation of the inducible knockout strain (Δ ARO^e/AROⁱ-Ty; hereafter termed ARO-iKO) with ARO deletion mutants. These non-functional mutants revealed a new role for TgARO in holding rhoptries in bundles, and also led to the detection of an extended structure that is most likely of membranous origin since it is only labelled with markers of the rhoptry membrane. Structural analyses show that TgARO closely resembles the myosin-binding domain of UNC-45 and has a highly conserved binding groove that would most likely accommodate an extended polypeptide chain. Finally the cross-genera complementation of the functions assigned to TgARO by *P. falciparum* ARO highlights the conservation of these aspects of rhoptry biogenesis across the phylum.

RESULTS

***T. gondii* adenylate cyclase beta is a rhoptry surface protein that binds to TgARO**

TgAC β was previously identified via mass spectrometric analyses as a potential partner of TgARO in co-IP experiments using a GFP-tagged copy of TgARO (Mueller et al., 2013). In order to confirm this interaction, we first determined the subcellular distribution of TgAC β by inserting three Ty epitope tags at the carboxy-terminus of the protein through single homologous recombination at the endogenous locus in the Δ KU80 strain (TgAC β -3Ty). In parallel, recombinant TgAC β (amino acids (aa) 1246-1600) was used to generate polyclonal anti-TgAC β -specific antibodies in rabbits (α -TgAC β). Western blot analyses of lysates from wild type RH strain (RH) and TgAC β -3Ty using α -TgAC β sera detected a strong band

approximating the predicted size of 220 kDa that was co-migrating with the band detected by α -Ty Abs in TgAC β -3Ty parasites (Fig. 1A). Fainter lower bands were also detected that likely correspond to degradation products, as well as non-specific background bands. Indirect immunofluorescence assay (IFA) using α -Ty and α -GAP45 Abs revealed that TgAC β is apically distributed and that the α -TgAC β staining was identical to the α -Ty staining. Co-labelling with α -ARO and α -ROP2 markers identified TgAC β as a rhoptry neck protein (Fig. 1B).

In order to validate the interaction of TgAC β with TgARO, we transiently transfected TgAC β -3Ty parasites with TgARO-GFP-Ty or a control construct expressing GFP. Parasites were harvested 24 h later and co-IP utilising the GFP-Trap system. Western blot analyses with α -Ty and α -GFP Abs showed that TgARO-GFP-Ty and not GFP pulled down TgAC β -3Ty, confirming that TgAC β is indeed associated with TgARO (Fig 1C).

Previous studies have indicated that TgAC β is closely related to PfAC β and both belong to the family of soluble adenylate cyclases (Baker, 2004). Fractionation experiments confirmed that TgAC β is a soluble protein (Suppl. Fig. S1A) and proteinase K protection assay showed that TgAC β does not reside in the rhoptry lumen, but like TgARO is present at the surface of organelle facing the parasite cytosol (Suppl. Fig. S1B). To determine whether the presence of TgAC β at the rhoptries is dependent upon its association with TgARO, ARO-iKO parasites were treated for 48 h with anhydrotetracycline (ATc) to deplete AROi-Ty and the localization of TgAC β was assessed by IFA using α -TgAC β Abs. In the absence of TgARO, TgAC β no longer localized to the neck of the dispersed rhoptries (Fig. 1D).

TgAIP, which has been described previously as a rhoptry neck protein (Mueller et al., 2013), perfectly co-localized with TgAC β (Fig. 1E). Upon closer examination, confocal images of TgAC β revealed that the protein does not cover the entire neck portion but instead occupies a distinct region of the neck adjacent to the rhoptry bulb, as shown by IFA carried out using α -TgAC β and α -RON9 Abs (Lamarque et al., 2012) (Fig. 1F). Interestingly, previous transmission electron microscopy (TEM) studies reported three sub-compartments of rhoptries delimited by a dark and electron-dense neck, an amorphous and less electron-dense bulb, and a region of intermediate electron density, which connects the bulb to the neck (Lemgruber et al., 2010). Taken together, these results demonstrate that TgARO interacts with TgAC β and that,

together with TgAIP, these two ARO partners constitute the first known markers restricted to the surface of an intermediate sub-compartment of the rhoptries.

TgAIP and TgAC β localize to the rhoptries only in the presence of TgARO

More insight into the association of TgAIP and TgAC β to the rhoptry surface was provided by a conventional disruption of *TgAIP* gene (AIP-KO) generated by double homologous recombination within the regions flanking the coding sequence. The genotype of the clones was confirmed by genomic PCR (Suppl. Fig. S1C, Table S1). IFA analyses of this strain using α -ARO, α -ROP2 and α -TgAC β Abs revealed that i) TgARO was still found at the rhoptries, ii) the organelles were properly localized to the parasite apex and iii) TgAC β was no longer detected at the rhoptry neck (Fig. 2A). Moreover, western blot analysis revealed that TgAC β was reduced to undetectable levels in AIP-KO lysates indicating that TgAC β is unstable in absence of AIP. In contrast, the level of TgARO remained constant between AIP-KO and wild type parasites (RH Δ HX; Fig. 2B). Functional complementation of AIP-KO by transient transfection with T8AIP-Myc returned TgAC β to the rhoptries in the presence of T8AIP-Myc but not in the negative control transiently expressing T8ARO-Myc (Fig. 2C). These results confirm that TgAIP is necessary both for stabilization of TgAC β and its targeting to the rhoptries. TgAIP does not appear to harbour any transmembrane domains (TMDs) or acylation motifs that would confer membrane association. Fractionation experiments with lysates of TgAIP-3Ty confirmed that this protein, like TgAC β was fully soluble in PBS (Suppl. Fig. S1D).

To further investigate whether the presence of TgAIP and ultimately TgAC β at the rhoptries is dependent upon the association with TgARO, we stably introduced a second copy of C-terminally Myc-tagged TgAIP controlled by the tubulin promoter in the ARO-iKO strain (ARO-iKO/T8AIP-Myc). IFA analyses revealed that the presence of T8AIP-Myc on the rhoptries was abolished upon AROi-Ty depletion. Furthermore, the expression of T8AIP-Myc in the absence of AROi-Ty failed to position TgAC β on the dispersed rhoptries (Fig. 2D). Taken together, TgARO likely interacts directly with TgAIP whereas TgAC β requires TgAIP to localize to the rhoptry intermediate compartment.

Each predicted armadillo repeat contributes to TgARO function in rhoptry positioning

Earlier studies predicted that TgARO comprises two proper ARM repeats (ARM3 and ARM4 in Fig. 3A) and at least three “degenerate” ARM repeats (ARM2, ARM5, ARM6 in Fig. 3A), preceded by an N-terminal region responsible for membrane association (Cabrera et al., 2012). To address the importance of each of the predicted ARM repeats for TgARO function, we generated deletion mutants for each of the ARM repeats 2-6 individually as well as both of the ARM3 and ARM4 domains together (Δ ARM3,4-Myc). The mutants were assessed for functional complementation by stable expression in the ARO-iKO strain (Fig. 3A). IFA analyses carried out with ARO-iKO expressing Δ ARM3,4-Myc (ARO-iKO/ Δ ARM3,4-Myc) grown $\pm$ ATc for 48 h demonstrated that AROi-Ty remained regulatable by ATc, while Δ ARM3,4-Myc was constitutively expressed (Fig. 3B). Δ ARM3,4-Myc localized to the rhoptries in the absence of ATc but failed to complement the phenotype in the presence of ATc. Rather unexpectedly this truncated form of TgARO did not co-localize with the ROP2 marker in the absence of AROi-Ty. In other words, in the presence of ATc there is no co-localization between this truncated form and the dispersed organelles, but instead it stained an extended membranous structure that has not been previously observed (Fig. 3B-C; Suppl. Fig. S2A). Of importance is the finding that upon ATc treatment of this strain, TgAC β was no longer detectable at the rhoptry neck (Fig. 3C). Western blot analyses indicated that constitutive expression of Δ ARM3,4-Myc did not significantly reduce the level of TgAC β following ATc treatment suggesting that the protein became cytosolic (Suppl. Fig. S2B). Similarly, all individual ARM deletion mutants labelled this unusual structure and failed to complement for the absence of TgARO leading to the organelle dispersion phenotype, and with the exception of one mutant lacking ARM6, to the delocalisation of TgAC β to the cytosol (data not shown). Importantly, the membranous structure was not only detected by expression of the different ARM repeat truncated proteins but also in the ATc treated “parental” ARO-iKO strain that lacks TgARO. In this case, detection of the structure was achieved by transiently expressing an epitope-tagged form of *T. gondii* carbonic anhydrase 1 (TgCAH1-Myc). TgCAH1 was originally identified in the rhoptry proteome (Bradley et al., 2005) and used here as the only other marker beside TgARO known to specifically label the

rhoptry membrane. TgCAH1 possesses hydrophobic segments at each extremity and was tagged in the central region by single homologous recombination at the endogenous locus, which has been confirmed by western blot (Suppl. Fig. S2C-E). TgCAH1-Myc co-localized with rhoptry markers by IFA and the central region between the two hydrophobic termini was found to be exposed to the cytosolic face of the organelle similarly to TgARO, based on proteinase K protection assay (Suppl. Fig. S2F-G). The membranous structure was only detectable in absence of TgARO (i.e. in ATc treated ARO-iKO) using the rhoptry membrane marker TgCAH1 (Fig. 3D and not by ER or mitochondrial markers (data not shown) thus suggesting that it is of rhoptry origin.

In line with the misplacement of rhoptry organelles, plaque assays revealed that deletion of the ARM motifs results in non-viable parasites (Fig. 3E). There was however a subtle difference between the ARO-iKO/ Δ ARM3,4-Myc strain that formed no plaques when grown in presence of ATc compared to another mutant, ARO-iKO/ Δ ARM6-Myc, which showed very tiny plaques formation.

TgARO is implicated in the clustering of the rhoptry organelles

Only the deletion mutant of ARM6 showed a distinct phenotype compared to the other mutants. Δ ARM6-Myc failed to bring the rhoptries to the apical pole, however the phenotype strongly contrasted with ARO-iKO parasites or ARO-iKO parasites expressing Δ ARM3,4-Myc. The organelles were not dispersed but commonly (up to 90%) remained in bundles (Fig. 4A-C). The preserved clustering of the rhoptries was more clearly evident from the TEM analyses performed on parasites expressing Δ ARM6-Myc grown in the presence of ATc for 48 h (Fig. 4B). As observed with the other deletion mutants, Δ ARM6-Myc localized to the membranous structure in the presence of ATc, while AROi-Ty was still tightly regulated by ATc (Suppl. Fig. S3A,B). Furthermore, Δ ARM6-Myc was also the only mutant wherein TgAC β was still present at the intermediate compartment of the mislocalized organelles (Fig. 4C). Although this phenomenon was clearly and repeatedly observed by IFA, IP experiments with Δ ARM6-GFPTy in AC β -3Ty did not pull down TgAC β (Fig. 4D). Besides this discrepancy, Δ ARM6-Myc, like the other mutants, failed to properly position the rhoptries, which we assumed to be caused by a lack of a productive interaction with TgMyoF. Rather unexpectedly Δ ARM3,4-GFPTy and Δ ARM6-GFPTy were still interacting with TgMyoF-3Ty based on IP experiments whereas

GFPTy alone was unable to bind to TgMyoF (Fig. 4E). Taken together, the functional dissection of TgARO revealed that each individual ARM repeat is required for proper functioning of TgARO in bringing the rhoptries to the apical pole. It appears that only the conventional conformation of TgARO allows the establishment of a functional unit with TgMyoF, while just binding to TgMyoF does not require the presence of all ARM motifs. In addition, the C-terminal ARM6 is dispensable for the separate function of TgARO in the maintenance of rhoptry clustering.

Importantly, the expression of Δ ARM3,4 and Δ ARM6 as second copies in wild type parasites (RH Δ HX) or in the ARO-iKO not treated with ATc, exhibited a dominant negative effect that resulted in a partial mislocalization of the rhoptry organelles (Suppl. Fig. S3C-E). The fact that these parasites are viable indicates that endogenous TgARO ensures that at least one rhoptry is properly positioned at the apical end to assist in invasion. This is indeed the case, as shown by TEM analyses of the ARO-iKO expressing Δ ARM3,4-Myc or Δ ARM6-Myc in the absence of ATc, in which some but not all of the rhoptries are clearly dispersed (Suppl. Fig. S3F,G).

TgARO shares with UNC-45 a conserved myosin-binding fold

To elucidate the folding and organization of the predicted ARM repeats in TgARO, we used a combination of SAXS and homology modelling to reconstruct the 3D structure of TgARO in solution. The independently constructed homology and SAXS models both show that TgARO is a globular, monomeric protein with a maximum intramolecular distance of ~ 9 nm, comprising at least five ARM repeats and an additional N-terminal segment that also resembles an ARM repeat and may prove to be such in the structure (Fig. 5A-C). The homology model and the SAXS *ab initio* model can be superimposed nearly perfectly (Fig. 5B), and both show very good fits (χ^2 values 1.27 and 1.43, respectively) to the measured scattering curve (Fig. 5D,E).

The ARM repeats of TgARO form a right-handed super-helix, and repeats 2-6 superimpose very well on the five C-terminal ARM repeats of the myosin chaperone UNC-45 (Gazda et al., 2013; Lee et al., 2011) (Fig. 5A,B). The third helices of ARM repeats 2-6 line up to form a shallow groove (Fig. 6A,B and Suppl. Fig. S4A,B), which in UNC-45 is implicated in myosin binding (Gazda et al., 2013; Lee et al., 2011). This groove is ideally suited for binding an extended polypeptide chain, and is likely to do so in UNC-45 (Barral et al., 2002; Gazda et al., 2013), although it has also

been suggested that a folded part of the myosin motor domain could bind this motif (Frater et al., 2013). A similar binding groove is also present in human importin $\alpha 7$ where it accommodates the extended tail of the influenza PB2 nuclear localization domain (Suppl. Fig. S4B) (Pumroy et al., 2015). The groove in TgARO is lined by several aromatic residues (Fig. 6C), and its deepest part is highly negatively charged (Fig. 6D). The importance of this surface for ligand binding is also demonstrated by the high degree of conservation of the aromatic and acidic residues along the groove (Fig. 6E).

TgARO has previously been shown to depend on acylation of the N-terminal glycine and two cysteine residues, as well as basic residues in the N-terminal α helix for membrane attachment (Cabrera et al., 2012). The solution model suggests that the N-terminal ARM-like repeat does not form a continuum of the superhelix with the 5 C-terminal proper ARM repeats (Figs. 5B, 6). In this arrangement, all the suggested membrane-interacting residues point out of the core structure and would allow for sufficient degrees of freedom for simultaneous binding of multiple ligands to the 5-ARM-repeat core (Fig. 6A).

Trans-genera complementation of ARO within the phylum Apicomplexa

ARO is uniquely conserved amongst Apicomplexa with 63% identity and 79% similarity at the amino acid level between *P. falciparum* and *T. gondii* (Fig. 5A). Since PfARO was shown to localize to the rhoptries of intraerythrocytic schizonts via acylation we postulated that the protein fulfils a shared function across the phylum (Cabrera et al., 2012). In light of the findings reported here, it became pertinent to determine whether PfARO could interact with the same partners as TgARO and accomplish its identified functions. Transient transfection of a vector expressing a synthetic and codon optimized *PfARO* cDNA (Suppl. Fig. S4C) in RH and ARO-iKO parasites confirmed that PfARO localizes to the rhoptries of *T. gondii* (Fig. 7A,B). Upon ATc-mediated depletion of TgAROi-Ty, PfARO complemented both the apical positioning of the organelles and the correct targeting of TgAC β to the rhoptries (Fig. 7B). To determine whether PfARO also rescues rhoptry secretion and complements the invasion defect, a stable line of ARO-iKO parasites expressing PfARO was generated (ARO-iKO/T8PfARO). As observed in transient experiments, upon AROi-Ty depletion the rhoptries were no longer dispersed but correctly attached apically

when PfARO was stably expressed, and no dominant negative effect was detected. Plaque assays confirmed that PfARO fully rescues the invasion phenotype in the absence of AROi-Ty (Fig. 7C).

DISCUSSION

Invasion and subversion of host cellular functions are two events that crucially depend upon the release of the rhoptry contents (Carruthers and Boothroyd, 2007; Kemp et al., 2012). Whilst these two functions have been extensively studied, there is a plethora of rhoptry-related questions that are yet to be fully addressed, *e.g.* what are the steps in rhoptry biogenesis and how/why are they club-shaped? How do proteins traffic to the rhoptries and how are they segregated into the specifically delineated organelle sub-compartments? How is the membranous material accumulated and organized within the organelles? How are these organelles anchored at the parasite apex and maintained in clusters? What are the triggers stimulating rhoptry secretion and what are the physical changes allowing such a large organelle to inject its contents within seconds? And finally, how are the rhoptry organelles from the mother recycled during parasite division?

Isolation and characterization of the rhoptry proteome and lipidome has been reported for *T. gondii*, *Plasmodium* spp. and *Eimeria tenella* (Besteiro et al., 2008; Blackman and Bannister, 2001; Bradley et al., 2005; Etzion et al., 1991; Leriche and Dubremetz, 1991; Oakes et al., 2013; Sam-Yellowe et al., 2004). These studies have been instrumental in developing a more comprehensive understanding of the function of this highly specialized organelle and have also served in approaching the questions stated above with specific molecular tools. While many RON and ROP proteins, especially ROP kinases and pseudokinases have been (and continue to be) identified and characterized (Peixoto et al., 2010), not many proteins present at the surface of the rhoptries have been reported to date. These proteins, which are not secreted during invasion, are likely to play important roles in morphology, signal sensing or attachment of the organelles to the parasite cytoskeleton. Identified proteins localizing to the surface of rhoptries via TMDs or acylation motifs include i) TgNHE2, a non-essential sodium hydrogen exchanger with 12 predicted TMDs and a potential role in pH regulation and osmotolerance, ii) TgARO, which is anchored to the rhoptries by the N-terminal myristoylation and palmitoylation and plays an essential role in

rhoptry positioning and hence invasion, iii) TgDHH7, an essential, four TMD-containing PAT that is responsible for the palmitoylation of TgARO and iv) a putative carbonic anhydrase (TgCAH1; TGME49_297070) predicted to contain a TMD or GPI anchor signal and shown here to localise to the surface of the rhoptry organelle (Beck et al., 2013; Frenal et al., 2013; Karasov et al., 2005; Mueller et al., 2013).

This study reports a thorough dissection of TgARO function, aiming to understand how this protein mediates apical rhoptry positioning. A detailed scheme recapitulating our findings is presented in Fig. 8. Based on combined biochemical and genetic evidence we have established that TgAC β interacts with TgAIP, which in turn binds directly or indirectly to TgARO. Both proteins are the first known markers of a morphologically defined intermediate compartment of the rhoptries that separates the neck from the bulb (Lemgruber et al., 2010). It is unclear at this point what restricts the two proteins to this sub-compartment, The complementation experiment with Δ ARM6-Myc suggests that the C-terminus of TgARO might not be involved in the interaction with TgAC β , since the latter was still present on the mislocalized rhoptries in AROi-Ty depleted parasites by IFA analysis. Albeit indirectly, this also indicates that TgAIP was still able to bind to the Δ ARM6-Myc mutant on the rhoptry neck. Concordantly, the C-terminal ARM repeat is markedly less conserved than the preceding repeats (Figs. 5A, 6E), and the aromatic patch that could mark a binding surface for protein ligands is located on a face remote from the C-terminal ARM repeat (Fig. 5C). However, IP experiments did not confirm the immunofluorescence observations, since Δ ARM6-Myc did not pull down TgAC β . The different experimental outcomes might be explained by the fact that two different strains were used (Δ ARM6-Myc versus Δ ARM6-GFP-Ty), or that other settings such as lysis and IP conditions might have been too harsh to preserve this particular possibly suboptimal protein-protein interaction. Although TgAIP function is still unclear, the stability of TgAC β is intimately associated to the presence of TgAIP since TgAC β is undetectable in AIP-KO parasites.

The functional complementation utilised a series of deletion mutants for the 5 C-terminal ARM motifs aimed at dissecting the putative multiple roles of TgARO. Importantly, none of the mutant constructs were able to mediate apical rhoptry positioning, suggesting that each domain contributes to the overall folding and structure of TgARO, which is a prerequisite for the recruitment of TgMyoF. This is

understandable from the folding of the five C-terminal ARM repeats into a superhelix containing a highly acidic and conserved groove important for ligand binding. Deriving from other homologous ARM repeat proteins, such as importin $\alpha 7$ (Pumroy et al., 2015), it seems likely that this groove would bind an extended polypeptide chain. Notably, all TgARO binding partners identified so far (TgMyoF, TgAIP, and TgAC β) are large multi-domain proteins that contain long disordered or extended stretches of amino acids. On the other hand, homology modelling suggests that the globular WD40 domain at the C-terminus of MyoF has a highly basic surface (data not shown). Such a positively charged folded protein could also be a putative ligand for the highly acidic TgARO surface groove. In yeast, Vac8p is an armadillo-repeat containing acylated protein involved in vacuolar membrane dynamics, e.g., vacuole inheritance and vacuolar membrane fusion (Fleckenstein et al., 1998; Wang et al., 1998). No structure of Vac8p is known to date, but homology modelling suggests a structure similar to importin $\alpha 7$, the UCS domain of UNC-45 and TgARO. Vac8p also interacts with the actin cytoskeleton (Wang et al., 1998). Thus, it seems that the molecular mechanisms behind vacuole inheritance in higher eukaryotes and rhoptry positioning in Apicomplexa may be conserved.

A striking observation that emerged from analysing the ARM deletion mutants was their localization to membranous structures. In fact, these structures were clearly apparent in either wild type or ARO-iKO parasites expressing the non-functional ARM deletion mutants, but also and importantly in ARO-iKO parasites treated with ATc using as rhoptry membrane marker for their detection. These structures are likely of membranous origin since they were only detectable with rhoptry membrane markers (non-functional ARM deletion mutants, 20ARO-GFP (Cabrera et al., 2012) and CAH1-Myc). We envision that these rhoptry-derived membranes originate from the improper recycling of mislocalized mature rhoptries from the mother cell during daughter cell formation. If this holds true, this would also suggest that the lipid fraction and rhoptry contents might be degraded/recycled by distinct mechanisms. To date, the machinery involved in the fast recycling of rhoptries is unknown. Intriguingly, *T. gondii* does not possess bonafide lysosomes, and autophagy has not been associated with the recycling of organelles that are produced *de novo* during the cell cycle (Besteiro, 2012).

Deletion of the C-terminal motif in Δ ARM6-Myc turned out to be informative, as this deletion does not prevent the remaining ARM units from folding into a stable structure that retains partial functionality. Although this mutant failed to rescue the apical rhoptry localization and invasion defect, the organelles were no longer dispersed. TEM analyses clearly showed that the 10-12 rhoptries remained neatly clustered together, as observed in wild type parasites, but were not targeted apically and hence were still unable to discharge their contents during invasion. These findings strongly suggest that TgARO not only positions the rhoptries to the apical pole, but also maintains them in bundles. It is intriguing that Δ ARM6-Myc is also the only mutant wherein the localization of TgAC β to the rhoptries is not affected, at least by IFA. In light of this it is tempting to speculate that the intermediate compartment is ideally placed to position the machinery required to maintain the organelles in clusters.

Given the panoply of TgARO partners and its implication in positioning, clustering and possibly recycling of rhoptries, it became relevant to determine whether the same complexity was preserved across the phylum of Apicomplexa. When expressed in *T. gondii*, PfARO-Myc localizes to the rhoptries, indicating that the PAT TgDHHC7 is capable of recognizing this heterologous substrate. Additionally, this successful trans-genera functional complementation implies that TgMyoF can functionally interact with PfARO. Furthermore, PfARO is also able to interact with TgAC β since it brings this protein back to the rhoptries and to the appropriate sub-compartment. These findings demonstrate that many processes related to rhoptry biogenesis and discharge are conserved across the phylum, and in consequence, we can capitalize on comparative analyses between members of the Apicomplexa to tackle the many remaining open questions regarding this fascinating organelle.

MATERIALS AND METHODS

Parasite transfection and selection of clonal stable lines

T. gondii RH lacking *HXGPRT* or *KU80* (RH Δ HX or Δ KU80) were used as parental strains (Huynh and Carruthers, 2009). Parasites with tetracycline-controlled gene expression were regulated by 0.5 μ g ml⁻¹ anhydrotetracycline (ATc) (Meissner et al., 2002). *T. gondii* transfections were performed as previously described (Soldati and Boothroyd, 1993). TgAC β -3Ty parasites were generated by transfecting Δ KU80

parasites with 50 µg of linearized (*Bgl*III) *TgAC*β-3Ty vector (Fox et al., 2009; Huynh and Carruthers, 2009). Selection of transgenic parasites was performed with mycophenolic acid (MPA) and xanthine for HXGPRT (HX) selection and cloning was via serial dilution (Donald et al., 1996; Soldati et al., 1995). The AIP-KO strain was generated by transfecting p2855-HXGPRT-5'3'*TgAIP* into ΔKU80, which was then subjected to MPA/xanthine selection and cloned by serial dilution. The previously generated parasite line ARO-iKO was transfected with 50 µg of the *Not*I linearized plasmid pT8AIP-Myc-BLE and selected with 30 µg/ml of phleomycin and then cloned by serial dilution (Mueller et al., 2013). ARO-iKO was used for transfection of *Sac*I linearized plasmids pT8ΔARM3-Myc-BLE, pT8ΔARM4-Myc-BLE, pT8ΔARM3,4-Myc-BLE, pT8ΔARM2-Myc-BLE, pT8ΔARM5-Myc-BLE and pT8ΔARM6-Myc-BLE (50 µg plasmid). Transgenic parasites were selected with 30 µg ml⁻¹ of phleomycin and subsequently cloned by serial dilution (Mueller et al., 2013). RHΔHX was transfected with 50 µg of *Sac*I linearized pT8ΔARM3,4-Ty-HX or pT8ΔARM6-Ty-HX. After selection with MPA/xanthine, parasites were cloned by serial dilution. ARO-iKO expressing T8PfARO-Myc-BLE was generated by transfecting 50 µg of *Sac*I linearized plasmid pT8PfARO-Myc-BLE. After selection with 30 µg ml⁻¹ of phleomycin the transgenic parasites were cloned by serial dilution. Plaque assays were performed as previously described (Mueller et al., 2013).

Cloning of DNA constructs

TaKaRa Ex Taq DNA polymerase (Clontech) and Phusion high-fidelity DNA polymerase (NEB) were used for all PCR reactions performed. The *TgAC*β-3Ty-HX plasmid was generated by amplification of tachyzoite gDNA using the primer set 4189/4190. The PCR product as well as pT8-TgMIC13-3Ty-HX was digested with *Kpn*I and *Nsi*I restriction enzymes and the PCR product was subsequently ligated into the digested vector (Friedrich et al., 2010). For recombinant *TgAC*β Abs production, the C-terminal coding part of *TgAC*β was amplified by PCR using the primers 4461 and 4358. The fragment was digested with *Nco*I and *Spe*I and cloned into the pETH Tb 6xHis expression vector, which had also been digested with these restriction enzymes. The plasmid pT8AIP-Myc-BLE was generated by amplifying the *TgAIP* open reading frame via two PCR reactions using primer sets 4275/4276 (product 1) and 4277/4278 (product 2). Product 1 was digested with *Mfe*I and *Nsi*I and product 2 with *Nsi*I. Both products were then ligated simultaneously into the *Eco*RI and *Nsi*I digested plasmid

pT8ARO-Myc-BLE (Mueller et al., 2013). P2855-HXGPRT was digested with *KpnI* and *XhoI* for insertion of *TgAIP* 5' UTR, amplified with primers 4433/4434, that was also digested with *KpnI* and *XhoI*. Following digestion of the resulting plasmid with *XbaI* and *NotI* and insertion of *TgAIP* 3' UTR, amplified with primer set 4435/4436 and also digested with *XbaI* and *NotI*, the plasmid p2855-HXGPRT-5'3'*TgAIP* was obtained. For deletion of the different armadillo motifs full length *TgARO* cDNA was amplified with primers 3796/3740 and cloned into pGEM-T Easy Vector (Promega). The entire plasmid was amplified with following primer sets a) 4192/4193 for deletion of ARM3; b) 4194/4195 for deletion of ARM4; c) 4192/4195 for deletion of ARM3+4; d) 4639/4640 for deletion of ARM2 and e) 4641/4642 for deletion of ARM5. Resulting amplified plasmids were first digested with *NheI*, afterwards ligated and then re-digested with *MfeI* and *NsiI* and ligated into *EcoRI/NsiI* digested pT8ARO-Myc-BLE. For deletion of ARM6, pT8ARO-Myc-BLE was used as a template to amplify a PCR product with the primers 3796/4643 that was subsequently digested with *MfeI/NsiI* and ligated into the *EcoRI/NsiI* digested pT8ARO-Myc-BLE. pT8ΔARM3,4-Ty-BLE and pT8ΔARM6-Ty-BLE were digested with *KpnI* and *NsiI* and subcloned into the *KpnI/NsiI* digested vector pT8ARO-Ty-HX, thereby generating pT8ΔARM3,4-Ty-HX and pT8ΔARM6-Ty-HX. Synthesis of the codon-optimized PfARO sequence was carried out by Invitrogen. The synthetic sequence was amplified with the primers 4931 and 4932 and the product as well as the plasmid pT8ARO-Myc-BLE were digested with *EcoRI* and *NsiI*, which allowed ligation of the product into the digested plasmid. Cloning of the GST-His-TgARO in the bacterial expression vector was carried out using pET42aTEV into which the full-length cDNA of TgARO was introduced using *NcoI/XhoI* restriction sites. TgARO cDNA was amplified using the primers 4820 and 4821. The TgCAH1-Myc-HX plasmid was generated by amplification of tachyzoite gDNA using the primer sets 4586/4587 and 4588/4589. The PCR product 4586/4587 was digested with *KpnI* and *BglII* restriction enzymes, the PCR product 4588/4589 was digested with *BglII* and *PacI* and the vector pT8-TgARO-GFP-Ty-HX was digested with *KpnI* and *PacI*. The two PCR products were subsequently ligated into the digested vector.

All primers mentioned here are listed in supplementary material Table S1.

Antibodies

Recombinant 6xHis-CtTgAC β protein was purified from *E. coli* BL21 strain and used as antigen to raise polyclonal Abs against TgAC β protein in rabbits. This was done according to standard protocols by Eurogentec. The polyclonal TgARO Abs were described previously (Mueller et al., 2013). The mAbs α -Ty tag BB2, α -Myc tag 9E10, α -actin, α -SAG1 T4-1E5, α -RON9 2A7, α -ROP1 T5-1A3, α -ROP2 T3-4A7, α -ROP5 T5-3E2 as well as the polyclonal Abs α -GAP45, α -CAT and the α -recombinant TgROP1 and TgROP2 rabbit sera were previously described (Brecht et al., 2001; El Hajj et al., 2007b; El Hajj et al., 2008; Leriche and Dubremetz, 1991; Plattner et al., 2008) (Lamarque et al., 2012). For western blot analysis, secondary peroxidase conjugated goat α -rabbit and α -mouse Abs (Molecular Probes, Paisley, UK) were used. For IFA analysis, the secondary Abs Alexa Fluor 488-conjugated goat α -rabbit IgG Abs and Alexa Fluor 594-conjugated goat α -mouse Abs (Molecular Probes, Invitrogen) were used.

Western blotting

Freshly egressed parasites were pelleted after complete host cell lysis. Parasites harbouring a tet-inducible copy of ARO (ARO i -Ty) were grown for 48 h $\pm$ 0.5 μ g ml $^{-1}$ ATc before harvesting. SDS-PAGE, semi-dry transfer to nitrocellulose and proteins visualised using ECL system (Amersham Corp) were performed as described before (Mueller et al., 2013). Proteinase K protection assay were performed as previously described Cabrera et al., 2012 and analysed by Western blots using different antibodies: α -Myc, α -ARO, α -ROP2-4 and α -profilin Abs.

Immunofluorescence assay and confocal microscopy

HFF seeded on coverslips in 24-well plates were inoculated with freshly released parasites. After 2-4 rounds of parasite replication, cells were fixed with 4% paraformaldehyde (PFA) or 4% PFA/0.05% glutaraldehyde (PFA/GA) in PBS depending on the antigen to be labelled, and processed as previously described (Plattner et al., 2008). Confocal images were generated with a Zeiss confocal laser scanning microscope (LSM700) using a Plan-Apochromat 63x objective with NA 1.4. All the images taken are maximal projections of confocal stacks spanning the entire parasites.

Transmission electron microscopy

ARO-iKO parasites expressing either Δ ARM3,4-Myc or Δ ARM6-Myc that had undergone or not a 24 h pre-treatment with ATc, were used to infect a monolayer of HFF and grown $\pm$ ATc for 24 h. The infected host cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, post-fixed in osmium tetroxide, dehydrated in ethanol and treated with propylene oxide prior to embedding in Spurr's epoxy resin. Thin sections were stained with uranyl acetate and lead citrate prior to examination using a Tecnai 20 electron microscope (FEI Company). Multiple thin sections from each of the two sample preparations were examined by the electron microscope.

Solubility assay

Freshly released TgAC β -3Ty and TgAIP-3Ty parasites were harvested and their pellets resuspended in PBS or PBS containing 1% Triton X-100 or 1M NaCl. Following 5 freeze-thaw cycles using liquid N₂ and a 37°C water bath the pellet (P) and soluble fractions (S1) were separated by centrifugation at 14'000 rpm, 45 min, 4°C. One pellet that initially was resuspended in PBS, was now resuspended in 0.1M Na₂CO₃ (pH11), incubated for 30 min at RT and the pellet and soluble (S2) fraction were separated by centrifugation at 14'000 rpm, RT, 10 min. The different samples were subsequently analysed by western blot. As control, solubility of CAT and GAP45 were tested.

Co-Immunoprecipitation

Freshly released tachyzoites were harvested, washed in PBS, and lysed in co-IP buffer (PBS containing 0.2% [v/v] Triton X-100, 150 mM NaCl, protease inhibitor cocktail (Roche)) and incubated on ice for 20 min. After centrifugation at 14000 rpm, 45 min, 4°C the supernatant was subjected to IP using α -GFP lama Abs (GFP-Trap_M Magnetic Agarose Beads from ChromoTek). Beads were washed in a co-IP buffer with 300 mM NaCl.

Small-angle X-ray scattering and homology modelling

GST-His-TgARO was expressed in *E. coli* BL21(DE3) cells at 37°C for 4 h using 1 mM IPTG for induction. The cell pellet was resuspended in lysis buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 20 mM imidazole, 10 mg ml⁻¹ DNase I, 0.1 mg ml⁻¹ lysozyme, an EDTA-free protease inhibitor cocktail (Roche), and 5 mM β -mercaptoethanol), and the cells were lysed using sonication. The clarified supernatant was loaded onto Ni-NTA matrix pre-equilibrated with the lysis buffer without DNase I, lysozyme, and protease inhibitors, and binding was carried out for 1 h at 4°C. The column was subsequently washed three times with 20 ml of buffer containing 20 mM HEPES, 300 mM NaCl, 20 mM imidazole, and 5 mM β -mercaptoethanol, and the protein was eluted with 300 mM imidazole in the lysis buffer. Imidazole was removed from the sample using a PD10 desalting column, and the fusion protein was subjected to TEV digestion overnight at 4 °C. The cleaved GST-His- tag and any uncleaved fusion protein were removed by passing the sample through a second Ni-NTA column. The unbound TgARO sample was concentrated and loaded onto a Hiload 16/60 Superdex 75 column pre-equilibrated with buffer containing 20 mM Tris-HCl pH 8.0, 300 mM NaCl, 5% glycerol, and 1 mM tris (2-carboxyethyl)phosphine. Fractions containing pure TgARO were concentrated, flash frozen in liquid nitrogen, and stored at -70°C until use.

Small-angle X-ray scattering (SAXS) data on TgARO at concentrations of 6.1, 3.1, and 1.5 mg ml⁻¹ were collected at the EMBL beamline P12 at PETRA III/DESY, Hamburg. Analysis of the data was carried out using the ATSAS package (Petoukhov et al., 2012). As there seemed to be no concentration-dependent effects on the scattering curves, the highest concentration data were used for further modelling. An *ab initio* model of TgARO was built using GASBOR (Svergun et al., 2001). A homology model was constructed based on the TgARO sequence using the Phyre² server (Kelley et al., 2015). The *ab initio* and homology models were superimposed both using SUPCOMB (Kozin and Svergun, 2001) as well as manually in Pymol. The fit of the homology model to the experimental scattering curve was calculated using CRY SOL (Svergun et al., 1995). The electrostatic surface potential was calculated using APBS (Baker et al., 2001) in Chimera (Pettersen et al., 2004) and the conservation plot using ConSurf (Ashkenazy et al., 2010).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

CM, P-MH and NK have performed the experiments reported in figures 1, 2, 3, 4, 7, S1-S4. AS, JPK and IK have performed the experiments and structural analyses reported in figures 5, 6 and S5. CH, DSF and IK have conceived and designed the experiments and written the manuscript.

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Figures

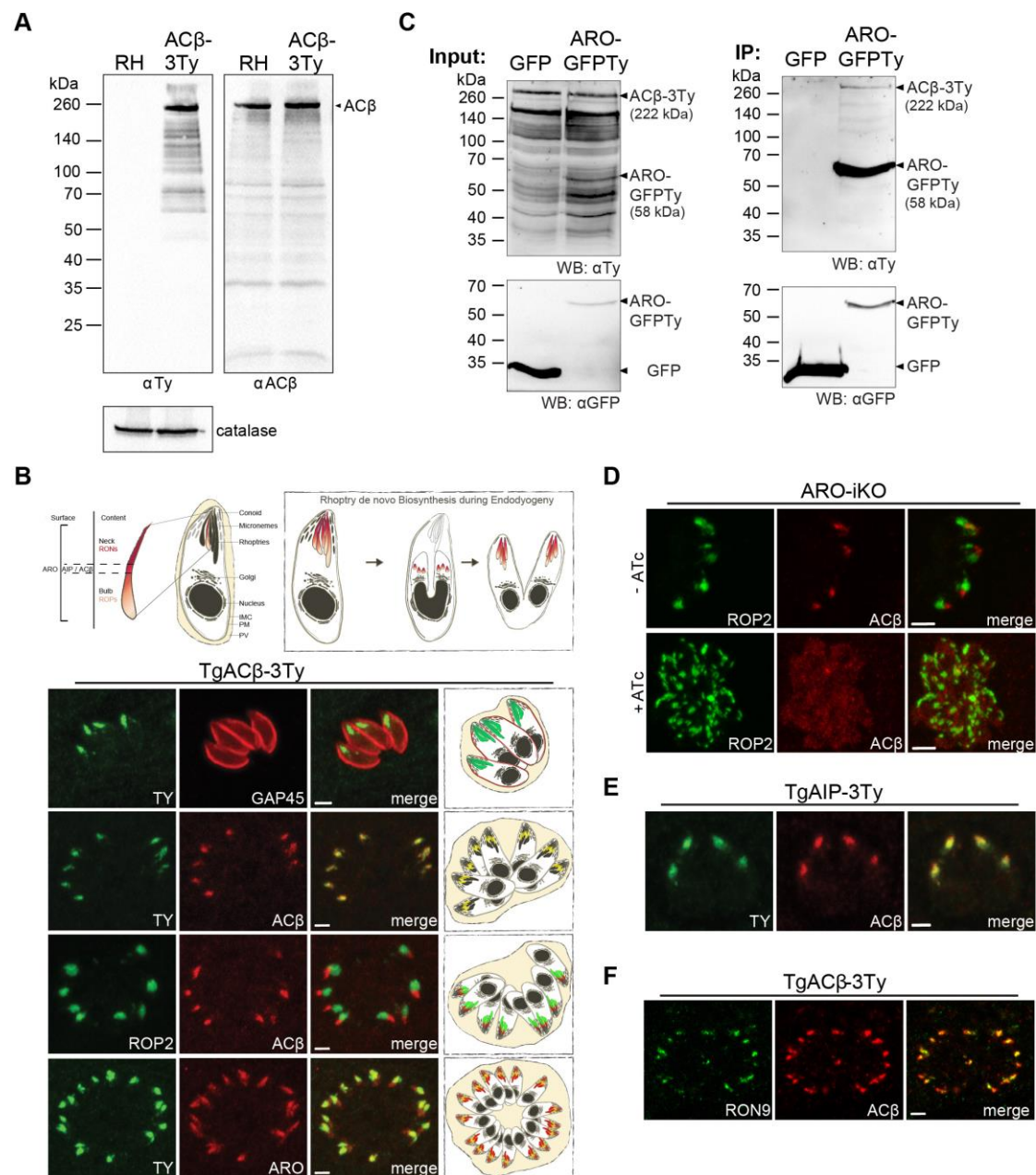


Fig. 1. Characterization of TgACβ, an interacting partner of TgARO

A. Western blot analyses of RH and TgACβ-3Ty parasite lysates using α-Ty and the polyclonal α-TgACβ Abs. TgACβ migrates at approximately 220 kDa as detected by the polyclonal Abs, and also in the TgACβ-3Ty by α-Ty Abs. Several lower bands are also detected and most likely represent degradation products. Catalase (CAT) was used as a loading control.

B. Scheme on the top: On the left the schematic representation shows an enlarged rhoptry organelle with the two sub-compartments, bulb and neck, delimited by ROP (beige) and RON (reddish) proteins. The intermediate region occupied by both, TgAC β and TgAIP (in bold) is indicated by dashed lines. TgARO distributes over the entire rhoptry surface. On the right (boxed) the disappearance of the mother rhoptries and the *de novo* formation of daughter rhoptries during parasite cell division is illustrated. Below: Localization of TgAC β in the TgAC β -3Ty strain by IFA and confocal microscopy. Parasites were stained with α -Ty, α -GAP45, α -ROP2, α -ARO and α -TgAC β Abs. α -GAP45 Abs were used to visualize the parasites, α -ARO Abs were used to visualize the whole rhoptry organelle and α -ROP2 Abs were used to label the bulbous region of the rhoptries. TgAC β localizes to the rhoptry neck region. Scale bars represent 2 μ m. The sketches depict localization of organelles and proteins within *T. gondii* and the parasitophorous vacuole.

C. Transient transfection of ARO-GFP-Ty or GFP in the TgAC β -3Ty strain, following co-IP using α -GFP Abs and western blot analyses using α -Ty and α -GFP Abs. ARO-GFP-Ty specifically interacts with TgAC β (α -Ty staining). The negative control GFP does not co-immunoprecipitate TgAC β (α -Ty and α -GFP staining).

D. IFA analyses and confocal microscopy of ARO-iKO parasites grown $\pm$ ATc for 48 h and stained with α -ROP2 and α -TgAC β Abs. In the absence of AROi-Ty, TgAC β is no longer detected at the rhoptries. Scale bars represent 2 μ m.

E. IFA analyses and confocal microscopy of the TgAIP-3Ty strain using α -Ty, α -TgAC β and α -ROP2 Abs. TgAC β perfectly colocalizes with TgAIP. Scale bars represent 2 μ m.

F. IFA analyses and confocal microscopy of the TgAC β -3Ty strain. Staining with α -RON9 and α -TgAC β Abs revealed that TgAC β localizes to a defined intermediate region that lies between the rhoptry bulb and the rhoptry neck.

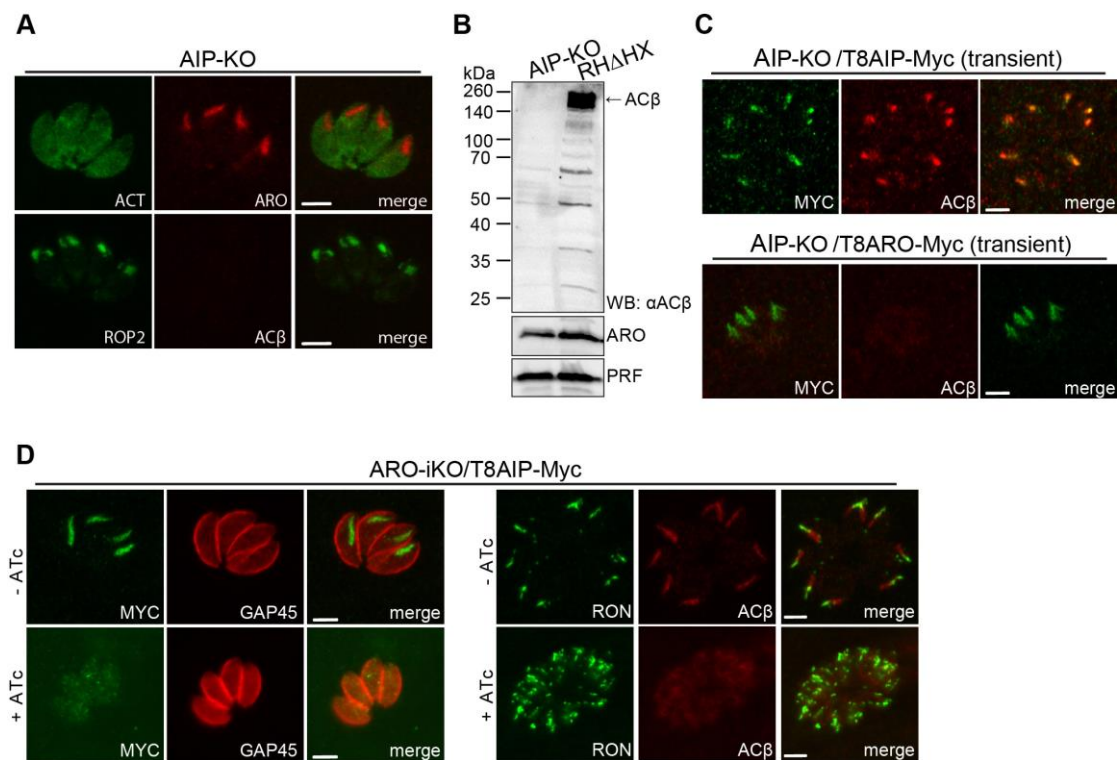


Fig. 2. Rhoptry localization of TgAIP and TgACβ is ultimately dependent upon TgARO

A. IFA analyses and confocal microscopy of AIP-KO parasites stained with α -ARO, α -actin (ACT), α -TgACβ and α -ROP2 Abs. In AIP-KO parasites the localization of TgARO is unaffected, however TgACβ is no longer found at the rhoptry neck. Scale bars represent 2 μm.

B. Western blot analyses of AIP-KO and RHΔHX parasite lysates. TgARO protein levels (α -ARO Abs) are comparable between the two lysates, while TgACβ is only detected in the RHΔHX but not the AIP-KO lysate (α -TgACβ Abs). Profilin (PRF) was used as a loading control.

C. AIP-KO was transiently transfected with either T8AIP-Myc or T8ARO-Myc constructs. Subsequent IFA analyses and confocal microscopy with α -Myc, α -TgACβ and α -ACT Abs revealed that only within parasites expressing T8AIP-Myc did TgACβ localize to the rhoptries. Scale bars represent 2 μm.

D. IFA analyses and confocal microscopy of ARO-iKO parasites expressing T8AIP-Myc, grown $\pm$ ATc for 48 h and stained with α -Myc, α -GAP45, uncharacterized α -RON and α -TgACβ1 Abs. In the absence of ATc, T8AIP-Myc localizes to the rhoptries, whereas upon knockdown of AROi-Ty, it is no longer detected in this

location. Staining with α -RON and α -TgAC β 1 Abs shows that in ATc treated parasites TgAC β is also absent from the rhoptries. Scale bars represent 2 μ m.

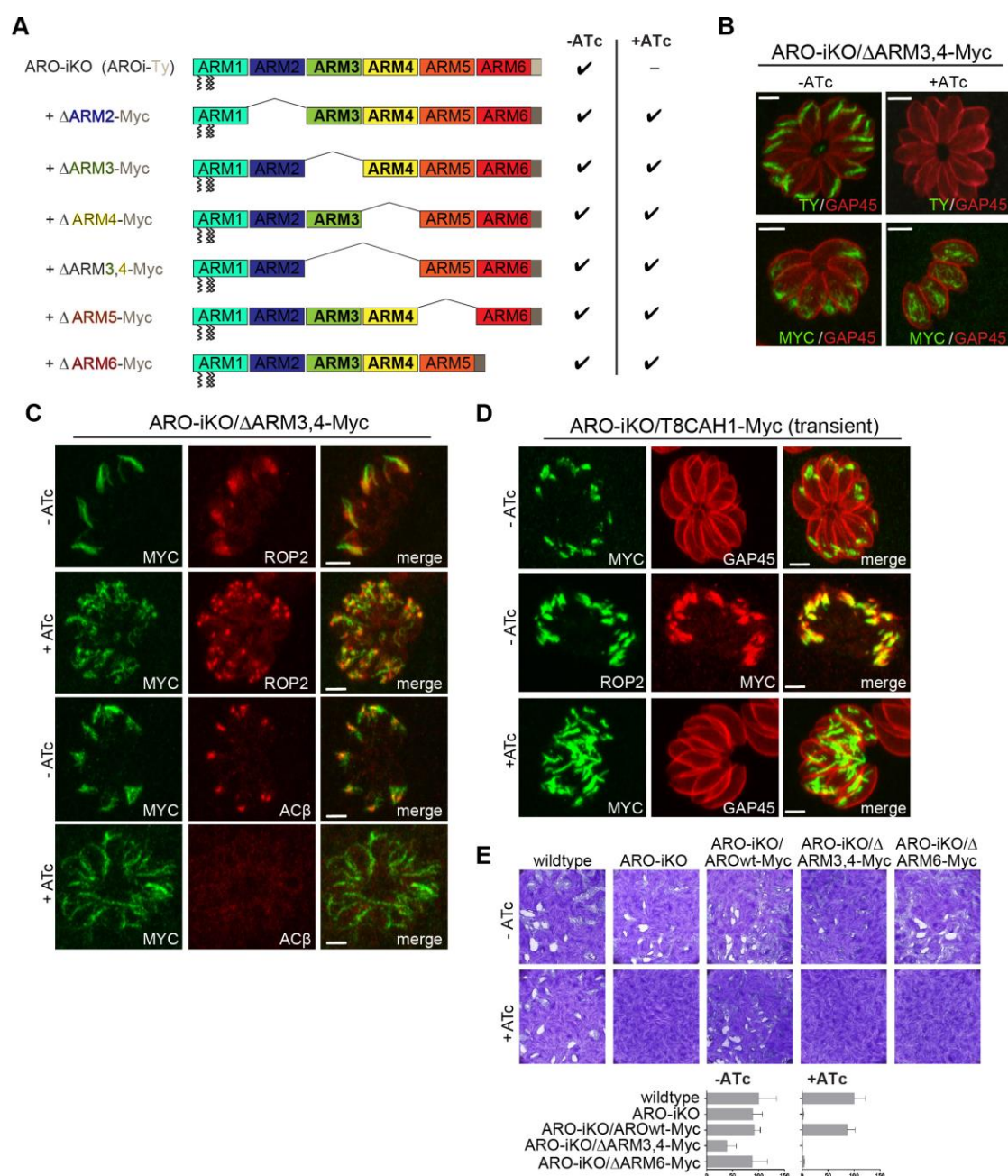


Fig. 3. ARM repeats are key to TgARO function and its interaction with partners

A. Schematic representation of TgARO showing the 6 putative ARM repeats, which are coloured as in Fig. 5A,B. The N-terminus (ARM1) harbours the glycine and cysteine residues important for TgARO acylation as reported in (Cabrera et al., 2012) (fatty acids are depicted as black zigzag lines). The C-terminal Myc-tags are highlighted in dark brown; the Ty-tag is highlighted in light brown. While AROi-Ty is only expressed in absence of ATc, the ARM deletion constructs are constitutively expressed under the control of the tubulin promoter (indicated by check marks).

B. IFA analyses and confocal microscopy of ARO-iKO stably expressing Δ ARM3,4-Myc grown for 48 h $\pm$ ATc. Staining with α -Ty Abs shows down-regulation of AROi-Ty and staining with α -Myc Abs shows that Δ ARM3,4-Myc is expressed in both, the absence and presence of ATc.

C. IFA analyses as described in B. Upon depletion of AROi-Ty the Δ ARM3,4-Myc mutant protein is found dispersed throughout the cytosol but the α -Myc staining does not resemble dispersed rhoptry organelles. Staining with α -ROP2 and α -AC β Abs shows that in presence of ATc i) the rhoptry organelles are dispersed and ii) the TgAC β signal is lost from the rhoptries. Scale bars represent 2 μ m.

D. IFA analyses and confocal microscopy of ARO-iKO transiently transfected with T8CAH1-Myc and treated $\pm$ ATc for a total of 48 h. IFA was performed with α -ROP2, monoclonal and polyclonal α -MYC and α -GAP45 Abs and revealed that the unusual membranous structures are most likely of rhoptry membrane origin. Scale bar represents 2 μ m.

E. Plaque assay at day 9 carried out with the strains Δ KU80 (wild-type), ARO-iKO and ARO-iKO stably expressing AROwt-Myc, Δ ARM3,4-Myc, Δ ARM6-Myc grown $\pm$ ATc during the entire assay. Mean area of 10 plaques $\pm$ s.d. is depicted.

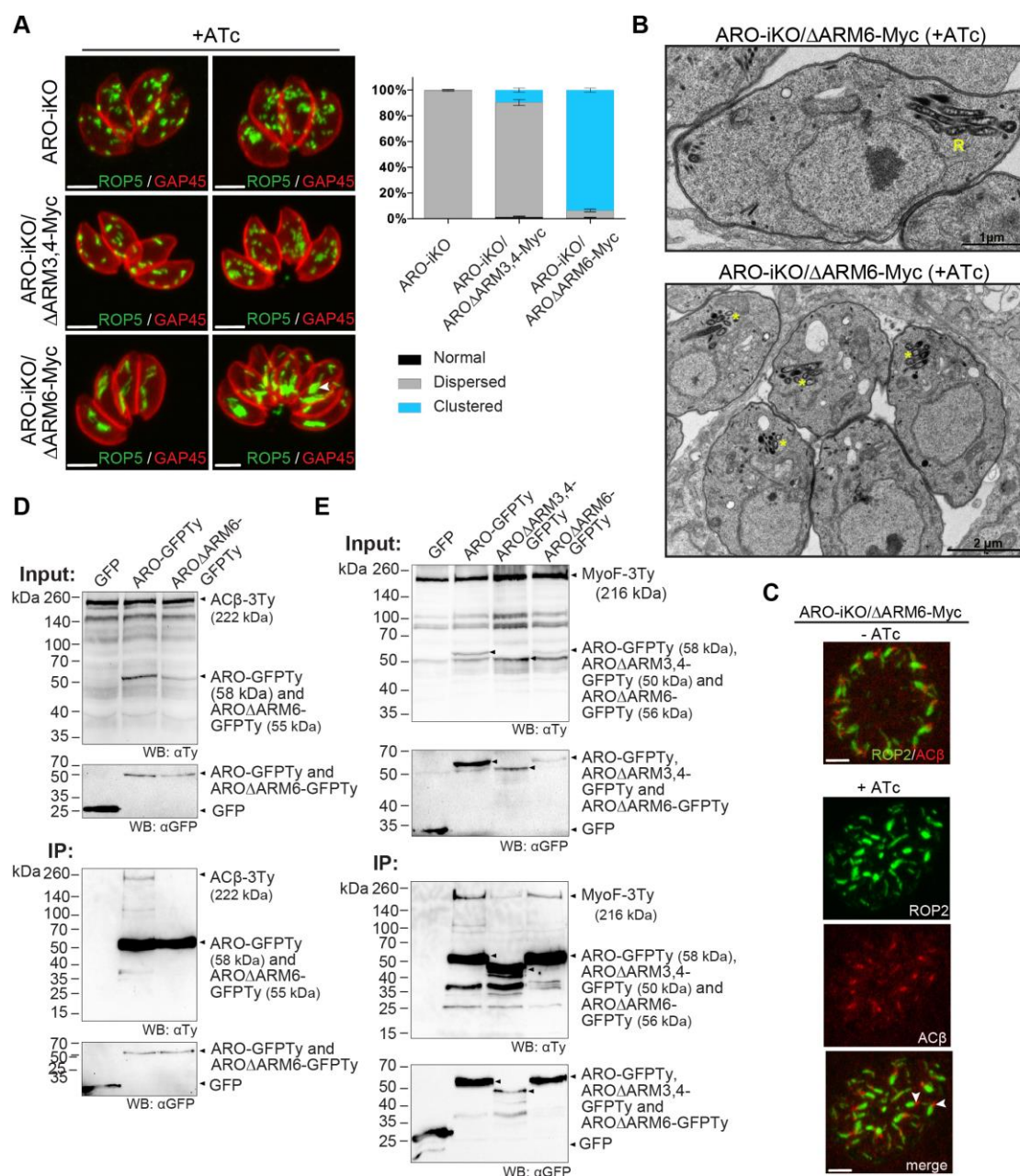


Fig. 4. The C-terminal ARM6 mutant reveals a novel function for TgARO

A. IFA analyses and confocal microscopy of ARO-iKO, ARO-iKO expressing Δ ARM3,4-Myc or Δ ARM6-Myc, previously grown in presence of ATc for 48 h and labelled with α -ROP5 and α -GAP45 Abs. The mislocalized rhoptry organelles look different, i.e. not dispersed but clustered in the ARM6 mutant. Arrowhead: mislocalized, clustered rhoptries. Rhoptries localization (normal, dispersed or in clusters) is reported for 100 vacuoles in 3 biological replicates from 2 independent experiments. Scale bar represents 2 μ m.

B. TEM analyses of ARO-iKO expressing Δ ARM6-Myc, grown in the presence of ATc for 48 h. Rhoptry organelles are mislocalized in the parasite but mostly remain clustered together. Scale bar 1 μ m. Conoid (C); Rhoptry (R and *).

C. IFA and confocal microscopy analyses of ARO-iKO stably expressing Δ ARM6-Myc grown $\pm$ ATc for 48 h and labelled with α -ROP2 and α -TgAC β Abs. Of all the ARM deletion mutants, Δ ARM6-Myc is the only mutant that upon AROi-Ty depletion does not cause the loss of the TgAC β signal at the mislocalized rhoptries (arrowhead). Scale bars represent 2 μ m.

D. Transient transfection of Δ ARM6-GFPTy, the positive control ARO-GFPTy or the negative control GFP in the TgAC β -3Ty knock-in strain following co-IP using the GFP-Trap system and western blot analysis with α -Ty and α -GFP Abs. Shown are the input and the eluted (IP) fractions. Arrows indicate the different constructs.

E. Same procedure as in D, but with the TgMyoF-3Ty knock-in strain and including the construct Δ ARM3,4-GFPTy.

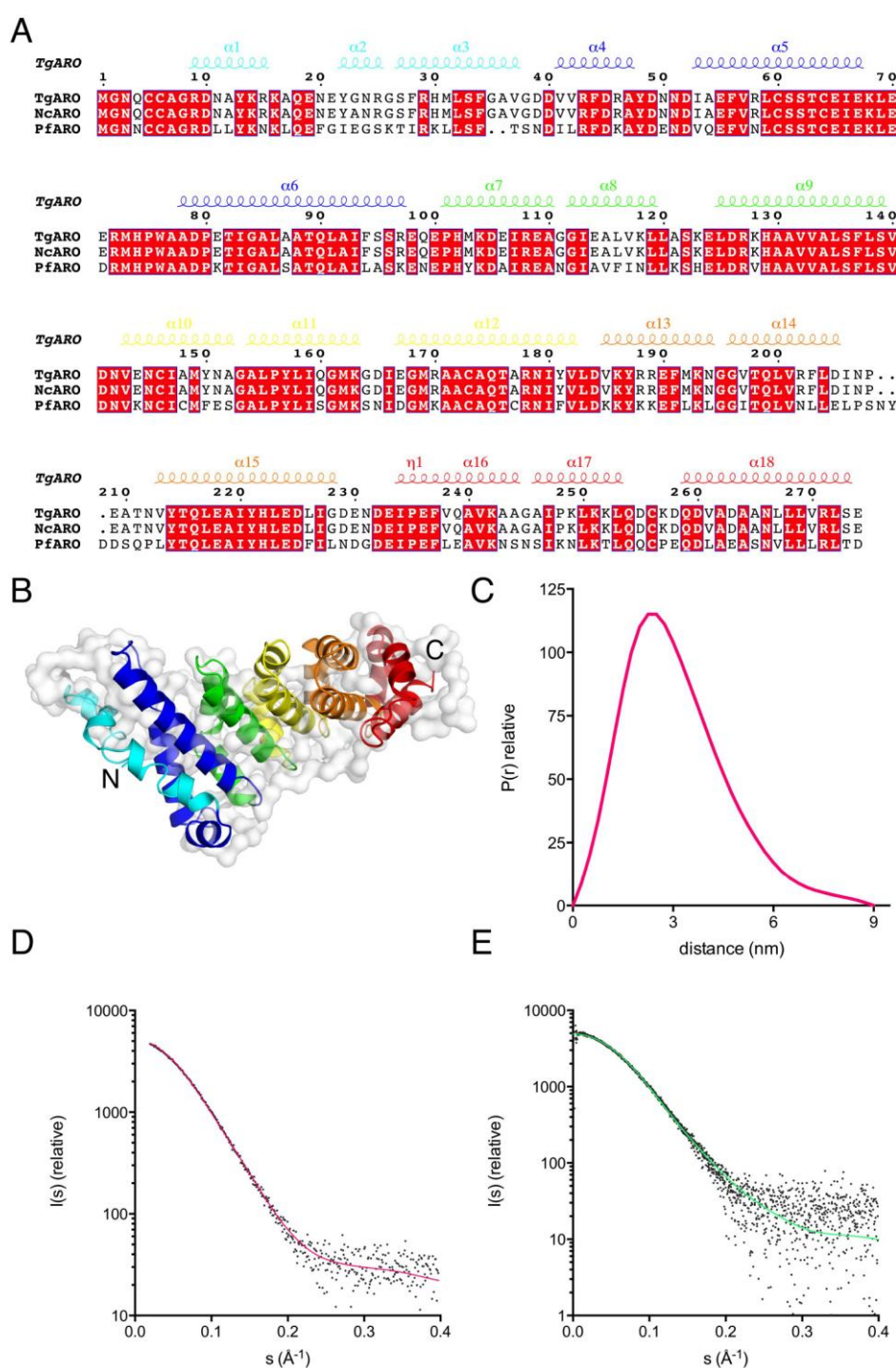


Fig. 5. Structure of TgARO

A. Sequence alignment of ARO proteins from *T. gondii* (Tg), *Neospora caninum* (Nc), and *P. falciparum* (Pf). Residues identical in all three sequences are highlighted on red background. The α helices from the TgARO homology model are indicated

above the alignment, and the ARM repeats are depicted with different colors (cyan, blue, green, yellow, orange, and red).

B. The homology model (cartoon) and the *ab initio* SAXS model (surface) of TgARO superimposed. The ARM repeats are colored as in panel A, and the N and C termini are labelled.

C. Distance distribution function of TgARO from the SAXS data.

D. Fit of the calculated scattering curve (pink line) from the *ab initio* model generated using GASBOR to the measured SAXS data processed by GNOM (Svergun, 1992) (black dots).

D. Fit of the calculated scattering curve (green line) from the homology model generated using Phyre² to the measured raw SAXS data (black dots).

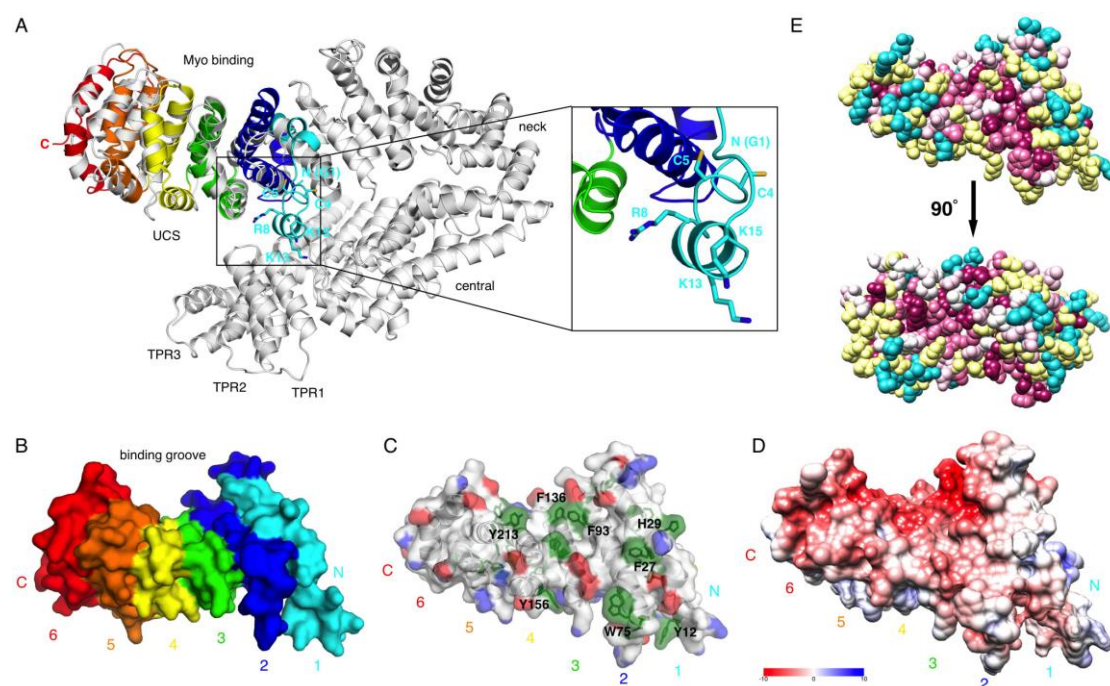


Fig. 6. Similarity of TgARO to UNC-45 and implications for MyoF binding

A. Superposition of the TgARO model (ARM repeats colored and labelled as in Fig. 5) on the UCS domain of UNC-45 (gray; PDB code 4I2Z; (Gazda et al., 2013)). The different domains and the myosin-binding groove of UNC-45 are labelled in black. The residues in the N-terminal segment earlier identified as important for the membrane attachment of TgARO are shown as sticks and labelled in cyan.

B. Surface representation of the TgARO model. Colouring is as above. The putative MyoF-binding groove is labelled.

C. TgARO surface with all aromatic residues shown as sticks, coloured green, and labelled. Acidic residues are colored red and basic residues blue.

D. Electrostatic surface potential of the TgARO model calculated by APBS. The scale is from -10 (red) to 10 kT/e (blue).

E. Conservation of TgARO against homologous ARM repeat proteins from different Apicomplexa (*Plasmodium*, *Eimeria*, *Babesia*, *Cryptosporidium*, *Theileria*) identified by a BLAST search. Magenta denotes highly conserved residues, cyan variable. For the yellow residues, there was not sufficient data in the alignment to assign a conservation grade. The highly conserved dark red belt marks the putative MyoF-binding site. The orientation in the upper panel is the same as in panels B-D. The view

in the lower panel is rotated by $\sim 90^\circ$ along the X-axis compared to the upper panel (the top of the upper panel rotated towards the viewer).

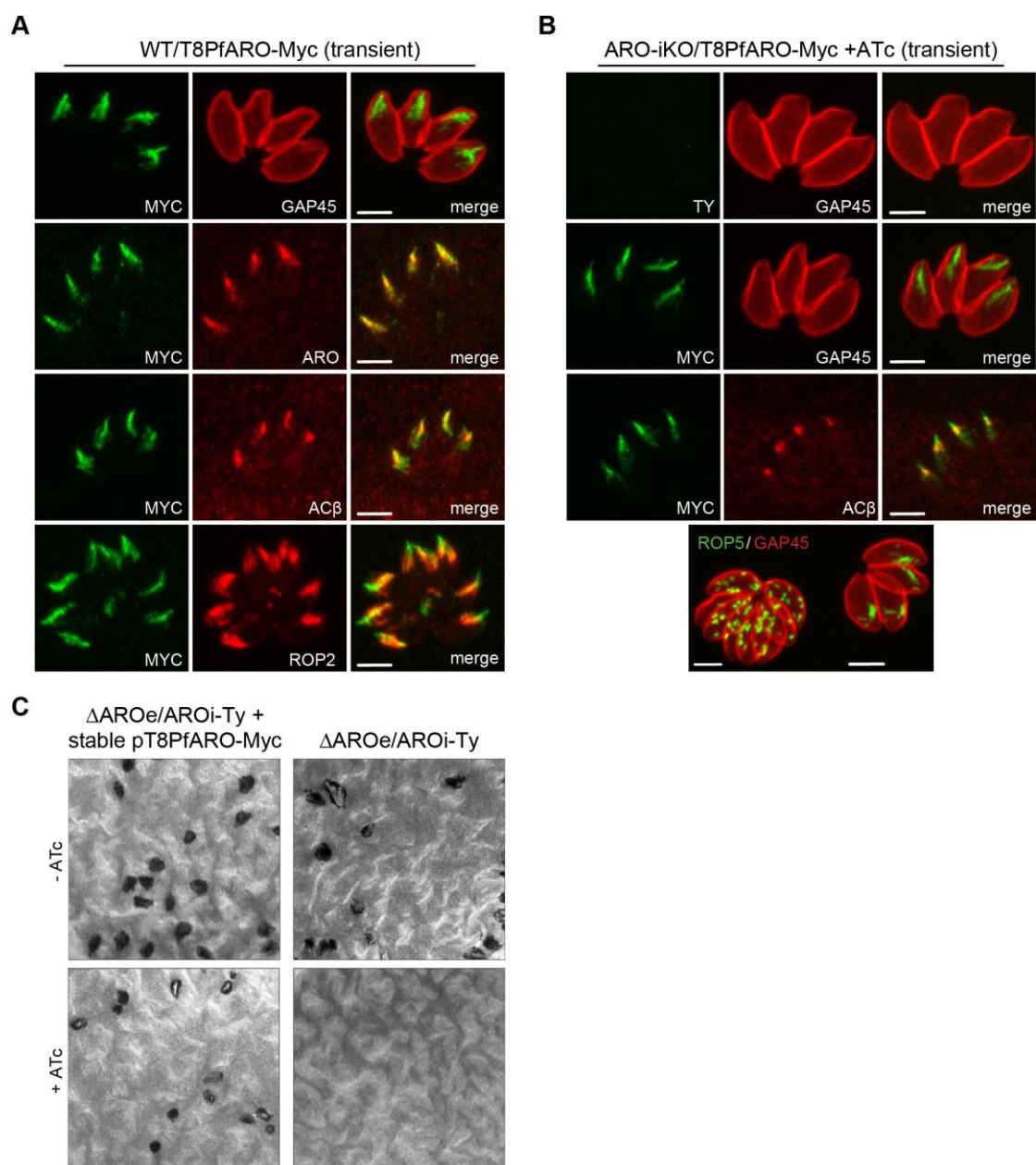


Fig. 7. PfARO complements the loss of ARO in *T. gondii*

A. Transient transfection of T8PfARO-Myc in RH and subsequent IFA analyses with α -Myc, α -GAP45, α -ARO, α -TgAC β and α -ROP2 Abs. As TgARO, PfARO (α -Myc staining) appears to localize to the rophtry organelles and colocalizes with ARO, AC β and ROP2. Scale bar represents 2 μ m.

B. Transient transfection of T8PfARO-Myc in ARO-iKO that prior to fixation were grown in the presence of ATc (48 h). IFA analyses using α -Ty Abs shows that AROi-Ty is no longer detected. Anti-Myc labelling reveals AC β localizes to the rophtries .

Staining with α -ROP5 Abs reveal that in the pool of transfected parasites some parasites retain normal rhoptry localisation. Scale bar represents 2 μ m.

C. Plaque assay of 9 days with the strains ARO-iKO (control) and ARO-iKO stably expressing T8PfARO-Myc, grown $\pm$ ATc during the entire assay.

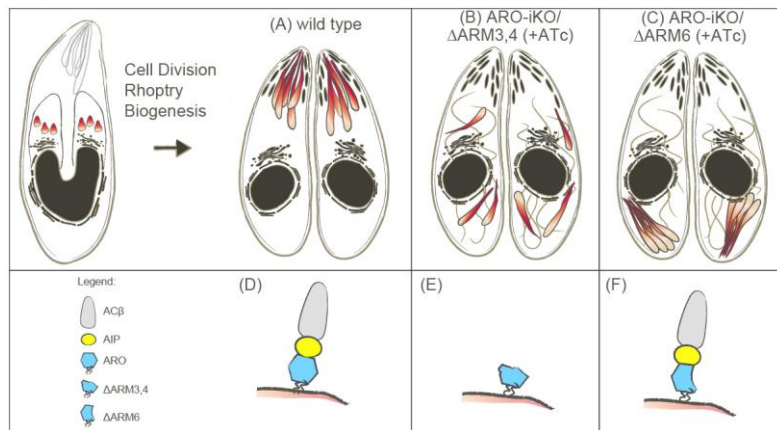


Fig. 8. Summarizing model: Don't mess with ARO

In the upper left box a parasite during endodyogeny is shown, a process in which rhoptry organelles are synthesised *de novo* in the forming daughter cells. The ultrastructural changes are shown for either (A) wild type parasites, (B) ARO-iKO/ Δ ARM3,4-Myc parasites depleted in AROi-Ty and (C) ARO-iKO/ Δ ARM6-Myc also depleted in AROi-Ty. In the situation (A) rhoptries are found at the apical end and parasites are able to invade host cells. In scenario (B) rhoptries are dispersed, indicating a non-productive interaction between TgARO and TgMyoF. In addition, a membranous structure is visible, which possibly originates from rhoptries of the mother cell that were not properly recycled. Scenario (C) also results in mislocalized rhoptries, however they remain bundled together. The membranous structure is also visible. In the lower boxes the interaction between TgARO and the soluble proteins TgAIP and TgAC β is shown in either wild type (D) or AROi-Ty depleted Δ ARM3,4 (E) or Δ ARM6 (F) parasites. Based on IFA data, in absence of AROi-Ty (+ATc), Δ ARM3,4 cannot recruit TgAIP and TgAC β at the rhoptries (E) whereas Δ ARM6 does (F).