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The apicomplexan glideosome and adhesins -- structures and function

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Abstract

The apicomplexan family of pathogens, which includes *Plasmodium spp*. and *Toxoplasma gondii*, are primarily obligate intracellular parasites and invade multiple cell types. These parasites express extracellular membrane protein receptors, adhesins, to form specific pathogen-host cell interaction complexes. Various adhesins are used to invade a variety of cell types. The receptors are linked to an actomyosin motor, which is part of a complex comprised of many proteins known as the invasion machinery or glideosome. To date, reviews on invasion have focused primarily on the molecular pathways and signals of invasion, with little or no structural information presented. Over 75 structures of parasite receptors and glideosome proteins have been deposited with the Protein Data Bank. These structures include adhesins, motor proteins, bridging proteins, inner membrane complex and cytoskeletal proteins, as well as co-crystal structures with peptides and antibodies. These structures provide information regarding key interactions necessary for target receptor engagement, machinery complex formation, how force is transmitted, and the basis of inhibitory antibodies. Additionally, these structures can provide starting points for the development of antibodies and inhibitory molecules targeting protein-protein interactions, with the aim to inhibit invasion. This review provides an overview of the parasite adhesin protein families, the glideosome components, glideosome architecture, and discuss recent work regarding alternative models.

Keywords

apicomplexa; invasion machinery; glideosome; adhesins; Toxoplasma; Plasmodium; Cryptosporidium; malaria

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1. Introduction

Apicomplexans are primarily obligate, intracellular parasites that can cause devastating human diseases such as malaria (*Plasmodium spp*.), toxoplasmosis (*Toxoplasma gondii*), and gastrointestinal illness (*Cryptosporidium spp*.). These parasites must invade host cells to continue their lifecycle. For example, in *Plasmodium spp*., the parasite must invade the human host's liver and red blood cells, as well as the mosquito vector's midgut. To accomplish this task, the parasite employs an actomyosin motor at the core of a larger complex known as the invasion machinery, or glideosome. The mechanical force generated by this motor is used to invade a host cell, to which parasites attach *via* a variety of adhesive proteins. In this review, a structural survey of proteins involved in this critical invasion process, focusing on *Toxoplasma gondii* and *Plasmodium spp*., where the most research has been done, is presented. If the readers are interested in the cellular and molecular mechanisms of attachment and invasion, they are directed to a review of *Plasmodium spp*. merozoite invasion, by Cowman *et al*. (2012). The first part of this structural review focuses on the extracellular interaction of the parasite with the host cell, covering crystal structures that identify mechanisms of binding. The second section covers the intracellular proteins that compose the core invasion machinery and discusses the key protein-protein interactions stabilizing the complex. The third section highlights new findings that call into question the essentiality of machinery components, and discusses compensatory glideosomes, alternative models, and questions that arise from this data.

2. Parasite interaction with the host cell

Apicomplexans employ extracellular transmembrane protein receptors to interact with receptors present on the host cell surface to attach and reorient, form a tight junction, and invade successfully. There are four primary families of adhesin proteins into which receptors from *Plasmodium spp*. and *T. gondii* can be grouped based on conserved protein domains or localization: the apical membrane antigen (AMA) family, the Duffy binding-like (DBL) family, the reticulocyte binding-like (RBL) family, and the thrombospondin related anonymous protein (TRAP) family. *T. gondii* and *Plasmodium spp*. both express proteins in the AMA and TRAP families, while the DBL and RBL proteins are specific to *Plasmodium spp*. A list of the receptors and their EuPathDB codes (Aurrecoechea et al., 2007), their known interaction partners with references, and any related structures that have been described and deposited in the PDB, are listed in Table 1. Additionally, Figure 1A depicts the domain organization and family grouping of the different receptor proteins. A recently published review by Malpede *et al*. (2013), following the publication of their structure of the *P. vivax* Duffy Binding Protein in complex with the Duffy Antigen/Receptor for Chemokines (DARC), provides an introduction to *Plasmodium spp*. adhesins, including some that are not covered in this review.

2.1 Apical membrane antigen (AMA) and the rhoptry neck (RON) proteins

Unlike other parasite receptor families, which are shared only amongst the species of a particular genera of apicomplexans, the apical membrane antigen 1 (AMA1) protein is shared by both *T. gondii* and *Plasmodium spp*., as well as *Babesia, Neospora* and *Theileria* (Peterson et al., 1989; Taylor et al., 1990)*; Cryptosporidium* does not have an AMA1

ortholog. AMA1 is involved in the tight-junction formation that occurs after reorientation of the parasite (Mitchell et al., 2004); however, there have been conflicting reports regarding the exact role of AMA1 and its necessity for invasion (Bargieri et al., 2013; Mital et al., 2005; Richard et al., 2010; Yap et al., 2014).

2.1.1 Apical membrane antigen 1 (AMA1)—The domain organization of AMA1 includes a signal sequence, a microneme targeting sequence, an ectodomain divided into three structural domains as defined by a pattern of eight disulfide bonds (Hodder et al., 1996), and a transmembrane helix followed by a cytoplasmic C-terminal tail (Figure 1A). Domains I and II are similar and belong to the PAN (plasminogen, apple, nematode) superfamily, a protein fold noted to be involved in receptor binding (Tordai et al., 1999).

The structure of TgAMA1 is the most complete of the AMA1 structures, with ~98% of the ectodomain modeled (Crawford et al., 2010) and the domain packing is shown in Figure 2A. Domain I is at the distal end of the protein with respect to the assumed orientation on the parasite membrane. Domain I packs alongside Domain II, which contains a loop that extends down to the distal end of the protein and occludes a portion of the receptor binding pocket. Domain III is membrane proximal and contains a stable cysteine knot comprised of 3 disulfide bonds.

2.1.2 Unique parasite derived, host cell receptor, rhoptry neck proteins (RONs)—AMA1 is a unique receptor because it does not bind to a host cell derived membrane receptor. Proteins secreted from the rhoptries, known as the rhoptry neck proteins (RONs) (Bradley et al., 2005), localize to the moving junction, and form a complex of RON2/4/5 that is inserted into the host cell, to which AMA1 then binds (Alexander et al., 2005; Lebrun et al., 2005). In *T. gondii*, RON8 is a key component of the complex (Besteiro et al., 2009), but is not required in *Plasmodium spp*. invasion. It was further shown that AMA1 binds only to RON2 directly (Cao et al., 2009). The RON2/4/5/8 complex is targeted to the host cell membrane, with RON4/5/8 exposed to the host cell cytoplasm, and RON2, predicted to contain at least one transmembrane helix and a large ectodomain, providing a receptor for AMA1 to bind to on the extracellular side (Besteiro et al., 2009) (Figure 2C).

To further investigate the RON2-AMA1 interaction and RON2 topology in the membrane, specific antisera were developed against different regions of RON2 (Lamarque et al., 2011). Experiments using the antisera to detect regions of RON2 exposed to either the host cell cytoplasm or extracellular environment, showed that RON2 is inserted in the host cell membrane as an integral membrane protein, with a cytoplasmic N-terminal domain followed by a transmembrane domain and ectodomain. It remains unclear whether the well-conserved C-terminal domain is exposed to the extracellular space or is located in the cytoplasm, based on these studies. Peptides corresponding to the predicted RON2 ectodomain were generated and co-crystallized with AMA1 (Tonkin et al., 2011; Vulliez-Le Normand et al., 2012). Both the *P. falciparum* and *T. gondii* AMA1 structures in complex with their respective RON2 peptides show that the peptide adopts a U-conformation, with an N-terminal helix and a cysteine loop, binding to a hydrophobic groove after displacement of the Domain II loop (Figures 2B, 2E).

2.1.3 sporoAMA1 and sporoRON2—Recently, sporozoite-specific, novel *T. gondii* paralogs of AMA1 and RON2 were identified and designated sporoAMA1 and sporoRON2 (Poukchanski et al., 2013). *Neospora* and *Eimeria* also have homologs of sporoAMA1 and sporoRON2 in addition to the generic AMA1 and RON2, while *Plasmodium, Babesia*, and *Theileria* only have homologs related to the generic forms of AMA1 and RON2. There is no detectable cross-interaction between the generic AMA1 and sporoRON2 and *vice versa*, due to different specific interactions in the hydrophobic groove, especially in the cysteine loop region (Poukchanski et al., 2013). In the crystal structure of sporoAMA1 bound to sporoAMA1, the position of Domain III is altered upon binding of RON2 in comparison to the apo structure (Figure 2D). In the apo AMA1 structures, as well as the generic Pf/Pv/TgAMA1-RON2 structures, Domain III lays along the base of Domains I and II (Figure 2C). In the sporoAMA1-sporoRON2 structure, Domain III rotates more than 90°, extending away from Domains I and II (Figure 2D). Whether this is physiologically relevant or can trigger a signaling event is still an open question. This movement, as previously mentioned, is not observed in the binding of RON2 peptides in the generic Pf/Pv/TgAMA1-RON2 structures. Additionally, Domain III in the sporoAMA1-sporoRON2 crystal structure is making a crystal contact with Domain III of a symmetry mate, like shaking hands, so it may be nothing more than a crystallization artifact.

2.1.4 Inhibition of the AMA1-RON complex—Recognition of this binding motif and further co-crystallization of PfAMA1 with antibodies shed light on their inhibitory mode of action. An IgNAR single-domain antibody was mutated to select for AMA1 from three *P. falciparum* strains (Henderson et al., 2007). It was shown in a crystal structure that the CD3 loops bind the hydrophobic groove where the RON2 peptide sits, and two β-strands of the IgNAR align with the two strands of RON2 stabilized by the cysteine loop (Figure 2F). Another growth and invasion inhibitory fragment antibody, 1F9, binds to the same end of the hydrophobic groove where the cysteine loop of the RON2 peptide binds (Figure 2G) (Coley et al., 2007). The binding of this antibody can be competed against using serum from individuals exposed to *P. falciparum*, indicating that acquired antibodies target the same epitope as 1F9. While these antibodies show growth inhibitory effects against laboratory strains, sequencing of lab and field strains has generated hundreds of non-redundant AMA1 sequences, with most polymorphisms concentrated in Domain I (Cortes et al., 2003; Escalante et al., 2001); this variability could pose a problem for vaccine development against this antigen.

A small molecule inhibitor of the AMA1-RON2 interaction in *P. falciparum*, was determined using a high throughput screen of a 21,000 compound library (Srinivasan et al., 2013). Addition of the compound reduced tight junction formation and reduced invasion levels. The compound was also shown to be effective against multiple lab strains, including the DD2 drug resistant strain, with an IC50 of 10–14 μM. The ultimate effectiveness of antibodies and compounds may rely on how essential the AMA1-RON2 interaction is to the parasite, which has been called into question, as is discussed later in section 4.2.

2.2 Reticulocyte binding-like homolog (RBL/RH) protein family

The reticulocyte binding-like protein family is specific to *Plasmodium spp*., and is composed of receptors needed for direct interaction with the reticulocyte and erythrocyte surface receptors. There are several subfamilies of reticulocyte binding-like proteins across *Plasmodium spp*. Most proteins in these families contain a signal sequence, N-terminal ectodomain, comprised of a nucleotide binding domain (Ramalingam et al., 2008) and erythrocyte binding domain (Gao et al., 2008; Gruber et al., 2011; Sahar et al., 2011), a transmembrane helix, and cytoplasmic C-terminal tail (Figure 1B).

2.2.1 Reticulocyte binding-like proteins (RBPs)—There has been little reported regarding the structure and function of the reticulocyte binding-like proteins (RBPs) from *P. vivax*, PvRBP1 and PvRBP2 (Galinski et al., 1992) or the *P. yoelii* homolog Py235 (Holder et al., 1991). PvRBP receptors have yet to be identified, in part because it is not possible to culture blood stages of *P. vivax*; however, PvRBP1 peptides that bind to reticulocytes, have been identified (Cantor et al., 2001; Urquiza et al., 2002). A short review by Li and Han (2012) describes the RBPs in further detail.

2.2.2 Reticulocyte binding-like homolog proteins (RH)—In *P. falciparum*, five variants of reticulocyte binding-like homolog proteins (RH) are involved in the invasion process: RH1, RH2a, RH2b, RH4, and RH5 (Kaneko et al., 2002; Rayner et al., 2000; Rayner et al., 2001; Triglia et al., 2001). Tham *et al*. (2012) review the function of *P. falciparum* RH proteins, and the potential of raising antibodies against these antigens to inhibit invasion. RH proteins 1–4 possess erythrocyte binding domains (EBD), nucleotide binding domains (NBD), and a C-terminal transmembrane helix and cytoplasmic tail.

Receptors for RH1, 2a and 2b have yet to be identified. The RH4 receptor interacts in a sialic acid-independent manner with complement receptor 1 (CR1/CD35) (Tham et al., 2010). Biochemical and biophysical analysis has narrowed down the RH4 interaction site to the N-terminal, complement control protein modules 1–3 (CCP 1–3) of CR1, which is the most membrane-distal CCP region (Park et al., 2014). Furthermore, all residues necessary to bind RH4 are contained solely in CCP-1.

Diverging from the typical architecture of RH proteins, is RH5, which lacks both an identified NBD and a transmembrane helix. To form a complex joining the parasite with the red blood cell, RH5 interacts with RIPR (RH5 interacting protein) (Chen et al., 2011), which also lacks a transmembrane domain. It is assumed that there is another binding partner with a transmembrane domain with which RIPR or RH5 forms a complex, anchoring RH5 to the parasite. RH5 interacts with basigin (CD147/EMMPRIN) (Crosnier et al., 2011) and a crystal structure of RH5 in complex with basigin has been determined (Wright et al., 2014a).

RH5 consists of two bundles of three α-helices each (Figure 3A). In the basigin-RH5 crystal structure, the asymmetric unit contains a 2:2 stoichiometry of basigin and RH5, forming a tetramer consisting of two hetero-dimers. One dimer of basigin-RH5 is formed by the interaction of one tip of the RH5 helix bundle with Regions II and III of basigin (Figure 3B). The tetramer is then formed by the other helical bundle tip interacting with Regions II and III of the other basigin molecule, on the opposite side of the second RH5 molecule (Figure

3C). There is no direct interaction between the two RH5 molecules. Region III of the basigin molecules, which are the most membrane-proximal, stabilize the dimer. This complex leaves an accessible surface for RIPR binding to RH5. SAXS (small angle X-ray scattering) and analytical ultracentrifugation (AUC) data indicate that the basigin-RH5 complex has a 1:1 stoichiometry (Wright et al., 2014a); however, the local effective concentrations at the membrane surface may make the dimer of dimers observed in the crystal structure physiologically possible, and one can speculate that this dimerization event could lead to a signaling event.

RH5 was also crystallized in the presence of two growth inhibitory Fabs that act through different mechanisms (Wright et al., 2014a). Fab 9AD4 is potent (Douglas et al., 2014), however, it does not block binding of basigin *in vitro*. In the co-crystal structures it is observed that the antibody does not bind to the tip of RH5's helical bundle, which would thereby prevent basigin binding, but instead binds to another interface (Figure 3D). This binding may present access problems for RH5 to interact with basigin due to steric hindrance at the membrane or it may interfere with RIPR binding to RH5. Monoclonal QA1, however, does disrupt basigin binding, and this effect is due to QA1 binding at the tip of the RH5 helical bundle (Figure 3E), blocking the basigin interaction, as observed *in vitro*.

2.3 Duffy binding-like proteins (DBP) and the erythrocyte binding-like (EBL) proteins

Another family of proteins involved in erythrocyte and reticulocyte invasion by *Plasmodium spp*. is the Duffy binding-like family, which consists of the Duffy-binding-like proteins (DBPs) and erythrocyte binding-like proteins (EBLs). DBP and EBL proteins contain a signal sequence, N-terminal ectodomain, a transmembrane helix, and a short C-terminal tail. The ectodomain is divided into 6 regions based on sequence homology between the DBPs and EBLs. Region II is cysteine rich and contains the Duffy binding-like (DBL) domains, responsible for binding to red blood cell receptors, followed by Regions III–V and Region VI, a second conserved cysteine rich domain (Adams et al., 1992) (Figure 1C). A review discussing the discovery and role of DBLs/EBLs is provided by Iyer *et al*. (2007) and the proteins are further explored by Li and Han (2012).

P. falciparum has multiple receptors for erythrocyte invasion including a subfamily of DBLs known as the erythrocyte binding-like (EBL) proteins, or erythrocyte binding antigens (EBA). This family consists of several proteins implicated in invasion including EBA-140 (Thompson et al., 2001), EBA-175 (Adams et al., 1992) and EBA-181 (Gilberger et al., 2003). The EBLs bind in a sialic acid-dependent manner to the erythrocyte. While the receptor for EBA-181 is unknown, EBA-175 binds to Glycophorin A (Orlandi et al., 1992) and EBA-140 binds Glycophorin C (Lobo et al., 2003).

2.3.1 EBA-175 (BAEBL)—Region II of EBA-175 was crystallized in the presence and absence of sialyllactose as a dimer (Tolia et al., 2005). Region II consists of two DBL domains, F1 and F2, each composed primarily of α-helices and a β-hairpin, and the domains are linked by three α-helices (Figure 4A). The two monomers interact along their entire extended length forming a "handshake", with the F1 domain of one monomer interacting with the F2 domain of the other and *vice versa* (Figure 4B). The dimer observed in the

crystal is consistent with *in vitro* data where dynamic light scattering (DLS) indicates a dimer at high concentrations; however, AUC experiments at lower concentrations indicate that EBA-175 is primarily monomeric. It is tempting to speculate that on the membrane, the higher effective concentrations of EBA-175 promote dimerization with help from the obligate dimer of glycophorin A recruiting two monomers of EBA-175 and stabilizing the interaction, which could lead to a signaling event.

While EBA-175 was crystallized in the presence of sialyllactose, poor density meant that the authors could not model the glycans (Tolia et al., 2005). Even though the glycans could not be unambiguously modeled, it was observed that all the binding sites lie along the dimer interface and that the glycan binding involved making contacts with both monomers, indicating that dimerization is likely necessary for glycan/receptor engagement. An inhibitory Fab, R217 (Chen et al., 2013), contacts proposed glycan binding residues as well as the F2 β-finger (Figure 4C). This structural information allows one to rationalize the method of inhibition in which the antibody prevents binding to glycophorin A by two mechanisms. The antibody blocks the glycan binding site directly. Second, the antibody prevents dimerization of EBA-175 by interacting with the F2 β-finger, which promotes dimerization by inserting into a cavity on F1 of the other monomer. By blocking dimerization, glycan binding would also be affected, as the glycan sites are formed between dimers.

2.3.2 EBA-140—A structure of the binding Region II of EBA-140 in the presence and absence of sialyllactose was determined (Lin et al., 2012; Malpede et al., 2013). Unlike the dimer form observed for EBA-175, EBA-140 in the presence and absence of sialyllactose crystalizes as a monomer in the asymmetric unit, consistent with SAXS data indicating that it is a monomer in solution. The two DBL domains, composed of α-helices, are connected *via* a short helical linker (Figure 4D). The EBA-140-sialyllactose co-crystal structure determined two unique glycan-binding pockets, one in each of the DBL domains, F1 and F2 (Malpede et al., 2013). Mutations of sialic acid binding residues in F1 led to a severely compromised binding to erythrocytes while similar mutations in F2 led to only a 10% binding reduction. Other mutations in F2 resulted in more severe binding defects, but not to the same level as mutations of F1. The authors concluded that while the F2 binding site is functional, that it may not be essential for invasion. Additionally, only four polymorphisms have been reported in EBA-140, and these differences all localize to the F1 domain (Maier et al., 2009) with one found in the glycan binding pocket (Malpede et al., 2013).

2.3.3 Duffy binding-like proteins (DBPs)—*P. vivax* which primarily invades reticulocytes, and *P. knowlesi*, which invades normocytes, express PvDBP1/2 (Wertheimer and Barnwell, 1989) and multiple PkDBPs (Haynes et al., 1988), respectively. The proteins share 70% homology and a conserved DBL domain. Recently a structure was published of PvDBP with a peptide from its host cell receptor, the Duffy antigen/chemokine receptor (DARC) (Batchelor et al., 2014; Wertheimer and Barnwell, 1989). PvDBP consists of two α-helical bundles and a β-hairpin near the N-terminus (Batchelor et al., 2011) (Figure 4E). A dimer of PvDBP with one DARC peptide bound forming a heterotrimer, and a heterotetramer of 2 PvDBPs and 2 DARC peptides (Figure 4F), were characterized. The

DARC peptide binds at the edge of the PvDBP dimer interface. Authors proposed that the multiple states observed in the crystal structures were representative of physiologically relevant states. SAXS experiments indicate that PvDBP is a monomer at low concentrations in the absence of ligand/DARC peptide, whereas at higher PvDBP concentrations, data suggests that there is a mixture of monomers and dimers present (Batchelor et al., 2011). Additionally, addition of DARC peptide drove PvDBP dimerization at both low and high concentrations. Using ITC (isothermal titration calorimetry), it was confirmed that the PvDBP dimer first binds one DARC peptide and then a second peptide, in agreement with the two crystal states observed (Batchelor et al., 2014).

2.4 Thrombospondin related anonymous protein (TRAP) family and MIC2 protein

The last family of receptors discussed here are the thrombospondin related anonymous proteins (TRAPs) (Robson et al., 1988). The TRAP protein is a pan-apicomplexan protein including *Plasmodium spp*. TRAP, *T. gondii* MIC2 (microneme-associated protein 2) (Brossier and David Sibley, 2005; Wan et al., 1997), *Cryptosporidium spp*. TRAPC1 (Spano et al., 1998a; Spano et al., 1998b), *Eimeria spp*. Etp100 (Tomley et al., 1991), and *Babesia spp*. TRAP (Gaffar et al., 2004). *Plasmodium spp*. have multiple TRAP variants necessary for motility and invasion at different stages (Sultan et al., 1997), including TRAP for liver invasion (Muller et al., 1993), CTRP for mosquito midgut invasion (Trottein et al., 1995), MTRAP for erythrocyte invasion (Baum et al., 2006), and TLP (TRAP-like protein) for gliding in the skin (Moreira et al., 2008). The typical domain organization is a signal sequence followed by an N-terminal ectodomain, containing at least one thrombospondin repeat type 1 (TSR) domain and a von Willebrand factor type-A (VWA) motif, a transmembrane helix, and a C-terminal tail (Figure 1D).

2.4.1 TSR and VWA domains and structures—Structures of PfTRAP, PvTRAP and TgMIC2 have been characterized and have provided insight into the mechanisms of TRAP function. In looking at the numerous structures of the VWA and TSR domains, it was observed that TRAP exists in two states, either an open or closed conformation, which is dependent on metal binding to a MIDAS motif (metal ion dependent adhesion sites) (Song and Springer, 2014; Song et al., 2012). Other VWA domains, specifically those in integrin I domains, also contain MIDAS motifs and can adopt open and closed conformations based on magnesium and ligand binding.

A PfTRAP construct containing the VWA and TSR domain was crystallized (Song et al., 2012), however, only the VWA domain could be modeled, even though it was confirmed *via* SDS-PAGE that the crystals contained the full-length construct. The VWA adopted an α/β Rossmann fold (Figure 5A). A void in the crystal packing at the C-terminus of the VWA domain was large enough to accommodate multiple conformations of the TSR domain, which was apparently disordered given the lack of electron density.

PfTRAP adopted a closed state conformation in the absence of magnesium and was structurally different, compared to the open state of PvTRAP with magnesium bound (Figure5A and 5B) (Song et al., 2012). In the closed, apo structure (Figure 5A), the TSR domain was disordered; however, in the open metal bound state, an extended β-ribbon is

formed and the TSR domain becomes ordered. An extended β-ribbon is formed between the N-terminal residues before the VWA domain start and residues that unwind from the last α-helix in the VWA domain (Figure 5B). This movement and extension can be seen in the positioning of the disulfide bond between C39 and C231 in the PvTRAP open state versus the position of the disulfide bond between C43 and C235 in the PfTRAP closed state (Figure 5C). Three disulfide bonds between C39 and C321, C240 and C261, and C253 and C289, stabilize the structure of the ordered TSR domain in the PvTRAP open state.

The homolog of PfTRAP, TgMIC2, was also crystallized with its VWA and TSR domains modeled (Song and Springer, 2014). The protein was modeled in the closed state, as can be seen by the positioning of the previously noted disulfide bond, which has not extended away from the VWA domain, preventing MIC2 from adopting an extended structure similar to that of the PvTRAP (Figure 5D). While the three disulfide bonds present in MIC2 correspond to those in the extended β-strand and TSR domains in the plasmodial proteins, there is an additional loop stabilized by a disulfide bond that may extend away from the VWA domain in the open state (Figure 5D). A magnesium bound, open state of TgMIC2 was not crystallized, but it is thought that binding to the MIDAS motif would cause an unwinding of the VWA helix and extension of the β-strand and TSR domain to adopt a conformation similar to that of PvTRAP.

It has been proposed by the authors that binding of a ligand/receptor to the VWA domain induces a conformational change, causing the extension of the TSR domain, creating a stable arm through which force generated by the actomyosin motor of the invasion machinery is transmitted to the host cell surface (Figure 5E) (Juliana et al., 2012). One caveat to this model is that a structure of PfTRAP with a magnesium bound to the MIDAS motif was crystallized in the closed state, indicating that this open/closed mechanism may not be conserved between PfTRAP and PvTRAP (Pihlajamaa et al., 2013). However, the construct used in crystallization did not contain the TSR domain and lacked the N-terminal residues, which form the β-strand preceding the VWA domain and forms part of the extended β-ribbon observed in the PvTRAP structure.

2.4.2 TRAP receptors—Receptors for the TRAP proteins have yet to be identified, except for PfMTRAP. The Semaphorin-7a (CD108) receptor was identified and characterization of the interaction between MTRAP and Semaphorin-7a ectodomains was performed *via* size exclusion chromatography and surface plasmon resonance (Bartholdson et al., 2012). Semaphorin-7a is a homodimer in solution according to SEC (size exclusion chromatography) and MALS (multi-angle light scattering), and MTRAP elutes as a monomer in SEC. Experiments indicate that the binding of MTRAP to Semaphorin-7a occurs in a 2:1 stoichiometry and that MTRAP interacts *via* its TSR domain, as it lacks the VWA domains that other family members possess.

3. The Invasion Machinery (Glideosome)

For motility and invasion, apicomplexan parasites employ an actomyosin based motor that is part of a larger invasion machinery complex known as the glideosome. Multiple interacting proteins are used to bridge and transmit the locomotive force of the motor to the parasite's

attachment point at the host cell surface and to anchor the motor within the parasite. Most of these proteins are conserved across apicomplexa. The proteins have been sorted into three groups: motor-receptor bridging proteins, motor complex proteins, and inner membrane complex (IMC) proteins and anchors. A list of the machinery components, accessory proteins and anchors, their known interaction partners with references, EuPathDB codes, and any structures that have been deposited with the PDB, are noted in Table 2. A schematic of the glideosome can be found in Figure 9A.

3.1 Motor-receptor bridging proteins

As has been discussed previously, apicomplexans use a wide range of extracellular receptors to attach to a host cell membrane for invasion. In order to transmit the force of the cytoplasmic actomyosin motor to the extracellular attachments, bridging proteins are required. In apicomplexans, two bridging proteins have been identified: aldolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In the *Plasmodium spp*. genomes there are single copies of both aldolase and GAPDH, while in *T. gondii*, there are annotations of the genome indicating two copies of each enzyme.

3.1.1 Aldolase—A key bridging protein is the enzyme, fructose 1,6 bisphosphate aldolase. In addition to catalyzing a reaction in the glycolytic pathway, this protein has a second non-enzymatic function; the aldolase tetramer interacts with actin on one face (Buscaglia et al., 2003), and the C-terminal tails of multiple receptor proteins *via* its active site. In *Plasmodium spp*., aldolase interacts with multiple receptors responsible for invasion: the TRAPs, AMA1, and RH1/2b/4, and in *T. gondii*, AMA1 and MIC2/6/12 (Baum et al., 2006; Bosch et al., 2007b; Boucher and Bosch, 2013; Buscaglia et al., 2003; Heiss et al., 2008; Jewett and Sibley, 2003; Pal-Bhowmick et al., 2012; Sheiner et al., 2010; Srinivasan et al., 2011). Alignment of the receptor tails (Figure 6A), mutational studies, and a PfAldolase-PbTRAP crystal structure (Bosch et al., 2007b) indicate that a conserved tryptophan residue is key to the binding (Kappe et al., 1999). A C-terminal PbTRAP peptide (EDNDWN) was co-crystallized with aldolase, with the last three residues modeled in the aldolase active site (Bosch et al., 2007b). To accommodate the peptide, there is a helix shift and R48 and R309 move to sandwich and stack with the key tryptophan (W605), which makes and additional hydrogen bond contact with E40 (Figure 6C). The structure of *T. gondii* aldolase 1 has been determined (Boucher and Bosch, 2014; Tonkin et al., 2014b). It was noted that the sequence identity in the active site/peptide binding site was 100% conserved and surface plasmon resonance experiments indicate that TgAldolase is competent to bind *P. falciparum* TRAP tails (data not shown).

In addition to aldolase-TRAP co-crystal structures, the ternary co-crystal structure, with a small molecule stabilizing the TRAP-peptide in the aldolase active site, has been deposited and will be described elsewhere (PDB: 4TR9, PCT/US2012/061875) (Cardozo et al., 2013). Briefly, when tested in parasite cultures, the stabilizing compound results in a gliding and invasion phenotype that is in agreement with inhibition of the glideosome at the aldolase-TRAP interface.

3.1.2 GAPDH—A second enzyme used as a connection between actin and the receptors is GAPDH. While the tetrameric structure of PfGAPDH (Robien et al., 2006; Satchell et al., 2005) and dimer of TgGAPDH (SSGCID et al., 2011) have been deposited, to date no co-crystals of GAPDH and a peptide have been structurally characterized. It has been demonstrated *via* biochemical and biophysical assays that GAPDH can bind the C-terminal peptide tails (Figure 6B) of RH1, RH4, EBA-175 and EBA-181 (Pal-Bhowmick et al., 2012).

3.1.3 Unknown bridging molecules—While interactions between aldolase/GAPDH and the C-terminal tails of multiple receptors has been demonstrated, there are some receptors that do not interact with these bridging partners. EBA-140, RH2a, RH5/RIPR, Pv/PkDBP, and PvRBPs have not been shown to interact with either aldolase or GAPDH. Lack of an apparent interaction partner may indicate that there is either an undiscovered bridging molecule or that the proteins are able to interact with the motor in the absence of a bridging molecule. Another possibility is that the primary function of the receptor is for attachment, and not motility, and therefore, it does not interact with the motor.

3.2 Motor-complex proteins

The locomotive force for invasion is generated by the actomyosin motor. Forward movement of the parasite is generated by myosin pulling back on short actin polymers, to which the extracellular receptors are bridged *via* aldolase, GAPDH, or yet to be identified components.

3.2.1 Actin—Actin forms the linear track of the glideosome motor. Typically, actin polymerizes in a head-to-tail fashion forming helical, parallel strands that are highly stable and range from several hundred nanometers to microns in length. However, both *Plasmodium spp*. and *T. gondii* form unstable actin polymers around 100 nm in length (Sahoo et al., 2006; Schmitz et al., 2005; Schuler et al., 2005b; Vahokoski et al., 2014). *Plasmodium spp*. express two variants of actin, actin 1 and actin 2 (Wesseling et al., 1988), whereas most apicomplexans, including *T. gondii*, express only one (Dobrowolski et al., 1997). Actin 2 forms filaments similar to canonical F-actin (Figure7B and 7C), whereas actin 1 is responsible for the short, fragile filaments observed in apicomplexans (Figure 7A) (Vahokoski et al., 2014). The dynamics of apicomplexan actins are unique. *Plasmodium spp*. actins 1 and 2 can hydrolyze ATP more efficiently and are able to oligomerize in response to ADP (Vahokoski et al., 2014), while TgActin 1 can form filaments at critical concentration 3–4 times lower than F-actin (Sahoo et al., 2006). Observations that TgActin 1 could polymerize at lower critical concentrations as well as without a lag phase, is the result of isodesmic polymerization, where the initial nucleation and polymerization steps have the same rate constants (Skillman et al., 2011).

Why do these actins not behave like canonical actins? Structures of plasmodial actins 1 and 2 have been determined, shedding some light on this question (Vahokoski et al., 2014). Structural alignment of actin 1, responsible for the short filaments and for forming the linear track of the glideosome, with a canonical actin shows that the overall structures do not differ radically (Figure 7D). In general, structural rearrangements of the plasmodial actins are

found in regions responsible for contacts between monomers in a filament, as well as known interfaces for regulatory protein binding. It was demonstrated that substitution of the DNase I (D-)loop from canonical F-actin into actin 1 could “rescue” polymerization and induce formation of longer filaments, similar to those formed by actin 2 (Vahokoski et al., 2014). However, the sequences of the D-loops of actin 1 and actin 2 are not very different, and yet actin 2 is able to form longer filaments, indicating that actin 2 has managed to develop a mechanism for more robust polymerization through other means. Additionally, the Y54F substitution at the base of the D-loop in actin 1, which differentiates it from actin 2 and canonical actins, could explain the lower levels of polymerization; however, mutation of this residue to tyrosine did not rescue polymerization.

3.2.2 Actin accessory proteins—In general, organisms have evolved a set of actin accessory proteins that aid in the nucleation of actin, stimulate polymerization, stabilize filaments, cap filaments, and sever filaments. Apicomplexans have a limited subset of the actin accessory proteins that are present in higher eukaryotes (Schuler and Matuschewski, 2006). For a detailed structure/function review of these actin binding proteins can be found in the review by Olshina *et al*. (2012). While these proteins are not discussed here, but have been listed in Table 2.

3.2.3 Myosin A—Myosin A (MyoA) pulls on the actin filament to move the parasite forward by generating mechanochemical force through the hydrolysis of ATP. TgMyoA and *Plasmodium spp*. MyoA are part of the Class XIV myosin family. This class of myosins possesses a head domain, but lack a neck composed of IQ motifs, and a tail. Apicomplexan MyoA diverges from this architecture as it does possess a tail domain (Heintzelman and Schwartzman, 1997; Heintzelman and Schwartzman, 1999; Pinder et al., 2000; Pinder et al., 1998). PfMyoA does not contain a canonical IQ-motif (IQxxxRGxxxR) present in other myosins, but does have a modified motif VQxxxRKxxx(A/V). It has been shown that TgMyoA moves at speeds of ~5 μm/s *in vitro*, despite lacking features of typical myosins such as a conserved glycine in the motor domain, a conserved residue in an actin binding loop, and possession of a degenerate IQ-motif.

Unfortunately, structures of the MyoA motor domain do not exist due to difficulty in expression, but recently the successful expression of TgMyoA in *Sf9* insect cells was accomplished *via* co-expression with TgMLC1 (myosin light chain 1), TgELC1 (essential light chain 1), and a chaperone, TgUNC, a myosin-specific co-chaperone from the UCS family, Unc45b (Bookwalter et al., 2014). This strategy will hopefully enable structural characterization of the protein in the future.

3.2.4 MTIP/MLC1—To anchor the actomyosin motor to the inner membrane complex, *T. gondii* encodes for the myosin light chain (MLC) protein (Herm-Gotz et al., 2002) and *Plasmodium spp*. express the ortholog, myosin-A tail interacting protein (MTIP) (Bergman et al., 2003). These proteins are distantly related to the myosin light chain proteins, but lack the calcium binding motif common to the family. Unique to the apicomplexan interaction of MyoA with MTIP/TgMLC1 is that the interaction is mediated *via* the MyoA tail containing a single degenerate IQ motif, instead of by a neck domain containing multiple, canonical IQ motifs (Bergman et al., 2003; Herm-Gotz et al., 2002). An N-terminal deletion mutant,

PkMTIPΔ78, was first crystallized at a low, non-physiological pH with a helical MyoA peptide containing the modified IQ motif (Bosch et al., 2006) (Figure 7E). The PkMTIPΔ78 structure possesses a central helix that connects the N- and C-terminal domains, forming one helix in each domain. The N-terminal domain of the truncated construct consists of four helices, forming two degenerate EF hands (no Ca^{2+} binding was observed), while the C-terminal domain is composed of four helices, forming two degenerate EF hands, as well as a small anti-parallel β-sheet. The MyoA peptide binds to the C-terminal domain *via* residues extending into hydrophobic pockets and *via* complementary electrostatic interactions.

A later structure of PfMTIPΔ60-MyoA was crystallized at physiological pH and a different conformation was observed (Bosch et al., 2007a). The long, continuous central helix previously observed at low pH is kinked at physiological pH, resulting in a closed MTIP structure, where the N- and C-terminal domains latch *via* an electrostatic interaction. This state enables a tight interaction of the MyoA peptide with both the C- and N-terminal domains and hinge region. This kink, or compacted structure, is facilitated by the deprotonation of MyoA residue H810, allowing hydrogen bonding interactions with an aspartate in the MTIP hinge and the MyoA lysine (K813) at the canonical glycine position of the IQ motif. This closed conformation is believed to be the functionally competent state.

Phosphorylation of MTIP was thought to play a role in regulation of the MTIP-MyoA interaction when studies determined that MTIP could be phosphorylated *in vitro via* CDPK1, and a phospho-proteomics study identified a phosphorylated S108 (Green et al., 2008; Treeck et al., 2011). To test whether phosphorylation affected MyoA binding, the MTIP S108E mutation was made. The S108E mutation affected the stability of the MyoA-MTIP complex as measured *via* ITC and DSF (differential scanning fluorimetry) (Douse et al., 2012). In the crystal structure it is seen that S108 of the N-terminal domain forms a latch with D173 of the C-terminal domain (Figure 7E). Phosphorylation of this residue would alter the charge state and disrupt the latch, which prevents formation of the closed state. This result indicates that phosphorylation may be regulating motor assembly.

As the MTIP-MyoA interaction is necessary to anchor the motor to the IMC, some work has been done to identify small molecules that disrupt the protein-protein interaction. Using the crystal structure of the MyoA-MTIPΔ60 complex, a hybrid virtual screening approach was implemented, first building a template pharmacophore based on key interactions of MyoA with MTIP, and then screening a large library for compounds matching the pharmacophore (Kortagere et al., 2010). Identified molecules were then docked against the crystal structure and several compounds tested for growth inhibition of *P. falciparum* cultures exhibited IC_{50} < 25 μM, leading to the identification of a pyrazole-urea compound that led to decreased growth and altered gliding phenotypes. However, proof of a direct interaction of the compound with MTIP was not established.

A more recent study used a fragment-based approach to screen molecules against the protein-protein interaction using DSF to screen an initial library, followed by cross-validation with ITC and NMR (Douse et al., 2014). Unfortunately, crystal structures of the compounds complexed with MTIP alone were not able to be obtained, likely due to the low binding affinities (K_D ~ mM) typical of fragments. To overcome the low binding affinity, a

MTIP-stabilizing fragment, which was demonstrated to interact with residues implicated in MyoA binding, was linked to the N-terminus of a truncated MyoA peptide to form a chimera. A crystal structure of MTIP in complex with the chimera was solved providing further insight into how the compound interacts with MTIP (Figure 7F). This structure may allow for rational expansion of the fragment to target other regions and residues of MTIP and development of a tighter binding compound.

3.2.5 ELC1—Calcium signaling has been demonstrated to be important for egress and invasion in apicomplexans, with CDPK1 phosphorylating multiple glideosome proteins (Green et al., 2008). Myosin activity is often regulated by calcium through calcium-dependent phosphorylation or calcium binding to EF hands of myosin regulatory elements (Himmel et al., 2009). As was previously mentioned, MTIP contains degenerate EF hands, and does not bind calcium. In the course of a phospho-proteomics study to identify calcium-dependent phosphorylation targets in *T. gondii* during invasion, a previously uncharacterized, putative calmodulin-like protein was identified (Nebl et al., 2011). Sequence searches revealed that the identified protein was most similar to the essential light chain of scallop myosin II. This protein was therefore termed the essential light chain 1 (ELC1). It was demonstrated that ELC1 co-purified with the MyoAMLC1-GAP40/45/50 glideosome and more strongly associated with the complex during calcium signaling. This light chain element may be responsible for direct calcium regulation of the myosin, as opposed to MLC1 phosphorylation by CDPK1.

3.3 Inner membrane complex (IMC)

The actomyosin motor is attached to the inner membrane complex (IMC) comprised of alveoli. These alveoli underlay the entirety of the parasite's plasma membrane, except at the base and apex, and are connected to the parasite's cytoskeleton. Several proteins connect the actomyosin motor to the IMC and are involved in attachment to the cytoskeleton. In this section, the structural features of the glideosome associated proteins (GAP), glideosome associated proteins with multiple membrane spans (GAPM), IMC sub-compartment proteins (ISPs), and the anchoring alveolins are discussed. A table with predicted and experimentally derived post-translational modifications that may affect assembly, localization, and the function of the motor, are listed in Table 3. An in depth review of the apicomplexan IMC architecture and involved proteins, has been published by Kono *et al*. (2013).

3.3.1 Glideosome associated proteins (GAP)—Three glideosome associated proteins (GAPs) are conserved between *T. gondii* and *Plasmodium spp*. (Frenal et al., 2010; Gaskins et al., 2004; Rees-Channer et al., 2006) and are notated by their apparent molecular weight, as determined by SDS-PAGE, as GAP40, GAP45, and GAP50. It has been demonstrated that GAP40, GAP45 and GAP50 co-localize with the MyoA-MTIP/MLC1 complex (Gaskins et al., 2004).

GAP45, MyoA, and MTIP/MLC1 form a precomplex mediated by the N-terminal domain of MTIP/MLC1 interacting with the C-terminus of the peripheral membrane protein GAP45 (Gaskins et al., 2004; Johnson et al., 2007; Rees-Channer et al., 2006). The structure of GAP45 is unknown, however, it is predicted that GAP45 consists primarily of a coiled-coil

domain. At the N-terminus, GAP45 is myristoylated and palmitoylated (Rees-Channer et al., 2006; Wright et al., 2014b). This acylation targets GAP45 preferentially to the plasma membrane and serves to prevent GAP45 from associating with the nascent IMCs (Frenal et al., 2010). GAP45 has additional predicted palmitoylation sites at its C-terminus, and this modification is expected to facilitate GAP45 localization to the IMC, allowing the MyoA-MTIP/MLC1 complex to also associate with the IMC (Rees-Channer et al., 2006). Phosphorylation is the final step that controls association of the complex with the IMC (Gilk et al., 2009; Ridzuan et al., 2012). Phosphorylation of residues in the C-terminal domain of GAP45, controls association with the IMC. In addition to GAP45, two related proteins, present only in coccidians (e.g. *T. gondii* and *Cryptosporidium spp*.), GAP70 (Frenal et al., 2010) and GAP80 (Frenal et al., 2014b), have been identified and have been shown to interact with MyoA-MLC1 and MyoC-MLC1, respectively, in a manner similar to the interaction of GAP45 and MyoA-MLC1.

There still remains a question whether or not GAP45 is contacting both the IMC and plasma membrane simultaneously, spanning the supra-alveolar space. Measurements of the distance between the plasma membrane and IMC *via* cryo-EM, have ranged from 23–30 nm in *P. berghei* sporozoites (Kudryashev et al., 2010). An I-TASSER (Roy et al., 2010) homology model of PfGAP45 predicts a coiled-coil with a 180° bend in the middle (Figure 8A). This structure is approximately 15 nm in length when folded, and if extended, could potentially span the supraalveolar space to make contact with both membranes simultaneously.

GAP45 also interacts with GAP50, a protein bound to the IMC (Gaskins et al., 2004). As previously mentioned, GAP50 contains a signal sequence for targeted secretion to the IMC, as well as a C-terminal transmembrane helix for insertion into the IMC vesicle. The likely orientation of GAP50 is with the N-terminal domain located within the lumen of the IMC (Johnson et al., 2007). The structure of GAP50 (Bosch et al., 2012) is most closely related to the human purple acid phosphatase; however, the recombinant protein shows no protein phosphatase activity *in vitro*, but does have activity against small molecules such as ATP and IPP (isopentenyl pyrophosphate), with a preference for di- and tri-phosphates (Müller et al., 2010). Due to the conserved nature of the protein and the lack of direct interaction other than *via* co-immunoprecipitation/ purification (co-IP) of GAP45 and GAP50, it is possible that there are other binding partners that are yet to be discovered.

The last member of the GAP family to be discussed is GAP40, a polytopic membrane protein (Frenal et al., 2010). GAP40 co-purifies with the MyoA-MTIP/MLC1-GAP45/50 complex. GAP40 is comprised of nine transmembrane spanning domains, and could act as an anchor of the motor to the IMC. The direct interactions of the GAP proteins with each other is still under investigation, but it has been noted that the glycosylation and phosphorylation states of the proteins play a role in their interactions with the other IMC components (Fauquenoy et al., 2011; Gilk et al., 2009).

3.3.2 Glideosome associated proteins with multiple membrane spans (GAPM)

—The actomyosin motor is attached to the outermost side of the IMC alveoli membranes. In cryo-EM freeze fracture studies, 9 nm particles on the innermost, cytoplasmic side of the membrane of the IMC were observed (Raibaud et al., 2001). Investigation of the makeup of

these particles led to the discovery of the glideosome associated proteins with multiple membrane spans (GAPMs) (Bullen et al., 2009). There are three GAPM variants (GAPM 1–3), which possess six transmembrane domains and are present at the IMC in large, detergent resistant oligomeric complexes. GAPM interacts with the alveolins, which form part of the parasite's cytoskeletal network. The proteins also co-IP with both GAP50 and GAP45 of the IMC, indicating that GAPM may interact with other IMC associated proteins across the lumenal space of the IMC.

3.3.3 IMC sub-compartment proteins (ISPs)—The IMC sub-compartment proteins (ISP) are conserved across apicomplexans and are unique because the proteins are localized to different parts of the IMC along the parasite (Beck et al., 2010; Fung et al., 2012; Poulin et al., 2013). *T. gondii* encodes for ISPs 1–4, while only orthologs of ISP1 and ISP3 have been identified in *Plasmodium spp*. In *T. gondii*, ISP1 localizes to the apical end of the parasite, ISP2 localizes along the central region of the IMC, and ISP3 is able to localize to both the basal and central regions.

The ISPs do not possess signal sequences or transmembrane sequences; however, all ISPs are predicted to have palmitoylation and myristoylation sites near the N-terminus. The working theory is that targeting of the ISPs relies on recognition by an IMC bound palmitoyl acyl transferases (PAT) (Beck et al., 2010). Myristoylation of ISP1–3 would lead to nonspecific membrane association. If an ISP comes in contact with a PAT, it would be palmitoylated and bound to that membrane. PATs located at different parts of the IMC would control the specific localization of each ISP by only palmitoylating their specific substrate protein. In *T. gondii*, there are three putative membrane-bound O-acyl transferases, which typically palmitoylate secreted proteins, and 18 DHHC-CRD PATs, which typically palmitoylate non-secreted proteins for targeting to membranes (Beck et al., 2013; Resh, 2006). This information raises the questions as to whether there are multiple PATs capable of recognizing specific substrates, and how in turn the PATs would be localized to different sub-compartments of the IMC. A recent review by Frenal et al. (2014a) discusses palmitoylation and depalmitoylation cycles in *T. gondii*, and their role in protein localization.

Crystal structures of TgISP1 and TgISP3 (Tonkin et al., 2014a) reveal that the proteins adopt a Pleckstrin homology (PH) fold (Figure 8B), that is typically involved in phospholipid binding; however, there was no evidence of phospholipid binding. This result is likely due to a mutation of the residues that bind the phospholipid. Analysis of surface electrostatics show that the binding site in the ISP proteins is more hydrophobic and less positively charged than the phospholipid binding site from phospholipase C (Figure 8B). Analysis of the surface of ISP1 reveals a hydrophobic groove and a sulfate molecule bound near the groove leading to speculation that the protein has adapted to bind a protein partner possibly mediated by phosphorylation.

3.4 Subpellicular Network (SPN)

The inner membrane complex, made up of flattened membranes and associated with multiple, acylated peripheral membrane proteins and integral membrane proteins, interacts

with the parasite's subpellicular network (SPN) (Mann and Beckers, 2001). It was found in *T. gondii* that a network of 8–10 nm fibers were associated with the cytoplasmic face of the IMC.

3.4.1 Alveolins—The alveolin protein, TgIMC1 (ALV1), was identified as the main protein present in the 2D lattice of the SPN (Mann and Beckers, 2001). IMC1 shares homology with articulins, which are cytoskeleton proteins found in protists. The TgIMC1 orthologs for *Plasmodium spp*. were annotated as IMC1a-h (Khater et al., 2004). The alveolins, which vary in size, contain multiple repeat domains with a common motif of (E/D)(K/R)ΦΦ(E/D)ΦPx (Φ=hydrophobic and x=any amino acid), flanked by nonconserved regions on either side, and are predicted to have extended coiled-coil domains (Gould et al., 2008). It has been shown that knockout of IMC1a, IMC1b and IMC1h negatively affect the gliding motility of sporozoites and ookinetes and reduce infectivity (Khater et al., 2004; Tremp and Dessens, 2011; Volkmann et al., 2012). Connecting this cytoskeleton to the actomyosin motor, gliding motility and invasion/infectivity is likely accomplished *via* a connection with the IMC and GAPM1–3 proteins. The GAPMs co-purify with the alveolins, forming detergent-resistant oligomeric complexes, likely providing an anchoring point for the IMC to the subpellicular network (Bullen et al., 2009).

4. Exploring the essentiality of the adhesins and glideosome components

In the past year, several groups have published data regarding conditional knockouts of proteins key to the glideosome model in *T. gondii*. Conditional knockouts, using a DiCre-recombinase system, of *ama1, aldolase, mic2, act1, gap45* and *myoA* have been generated in *T. gondii* and it has been shown that invasion still progresses, albeit at severely reduced levels (Andenmatten et al., 2013; Egarter et al., 2014; Frenal et al., 2014b; Shen and Sibley, 2014). Discussed below are these experiments and what the results mean in our understanding of the actomyosin motor based invasion by *T. gondii*, how this work translates to other apicomplexans, specifically *Plasmodium spp*., and alternative models that have been proposed for invasion. A summary of the conditional knockouts and the observed phenotypes, is presented in Table 4.

4.1 AMA1 knockouts

A clonal knockout of *ama1* in *T. gondii* tachyzoites and non-clonal knockout in *P. berghei* merozoites, were produced, which negatively affected host cell attachment (Bargieri et al., 2013); however, these parasite lines were still able to form a tight junction and invade cells in a smooth, one-step fashion. The merozoites and tachyzoites displayed a 3–5 fold lower invasion efficiency overall, but when normalized to the number of merozoites attached to cells, the parasites could invade cells better. It was concluded that AMA1 is dispensable for invasion by both *P. berghei* merozoites and sporozoites and *T. gondii* tachyzoites and opened up the possibility that the model of AMA1 and the RON complex as an essential component of the moving junction may be incorrect.

Following the study in *T. gondii* and *P. berghei*, an *ama1* conditional knockout in *P. falciparum* was produced (Yap et al., 2014). Authors of the study concluded that AMA1 is not dispensable in merozoite invasion by *P. falciparum*. The *ama1* gene was placed under

conditional control in merozoites using the DiCre-recombinase system. Efficiency of deletion was ~80%. Because the deletion occurred after DNA-replication, due to the trophozoite specific promoter used to express the recombinase, and in combination with the imperfect deletion efficiency, there was a resulting mix of genetically null and wild-type (WT) merozoites. However, because micronemes, where AMA1 resides, form prior to segmentation, it was possible for genetically null parasites to have a range of AMA1 levels, which was observed with levels ranging from undetectable to near WT. The invasion efficiency of the PfAMA1 deficient merozoites was very low, and it was explained that there was likely insufficient AMA1 present to form a tight junction or to seal the invasion pore if a merozoite managed to have high enough levels of AMA1. One explanation offered by the authors to reconcile the *P. falciparum* results with the *P. berghei* and *T. gondii* data (Bargieri et al., 2013) is that both *T. gondii* and *P. berghei* possess orthologs/paralogs of AMA1, which could act as redundant adhesins; however, no redundancies have been identified in *P. falciparum*, which would make it essential for the model in which AMA1 plays a role in the moving junction. A more thorough detailing of this controversy is found in a review by Harvey *et al*. (2014).

4.2 Glideosome component knockouts

A conditional DiCre-recombinase system in *T. gondii* has been established and used to investigate the role of key invasion machinery components (Andenmatten et al., 2013; Egarter et al., 2014). Conditional/induced knockouts (iKO) were generated for *mic2*, *myoA*, *gap40*, *gap45*, *gap50*, *mlc1*, and *act1*. In the process of creating conditional knockouts, it was possible to isolate clonal knockouts of *mic2* and *myoA*, whereas clonal knockouts could not be obtained of *gap40*, *gap45*, *gap50*, *mlc1* or *act1*. It is important to remind readers that the following experiments were performed with *T. gondii* tachyzoites, and not *Plasmodium spp*. parasites.

4.2.1 MyoA knockout—In the *myoA* knockout, the Meissner group observed that the invasion rate was only 16% that of wild-type (WT) (Andenmatten et al., 2013; Egarter et al., 2014). Gliding motility was reduced by ~65% and tachyzoites moved mostly by circular gliding and in short steps. The *myoA* iKO parasites displayed different kinetics of invasion, with a few parasites entering smoothly and efficiently like WT in ~25 seconds, while the majority entered in a “stop-and-go” manner and could take up to 10 minutes to invade. The observation that *myoA* iKO parasites could still invade, albeit more slowly, suggested that MyoA is important for efficient invasion, but that there could also be an alternate force generation model. An explanation for the ability of *myoA* iKO invasion is that other myosins could act as a redundancy mechanism. The Soldati group has indicated that MLC1 is able to interact with MyoC (Jacot et al., 2014), and may be able to substitute for MyoA. To test this possibility, a triple conditional knockout of *myoA/B/C* was produced, resulting in a more severe phenotype than the single *myoA* iKO, however, parasites could still invade. It is still possible that the other myosins such as MyoD and MyoE, which were not knocked out, could also complement *myoA/B/C* function, providing *T. gondii* with multiple compensatory myosins.

4.2.2 MLC1 knockout—The *mlc1* iKO caused mislocalization of MyoA, as expected, and reduced the ability to egress to 5% that of WT, and led to ~70% reduction in invasion (Egarter et al., 2014). This data suggests that MLC1 is important for host cell egress and MyoA localization, and that other MLCs are unable to fully complement this function. While there may not be another MLC capable of fully complementing the role of MLC1, it was noted by the authors that it was not possible to rule out the possibility that the MyoD-MLC2-motor (Polonais et al., 2011) could complement loss of the MyoA-MLC1-motor.

4.2.3 GAP45 knockout—The *gap45* iKO resulted in cytosolic localization of the MyoA/MLC1 complex and affected the parasite morphology, due to separation of the IMC from the plasma membrane (Egarter et al., 2014). Along with a change in localization and morphology, depletion of GAP45 resulted in ~95% reduction in egress as well as invasion. However, gliding remained at 100% that of WT parasites, suggesting that morphology is the cause of invasion defects and not motility.

4.2.4 Actin 1 knockout—To further investigate the requirement of the actomyosin motor, the Meissner group generated a conditional knockout strain of *act1* (Andenmatten et al., 2013; Egarter et al., 2014). A clonal *act1* KO could not be isolated and conditional knockouts resulted in a defect in apicoplast segregation. Unlike in *Plasmodium spp*., it is not possible to supplement *T. gondii* cultures with IPP to determine if the *act1* clonal KO is unable to be obtained solely due to an apicoplast defect. The conditional *act1* knockout displayed gliding and invasion defects, but was still motile and could invade at ~10% that of WT, It was reasoned that actin 1 is not essential for invasion, only for apicoplast segregation or some other replicative process. As there is only one actin in *T. gondii*, if this actin is dispensable, an alternative to the glideosome would be needed.

To account for the ability to invade without actin, the authors propose a gelation-solation osmotic motor comprised of actin-like filaments (Egarter et al., 2014). However, if actin 1 is not required for invasion, as suggested by their data, and unlike *Plasmodium spp*., *T. gondii* does not possess actin 2, then what is the makeup of the gel? There is a family of actin-related proteins (Arps), also known as actin-like proteins (ALPs), members of the actin superfamily, which may be able to form this gel (Gordon and Sibley, 2005). Apicomplexans have 8–10 of these Arps and knockouts of the associated genes could answer the question of whether or not these proteins are involved in invasion and gliding motility. There is also a question of whether there is any remaining actin around. While it was reported that actin levels were undetectable *via* Western blot and fluorescence, and that would mean the concentration was lower than the critical concentration needed for filament formation (Egarter et al., 2014), it was noted previously that *T. gondii* actin 1 can form filaments at a concentration 3–4 times lower than canonical actin (Sahoo et al., 2006). So, it may be possible that filaments could still form from any remaining actin pools. More experiments will hopefully reveal the existence of either complementary motors or evidence for alternative motor models.

4.2.5 Aldolase conditional knockout—In addition to the knockouts performed by the Meissner group, the Sibley group was able to generate an *aldolase* conditional knockout in *T. gondii*, that was able to invade fibroblasts under low glucose conditions (Shen and Sibley,

2014). The low glucose conditions prevented accumulation of toxic glycolysis intermediates, enabling the *aldolase* iKO tachyzoites to invade in the absence of aldolase. The glucose data is interesting, but explanations of possible redundancies, we believe, are not adequately addressed.

Only briefly discussed in the Sibley paper (2014) is the fact that GAPDH, another known bridging protein, could possibly rescue the invasion of the parasite in the absence of aldolase. Reported mutations that can disrupt the AMA1 interaction with GAPDH, did not lead to inhibition of invasion, supporting the case that GAPDH is not necessary for invasion. However, it is known in *T. gondii* that the AMA1 pathway is nonessential and is likely a redundant pathway to invasion (Bargieri et al., 2013). So, a clone with an AMA1 defective in both aldolase and GAPDH binding that can invade, does not prove the non-essentiality of GAPDH. A conditional knockout of both GAPDH and aldolase would be needed to provide more evidence that bridging to this motor is not necessary. Also, this point recalls the evidence presented earlier that while AMA1 seems to be dispensable for *T. gondii* and it is possible that paralogs/orthologs to AMA1 are able to compensate for AMA1 loss, that AMA1 is essential for tight junction formation and pore closure in *P. falciparum* (Yap et al., 2014).

4.2.6 Egress phenotypes—Discussion has focused on the invasion and gliding phenotypes, yet many of these conditional knockouts blocked host cell egress. Host cell egress is blocked in *myoA/B/C* triple knockouts as well as the *act1*, *mlc1*, *gap40*, *gap45*, and *gap50* conditional knockouts (Andenmatten et al., 2013; Egarter et al., 2014). The question that arises is this: Why is egress blocked when proteins, traditionally associated with the invasion machinery, are knocked out? Clearly, while these proteins could be considered nonessential for invasion based on the ability of the conditional knockouts to invade host cells, it is also likely that either proper localization or expression of the invasion machinery associated proteins, acts as a regulator of egress. It is possible that proper glideosome assembly acts as a checkpoint, so that only parasites that are fully competent for invasion are released.

4.2.7 Discussion of alternative invasion mechanisms and knockouts—It is important to keep in mind that the conditional knockout of glideosome components discussed here has only been performed in *T. gondii* (Andenmatten et al., 2013; Egarter et al., 2014), and that while it has been presented that apicomplexans share these invasion proteins, that the mechanisms of invasion may be different between the genera. However, it is also important to take these data and begin to look for alternative models and glideosomes to explain a mechanism of host cell invasion.

One possibility to explain conditional knockouts of the MyoA glideosome components in *T. gondii*, is that the MyoA-MLC1 complex is only one of multiple, redundant motors (e.g. MyoD-MLC2, MyoC-MLC1). Recent work has described a novel glideosome in *T. gondii* involving MyoC (Frenal et al., 2014b). It has been demonstrated that GAP80, a member of the GAP45 family, is able to recruit MyoC to the basal polar ring with the help of IMC-associated protein 1 (IAP1). Previous work had already shown that MyoC could interact with MLC1 (Polonais et al., 2011). MyoC, like MyoA, also interacts with the IMC

embedded proteins, GAP40 and GAP50, and forms the MyoC-MLC1-GAP80-IAP1-GAP40-GAP50 glideosome (Frenal et al., 2014b).

In WT parasites, the MyoA-MLC1-GAP45-GAP40-GAP50 glideosome functions along the parasite periphery, while the MyoA-MLC1-GAP70-GAP40-GAP50 glideosome functions at the apical cap and the MyoC-MLC1-GAP80-IAP1-GAP40-GAP50 glideosome functions at the basal polar ring (Figure 9A, 9B). Depletion of MyoA in a conditional knockout strain leads to a redistribution of the MyoC glideosome along the parasite periphery and into the apical cap, likely accounting for the observed low levels of invasion. In *gap80* and *gap70* iKOs, GAP45 can assume their roles, but neither GAP80 nor GAP70 could complement GAP45 function. If the conditional knockout work done in *T. gondii* is replicated in *Plasmodium spp*. and invasion is observed, these alternative glideosomes with GAP70 and GAP80, would not be a possible explanation, since GAP70 and GAP80 are only present in coccidians. However, the demonstrated plasticity of the glideosomes in *T. gondii*, and the ability of the MyoC glideosome to redistribute along the parasite in the absence of the MyoA glideosome, would lead one to speculate that there is a similar plasticity and redundancy mechanisms for *Plasmodium spp*. to allow for invasion.

Alternatively, it has been suggested that there are other modes of invasion (linear glideosome versus gelation-solation model). Perhaps multiple motors exist and have different roles in invasion. It has been suggested that a linear glideosome motor is needed for efficiency in reorientation, tight junction formation, and the start of invasion and another motor completes invasion once it is going. Another possibility is that invasion is regulated by the host cell *via* a host cell mediated uptake or autophagy/phagocytosis pathway. Regardless, these conditional knockouts of the glideosome components do cause severe phenotypes, with a 95% reduction in invasion in some cases (Andenmatten et al., 2013; Egarter et al., 2014; Frenal et al., 2014b; Shen and Sibley, 2014), and are still worthy of continued study and translational work to develop inhibitors of these glideosomes.

5. Conclusion

When this structural survey was performed, approximately 50 protein structures of apicomplexan adhesin proteins and host cell receptors had been deposited with the PDB. Many of these structures concern key domains that have been previously implicated in receptor interactions or have been targeted by antibodies during the course of vaccine development. Antibody-adhesin co-structures are useful to structurally identify their mechanism of interaction and inhibition, to help guide antibody development to regions of the adhesin that are both accessible and needed for receptor engagement or adhesin function. Structures of receptor domains bound to ligand or peptide have provided insight into assembly and recognition as well as clues as to how to disrupt them, which may lead to new therapeutics. Structural, biochemical, and cellular/genetic investigations are needed in the future to understand how apicomplexan adhesin proteins interact with ligands, as well as to discover their interacting partners.

Around 35 structures related to the glideosome machinery have been deposited, accounting for ~60% of the glideosome complex. Lacking are apicomplexan structures of the unique

Myosin A, as well as ISP2, GAP40, GAP45 and GAPM1–3. With successful purification of MyoA, it may not be much longer before a structure is obtained, providing a better understanding of how this unique myosin operates. Structures of GAPM1–3 may be difficult due to the multiple transmembrane passes and GAP45 due to the predicted extended-coiled-coil structure. Additionally, structures can provide key information regarding complex formation that enables the targeting of protein-protein interactions of the invasion machinery with small molecule disruptors or stabilizers.

Multiple papers were released implicating that many components of this machinery were not essential in *T. gondii* and called for the proposal of alternate mechanisms of invasion. This review has summarized the results of these papers and presented evidence that seems to be contradictory, including the knockout results from the Soldati group, which showed plasticity of the glideosomes and identification of novel glideosome complexes. Further work to use a conditional knockout system in *P. falciparum* should address whether these components are essential for invasion in other apicomplexans. There are still unanswered questions such as why there is an egress block associated with machinery component knockouts, and what is the composition of an alternative motor. Keeping an open mind to the possibility that the established model may represent only one of several mechanisms of invasion, is important. However, the reports of the invasion machinery's non-essentiality seem to be premature in light of recent work, meaning that further investigation will be necessary and the glideosome is still a viable area for study to develop novel anti-parasitics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Pf	*Plasmodium falciparum*
Pb	*Plasmodium berghei*
Pv	*Plasmodium vivax*
Py	*Plasmodium yoelii*
Pk	*Plasmodium knowlesi*
Tg	*Toxoplasma gondii*
ACT1	actin 1

AMA1	apical membrane antigen 1
AUC	analytical ultracentrifugation
DARC	Duffy antigen/chemokine receptor
DBL	Duffy binding-like domain
DBP	Duffy binding-like protein
DLS	dynamic light scattering
DSF	differential scanning fluorimetry
EBA	erythrocyte binding antigen
EBD	erythrocyte binding domain
EBL	erythrocyte binding-like protein
EGF	epidermal growth factor
Fab	fragment antibody
GAP	glideosome associated protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAPM	glideosome associated proteins with multiple membrane spans
IgNAR	immunoglobulin new antigen receptor
IAP1	IMC associated protein 1
iKO	DiCre-recombinase induced knockout
IMC	inner membrane complex
IMC1-x	inner membrane complex protein 1-x
IPP	isopentenyl pyrophosphate
ISP	IMC sub-compartment proteins
ITC	isothermal titration calorimetry
MALS	multi-angle light scattering
MIC2	microneme-associated protein 2
MLC	myosin light chain
MSP	merozoite surface protein
MTIP	myosin tail interacting protein
MTRAP	merozoite thrombospondin related anonymous protein
MyoA	myosin A
NBD	nucleotide binding domain
PAN	plasminogen, apple, nematode

RBP	reticulocyte binding-like protein
RH	reticulocyte binding-like homolog
RIPR	RH5 interacting protein
RON	rhoptry neck protein
TSR	thrombospondin repeat domain
TRAP	thrombospondin related anonymous protein
SAXS	small angle X-ray scattering
SEC	size exclusion chromatography
SPN	subpellicular network
VWA	von Willebrand factor type A domain
WT	wild-type

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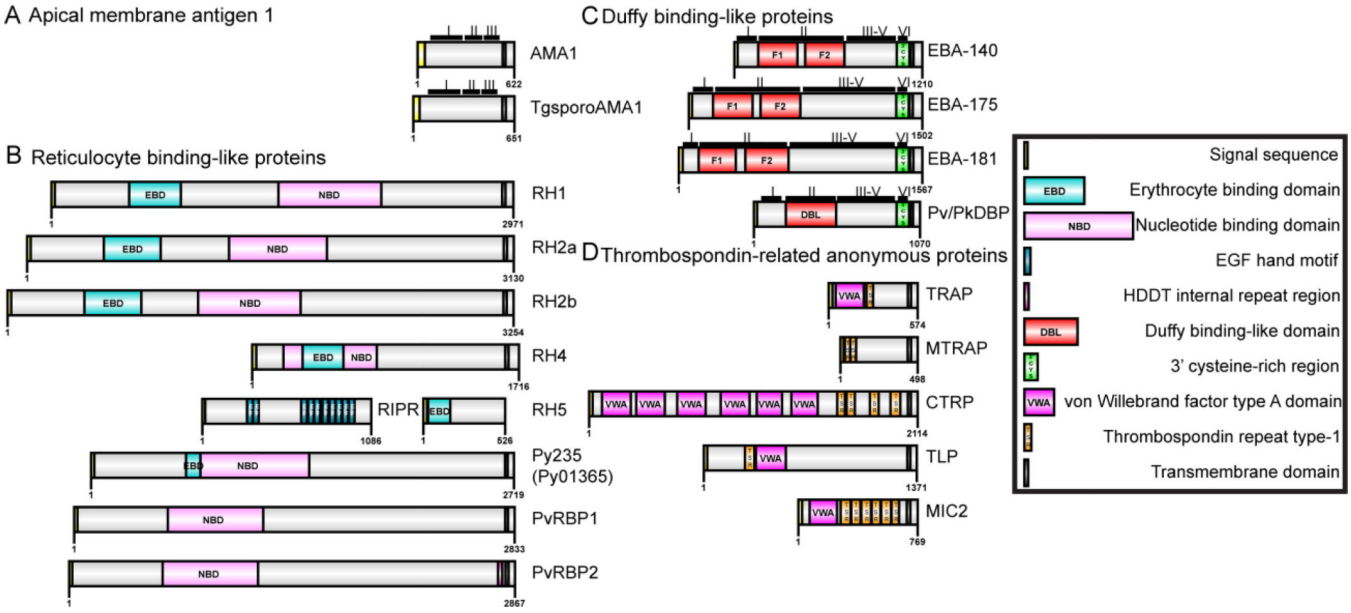

Figure 1. Domain organization of parasite receptors

(A) Apical membrane antigen family of receptors including AMA1 from *P. falciparum* and the sporozoite specific AMA1 from *T. gondii*. (B) Reticulocyte binding-like proteins including the RH proteins from *P. falciparum* and adapter proteins, RBPs from *P. vivax*, and Py235 from *P. yoelii*. (C) Duffy binding-like proteins including the EBAs from *P. falciparum* and DBPs from *P. vivax* and *P. knowlesi*. (D) Thrombospondin-related anonymous protein family including the TRAPs from *P. falciparum* and MIC2 from *T. gondii*. A key is provided for the various key features determined from sequence homology or from biochemical and structural information, with region and domain boundaries are marked according to literature reports.

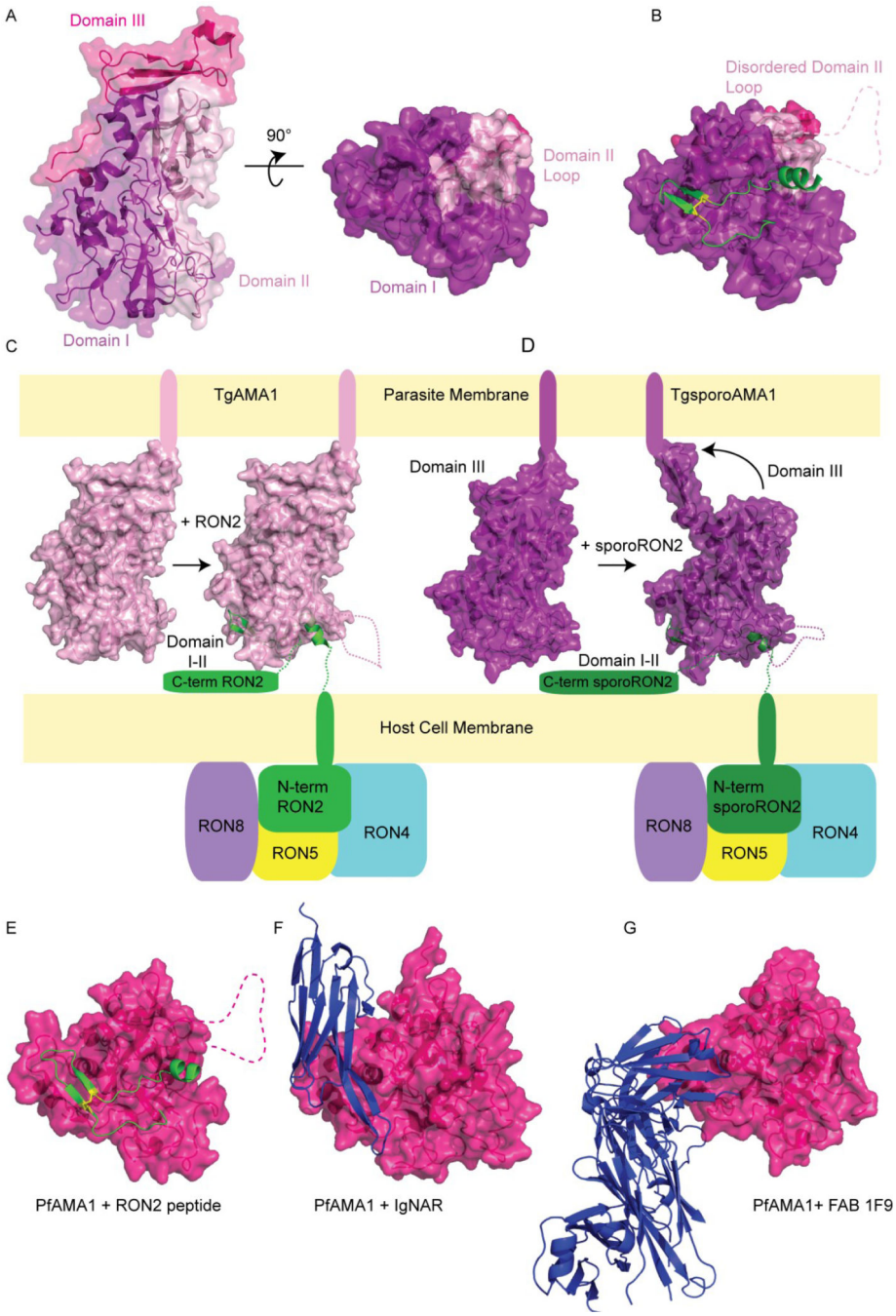

Figure 2. Apical membrane antigen 1 structure

(A) TgAMA1 (surface and cartoon) depicted in two orientations rotated 90° from the other about the x-axis. The three domains of TgAMA1 are highlighted in purple, light pink, and magenta to distinguish Domains I, II and III, respectively. (B) TgAMA1 (surface) bound to the TgRON2 peptide (green, cartoon). The disulfide bond that stabilizes the cysteine loop is shown as yellow sticks. The dotted pink line represents the disordered loop from Domain II that is displaced from its contact with Domain I upon RON2 binding. Depiction of the orientation of (C) TgAMA1 (light pink, surface) and (D) TgsporoAMA1 (purple, surface) in

the unbound and RON2 peptide-bound (green, cartoon) state on the parasite membrane surface. The parasite derived RON2/4/5/8 complex is shown inserted into the host cell membrane. An arrow indicates the Domain III movement in sporoAMA1 seen in the crystal structure when it is unpacked from Domains I and II upon binding of sporoRON2. (E) PfAMA1 (magenta, surface) bound to RON2 peptide with disulfide bond (yellow, sticks), and disordered loop (dashed). Structure of PfAMA1 in complex with inhibitory (F) IgNAR and (G) Fab 1F9, shown binding to Domain I where the RON2 cysteine loop binds.

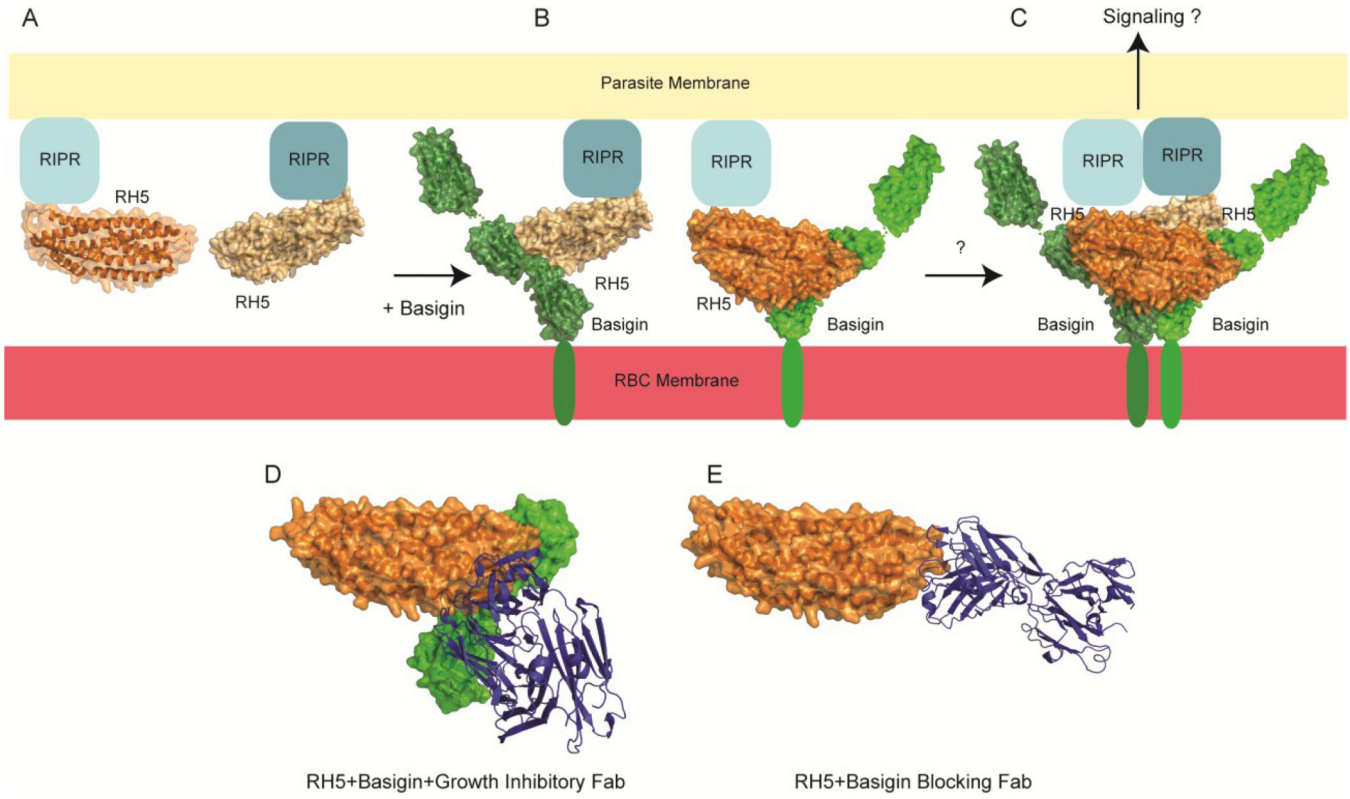

Figure 3. RH5 in complex with human receptor basigin

Schematic of (A) RH5 (orange and light yellow, surface and cartoon) interaction with the parasite membrane *via* RIPR (blue squares). Addition of basigin (green, surface) results in a (B) 1:1 complex, as confirmed in SAXS and AUC experiments. The crystal structure of the basigin-RH5 complex shows a (C) 2:2 stoichiometry in the ASU. RH5 in complex with a (D) growth inhibitory antibody (blue, cartoon) that does not prevent basigin binding. Basigin is modeled, not present in the crystal structure, to indicate that the binding sites do not overlap. RH5 in complex with a (E) growth inhibitory antibody (blue, cartoon) that does block basigin binding.

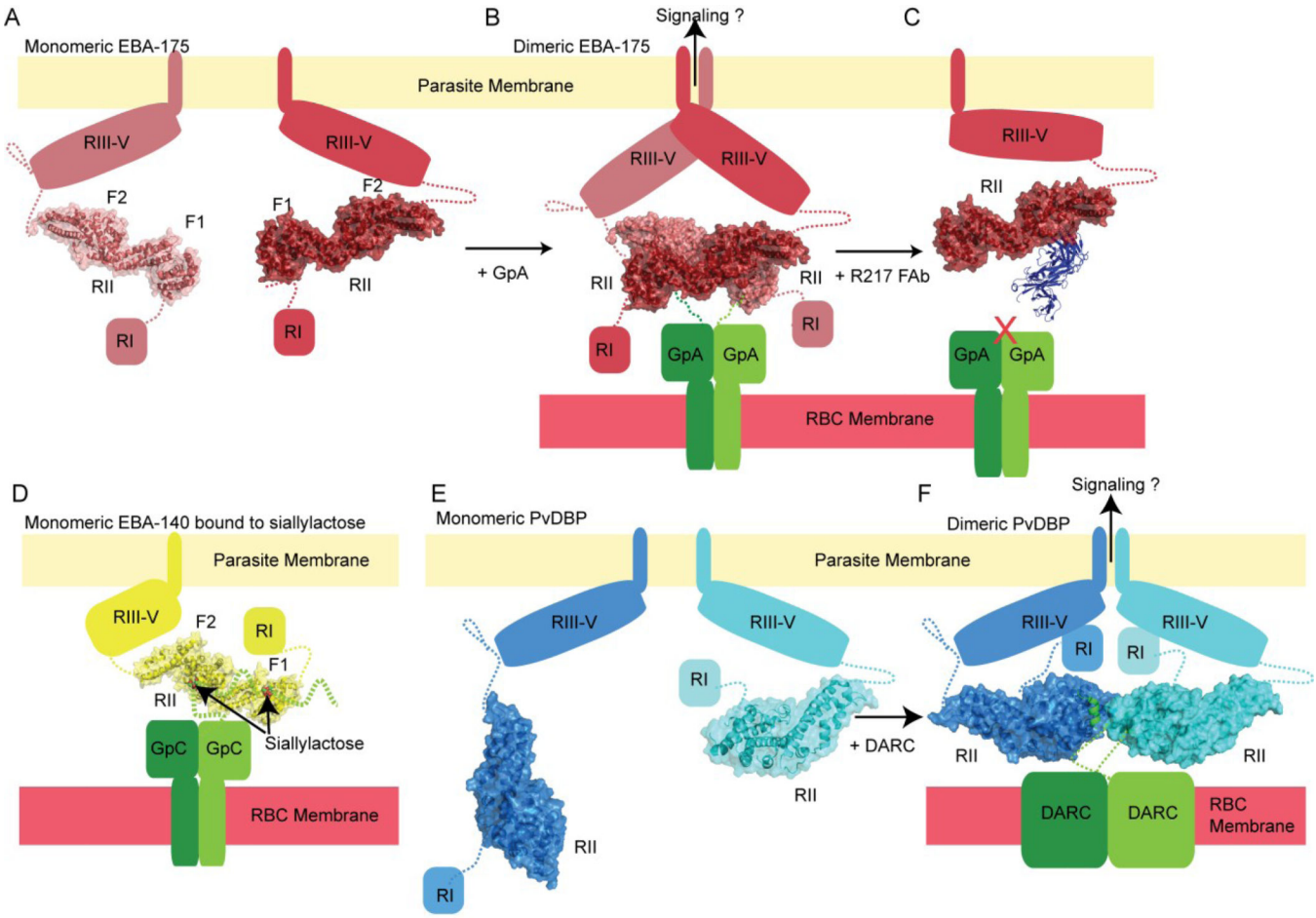

Figure 4. Duffy binding-like protein structures

Schematic depicting orientation of EBA-175 Region II (red, surface and cartoon) (A) before receptor engagement and (B) and dimerization after engagement of dimeric glycophorin A on the red blood cell surface. (C) An inhibitory Fab (blue, cartoon) blocking the sialyllactose binding pocket as well as contacts needed for EBA-175 dimerization. (D) Crystal structure of monomeric EBA-140 with a sialyllactose molecule bound in both the F1 and F2 domains of Region II, which engage the dimeric glycophorin C receptor on the red blood cell surface. Schematic of (E) PvDBP (blue, cartoon and surface) (F) dimerization upon binding of the DARC peptide, part of the dimeric DARC receptor (green) on the red blood cell surface.

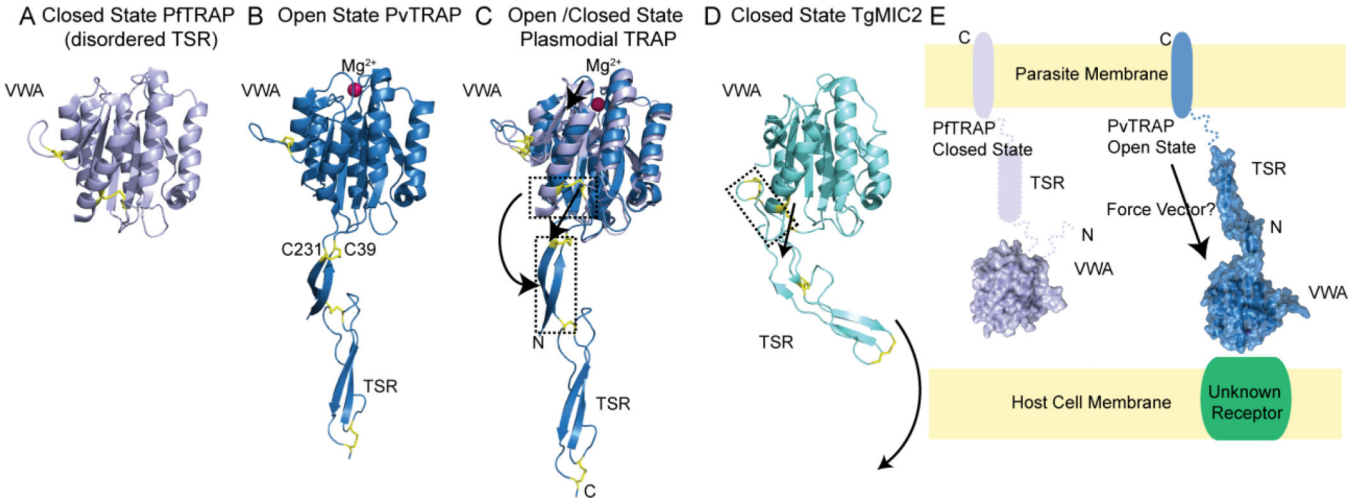

Figure 5. Open and closed state of TRAP proteins

(A) VWA domain of PfTRAP (grey, cartoon) in the closed state, with key disulfide bonds in yellow. (B) Open state of PvTRAP (blue, cartoon) depicting the VWA with Mg_{2+} (magenta, sphere) in the MIDAS site, and TSR domain connected by an extended β-strand formed by residues up- and downstream of the VWA domain. (C) Alignment of the open and closed TRAP state showing the unwinding of the helix allowing the disulfide bond between C231 and C39 (PvTRAP) to shift down and away from the VWA domain to stabilize the extended β-strand before the TSR domain. (D) Closed state of TgMIC2 depicting an unextended, ordered TSR domain, however, the helix has not unwound allowing the disulfide bond to shift down and away from the VWA domain and forcing an extension of the TSR domain into the open state as indicated by the arrow. (E) Schematic of the orientation of TRAP relative to the parasite membrane with the N-terminal VWA domain farthest from the membrane followed by a disordered TSR and C-terminal transmembrane domain. Binding of ligand in the membrane distal VWA domain induces a switch to the open state with an extended TSR. This open state may allow force (arrow) to be transmitted along the more rigid TSR domain to the host cell attachment at the unknown receptor.

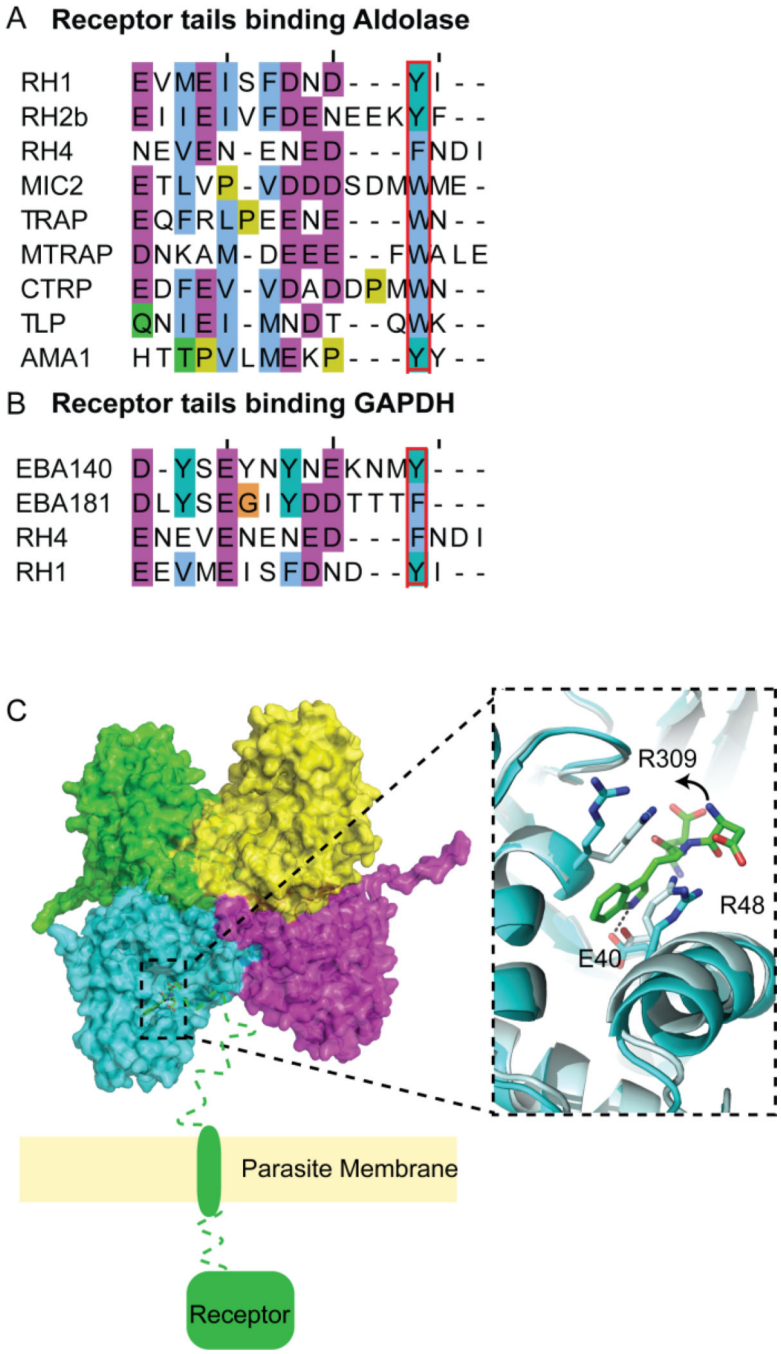

Figure 6. Binding of adhesin tails to aldolase and GAPDH

Alignment of C-terminal tail sequence of receptors known to bind (A) aldolase and (B) GAPDH, highlighting a key residue for binding in red. (C) Tetrameric PfAldolase (multicolored chains, surface) with PbTRAP peptide residues, EWN, bound and modeled in the active site, and an overlay of the binding site of apo (grey) and bound (cyan) aldolase (cartoon). The key tryptophan of the TRAP tail is sandwiched between R48 and R309, after R309 swings out of the active site and the helix containing R48 shifts to allow binding. This positioning allows the tryptophan to make a hydrogen bond to E40.

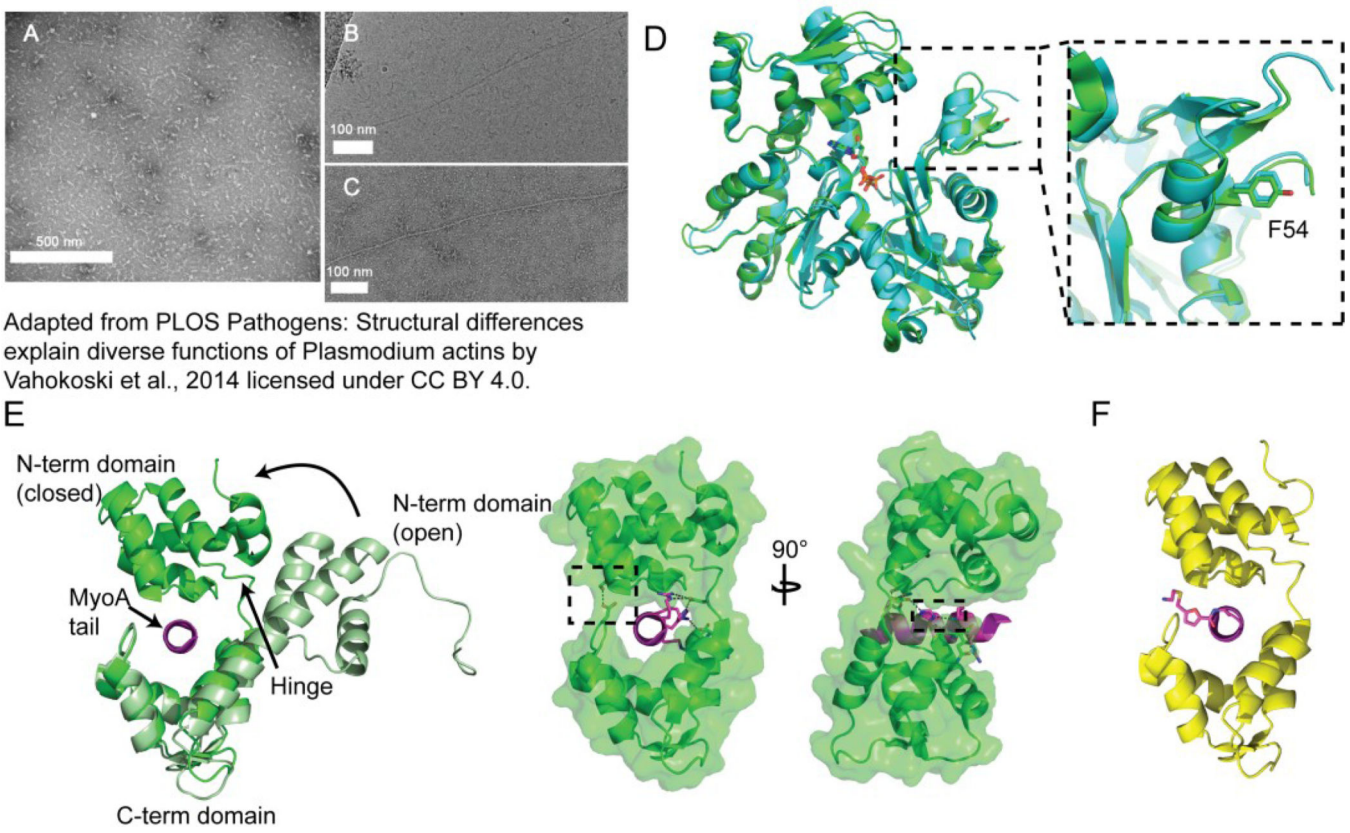

Figure 7. Motor components actin and MTIP bound to MyoA tail

EM images of (A) PfActin 1 forming short 100 nm fibers and (B and C) PfActin 2 forming long filaments, adapted from PLOS Pathogens "Structural differences explain diverse functions of *Plasmodium* actins" by Vahokoski *et al*. (2014) licensed under a Creative Commons Attribution 4.0 International License. (D) Overlay of the canonical actin (green, cartoon) with PfActin 1 (cyan, cartoon), and ATP and a nonconserved F54 depicted as sticks. (E) MTIP (cartoon) in the open (light green) and closed (dark green) states with the MyoA peptide tail (magenta, cartoon) bound. The hinge, which does not have secondary structure in the closed state but is part of the long central helix in the open state is marked. Two views of the closed MTIP structure, separated by 90° rotation about the y-axis, show the serine-aspartate latch (sticks and boxed) that stabilizes the closed structure. The second orientation depicts the hydrogen bonding network (sticks and dashes), stabilizing the MTIP-MyoA complex, formed between the aspartate residue in the hinge, the arginine, histidine, and lysine along the MyoA helix, the asparagine carboxy terminus of MTIP, and the last arginine on the MyoA peptide. (F) MyoA-tail chimera (magenta, cartoon and stick) bound to MTIP (yellow, cartoon).

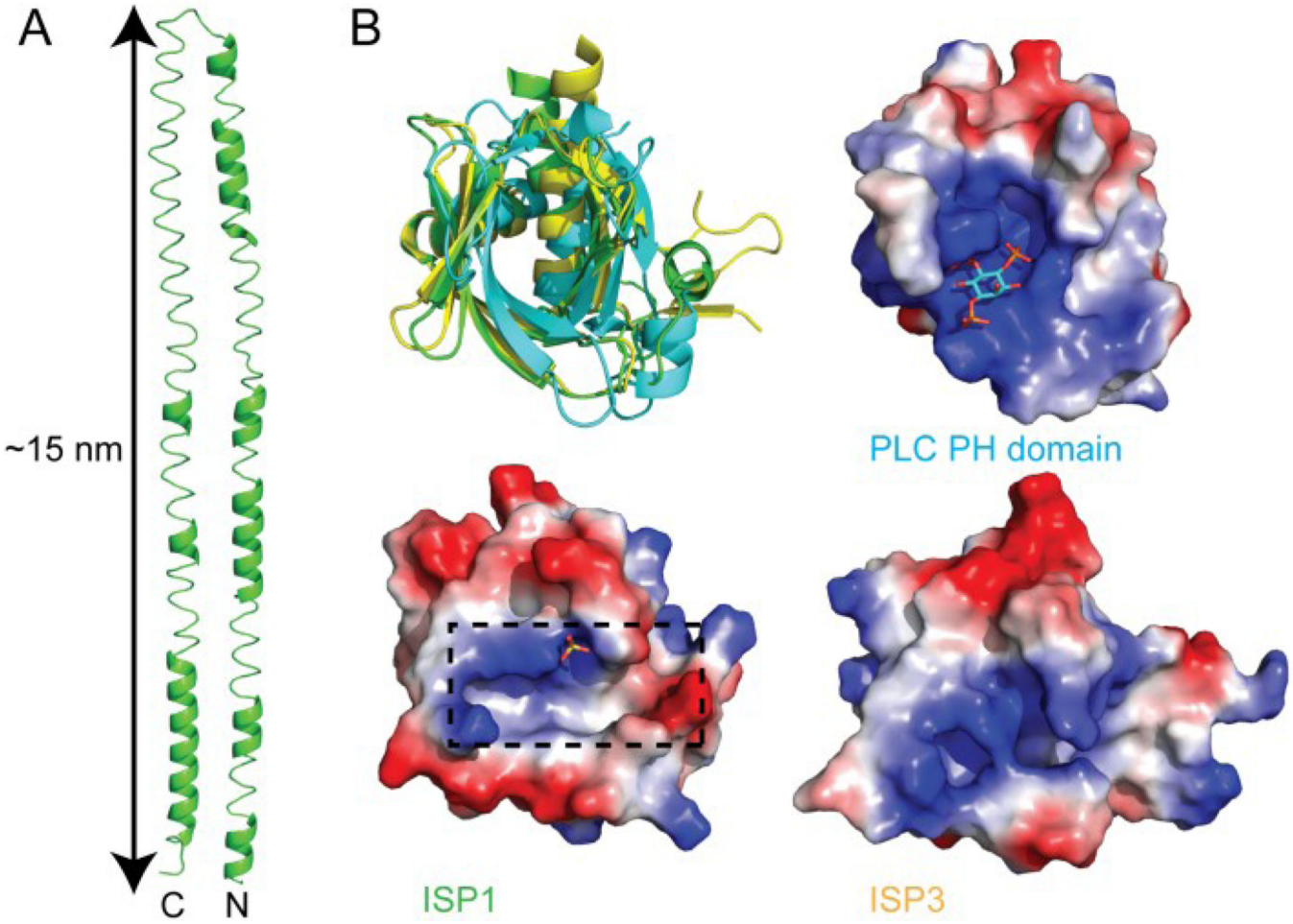

Figure 8. IMC associated proteins GAP45 and ISP1 and 3

(A) I-TASSER homology model of PfGAP45 (green, cartoon) composed of coiled-coils spanning 15 nm. (B) Structures of the Pleckstrin homology domain of ISP1 (green, cartoon), ISP3 (yellow, cartoon), and structurally similar phospholipase C (green, cartoon). Calculated electrostatic surface potential for PLC and ISP1 and 3 with phospholipid bound to PLC and sulfate to ISP1 (sticks). Box indicates a potential protein binding site.

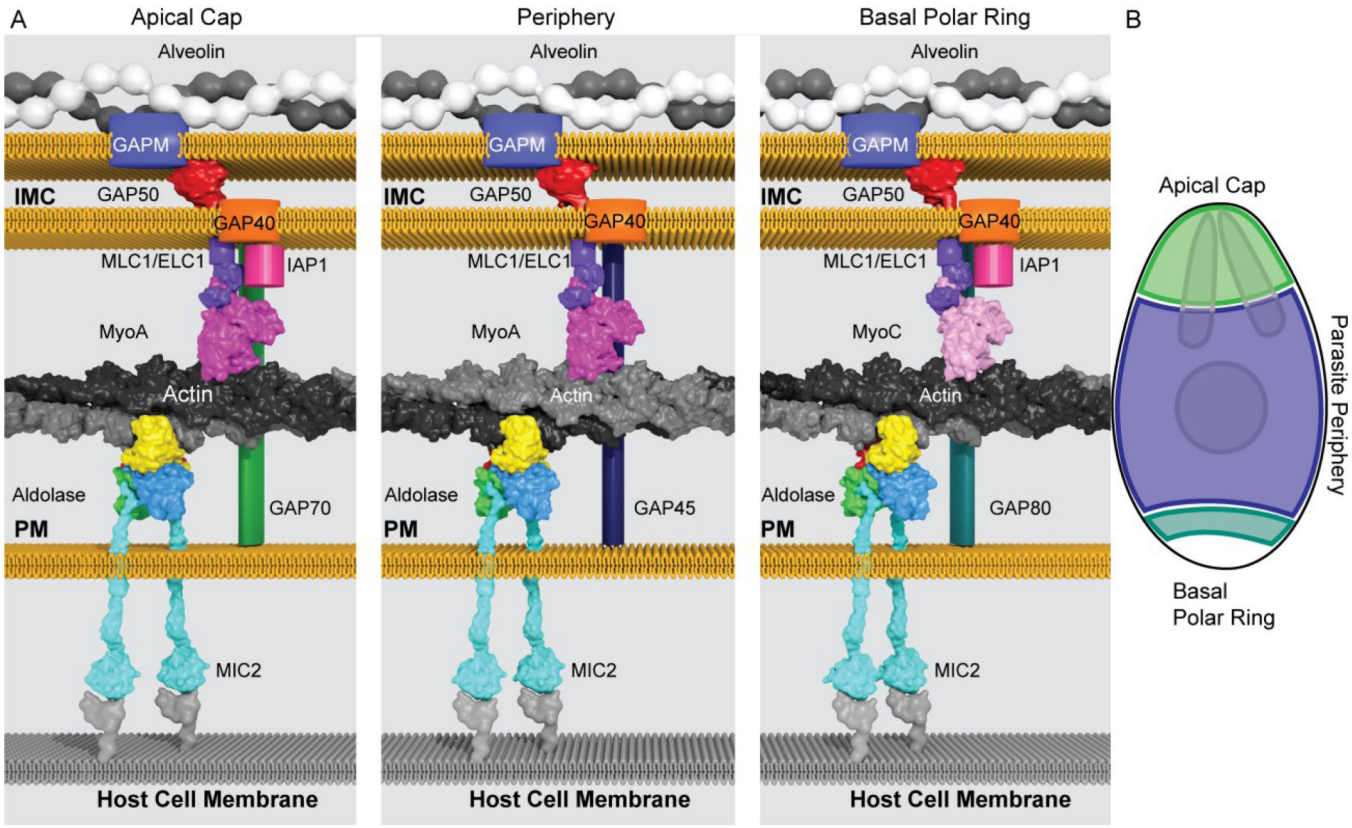

Figure 9. Schematic of apicomplexan glideosomes

Protein structures solved from both *T. gondii* and *Plasmodium spp*. and homologous protein structures were combined with the results of biochemical and genetic data to depict the (A) glideosome model. The three panels illustrate the motor complexes present in the apical cap (left), along the parasite periphery (middle), and at the basal polar ring (right). The actin-myosin motor (grey/black and magenta/light pink) is bridged by tetrameric aldolase (multicolor) to the extracellular adhesins (cyan), which connects to unknown cellular receptors on the host cell membrane (grey). The motor is anchored to the IMC *via* its interaction with MTIP (*Plasmodium spp*.) or MLC1/ELC1 (*T. gondii*) (purple). In *T. gondii*, the MyoA (magenta) motor is located in the apical cap and periphery of the parasite while the MyoC (light pink) motor acts at the basal polar ring. The myosin-MLC1 motors interact with either lipidated GAP70 (green), GAP45 (dark blue), or GAP80 (teal), which span the supra-alveolar space between the plasma and IMC membranes, at the apical cap, periphery or basal polar ring, respectively. At the apical cap and basal polar ring, IAP1 (hot pink) interacts with the MyoA/C-GAP70/80-MLC1-ELC1 complex. These motor complexes interact with GAP40 (orange) and GAP50 (red) located in the IMC. GAPMs (blue) are located on the cytosolic side of the IMC membrane and interact with the alveolins (white/grey). (B) Schematic of *T. gondii* tachyzoite highlighting the apical cap (green), periphery (blue), and basal polar ring (teal) with nucleus and rhoptries depicted.

Table 1

Parasite and host cell receptors of invasion.

Parasite Receptor	EuPathDB	Host Cell Receptor	Structure
Duffy binding-like (DBL) family and erythrocyte binding-like (EBL) proteins			
PfEBA-140	PF3D7_1301600	Glycophorin C	4GF2 (Lin et al., 2012) (Region II)
			4JNO (Malpede et al., 2013) (Region II + sialyllactose)
PfEBA-175 (BAEBL)	PF3D7_0731500	Glycophorin A	1ZRL (Tolia et al., 2005) (Region II)
			1ZRO (Tolia et al., 2005) (Region II in presence of sialyllactose)
			2RJI (Withers-Martinez et al., 2008) (Region VI)
			4QEX, 4K4M, 4K2U (Chen et al., 2013) (Region II + Fab)
PfEBA-181 (JESEBL)	PF3D7_0102500	unknown	--
PvDBP	PVX_110810	DARC	3RRC (Batchelor et al., 2011) (Region II)
			4NUU (Batchelor et al., 2014) (Region II + DARC dimer)
			4NUV (Batchelor et al., 2014) (Region II dimer + DARC dimer)
PkαDBP	PKH_062300	DARC	2C6J (Singh et al., 2006) (Region II)
Reticulocyte (homolog) binding-like (RBL/RH) family			
PfRH1	PF3D7_0402300	sialic-acid containing putative erythrocyte receptor Y	--
PfRH2a	PF3D7_1335400	chymotrypsin sensitive erythrocyte receptor Z	--
PfRH2b	PF3D7_1335300	chymotrypsin sensitive erythrocyte receptor Z	--
PfRH4	PF3D7_0424200	Complement Receptor 1 (CD35)	2Q7Z (Furtado et al., 2008) (30 SCR domains of HsCR1)
			2MCZ (Park et al., 2014) (SCR domains 1, 2 of HsCR1)
			2MCY (Park et al., 2014) (SCR domains 2, 3 of HsCR1)
PfRH5/PfRIPR	PF3D7_0424100	Basigin (CD147/EMMPRIN)	4U0Q (Wright et al., 2014a) (Region II + Basigin Domain Ig1&Ig2)
			4U0R, 4U1G (Wright et al., 2014a) (Region II + Fab)
			3QR2 (Redzic et al., 2011) (Basigin Ig0)
PvRBP1/2	PVX_098585	unknown	--
Py235	PY17X_1468100	unknown	3HGF (Gruber et al., 2010) (NBD94)
Thrombospondin related anonymous protein (TRAP) family			
PfTRAP	PF3D7_1335900	unknown	4HQF, 4HQK (Song et al., 2012) (Closed form VWA + TSR domain)
			2BBX (Tossavainen et al., 2006) (TSR domain)
PvTRAP	PVX_082735	unknown	4HQL, 4HQN (Song et al., 2012) (Open form w/ Mg^{2+}/Mn^{2+})
			4HQO (Song et al., 2012) (VWA + TSR)
			4F1K (Pihlajamaa et al., 2013) (VWA domain)
			4F1J (Pihlajamaa et al., 2013) (VWA domain+ Mg^{2+})
TgMIC2	TGME49_201780	unknown	4OKR, 4OKU (Song and Springer, 2014)

Parasite Receptor	EuPathDB	Host Cell Receptor	Structure
			2XGG (Tonkin et al., 2010) (VWA domain)
PfMTRAP	PF3D7_1028700	Semaphorin-7a (CD108)	3NVQ (Liu et al., 2010) (Semaphorin-7a in complex with Plexin-C1)
PfTLP	PF3D7_0616500	unknown	--
PfCTRP	PF3D7_0315200	unknown	--
Apical membrane antigen (AMA)			
PfAMA1	PF3D7_1133400	PfRON2*	1Z40 (Bai et al., 2005) (Domains I&II)
			1YXE (Feng et al., 2005) (Domain II)
			1HN6 (Nair et al., 2002) (Domain III)
			3ZWZ (Vulliez-Le Normand et al., 2012) (Domains I&II + RON2 39 aa peptide)
			3SRI (Vulliez-Le Normand et al., 2012) (Domains I&II + RON2 29 aa peptide)
			3SRJ (Vulliez-Le Normand et al., 2012) (Domains I&II + inhibitory peptide)
			2Z8W, 2Z8V (Henderson et al., 2007) (Domains I&II + IgNAR)
			2Q8B, 2Q8A (Coley et al., 2007) (Domains I&II + Fab)
			2J5L (Igonet et al., 2007) (Fab + AMA1 region III peptide)
PvAMA1	PVX_092275	PvRON2*	1W81, 1W8K (Pizarro et al., 2005) (Domains I,II,III)
			2J4W (Igonet et al., 2007) (Fab + AMA1 region III peptide)
			4UXK[TBP](+RON2 29 aa peptide)
TgAMA1	TGME49_255260	TgRON2*	2Y8R (Tonkin et al., 2011) (Y230A Domains I,II,III)
			2X2Z (Crawford et al., 2010) (Domains I,II,III)
			2Y8T (Tonkin et al., 2011) (Domains I,II,III + RON2 37 aa peptide)
			2Y8S (Tonkin et al., 2011) (Y230A Domains I,II,III + RON2 37 aa peptide)
TgsporoAMA1	TGME49_115730	TgsporoRON2*	3ZLE (Poukchanski et al., 2013) (Domains I,II,III)
			3ZLD (Poukchanski et al., 2013) (Domains I,II,III + RON2 36 aa peptide)
BdAMA1	BBOV_IV011230	BdRON2	4APM (Tonkin et al., 2013) (Domains I,II,III)
NcAMA1	NCLIV_028680	NcRON2	4APL (Tonkin et al., 2013) (Domains I,II,III)

*RON2 is inserted into host cell membrane along with RON4, RON5, and RON8 (RON8 is not needed for *Plasmodium spp.*) forming a receptor for the parasite. RON2 is the only extracellular exposed protein.

TBP Deposited, Hold for Publication

Table 2

Proteins of the invasion machinery complex.

Protein	EuPathDB	Interacting Proteins	Structure
Motor-Receptor Bridge			
GAPDH	PF3D7_1462800	PfRH1, PfRH4, EBA-175, EBA-181 (Pal-Bhowmick et al., 2012)	1YWG (Satchell et al., 2005) (Pf tetramer)
	TGME49_289690		3STH (SSGCID et al., 2011) (Tg dimer)
	TGME49_269190	Actin	2B4R, 2B4T (Robien et al., 2006) (Pf tetramer + protease inhibitor near NAD+ site)
Aldolase	PF3D7_1444800	PfTRAP/PvTRAP/PbTRAP/TgMIC2 (Bosch et al., 2007b; Boucher and Bosch, 2014; Buscaglia et al., 2003; Jewett and Sibley, 2003)	1A5C (Kim et al., 1998) (apo Pf tetramer)
	TGME49_236040		2PC4, 2EPH (Bosch et al., 2007b) (Pf tetramer + PbTRAP peptide)
	TGME49_321900		
		Actin (Buscaglia et al., 2003)	4TU1 (Boucher and Bosch, 2014) (Tg tetramer)
		PfMTRAP (Baum et al., 2006)	4D2J (Tonkin et al., 2014b)
		PfCTRP & PfTLP (Heiss et al., 2008)	4TR9^{NR} (Pf tetramer + PbTRAP peptide + small molecule)
		TgMIC6 & TgMIC12 (Sheiner et al., 2010)	
		PfAMA1/TgAMA1 (Sheiner et al., 2010; Srinivasan et al., 2011)	
		PfRH1, PfRH2b, PfRH4 (Pal-Bhowmick et al., 2012)	
Motor Complex			
Actin	PF3D7_1246200	Aldolase (Buscaglia et al., 2003)	4CBU (Vahokoski et al., 2014) (PfActin1)
	PF3D7_1412500	Myosin A (Bergman et al., 2003; Heintzelman and Schwartzman, 1999; Herm-Gotz et al., 2002)	4CBX (Vahokoski et al., 2014) (PbActin2)
	TGME49_209030		4CBW (Vahokoski et al., 2014) (PbActin1 + muscle actin D-loop*)
		Profilin (Kursula et al., 2008; Plattner et al., 2008)	
		ADF1/2 (Allen et al., 1997; Schuler et al., 2005a)	
		Capping Proteins (Ganter et al., 2009; Tardieux et al., 1998)	
		Formin (Baum et al., 2008; Daher et al., 2010)	
Myosin A	PF3D7_1342600	Actin (Bergman et al., 2003; Heintzelman and Schwartzman, 1999; Herm-Gotz et al., 2002)	
	TGME49_235470		
		PfMTIP/TgMLC1 (Bergman et al., 2003; Herm-Gotz et al., 2002)	
PfMTIP/TgMLC1	PF3D7_1246400	Myosin A (Bergman et al., 2003; Herm-Gotz et al., 2002)	4AOM (Douse et al., 2012) (PfMTIP + PfMyoA peptide)
	TGME49_257680		
		GAP40/45/50 (Baum et al., 2006; Frenal et al., 2010; Gaskins et al., 2004)	2QAC (Bosch et al., 2007a) (PfMTIP + PfMyoA peptide, closed form)
			2AUC (Bosch et al., 2006) (PkMTIP + PyMyoA peptide, open form)
			4GGN (Turley et al., 2013) (PkMTIP + PyMyoA peptide, closed form)
			4MZK, 4MZJ (Douse et al., 2013) (PfMTIP + MyoA stapled tail peptide)

Protein	EuPathDB	Interacting Proteins	Structure
			4MZL (Douse et al., 2013) (PfMTIP + HBS mimetic peptide)
			4GFT (Khamrui et al., 2013) (PfMTIP + nanobody)
			4R1E (Douse et al., 2014)
Inner Membrane Complex (IMC)			
GAP40/45/50	PF3D7_0515700	MTIP/MyoA (Baum et al., 2006; Frenal et al., 2010; Gaskins et al., 2004)	3TGH (Bosch et al., 2012) (PfGAP50)
	PF3D7_1222700		
	PF3D7_0918000	Alveolin (Bullen et al., 2009)	
	TGME49_249850		
	TGME49_223940		
	TGME49_219320		
GAPM1/2/3	PF3D7_1323700	Alveolin & GAP45/50 (Bullen et al., 2009)	--
	PF3D7_0423500		
	PF3D7_1406800		
IMC Sub-compartment	TGME49_260820	Unknown	4CHM (Tonkin et al., 2014a) (TgISP1)
	TGME49_237820		4CHJ (Tonkin et al., 2014a) (TgISP3)
Proteins (ISPs) 1–3	TGME49_316540		
Subpellicular Network (SPN)			
Alveolin	PF3D7_0304000	GAPM1/2/3 (Bullen et al., 2009)	--
	PF3D7_1221400		
	PF3D7_1141900		
	TGME49_231640		
Actin polymerization factors			
Profilin	PF3D7_0932200	Actin (Kursula et al., 2008; Plattner et al., 2008)	2JKF (Kursula et al., 2008) (Pf)
	TGME49_293690		2JKG (Kursula et al., 2008) (Pf + Mg^{2+})
			3NEC (Kucera et al., 2010) (Tg)
Formin	PF3D7_0530900	Actin (Baum et al., 2008; Daher et al., 2010)	--
	PF3D7_1219000		
	TGME49_206430		
	TGME49_206580		
	TGME49_213370		
ADF/cofilin	PF3D7_0503400	Actin (Allen et al., 1997; Schuler et al., 2005a)	3Q2B (Wong et al., 2011), 2XF1 (Singh et al., 2011)(PfADF1)
	PF3D7_1361400		
	TGME49_220400		2XFA (Singh et al., 2011) (PbADF2)
			2L72 (Yadav et al., 2011) (TgADF)
CAP	PF3D7_0105300	Actin	2B0R (Hliscs et al., 2010) (CpC-CAP)

TBP Deposited, Held for Publication.

Table 3

Confirmed and predicted post-translational modifications of the invasion machinery components.

Protein	Myristoylation	Palmitoylation	Phosphorylation
Aldolase	-	+ Pf[1]	+ Pf/Tg[2,3,4]
GAPDH	-	-	+ Pf[2,4]
Actin 1	-	-	+ Pf/Tg[2,3,4]
MyoA	-	-	+ Pf/Tg[2,3,4,5]
MyoB	-	-	+ Pf[3,4,5]
MyoC	-	-	+ Pf/Tg[2,4]
MyoD	-	-	
MyoE	-	-	+ Pf[2,3,4,5]
MTIP	-	+ Pf[1]	+ Pf[4,6]
ELC1	-	+ Tg predicted*	
MLC1	-	+ Tg[13]	+Tg[4]
MLC2	-	+Tg[12]	+Tg[4]
GAP40	-	+ Pf[1]	+ Pf[2,3]
GAP45	+ Pf[7]	+ Pf[1,8,13]	+ Pf[2,3,5]
GAP50	-	+ Pf[1]	+ Tg[4]
GAP70	+Tg predicted[15]	+Tg predicted[15]	+ Tg[4]
GAP80	+Tg predicted[15]	+Tg predicted[15]	+ Tg[4]
GAPM1	-	+ Pf predicted*	+ Pf[4]
GAPM2	-	+ Pf[1]	+ Pf[2,3,4]
GAPM3	-	+ Pf[1]	+ Pf[5]
IAP1	-	+Tg[15]	+Tg[4]
ISP1	+ Tg[9]+ Pf[10]	+ Tg[9]+ Pf[10]	+ Pf[10]
ISP2	+ Tg[9]	+ Tg[9]	+ Tg[4]
ISP3	+ Tg[9]+ Pf[10]	+ Tg[9]+ Pf[10]	+ Tg[9]+ Pf[10]
ISP4	-	+ Tg[11]	
TgIMC1, 4, 6, 9, 10, 11, 12, 13, 14, 15		+ Tg predicted[14]	+ Tg[4]
IMC1-c (ALV5)		+ Pf[1]	+ Pf[2,3,4,5]
IMC1-e (ALV2)		+ Pf[1]	+ Pf[4,5]
IMC1-f (ALV6)		+Pf predicted*	+ Pf[3]
IMC1-g (ALV4)		+ Pf[1]	+ Pf[2,3,4,5]

1. (Jones et al., 2012)

2. (Collins et al., 2014)

3. (Lasonder et al., 2012)

[4]. (Treeck et al., 2011)

[5]. (Pease et al., 2013)

[6]. (Douse et al., 2012)

[7]. (Wright et al., 2014b)

[8]. (Rees-Channer et al., 2006)

[9]. (Beck et al., 2010)

[10]. (Poulin et al., 2013)

[11]. (Fung et al., 2012)

[12]. (Polonais et al., 2011)

[13]. (Frenal et al., 2010)

[14]. (Anderson-White et al., 2011)

[15]. (Frenal et al., 2014b)

[*] Palmitoylation predicted using CSS-Palm (Ren et al., 2008)

Table 4

DiCre-recombinase inducible knockouts in *T. gondii* effects on egress and invasion phenotypes.

Induced Knockout	Egress	Invasion	Gliding	Compensatory Mechanism	Reference
MyoA	2%	16%	37%	MyoC glideosome	Egarter et al., 2014
MyoB/C	100%	100%	100%	MyoA glideosome	Egarter et al., 2014
MyoA/B/C	2%	5%	N.D.	MyoD? MyoE? (untested)	Egarter et al., 2014
MLC1	5%	28%	42%	MyoD-MLC2? (untested)	Egarter et al., 2014
GAP45	4%	6%	100%	GAP80 over-expression Frenal et al., 2014b	Egarter et al., 2014
GAP80*	85%	80%	N.D.	GAP45	Frenal et al., 2014b
GAP40	N.D.	N.D.	N.D.	essential	Egarter et al., 2014
GAP50	N.D.	N.D.	N.D.	essential	Egarter et al., 2014
Actin 1	2%	10%	10%	?	Egarter et al., 2014
Aldolase**	N.D.	100%	100%	GAPDH?	Shen and Sibley, 2014
AMA1	N.D.	~25%	100%	Other adhesins	Shen and Sibley, 2014

* Phenotype from GAP80 clonal KO line with GAP45 iKO in absence of ATc. All other clones are inducible knockouts *via* the DiCre-recombinase system established in *T. gondii*.

** Performed in low glucose conditions to alleviate toxicity of glycolysis intermediates.