TWO EVOLUTIONARY DISTINCT TRANSLOCONS IMPORT PROTEINS INTO THE APICOPLAST OF TOXOPLASMA GONDII.

by

SWATI AGRAWAL

(Under the Direction of Boris Striepen)

ABSTRACT

Apicomplexa are a diverse and medically important group of obligate intracellular parasites. Most apicomplexans harbor a non-photosynthetic plastid (called apicoplast) that was derived by secondary endosymbiosis of a red alga. Despite being tucked away in the dark (unlike the photosynthetic plastids) the apicoplast is home to several biochemical pathways that are essential for parasite growth. Beyond its popularity as the ideal drug target, the interesting evolutionary past of the apicoplast makes it a fascinating model to explore the cell biology of endosymbiosis. In the process of synchronizing its life with the host, majority of the endosymbiont genome was transferred to the host. The nuclear-encoded apicoplast proteome must now be re-routed back to the organelle to perform its function. The mechanisms and molecules that mediate import of such large numbers of cargo proteins across the four membranes surrounding the plastid were largely unknown. It was suspected that the apicoplast might have retained all or parts of the import machinery from the ancestral endosymbiont. The research presented here focuses mainly on endosymbiont derived ERAD and ubiquitination machinery that mediate protein import across periplastid membrane of the apicoplast. By constructing conditional mutants in the $Der1_{Ap}$ and Ubc_{Ap} genes we show that these proteins are required for import and biogenesis of the organelle

and ultimately affect parasite viability. We also provide evidence for presence of additional accessory proteins such as $Cdc48_{Ap}$, $Ufd\text{-}1_{Ap}$ and Uba_{Ap} in the peripheral sub-compartment of the organelle. Additionally plant like Tic and Toc translocon components were also identified during the course of this study. Similar genetic interference studies with TgTic20, TgTic22 and TgToc75 show that a highly divergent Tic/Toc machinery mediates protein trafficking through these two innermost membranes of the apicoplast.

INDEX WORDS: Apicoplast, ERAD, Tic, Toc, periplastid membrane.

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DEDICATION

I would like to dedicate this dissertation to my parents V.K. Agrawal and Shashikala Agrawal, my sister Vasundhara and my husband Nitya for their unconditional love and persistent encouragement in every aspect of my life.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Apicomplexan parasites are responsible for some of the most important human and animal parasitic diseases including including malaria, toxoplasmosis, cyclosporidiosis, East coast fever and Babesiosis. Treatment of the aforementioned diseases face constant threats either due to drug resistance (in case of malaria), inefficacy of the available drugs in treatment of the latent parasite stages (in case of toxoplasmosis) or undesirable side affects of chemotherapy used (such as side effects associate with sulfadoxine/pyrimethamine therapy). Amongst several unifying characteristics in the phyla, like the presence of an apical complex mediating locomotion and cellular invasion, the closely apposed inner membranes of the pellicle, one of the defining characteristic of apicomplexan parasites is the presence of a non-photosynthetic plastid called "apicoplast" (with the exception of *Cryptosporidium* parasites that seem to have lost the organelle). The apicoplast was acquired during secondary endosymbiosis of a red alga by a heterotrophic eukaryote (Keeling 2009). Despite the loss of photosynthesis, apicoplast is indispensible to the parasites as it houses a range of essential biosynthetic pathways such as fatty acid, haem and isoprenoid biosynthesis (Ralph et al. 2004). Inhibition of metabolic function or interference with its DNA replication has been shown to be lethal for the parasite (Fichera and Roos 1997). The majority of the apicoplast proteome is nuclear-encoded, and targeted to the apicoplast via the secretory pathway. Very little is known about the molecular machinery that guides nuclear-encoded proteins across the four membranes surrounding the apicoplast. Three

types of translocons have been speculated to potentially act in apicoplast protein import: primary plastid-derived Tic and Toc complexes, and Der1 complexes re-tooled from their original role in translocation across the ER membrane. In this work we provide experimental evidence for the presence of two evolutionary divergent translocons in the membranes of the apicoplast and demonstrate their role in protein import.

1.2 Structure of the Dissertation

The dissertation is subdivided into five chapters. Chapter 2 is a published review article that provides an overview of our present understanding of protein import mechanisms adapted by apicoplast and other secondary plastids, in evolutionary related organisms (Agrawal and Striepen 2010). Chapter 3 presents the characterization of a novel ERAD (Endoplasmic Reticulum Associated degradation) derived complex adapted for protein import into the apicoplast. This work was published in the Journal of Biological Chemistry (Agrawal et al. 2009). In Chapter 4 we investigate the localization of two apicoplast-targeted enzymes, ubiquitin activating enzyme (Uba_{Ap}) and ubiquitin conjugating enzyme (Ubc_{Ap}) involved in substrate ubiquitination. In the absence of protein degradation in the apicoplast the function of these enzymes was not immediately clear. We sought to test if the endosymbiont acquired ubiquitylation machinery had been similarly remodeled for assisting the $Der1_{Ap}$ complex in the import process. We generated a regulatable cell line in the Ubc_{Ap} gene and demonstrated that the enzyme is required for import of apicoplast cargo and biogenesis of the organelle. Additionally there are two appendix chapters, one a published article from PNAS which highlighted the primary evidence for presence of plant like Tic complex in the innermost membrane of the apicoplast (van Dooren et al. 2008). *Tg*Tic20 was the first experimentally validated protein import factor identified in apicoplasts, and secondary plastids generally, and our studies provide the first experimental

evidence for a common evolutionary origin of import mechanisms across the innermost membranes of primary and secondary plastids. Since its discovery the apicoplast has stirred the curiosity of many researches because of its fascinating biology and pharmacological relevance. The second appendix chapter is a book chapter summarizing our present understanding of the origin, anatomy and function of the organelle (Agrawal and Striepen 2010).

The work presented in this dissertation, sheds new light on the complexity of *T. gondii* apicoplast (and other secondary plastids) protein import mechanisms. We anticipate that the advances made in this research will enable us to piece together the fascinating evolutionary puzzle.

CHAPTER 2

MORE MEMBRANES, MORE PROTEINS: COMPLEX PROTEIN IMPORT MECHANISMS INTO SECONDARY PLASTIDS[1]

[1] Agrawal, Swati and Boris Striepen. 2010. More Membranes, more Proteins: Complex Protein Import Mechanisms into Secondary Plastids. *Protist.* 161(5):672 – 687.

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Abstract

Plastids are found across the tree of life in a tremendous diversity of life forms. Surprisingly they are not limited to photosynthetic organisms but also found in numerous predators and parasites. An important reason for the pervasiveness of plastids has been there ability to move laterally and to jump from one branch of the tree of life to the next through secondary endosymbiosis. Eukaryotic algae have entered endosymbiotic relationships with other eukaryotes on multiple independent occasions. The descendants of these endosymbiotic events now carry complex plastids, organelles that are bound by three or even four membranes. As in all endosymbiotic organelles most of the symbiont's genes have been transferred to the host and their protein products have to be imported into the organelle. As four membranes might suggest this is a complex process. The emerging mechanisms display a series of translocons that mirror the divergent ancestry of the membranes they cross. This review is written from a parasite biologist viewpoint and seeks to provide a brief overview of plastid evolution in particular for readers not already familiar with plant and algal biology and then focuses on recent molecular discoveries using genetically tractable Apicomplexa and diatoms.

2.1 Introduction

The massive expansion of cyanobacteria and oxygenizing photosynthesis, began to transform the atmosphere of our planet about 2.4 billion years ago. This new atmosphere gave birth to an explosion of complex life forms that took advantage of molecular oxygen and the large amount of energy that can be gained through oxidative phosphorylation. Cyanobacteria also gave rise to the chloroplasts of plants and algae further increasing their numbers and ecological impact (Cavalier-Smith 1982; Gray 1993). There is now broad support for an endosymbiosis model of plastid genesis (Gould et al. 2008). According to this model a cyanobacterium was taken up by

an early eukaryote and subsequently domesticated into a dependent organelle (Figure 2.1A). It appears that a single endosymbiotic event was responsible for the origin of the three major extant lineages of photosynthetic eukaryotes namely the red and green algae (including their progeny the green plants) and the glaucophytes. Members of these groups possess plastids surrounded by two membranes and we will refer to these as primary plastids (Adl et al. 2005).

2.2 Endosymbiosis and the Complex Ancestry of Complex Plastids

Primary plastids were massively successful but do not yet represent the end of the journey cyanobacteria have taken through the eukaryotic tree of life. In addition plastids were acquired laterally by secondary endosymbiosis to give rise to "secondary" or "complex" plastids spreading them further into previously non-photosynthetic eukaryotes (Cavalier-Smith 1982). Although the plastid is now the most conspicuous remnant of these events, it is important to bear in mind that as depicted in Figure 2.1A, an entire eukaryotic alga was the initial endosymbiont in these events. This eukaryotic cell within a second eukaryotic cell was then gradually reduced to the feature most useful to the host, the plastid. This origin is still reflected in the additional membranes found in secondary plastids, and, as we will discuss in more detail below, also in the origin and mechanisms of some of the protein import machinery. Secondary acquisition of plastids appears to have occurred in at least three independent incidents and gave rise to major branches of the eukaryotic tree. The plastids of euglenids and chlorarachniophytes arose by enslavement of two different green algae with chlorophyll *a* and *b*. Chlorarachniophytes are a group of unicellular green protists with some purely photosynthetic members (*Lotharella globosa),* and other species that are both photosynthetic and phagotrophic (*Chlorarachnion*). Euglenids are nutritionally even more diverse, ranging from photosynthetic species like *Euglena*, to phagotrophic petalomonads and peranemids (specialized on bacterial or eukaryotic prey

respectively), to saprotrophs, which show no trace of plastids or have lost photosynthesis but retain a colorless plastid with a reduced genome. Initial studies postulated a common origin for chlorarachniophyptes and euglenids, but this hypothesis was later refuted (Rogers et al. 2007). Phylogenetic analysis of plastid proteins from the chlorachniophyte *Bigelowiella natans* supported an independent origin of plastids in these two groups (Rogers et al. 2007). In contrast, the plastids found in cryptomonads, haptophytes, stramenopiles, dinoflagellates and Apicomplexa are of red algal origin. The discovery of apicoplasts, plastids in the non-photosynthetic Apicomplexa, has fostered the idea of a common origin, for an at first sight rather incoherent group of organisms. The chromalveolate hypothesis proposes that a single endosymbiotic event followed by subsequent diversification was responsible for acquisition of the present day complex plastids in all these taxa (Cavalier-Smith 1999). The resulting super-phylum Chromalveolata (joining chromists and alveolates) represents as much as half of the thus far described protists and also includes many multicellular groups (see Figure 2.1B). In addition to the plastid containing taxa mentioned above the Chromalveolata also include groups such as the oomycetes or ciliates that may have possessed plastids in the past but have subsequently lost them (Cavalier-Smith and Chao 2006). Overall this represents a highly diverse group that has adapted to a tremendous breadth of ecological niches including autotrophy, predation and parasitism. Additional evidence in support of this hypothesis came from phylogenetic studies on glyceraldehyde 3- phosphate dehydrogenase (Fast et al. 2001; Harper and Keeling 2003; Harper et al. 2005). The common ancestor of different chromalveolates groups, cryptomonads, dinoflagellates and Apicomplexa seem to have replaced their plastid GAPDH gene with a cytosolic form that underwent duplication and acquired plastid targeting signal. It is unlikely that four different groups of organisms independently followed this complex path of locus evolution

and it appears more parsimonious to conclude that diversification of chromalveolates was preceded by a single common endosymbiotic event involving a red alga. Further support for the red algal origin of chromalveolate plastids comes from the recent description of a photosynthetic apicomplexan, *Chromera velia*, a coral symbiont (Moore et al. 2008). This discovery has also produced interesting insights on the common origin of apicomplexan and dinoflagellate plastids (Janouskovec et al. 2010). *C. velia* plastids have features found in dinoflagellate plastids including a peculiar rubisco that appears to be acquired by horizontal transfer, transcript polyuridylation, and triplet stacking of thylakoids. In contrast, the plastid encoded *psbA* gene of *C. velia* utilises the canonical UGA stop codon to encode tryptophan similar to its counterpart in the apicoplast genome and furthermore the gene order in the ribosomal superoperon is highly conserved. Careful phylogenetic anaysis of *C. velia* plastid genes shows an overall closer affiliation to apicomplexan plastids and confirms a common red algal origin of both apicomplexan and peridinin-containing dinoflagellate plastids (Janouskovec et al. 2010; Moore et al. 2008).

2.3 All plastids import the bulk of their proteome

Plastids are important organelles and home to numerous essential cellular functions. Photosynthesis is an obvious plastid function and its benefits likely drove the initial endosymbiosis. However, plastids are central anabolic hubs that provide cells with additional key metabolites such as heme, aromatic amino acids, fatty acids, isoprenoids, and phospholipids, and this is true for primary as well as secondary plastids. These metabolic functions appear responsible for plastid persistence even after loss of photosynthesis in taxa like the Apicomplexa (Seeber and Soldati-Favre 2010). The metabolic functions of the apicoplast are heavily pursued as potential drug targets in particular for the treatment of malaria. Compatible with their complex

functions, plastids have a complex proteome made up of hundreds of proteins. Although all currently known plastids maintain a organellar genome, this genome encodes only a relatively small number of proteins. As an example, the chloroplast proteome of higher plants is estimated to be comprised of about 4000 polypeptides (Leister 2003), a number far in excess of the 130 gene products encoded by the organellar genome (Wakasugi et al. 2001). Much of the symbiont's genetic information has been transferred to the host thus providing central control over function and inheritance (Kleine et al. 2009; Martin et al. 1993; Martin and Herrmann 1998). This applies to primary and secondary endosymbiosis. In the case of secondary plastids, genes were largely transferred from the nucleus of the algal symbiont to the nucleus of the host, however additional transfer may also have occurred from the symbiont's plastid genome to the host nucleus (McFadden 1999). The endosymbiont nucleus underwent severe reduction through this process and in most cases (as in the apicomplexan plastid) was lost entirely. Fascinatingly, in cryptophytes and chlorarachinophytes a small remnant of the nucleus, the nucleomorph, resides between the second and third membrane of the organelle (Douglas et al. 2001; Gilson and McFadden 1996) (see figure. 2.2 B and E for a schematic representation). The nucleomorph genomes of both groups are very compact and organized into three small linear chromosomes with telomeres at their ends (Gilson and McFadden 1995; Gilson and McFadden 2002). An obvious consequence of gene transfer is that the bulk of the proteome now has to be post-translationally imported. The relative complexity of establishing protein import mechanisms to accomplish this might have limited the number of endosymbiotic events throughout evolution (Cavalier-Smith 1999).

Our understanding of the molecular detail of protein import mechanisms in different complex plastids has benefited tremendously from the quickly growing number of complete genome

sequences (nuclear, nucleomorph and plastid) and the successful establishment of genetic transfection models in apicomplexan parasites (mostly *Toxoplasma* and *Plasmodium*), diatoms (*Phaeodactylum* and *Thalassiosira*), and most recently in chlorarachniophytes (*Lotharella amoebiformis*). In this review we will try to capture the complex journey of nuclear encoded plastid proteins, the similarities and differences in the pathways between different taxa and between plastids bounded by four versus three membranes.

2.4 N-terminal leaders guide trafficking to the plastid

Most plastid proteins initially possess sequences at their N-termini that guide targeting to the organelle, and these are commonly referred to as transit peptides. Plant chloroplast transit peptides are characterized by an overall positive charge and are enriched for the hydroxylated amino acids, serine and threonine. These transit peptides have been shown to be necessary and sufficient for targeting GFP and other reporters to the chloroplast. Secondary plastid proteins of heterokonts, cryptomonads, Apicomplexa, chlorarachinophytes, dinoflagellates and euglenoids all seem to require additional targeting information that is also encoded at the N-terminus. The targeting information for complex plastids consists minimally of a bipartite signal made up of at least two sequential elements, the signal peptide and the transit peptide. The first part is the signal peptide that allows co-translational insertion of preproteins into the ER (Bhaya and Grossman 1991). This is thought to occur using the Sec61 translocon, a mechanism described for a large variety of secretory proteins (Rapoport 2007). Numerous studies have shown that this signal peptide once isolated confers secretion to a reporter (DeRocher et al. 2000; Waller et al. 2000). Furthermore, in the bipartite context, the signal peptide can be replaced with a canonical signal peptide from a secretory protein without loss of plastid targeting (Tonkin et al. 2006b). The cleavage of the signal peptide, which is typical for this process would then reveal the second

part, the transit peptide, which appears to be responsible for the remainder of the journey of plastid proteins with the stromal compartment as final destination. Several groups have now confirmed that transit peptides from secondary plastids (euglenids, Apicomplexa, cryptomonads and heterokonts) when fused to a reporter such as GFP can mediate protein import into isolated primary plastids of higher plants in vitro suggesting that they share structural and functional characteristics with plant transit peptides (Ralph et al. 2004b). For example a truncated form of plastid GAPDH from the cryptophyte *G. theta* that lacks a signal peptide is translocated across the two membranes of the pea chloroplast (Wastl and Maier 2000). The processing of signal peptides is thought to be fast and likely occurs before the protein is synthesized to full length; transit peptide processing is slower. Using pulse chase labeling with radioactive amino acids and immuno-precipitation of an apicoplast-targeted acyl carrier protein-GFP fusion protein in *P. falciparum,* van Dooren et al. estimated that plastid proteins take an average of 90 minutes to reach the stroma (van Dooren et al. 2002).

Although the transit peptide is not clearly defined at the primary sequence level, some common features have emerged. The transit peptide of apicoplast proteins in *T. gondii* and *P. falciparum* are characterized by an overall abundance of positively charged residues. Analysis of transit peptide variants generated by mutagenesis or *de novo* design suggest that a net positive charge is necessary for import but that the exact position of positively charged residue can be flexible (Foth et al. 2003; Tonkin et al. 2006a). Complex plastids with their multiple membranes have numerous destinations that require differential targeting. An important example is the space between the 2nd and 3rd membrane, the periplastid compartment or PPC. In cryptomonads and chlorarachniophytes this is home to the reduced algal nucleus and thus likely represents the remnant of the algal cytoplasm. The nucleomorph genome encodes many of the housekeeping

proteins required to maintain this nucleus (in addition to a smaller subset that targets to the stroma of the plastid). However, a significant number of proteins essential for DNA replication and protein synthesis in this space are encoded by the host nucleus. Proteins targeted to the stroma in cryptophytes are distinguished from those targeted to the periplastid compartment by the presence of an aromatic amino acid at the +1 position of the transit peptide (Gould et al. 2006; Kilian and Kroth 2005). A similar pattern was subsequently found in plastid proteins from organisms that have lost the nucleomorph, like diatoms (Kilian and Kroth 2005), haptophytes (Patron et al. 2006), dinoflagellates (Patron et al. 2005) and *P. falciparum* (Ralph et al. 2004a). Recently Ishida and colleagues found that in the chlorarachniophyte *L. amoebiformis,* the presence of negative charges in the transit peptide of an elongation factor-like protein was important for accurate targeting to the PPC (Hirakawa et al. 2010).

Protein targeting to the three membrane bound plastid of *Euglena* occurs via the secretory pathway analogous to other complex plastids, however in some cases this requires a tripartite presequence (Durnford and Gray 2006; Patron et al. 2005). In addition to the signal and transit peptide sequences found in other complex plastids which ensure transport into the ER and across inner membranes of the plastid, *Euglena* plastid proteins feature a third domain (also present in some dinoflagellates plastid proteins). This domain acts as a hydrophobic stop transfer signal and allows precursor proteins to be transported to the plastid as integral membrane proteins (Sulli et al. 1999; van Dooren et al. 2001). Experiments using the *Euglena* light harvesting chlorophyll *a/b*-binding protein of photosystem II and a canine microsome membrane system have validated this model (Kishore et al. 1993). The first segment (a signal peptide) is required for co-translation insertion and is cleaved. The transit peptide is followed by the second hydrophobic region that prevents full translocation. Thus plastid directed proteins remain associated with the

ER (and Golgi) membranes, instead of being soluble in the lumen of the endomembrane system. Note that the bulk of the proteins remains exposed to the cytoplasm under this model, a prediction that is supported by protease digestion experiments (Sulli et al. 1999). Dinoflagellate plastid-directed proteins feature leader sequences that are functionally similar to those found in *Euglena* suggesting that the second hydrophobic region might be linked to the three membrane topology of euglenid and dinoflagellate plastids (Nassoury et al. 2003; Patron et al. 2005). It appears that the transit peptide is required and sufficient to cross the remaining inner membranes in all complex plastids. Once inside the plastid stroma, a protease related to plant stromal processing peptidase is thought to cleave the transit peptide to release the mature protein. A SPP homolog has been identified in *P. falciparum* and is speculated to reside in the apicoplast lumen (van Dooren et al. 2002).

While bipartite leader sequences have been identified and validated in many proteins in different organisms they are not found on all plastid proteins. Several apicomplexan proteins, in particular membrane and intermembrane proteins have been reported that lack a canonical plastid signaling sequences. Examples of such proteins include FtsH1, a zinc finger metalloprotease, that undergoes both N and C-terminal processing, (Karnataki et al. 2007b; Karnataki et al. 2009) ATRX1, a thioredoxin like protein that is targeted to the outermost apicoplast membrane (DeRocher et al. 2008) and apicoplast phosphate translocators that show differential targeting in *T. gondii* and *P. falciparum* (Karnataki et al. 2007a; Mullin et al. 2006). It is not clear how these proteins traverse the plastid membranes at this time but one of the transmembrane domains of these proteins may act as cryptic signal anchor. It is important to consider here that there are many distinct destinations within secondary plastids (4 membranes and 4 luminal or intermembrane compartments) that proteins need to be targeted to. The differences in the

structure and topology of transit peptides might be an important clue for differential targeting mechanisms.

2.5 Crossing multiple membranes using specific and distinct translocons

A variety of mechanistic models have been developed to explain how nuclear encoded proteins cross the many membranes that define complex plastids. Over time these models invoked internal vesicular trafficking and membrane fission and fusion (Gibbs 1981; Kilian and Kroth 2005), transport by an unspecific pore, and more recently a series of protein translocons of varied origin (Bodyl 2004; Cavalier-Smith 1999). Our view of the precise nature and sequence of these translocons has evolved rapidly over recent years as more and more molecular and functional data became available. An important observation from this work is that in general membranes are crossed with the help of translocons derived from the organism that "donated" the membrane to the complex organelle. In other words evolution matters, and mechanisms set up at the beginning are very stable and remain in place over hundreds of millions of years despite dramatic cellular reduction and reorganization. To fully appreciate this it might be helpful to the reader less familiar with the diverse set of organisms that harbor secondary plastids to quickly reconsider the origin of the various membranes involved. During secondary endosymbiosis an alga was endocytosed into a vacuole most likely derived from the host endomembrane system (Figure 2.1A), (Archibald and Keeling 2002; Delwiche 1999; Gould et al. 2008; van Dooren et al. 2001). This is believed to now represent the outermost membrane. Following this line of thought the next membrane (the periplastid membrane) would be a derivative of the plasma membrane of the alga. The two innermost membranes are likely equivalent to the two chloroplast envelope membranes. Note that as in the original chloroplast these two membrane show tight physical apposition (Tomova et al. 2006)**.** Chlorarachinophytes, heterokontophytes, haptophytes,

cryptophytes and Apicomplexa have four membrane bound plastids. Phototrophic euglenophytes and peridinin containing dinoflagellates posses plastids that are bound by three membranes (Cavalier-Smith 1999). The third membrane of euglenid plastids has been argued to be either a remnant of the algal plasma membrane, or the host food vacuole membrane (Whatley et al. 1979). It is not inconceivable that the difference in numbers of membranes may be linked to a particular form of predatory "vampirism" found in members of these taxa called myzocytosis. In this process the membrane of a prey organism is punctured and the cellular contents are sucked into a phagosome leaving the empty membrane behind (Lukes et al. 2009).

2.6 The search for Tic and Toc in secondary plastids

As detailed above the two innermost membranes of all secondary plastids are considered to be equivalent to membranes of the algal chloroplast. Plant chloroplast protein import has been studied in considerable detail and is mediated by two macromolecular protein complexes that translocate proteins across the two membranes. These are: the translocon of the outer chloroplast membrane and the translocon of the inner chloroplast membrane (Tic) (Soll and Schleiff 2004). Numerous proteins have been identified in these complexes; we focus here on a smaller subset that have also been found in secondary plastids. Critical components of the Toc complex in plants are the receptor proteins Toc34 and Toc159 that recognize transit peptide bearing proteins and direct them to the β barrel pore in the outer membrane Toc75 (Schleiff et al. 2003). Only homologs of Toc75 and Toc34 have been identified so far in the genome of the red alga *Cyanidioschyzon merolae* (McFadden and van Dooren 2004). Once through the outer membrane, proteins interact with and subsequently pass through the Tic complex. Tic 22 is located in the space between the two membranes and interacts with both Tic and Toc components and may function to hand over proteins from one to the other complex (Becker et al. 2004; Kouranov et

al. 1998). Tic20 is an integral membrane protein and together with Tic 110 has been thought to function as part of the protein pore that facilitates protein transport across the inner membrane. Note that some researchers think of Tic20 as the actual pore while others favor Tic110 (Becker et al. 2005). Inside the chloroplast lumen, the AAA-ATPase chaperon ClpC interacts with Tic40 and Tic110 and appears to provide the mechanical force needed for pulling proteins across the inner membrane (Kovacheva et al. 2005). On arrival into the chloroplast stroma the transit peptide is removed by the stromal processing peptidase (Richter and Lamppa 1998) thus releasing the mature protein to be folded properly by the stromal chaperones (Li and Chiu 2010). The transit peptide is further degraded by the presequence peptidase (Stahl et al. 2005).

Genome sequencing efforts for numerous organisms with secondary plastids has opened the hunt for components of protein translocons using bioinformatic approaches. Comparative genome-wide searches have since identified several members of the Tic complex in secondary plastid containing organisms (see Table 2.1 for a list of Tic and Toc components). The nucleomorph genome of the cryptophyte *G. theta* encodes homologs of Tic110, Tic22 and a chaperone-binding Tic complex-associated protein called IAP100 (McFadden and van Dooren 2004), while a Tic20 gene is present on the *B. natans* nucleomorph (Gilson et al. 2006)**.** Importantly, all of these components are also found in the genome of the red alga *C. merolae* (McFadden and van Dooren 2004). Homologs of Tic20 and Tic22 have also been identified in a variety of Apicomplexa with the marked exception of the plastid-less genus *Cryptosporidium* (van Dooren et al. 2008). *Toxoplasma* Tic20 (*Tg*Tic20) possesses a canonical N-terminal bipartite signal sequence that is cleaved and has four transmembrane domains. Adapting split-GFP technology van Dooren and colleagues further demonstrated that *Tg*Tic20 is an integral protein of the innermost membrane with both the N- and C-terminus of the protein facing the inside of

apicoplast stroma (van Dooren et al. 2008). The split-GFP assay uses two fragments of GFP that are inserted into candidate genes to produce translational fusions (Cabantous and Waldo 2006). If the resulting proteins co-localize to a particular compartment they assemble into a fluorescent reporter. This system has proven to be very useful to dissect the complex compartmentalization of secondary plastids in Apicomplexa and more recently in diatoms. The human parasite *Toxoplasma* has become a somewhat surprising yet quite powerful experimental model organism to study plastid biology. One of the most helpful tools is the ability to construct conditional null mutants. Such genetic analysis provides a rigorous test for the importance and function of presumptive translocon components. This is particularly important for components that only share modest sequence conservation across taxa or that are specific to secondary plastids. Genetic ablation of *Toxoplasma Tg*Tic20 leads to parasite death (van Dooren et al. 2008). While the *Toxoplasma* plastid cannot be isolated in significant quantity at this point several assays have been recently developed to measure protein import following posttranslational modification of reporter proteins. These analyses demonstrate that the *Tg*Tic20 mutant shows profound loss of protein import followed by organelle loss and cell death (van Dooren et al. 2008). Similarly a second putative member of the Tic complex, Tic22, has been identified in *P. falciparum* and *T. gondii* apicoplast (Kalanon et al. 2009). Inducible knock down of *Tg*Tic22 results in loss of import and parasite viability similar to *Tg*Tic20 ablation suggesting an important role in the apicoplast protein import apparatus (Giel van Dooren, Swati Agrawal and Boris Striepen unpublished). It remains to be tested whether Apicomplexa have lost other Tic components, which may suggest fundamental differences in protein import mechanisms between the chromists and Apicomplexa, or if these components have simply diverged sufficiently to preclude identification by primary sequence comparison.

In contrast to the Tic components, homologs of the Toc complex have been surprisingly hard to find in complex plastids. This observation could be attributed to the loss of Toc components in which case a different translocon might have replaced the Toc complex (a second Der1 translocon has been speculated to serve in this function (Tonkin et al. 2008)). Alternatively it is possible that the Toc protein homologs in secondary plastids are too divergent to be identified. Nonetheless, Bullmann and colleagues recently reported the presence of an omp85 like protein in the second innermost membrane of the complex plastid of the diatom *Phaeodactylum tricornutum* (Bullmann et al. 2010). Physiochemical studies using recombinant protein reconstituted into membranes show that *pt*Omp85 shares electrophysiological characteristics with its cyanobacterial counterparts and the Toc75 protein from land plants. Taken together the size and gating behaviour of the pore and the presence of primary and secondary sequence features suggest that this protein is a strong candidate for a Toc component in complex plastids. There are similar beta barrel proteins in Apicomplexa, their localization and function is currently unknown. A Toc75 homolog is also encoded on the nucleomorph genome of the chlorarachniophyte *B. natans* (Gilson et al. 2006). Lastly, a putative Toc34 homolog has also been described in *P. falciparum,* but its function and role in import has not been tested (Waller and McFadden 2005). The overall working model that emerges is that the two innermost membranes of complex plastids share the import machinery of primary plastids in broad strokes, but that the system has experienced considerable diversification and adaptation in different taxa.

2.7 Crossing the algal plasma membrane by retooling an ER protein extraction machinery

The second outermost membrane (in plastids with four membranes) or periplastid membrane is thought to be a remnant of the algal plasma membrane. The establishment of a protein import apparatus across this membrane can be considered a key event of secondary endosymbiosis as it

directly links host and endosymbiont opening the door for communication and gene transfer. Several hypotheses have been put forward in the past to explain traffic across this border, but the lack of experimental evidence made it hard to distinguish between these different models. Recently, substantial insights into the molecular determinants that might mediate import across this membrane have emerged. A key observation came from sequencing the nucleomorph genome of the cryptophyte *G. theta*. The highly reduced nucleomorph genome surprisingly retains the genes for key elements of the endoplasmic reticulum associated degradation system (Sommer et al. 2007) (see Table 2.1 for list of plastid specific ERAD and ubiquitination components in cryptomonads, diatoms and Apicomplexa). The ERAD pathway is generally responsible for ER homeostasis by retro-translocating misfolded secretory proteins from the ER followed by degradation by the proteasome in the cytosol. Elements of the ERAD machinery found in the cryptomonad nucelomorph genome are Der-1 (a candidate for the translocation channel), the AAA-ATPase Cdc48 and its cofactor Ufd-1. Note that there is no indication of Sec61, an alternative candidate for the ERAD pore. Der-1 is a small four trans-membrane domain containing protein, shown to be essential for retrotranslocation of misfolded luminal proteins from the ER in yeast and human cells (Ye et al. 2004). Translocating proteins are marked for degradation by conjugation of ubiquitin resulting in polyubiquitin chains. This appears to occur during translocation on the cytoplasmic face of the membrane. Cdc48 subsequently extracts these substrate proteins from the pore with the help of its cofactors, Ufd-1–Npl4 complex (Ye et al. 2004). Cdc48 has additional functions in protein extraction and degradation and many of these functions are linked to ubiquitination. Sommer and colleagues argued that protein degradation might not be the primary function of these proteins in secondary plastids and proposed a new role for this ERAD derived translocon (Sommer et al. 2007). They

speculated that components of this ERAD complex might be involved in transport across the periplastid membrane in complex plastids. The hypothesis was subsequently tested in several organisms including cryptomonads, diatoms, and Apicomplexa (Agrawal et al. 2009; Kalanon et al. 2009; Sommer et al. 2007; Spork et al. 2009). As an example we demonstrated that the *T. gondii* nuclear genome encodes multiple paralogs of key components of ERAD including the pore candidate Der1, Cdc48 and Ufd-1. Immunofluorescence analysis of epitope tagged fusion products of these genes demonstrated the presence of two putative complexes. One set of components localizes to the ER and the cytoplasm; consistent with the well-described role of ERAD in degradation of misfolded ER proteins. A second set of ERAD components however localized to the apicoplast. EM analysis further confirmed peripheral localization of these components in the apicoplast. Using split GFP assays in the diatom *P. tricornutum* Hempel and colleagues confirmed that Der-1 is associated with the third membrane as proposed in the initial hypothesis (Spork et al. 2009; Hempel et al. 2009). To further investigate the role of these plastid localized proteins, we generated a conditional knock out in the apicoplast specific Der-1 paralog in *Toxoplasma*. Genetic ablation of the protein leads to a rapid loss of protein import as measured by pulse chase assay of post-translational modification associated with plastid targeting (Agrawal et al. 2009). Loss of protein import results in apicoplast biogenesis defects and ultimately parasite death. Phylogenetic analysis of the two Cdc48 paralogues found in organisms with complex plastids showed a divergent evolutionary origin of these two proteins. The plastid specific Cdc48 paralogs of Apicomplexa and diatoms show strong affinity with their red algal counterparts (Agrawal et al. 2009) while the cytoplasmic proteins of Apicomplexa are most closely related to Cdc48 proteins of their sister phyla of ciliates and dinoflagellates. This phylogeny, along with the presence of ERAD genes on the nucleomorph genome, strongly

suggests that the plastid system evolved through the adaptation of the endosymbiont's ERAD machinery. Interestingly, such an adaptation might have been relatively straightforward. Duplication of Der1 and relocation to the plasma membrane of the endosymbiont likely represented the first step. Topologically the ERAD system still imported into same compartment, the cytoplasm of the endosymbiont, and the numerous factors required to pull, fold and modify cargo proteins were already in place.

As mentioned earlier, classical ERAD is coupled with ubiquitination to mark proteins for subsequent degradation by the proteasome. Ubiquitination is a highly regulated process that in most systems requires the activity of three key proteins, namely the ubiquitin activating (E1), conjugating (E2) and ligase (E3) enzymes to transfer ubiquitin onto target proteins. The covalent addition of ubiquitin to a substrate starts with the activation of ubiquitin using ATP to form an ubiquitin-adenylate intermediate followed by transfer to the active site cysteine residue of E1 enzyme and the release of AMP. Ubiquitin is then transferred to the next enzyme of the pathway, E2 by a trans-thioesterification reaction. In the final step the C-terminal glycine of ubiquitin is ligated through an isopeptide bond to a lysine residue of the target protein. This step is mediated by an E3 enzyme and this step confers specificity for the final substrate (Haas and Siepmann 1997; Siepmann et al. 2003). Through this process single as well as multiple ubiquitin molecules can be added to a substrate protein. Polyubiquitin chains form by addition of ubiquitin on the lysine residues of a previously attached ubiquitin. Although the earliest reported functions of ubiquitin focused on its role in proteasomal degradation (Elsasser and Finley 2005; Miller and Gordon 2005), we now know that ubiquitin is important for many other physiological processes, including endocytosis, vesicular trafficking (Hicke 2001; Raiborg et al. 2003; Staub and Rotin 2006), cell-cycle control, stress response, DNA repair, signalling, transcription and gene

silencing (Di Fiore et al. 2003; Haglund and Dikic 2005; Huang and D'Andrea 2006). Ubiquitin conjugation has been reported to be critical for both the ERAD translocation step and the subsequent degradation in the proteasome. There is accumulating evidence that cryptomonads, Apicomplexa and diatoms, all possess plastid-specific ubiquitination factors (Hempel et al. 2010; Spork et al. 2009; Swati Agrawal, Giel van Dooren, and Boris Striepen unpublished). Biochemical studies using recombinant proteins further suggest that these enzymes have the ability to activate and transfer ubiquitin. However, some questions remain. Most importantly, is ubiquitination required for plastid import? The model would suggest that plastid-targeted preproteins are ubiquitinated at some point of their journey. Unfortunately this has not been robustly demonstrated so far. However, there may be technical reasons for this, e.g. modification is only transient and therefore difficult to detect. It may be interesting to note in this context that plastid specific deubiquitinases have been reported in the *P. tricornitum* (Hempel et al. 2010). Ubiquitylation of plastid cargo may serve multiple functions. The first function that comes to mind is as an additional signal for movement of preproteins across the membranes, and this may be a requirement for the activity of Cdc48 in this process. Alternatively, ubiquitination may provide the signal necessary for retention of proteins in intermediary compartments. Ubiquitin may also serve as a specific signal for only a subset of plastid proteins. Ubiquitin has emerged at the center of numerous sorting and transport processes (Mukhopadhyay and Riezman 2007), and further work is needed to fully define its specific role(s) in the biology of complex plastids.

2.8 From ER to the outermost plastid membrane

Protein targeting in three membrane bound complex plastids like euglenophytes and dinoflagellates appears to proceed from the ER to Golgi and finally to the plastid (Inagaki et al. 2000; Nassoury et al. 2003; Sulli et al. 1999). This model is supported by strong biochemical

evidence using homologous and heterologous cell free protein trafficking systems in *Euglena*. Slavikova and colleagues further suggested that in euglenophytes vesicles bud off from the Golgi and fuse with the outermost plastid membrane in a fashion independent of SNARE proteins (Slavikova et al. 2005). Although plastids of these two groups have divergent phylogenetic origins (Euglenophytes have a plastid of green algal lineage whereas the dinoflagellates plastids have a red algal origin) they appear to have evolved similar transport mechanisms (Yoon et al. 2002). The plastid takes its place as a "vacuole type" organelle and proteins are sorted via the Golgi apparatus following typical vesicular steps. These initial steps may be less conventional in plastids with four membranes. The outermost membrane in cryptophytes, haptophytes and heterokontophytes is decorated with 80S ribosomes and is thought to have evolved by fusion of the membrane of the phagosome surrounding the symbiont with the host ER (Cavalier-Smith 2002). It may be worthwhile here to also consider recent studies that suggest a more direct functional link between the ER in certain types of endocytosis dependent on synagmotagmin VII (Idone et al. 2008; Lewis and Lazarowitz 2010). Under such a scenario an endosymbiont might reside within the ER of the host cell from the start. Nucleus encoded plastid proteins in these organisms are imported co-translationally into the ER lumen (Bhaya and Grossman 1991). Once in the ER, proteins have crossed the outermost membrane of the complex plastid and the transit peptide could then interact with the ERAD system of the periplastid membrane. In vitro experiments in canine microsomes demonstrated that signal peptide is required for microsomal import, which mimics protein import into the ER lumen. Subsequent in vivo experiments using reporter constructs that fuse signal peptides of plastid proteins from heterokontophytes, cryptophytes and Apicomplexa, showed targeting to the ER lumen or the secretory system (Gould et al. 2006; Kilian and Kroth 2005). How do proteins reach the plastid in organisms

where the plastid does not reside within the ER? There is no solid evidence for permanent connections between the ER and the apicoplast but some electron microscopy studies suggested that the two organelles come into close contact, which may reflect functional interaction (Tomova et al. 2006; Tomova et al. 2009). One might caution that connections may nonetheless be present, but too transient or too labile to be detected by electron microscopy. Brefeldin A is a fungal metabolite known to result in the redistribution of the Golgi apparatus thus blocking Golgi mediated transport steps. Interestingly in apicomplexan parasites like *T. gondii* and *P. falciparum* Brefeldin A does not perturb the trafficking of GFP reporter constructs destined to the apicoplast (DeRocher et al. 2005). Nonetheless, vesicles carrying apicoplast proteins have been reported by several independent groups using light and electron microscopy, in particular in apicoplast import mutants or mutants that have apicoplast biogenesis defects (DeRocher et al. 2008; Karnataki et al. 2007a; van Dooren et al. 2008; van Dooren et al. 2009). Overall these observations have been interpreted as evidence for a vesicular step in trafficking from the ER to the plastid that sidesteps the Golgi and is thus resistant to Brefeldin A. Such a mechanism would require a system to sort plastid-destined proteins into vesicles budding from the ER, and further imply a likely SNARE based system to ensure correct fusion of these vesicles with the plastid. There may be alternatives to this model. A new study in *P. falciparum* has detected phosphatidylinositol 3-monophosphate on the membrane of the apicoplast, the food vacuole and a subset of vesicles (Tawk et al. 2010). Additional experiments showed that interference with PI3P in *T. gondii* either by drug treatment or overexpression of PI3P binding proteins leads to severe plastid biogenesis defects (Maryse Lebrun, personal communication). More work is needed to understand the nature of these defects but they might suggest that one should consider the mechanisms active in the endosomal pathway and not solely focus on models that assume

direct forward targeting from the ER.

2.9 Conclusion

The understanding of protein import into complex plastids has made several important leaps in recent years. The ability to perform genetic experiments in an ever-growing number of systems has produced quite detailed molecular models involving numerous protein factors. The list of new candidates and new candidate mechanisms is constantly fueled by the ongoing genome mining effort. Although the process is not fully understood in any of the models collectively, a working model of subsequent translocons is gaining more and more support. Although individual translocons appear well supported, where and when they act exactly and how they are coordinated is less clear. The tools available now should be suitable to tackle open questions like the precise role of ubiquitination or phosphatidylinositol phosphorylation in plastid protein import. Overall the mechanisms at work appear remarkably conserved, in some cases there are similar mechanisms even when the organelles emerged from distinct endosymbioses. Interestingly, how proteins take the first step, from ER to the plastid's outermost compartment, shows the most diversity. For some systems like the apicoplast this also remains the least understood part of the journey. There is a fundamental distinction between three and four membrane systems. Three membrane systems follow the conventional secretory route of ER to Golgi to final target, and four membrane systems bypass the Golgi. Shedding light on the initial routing of plastid proteins and the different choices made by different organisms in this step may hold significant insights into the evolution of plastids and the eukaryotic cell in general.

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Figure Legends

Figure 2.1

(A) Diagramatic representation of the complex evolutionary process that gave rise to present day primary and secondary plastids. A cyanobacterium was engulfed by a heterotrophic eukaryote giving rise to the plastids of red and green algae (including land plants) and glaucophytes. In a second endosymbiotic event an alga was taken up by another eukaryote (note that this occurred multiple independent times). The resulting complex plastids are surrounded by four (and sometimes three) membranes. (**B)** Schematic tree of eukaryotic life highlighting the three major secondary endosymbiotic events that are thought to be responsible for present day complex plastids (*note that we excluded multiple events in dinoflagellates for simplicity). The relationships shown here are based on phylogenetic analyses summarized by Keeling and colleagues (Keeling et al. 2005). The ancestor of present day chromalveolates acquired their plastid through endosymbiotic uptake of a red alga. Diversification and adaptation to different ecological niches led to subsequent loss of photosynthesis (as in Apicomplexa) or loss of entire plastids (as in ciliates or oomycetes). Plastids of euglenids and chlorarachniophytes were acquired by two independent endosymbiotic events involving green algae.

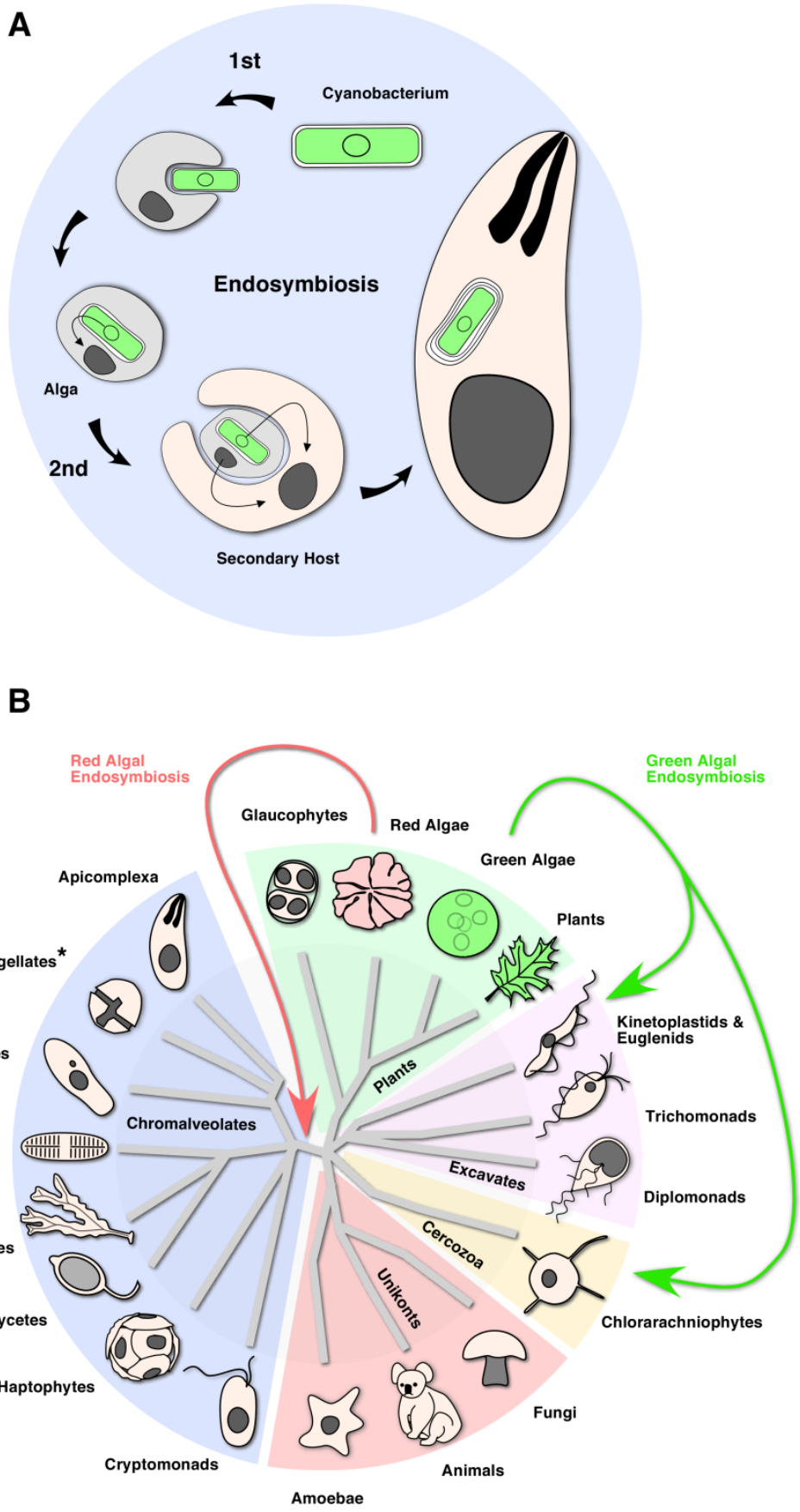
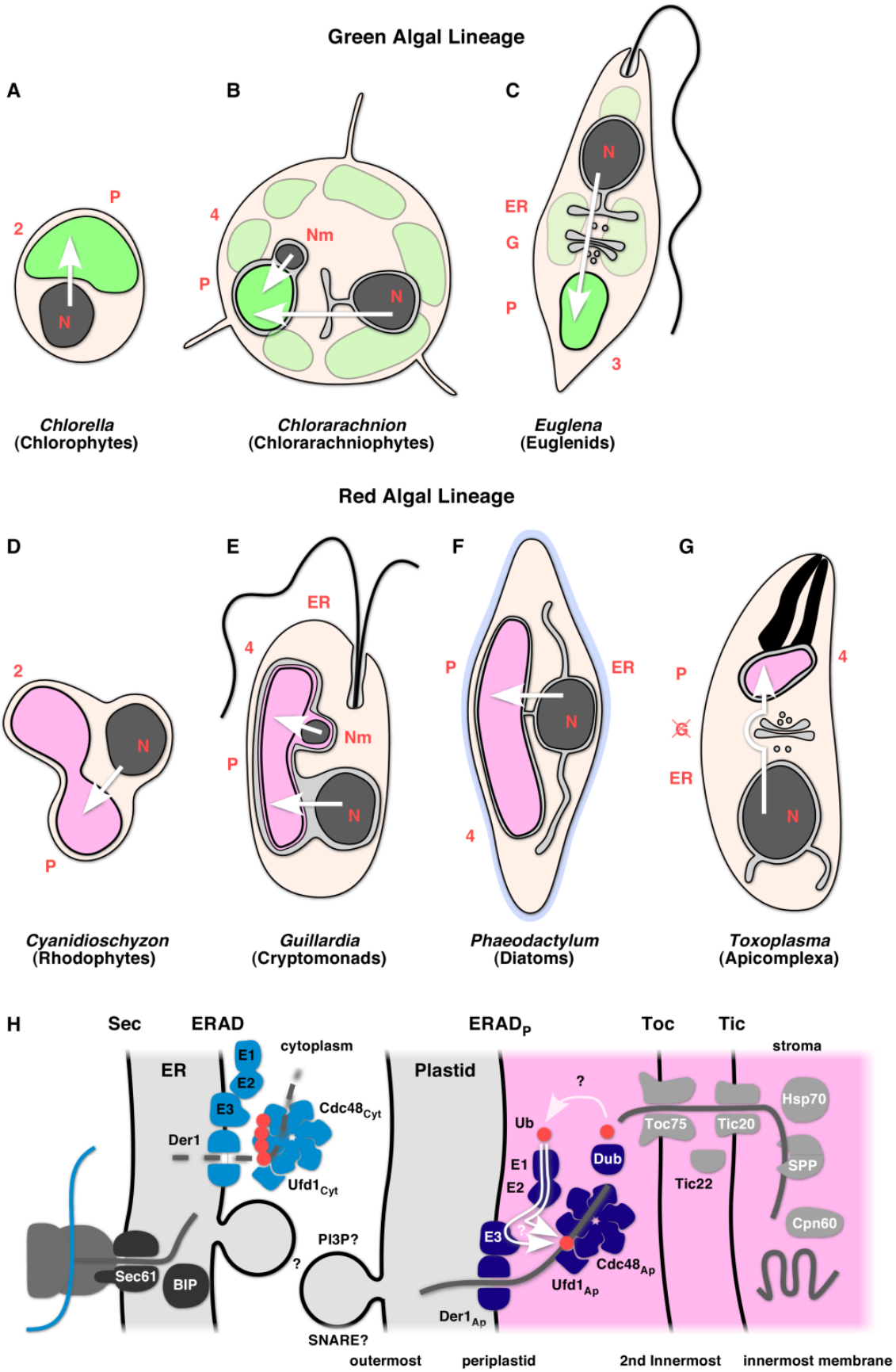

A
1st
Cyanobacterium
Endosymbiosis
Alga
2nd
Secondary Host
B
Red Algal Endosymbiosis
Green Algal Endosymbiosis
Glaucophytes
Red Algae
Green Algae
Plants
Apicomplexa
Dinoflagellates*
Ciliates
Diatoms
Phaeophytes
Oomycetes
Haptophytes
Cryptomonads
Chromalveolates
Plants
Kinetoplastids & Euglenids
Trichomonads
Diplomonads
Excavates
Cercozoa
Chlorarachniophytes
Unikonts
Fungi
Animals
Amoebae

Figure 2.2

(A) Schematic depiction of cellular morphology and the trafficking routes to the plastid for a selection of plastid containing organisms. Arrows indicate the path taken by nuclear/nucleomorph encoded proteins to reach their final destination in the plastid. (**A, D)** The green algae *Chlorella* and the red alga *Cyanidioschyzon*: nuclear encoded plastid proteins are synthesized in the cytosol and transported across the two plastid membrane with the help of Tic-Toc translocons. Engulfment of a red or green plastid by a heterotrophic eukaryote gave rise to organisms with multiple membrane bound secondary plastids. Secondary symbiosis gave rise to two main green lineages, chlorarachniophytes (**B)** euglenids **(C).** The main secondary red lineage has a common origin and includes cryptomonads **(E)**, diatoms **(F)** and Apicomplexa **(G).** A remnant of the algal nucleus, the nucleomorph (Nm) resides between the second and third outermost compartments in chlorarachniophytes and cryptomonads **(B , E).** Note that in cryptomonads and diatoms the plastid resides within the endoplasmic reticulum **(E, F).** N, nucleus, G, Golgi, P, plastid, ER, endoplasmic reticulum. **H**. Schematic outline of a molecular model of the plastid protein import machinery based on results from Apicomplexa and diatoms. Note that not all elements have been experimentally validated (several such points are highlighted by a question mark). The pathway taken by apicomplexan proteins from the ER to the apicoplast remains highly speculative. Cargo proteins are shown as grey lines, proteins destined for degradation as dashed lines. Please refer to Table 1 for further detailed reference on specific proteins.


Green Algal Lineage
A
B
C
P
2
N
4
Nm
P
N
N
ER
G
P
3
Chlorella
(Chlorophytes)
Chlorarachnion
(Chlorarachniophytes)
Euglena
(Euglenids)
Red Algal Lineage
D
E
F
G
2
N
P
ER
4
Nm
P
N
P
ER
N
4
P
G
ER
4
N
Cyanidioschyzon
(Rhodophytes)
Guillardia
(Cryptomonads)
Phaeodactylum
(Diatoms)
Toxoplasma
(Apicomplexa)
H
Sec
ERAD
ERADP
Toc
Tic
cytoplasm
stroma
ER
Plastid
E1
E2
E3
Cdc48Cyt
Der1
Ufd1Cyt
Sec61
BIP
PI3P?
?
SNARE?
?
Ub
E1
E2
E3
?
Dub
Cdc48Ap
Ufd1Ap
Der1Ap
Toc75
Tic20
Tic22
Hsp70
SPP
Cpn60
outermost
periplastid
2nd Innermost
innermost membrane

Tables

Table 2.1 : Proteins associated with membrane translocons in secondary plastids.

Note that not all proteins have been experimentally validated. Numbers refer to PlasmoDB, ToxoDB, NCBI (*G. theta* and where indicated), or PhatrDB protein identifiers respectively.

	P. falciparum	***T. gondii***	***G. theta***	***P. tricornutum***
Innermost membrane				
Tic20	PF11_0459	EU427053 (NCBI)[1]		
Tic22	PFE1460w[7]	TGME49_086050		
Second innermost membrane				
Toc75	ADG29123[2] (NCBI)	TGME49_072390[2]		1288148[2]
Periplastid membrane				
Der1-1	PF14_0498[3,7]	TGME49_081940[4]	AAK39810[5]	31697[6]
Der1-2	PFC0590c1[3]	TGME49_037460[4]		35965[6]
Cdc48	PF07_0047[3]	TGME49_121640[4]	AAK39773[5]	50978[6]
Ufd-1		TGME49_85700[4]	AAF24006[5]	49319[5]
E1	PF13_0182 PF13_0344[3]	TGME49_114890		54460[5]
E2	Mal13P1.227[3]	TGME49_095990		56431[5]
E3			CAC27064[5]	48034[6]
DeUb				ADK55599 (NCBI)[6]
Ub	PF08_0067[3]			

CHAPTER 3

GENETIC EVIDENCE THAT AN ENDOSYMBIONT-DERIVED ERAD SYSTEM FUNCTIONS IN IMPORT OF APICOPLAST PROTEINS[2]

[2] Swati Agrawal, Giel G. van Dooren, Wandy L. Beatty, and Boris Striepen.2009. *Journal of Biological Chemistry*. Nov. 27. 284(48):33683-91.

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Abstract

Most apicomplexan parasites harbor a relict chloroplast, the apicoplast, that is critical for their survival. Whereas the apicoplast maintains a small genome, the bulk of its proteins are nuclear encoded and imported into the organelle. Several models have been proposed to explain how proteins might cross the four membranes that surround the apicoplast; however, experimental data discriminating these models are largely missing. Here we present genetic evidence that apicoplast protein import depends on elements derived from the ER-associated protein degradation (ERAD) system of the endosymbiont.We identified two sets of ERAD components in Toxoplasma gondii, one associated with the ER and cytoplasm and one localized to the membranes of the apicoplast. We engineered a conditional null mutant in apicoplast Der1, the putative pore of the apicoplast ERAD complex, and found that loss of Der1Ap results in loss of apicoplast protein import and subsequent death of the parasite.

3.1 Introduction

Apicomplexa are a phylum of obligate parasites that includes the causative agents of malaria, toxoplasmosis and cryptosporidiosis. Recent evidence suggests that apicomplexans evolved from a free-living photosynthetic ancestor (Moore et al. 2008). This ancestry is reflected in the presence of a chloroplast-like organelle, the apicoplast (Kohler et al. 1997). While no longer engaged in photosynthesis, the apicoplast is essential to parasite survival and home to several critical biosynthetic pathways. Bioinformatic and experimental evidence suggests that the apicoplast is engaged in the synthesis of fatty acids, isoprenoids, and heme (Ralph et al., 2004 and Fichera and Roos, 1997). Genetic or pharmacological ablation of these pathways blocks parasite growth and the apicoplast therefore is currently considered a prime target for anti-parasitic drug development (Mazumdar et al. 2006). The organelle was derived by secondary

endosymbiosis and reflects the successful union of a red alga and a heterotrophic eukaryote (Gould et al. 2008). A large number of algal genes were transferred to the host's nucleus and their products must now be routed back into the organelle. Trafficking occurs via the secretory pathway and is guided by a N-terminal bipartite targeting sequence (Waller et al. 1998). Transport from the ER to the apicoplast is believed to be vesicle mediated and to sidestep the Golgi apparatus (Derocher et al 2005; and Tonkin et al. 2006). Once delivered to the apicoplast, proteins have to cross three additional membranes. Recently we demonstrated that a homolog of Tic20, a component of the translocon of the inner chloroplast membrane (Tic) complex in plants, is likely required for protein import across the innermost membrane of the apicoplast (van Dooren et al. 2008). To date bioinformatics searches have failed to identify a matching translocon of the outer chloroplast membrane in apicomplexans (Tonkin et al. 2008). Analysis of the genome of the remnant nucleus of the algal endosymbiont in cryptomonads showed the presence of an ER associated protein degradation (ERAD) system and offered a candidate for a translocon across the third and potentially the second membrane (Sommer et al. 2007). Classically, ERAD functions in the homeostasis of the secretory pathway by retrieving misfolded proteins from the ER that are subsequently degraded by the cytoplasmic proteasome. Der1 is a candidate for the membrane protein pore of the translocon (Ye et al. 2004). The ATPase Cdc48 together with its cofactors Ufd1 and Npl4 recognizes substrate emerging on the cytosolic side of the ER(Meyer et al. 2000) (Figure 3.1a). ATP hydrolysis then drives extraction into the cytoplasm followed by degradation in the proteasome (Flierman et al. 2003). Recently Der1 homologs have been shown to localize to the plastids of a range of secondary plastid-containing organisms (Sommer et al 2007; Sporks et al. 2009; Kalanon et al. 2009), but evidence linking these proteins to a functional role in apicoplast import is lacking. Here we use *Toxoplasma* as a

genetic model to rigorously test the function of an ERAD derived system in apicoplast biogenesis. We demonstrate the presence of two differentially localized systems, one in the ER/cytoplasm and one in the apicoplast. These systems are phylogentically distinct and the apicoplast ERAD is derived from the algal endosymbiont. Importantly, we provide direct genetic and biochemical evidence for an essential function of the ERAD membrane component $Der1_{Ap}$ in apicoplast protein import.

3.2 Material and Methods

3.2.1 Gene Identification And Gene Tagging.

Yeast sequences for Der1, Cdc48 and Ufd-1 (Genbank IDs P38307, NP_010157, and NP_011562 respectively) were used as query sequences for BLAST searches against the *T. gondii* genome (toxoDB.org) and Genbank databases (ncbi.nlm.nih.gov/blast/Blast.cgi). Initial RT-PCR experiments indicated that the automated gene predictions for the respective *T. gondii* homologs did not identify the beginning and end for all genes correctly. We performed 5' and 3'-RACE using the SMART RACE cDNA amplification kit (BD Biosciences) and the resulting PCR products were cloned and sequenced. The sequences of primers used are listed in supplementary Table 3.1. Using this approach we experimentally verified the ends of six genes and the 3' end of Ufd_{Ap}. The 5' end of the UfdAp gene was identified by RT-PCR taking advantage of array based promoter predictions (toxoDB.org and Gissot et al. 2007). The experimentally validated *T. gondii* genes described in this study are Der1-1_{ER} (FJ976521), Der1-2_{ER} (FJ976522), $Cdc48_{Cy}$ (FJ976518), $Ufd1_{Cy}$ (FJ976516), $Der1_{Ap}$ (FJ976520), $Cdc48_{Ap}$ (FJ976519) and $Ufd1_{Ap}$ (FJ976517). The full-length coding sequences were amplified from *T. gondii* cDNA using primers introducing flanking *Bgl*II and *Avr*II restriction sites, subcloned into plasmid pCR2.1 (Invitrogen) and subsequently introduced into the equivalent sites of either

plasmid pCTH or $pCTM_3$ (GvD, unpublished) placing them under the control of the *T. gondii* α-tubulin promoter and fusing a 3x HA-tag (pCTH) or a 3x cmyc tag ($pCTM_3$), respectively to the 3' end. These constructs were stably introduced into RH strain *T. gondii* parasites using chloramphenicol selection (Striepen and Soldati, 2007).

To study the localization of Ufd_{Ap} we tagged the 3' end of the gene by targeting the native locus with a cosmid clone of the respective locus (ToxoX83, see ToxoDB and Gubbels et al. 2008) modified to contain a 3xHA tag by recombineering (C.F. Brooks, GGvD and BS, unpublished). Briefly, a modification cassette containing a 3xHA tag, a phleomycin marker for selection in *T. gondii* and a gentamycin marker (Poteete et al. 2006) for selection in bacterial cells was amplified from the plasmid template pH_3BG. The modification cassette was flanked by 50 bp of targeting sequence introduced in the primer sequence to guide recombination into the cosmid at the appropriate sites at the 3' end of the Ufd1 gene (see supplemental Table 3.2). The resultant PCR product was electroporated into *E. coli* EL250 cells (Lee et al. 2001) previously transfected with the ToxoX83 cosmid. Recombination was induced by heat shock and recombinant clones were isolated by double selection on kanamycin and gentamycin. The resulting modified cosmid clone was transfected in RH strain *T. gondii* parasites and stable clones where obtained through phleomycin selection.

3.2.2 Parasite Culture And Isolation Of $Der1_{Ap}$ Conditional Mutant.

Parasites were cultured and genetically manipulated as described (Striepen and Soldati, 2007). To generate a conditional knockout of $Der1_{Ap}$, we generated a parental strain expressing an inducible copy of $Der1_{Ap}$. The $Der1_{Ap}$ coding sequence was introduced into the pDt7s4H vector (van Dooren et al. 2008) and the resulting construct was transfected into the *T. gondii* TATi strain, which expresses a tetracycline transactivator protein (Meissner et al. 2002) using

pyrimethamine selection. In this background we disrupted the native $Der1_{Ap}$ locus. To engineer a targeting construct we amplified approximately 2 kb up- and downstream of the $Der1_{Ap}$ coding sequence (see Supplemental Table 3.3 for primers) and ligated the amplicon into the *Sal*I and *Kpn*I and *Spe*I and *Aat*II sites, respectively, of vector pTCY. Linearised plasmid was transfected into the parental strain and selected on chloramphenicol. Chloramphenicol-resistant YFP-negative parasites were cloned by cell sorting as described (Mazumdat et al. 2006). To complement the Der1Ap knockout, we introduced a $Der1_{Ap}$ minigene under the control of a heterologous Tom22 promoter and a phleomycin-resistance cassette in the knockout parasite line by phleomycin selection (Messina et al. 1995). To generate a $Der1_{Ap}$ knockout cell line expressing apicoplast-targeted RFP, we digested the ptubFNR-RFP/sagCAT vector (Striepen et al. 2000) with *Bgl*II and *Not*I and ligated the FNR-RFP containing fragment into the equivalent sites of pBTH($Der1_{Ap}$). The resultant pBTR(FNR) construct was stably transfected into the $Der1_{Ap}$ knockout and parental lines using phleomycin selection.

Parasite growth was measured by fluorescence and plaque assay (Striepen and Soldati, 2007) in the presence and absence of 0.5 μM ATc.

3.2.3 Microscopy.

For immunofluorescence analysis human fibroblasts were infected with the indicated parasite strain, and fixed 24 hours after infection with 3% paraformaldehyde and permeabilized with 0.25% Triton X100 in PBS. Primary antibodies used were rabbit anti-ACP (1:1000 dilution), rat anti-HA (1:100 to 1:500), mouse anti-GFP (1:400) and mouse anti-cmyc (1:200). Secondary antibodies used were goat anti- rabbit Alexa Fluor 546 (1:500), goat anti-rat Alexa Fluor 488 and rabbit anti-mouse Alexa Fluor 488 (1:200; Invitrogen). Images were collected on an Applied

Precision Delta Vision or a Leica DIRBE microscope and images were deconvolved and adjusted for contrast using Softworx and Openlab software.

For cryo-electron microscopy infected cells were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc.) in 100mM PIPES buffer. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES at 4°C. Samples were frozen in liquid nitrogen and sectioned with a cryo-ultramicrotome. Sections were probed with the indicated primary antibodies followed by the appropriate secondary antibody conjugated to 12 or 18 nm colloidal gold, stained with uranyl acetate/methylcellulose, and analyzed by transmission EM as described previously (Brossier et al. 2008).

3.2.4 Western Blotting

Protein samples were loaded onto precast 12 % Bis-Tris and 3-8% Tris-Acetate NuPAGE gels (Invitrogen). After electrophoresis, proteins were transferred to nitrocellulose membrane. Blots were probed with antibodies against ACP (1:1000 to 1:2000 dilution; a kind gift from Geoff McFadden, U. Melbourne(9), GRA8 (1:200 000), a kind gift from Gary Ward, U. Vermont (Carey et al. 2000); anti-HA (1:100 to 1:500; clone 3F10, Roche Applied Science), anti c-myc (1:50 to 1:100; clone 9E10, Roche Applied Science), GFP (1:1000; Torry Pines Biolabs), and anti-$CDC48_{Ap}$ (1:500; generated in this study see supplement). Horseradish peroxidase-conjugated anti-rat and anti-rabbit antibodies (Pierce) were used at 1:5000 to 1:10000 dilutions, while HRP-conjugated anti- mouse antibodies (TrueBlot, eBioscience) were used at a 1:1000 dilution.

3.2.5 Pulse-Chase Labeling And Immunoprecipitation

Pulse-chase experiments were carried out essentially as described previously (van Dooren et al. 2008). Briefly, host cell cultures were infected with 2 x 10^6 parasites and grown in the presence or absence of ATc. 2 days after infection, pulse-chase labeling with 100 μCi/ml ^{35}S cysteine and methionine (MP biomedicals) was conducted as described in the results section. To test parasites that were exposed to ATc for three and four days culture were preincubated with drug in the previous passage for the appropriate time. Proteins of interest were purified by immunoprecipitation, separated by SDS-PAGE and visualized by autoradiography. Antibodies used were anti-MIC5, a kind gift from Vern Carruthers, U. Michigan (Brydges et al. 2008); anti-lipoic acid (Calbiochem), anti-RFP (Roche Applied Science), and anti-apicoplast-Cpn60 (rabbit serum raised against recombinant protein for this study).

3.2.6 Phylogenetic Analyses

For the phylogentic analysis of Cdc48 we used sequences from 30 taxa (Accession numbers for Cdc48 proteins are provided in the supplementary materials) and generated a multiple sequence alignment in ClustalX. 900 unambiguously aligned amino acid positions were used for further analysis (alignments available on request). This data set was subjected to maximum likelihood phylogenetic analysis using RAxML version 7.0.4 (Stamatakis, 2006). A phylogenetic tree was constructed using the GAMMA+P-Invar evolutionary model and the model parameters were alpha: 0.981286, invar: 0.114318 and Tree-Length: 3.905532. Bootstrap analyses were conducted using 100 replicates (Stamatakis, et al. 2008).

3.3 Results

3.3.1 Two Differentially Localized Sets Of ERAD Components In *Toxoplasma Gondii*

Using the ERAD components from yeast and their recently identified cryptophyte homologs (Sommer et al. 2007) as query sequences, we identified the genes for four putative Der1, two Ufd1 and two Cdc48 homologs in the *T. gondii* genome. Interestingly, the plastid-less apicomplexan *Cryptosporidium* retains only two Der1 homologs, and a single Ufd1 and Cdc48. The coding sequences of seven of these candidates were amplified by PCR from *T. gondii* cDNA and introduced into parasite expression constructs that result in C-terminal fusion with an epitope tag (see "Material and Methods" and supplemental Table 3.1). The resulting constructs were transfected into parasites and stable transgenic lines were obtained by drug selection. Ectopic expression of proteins from a non-native promoter can potentially impact their localization. To ensure that the observed localization patterns are physiological we also introduced an epitope tag directly into the genomic locus of one gene ($Ufd1_{AP,}$ see "Material and Methods" for more detail) and we raised antibodies to recombinant protein for a second gene ($Cdc48_{Ap}$, see supplemental methods).

Using immunofluorescence assays we found that different *T. gondii* ERAD proteins localize to distinct subcellular compartments. Figures 3.2a and 3.2b show the localization of two Der1 homologs to a perinuclear structure that co-localizes with P30-GFP-HDEL, a marker for the *T. gondii* ER (Hager et al. 1999). One homolog each of Cdc48 and Ufd1 are found in the cytosol. The localization of these four proteins is comparable with the localization of their homologs in yeast and mammalian cells, and is consistent with their likely role in the classical ERAD pathway. A second set of ERAD components localized to the apicoplast. Figures 3.2e, 3.2f and 3.2g show localization of $Der1_{Ap}$-HA, $Cdc48_{Ap}$-cmyc, and $Ufd1_{Ap}$-HA and the apicoplast acyl-

carrier protein (ACP) for comparison. Apicoplast proteins typically contain an N-terminal signal for apicoplast targeting which is cleaved upon arrival (Waller et al. 1998); van Dooren et al. 2002). Sequence analysis predicts the presence of such a signal in the apicoplast ERAD proteins. Western blot analysis reveals two bands for each protein suggesting N-terminal processing (Figure 3.2 h-j) and the respective molecular masses are consistent with the predicted full-length precursor and the processed smaller mature form of the proteins.

While ERAD proteins localize to the apicoplast, they do not colocalize fully with the luminal marker ACP (see insets in Figure 3.2 e-g). *In silico* modeling of the protein structure of $Der1_{Ap}$ suggests the presence of transmembrane domains whereas $Cdc48_{Ap}$ and $Ufd1_{Ap}$ are predicted to be soluble. To study their intraorganellar localization, we performed immuno-electron microscopy. As shown in Figure 3.3, $Der1_{Ap}$ and $Cdc48_{Ap}$ localise to an organelle that is bound by four membranes and can be labelled for the apicoplast marker ACP. $Der1_{Ap}$ and $Cdc48_{Ap}$ were confined to the periphery of the organelle, where both proteins colocalize, while ACP was found mainly in the lumen of the apicoplast. We conclude that a set of ERAD components is likely associated with the apicoplast membranes.

3.3.2 $Der1_{Ap}$ Is Essential For Parasite Growth

To genetically dissect the function of apicoplast ERAD components, we engineered a conditional null mutant in $Der1_{Ap}$, the presumptive membrane pore of the putative translocon. We introduced an epitope-tagged $Der1_{Ap}$ minigene under the control of a tetracycline-regulatable promoter into a parasite strain expressing the tetracycline transactivator protein (Meissner et al. 2002). In this background, we replaced the coding sequence of the native $Der1_{Ap}$ locus with a chloramphenicol acetyl transferase (CAT) selectable marker by double homologous recombination (Mazumdar et al. 2006; van Dooren et al. 2008). We confirmed disruption of the locus in chloramphenicol-

resistant clonal parasites by PCR and Southern blot analysis (Figure 3.4). In this mutant line $Der1_{Ap}$ expression can be suppressed by culture in the presence of anhydrotetracycline (ATc). To measure inducible $Der1_{Ap}$-HA expression, we grew parasites for 0 to 4 days on ATc, harvested parasites and detected $Der1_{Ap}$-HA protein by Western blot. We found that $Der1_{Ap}$-HA levels were greatly reduced after 1 day on ATc and undetectable after two days (Figure 3.5a).

We next asked whether $Der1_{Ap}$ was essential for parasite growth. We introduced a red fluorescent protein into both mutant and parental lines, and monitored parasite growth by measuring fluorescence intensity. In the absence of ATc, both cell lines grow at the same rate (Figures 3.6a and 3.6b). While ATc appears to have no effect on the parental strain, growth of the mutant slows dramatically after 3 days of treatment. Pretreatment for 3 days prior to the assay blocks growth entirely. Growth of the mutant is restored when we reintroduce the $Der1_{Ap}$ coding sequence under a constitutive promoter (Figure 3.6c), demonstrating the specificity of the observed phenotype. As a separate measure for parasite growth, we performed plaque assays. In this assay confluent host cell cultures are infected with a small number of parasites and incubated for 8 days. Repeated rounds of invasion, growth and egress result in the formation of clearings in the host cell monolayer that can be visualized by crystal violet staining. These experiments demonstrated minimal plaque formation in cultures infected with mutant parasites when these were grown in the presence of ATc, while parental and complemented strains showed no attenuation in growth (Figures 3.6d-f). We conclude that $Der1_{Ap}$ function is essential for parasite growth.

3.3.3 Genetic Knockdown Of $Der1_{Ap}$ Ablates Apicoplast Protein Import

While recent studies suggest that ERAD derived proteins are found in several organisms harboring secondary plastids (Spork etl a. 2009; Kaalanon et al. 2009; Hempel et al. 2009)

experimental evidence for their function is largely missing. The apicoplast ERAD system could act equivalent to the ER system in quality control-associated protein export from the apicoplast, or alternatively in protein import into the organelle (Sommer et al. 2007). To test latter possibility, we measured apicoplast protein import in the $Der1_{Ap}$ mutant by assaying the maturation and posttranslational modification of cargo proteins (van Dooren et al. 2008). We introduced a transgenic marker for the apicoplast lumen, FNR-RFP (Striepen et al. 2000), into the mutant background. This strain was then used to perform pulse-chase labeling experiments. We labeled parasites for 1 hour with ^{35}S-labeled amino acids (pulse; P), followed by washout and incubation in nonradioactive medium for 2 hours (chase; C). We next measured processing or modification of several apicoplast-targeted and control proteins by immunoprecipitation, gel electrophoresis and autoradiography. As shown in Figure 3.5b, FNR-RFP occurs as a 38 kDa precursor during the pulse and is processed during the chase to yield the mature form of 27 kDa. ATc treatment results in a significant reduction and subsequent loss of FNR-RFP maturation after 1 and 2 days of treatment, respectively (Figures 3.5b and 3.5c). Equivalent results were obtained using an antibody against Cpn60, a native luminal apicoplast chaperone, while treatment had no apparent effect on the maturation of MIC5, a secretory protein targeted to the micronemes (Brydges et al. 2008) (Figures 3.5b and 3.5c). As an independent measure of protein import we determined the level of lipoylation of the apicoplast pyruvate dehydrogenase E2 subunit (PDH-E2) (Mazumdar et al. 2006; van Dooren et al. 2008). Protein import is a pre-requisite for lipoylation of PDH-E2 as the process requires two apicoplast resident enzymes, and the precursor molecule octanoyl-ACP that is synthesized de novo in the lumen of the organelle by the type II fatty acid synthesis system (Mazumdar et al. 2006; Thomsen-Zeiger et al. 2003). An antibody was used to immunoprecipitate specifically lipoylated proteins from parasite lysate.

After 1 day on ATc the level of lipoylated PDH-E2 was reduced, falling to undetectable levels after two days (Figures 3.5b and 3.5c). Several mitochondrial enzymes are similarly lipoylated and modification relies on successful import into the mitochondrion. This process showed no observable defect in the $Der1_{Ap}$ mutant. We conclude that ablation of protein import in the $Der1_{Ap}$ mutant is specific to the apicoplast. To ensure that the observed defects are not an artifact of ATc treatment we performed control import experiments using the $Der1_{Ap}$ parental strain. In this strain ATc treatment for four days had no apparent effect on the maturation of Cpn60 or the lipoylation of PDH-E2 (see supplemental Figure 3.9).

Defects in apicoplast replication have been shown to affect apicoplast protein import (van Dooren et al. 2009). To test whether $Der1_{Ap}$ has a role in apicoplast replication, we performed live imaging of $Der1_{Ap}$ mutant parasites expressing FNR-RFP. We observe no defects for the first two days, a decrease in plastid numbers after 3 days and widespread organellar loss after 4 days (Figure 3.5d and supplemental Figure 3.10). We conclude that $Der1_{Ap}$ does not directly affect apicoplast replication and we establish the following sequence of consequences of ATc treatment in the $Der1_{Ap}$ mutant: $Der1_{Ap}$ protein levels are severely depleted after one day, directly coinciding with a loss of protein import, leading to subsequent defects in organellar biogenesis and parasite growth on days 3 and 4 post-treatment. Our data strongly support a direct role for $Der1_{Ap}$ in protein import into the apicoplast.

3.3.4 The Apicoplast ERAD System Is Derived From The Red Algal Endosymbiont

The apicoplast ERAD system could have evolved by duplication of ERAD genes of the host or alternatively could be derived by horizontal gene transfer from the algal endosymbiont. We performed phylogenetic analyses for the *T. gondii* ERAD components. The strong sequence divergence of Der1 proteins precluded the construction of meaningful alignments and trees.

However, Cdc48 proteins are highly conserved providing 900 unambiguously aligned residues for robust analysis. We find that the two *T. gondii* proteins are of divergent phylogenetic origin (Figure 3.7). The cytoplasmic protein forms a well-supported clade with homologs from chromalveolates (including photosynthetic and plastid-less taxa). This placement is consistent with the current model of vertical evolution of Apicomplexa as established by phylogenetic analyses using numerous protein and ribosomal RNA sequences (Keeling, 2009; Cavalier-Smith, 2002). In contrast, $Cdc48_{Ap}$ clusters with proteins from organisms harboring secondary plastids of red algal origin. While analysis of Ufd1 did not provide full resolution of the tree of life due to a lower level of sequence conservation when compared to Cdc48, it fully supported divergent ancestry of the cytoplasmic and apicoplast protein (Figure 3.3). We conclude that the apicoplast ERAD system is likely derived from the red algal endosymbiont while the classical ER resident system was inherited vertically.

3.4 Discussion

Endosymbiosis is now well established as a mechanism that has played a crucial role in the evolution of eukaryotic cells. One organism, the symbiont, is engulfed by a second organism, the host, and a stable symbiotic relationship ensues in which the endosymbiont loses its independence and gradually evolves into an organelle that is controlled by the host and serves the host's metabolic needs. A common hallmark of this process is massive horizontal gene transfer from the endosymbiont to the host (Kleine et al. 2009). This gene transfer affords control to the host but also requires the establishment of mechanisms to reroute proteins that are now encoded and synthesized by the host back into the symbiont. A large body of work on mitochondria and chloroplasts has demonstrated the presence of elaborate protein translocons in the inner and outer membranes of these organelles (Neupert and Herrmann, 2007; Jervis, 2008)

that specifically recognize targeting information and deliver cargo proteins accordingly to the organellar lumen or various membrane compartments.

The apicoplast is the product of secondary endosymbiosis, the enslavement of a single celled eukaryotic alga. Compared with their primary progenitors, secondary plastids are surrounded by additional membranes that must be traversed by nuclear-encoded proteins (a total of four membranes surround the apicoplast). The evolution of mechanisms to traverse these additional membranes must have occurred early in organelle acquisition, and a simple solution would have been to use existing protein transport complexes. Our previous studies support this model and have shown that transport over the innermost apicoplast membrane is dependent on elements derived from the translocon of the inner chloroplast membrane of the endosymbionts chloroplast (van Dooren et al. 2008). The conservation of the Tic complex would make it appear likely that the Toc complex is equally conserved. However, so far genome searches have failed to identify Toc components in Apicomplexa or diatoms (Tonkin et al. 2008). The presence of the Toc complex might be masked by a high level of sequence divergence of its components or alternatively indicate that it has been replaced by a different mechanism. How proteins might cross the third or periplastid membrane is of particular interest as this membrane is thought to be a derivative of the endosymbionts plasma membrane. Based on the discovery of genes encoding elements of the ERAD system in the nucleomorph in cryptophytes, Sommer and colleagues proposed that this complex was retooled to function in trafficking of plastid proteins (Sommer et al. 2007) {proteins of ERAD origin have also been speculated to be part of the peroxisomal proteome (Gabaldon et al. 2006)}. Most recently this model has received support from studies on a variety of organisms bearing secondary plastids including the current study. ERAD-associated proteins are found in the membranous compartment surrounding secondary plastids in the

apicomplexans *Plasmodium* and *Toxoplasma* (Spork et al. 2009; Kalanon et al. 2009) (and Figure 3.2, Figure 3.3 and supplemental Figure 3.8), the diatom *Phaeodactylum* (Spork et al. 2009), and the cryptophyte *Guillardia* (Sommer et al. 2007). Light and electron microscopic experiments indicate that these proteins are associated with the outer membranes of the plastid, but they lack resolution to tie the system to a specific membrane. Interestingly, split GFP assays in *Phaeodactylum* demonstrate the presence of two Der1 homologs in the third membrane. However, it is important to note that these experiments do not exclude the presence of Der1 homologs in other plastid membranes. Additional markers are needed to establish if the ERAD system is limited to the third membrane or might also be found in the second membrane and thus have replaced the Toc complex previously present in this membrane (Figure 3.1d).

While the presence of an ERAD translocon in the membranes of plastids is consistent with a role in plastid biology, it was unclear what this role of the ERAD complex might be. Functional data discriminating between a role in protein import versus protein export and quality control have so far been missing. While symbiont-derived Der1 proteins in *Phaeodactylum* have been shown to interact at a steady state level with fusion proteins targeted to the periplastid compartment no interaction was observed with fusion proteins targeted to the plastid lumen (Hempel et al. 2009). In the current study we have used the ability to construct conditional mutants in *T. gondii* to devise a rigorous test of the protein import hypothesis. We isolated a *T. gondii* Der1_{Ap} mutant and demonstrate that this protein is essential for apicoplast protein import and parasite survival. We have previously shown that apicoplast protein import across the innermost membrane is essential for *Toxoplasma* survival (van Dooren et al. 2008). In contrast, genetic disruption of classical ERAD in yeast or mammalian systems does not affect cell viability (Travers et al. 2000) and mutant cells have to be subjected to stressors resulting in the accumulation of misfolded

proteins to produce a viability phenotype. More directly, we have developed and validated assays that track posttranslational modifications of reporters that are restricted to the apicoplast lumen and thus require successful import (van Dooren et al. 2008). Applying these assays to the current study, we demonstrate a direct correlation between the loss of $Der1_{Ap}$ and a complete loss of apicoplast protein import (Figure 3.5). These genetic and biochemical experiments provide the strongest evidence to date that the novel plastid ERAD system has a direct and essential role in plastid protein import.

Our phylogenetic analyses of CDC48 and Ufd1 indicate that the *T. gondii* ERAD systems are phylogentically distinct and that the apicoplast system is derived from the red algal endosymbiont (Figure 3.7 and supplemental Figure 3.11). This is consistent with the presence of homologs in the cryptophyte nucleomorph, a remnant of the algal nucleus (Sommer et al. 2007). It thus appears that the Der1 protein of the red algal symbiont was re-targeted from its original location in the ER to the symbiont to its plasma membrane, where it could now function in importing plastid-targeted proteins from the host secretory pathway (Figures 3.1a-c). This represents a remarkably simple and elegant solution for the complex problem of how to evolve protein exchange between host and endosymbiont at the beginning of their relationship. Once targeted to the endosymbionts cytoplasm proteins could take advantage of established mechanisms, namely the Toc and Tic, to gain access to the chloroplast. We hypothesize that the apicoplast employs a series of specific translocons that reflect the diverse evolutionary origin of the membranes in which they reside (Figure 3.1d).

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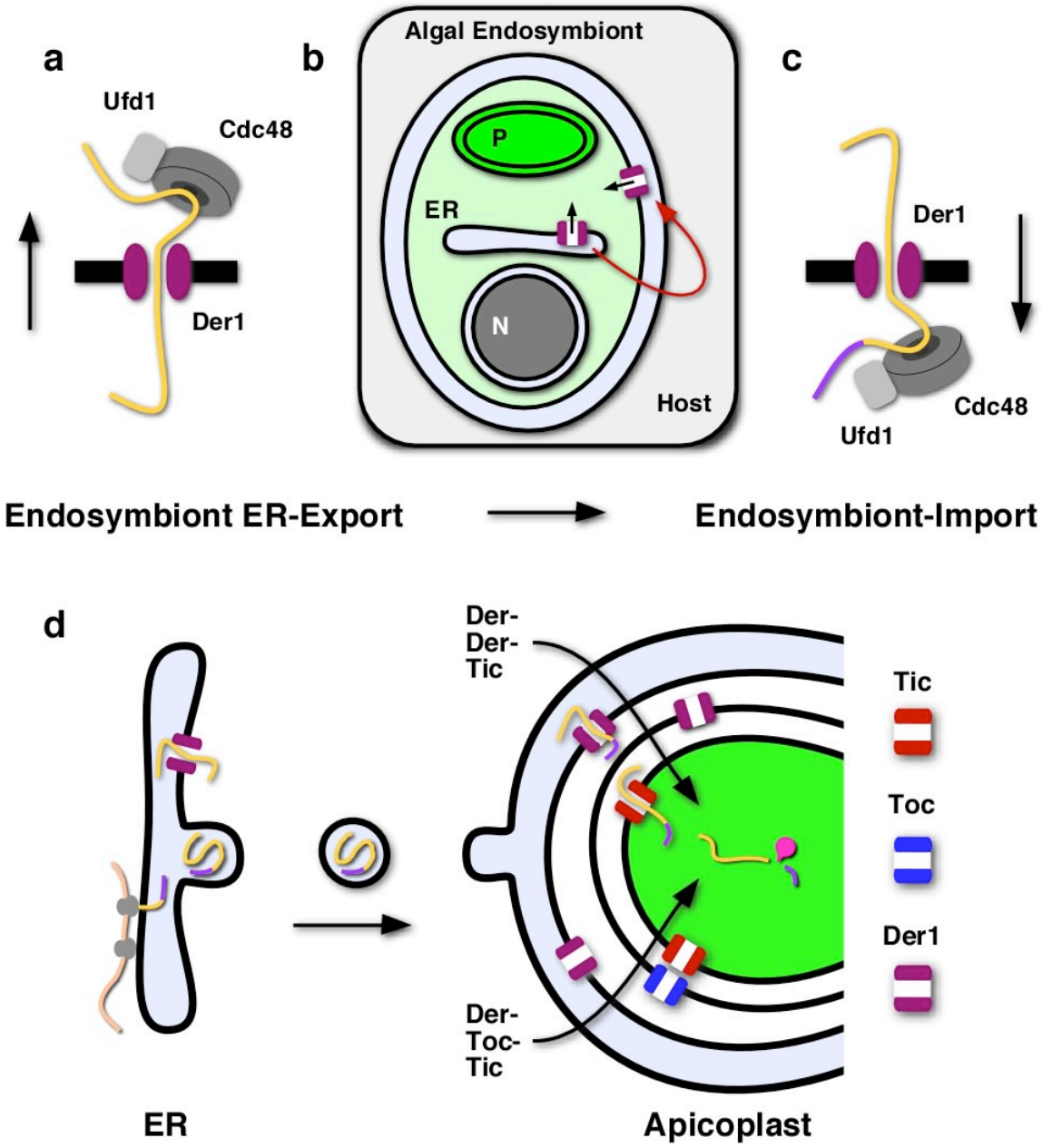
Figures

Figure 3.1: Multiple distinct translocons act in apicoplast protein import

Multiple distinct translocons act in apicoplast protein import. **a**, Der1, Cdc48 and Ufd1 are believed to be core components of the translocon of the endoplamic reticulum-associated degradation (ERAD) pathway. **b**, Duplication of the Der1 gene in the algal endosymbiont and relocation of its protein product to its plasma membrane enables **c**, protein import from the host endomembrane system. **d**, Schematic representation of trafficking of nuclear encoded proteins to the lumen of the apicoplast comparing a model employing subsequent Der1, Toc and Tic translocons with a model in which the Toc translocon has been subsequently replaced by a second Der1 translocon. Cargo proteins are shown in yellow. The transit peptide (purple) is cleaved upon arrival. Note that trafficking of certain apicoplast membrane proteins appears not to require a bipartite signal sequence (48) and might occur through a different mechanism.


a
Ufd1
Cdc48
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b
Algal Endosymbiont
P
ER
N
Host
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Der1
Cdc48
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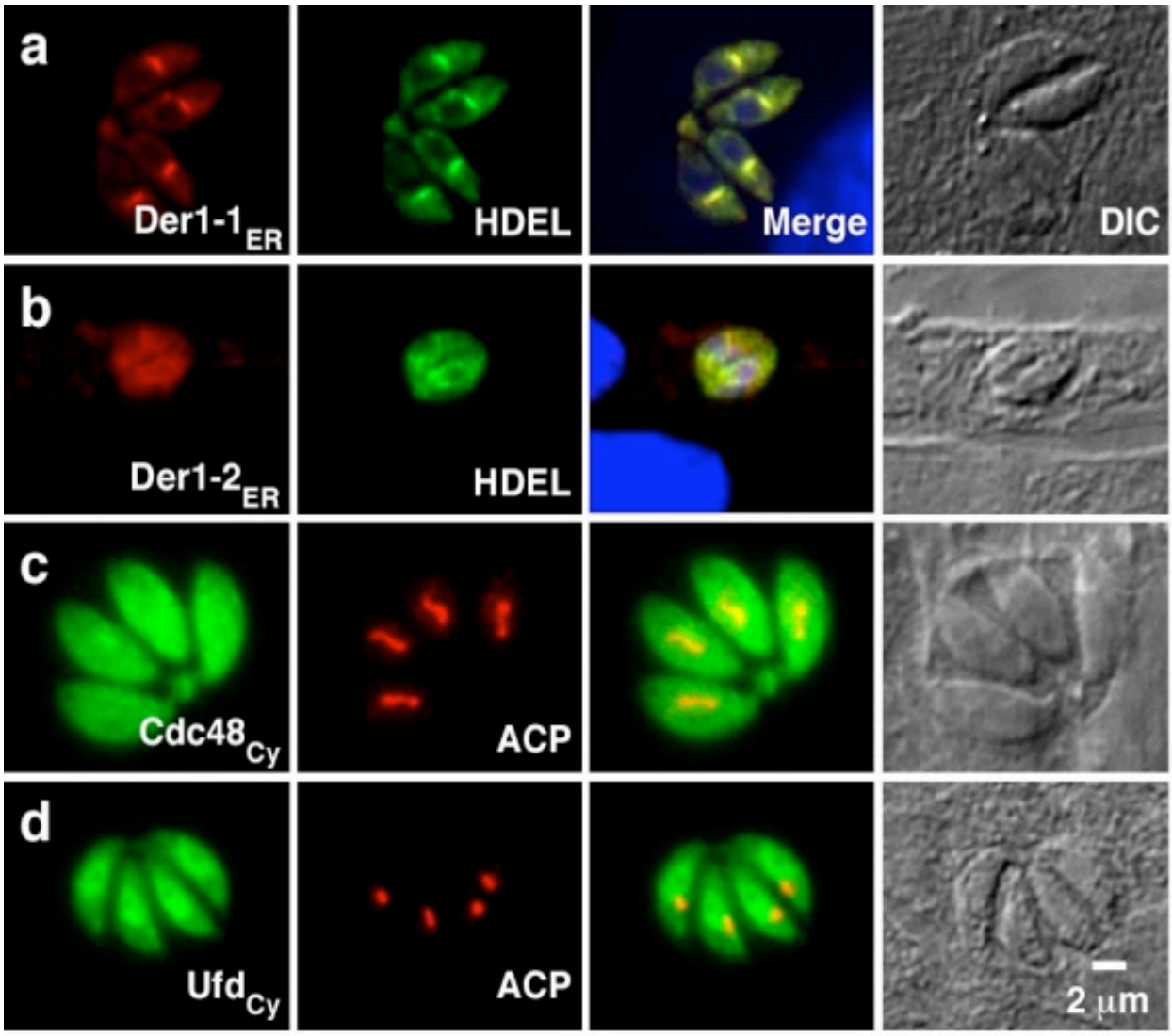
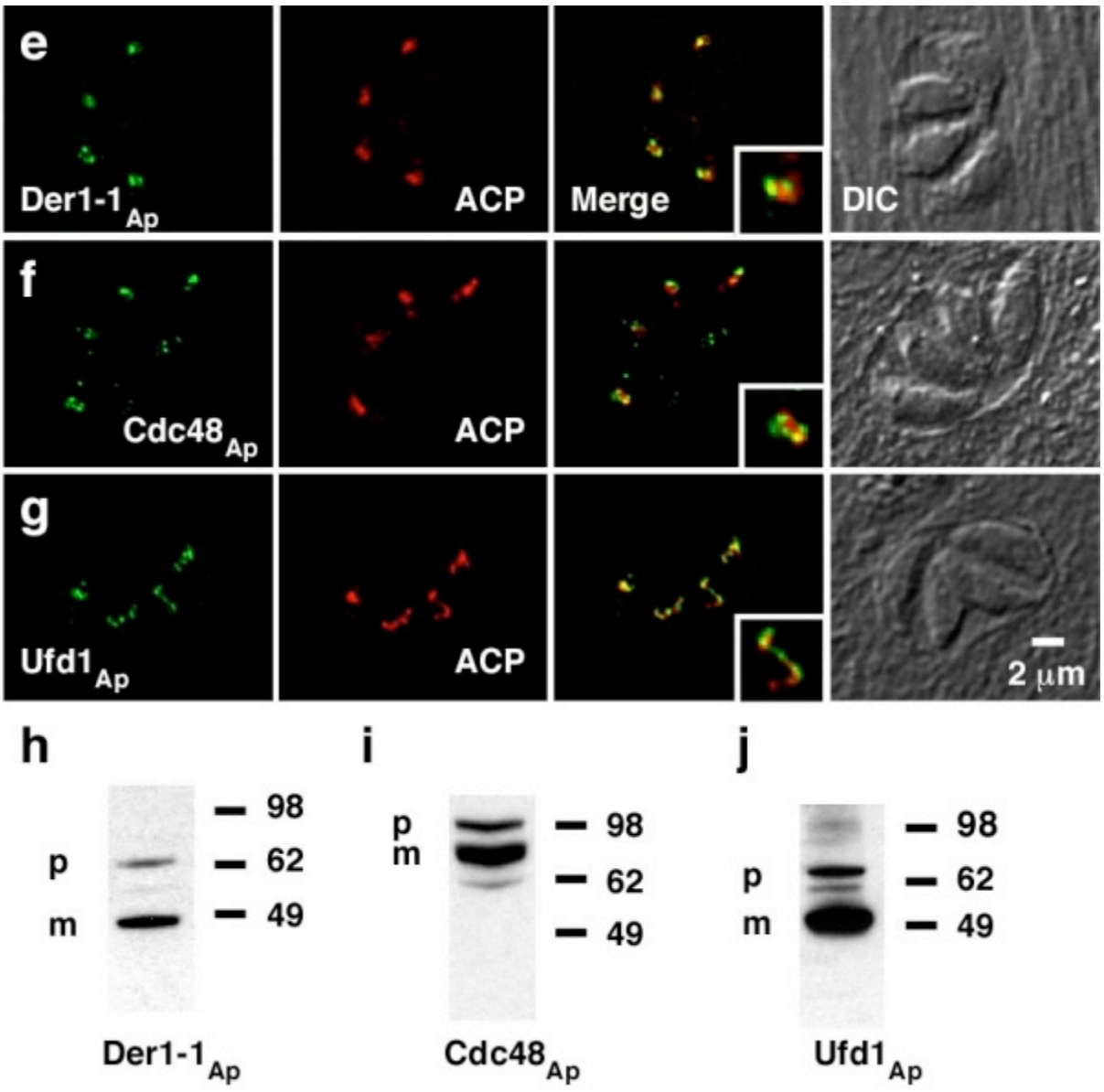
Endosymbiont-Import
d
Der-
Der-
Tic
Tic
Toc
Der1
Der-
Toc-
Tic
ER
Apicoplast

Figure 3.2: *Toxoplasma gondii* ERAD components are associated with the ER/cytoplasm and the apicoplast.

Toxoplasma gondii ERAD components are associated with the ER/cytoplasm and the apicoplast. **a-g**, Immunofluorescence analysis of parasites transfected with the coding sequences of seven putative *T. gondii* ERAD proteins carrying a c-terminal epitope tag (**g**, the $Ufd1_{Ap}$ epitope tag was constructed by inserting the tag directly into the genomic locus, see supplementary Methods). We also expressed $Cdc48_{Ap}$ as recombinant protein and raised a specific anti-serum. Localization of the native protein detected with this antibody is indistinguishable from the protein derived from the tagged transgene (Figure 3.5 and Figure 3.11). Insets in the merge in panel **e-g** show a two-fold higher magnification. **h-j**, Western blot analysis of apicoplast ERAD components. As for most apicoplast proteins two bands are apparent (p, precursor; m, mature protein (9,36)). ACP, acyl carrier protein (luminal apicoplast marker), HDEL; *T. gondii* ER marker P30-GFP-HDEL (35).




e
Der1-1Ap
ACP
Merge
DIC
f
Cdc48Ap
ACP
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Ufd1Ap
ACP
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Der1-1Ap
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Ufd1Ap

Figure 3.3: The ERAD components $Der1_{Ap}$ and $Cdc48_{Ap}$ localize to the membrane compartment surrounding the apicoplast.

The ERAD components $Der1_{Ap}$ and $Cdc48_{Ap}$ localize to the membrane compartment surrounding the apicoplast. Host cells infected with a transgenic *T. gondii* line expressing a HA-tagged version of $Der1_{Ap}$ were fixed, frozen and sectioned with a cryo-ultramicrotome. Sections were incubated with a rat antibody to HA (**a-b**, **d,** black arrowheads), a rabbit serum to ACP (9) (**a** and **b,** white arrowhead), and a newly developed rabbit antibody against recombinant $Cdc48_{Ap}$ (**c-d**, white arrowhead with black outline, see also supplementary Fig. S1) followed by secondary antibody conjugated to 12 or 18 nm colloidal gold, stained with uranyl acetate/methylcellulose, and analyzed by transmission electron microscopy. Panel **b**, shows a higher magnification of the cell shown in panel **a**, the four membranes of the apicoplast are indicated by black arrowheads outlined in white. Ap, apicoplast; Go, golgi; Nu, nucleus.

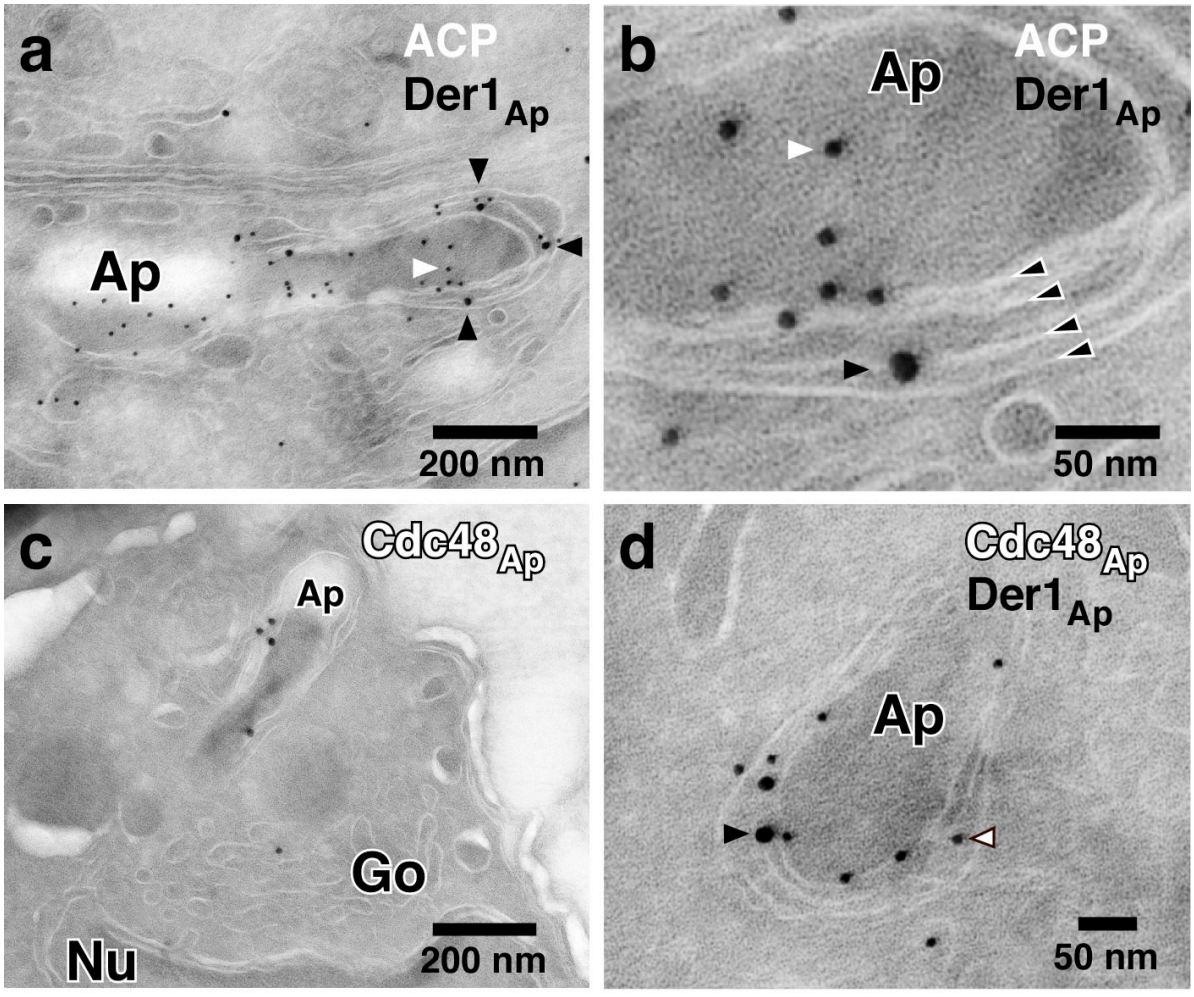


Figure 3.4: Disruption of the native $Der1_{Ap}$ locus.

Disruption of the native $Der1_{Ap}$ locus. **a**, The $Der1_{Ap}$ locus was disrupted by homologous recombination using a targeting construct carrying a chloramphenicol acetyl-transferase (CAT) marker flanked by 2 kb of gene specific upstream and downstream sequences. Recombinants were isolated using chloramphenicol to select for CAT expression, and cell sorting to select against YFP expression. Wild type (RH-WT), parental (carrying the native ($Der1_{Ap}$) as well as an inducible minigene version ($iDer1_{Ap}$), and $DDer1_{Ap}$ (carrying only the inducible version) were tested for the presence of the native locus by **b**, PCR (position of primers is indicated in **a**), or **c**, Southern blot using the radiolabeled coding sequence of $Der1_{Ap}$ as probe and Nde1 restriction to distinguish the native and inducible locus.

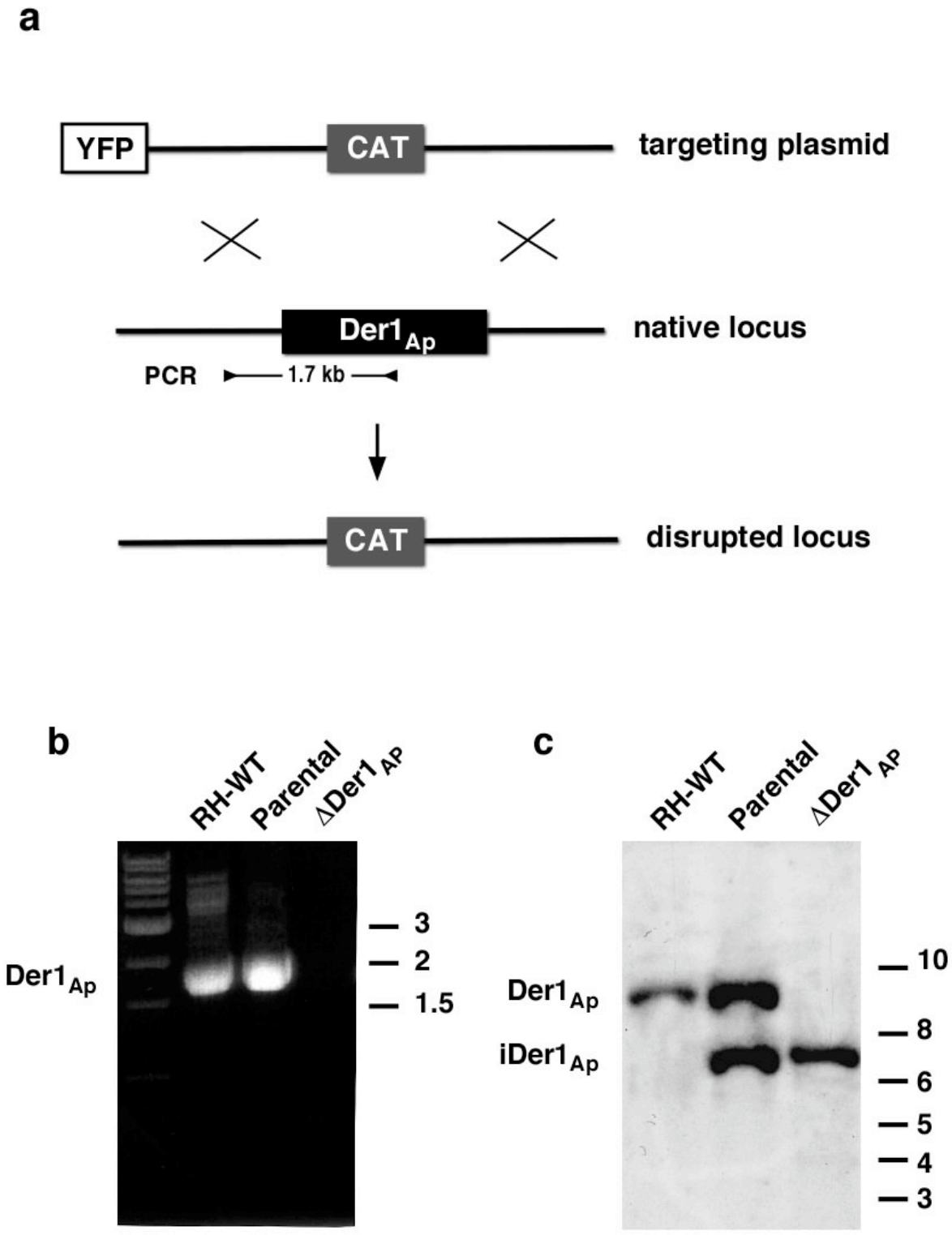

a
YFP
CAT
targeting plasmid
Der1Ap
native locus
PCR
1.7 kb
CAT
disrupted locus
b
RH-WT
Parental
ΔDer1AP
Der1Ap
3
2
1.5
c
RH-WT
Parental
ΔDer1AP
Der1Ap
iDer1Ap
10
8
6
5
4
3

Figure 3.5: Genetic ablation of $Der1_{Ap}$ results in loss of apicoplast protein import and organellar defects.

Genetic ablation of $Der1_{Ap}$ results in loss of apicoplast protein import and organellar defects. **a**, Western blot analysis of $Der1_{Ap}$-HA levels in $DDer1_{Ap}$ in response to ATc treatment (the dense granule protein GRA8 serves as loading control). **b**, Pulse-chase (P/C) analysis of posttranslational modification of apicoplast (FNR-RFP, Cpn60, PDH-E2), mitochondrial (mito(E2)), and secretory (MIC5) proteins. The data shown is representative of three independent experiments. Bands labeled with * likely represent RFP expression from an internal translation start site, mitochondrial Cpn60, and human PDH-E2 (12), respectively. Note that ATc treatment in parental parasites has no effect on apicoplast protein import (see Figure 3.9 and (12)) **c**, Phosphorimager quantification of mature apicoplast targeted protein bands shown in **b**. **d**, Number of "four parasite" vacuoles in which each parasite has a clearly discernable apicoplast (n= 100 for each data point, see supplementary Figure 3.2 for additional detail).

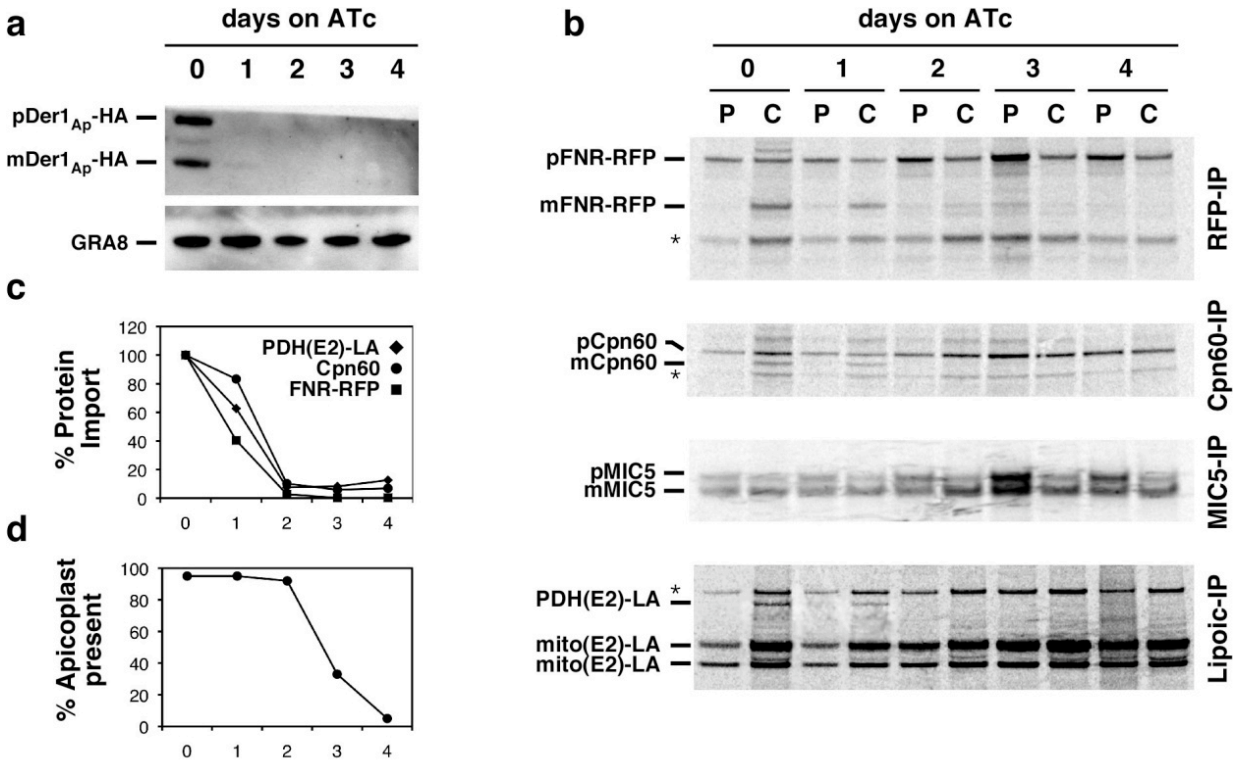


Figure 3.6: $Der1_{Ap}$ is essential for parasite growth.

$Der1_{Ap}$ is essential for parasite growth. **a-c**, fluorescence growth assays for the $DDer1_{Ap}$ mutant carrying the inducible copy only (**a**), the parental strain carrying both inducible and native $Der1_{Ap}$ (**b**), and a $DDer1_{Ap}$ clone complemented with the $Der1_{Ap}$ gene driven by a constitutive promoter (**c**; all strains were engineered to express RFP). Assays were performed in triplicate (error bars reflect standard deviation, note that error bar is only shown if larger than symbol (>3%)) in the absence (circles) and presence (squares) of 0.5 mM ATc, or after three days of ATc preincuabtion (triangles). **d-f** Plaque assays measuring impact of loss of $Der1_{AP}$. Confluent HFF cultures were infected with 400 parasites of the $DDer1_{Ap}$ mutant (**d**), parental (**e**), or complemented (**f**) strain respectively and cultured for 9 days in the absence (-ATc) or presence (+ATc) of anhydrous tetracycline. Cultures were fixed and stained as described in the methods section. Note loss of growth in the mutant under ATc that is restored by gene complementation.

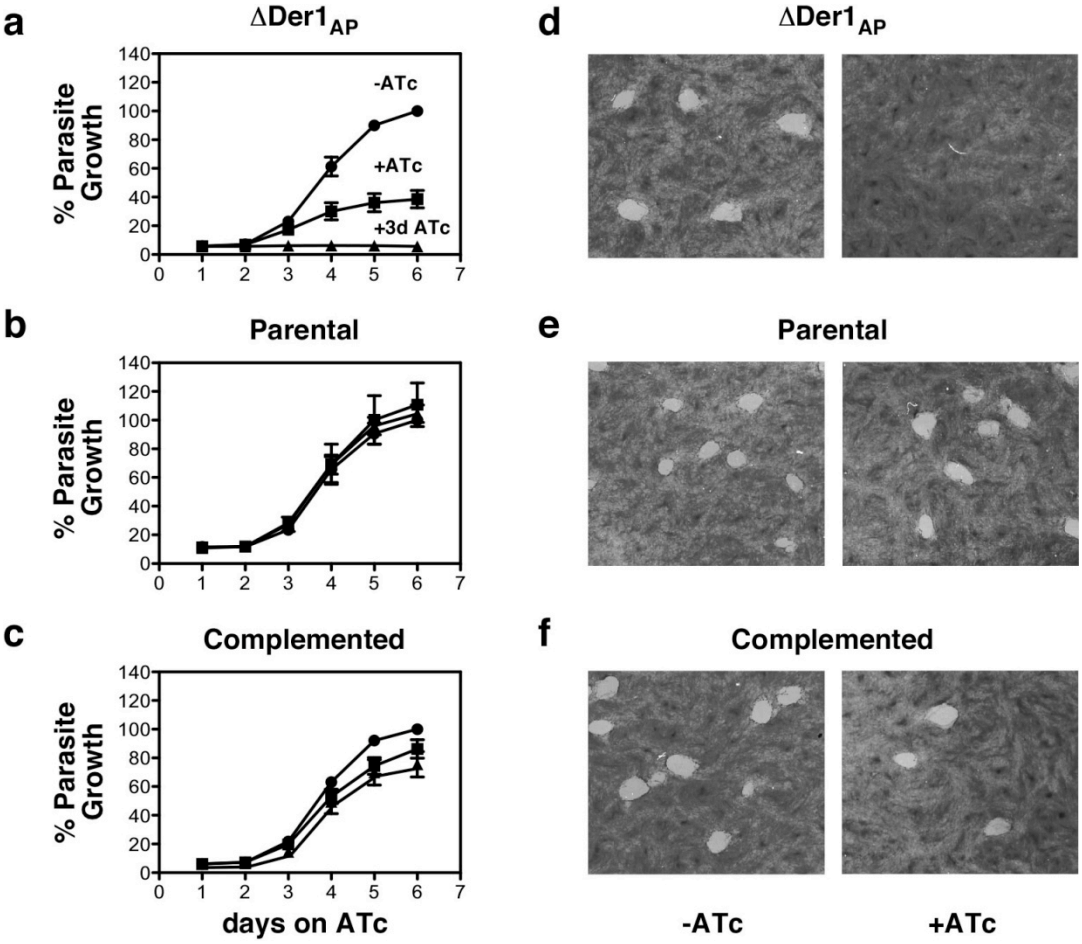
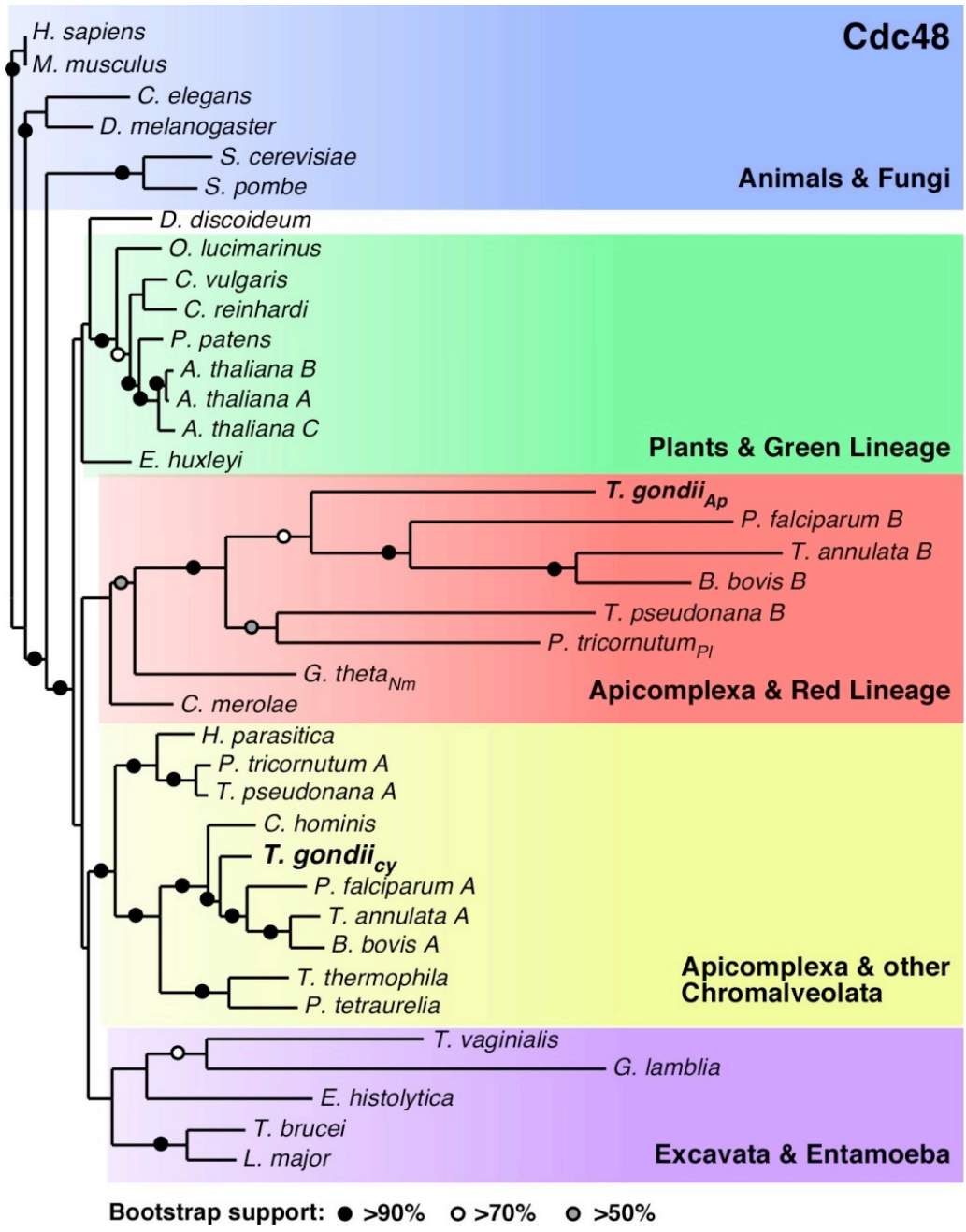


Figure 3.7: Divergent origins of *T. gondii* Cdc48 proteins. RAxML maximum likelihood tree derived from an alignment of Cdc48 proteins from 30 taxa

Divergent origins of *T. gondii* Cdc48 proteins. RAxML maximum likelihood tree derived from an alignment of Cdc48 proteins from 30 taxa (900 unambiguously aligned amino acid characters were used after manual inspection, accession numbers are provided in the supplement). Bootstrap analyses were conducted using 100 replicates. Nm, nucleomorph (remnant endosymbiont nucleus), Pl, plastid, Ap, apicoplast, Cy, cytoplasm.



Supplemental Figures

Figure 3.8

Antibodies raised against recombinant $Cdc48_{AP}$ and Cpn60. Fragments of the $Cdc48_{Ap}$ and Cpn60 genes were expressed as His-tagged recombinant fusion proteins in *E. coli*. The proteins were purified by affinity chromatography and used to immunize rabbits. The resulting anti-sera were tested against *T. gondii* by **a**,**b**, Immunofluorescence and **c**, **d** Western blot. Both sera (aCpn60, $aCdc48_{Ap}$) detect protein products of the expected molecular weight and show co-labeling with the apicoplast marker FNR-RFP.

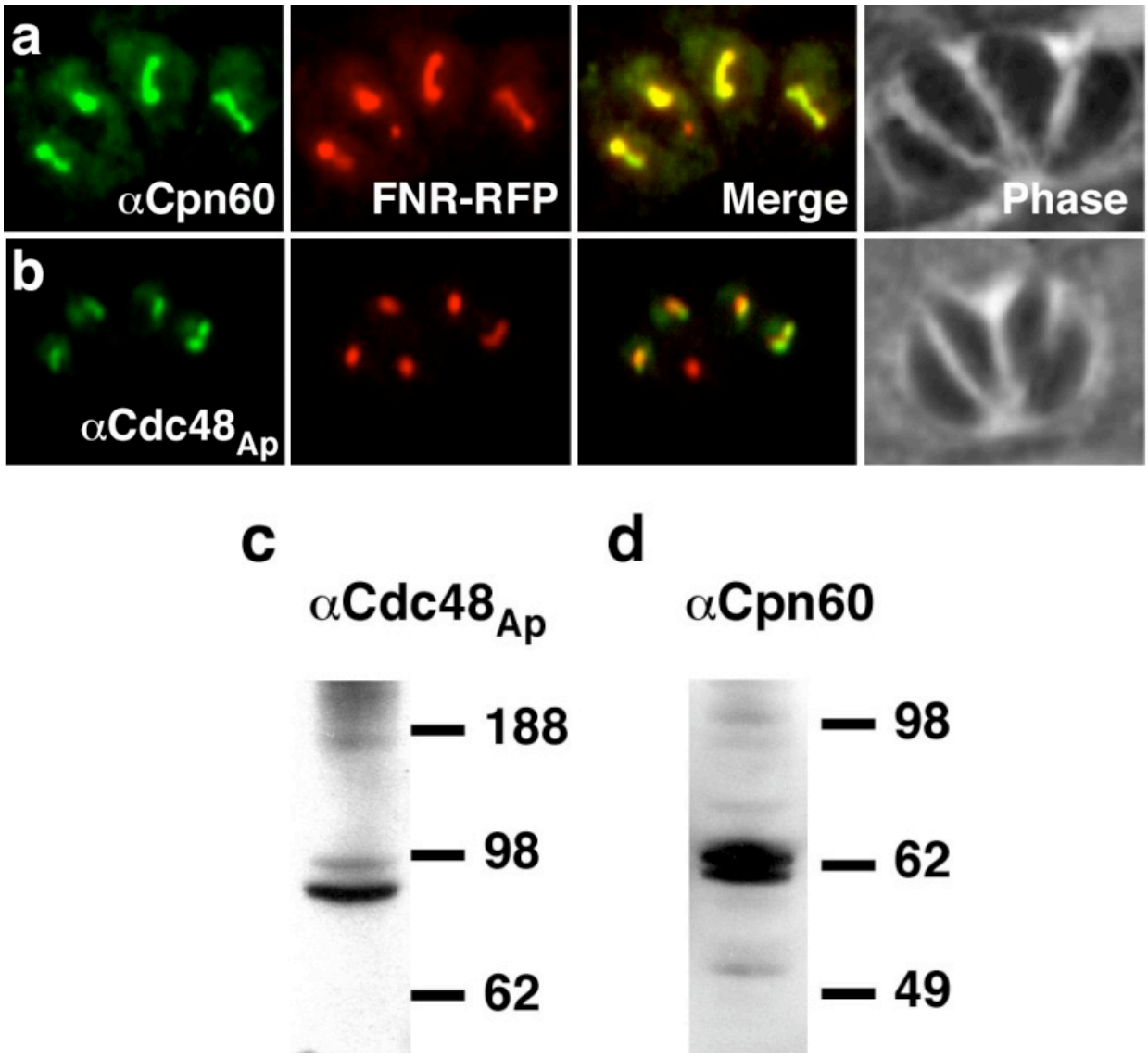


Figure 3.9

ATc treatment alone has no significant effect on apicoplast protein import. As additional control for experiments shown in Fig. 5 we performed pulse-chase analysis using the parental $Der1_{Ap}$ line, which still carries the native copy of $Der1_{Ap}$. (**a**) Post-translational modification of Cpn60 and PHD-E2 were analyzed after 0 or 4 days of ATc treatment as detailed in Fig. 5 by pulse-chase labeling, immunoprecipitation, SDS-PAGE and autoradiography (* human PDH-E2). (**b**) Maturation of Cpn60 and lipoylation of PDH-E2 were quantified by phosphorimager analysis of the respective bands in the autoradiographs shown in (**a**) followed by calculation of the ratio of mature to precursor protein (Cpn60) and ration of PDH-E2 to mitochondrial E2 (lipoylation; untreated control ratios were arbitrarily set to 100%).

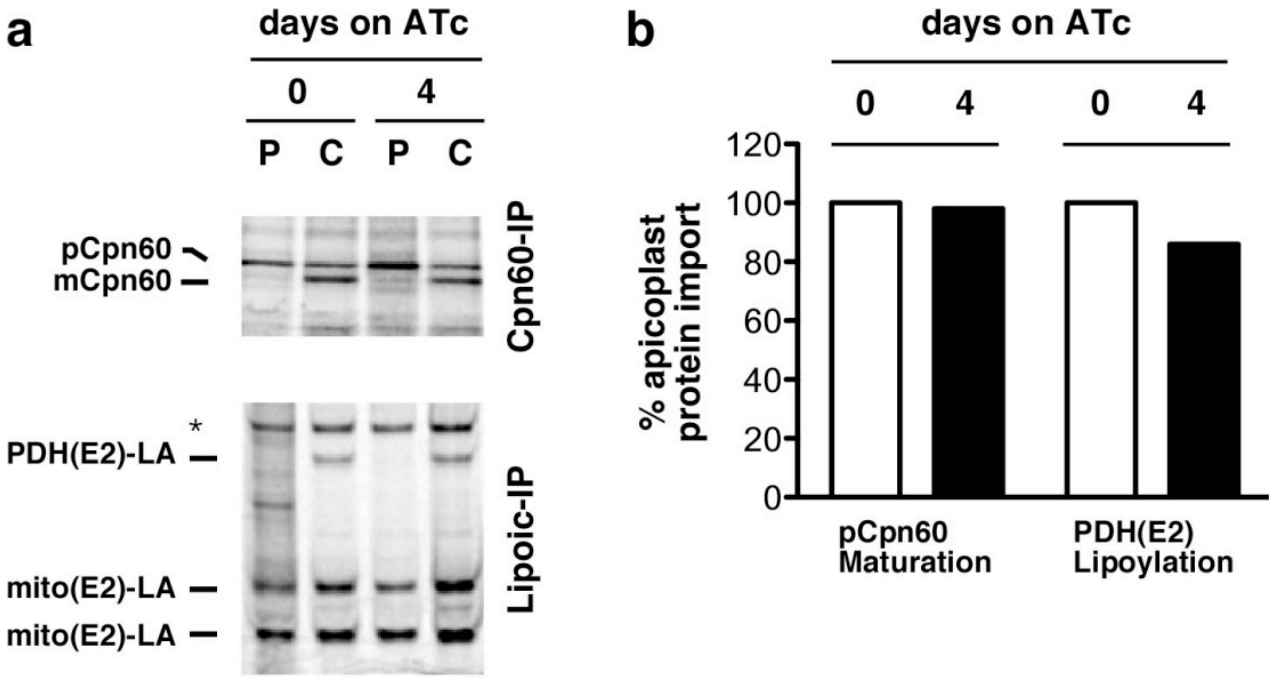
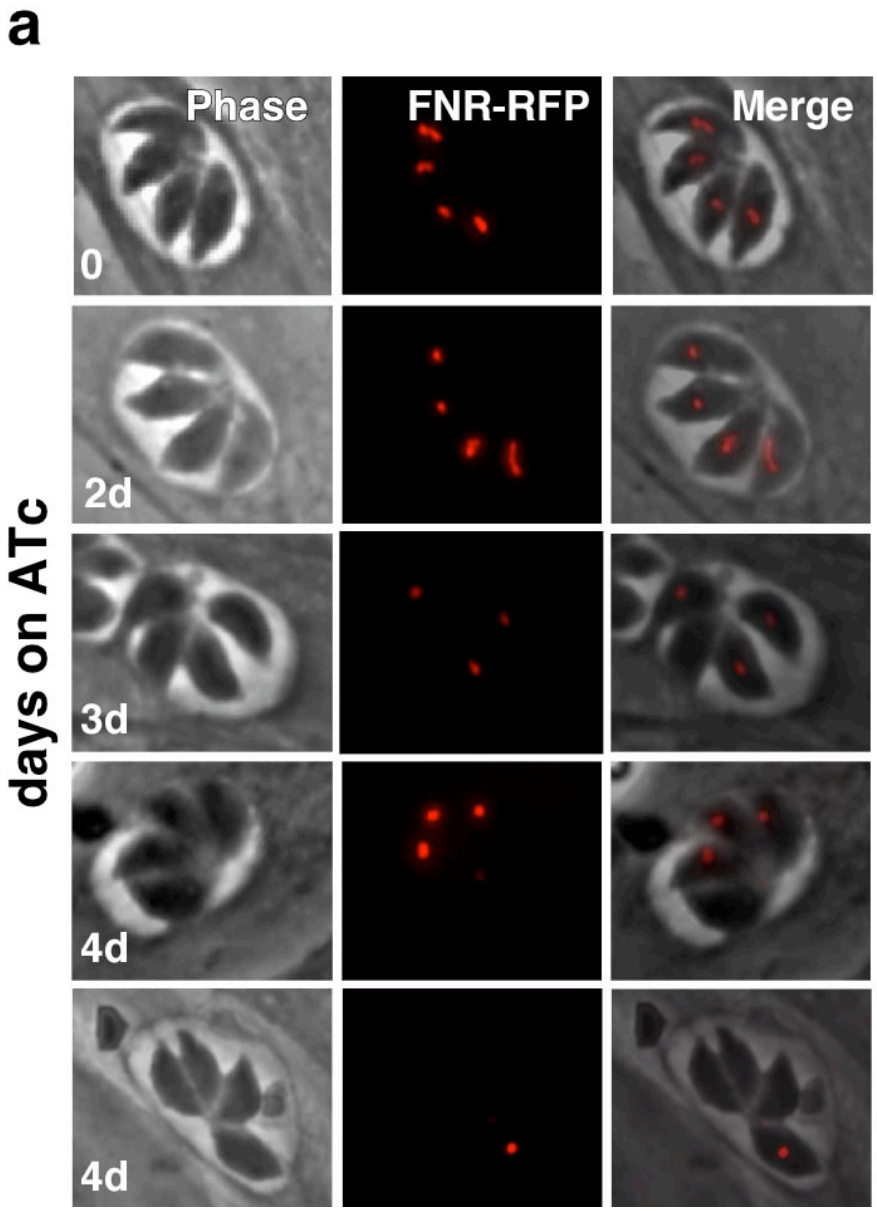


Figure 3.10

Loss of $Der1_{Ap}$ results in apicoplast biogenesis defects. $Der1_{Ap}$ mutant parasites expressing the apicoplast marker FNR-RFP were grown in the presence of ATc for 0-4 days. The presence of apicoplast was scored by life cell microscopy (1) in 100 vacuoles (each containing four parasites (see **a**). We note plastid counts indistinguishable from controls for the first two days with a dramatic decline on day 3 and 4. Panel **a,** shows representative micrographs and **b**, shows quantification of plastid numbers for 100 4-cell vacuoles for each sample. Note that plastid biogenesis is not affected by ATc treatment in wild type parasites (1,2).



b

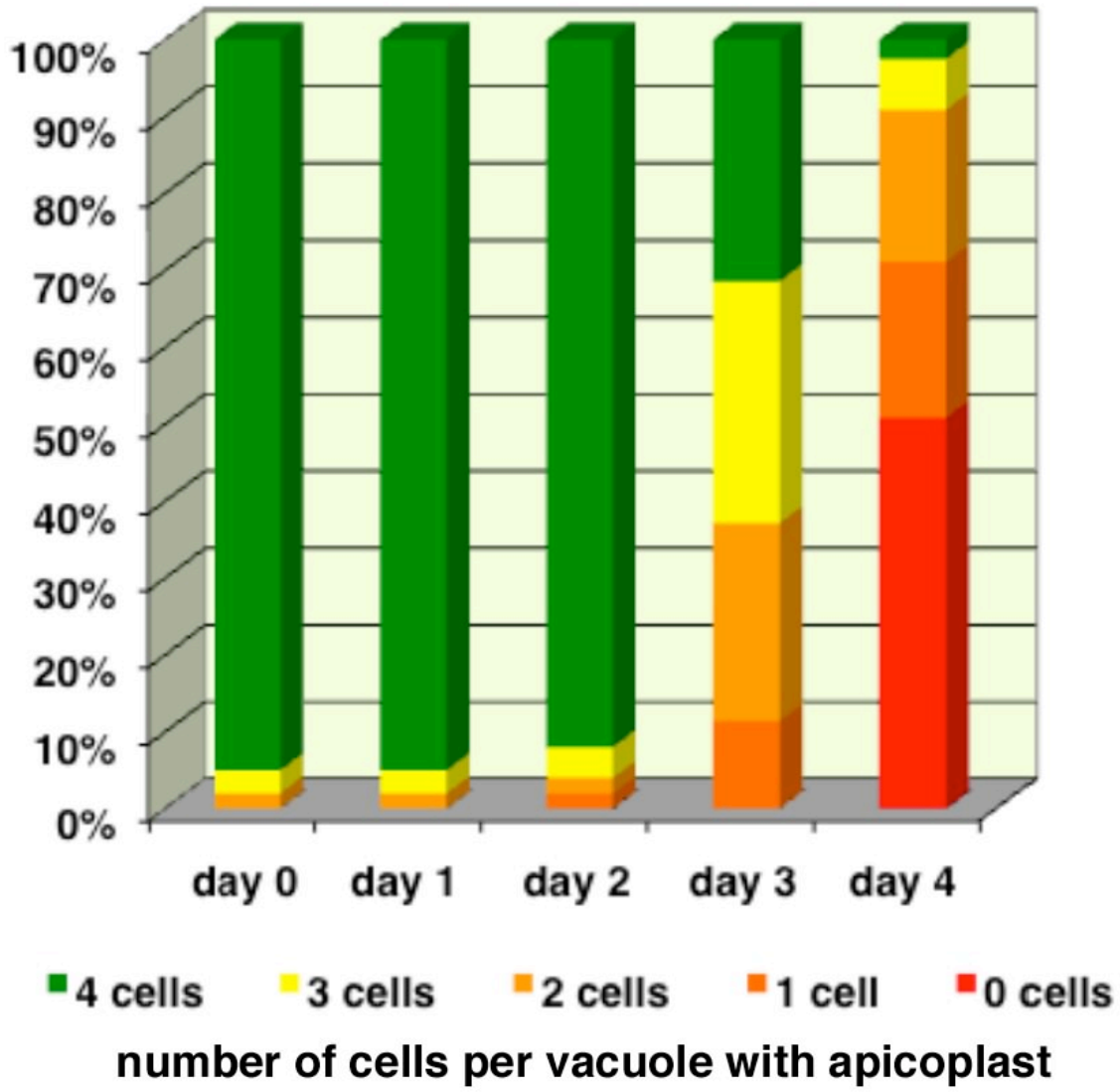
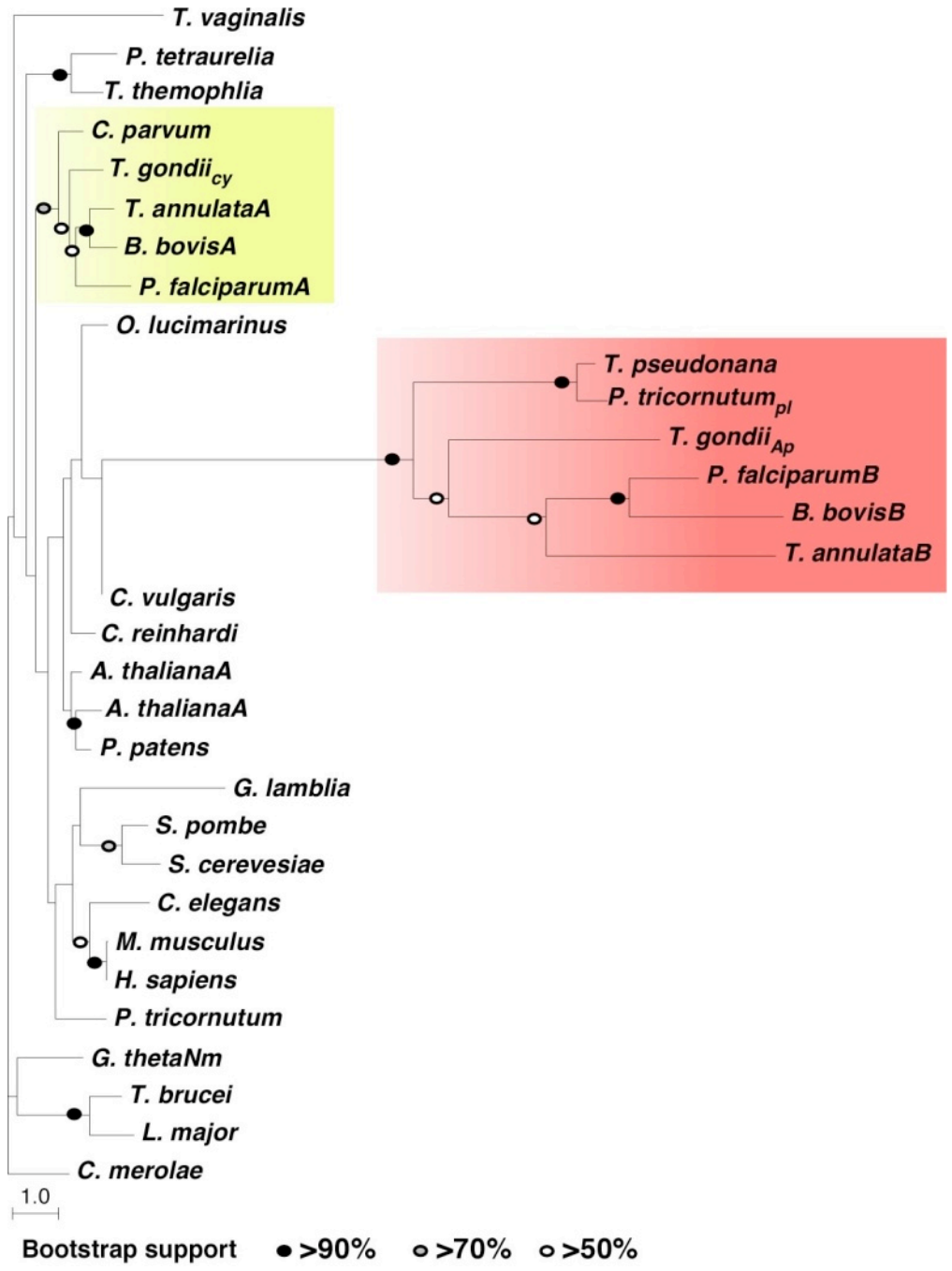

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
day 0
day 1
day 2
day 3
day 4
4 cells
3 cells
2 cells
1 cell
0 cells
number of cells per vacuole with apicoplast

Figure 3.11

Divergent origins of *T. gondii* Ufd proteins. RAxML maximum likelihood tree derived from alignments of Ufd proteins from 25 taxa (480 aligned amino acid characters were used after manual inspection, accession numbers are provided in the supplement). Bootstrap analyses were conducted using 100 replicates. Nm, nucleomorph (remnant endosymbiont nucleus), Pl, plastid, Ap, apicoplast, Cy, cytoplasm. Der1 protein sequences were to divergent to construct multiple sequence alignments.



Supplemental Methods

Phylogenetic analyses

Cdc48 homologues included in the analyses were ([genbank accession number], [*joint genome institute accession numbers]) from human [AAI21795], *Mus musculus* [NP_033529], *Caenorhabditis elegans* [NP_496273], *Drosophila melanogaster* [NP_477369), *Saccharomyces cerevisiae* [NP_010157], *Schizosaccharomyces pombe* [NP_593287], *Dictyostelium discoideum* [XP_636910], *Ostreococcus lucimarinus* [XP_001415566], *Chlorella vulgaris* [42408*], *Chlamydomonas reinhardtii* [XP_001696503], *Physcomitrella patens* [XP_001777213], *Arabidopsis thaliana* (A. thalianaA [P54609], A. thalianaB [NP_190891], (A. thalianaC [NP_568114]), *Emiliania huxleyi* [557511*], *Hyaloperonospora* parasitica [AAY58902], *Toxoplasma gondii* ($TgCdc48_{Cy}$ [1207706] and $TgCdc48_{Ap}$ [FJ976519]), *Plasmodium falciparum* (P. falciparumA [CAG25009] and P. falciparumB [XP_001349023]), *Theileria annulata* (T.annulataA [XP_953837] and T.annulataB [XP_955188]), *Babesia bovis* (B. bovisA [XP_001610758] and B. bovisB [XP_001609335]) and *Cryptosporidium hominis* [XP_667275], *Thalassiosira pseudonana* (T.pseudonanaA [XP_002286617] and T.pseudonanaB [XP_002289352] and *Phaeodactylum tricornutum* (P. tricornutumA [XP_002185883] and P. tricornutumB [50978*]), *Guillardia theta* [XP_001713564], Cyanidioschyzon *merolae* (http://merolae.biol.s.u-tokyo.ac.jp/ CmCdc48A [c12f0001], *Tetrahymena thermophila* [XP_001007447] and *Paramecium tetraurelia* [XP_001456132]), *Trichomonas vaginalis* [XP_001317755], *Giardia Lamblia* [XP_001704687], *Entamoeba histolytica* [AAF74998]), *Trypanosoma brucei* (TbCdc48 [AAC02215]) and *Leishmania major* [XP_001686709]).

Ufd protein sequences from 25 eukaryote taxa were used to generate a multi-sequence alignment using ClustalX version 2.0. Upon manual inspection 480 aligned amino acid characters were selected for further analysis (alignments are available on request from the authors). We note that Ufd1 is not sufficiently conserved to resolve the tree of live, however the divergent origin of the cytoplasmic and apicoplast proteins are evident. The data set was subjected to maximum likelihood-based phylogenetic analysis using RAxML (3) version 7.0.4 available at http://8ball.sdsc.edu:8889/cipres-web. A phylogenetic tree was constructed employing the GAMMA+P-Invar evolutionary model and the model parameters used were alpha: 1.636618, invar: 0.000117 and Tree-Length: 21.567051. Bootstrap analyses were conducted using 100 replicates as described previously(4). Ufd homologues included in the analysis are from human [NP_005650], *Mus musculus* [AAH06630], *Caenorhabditis elegans* [NP_502348], *Saccharomyces cerevisiae* [NP_011562], *Schizosaccharomyces pombe* [NP_596780], *Ostreococcus lucimarinus* [XP_001419494], *Chlorella vulgaris* [28219*], *Chlamydomonas reinhardtii* [XP_001699038], *Physcomitrella patens* [XP_001778779], *Arabidopsis thaliana* (A. thalianaA [NP_001077933], *A. thalianaB* [NP_973557], *Toxoplasma gondii* (T. gondiiCy [FJ976516] and *T. gondii*Ap [FJ976517]), *Plasmodium falciparum* (*P. falciparumA* [XP_001348351] and *P. falciparum*B [CAX64217]), *Theileria annulata* (*T. annulataA* [XP_952190] and *T. annulataB* [XP_953638]), *Babesia bovis* (*B. bovisA* [XP_001610110] and *B.bovis*B [XP_001611093]) and *Cryptosporidium parvum* [XP_625717], the *Thalassiosira pseudonana* (*T. pseudonana* [XP_002289664]) and *Phaeodactylum tricornutum* (*P. tricornutum*A [XP_002178490] and *P. tricornutum*B [49319*]), *Guillardia theta* nuleomorph (*G. theta*Nm [AAF24006.1], *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp/ CmCdc48A [11f0001], the *Tetrahymena thermophila* [XP_001010411] and *Paramecium*

tetraurelia [XP_001437627]), *Trichomonas vaginalis* [XP_001321792], *Giardia Lamblia* [XP_001706512] *Trypanosoma brucei* (TbCdc48 [XP_823013]) and *Leishmania major* [XP_001687240]).

Expression of recombinant $Cdc48_{Ap}$ and Cpn60 and antibody production

T.gondii $Cdc48_{Ap}$ and Cpn60 sequences (primers used for amplification are listed in ST3) were amplified and introduced into the bacterial expression plasmid pAVA421(5) by ligation independent cloning as described previously (6) to generate a fusion protein carrying a six histidine tag at the N-terminus. Plasmids were introduced into the *E. coli* BL21 strain. Recombinant fusion proteins were purified by affinity chromatography on Ni2+NTA resin in presence of 6M urea according to the manufacturer's protocol (Qiagen). A polyclonal anti-serum was generated by rabbit immunization (Cocalico Biologicals, Inc. PA, USA) following a standard protocol. The antisera were tested by Western blot and IFA against *T. gondii* (see Fig. S1).

Supplemental Tables

Table 3.1: Identification of the full coding sequences for *T. gondii* ERAD components.

For seven genes we experimentally evaluated the gene model derived from the automatic annotation process (toxoDB.org). We identified additional exons and/or divergent 5' and 3' termini by RACE and RT-PCR, subcloning and sequencing. We provide the ToxoDB genome identifiers below for reference but note that further work should be based on the validated coding sequences available from genbank using the indicated accession numbers.

Name	Genbank - accession number	ToxoDB identifier	Primers for 5'RACE	Primers for 3'RACE
Der1-1$_{ER}$	FJ976521	TGME49_094290	EST's covering 5' end were present	EST's covering 3' end were present
Der1-2$_{ER}$	FJ976522	TGME49_017160	5'-TGAAAAAGTACGAACCGACGCCA AAGATATGCGACAGTCC	5'-TTTGGCGTCGGTTCGTACTTTTT CAGCGG
Cdc48$_{cyt}$	FJ976518	TGME49_073090	5'-GGCAAGTAAATCAGGTCCCCCAC GGTCAG Nested 5'-AAAGTGGCAAGTTCC TTCCTCCGCAACGAA	5'-TCCCGAGAGCGCACCAGCGAAGA AGGAAGAG Nested 5'-ACTCGGGAGCCGTGGAAG AACCGGATCAAAA
Ufd$_{cyt}$	FJ976516	TGME49_070530	5'-GGCAAGTAAATCAGGTCCCCAC GGTCAG nested 5'-AAAGTGGCAAGTTCCTTC CTCCGCAACGAA	5'TCCCGAGAGCGCACCAGCGAAGAAGGAAGAG nested 5'-ACTCGGGAGCCGTGGAAGAA CCGGATCAAAA
Der1$_{Ap}$	FJ976520	TGME49_081940	5'-CGACGAAGAAAAAGACGACAGG AGCGACAGTG nested 5'-CTTTGTCAGCTTCGGCGTCGCCTTCCACT	5'-CACTGTCGCTCCTGTCGTCTTTTTC TTCGTCG nested 5'-CCTGCTTTCTTCGCTATTCTT CCTCGGCCCGTT

Cdc48$_{Ap}$	FJ976519	TGME49_121640	5'ATCCGCCGCAAATTCG CCTCGCTCTCCCCAGCC AACT nested 5'-GTGAAGCAATACGCCCC G CGGTGTCTGT	5'-CGCAAACCTATCTTCGTC ATCGGAG CCACCAATCG nested 5'-CGTGGACATTGAAGACAT GG CTCGGAGACTCGAAGGCT TTTC
Ufd$_{Ap}$	FJ976517	TGME49_085700	RACE unsuccessful	5'-CTTCCTCCCCTTATGGGTC ATGAAG GCACTCGACTTGCG nested 5'-AATGGGAGCGCCTTC CGCTGGCGGGTCACGT

Table 3.2: Construction of tagged genes for subsequent localization of their protein products.

Name	Primers used for cloning	Vector used for cloning	Method of localization	Localization
Der1-1$_{ER}$	for 5'-AGATCTAAAATGGGGTTCCCTTCA GCTCTCT rev5'-CCTAGGGTACGAGTCTGACGGC TTCTCGCCGAT	pCTH	Cloned and introduced as minigene	ER
Der1-2$_{ER}$	for5'-CTAGAGATCTAAAATGGCGCAGG TGGACTTGTTCTTC rev5'-CTAGCCTAGGCTCTCTTGGGGTGA GGCGCGACCTC	pCTH	Cloned and introduced as minigene	ER
Cdc48$_{cyt}$	for 5'-CTAGGGATCCAAAATGGCCGGCG GCATTCGCAG rev 5'-CTAGCCTAGGCGAGTAGAGGTCAT CGTCATCCGCG	pCTM3	Cloned and introduced as minigene	Cytosol
Ufd$_{cyt}$	for5'-CTAGAGATCTAAAATGTTCAGTCG CCACGTAGCGAATCTG rev 5'-CTAGCCTAGGCTCGCAGGTATTG CCTTTTCCAAAG	pCTH	Cloned and introduced as minigene	Cytosol
Der1$_{Ap}$	for 5'-AGATCTAAAATGGAAAGAGGGGA TTTTTTCTCACT rev 5'-CTAGTCCGTTTCCAACGGCGTCCTCGTTTAA	pDT7S4HA	Cloned and introduced as minigene	Apicoplast

Der1$_{Ap}$	for 5'-AGATCTAAAATGGAAAGAGGGGA TTTTTTCTCACT rev 5'-CTAGTCCGTTTCCAACGGCGTCCTCGTTTAA	pDT7S4HA	Cloned and introduced as minigene	Apicoplast
Cdc48$_{Ap}$	for 5'-CTAGCAAAATGGGGACTGCGTGG TGCCCTCTCG rev 5'-CTAGCCTAGGCTTTGTTTCCTTC GCCGTCTCCGT	pCTM3	Cloned and introduced as minigene (antiserum was raised against recomb. protein)	Apicoplast
Ufd$_{Ap}$	for 5'-AACAAGAGAAACAGAGAGAGAA GAAGAGAACAGAAGACACGAAACAC GCAAGGTACCCGTACGACGTCCCGGACTAC rev 5'-	Cosmid TOXOx83	Tagged 3'end of gene in cosmid	Apicoplast

Table 3.3: Construction of $Der1_{Ap}$ KO construct and $Cdc48_{Ap}$ and Cpn60 antibody expression vectors

Name	Primers used for cloning	Vector used
$Der1_{Ap}$ 5' flank for KO vector	For 5'-CTAGACTAGTGGCTGTTCCTTCCCCACTGTATTAT Rev 5'-CTAGGACGTCGCTGGTGAACAGGAAGCACGACCTT	pTCY *Spe*I and *Aat*II sites
$Der1_{Ap}$ 3' flank for KO vector	for 5'-CTAGgtcgacACGCTTCGATGTCTTTATCGGCATC and rev 5'-CTAGGGTACCCGAGCATTGCACACGCTCGTCCTCC	pTCY *Sal*I and *Kpn*I sites
$Cdc48_{Ap}$ Antibody	Primers for 5'-GGGTCCTGGTTCGATGGATCCTTCAGTGGTTTTCCTCTCGCC and rev 5'-CTTGTTCGTGCTGTTTATTAGTCTTCCCAGCGAACGTCTGGCACTT	pAVA421, Cloned by LIC
Cpn60 Antibody	For 5'-gggtcctggttcgatgAAAGATCGCACGTCGATTCTTACAAGG rev5'-cttgttcgtgctgtttattaTGCCATTGGCATGTCTGGTACATC.	pAVA421, Cloned by LIC

CHAPTER 4

UBIQUITINATION MACHINERY LOCALIZED TO THE PERIPHERAL COMPARTMENT OF APICOPLAST IS REQUIRED FOR PROTEIN IMPORT

4.1 Introduction

The plastid of apicomplexans is the product of a secondary endosymbiosis event, where a chloroplast bearing red algal was engulfed by a eukaryote. As a result, the organelle is surrounded by four membranes of divergent phylogenetic origin. Theres accumulating evidence that import across the second outermost membrane of apicoplast utilizes a endosymbiont derived ERAD like machinery (Sommer et al. 2007, Agrawal et al. 2009). A careful look at the nucleomorph genome of cryptophytes led to the observation that not only were the $Der1_{Ap}$ and associated ERAD components retained from the algal endosymbiont, a few key enzymes that participate in substrate ubiquitination were also present in the genome (Sommer et al. 2007). Also present in the genome are 21 nucleomorph genes for different subunits of the 20S core proteasome and its 19S cap components. Thus it was initially proposed that the ubiquitination and proteasomal components probably function in the cell cycle control during the replication of the nucleomorph genome (Douglas et al. 2001). However during the early stages of this research we and other groups discovered several nuclear encoded plastid homologs of ubiquitin associated proteins (Ufd, Uba, Ubc, E3 and DUB) in the nuclear genomes of alveolates and chromists with secondary plastids like *T. gondii, P. falciparum and P. tricornitum* (Hempel et al. 2010; Spork et al. 2009). The plastids of these organisms no longer possess the algal genome. Most of the endosymbiont's gene were either relocated to the nuclear genome of the host or lost in the course

of evolution. Thus the endosymbiont derived ubiquitin machinery likely posesses a novel role in this new cellular setting. During ERAD substrate translocation across the Der1 pore is followed by modification by ubiquitin in preparation for proteasomal degradation of the target protein. Ubiquitination is essential for almost all proteins to be successfully retro-translocated across the ER prior to degradation by ERAD process. Ubiquitination is a post-translational protein modification that requires sequential action of three enzymes namely ubiquitin activating (E1) enzyme, ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). Ubiquitin is activated in a two-step reaction by an E1 ubiquitin-activating enzyme utilizing ATP as the energy source and transferring ubiquitin to the E1 active site cysteine residue, with concomitant release of AMP. Transfer of ubiquitin from E1 to the active site cysteine of a ubiquitin-conjugating enzyme E2 occurs via a trans-thiol esterification reaction. The final step of the ubiquitination cascade creates an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin achieved by the activity of E3 ubiquitin-protein ligases. However proteasomal degradation is not the sole purpose of substrate ubiquitination. Over the past two decades other fascinating and physiologically important roles of ubiquitin in intracellular protein trafficking and internalization of substrates have reported. Ubiquitin plays an essential role in intracellular protein trafficking, either from the Golgi or from the plasma membrane via the endosomal system to the multi vesicular bodies (MVBs) in the vacuolar lumen. An array of plasma membrane proteins with widely different functions and spatial distributions are regulated by the ubiquitin system. Some examples include, receptors (yeast pheromone receptor Ste2, mammalian T-cell receptors, cytokine receptors, receptor tyrosine kinases) (Hicke and Riezman 1996; Ihle et al. 1994; Mori et al. 1993), transporters (ABC transporter, yeast permeases) (Bonifacino and Weissman 1998; Kuchler et al. 1989) and ion channels (epithelial Na^+channel (ENaC), CFTR chloride channel)

(Harvey et al. 2001; Jensen et al. 1995). Conjugation of ubiquitin regulates the fate of substrate proteins by either initiating internalization or sorting of the target or both. The final fate of these ubiquitinated membrane cargoes however is degradation, similar to proteasomal degradation.

Is the $Der1_{Ap}$ translocation machinery in the apicoplast coupled with ubiquitination machinery? The apicoplast specific ubiquitination components could be involved in several interesting functions adapted for this endosymbiotic organelle such as: translocation of cargo protein for successful trafficking through the Der1 pore. Alternatively, ubiquitination in the apicoplast may be only limited to a subset of cargo proteins and therefore act as a specific sorting signal e.g, as a retention signal for the periplastid space preventing further transport of these proteins to the lumen (Abrami et al. 2008). On the other hand, ubiquitination may act in a process independent of protein import (e.g, apicoplast division, protein folding and quality control, etc).

We approached this question by identifying and localizing the apicoplast specific ubiquitination factors in *T. gondii*. We generated a mutant in the apicoplast localized ubiquitin-conjugating enzyme, Ubc_{Ap} and tested for apicoplast import competence in the mutant parasites. In the present work we provide evidence for localization of two key components, the ubiquitin activating and conjugating enzyme to the *T. gondii* apicoplast. We further demonstrate through functional charcterization of the Ubc_{Ap} mutant, that the machinery is indispensible for apicoplast protein import and ultimately parasite survival.

4.2 Materials and Methods

4.2.1 Plasmids, cell line and cell culture

A Ubc_{Ap}-HA expression vector was constructed by cloning the full length cDNA of Ubc_{Ap} into BglII/ AvrII sites of the pDT7S4HA vector. The resulting plasmid was transfected into TATi strain as previously described (Donald and Roos 1993). Ubc_{Ap}-HA parasites were selected by

under drug pressure with pyrimethamine. To endogenously tagg Uba_{Ap} a 1.5Kb sequence upstream of the stop codon from genomic DNA was cloned into the pLIC-HA-CAT vector by ligation independent cloning as described by Huynh and colleagues (Huynh and Carruthers 2009). The final construct was linearlized and transfected in Ku80Δ parasites (Huynh and Carruthers 2009). Uba-HA expressing parasites clones were selected with chloramphenicol. Integration of the tag and expression of the chimeric protein was confirmed by PCR analysis (not shown here) and immunoflurescence assay with anti-HA antibody respectively.

For targeted insertion of an inducible promoter at the endogenous locus of Ubc_{Ap} a 1.5Kb region of the genomic sequence from the start of the gene was PCR ampilifed. This fragment was cloned in BglII/ AvrII sites of the pDT7S4HA vector. Next a 1kb flank immediately upstream of the gene (putative promoter region) was PCR amplified with NdeI cut sites and cloned into equivalent sites in the pDT7S4 plasmid containing the gene specific sequence. The transformants were screened for integration in correct orientation. The resulting construct was linearized by digestion with AvrII and transfected into pyrimethamine-sensitive tetracycline transactivator expressing KU80 knockout parasites (Ku80Δ-TATi) (refer to Fig 4.5 A) (Sheiner et al in preparation). We selected for pyrimethamine-resistant parasites and cloned parasites by limiting dilution. Successful modification of the genetic locus was confirmed by PCR analysis of isolated clones. Single site integration into genomic DNA was confirmed by Southern blot hybridization as described in (Agrawal et al. 2009). Briefly gDNA was extracted using the Qiagen DNA-easy kit, from Ku80Δ-TATi and $iUbc_{Ap}$ cell lines. The DNA was digested overnight with NsiI/NdeI restriction enzyme. The digested DNA was separated on 1% agarose gel, blotted on nitrocellulose membrane and probed with radiolabeled Ubc_{Ap} cDNA amplified by PCR as the probe.

For complementation of the Ubc_{Ap} with wild type and point mutants, the Ubc_{Ap} minigene was expressed in the $iUbc_{Ap}$ cell line under sag1 constitutive promoter. The gene was cloned into NsiI/NheI restriction sites downstream of the sag1 promoter in a plasmid containing 2 kb flanking regions upstream and downstream of the uracil phosphoribosyl transferase (UPRT) gene, which was used for targeting the endogenous UPRT locus by double homologus recombination. Point mutants were generated with the Quik-Change II XL Site-Directed Mutagenesis Kit (Stratagene) using the plasmid described above as template (Brooks et al. 2010). Different variants of this construct were then transfected into the inducible $iUbc_{Ap}$ cell line and selected with 5μM 5-FUDR after 48 hrs of transfection.

Measurements of parasite growth were performed by plaque assays and tandem tomato RFP fluorescence plate assays as previously described in (Gubbels et al. 2003; Hempel et al. 2010; van Dooren et al. 2008).

4.2.2 Apicoplast import assay

Apicoplast import assays were performed as described in (Agrawal et al. 2009; van Dooren et al. 2008). Briefly infected host cells were starved for one hour in cysteine and methionine-free Dulbecco's Modified Eagles medium supplemented with 1% foetal bovine serum and antibiotics. Infected host cells were then radiolabelled with 100 mCi/mL of 35-S methionine and cysteine (MP biomedicals) for one hour. Cells were either harvested (pulse) or washed twice with 10 ml of parasite growth medium, and incubated in 10 ml parasite growth medium for 2 to 8 hours (Vorobiof et al.) before harvesting. Proteins of interest were purified by affinity purification and separated by SDS-PAGE as described above. Gels were dried and bands were visualised by autoradiography or using a Storm 860 PhosphorImager (GE Healthcare). Band intensities were quantified using Image Quant TL software (GE Healthcare).

For immunoprecipitations, parasites were lysed from host cells by passage through a 26 gauge needle, and pelleted by centrifugation at 1500g for 10 minutes. Pellets were washed in PBS then lysed for 30 minutes on ice in immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) noniodet P-40 substitute (Fluka), 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulphate, 2 mM EDTA) supplemented with protease inhibitors (Complete protease inhibitor cocktail, Roche Applied Science). Samples were centrifuged to remove insoluble material. Proteins-of-interest were purified by immunoprecipitation using antibodies bound specifically to protein A-sepharose CL-4B beads (GE Healthcare). Samples were pre-cleared by incubation in 30 to 40 mls of a 50 % slurry of Protein A-Sepharose CL-4B beads. Anti-lipoic acid (Calbiochem) was bound to protein A-sepharose CL-4B beads for one hour at 4^{o}C before addition to pre-cleared lysates. All samples were incubated overnight at 4^{o}C then washed 4 times in immunoprecipitation wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) noniodet P-40 substitute, 0.5 % (w/v) sodium deoxycholate, 0.25 % w/v bovine serum albumin, 2 mM EDTA) and twice in PBS. Samples were eluted by boiling in reducing or non-reducing sample buffer then separated by SDS-PAGE as described above. For affinity purification of biotinylated proteins, lysates were incubated overnight with immobilised streptavidin-agarose beads (Pierce). Samples were washed 4 times in Streptavidin wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) noniodet P-40 substitute, 0.5 % (w/v) sodium deoxycholate, 0.1 % w/v sodium dodecyl sulfate, 2 mM EDTA) and twice in PBS, before elution by boiling in reducing sample buffer.

For mass spectrometry (MS) analysis, approximately 10e8 parasites were lysed with 500µl RIPA buffer on ice for 30 mins. Samples were centrifuged to remove insoluble material and then incubated with 50µl µMACS™ anti-HA micro beads overnight. The microbeads were

magnetically precipitated, washed and eluted using columns according to the manufacturer's instructions (μMACS, Miltenyi Biotec Inc). For MS analysis, samples were separated on SDS-PAGE gel and stained with a mass spectrometry-compatible silver staining kit (thermo-scientific). Bands of interest were then excised and send for MS analysis.

4.3 Results

The *T. gondii* genome encodes several proteins involved in the ubiquitination pathway. We identified candidates for E1, E2 and RING finger domain containing E3 proteins, with a predicted N-terminal signal peptide. The presence of a signal peptide is a pre-requisite for entry into the secretory pathway and the majority of apicoplast proteins feature an N-terminal extension consisting of a signal peptide followed by a short leader sequence, which is revealed upon processing of the signal peptide in the ER. We investigated the localization of four potential apicoplast targeted ubiquitin-associated proteins (Uba_{Ap}-TGGT1_092180, Ubc_{Ap} TGGT1_021600, $E3_{cyc}$-TGGT1_006600, and TGGT1_082020). We either cloned the full length coding sequence of the genes into *T. gondii* expression plasmid with a C-terminal epitope tag or prepared single homologous targeting constructs for endogenous tagging of the locus at the C-terminus. These constructs were used for transfection, and selected using appropriate antibiotic selection marker to generate stable transgenic cell lines. Fig. 1 shows the localization pattern of HA epitope tagged clones constructed by either direct tagging of the genomic locus, in the case of Uba_{Ap} (Fig.1a) and $E3_{cyc}$ (Fig.1c) (Huynh and Carruthers 2009) or tagging of a cDNA clone in the case of Ubc_{Ap} (Fig. 1b). We identify one protein with cytoplasmic localization, a putative E3 and two proteins an E1, and an E2, that show a pattern indistinguishable from the three apicoplast ERAD proteins that we identified earlier (Agrawal et al. 2009). In the case of TGGT1_082020 (a putative RING domain containing E3) we did not detect any expression of

the transgene upon endogenous C-terminal tagging. We are presently evaluating the annotation of this gene by 5' and 3' RACE experiments to determine the correct coding sequence of the gene. Immuno-electronmicroscopy analysis revealed that Ubc_{Ap} localizes to the same peripheral sub-compartment of the apicoplast as the other apicoplast ERAD components.

Previously we demonstrated that *T. gondii* apicoplast possess three important components of the ERAD complex, namely the pore Der1-1, the motor Cdc48 and its adapter Ufd-1, and $Der1_{Ap}$ is required for protein import into apicoplast (Agrawal et al. 2009). Thus it is possible that similar to the ERAD components, ubiquitination enzymes have also been adapted for protein import in apicoplast. We sought to test this experimentally by directly challenging the parasite's need for ubiquitination enzymes in the apicoplast. Ubiquitin conjugating enzymes (E2s) are responsible for a great deal of diversity in choice of substrate for ubiquitination, as they are directly responsible for E3 selection and substrate modification. Additionally, in many cases E2 alone can transfer activated ubiquitin to the target, thus E2 can play an important role in substrate recognition. To test the function of apicoplast specific ubiquitination machinery we engineered a conditional mutant in the Ubc_{Ap} gene. The targeting construct consisted of genomic sequence upstream of the translation start site of the Ubc_{Ap} gene, dhfr selectable marker followed by tetracycline inducible (tet-off) promoter and a partial 1.5Kb region of the gene (from the translation start site) (refer to Fig 4.5 A) (Sheiner et al. in preparation). After transfection and drug selection with pyrimethamine, mutant clones were picked that tested positive for correct insertion of the tetracycline inducible promoter (see PCR and southern blot results in Fig 4.2 B and C). The resulting transgenic parasite line encodes for only one copy of the gene that can be tightly regulated by the addition of the tetracycline analogue ATc (for more details about the promoter see references (Mazumdar et al. 2006; Meissner et al. 2002)). Once a mutant clone was

established we first tested the parasite viability in the absence of Ubc_{Ap} gene using plaque assay and real-time fluorescence assay (Gubbels et al. 2003; van Dooren et al. 2008)). For the fluorescence assay, tandem tomato red fluorescent protein construct was transfected into the tet-inducible Ubc_{Ap} cell line (from now on referred to as $iUbc_{Ap}$). Parasite growth was then monitored daily for over a period of seven days using overall fluorescence intensity as the read out for growth. Initially the growth of parasites was indistinguishable in the absence and presence of ATc (Fig 4.3 A) but preincubation of parasites in ATc for 3 days prior to the growth assay completely abolished growth suggesting that Ubc_{Ap} is essential for parasite growth. We also performed plaque assays with the $iUbc_{Ap}$ cell line in the presence or absence of ATc. Parasites grow normally in the absence of ATc indicated by formation of plaques, however they fail to form plaques when Ubc_{Ap} is downregulated upon addition of ATc (Fig 4.3 B).

Substrate ubiquitination is required not only for proteasomal degradation but also for the retrotranslocation process during ERAD. Addition of polyubiquitin chains on the cargo prompts recruitment of ATP dependent Cdc48-Ufd1-Npl4 complex and causes mobilization of the ubiquitinated substrate from the ER membrane (Flierman et al. 2003; Ye et al. 2003). Similarly in the ESCRT process fusion of ubiquitin to the cytoplasmic tail of a normally stable plasma membrane protein induces internalization (Haglund et al. 2003; Hicke and Dunn 2003; Nakatsu et al. 2000; Roth and Davis 2000; Shih et al. 2000). Ubiquitination also controls the sorting and localization of certain proteins in a reversible manner, in a manner similar to how phosphorylation modulates changes in the structure, activity and the localization of the target proteins. Ubiquitin and the associated enzymes required for activation and synthesis of polyubiquitin chains are directly involved in translocation of proteins in different subcellular environments. We hypothesized that if Ubc_{Ap} is indeed associated with the $Der1_{Ap}$ machinery (as

suspected from the localization results), interference with Ubc_{Ap} expression would block apicoplast protein import as seen in the case of the $Der1_{Ap}$ mutant. We tested the ability of $iUbc_{Ap}$ parasite to import apicoplast proteins in the absence or presence of ATc using previously described import assays. $iUbc_{Ap}$ parasites were treated with ATc for 0, 2, 3, 4 and 5 days respectively and labeled for 1 hour at 37°C with 250 μCi of [35S] methionine/cysteine. For the chase samples the radioactivity was washed off, and cells further incubated for 2 hours in normal DMEM media. The samples were then used for immunoprecipitation with anti-Lipoic acid antibody followed by separation on SDS-PAGE. Treatment of cells with ATc for 2 days resulted in attenuation of import eventually leading to complete loss of import after 4 days when compared to control non treated cells (Fig 4.4). We conclude that apicoplast protein import is impaired in the absence of Ubc_{Ap}.

In order to confirm that the defect was specifically due to disruption of Ubc_{Ap}, we complemented the conditional mutant by ectopically expressing Ubc_{Ap} from a constitutive promoter ($iUbc_{Ap}/cUbc_{Ap}$). Complementation of the gene resulted in restoration of parasite growth in the presence of ATc as measured by plaque assay (Fig. 4.6 B).

4.4 Discussion

So far the evidence to support the presence of ubiquitin in the apicoplast has been slim. Spork and colleagues show that a N-terminal sequence of putative ubiquitin gene in *P. falciparum* can confer plastid targeting to GFP (Spork et al. 2009). However there has been no direct experimental evidence for the presence of ubiquitin or ubiquitinated apicoplast cargo in apicomplexan or algal systems with secondary plastids (Hempel et al. 2009; Sommer et al. 2007). While we are currently testing a few candidate ubiquitin genes for apicoplast localization, we decided to take an alternative approach to answer this question. During the catalytic process

the E2 enzyme becomes covalently linked to ubiquitin. A western blot analysis of the Ubc_{Ap} candidate typically reveals three bands (Fig. 3B) (instead of the conventional premature and mature species detected for most apicoplast proteins). The sizes of two of these bands are consistent with the predicted precursor and mature form of the protein (before and after removal of the apicoplast transit peptide). The third band is of intermediate size and may be consistent with an ubiquitinated form (+ 8 kDa). To evaluate the identity of these three bands we are currently performing affinity purification with the HA tagged Ubc_{Ap} cell line using μMACS™ anti-HA columns along with a mock purification from wild type RH parasites. After separating the proteins on a SDS –PAGE gel and silver staining we will excise bands of interest for further mass spectrometry analysis. As a backup experiment we have also replaced the HA epitope tag in the expression construct with a tandem affinity purification (TAP) tag. A clonal parasite cell line expressing this transgene has been isolated. The localization and Western pattern does not seem to be perturbed by the tag (data no shown here). We plan to conduct LC-MS-MS experiments on trypsinized gel slices to generate peptide sequence information. Our hypothesis suggests that the intermediate band will yield peptides consistent with ubiquitin or a ubiquitin like modifier that may have especially evolved to cater to the needs of the apicoplast environment. Pull down of Ubc_{Ap} and mass spectrometric analysis will allow us to identify such a modifier de novo.

In a second independent approach we will use mutational analysis of Ubc_{Ap} to characterize the putative ubiquitin/ ubiquitin like modifier on Ubc_{Ap}. Many proteins are tagged upon misfolding or at the end of their cellular lifetime. However during activation E2 is typically conjugated to ubiquitin through a thiolester linkage at the active site cysteine residue. In contrast during the degradation process substrates are modified by a peptide linkage at their lysine residues.

(Ishikura et al.). If the middle band of Ubc_{Ap} is indeed an ubiquitinated form of the enzyme, mutation of the active site cysteine residue would possibly abolish thiolester formation and may result in disappearance of the band. This has been shown to be the case in other systems where cysteine to alanine mutation disrupts E2 activity while preserving the overall structure and stability of the enzyme e.g. (Sung et al. 1990). In some cases catalytic cys mutants have been shown to have dominant negative effects (Pickart 2001; Sung et al. 1990). Our alignments identify cys573 as the likely active site cysteine surrounded by a number of highly conserved residues (see fig 4.5). We have constructed a cys/ala point mutation. We are currently testing these point mutants in complementation assays in the $iUbc_{Ap}$ mutant to formally establish if enzymatic activity is required for biological function. The complementation assays will also confirm whether our assignment of the catalytic site cysteine is correct. However it has been demonstrated with some other E2 enzymes that dominant negative effect may have a stringent requirement for serine substitution at the active site cysteine position. In normal E2 enzymes, the active site cysteine forms a thiol ester linkage with the C-terminal carboxyl of ubiquitin. This thiol ester-linked ubiquitin is subsequently transferred either to an E3 protein or to substrates directly. Substitution of the active site cysteine by a serine has been shown to inactivate other ubiquitin-conjugating enzymes (Seufert et al. 1995; Sung et al. 1991) because the more stable oxygen ester-linked ubiquitin is not further transferred. The requirement for a serine may be due to the unique ability of a serine to form a more stable linkage with ubiquitin. E2 mutants containing a stably linked ubiquitin thus act as better inhibitors than inactive enzymes (with alanine substitutions) that cannot be linked with ubiquitin. Additionally In yeast Cdc34 (E2 enzyme involved in cell cycle G_1/S transition) mutation of active site cysteine (cys95ser) residue alone does not qualify for a dominant negative phenotype. Mutation of a leucine residue (leu99)

located 4 amino acid downstream of the active site cysteine together with cys95 is required to confer any growth defects (Banerjee et al. 1995). Mutation of the leu99ser causes ubiquitin to be linked to ser95 at a faster rate. A matching conserved leucine residue (leu577) exists in $Ubc_{Ap.}$ Our future studies will aim at creating single cys573ser and leu577ser and double mutants and analyzing them for dominant negative defects or in complementation assays.

4.5 Conclusion and future direction

The present study we demonstrates for the first time the role of especially evolved ubiquitination machinery in apicoplast import. We expect that the analysis of point mutants will shed important insight into the molecular basis of Ubc_{Ap} function. Additionally the Ubc_{Ap}-TAP cell line will be an important tool for characterization of other components of this sophisticated enzymatic machinery. Recent work on diatom suggests that a plastid targeted ubiquitin ligase and a deubiquitinating enzyme are also part of this complex (Hempel et al. 2010). Although, several putative candidates can be identified in *T. gondii* genome based on BLAST scores and presence of signal peptide (see table 4.1), initial attempts to localize these proteins did not produce any convincing results. Further annotation of gene models and reassessment of constructs used to epitope tag (N vs. C-terminal tag, as the tag might interfere with the protein function) will be required to get conclusive data.

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Figure Legends

Figure 4.1 Localization of Ubc_{Ap}, Uba_{Ap} and $E3_{ER}$

Ubc_{Ap} and Uba_{Ap} are two peripheral apicoplast proteins while $E3_{ER}$ is an ER protein. Immunofluorescence assay depicting endogenously tagged Uba_{Ap}HA cell line (A) and full length Ubc_{Ap} cDNA expressing cell line (B) with a c-terminal HA epitope tag. The HA labelling (Chavagnac et al.) co-localises with the apicoplast marker Cpn60 (red). The insets in the merge panel show peripheral HA staining that does not overlap completely with Cpn60. (C) Western blot of protein extracts from the Ubc_{Ap}-HA line with anti-HA antibodies. Three major bands can be seen, a ~62 KDa species (upper most) representing the protein with intact apicoplast targeting transit peptide sequence, a ~49 KDa band represents the processed form of the protein and a third intermediate band probably represents a post-translationally modified form of the mature protein. (D) Endogenously $E3_{ER}$–HA tagged cell line showing ER localization of the protein. (E) Transmission electron micrograph of the Ubc_{Ap} -HA cell line, where Ubc_{Ap}-HA labelling is restricted to the periphery of the organelle.

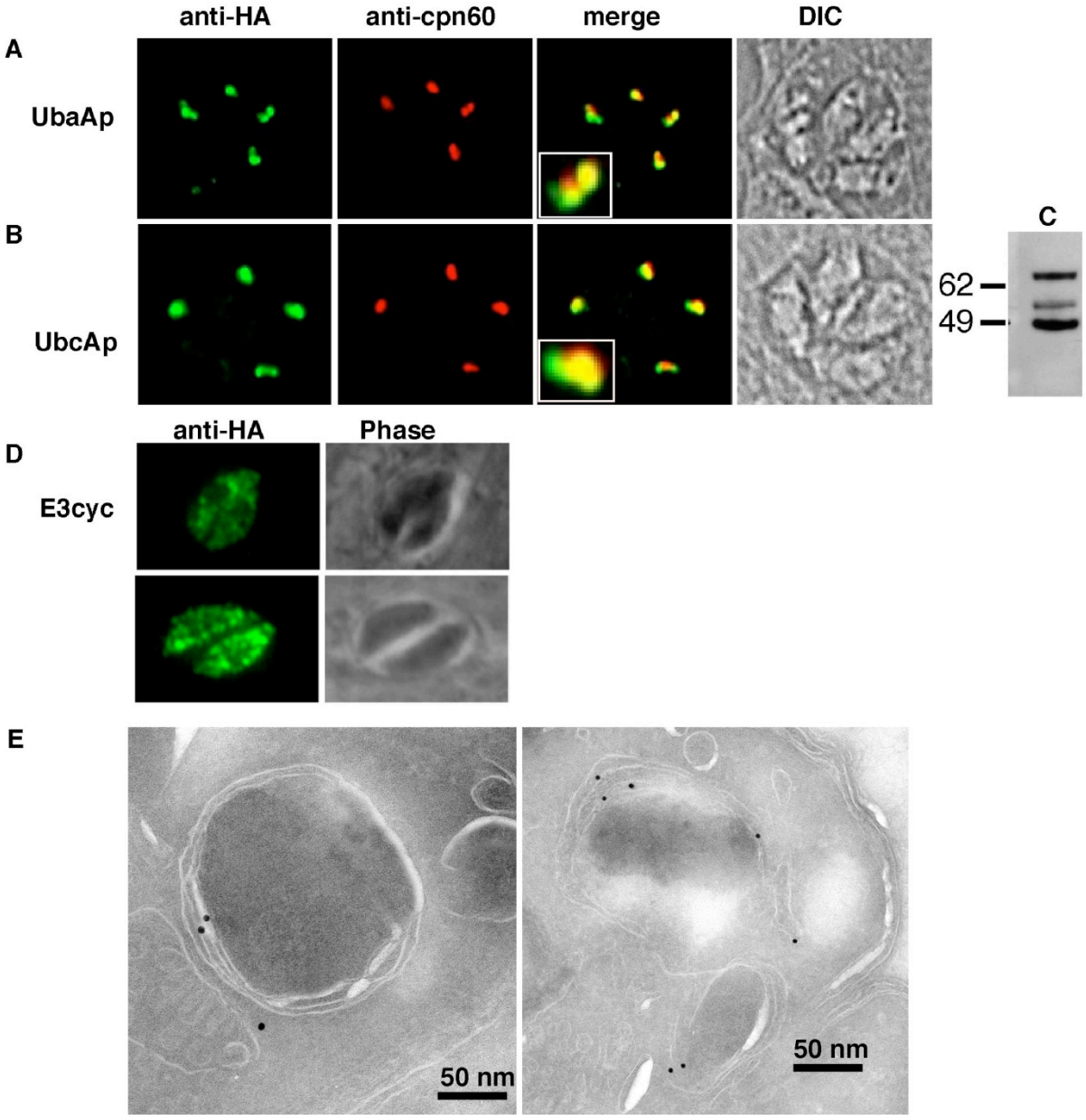

anti-HA
anti-cpn60
merge
DIC
A
UbaAp
B
UbcAp
C
62
49
anti-HA
Phase
D
E3cyc
E
50 nm
50 nm

Figure 4.2 Generation of an inducible Ubc_{Ap} cell line.

(A) The integration construct consists of genomic region immediately upstream of the start codon of Ubc_{Ap}, Tet operator elements, sag4 promoter and 1.5Kb of genomic sequence from the start of the gene. After double homologus recombination at the native locus a chimeric Ubc_{Ap} controlled by the T7S4 promoter is generated. (B) Southern blot hybridisation using radiolabeled Ubc_{Ap} probe against purified untransfected parasite gDNA and transfected $iUbc_{Ap}$ parasites detects the expected DNA fragments. PCR analysis of clones shows integration of the promoter.

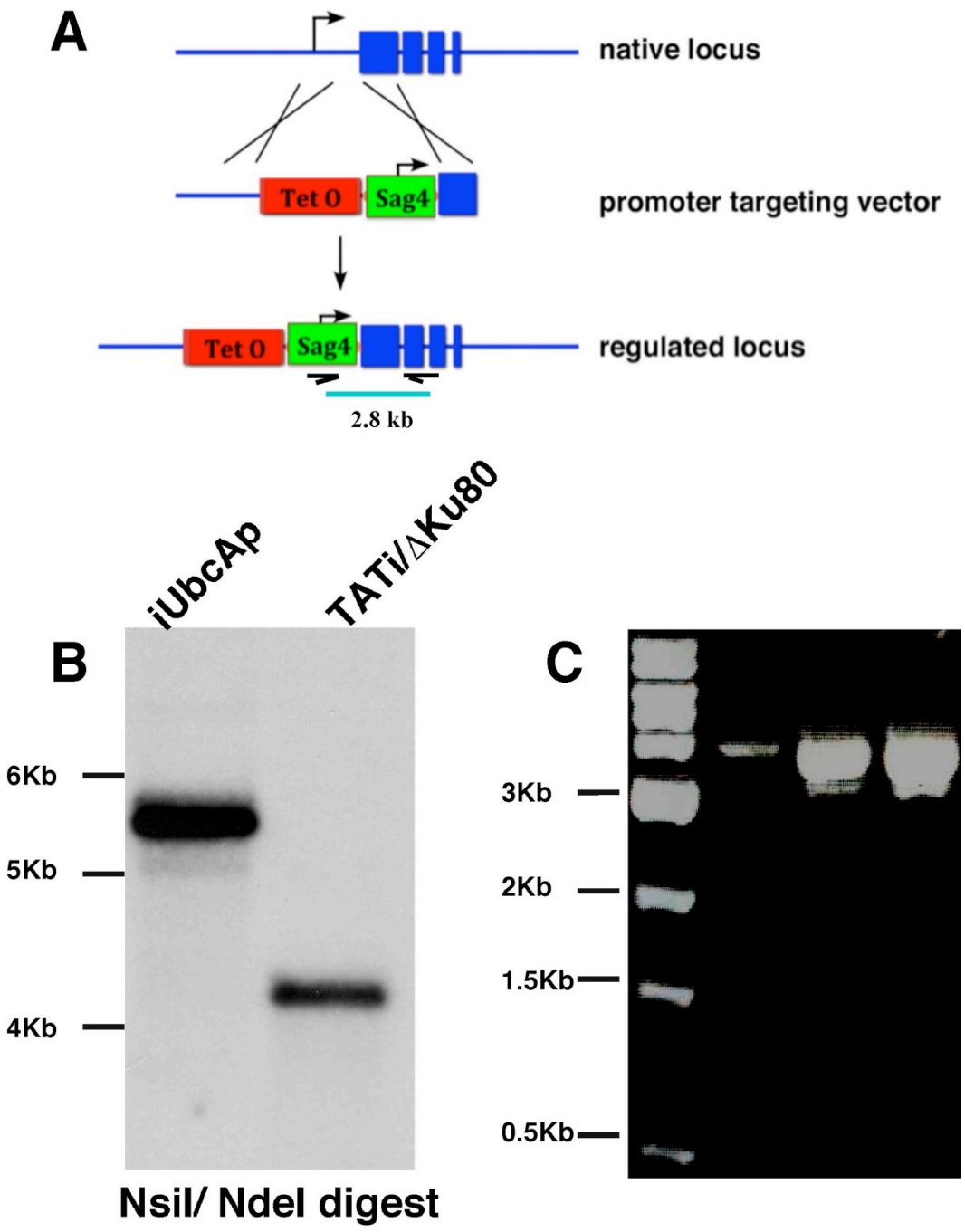

A
native locus
Tet O
Sag4
promoter targeting vector
Tet O
Sag4
regulated locus
2.8 kb
iUbcAp
TATi/ΔKu80
B
6Kb
5Kb
4Kb
NsiI/ NdeI digest
C
3Kb
2Kb
1.5Kb
0.5Kb

Figure 4.3: Growth and viability assay of iUbc$_{Ap}$ parasites. (A) 500 iUbc$_{Ap}$ parasites were added to a confluent monolayer of Human fibroblast cells in a T-25 flask. After 8 days of incubation with or without ATc the monolayer was fixed and stained with crystal violet to visualize plaques produced by parasite growth. (B) Fluorescent growth assay of tandem tomato expressing iUbc$_{Ap}$ parasites. The assay was conducted by adding 5000 parasites to host cells grown in a 96 well plate and incubating them either in absence (blue circles) or presence (red squares) of ATc or after pre-treating the parasites for 3 days in ATc (green squares).

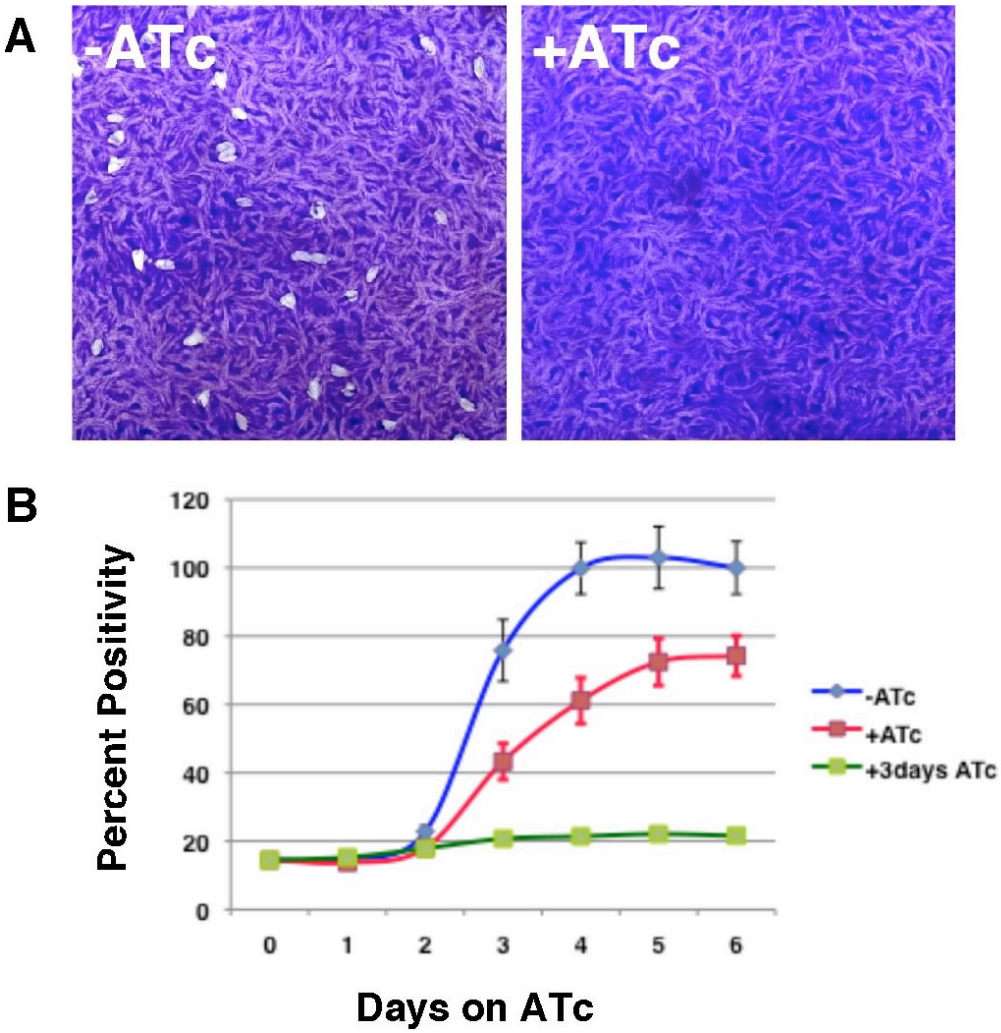


Figure 4.4: Loss of Ubc_{Ap} results in loss of Apicoplast import. (A) Schematic depiction of the lipoylation assay used to measure apicoplast protein import in Ubc_{Ap} mutant. The E2 subunit of apicoplast PDH is lipoylated by the apicoplast resident enzymes LipB and LipA. This post-translational modification of PDH-E2 event requires successful import into the apicoplast lumen. The E2 subunit of mitochondrial enzymes are similarily modified after translocation across the mitochondrial membranes by a luminal enzyme LplA. (B) Pulse chase assay for measurement of apicoplast protein import in $iUbc_{Ap}$ cell line. $iUbc_{Ap}$ parasites were incubated for 0, 2, 3, 4, 5 and 6 days on ATc followed by radiolabelling of parasites and immunoprecipitation of lipoylated proteins. Lanes marked with "P" represent the pulse samples and the ones with "C" represent chase samples. 3 days post incubation with ATc $iUbc_{Ap}$ parasites show reduced levels of PDHE2 (top band labelled api-PDHE2) whereas the mitochondrial proteins (two lower bands labelled as mito-E2) are unaffected suggesting organellar specific import impairment.

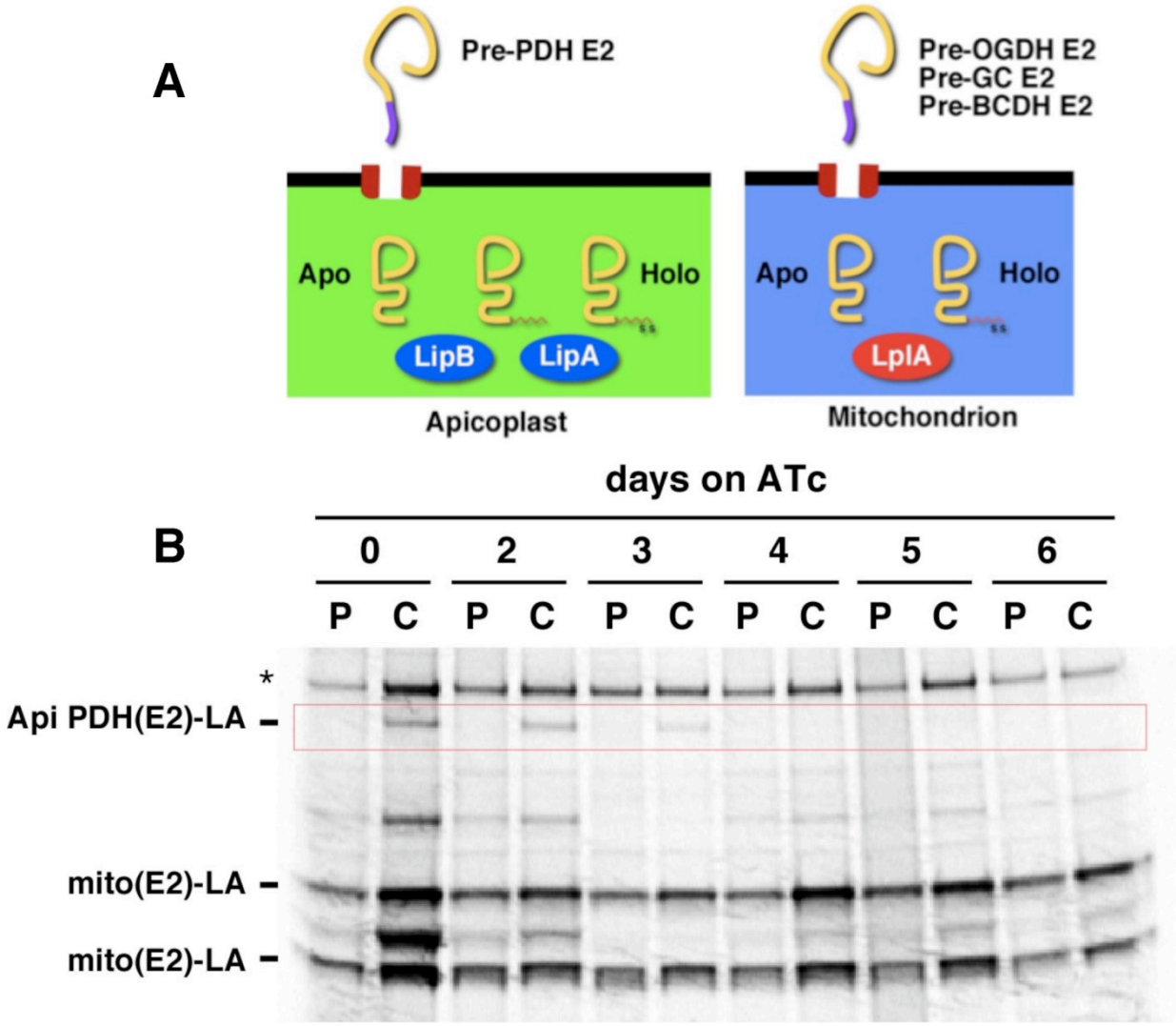
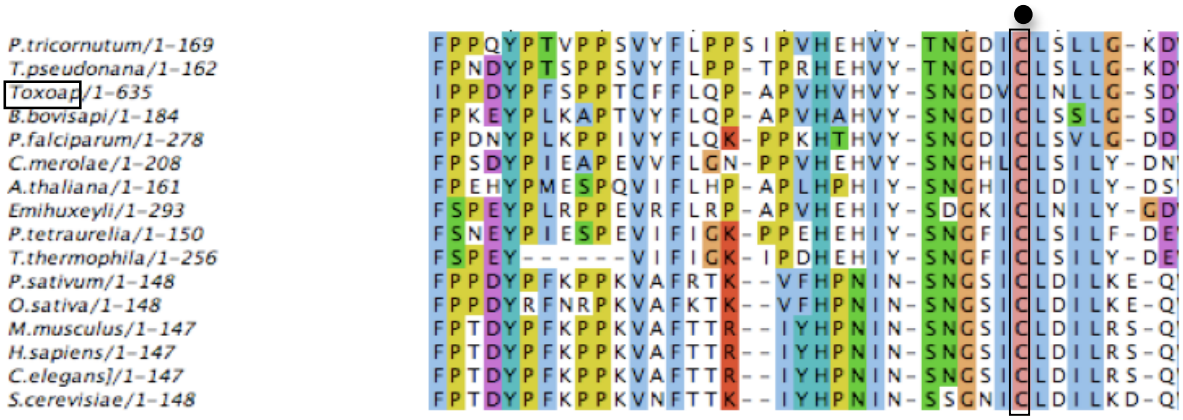

A
Pre-PDH E2
Pre-OGDH E2
Pre-GC E2
Pre-BCDH E2
Apo
Holo
LipB
LipA
Apicoplast
Apo
Holo
LplA
Mitochondrion
B
days on ATc
0
2
3
4
5
6
P C P C P C P C P C P C
*
Api PDH(E2)-LA
mito(E2)-LA
mito(E2)-LA

Figure 4.5: Ubc_{Ap} multiple sequence alignment showing the catalytic core of the enzyme and important adjoining amino acid residues that have reported to play. The Circle marks the position of active site cysteine residue.



Table

Table 4.1: List of putative ubiquitin associated proteins in the GT1 strain of *T. gondii*. Gene id's with "*" have not been experimentally verified for apicoplast localization.

Ubiquitin-activating Enzyme E1	**Ubiquitin-conjugating enzyme E2**	**Ubiquitin-ligase**	**Deubiquitinating enzyme**	**Ubiquitin**
TGGT1_092180	TGGT1_021600	TGGT1_006600 * TGGT1_082020 *	TGGT1_033890 *	TGGT1_033460 *

CHAPTER 5

CONCLUSION

5.1 Summary of Key Findings

At the commencement of this thesis the mechanisms for protein translocation across the four membranes of the *T. gondii* apicoplast were poorly understood. Previous work had shown that the nuclear encoded plastid proteome is targeted to the organelle via the secretory pathway and that this was guided by a N-terminal bipartite targeting sequence. The two innermost membranes of the apicoplast are phylogenetically similar to chloroplast membranes. Both these membranes were likely derived from the cyanobacterium during primary endosymbiosis. Thus it had been proposed earlier that protein translocation across these inner membranes would probably engage plant like Tic/Toc machinery (Cavalier-Smith 1999; McFadden 1999). Identification of matching Tic and Toc components by sequence similarity in secondary plastid bearing organisms (including the *T. gondii* genome) was challenging, likely due to the large evolutionary gap. However in a collaborative project I identified a highly divergent homologue of Tic20 from *T. gondii* genome using iterative BLAST algorithm. The protein was localized to the innermost membrane of the apicoplast and genetic attenuation of this protein led to perturbation of apicoplast protein import (van Dooren et al. 2008). A Tic22 homologue was also subsequently identified in both *T. gondii* (van Dooren and Agrawal et al. data unpublished) and *P. falciparum* (Kalanon et al. 2009). Knock down of the protein results in loss of parasite growth and viability and severe impairment of apicoplast protein import (van Dooren and Agrawal et al. data unpublished). A recent publication from an algal model organism suggests that a Toc (tranlocon

of the outer chloroplast membrane) derived mechanism might be responsible for the transport across the second innermost membrane (Bullmann et al. 2010). In plants Toc75 is at the centre of the outer membrane import apparatus, functioning as the central preprotein tranlocation pore. Bullmann and colleagues describe the localization of a Toc75 protein to the second innermost membrane of the diatom plastid. They further conducted electrophysiological studies that show that the plastid Toc75 homolgue behaves like Omp85 proteins involved in protein translocation. A Toc75 homologue can also be identified in the apicomplexan parasites with the exception of the plastid-less genus, Cryptosporidium. The protein posseses a canonical signal peptide and a signature N-terminal PORTA domain (polypeptide-transport-associated). We have constructed a conditional mutant in the *T. gondii,* Toc75 gene. Our preliminary data strongly suggests that Toc75 plays an important role in the import process.

Several models had been proposed for protein translocation across the periplastid membrane (the former algal plasma membrane) however there was no experimental evidence to distinguish them. Sequencing of the nucleomorph genome of the cryptophyte *Guillardia theta* and the identification of key proteins involved in the ERAD process in the organellar genome led to the formulation of a new model by Sommer and colleagues. Given their established role in protein translocation across the ER membrane, Sommer et al. proposed that during plastid acquisition, the algal endoplasmic reticulum-associated degradation (or ERAD) complex that normally functions in the export of misfolded proteins was retooled to instead import proteins across the periplastid membrane (Sommer et al. 2007). Studies from several groups including those described in this thesisbolstered this idea by providing experimental evidence for a $Der1_{Ap}$ based translocon in the periplastid space of secondary plastid bearing organisms *T. gondii,* (Agrawal et al. 2009) *P. falciparum* (Kalanon et al. 2009; Spork et al. 2009) and *P. tricornitum* (Hempel et al.

2009; Sommer et al. 2007). These studies demonstrated the presence of putative translocation components in the plastid. In this thesis I provide direct experimental for the function of $Der1_{Ap}$ and in import. We engineered a conditional mutant in the $Der1_{Ap}$ gene and demonstrated that $Der1_{Ap}$ mutant parasites are severely impaired in apicoplast protein import (Agrawal et al. 2009). Additionally, in this chapter/article the phylogenetic analysis of Cdc48 and Ufd1 support the monophyly of the disparate Chromalveolate lineages. This analysis further confirms that diatoms, cryptophytes and apicomplexa all seem to have inherited the plastid ERAD machinery from the red algal endosymbiont. In Chapter 4 we identify and characterize apicoplast localized ubiquitination machinery and demonstrate that it functions in apicoplast protein import.

5.2 Future direction

5.2.1 How are proteins extracted through the apicoplast ERAD pore?

Cdc48 is an indispensable component of the ERAD complex and is known to pull proteins through the pore utilizing its ATPase activity. We and others have demonstrated that a plastid specific homolog of Cdc48 ($Cdc48_{Ap}$), that localizes to the outer compartments of the endosymbiont (Agrawal et al. 2009; Hempel et al. 2009). Translocation across biological membranes typically requires energy to either "push" or "pull" the proteins through the translocation apparatus. During ERAD the catalytic hydrolysis of ATP by Cdc48 along with its cofactors Ufd-1 and Npl4 helps in retrieval of ubiquitin tagged proteins into the cytosol. We hypothesize that the apicoplast localized Cdc48 functions similarly in the extraction of proteins out of the $Der1_{Ap}$ translocon. The construction of conditional null and dominant negative mutants of $Cdc48_{Ap}$ in its ATPase domain are underway to assess if and how the $Cdc48_{Ap}$ complex is involved in import.

5.2.2 What are the substrates for apicoplast ubiquitination?

With accumulating evidence for the presence of a ubiquitination machinery in the apicoplast, a key question that needs to be addressed is, what are the substrates of ubiquitination? Do all apicoplast proteins become modified or is protein ubiquitination mostly required for retention of ERAD and associated periplastid space proteins at their location. In the normal cellular environment, the rapidly changing dynamics of substrate ubiquitination and deubiquitination does not allow robust detection of ubiquitinated intermediates. Genetic interference with proteasome associated deubiquitinase or addition of specific protease inhibitors such as NEM have been shown in the past to block the deubiquitination activity resulting in easier detection of ubiquitinated proteins (Mines et al. 2009; Van der Veen et al. 2011). Recently Hempel et al. demonstrated that the periplastid compartment of the diatom *P. tricornitum* possesses a deubiquitinase enzyme that belongs to the C19 peptidase family (Hempel et al. 2010). The plastid deubiquitin could be required either for processing of ubiquitin propeptides or for removal of ubiquitin from apicoplast cargo to allow transport through following membranes. We searched the *T. gondii* genome database for homologs of C-19 peptidases using the *P. tricornitum* plastid DUB as query sequence. The gene products with most significant BLAST hits were evaluated by epitope tagging of the endogenous locus at the C-terminus followed by immunofluorescence assay to study their localization. So far we have evaluated the localization of three of these enzymes, which are confined to the ER. TGGT1_033890 is the best hit to the *P. tricornitum* DUB, however similar epitope tagging experiments did not provide any fluorescence signal. Reasons for the absence of labeling may include incorrect gene models or the fact that an epitope tag interferes with protein function. We are presently verifying the gene annotation by RACE analysis and expect to be able to test its expression either by endogenous or episomal

tagging of the gene. Assuming that such a plastid specific DUB is present in *T. gondii,* a knockdown of the enzyme may presumably leave proteins in a state where they remain modified by ubiquitin.

Over the course of this study, many useful markers for different sub compartments of the apicoplast have become available through the efforts of several research groups (including ours), in the form of antibodies or epitope tagged constructs (e.g., luminal proteins like ACP, Cpn60, DOXPRI, LytB, FNR-RFP; periplastid proteins like $Der1_{Ap}$, $Cdc48_{Ap,}$ Ufd_{Ap}, Ubc_{Ap}, Uba_{Ap} and outer membrane proteins like TPT, FtsH1) (Agrawal et al. 2009; Karnataki et al. 2007; Mazumdar and Striepen 2007; van Dooren et al. 2008). Immunoprecipitation of these proteins from a cell line where the apicoplast DUB is no longer expressed, followed by mass spectrometric analysis of different trafficking intermediates will provide vital insights into function of apicoplast ubiquitination.

APPENDIX 1

THE APICOPLAST: AN ANCIENT ALGAL ENDOSYMBIONT OF APICOMPLEXA[3]

[3] S. Agrawal, S. Nair, L. Sheiner and B. Striepen. 2010. In *Structures and Organelles in Pathogenic Protists, Microbiology Monographs 17.* Eds. W. de Souza. Springer-Verlag Berlin Heidelberg.

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Abstract

The discovery of a chloroplast in the Apicomplexa came as a surprise as these are nonphotosynthetic parasites that historically had been the domain of zoologists. This organelle, the apicoplast is essential for parasite survival and its metabolism is intensively pursued as the source of new targets for antiparasitic drugs, in particular new antimalarials. The apicoplast has a remarkable evolutionary history, and this history is reflected in its complex structure and cell biology. A cyanobacterium and two eukaryotes have contributed to the genesis of this organelle and their contributions can still be traced today. This chapter sets out by briefly summarizing the studies that led to the discovery of the apicoplast followed by an overview of our most current knowledge of the molecular mechanisms of apicoplast protein import, apicoplast division and replication and apicoplast metabolism.

A1.1 The surprising photosynthetic past of Apicomplexa

Apicomplexa are a phylum of unicellular eukaryotes that live as obligate intracellular parasites and maintain a complex life cycle that includes sexual and asexual reproduction. Over their long evolutionary history Apicomplexa have adapted to a tremendous variety of invertebrate and vertebrate hosts. Infection with many of these parasites, results in severe disease of the host. Members of the genus *Plasmodium* are the etiological agents of malaria, and *Toxoplasma gondii* causes neurological disease in immunosuppressed patients and upon congenital transmission. Parasites of the genera *Babesia* and *Theileria* are responsible for Texas and East Coast fever in cattle, and *Cryptosporidium*, *Cyclospora* and *Eimeria* cause gastro-intestinal diseases in humans and various domestic animals. Phylogenetic and morphological studies robustly position Apicomplexa within the Alveolata. In addition to Apicomplexa this group includes ciliates and dinoflagellates that share cortical alveoli as a defining morphological feature. These are flatted

membranous cisternae that underly the plasma membrane and form part of the complex pellicle (Gould et al, 2008). Following a hypothesis initially formulated by Cavalier-Smith, these organisms are thought to be part of a major branch of the eukaryotic tree of life, the chromalveolates, that includes single and multicellular organisms which at first sight show little resemblance and occupy a tremendous variety of ecological niches (Cavalier-Smith, 2002). The origin of this super-phylum is thought to lie in the symbiotic union of two single celled eukaryotes, a heterotroph and a red alga. While not without critics the chromalveolate hypothesis has steadily gained support over the years (Keeling, 2009). One of the key elements of this support was the discovery of a plastid in Apicomplexa – the apicoplast. As indicated by their historical phylum name Sporozoa, Apicomplexa have long been the domain of zoologist, however, it appears now well established that Apicomplexa have a photosynthetic past in the ocean (Moore et al, 2008). This chapter briefly describes the discovery of the apicoplast and then outlines our current understanding of its evolution, cell biology, and metabolic function.

A1.1.1 The discovery of the apicoplast – from vacuole to endosymbiont

The discovery of a plastid in Apicomplexa emerged from three observations made by researchers from different disciplines that initially did not appear to be related: a multi-membranous structure seen on electron micrographs, a third density species of parasite DNA apparent by cesium chloride gradient centrifugation, and the puzzling sensitivity of eukaryotic parasites to antibiotics that target prokaryotic-type protein translation.

Ultrastructural studies reported the presence of a multi-membrane bound organelle in a variety of apicomplexan parasites. This mysterious organelle received numerous descriptive names (reviewed in (Siddall, 1992)), yet its identity and cellular function remained elusive. In an independent effort that was aimed at isolating mitochondrial DNA, a series of studies in different

parasites described a circular DNA molecule distinct of nuclear chromosomal DNA (Borst et al, 1984; Dore et al, 1983; Gardner et al, 1988; Kilejian, 1975; Williamson et al, 1985). The size of the molecule and investigator expectation initially let to its assignment as putative mitochondrial genome. However doubts in this interpretation emerged in 1987 when Vaidya and Arasu described another even smaller non-nuclear DNA molecule in *Plasmodium yoelii* (Vaidya & Arasu, 1987).

Step by step the distinct nature of the two extra-chromosomal DNA molecules was uncovered in *Plasmodium*: one a 6kb repetitive linear molecule, which was assigned to the mitochondrion based on its genetic content, and the other 35kb circular molecule of unknown function. Phylogenetic evidence suggested that the two molecules were of different evolutionary origin (Feagin et al, 1992; Gardner et al, 1991a; Williamson et al, 1994), and fractionation data implied that they were likely located in different subcellular compartments (Wilson et al, 1992). Based on these insights Wilson and coworkers proposed the presence of an organelle corresponding to a residual plastid in apicomplexans (Gardner et al, 1994a). Features of the genome that supported this idea were an inverted tail to tail repeat of small and large subunit ribosomal RNA, which is reminiscent of red algal plastid genomes (Gardner et al, 1988; Gardner et al, 1993; Gardner et al, 1991b), and genes for elements of plastid-type transcription and translation machinery (Gardner et al, 1991a; Gardner et al, 1994b; Preiser et al, 1995; Wilson et al, 1996; Wilson et al, 1994). The notion of plastid protein translation also explained the observation that Apicomplexa are sensitive to certain antibiotics (see below).

As soon it was clear that apicomplexans harbor a plastid genome, the obvious question became: 'where does it reside'? Maternal inheritance of the genome strongly suggested a non-nuclear residence (Creasey et al, 1994). The previously enigmatic multimembranous organelle seen in so

many micrographs emerged as the favorite candidate. *In situ* hybridization experiments had the final word in confirming this hypothesis in *Plasmodium* and in *Toxoplasma* (Kohler et al, 1997; McFadden et al, 1996) and the apicoplast was formally identified and named. Apicoplasts have been described in many Apicomplexa with the marked exception of *Cyptosporidium* (Zhu et al, 2000).

A1.1.2 The apicoplast's endosymbiotic origin is the source of its complex biology

Plastids like mitochondria are thought to be the product of endosymbiosis. A eukaryotic cell engulfed a cyanobacterium, the metabolism and genomes of both partners became progressively intertwined ultimately resulting in a double membrane bound organelle that we now know as chloroplast (reviewed in (Gray, 1993; McFadden & van Dooren, 2004)). This early event gave rise to the common ancestor of three major groups of photosynthetic eukaryotes: glaucophytes, red algae and green algae. In several cases this was not the end of the journey though and plastids seem to have moved laterally from one lineage to another by secondary endosymbiosis. There is now robust evidence that plastids have been acquired secondarily several times during eukaryotes evolution giving rise to additional photosynthetic clades (Keeling, 2009; Lane & Archibald, 2008). These events occurred through the uptake of a single-celled alga carrying a primary plastid by a second eukaryote. As in the case of primary plastids and mitochondria, lateral gene transfer to the host nucleus resulted in subjugation of the symbiont into a dependent organelle (Howe & Purton, 2007), in this case likely involving transfer from the plastid as well as the algal nuclear genome. A telltale of this evolutionary history are the additional membranes that surround secondary plastids which often are referred to as 'complex' plastids (four in the case of the apicoplast see Fig. 1A and below for further detail). We know that this process occurred more than once as there are organisms with complex plastids that trace back to the

green as well as the red algal lineage. Some of the most intriguing evidence for secondary endosymbiosis comes from the discovery and sequencing of nucleomorphs (Moore & Archibald, 2009), these are remnant nuclei of the ancestral algal endosymbiont that are localized between the second and third membrane of the complex plastid.

Whereas most researchers agree that the apicoplast is the product of a secondary endosymbiotic event (with some exceptions (Kohler, 2005)), the identity of the alga involved remains disputed between a red and a green lineage camp. Numerous genes and proteins have been subjected to phylogenetic analysis to trace the origin of the endosymbiont. These studies have focused on genes found in the apicoplast genome itself, nuclear genes that encode apicoplast proteins, and lastly nuclear genes that encode proteins that do not target to the apicoplast but might have been acquired from the endosymbiont's nucleus nonetheless. While the majority of studies pointed to a red algal origin a significant number of analyses favored an ancestor from the green algal lineages (see (Feagin, 2005) for a comprehensive summary). These analyzes may be complicated by extensive gene loss in certain taxa and the potential lateral gene transfer between organellar genomes (Obornik et al, 2002). Fast and coworkers made an interesting breakthrough by noting that the plastid glyceraldehydes-3-phosphate dehydrogenase (GAPDH) from dinoflagelattes and apicomplexans are of eukaryotic origin, as opposed to the cyanobacterial-type targeted to the plastids of plants and green and red algae (Fast et al, 2001). Plastid-GAPDH phylogenies then grouped apicomplexans as well as dinoflagellates tightly together with heterokonts and cryptomonads. This suggests that the apicoplast originated early in evolution, and that a single endosymbiosis gave rise to plastids in these taxa. Given that well established phylogenies, based on plastid genes as well as pigmentation strongly support a red algal origin of both heterokont and cryptomonad plastids (Delwiche, 1999), these findings meant that a red alga is also the likely

origin of the apicoplast. All together this picture sits well with the chromalveolate hypothesis, which groups these lineages together based on a common secondary endosymbiosis involving a red alga (Cavalier-Smith, 1999; Cavalier-Smith, 2004; Keeling, 2009). Additional support for red ancestry emerges from the details of apicoplast cell biology and metabolism detailed later in this chapter. This includes the association of its genome with histone-like HU proteins found only in Eubacteria and red algae (Kobayashi et al, 2002), the apparent red algal origin of elements of the apicoplast protein import machinery (Agrawal et al, 2009), and a cytoplasmic starch pathway that resembles red alga and is distinct from the chloroplast localized starch metabolism in the green lineage (Coppin et al, 2005).

A1.1.3 The apicoplast genome is mostly concerned with itself

The apicoplast genome is reduced in size and highly focused in the functions of the encoded proteins. Excluding seven hypothetical genes and two known genes, all encode components of the apicoplast transcription and translation machinery (Wilson et al, 1996). These include three subunits of an eubactrial-type RNA-polymerase, 17 ribosomal proteins, a complete set of tRNAs and the translation elongation factor Tu (Wilson et al, 1996). The apicoplast genome encoded subunits (rpoB, rpoC1 and rpoC2) together with a nuclear encoded rpoA homologue (Bahl et al, 2003) could compose a chloroplast-like RNA polymerase (Wilson & Williamson, 1997) which is in agreement with the sensitivity of the parasite to rifampin, an inhibitor of eubacterial RNA polymerases (Dahl et al, 2006; McConkey et al, 1997). Antibiotics that typically only interfere with bacterial translation machinery have been known to show some efficacy in the treatment of malaria and toxoplasmosis for many years (Coatney & Greenberg, 1952; Tabbara & O'Connor, 1980). Subsequent studies in *Toxoplasma* connected this sensitivity to the sequence of the apicoplast ribosomal RNA (Beckers et al, 1995). Further experiments showed apicoplast specific

effects following clindamycin treatment of *Toxoplasma* (Fichera & Roos, 1997), and demonstrated association between resistance to this drug and mutations in the apicoplast ribosomal RNA genes (Camps et al, 2002). In addition to these reports, studies in *Plasmodium* linked the effects of thiostrepton and azithromycin to the apicoplast ribosomal RNA (Clough et al, 1997; Sidhu et al, 2007). As we will see below, most genes have been transferred to the nucleus. If the apicoplast genome largely encodes proteins concerned with itself, why has it not been lost? One idea is that actually it is in the process of loss but that this process is not concluded yet in all Apicomplexa. Another is that a small number of proteins to be made in place because they cannot be transported (e.g. due to particular protein folding or sensitivity to changing redox environments). Potential candidates are two genes encoding a putative chaperone (ClpC) and an iron-sulfur cluster biogenesis protein (SufB).

A1.1.4 The long journey from nucleus to apicoplast

As described above the proteome encoded by the apicoplast genome is small and restricted to housekeeping functions, including transcription and translation. However, about 500 proteins are predicted to populate the apicoplast stroma (Foth et al, 2003) and the biochemical pathways that have been localized to the organelle (see below). A closer look revealed that like mitochondria and chloroplasts, the biosynthetic and metabolic functions of the apicoplast are mediated in large part by nuclear encoded proteins. This is likely the result of massive gene transfer from endosymbiont to host. These proteins must now be translated in the cytosol from where they have to travel to their destination in order to perform their metabolic functions.

A1.2 A bipartite leader peptide is required for apicoplast targeting

When compared to their cyanobacterial orthologs most nuclear encoded apicoplast proteins possess a pronounced N-terminal extension. Such extensions are known to mediate import of

nuclear encoded chloroplast proteins in plants. Experimental work confirmed that the N-termini of several apicoplast proteins are necessary and sufficient to target a GFP reporter to the lumen of the organelle (Foth et al, 2003; Waller et al, 1998; Waller et al, 2000). Compared to their chloroplast counterparts the N-terminal extension of apicoplast proteins are longer and have a bipartite structure. Their beginning resembles the signal peptide present in secretory proteins (Waller et al, 1998), while the second portion (the transit peptide) appears to have features reminiscent of the transit peptides of chloroplast proteins. In fact, GFP engineered to carry the transit peptide of a *T. gondii* apicoplast ribosomal protein was shown to be imported efficiently into pea chloroplast (DeRocher et al, 2000). Similar bipartite leaders had been described for a variety of algae harboring secondary plastids and the initial trafficking in these systems appears to occur via the secretory pathway (Grossman et al, 1990; Sulli & Schwartzbach, 1995). The bipartite model has been extensively tested using transgenic reporters in *Plasmodium* and *Toxoplasma* in studies that deleted each component of the leader. In the absence of the transit peptide, the signal sequence of apicoplast proteins targets a GFP reporter to the parasitophorous vacuole, the default secretory route in these parasites. Deletion of the signal peptide results in cytosolic (and in some cases mitochondrial) localization of the reporter (DeRocher et al, 2000; Harb et al, 2004; Waller et al, 2000). Transit peptides of both primary and secondary plastid proteins are enriched in hydrophilic and basic amino acids, have very low sequence conservation and vary in length from 50-200 amino acids (Bruce, 2001; Claros et al, 1997). Similar to their choloroplast counterparts, apicoplast protein transit peptides have very few acidic and hydrophobic residues and possess a net positive charge. Detailed mapping of the transit peptide by point mutation and serial deletion revealed that a net positive charge at the N-terminus is essential for proper apicoplast targeting, but that their exact position can be varied (Foth et al,

2003; Harb et al, 2004; Tonkin et al, 2006a). Apicoplast transit peptides further appear to possess Hsp70 chaperone-binding sites suggesting that maintenance of preprotein in an unfolded state might be a prerequisite for successful import. Interestingly a stretch of 26 amino acids that separates Hsp70 binding sites in plant transit peptides is also present in apicoplast transit peptides suggesting a similar mechanism by which molecular chaperones facilitates the translocation of cargo proteins through membrane pores.

The bipartite leader is thought to guide preproteins to the apicoplast in a sequential fashion. The signal peptide is believed to result in the cotranslational insertion of the nascent peptide into the endoplasmic reticulum (ER) followed by its removal (full-length proteins with intact signal peptides have not been detected (Waller et al, 1998; Waller et al, 2000)). Cleavage of the signal peptide then would expose the transit peptide. The presence of the transit peptide and its processing has been observed both in *T. gondii* and *P. falciparum* (Vollmer et al, 2001; Waller et al, 1998; Waller et al, 2000; Yung et al, 2001). In Western blot analyses apicoplast proteins typically have a slower migrating precursor band, corresponding to the size of proteins with intact transit peptide, and a faster migrating mature band corresponding to the processed form. To understand the kinetics of transit peptide processing van Dooren and colleagues performed pulse-chase labeling with radioactive amino acids and immuno-precipitation of apicoplast-targeted proteins in *P. falciparum* (van Dooren et al, 2002). They demonstrated that processed apicoplast proteins appear after 45 min of labeling and complete processing might take as long as four hours. Processing of transit peptides in plants is mediated by a specific peptidase in the chloroplast stroma. A nuclear encoded stromal peptidase homologue with a bipartite targeting sequence is present in *T. gondii* and *P. falciparum* (He et al, 2001; van Dooren et al, 2002),

however direct experimental evidence that this protein is capable of transit peptide cleavage, is still lacking.

A1.2.1 Chloroplast derived membranes are crossed using chloroplast translocons

The apicoplast is surrounded by four membranes of divergent evolutionary origin. A number of intriguing models have been proposed to explain how proteins might cross these four membranes in Apicomplexa and other organisms bearing secondary plastids (Tonkin et al, 2008). These have invoked fusion and fission of membranes (Gibbs, 1979), non-selective pores (Kroth & Strotmann, 1999), or the activity of protein translocons derived from either the chloroplast envelope (van Dooren et al, 2008a) or the endoplasmic reticulum (Sommer et al, 2007). Until recently there was little evidence to distinguish and test the competing hypotheses. Two developments have dramatically accelerated research in this area. Full genome sequence information is now available for a growing number of apicomplexan and algal species, and increasingly sophisticated genetic tools can be used in species that were previously experimentally inaccessible. The model that emerges from these studies is that protein translocons are crucial to import, and that these translocons are divergent in their origin reflecting that contributed the membrane they cross. We will describe our current knowledge of translocons as it emerged from the inside out.

The two innermost membranes are homolgous to the membranes of the red algal chloroplast. Protein import across chloroplast membranes has been studied in great detail and is mediated by two multi-protein complexes: the translocon of the outer chloroplast membrane (Toc) and the translocon of the inner chloroplast membrane (Tic). These complexes and their protein components function in recognition and binding of the transit peptide, form the translocation channel, provide mechanical energy to drive translocation, and act as chaperones aiding in

unfolding and refolding of cargo proteins (see (Hormann et al, 2007; Jarvis, 2008) for recent reviews of the extensive literature). A homolog of the plant import component Tic20 has been identified in a variety of Apicomplexa with the marked exception of plastid-less genus *Cryptosporidium* (van Dooren et al, 2008a). While the primary sequence conservation between these proteins and their plant counterparts is very low, they share the overall topology of a four-pass transmembrane protein. *Tg*Tic20 features a canonical bipartite signal that is processed. Biochemical and immuno-electron microscopic studies demonstrated that *Tg*Tic20 is a component of the apicoplast membranes. To further define its localization van Dooren *et al.* employed a split-GFP assay. In this experiment GFP is split into two segments that are fused to either the test protein or marker proteins of known subcellular localization (Cabantous & Waldo, 2006). By themselves these fragments are non-fluorescent, however, they regain fluorescence by direct molecular interaction when the two fusion proteins are targeted to the same compartment. The analysis of *Tg*Tic20 split-GFP transgenics showed that the protein localizes to the innermost membrane of the organelle with both N and C-terminus projecting into the apicoplast stroma. Further functional insights emerged from a *Tg*Tic20 conditional mutant. Inducible knockdown of *Tg*Tic20 leads to impairment of protein import into the plastid, and subsequent defects in plastid biogenesis and parasite survival (van Dooren et al, 2008a). A homolog of a second putative member of the Tic complex, Tic22, has been identified in the *P. falciparum* and *T. gondii* apicoplast ((Kalanon et al, 2009) and G.G. van Dooren, SA and BS unpublished). Tic22 in plants has been described as a small soluble protein that peripherally associates with the Tic and the Toc complex and interacts with pre-proteins in transit. It was proposed that Tic22 serves as an adaptor that facilitates pre-proteins translocation from the Toc translocon towards the Tic complex (Kouranov et al, 1998). Conditional knockout of Tic22 in *T. gondii* demonstrates that

this protein is essential for apicoplast protein import and parasite survival (G.G. van Dooren, SA and BS unpublished).

Extensive genome mining so far has failed to conclusively identify homolgs of the proteins that make up the Toc complex in apicomplexan parasites (a putative Toc34 has been described in *P. falciparum* but this assignment has not been experimentally evaluated (Waller & McFadden, 2005). It is possible that apicomplexan homolgs of Toc proteins are too divergent to be identified. Alternatively a different translocon might have subsumed its function. While the details (in particular with respect to Toc) are still emerging it appears that transport across the membranes that are derived from the chloroplast employs conserved machinery that is homologous to the machinery that performed this task in the algal ancestor of the apicoplast.

A1.2.2 Retooling an endosymbiont translocon from export to import

The third membrane, also known as periplastid membrane, is of particular interest as it represents the plasma membrane of the alga and as such the direct interface between host and endosymbiont. Establishing transport across this membrane was likely a prerequisite for gene transfer, an crucial event in the progressive adaptation of the endosymbiont to intracellular life. Key to the discovery of the mechanism that allows proteins to cross this membrane was the sequencing of the nucleomorph genome of *Guillardia theta* (the nucleomorph is the "fossil" remnant of the algal nucleus). Sommer and coworkers noted that this highly reduced genome encodes core elements of the endoplasmatic reticulum associated degradation (ERAD) system (Sommer et al, 2007). ERAD usually acts in ER homeostasis by retrieving misfolded secretory proteins from the ER and funneling them for degradation to the proteasome in the cytosol. The core components of the ERAD transport machinery are Der-1, the ATPase Cdc48 and its co-factor Ufd-1. Der-1 is a favored candidate for the proteinaceous pore in the ER membrane and

has been shown to be essential for retrotranslocation of misfolded luminal proteins (Ye et al, 2004). Protein substrates destined to be degraded are polyubiquitinated and subsequently extracted from the pore by the Cdc48-Ufd-1-Npl4 complex (Ye et al, 2001). What could be the function of such a system in an endosymbiont that appears to have long lost its ER? In a bold stroke Sommer and colleagues formulated the hypothesis that an ERAD translocon had been retooled to import proteins into complex plastids (Sommer et al, 2007). Relocation of an existing translocation machinery to a different membrane seems to be remarkably simple and elegant solution to engineer import into a newly acquired organelle.

The ERAD hypothesis has accumulated considerable support from a recent flurry of publications reporting the identification and plastid localization of ERAD components in cryptomonads, diatoms, and Apicomplexa (Agrawal et al, 2009; Kalanon et al, 2009; Sommer et al, 2007; Spork et al, 2009). In our own work we have demonstrated that the *T. gondii* genome encodes multiple homologs of Der1, Cdc48 and Ufd-1. Immunofluorescence analysis of parasite cell lines expressing epitope tagged forms of these proteins reveal that while one complete set of components is associated with the ER and likely performs their classical role in ERAD, at least one homolog of each of these components localizes to the outer membranes of the apicoplast. Split GFP assays performed in the diatom *Phaeodactylum tricornitum*, suggest that Der-1 is indeed associated with the third membrane as predicted by the Sommer hypothesis (Hempel et al, 2009). Furthermore phylogenetic analysis of the two *T. gondii* Cdc48 proteins demonstrates that they are of divergent evolutionary origins. The apicoplast localized Cdc48 forms a well-supported clade with its red algal lineage counterparts (including the protein encoded on the *G. theta* nucleomorph) while the cytoplasmic protein branches with proteins that reflect the current view of vertical evolution for Apicomplexa (Agrawal et al, 2009). Genetic ablation of $Der1_{Ap}$ in

T. gondii results in swift and complete ablation of apicoplast protein import as measured using a variety of biochemical assays (Agrawal et al, 2009; van Dooren et al, 2008a) demonstrating a direct role of $Der1_{Ap}$ and the endosymbiont derived ERAD system in apicoplast protein import.

ERAD mediated protein retrotranslocation across the ER membrane coincides with poly-ubiquitination or the cargo protein. This modification is critical for the subsequent degradation of the protein but also appears to have a potential role in the translocation step. The amino terminus of Ufd-1 is known to bind poly-ubiquitin. Deletion of yeast Ufd-1 amino terminus results in disruption of translocation of proteins across the ER membrane (Park et al, 2005; Walters, 2005). Ubiquitylation is brought about by the sequential action of three enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). Genome mining and emerging experimental studies point to the likely presence of plastid-specific ubiquitylation factors in cryptomonads, Apicomplexa and diatoms ((Hempel et al, 2009; Spork et al, 2009) SA & BS unpublished). At this point the mechanistic role that ubiquitylation might play in the apicoplast is not understood. It is possible that ubiquitylation provides the necessary signal for movement of preproteins across the membrane. However, it remains to be experimentally demonstrated whether apicoplast targeted preproteins are ubiquitylated and whether the organelle possesses all the other necessary components for ubiquitylation.

A1.2.3 Still a black box: how do apicoplast proteins find the apicoplast?

While we now have some understanding of how proteins translocate across the inner membranes of the apicoplast, we know little about their way from the ER to the outermost compartment of the organelle. This part of the journey shows some diversity when comparing the targeting to different complex plastids. In heterokonts and cryptophytes the outermost compartment of the plastid is directly continuous with the ER and the nuclear envelope (Gibbs, 1979; Gould et al,

2006a; Kilian & Kroth, 2005; Wastl & Maier, 2000). This setup makes plastid targeting straightforward. The signal peptide provides access to the ER, and once in ER proteins have direct access to the periplastid membrane and its translocon (Gould et al, 2006a; Gould et al, 2006b). Targeting to complex plastids that are surrounded by three membranes in the euglenophytes and dinoflagellates appears to proceed from the ER to the Golgi and then the plastid. The hallmark of his route is sensitivity to the drug brefeldin A and the presence of a second hydrophobic segment at the end of the transit peptide that acts as a stop transfer signal thus exposing a potential sorting motif on the cytoplasmic face of vesicles (Durnford & Gray, 2006; Nassoury et al, 2003; Patron et al, 2005; Sulli & Schwartzbach, 1995; Sulli & Schwartzbach, 1996). In the case of Apicomplexa, brefeldin A treatment or low temperature incubation (both known to block Golgi trafficking) do not affect the steady state distribution of apicoplast targeted GFP reporters (DeRocher et al, 2005; Tonkin et al, 2006b). This would argue for direct trafficking from the ER to the apicoplast side-stepping the Golgi. Electron tomographic studies have found no evidence for a permanent connection between ER and the apicoplast, however, they noted sites of close apposition between the membranes of both organelles, which may reflect functional interaction (Tomova et al, 2006; Tomova et al, 2009). Evidence for a vesicular step at this point is mostly circumstantial. A number of groups noted what appear to be vesicles carrying apicoplast proteins. These can be more apparent when markers are overexpressed, or when import is blocked due to loss of the import machinery or loss of the apicoplast (DeRocher et al, 2008; Karnataki et al, 2007a; van Dooren et al, 2009; van Dooren et al, 2008b). If these structures truly represent transport vesicles *en route* remains to be studied in greater detail. If apicoplast proteins are packed into vesicles for transport from the ER, what

directs these vesicles to the apicoplast? Identifying specific marker proteins for such transport vesicles would be a major step towards a deeper mechanistic understanding.

A1.2.4 Additional signals and mechanisms may be involved in the trafficking of apicoplast membrane proteins

While the bulk of our understanding of apicoplast protein import is based on the study of luminal proteins, insights into proteins that target to the membranous compartments that surround the organelle are emerging. Some of these proteins, like the recently identified ERAD and Tic components appear to have canonical bipartite leaders and undergo processing at the N-terminus (Agrawal et al, 2009; Kalanon et al, 2009; Spork et al, 2009; van Dooren et al, 2008b). However, other membrane proteins lack identifiable leader peptides. Most of these proteins feature transmembrane domains that potentially could serve as a signal anchor. One example is FtsH1 a putative zinc metalloprotease that localizes to several apicoplast membranes and appears to undergo complex processing at the N-terminus and C-terminus (Karnataki et al, 2007b; Karnataki et al, 2009). Similarly, the thioredoxin-like protein Atrx-1 is targeted to the outer membranes of the apicoplast in the absence of a canonical bi-partite signal (DeRocher et al, 2008). At this moment it not clear how exactly these differences in transit peptide structure relate to differential targeting. As another example, in *P. falciparum* two phosphate translocators have been identified, one has a leader and is targeted to the innermost membrane and a second protein that lacks a leader and is found in the outer membranes (Mullin et al, 2006). In *T. gondii* a single translocator appears to traffic to all membranes, and it does so without a leader (Fleige et al, 2007; Karnataki et al, 2007a). The question as to how different proteins are restricted to different apicoplast subcompartments is still wide open. Mechanistic models could invoke defined positive forward signals, negative signals that block proteins from being substrates for certain translocons (and thus restrict them to outer compartments), or invoke import to the lumen and

subsequent re-export to outer compartments. Such rexport is seen for elements of the outer chloroplast membrane in plants (Tranel et al, 1995; Tranel & Keegstra, 1996). The fact that many proteins are processed by what is believed to be a luminal signal peptidase might provide some support for the latter. Clearly more work is needed to unravel the considerable complexity of the system.

A1.3 Replicating and dividing the apicoplast

As the apicoplast posses its own gnome, *de novo* formation is not possible, and replication and partition must occur in each cell division cycle to ensure inheritance to all daughter cells. Similar to apicoplast protein import the machinery that replicates the organelle is a phylogenetic mosaic with the most ancient components at its core and more recent additions acting at the periphery.

A1.3.1 The apicoplast genome is replicated by prokaryotic-type machinery

Apicoplast genome replication has been studied in *Plasmodium* and *Toxoplasma*. In both species the genome is present in multiple copies per cell but there is an important difference in their structure. In *Plasmodium* most apicoplast genome molecules are circular, while in *T. gondii* they are organized in linear tandem arrays (Williamson et al, 2001; Williamson et al, 2002). Branch point tracking through two dimensional gel electrophoresis indicated that in *Plasmodium* replication initiates at two sites within the inverted ribosomal RNA repeats and proceeds, via a D-loop intermediate, towards a circular replicate (Singh et al, 2005; Williamson et al, 2002). Williamson and coworkers also reported an additional, rolling circle, mechanism that starts at sites found outside the inverted repeats (Williamson et al, 2002). Rolling circle is the mechanism for replication proposed for the linear genome found in the *T. gondii* apicoplast (Williamson et al, 2001). The apicoplast genome itself does not seem to encode proteins involved in its replication, and those are therefore presumably imported. Indeed, several nuclear-encoded

homologues of such components were identified: Prex (**p**lastid-DNA **r**eplication **e**nzyme comple**x**), a multi-domain protein showing DNA-helicase, primaes and polymerase activities was shown to target to the apicoplast (Seow et al, 2005). Evidence for the potential activity of gyrase, a strand relaxation topoisomerase typical for bacteria and plastids, initially came from pharmacological experiments with ciprofloxacin in *Plasmodium* and *Toxoplasma* (Fichera & Roos, 1997; Weissig et al, 1997). Indeed, both the A and B subunits of gyrase were later identified in *Plasmodium* and their activity and interaction with each other have been confirmed (Ahmed & Sharma, 2008; Dar et al, 2007; Raghu Ram et al, 2007). DNA replication is a complex process and additional components of the machinery undoubtedly remain to be identified. Our efforts to systematically identify all apicoplast proteins in *T. gondii* by experimental localization have recently yielded several candidate genes. These include a DNA ligase, the gyrase B subunit, a helicase and two hypothetical proteins with domains reminiscent of with DNA-repair components (LS & BS unpublished).

A1.3.2 Organelle division and segregation – fission with a twist

Research into the mechanisms of plant chloroplast division has shown significant similarities between chloroplasts and their cyanobacterial ancestors. The most conserved element in this process is FtsZ (Armbrust et al, 2004; Fraunholz et al, 1998; Miyagishima, 2005; Osteryoung & Nunnari, 2003). FtsZ is a GTPase, structurally related to tubulin, and localizes to the division ring in bacteria and chloroplasts (Miyagishima, 2005; Stokes et al, 2000). The site of assembly of the FtsZ ring is controlled by homologues of the bacterial MinD and MinE proteins which have been found in the nuclear genome of plants and certain algae (Colletti et al, 2000; Itoh et al, 2001). Chloroplast division also has eukaryotic elements, most importantly a constrictive ring formed by the dynamin-related protein ARC5. The position of the FtsZ ring is ingeniously

transduced and coordinated from the stroma to the outer membrane through two interacting membrane proteins (Glynn et al, 2008). While there is some variation with respect to particular elements the overall mechanism and the central role of FtsZ seems conserved among all plastids including secondary plastids. This is to the marked exception of Apicomplexa which lack FtsZ and any of its associated other factors (Vaishnava & Striepen, 2006). How does the apicoplast divide in the absence of the conserved machinery?

An important initial observation was that in Apicomplexa plastid development is tightly associated with the development of the nucleus. While extracellular forms of various species carry small ovoid organelles that resemble each other they show significant morphological diversity in intracellular stages (Stanway et al, 2009; Striepen et al, 2000; Vaishnava & Striepen, 2006; van Dooren et al, 2005; Waller et al, 2000). In species that replicate through large polyploid intermediates through schizogongy or endopolygeny (see (Striepen et al, 2007) for detail) the apicoplast grows into a large tubular or branched reticulate structure. These structures then fragment during the final budding process that produces new infectious cells. Imaging experiments in *Toxoplasma* and *Sarcocystis* demonstrated that apicoplast division coincides with nuclear division and that this coordination is due to physical association between the apicoplast and the centrosome of the intranuclear spindle (Striepen et al, 2000; Vaishnava et al, 2005). This has been studied in greatest detail in *Toxoplasma*. Early in M-phase the apicoplast associates with the recently divided centrosomes and is suspended between be two poles of the spindle. As mitosis and budding proceeds both the nucleus and the apicoplast appear U-shaped, and this U elongates until fission occurs concurrently with buddy, leaving each new organelle in a separate new cell (Striepen et al, 2000; van Dooren et al, 2009). Centrosome associaton provides a satisfactory model to explain faithful segregation into daughter cells yet does not fully explain

fission. Ultrastructural studies have reported the observation of plastid constrictions that may be indicative of a division ring (Ferguson et al, 2005; Matsuzaki et al, 2001) but did not address the composition of this putative rings. A first candidate emerged with the description of a constrictive cytokinetic ring marked by the recently described repeat protein MORN1 (Gubbels et al, 2006). The position of the MORN1 rings coincides with apicoplast constrictions lending support to a pull and cut model in which centrosomes and cytokinetic ring cooperate in apicoplast division (Vaishnava & Striepen, 2006; van Dooren et al, 2009). Most recently another player was discovered, the dymanim-related protein DrpA. Genetic and cell biological studies using dominant negative mutants demonstrated that DrpA is essential for the final apicoplast fission step. In these mutants plastids segregate and pinch, however they remain continuous tubules connecting recently divided daughter cells (van Dooren et al, 2009). The assembly of dynamin-related proteins into mulitmeric active fission complexes is thought to require an initial constriction of the target area (Legesse-Miller et al, 2003). A new unified model shown in Fig. 1E takes this into account and proposes that centrosome and MORN1 rings generate constrictions that are then the site of DrpA activity. Interestingly, TgDrpA is phylogenetically distinct from the ARC5 dynamin involved in chloroplast division (van Dooren et al, 2009). This suggests that dynamins have been recruited independently multiple times to aid in the division of endosymbiont organelles.

A1.4 What are the metabolic functions of apicoplast?

With the discovery of *Chromera* it now appears well established that Apicomplexa are derived from a photosynthetic ancestor (Moore et al, 2008). In all likelihood the acquisition of photosynthesis was the driving benefit in the relationship between the ancestor of apicomplexans and the red algal endosymbiont. An obvious question is why Apicomplexa maintained the

apicoplast, through a dramatic change of ecological niche, from a life in the ocean to a life as an obligate intracellular parasite? Chloroplasts in plants and algae not only harvest the energy of light but are also home to several anabolic pathways that use precursors generated through photosynthesis. A plausible reason for the continued presence of the apicoplast is metabolic dependence. We dub this the 'addiction to free candy' hypothesis. Under this hypothesis, Apicomplexa evolved to rely on the anabolic capabilities of the algal endosymbiont. It might have been energetically more favorable to rely on endosymbiont pathways that were directly tied to primary production through photosynthesis. In this adaptation process redundant host pathways might have been lost thus generating dependence that persisted after the loss of photosynthesis. The most important insights into apicoplast metabolism arose from mining the genomes of Apicomplexa. This included directed searches for the genes of enzymes known to be chloroplast localized in plants and algae (Jomaa et al, 1999; Waller et al, 1998) as well as broader screens that attempted to identify all proteins that carry a potential bipartite leader (Foth et al, 2003; Ralph et al, 2004b). The metabolic map built in this effort now attributes three main functions to the apicoplast: fatty acid, isoprenoid and heme biosynthesis. These pathways trace their evolutionary history back to the initial primary endosymbiosis and are of cyanobacterial origin. As detailed below there are significant differences between these apicoplast pathways and those used by mammals to make equivalent metabolites. These differences have made the apicoplast one of the prime targets for the developments of new drugs for Apicomplexa. Pharmacological studies suggest that the pathways are essential (at least in certain life cycle stages) and that inhibitors of key enzymes involved in these pathways can successfully arrest the growth of the parasites *in vitro* and *in vivo* (Goodman et al, 2007; Wiesner et al, 2008). This assertion has been reinforced (and in part modified) by recent genetic studies that have targeted

specific enzymes. The following section will review studies on the three main pathways in detail. We will also highlight new insights into how the apicoplast anabolic pathways are supplied with energy, carbon and reduction power through import of metabolites from the cytoplasm of the parasite.

A1.4.1 Type II fatty acid biosynthesis (FASII) – a slippery target

In most organisms *de novo* synthesis of fatty acids is achieved using one of two types of fatty acid synthetases (FAS). FASI is found typically in animals and fungi and combines all required enzymatic activities on a single large polypeptide. This megasynthase architecture is shared with the related polyketide synthases (Smith et al, 2003). In contrast, in the FASII system, each enzyme is expressed as an independent protein. This system is found in many Eubacteria and the plastids of plants. The key feature of any fatty acid biosynthesis is the sequential extension of an alkalonic chain, two carbons at a time, by a series of decarboxylative condensation reactions (Smith et al, 2003). The central molecule in the process is a small protein called acyl-carrier protein (ACP). A primer substrate is generated by the transfer of a malonyl group from malonyl-CoA to ACP by FabD. The decarboxylative condensation of this primer with acetyl-CoA by FabH yields a two carbon extension of the acyl chain that is subsequently reduced, dehydrated, and reduced again by the sequential actions of FabG, FabZ and FabI. This cycle is repeated and progressive sequential decarboxylative extension using malonyl-ACP as donor yields even chained fatty acid molecules. All enzymes of the *P. falciparum* FASII pathway have now been expressed heterologously and the recombinant proteins have been studied in considerable biochemical and structural detail. Particular effort, including extensive medicinal chemistry, has been placed on FabI the target of the antibiotic triclosan and FabH the target of thiolactamycin (see (Goodman & McFadden, 2007; Mazumdar & Striepen, 2007) for specific reference).

Acetyl-CoA forms the primary carbon source of fatty acid biosynthesis. Acetyl-CoA is generated in the apicoplast lumen from pyruvate by the pyruvate dehydrogenase complex (PDH). Pyruvate, in turn, is synthesized from imported phosphoenol pyruvate (PEP) by the enzyme pyruvate kinase. An apicoplast localized PDH complex as well as pyruvate kinase has been reported in both *Plasmodium* and *Toxoplasma* (Fleige et al, 2007; Foth et al, 2005; Maeda et al, 2009), and it is important to note that this is the sole PDH in the cell. Subsequent conversion of acetyl-CoA to malonyl-CoA is the first committed step in the FASII pathway. This conversion is mediated by acetyl-CoA carboxylase (ACCase) a large polypeptide carrying a biotin prosthetic group. The genomes of both *Toxoplasma* and *Plasmodium* encode a putative ACCase that was shown to localize to the apicoplast (Gardner et al, 2002; Jelenska et al, 2001; Zuther et al, 1999) and a biotinylated protein of suitable size has been documented in the organelle in *T. gondii* (van Dooren et al, 2008a). *Toxoplasma* also has a cytosolic FASI system which is likely served by a second and cytosolic ACCase (Mazumdar & Striepen, 2007).

Bulk production of fatty acids supplying parasite lipid synthesis would appear to be the most obvious function of the FASII pathway. Initial pharmacological studies in *Plasmodium* lent support to this hypothesis. Following metabolic labeling with ^{14}C-acetate, radiolabeled fatty acids were apparent in extracts from *Plasmodium* infected red blood cells. This synthetic activity was linked to the apicoplast based on its sensitivity to triclosan, an inhibitor of the FASII enzyme FabI/enoyl-reductase (Surolia & Surolia, 2001). Furthermore, triclosan inhibited the growth of *Plasmodium* and *Toxoplasma* in culture supporting the critical importance of the pathway (McLeod et al, 2001; Surolia & Surolia, 2001). A conditional knockout study in T*oxoplasma* targeting ACP has shown that the pathway is essential for parasite growth in culture and in infected animals. However, and somewhat surprisingly, mutants show no difference in their

ability to synthesize ^{14}C-actetate labeled fatty acids (Mazumdar et al, 2006). More recently, genetic studies in *Plasmodium* have targeted the enzymes FabI and FabB/F and showed that FASII, while essential for liver cell development, is not required for the blood stage (Vaughan et al, 2009; Yu et al, 2008). Furthermore, triclosan will kill parasites in which its presumptive enzyme target FabI has been genetically ablated (Yu et al, 2008) or is naturally absent as in *Theileria* (Lizundia et al, 2009). This indicates strong off-target effects of triclosan. From a drug development perspective FASII appears to be a strong target in *Toxoplasma* and *Plasmodium* liver stages but not for the treatment of blood stage malaria.

A number of genetic and pharmacological studies have shown that FASII is essential for the lipoylation of the apicoplast PDH-E2 subunit (Crawford et al, 2006; Cronan et al, 2005; Mazumdar & Striepen, 2007; Mazumdar et al, 2006; Thomsen-Zieger et al, 2003; Wrenger & Muller, 2004). Lipoic acid is an essential prosthetic group and its synthesis depends on a FASII derived precursor (octanoic acid-ACP) and two plastid localized enzymes, LipA and B that constitute the *de novo* synthesis pathway. In contrast, lipoylated mitochondrial enzymes depend on a salvage system using LplA. Both pathways are independent and it appears that the mitochondrial enzyme uses lipoic acid derived from the host cell rather than the apicoplast (Crawford et al, 2006; Mazumdar et al, 2006). *Toxoplasma* is fully dependent on FASII for the lipoylation of PDH. In contrast, in *Plasmodium*, LipB, the initial step of *de novo* lipoylation of apicoplast PDH was found to be not essential in blood stages (Gunther et al, 2007). Careful measurements suggested that despite the absence of LipB, PDH still showed a significant amount of lipoylation, and that this might be due to the activity of a dually targeted second LplA protein. While there is now excellent experimentalsupport for the hypothesis that FASII provides essential precursors for PDH lipoylation, this does not provide a fully satisfactory answer to the

question as to what the essential role of FASII might be. At the moment the main function of PDH appears to be to supply FASII with acetyl-CoA generating a circular argument. There are potential solutions to this conundrum that await experimental validation: 1) PDH activity might be required for processes other than FASII 2) fatty acid synthesis might be the main function of FASII but this activity is poorly measured by acetate labeling experiments or 3) FASII might be required for the synthesis of specialized lipids that are not needed in large quantities, but are still essential for parasite survival. Deeper biochemical and metabolomic analysis of the various mutants might provide a resolution to this puzzle.

A1.4.2 Prokaryotic isoprenoid biosynthesis – the pathway found in all apicoplasts

Isoprenoids function in many aspects of cell metabolism as well as membrane structure and function (Moreno & Li, 2008; Wanke et al, 2001). Despite their enormous structural and functional diversity (sterols, cholesterol, retinoids, carotenoids, ubiquinones and prenylated proteins), all isoprenoids are derived from a simple five carbon precursor: isopentenyl pyrophosphate (IPP) and its allyl isomer dimethylallyl pyrophosphate (DMAPP). Until recently, IPP and DMAPP were thought to be synthesized exclusively via the mevalonate pathway, which had been extensively characterized in mammals and yeast. Yet unexpectedly the existence of an alternate pathway was discovered through isotope incorporation studies in a number of bacteria and plant species (Arigoni et al, 1997; Rohmer et al, 1993). This pathway is known by its key intermediates either as MEP (2-C-methyl-D-erythritol-4-phosphate) or DOXP (1-deoxy-D-xylulose-5-phosphate) pathway. We know now that many eukaryotes, archaebacteria and certain eubacteria rely on the classical mevalonate pathway, while most eubacteria and plastids use the MEP pathway (Rohdich et al, 2001). The pathway is initiated by the condensation of pyruvate and glyceraldehyde-3-phosphate to yield DOXP catalyzed by the enzyme DOXP synthase.

DOXP is then rearranged and reduced to MEP by the enzyme DOXP reductoisomerase. The activity of three additional enzymes results in the formation of a cyclic diphosphate that is transformed to yield either IPP or DMAPP in the final step (see (Eisenreich et al, 2004) for detailed review of the enzymology). Genomic and experimental analyses have shown that apicomplexan parasites harbor the DOXP pathway in the apicoplast but lack a cytoplasmic mevalonate pathway (Clastre et al, 2007; Coppens et al, 2000; Jomaa et al, 1999; Ralph et al, 2004b). In a seminal paper Jomaa and colleagues showed evidence for two enzymes of the DOXP pathway in the genome of *Plasmodium falciparum*. They demonstrated apicoplast localization of the protein product when expressed in *T. gondii* and also showed that *Plasmodium* is highly susceptible to fosmidomycin, an antibiotic that inhibits DOXP reductoisomerase (Jomaa et al, 1999). Using radiolabeling and HPLC analyses Cassera and colleagues then demonstrated the presence of diagnostic intermediates of the DOXP pathway in extracts derived from *Plasmodium* infected red blood cells and such labeling could be abolished by pretreatment with fosmidomycin (Cassera et al, 2004). Subsequent studies have now identified all enzymes of the pathway itself as well as enzymes to furnish the required substrates (Fleige et al, 2007; Wiesner & Jomaa, 2007). While some eubacteria encode an isomerase that mediates the inter conversions of IPP and DMAPP, in apicomplexan parasites the final enzyme LytB, is generating both isomers (Wiesner & Jomaa, 2007). The last two enzymes of the pathway contain a 4Fe-4S cluster that acts in single electron transfers. Interestingly, LytB has been shown to interact with and accept electrons from the apicoplast localized ferredoxin/ferredoxinNADP-reductase system, a hold out of the electron transfer system associated with the chloroplast photosystem I (Rohrich et al, 2005).

In *Plasmodium* the DOXP pathway appears to be essential based on the sensitivity of the parasites to fosmidomycin in culture. In combination with clyndamycin, fosmidomycin has also shown promising efficacy in the treatment of clinical malaria (Borrmann et al, 2006). The DOXP pathway is conserved in all Apicomplexa that have apicoplasts making it an attractive target for drug development and a candidate for the raison d'être of the apicoplast. Surprisingly however, fosmidomycin kills *Plasmodium* and *Babesia*, yet has no effect on *Theileria*, *Toxoplasma* or *Eimeria* (Clastre et al, 2007; Lizundia et al, 2009; Sivakumar et al, 2008). It is conceivable that different host cell environments (i.e. red blood cells versus nucleated cells) provide different levels of access to isoprenoids and are responsible for this difference. This could make the pathway dispensable in some parasites. To test this we recently constructed conditional mutants in LytB and DOXP reductoisomerase in *T. gondii*. The studies demonstrated that the DOXP pathway is essential for *T. gondii* and that fosmidomycin resistance is caused by differences in drug uptake, potentially at the level of the apicoplast membranes (SN & BS unpublished). Fosmidomycin is a phosphorylated compound that likely depends on transporters to cross membranes. Our observations suggest that drugs targeting the DOXP pathway that do not depend on specific transporters like fosmidomycin might be highly active in other Apicomplexa. Furthermore fosmidomycin could be vulnerable to the rapid devolopment of drug resistance in malaria by import transporter mutation as seen in a variety of bacterial infections against fosfomycin which is structurally related and shares resistance mechanisms e.g. (Castaneda-Garcia et al, 2009). Malaria parasites resistant to fosmidomycin have recently been generated in the laboratory. Resistance in these strains was associated with amplification of the target and a drug efflux pump, however, the level of this resistance was more modest than that observed in *T. gondii* (Dharia et al, 2009).

A1.4.3 Heme biosynthesis has many homes and remains to be fully characterized

Heme is a porphyrin, a complex molecule made up of a four modified pyrroline rings, that coordinate iron in the center of the molecule. Heme forms the prosthetic group of numerous important proteins and enzymes acting in catalysis, electron transfer and oxygen transport. In plants tetrapyrrole biosynthesis is plastid localized and provides both heme and chlorophyll (Heinemann et al, 2008). With the loss of photosynthesis the apicoplast has also lost the ability to synthesize chlorophyll, however, some apicomplexan parasites have maintained the ability to synthesize heme (Ralph et al, 2004a). *De novo* synthesis was detected in *Plasmodium falciparum* before the discovery of the apicoplast (Surolia & Padmanaban, 1992). The different parts of this complex pathway are localized to three different compartments (mitochondrion, apicoplast, and cytoplasm). The pathway is initiated in the mitochondrion of the parasite where glycine and succinyl-CoA are converted to δ-aminolevalonic acid (ALA) by delta aminolevolonate synthase (ALAS) (Varadharajan et al, 2002). The next step is the conversion of ALA to porphobilinogen by δ-aminolevulinate dehydratase (ALAD or HemB), which is an apicoplast localized enzyme. This obviously implies that ALA generated in the mitochondrion is transported to the lumen of the apicoplast, which could be one of the reasons for the observed close proximity of the two organelles (Rao et al, 2008). An apicoplast localized HemC (porphobilinogen deaminase) has been identified in *Plasmodium* which has been shown to catalyze the two next steps to yield uroporphynogenIII (Nagaraj et al, 2009a). The hydrophilic uroporphynogenIII is converted to hydrophobic coproporphinogenIII by HemE and in *Plasmodium* this step is clearly localized to the apicoplast (Sato et al, 2004). The last three steps of the pathway are catalyzed by the enzymes HemF, HemG and HemH to yield functional heme. HemF and G lack obvious targeting signals and might be cytosolic. *P. falciparum* HemH (ferrochelatase) can complement the

respective *E. coli* mutant (Sato & Wilson, 2003) and has recently been shown to target to the mitochondrion (Nagaraj et al, 2009b). Interestingly, there have also been reports that *Plasmodium* might import enzymes from its host cell including ALAD and ferrochelatase. (Bonday et al, 2000; Bonday et al, 1997; Varadharajan et al, 2004). Such a mechanism could generate redundancy and limit the potential value of heme biosynthesis as a target for antimalarials. How important this concern might be remains to be established.

A1.4.4 Apicoplast transporters: feeding a chloroplast in the dark

Establishing metabolic exchange between host and endosymbiont through proteins that allow for metabolite transport across the membranes of the endosymbiont likely was an early and important step in endosymbiosis (Cavalier-Smith, 2000; Weber et al, 2006). In higher plants, carbon skeletons generated through photosynthesis and Calvin cycle are exported in the form of triose phosphates by the triose phosphate/phosphate translocator (TPT) (Flügge et al, 2003). TPT is a member of a larger family of plastid phosphate translocators (Knappe et al, 2003). These translocators act as antiport systems exchanging inorganic phosphate for phosphorylated C3, C5 or C6 compounds. The TPT is involved in the export of carbon, in contrast, the other subfamilies import metabolites into plastids, namely phosphoenol pyruvate (PPT; (Fischer et al, 1997)), glucose-6-phosphate (GPT, (Kammerer et al, 1998)) and xylulose-5-phophate (Eicks et al, 2002). Proteins homologous to chloroplast phosphate translocators have been identified in Apicomplexa and have been shown to localize to the membranes of the apicoplast (Fleige et al, 2007; Karnataki et al, 2007a; Mullin et al, 2006). As the apicoplast has lost its photosynthetic capacity its anabolic pathways must now be supplied from the cytoplasm. To test this idea the *T. gondii* apicoplast phosphate translocator *Tg*APT was expressed in yeast and reconstituted into artificial liposomes (Brooks et al, 2009). Using this system transport substrates could be tested in a

controlled biochemical environment. Unique among phosphate translocators *Tg*APT combines the activities of chloroplast PPT and TPT by robustly exchanging phosphate against PEP, glyceraldehyde-3- phosphate and triose phosphate thus providing crucial substrates for at least two apicoplast pathways (FASII and DOXP). A conditional knockout mutant in *T. gondii* revealed that parasites die very quickly once the protein is lost. Interestingly, the phenotype of *Tg*APT loss is more severe than that of loss of FASII reinforcing a potential crucial role of the DOXP pathway in *Toxoplasma* (Brooks et al, 2009). Obviously the apicoplast has additional metabolic needs that in all likelihood are met by a number of additional transporters (nucleotides for RNA and DNA synthesis, or iron for iron-sulfur cluster assembly are only two examples). Studies on such transporters might take leads from extensive work on plant chloroplasts and such transporter may be important as targets for drugs or as indirect determinants of drug sensitivity or resistance.

A1.5 Conclusions

The apicoplast is a joint venture of three organisms (the alveolate host, the red alga and the cyanobacterium) and this is apparent in many aspects of its unique biology. Despite millions of years of cohabitation the boundaries between them, as demarcated by the various membranes, are still important divides. The most obvious indication of this divided governance is that the proteins that execute and control apicoplast biology (e.g. those involved in biogenesis and replication) in the different subdomains of the organelle are derived from the organism that contributed this domain. Why did the complex multimembrane structure persist? One of several hypotheses to test is that the organelle has locked itself into a biochemical compartmentalization that is not easily undone. Understanding the structural and functional differences between the different domains will be key to answering this question. A prime interest in this organelle stems

from its potential value as a drug target. The first phase of this pursuit shows that rigorous genetic evaluation of targets is an absolute requirement. The fortune of some targets rose and those of others fell in these analyses. While the apicoplast has not yet delivered a pharmacological silver bullet, this promise is still unbroken. Significant advances were made dissecting the apicoplast metabolism and these will undoubtedly guide and accelerate the drug development process.

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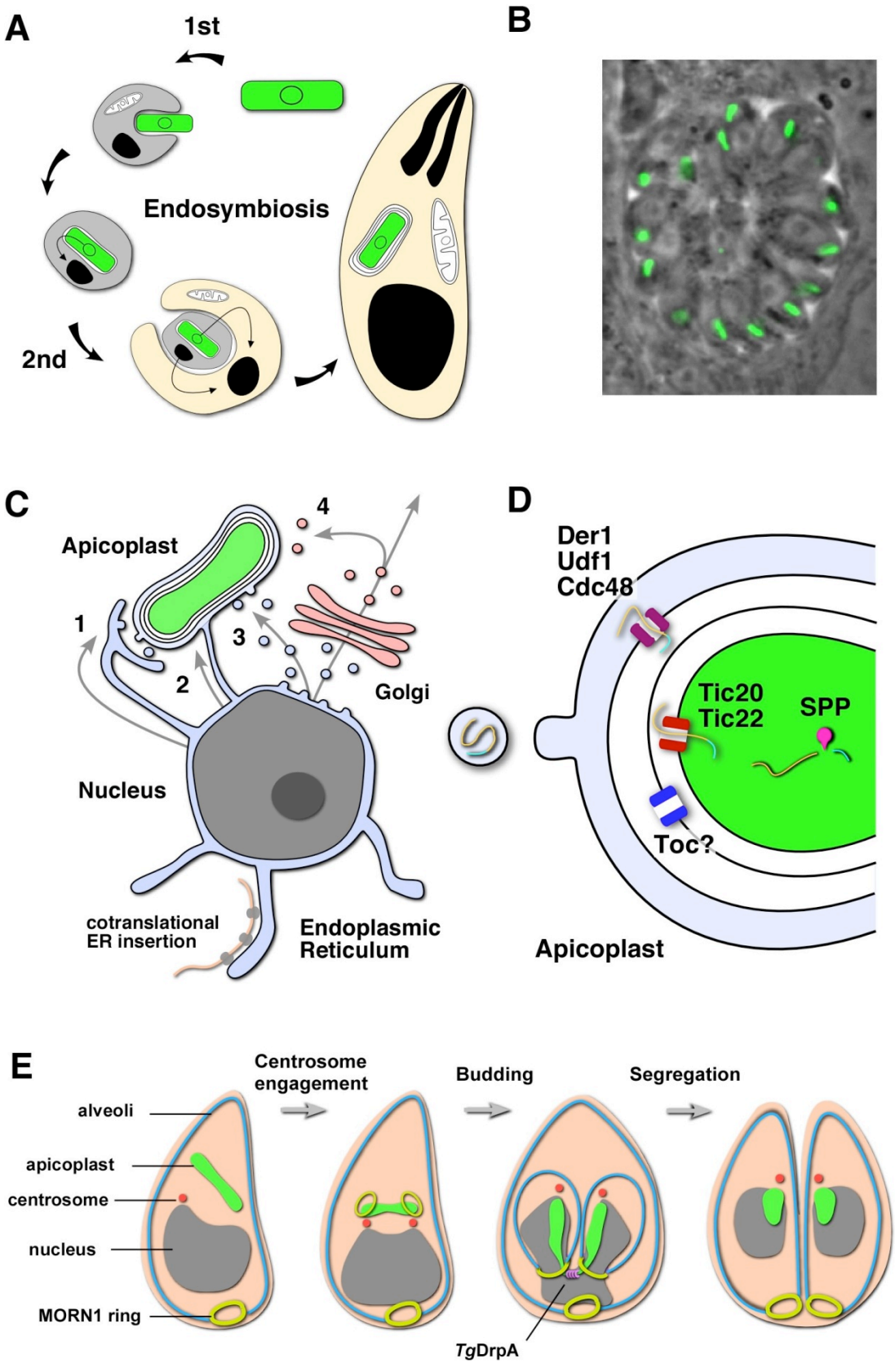
Figure A1: **A**. Schematic representation of the evolutionary origin of the apicoplast depicting primary and secondary endosymbiosis. **B**. Toxoplasma gondii parasites, the apicoplast is visible in green due to expression of ACP-GFP. **C**. Hypothetical routes of nuclear encoded apicoplast proteins along the secretory pathway in Apicomplexa. Vesicular trafficking directly from the ER to the apicoplast is a currently favored yet not fully validated hypothesis (from (Vaishnava & Striepen, 2006)). **D**. Simplified diagram of the four membranes of the apicoplast and the translocons that have been identified (modified from (Agrawal et al, 2009)). **E.** Schematic representation of apicoplast division in *T. gondii* (modified from (van Dooren et al, 2009)).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Der1
Udf1
Cdc48
Tic20
Tic22
SPP
Toc?
Apicoplast
E
Centrosome
engagement
Budding
Segregation
alveoli
apicoplast
centrosome
nucleus
MORN1 ring
TgDrpA

APPENDIX 2

TOXOPLASMA GONDII TIC20 IS ESSENTIAL FOR APICOPLAST PROTEIN IMPORT[4]

[4] Giel G. van Dooren, Cveta Tomova, Swati Agrawal, Bruno M. Humbel and Boris Striepen. 2008. *PNAS*. September 9, 105(36): 13574 – 13579.

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Abstract

Apicomplexan parasites harbor a secondary plastid that has lost the ability to photosynthesise yet is essential for the parasite to multiply and cause disease. Bioinformatic analyses predict that 5-10% of all proteins encoded in the parasite genome function within this organelle. However, the mechanisms and molecules that mediate import of such large numbers of cargo proteins across the four membranes surrounding the plastid remain elusive. In this study, we identify a highly diverged member of the Tic20 protein family in Apicomplexa. We demonstrate that Tic20 of *Toxoplasma gondii* is an integral protein of the innermost plastid membrane. We engineer a conditional null-mutant and show that *Tg*Tic20 is essential for parasite growth. To functionally characterize this mutant we develop several independent biochemical import assays to reveal that loss of *Tg*Tic20 leads to severe impairment of apicoplast protein import followed by organelle loss and parasite death. *Tg*Tic20 is the first experimentally validated protein import factor identified in apicoplasts, and our studies provide the first experimental evidence for a common evolutionary origin of import mechanisms across the innermost membranes of primary and secondary plastids.

A2.1 Introduction

Organelle acquisitions through endosymbiotic events have been major drivers of eukaryotic evolution. The incorporation of a cyanobacterium into a heterotrophic eukaryote led to the formation of plastids (e.g. chloroplasts), enabling eukaryotes to become autotrophic. It is thought that a single, so-called “primary”, endosymbiotic event led to the acquisition of chloroplasts in a lineage that later evolved into eukaryotic phyla such as red algae, green algae and plants (Rodriguez-Ezpeleta N, et al., 2005). An alternative means by which eukaryotes have obtained plastids is through a process of “secondary” endosymbiosis. Here, a eukaryote containing a

primary plastid is incorporated into a heterotrophic eukaryote. Secondarily-derived plastids are found in numerous lineages of ecologically, economically and medically important organisms, including diatoms, dinoflagellates and Apicomplexa. Apicomplexa are a phylum of obligate intracellular parasites that include *Plasmodium* species, the causative agents of malaria, and *Toxoplasma gondii*, which causes severe encephalitis upon congenital infection and in immunocompromized patients. The plastids of apicomplexans are known as apicoplasts, and are thought to function in several essential metabolic pathways such as fatty acid, haem and isoprenoid biosynthesis (Mazumdar J. et al., 2006; Ralph S.A. et al., 2004). Due to the phylogenetic and biochemical divergence of these pathways from their host counterparts, they are heavily pursued as potential targets for anti-parasitic drugs.

A key step in the conversion of an endosymbiont into a fully-fledged plastid is the transfer of endosymbiont genes to the nucleus of the host. This affords the host cell control over its endosymbiont, but requires the evolution of molecular machinery to enable the import of proteins encoded in the nuclear genome back into the organelle to carry out their role. This targeting process has been well studied in plants, where multi-subunit protein complexes mediate protein translocation across both membranes that bound the organelle (Soll J. and Schleiff E., 2004). However, protein translocation into secondary plastids is a fundamentally different process. Secondary plastids are surrounded by 3 or 4 membranes and reside within the endomembrane system, with plastid-targeted proteins requiring an N-terminal hydrophobic signal peptide to mediate the first step of protein import (Hempel F. et al., 2007). While the protein motifs required to direct proteins into secondary plastids are reasonably well characterised (Tonkin C.J. et al., 2008), little is known about the molecular mechanisms that mediate import. A fascinating evolutionary question is whether protein import into secondary

plastids required the evolution of novel machinery or whether organisms were able to re-tool existing mechanisms to accommodate novel means of protein targeting. Four membranes surround the apicoplast of *T. gondii* and in this study we demonstrate that protein import across the innermost membrane of this secondary plastid requires machinery derived from primary plastids.

A2.2 Results and Discussion

A2.2.1 Tic20 homolog in Apicomplexa. We performed iterative BLAST searches to identify homologs of the plant inner chloroplast membrane translocase component Tic20 in apicomplexan parasites. We identified Tic20 homologs from all the available genomes of apicomplexans, with the noted exception of the plastid-less genus Cryptosporidium. Alignments of apicomplexan Tic20 homologs with plant, red algal, diatom and cyanobacterial counterparts (supporting information (SI) Fig. A2-S1) reveal the presence of an N-terminal extension with characteristics of a bipartite apicoplast targeting sequence. Similarity to plant and algal Tic20 homologs resides in the C-terminal portion of the protein, although very few residues are conserved between all homologs depicted (Fig. A2-S1).

A2.2.1 *Tg*Tic20 is an integral protein of the inner apicoplast membrane.

Cloning of the full open reading frame of *Tg*Tic20 revealed the presence of three introns, and a predicted protein size of 43.4 kDa. We generated a transgenic parasite line expressing *Tg*Tic20 fused to a C-terminal HA tag and monitored its localisation by immunofluorescence assay (Fig. A2-1*A*). *Tg*Tic20-HA (green) localised to a small, apical organelle that overlapped with acyl carrier protein (ACP; red), a marker for the apicoplast stroma. A Western blot of cells expressing the *Tg*Tic20-HA transgene revealed a major protein species of approximately 23 kDa, and a less abundant species of around 40 kDa (Fig. A2-1*B*). This suggests that the N-terminal portion of the

protein is cleaved to yield the mature protein of around 20 kDa, consistent with the N-terminus of the protein functioning as an apicoplast-targeting domain that is processed upon import into the apicoplast (Waller R.F. et al., 1998; van Dooren G.G. et al., 2002). *In silico* modelling of the protein structure of *Tg*Tic20 suggests the presence of four transmembrane domains in *Tg*Tic20, found in close succession at the C-terminus of the protein (Fig. A2-S1). To determine whether *Tg*Tic20 is an integral membrane protein, we performed sodium carbonate extractions and Triton X-114 (TX-114) phase partitioning. Sodium carbonate extractions resulted in *Tg*Tic20 localising to the membrane fraction, much like the previously characterised apicoplast phosphate transporter (APT1; Karnataki A. et al., 2007) and unlike the soluble ACP (Fig. A2-1*C*). Much, but not all, *Tg*Tic20 localised to the detergent (i.e. membrane) phase during TX-114 phase partitioning, again consistent with a membrane localisation for *Tg*Tic20.

To further characterise the subcellular localisation of *Tg*Tic20 we performed transmission immuno-electron microscopy on parasites expressing *Tg*Tic20-HA, labelling with anti-HA and anti-ACP antibodies. This revealed the localisation of *Tg*Tic20-HA to membrane-bound organelles that also contained ACP (Fig. A2-1*D*), consistent with the apicoplast localisation of this protein. Localisation of *Tg*Tic20-HA within the apicoplast was generally confined to the membranes of the organelle, consistent with the membrane localisation of *Tg*Tic20, while ACP was distributed throughout the entire organelle.

Four membranes surround the apicoplast, and until now it has been difficult to determine to which membrane a given apicoplast membrane protein localises. To determine the membrane to which *Tg*Tic20 localises, we developed a novel green fluorescent protein (GFP) assay, making use of a previously established self-assembling split GFP (Cabantous S., T.C. Terwilliger and G.S. Waldo, 2005). In this system, the C-terminal β strand of GFP (GFP-11) was removed from

the remaining 10 β-strands (GFP 1-10) of the molecule. This GFP-11 was engineered with the ability to self-assemble with GFP 1-10 if both molecules localise to the same compartment (Cabantous S., T.C. Terwilliger and G.S. Waldo, 2005). As a proof of principle, we first targeted GFP 1-10 to the apicoplast stroma by adding the N-terminal apicoplast-targeting domain of ferredoxin-NADP+ reductase (FNR). By itself, this protein was unable to fluoresce (not shown). We next fused GFP-11 to the C-terminus of ACP and transfected this into the FNR-GFP 1-10 line. The resultant parasites revealed GFP fluorescence in the apicoplast (Fig. A2-2*A*). This indicated that when both components of the split GFP are targeted to the apicoplast stroma, we observe apicoplast fluorescence. It is possible that the two GFP domains interact in the secretory pathway before entry into the apicoplast, thus limiting the predictive value of the assay. To test this, we generated constructs where we fused both GFP 1-10 and GFP-11 to the C-terminus of P30, a previously established secretory marker protein for *T. gondii* (Striepen, B. et al., 1998). Expressed together, secreted GFP 1-10 and GFP-11 result in fluorescence in the parasitophorous vacuole that surrounds *T. gondii* parasites (Fig. A2-2*C*), indicating that the split GFP domains are capable of interacting when targeted to the same compartment in the secretory system. However, when we express the P30-GFP-11 construct in the FNR-GFP 1-10 line, we see no fluorescence (not shown), suggesting that any interaction of the split GFP components in the secretory pathway does not result in mis-targeting of the component proteins.

Having established the split GFP assay for determining whether proteins are localised to the apicoplast stroma, we next fused GFP-11 to the C-terminus of *Tg*Tic20 and transfected this into the FNR-GFP 1-10 cell line. The resultant line revealed fluorescence that co-localised with an apicoplast red fluorescent protein (RFP) marker (Fig. A2-2*B*), consistent with the C-terminus of *Tg*Tic20 residing in the apicoplast stroma. Flow cytometric analyses of the various cell lines

described above supported the results obtained by microscopic analysis (Fig. A2-S2*A*). One concern was that the observed apicoplast fluorescence might result from retention of FNR-GFP 1-10 in an outer membrane. To control for this, we monitored cleavage of the apicoplast-targeting leader of FNR-GFP 1-10, a measure for whether proteins are accessible to the stromal processing peptidase enzyme that likely resides in the apicoplast stroma (van Dooren G.G. et al., 2002). We found no difference in processing of FNR-GFP 1-10 whether expressed by itself or with interacting components (Fig. A2-S2*B*), indicating that most FNR-GFP 1-10 protein resides in the stroma, and consequently that *Tg*Tic20-GFP-11 does not prevent targeting of FNR-GFP 1-10 to the stroma.

We conclude that *Tg*Tic20 is an integral protein of the inner apicoplast membrane, with its C-terminus residing in the apicoplast stroma. Assuming that the predictions of four transmembrane domains is correct, the N-terminus would also be in the stroma, resulting in the model for *Tg*Tic20 topology presented in Fig. A2-2*D*. Recently, candidate proteins that likely localise to outer membranes of the apicoplast have been identified (Karnataki, A. et al., 2007; Sommer M.S. et al., 2007), and the split GFP assay may help to pinpoint the residence of these and other proteins to a specific membrane or apicoplast compartment.

A2.2.2 *Tg*Tic20 is essential for parasite viability.

To characterise the function of *Tg*Tic20, we generated a conditional *Tg*Tic20 mutant parasite cell line using a recently described tetracycline-based system (Mazumdar, J. et. al.,2006; Meissner M., D. Schluter and D. Soldati, 2002). We generated a parental cell line (iTic20/eTic20) that contains both endogenous (eTic20) and inducible (iTic20) copies of the *Tg*Tic20 gene (Fig. A2-S3*A*), where transcription of inducible genes can be down-regulated by the addition of the tetracycline analogue anhydrotetracycline (ATc) to the growth medium. We generated a

conditional *Tg*Tic20 mutant cell line (iTic20/ΔTic20) by disrupting the endogenous gene through homologous replacement of native *Tg*Tic20 with a selectable marker, verifying successful disruption of the native locus through polymerase chain reaction-based screening and Southern blotting (Fig. A2-S3*B*-*C*).

To establish whether *Tg*Tic20 is essential for parasite growth and viability we measured parasite growth in the parental and knockout cell lines using a real-time fluorescence assay (Gubbels, M.J., C. Li and B. Striepen, 2003). We introduced tandem tomato red fluorescent protein constructs into parental and knockout cell lines. Parasites were added to a 96-well plate and wells were monitored daily for overall fluorescence intensity, a correlate of parasite growth. Both strains initially grew at similar levels in both the absence and presence of ATc (Fig. A2-3*A*-*B*; green diamonds and blue squares). However, preincubation of parasites in ATc for 3 days prior to the growth assay revealed reduced parasite growth in the knock-out cell line but not in the parental cell lines (Fig. A2-3*A*-*B*, red triangles). After 6 or 7 days incubation in ATc, the conditional mutant ceases to grow. To confirm that this defect was specifically due to disruption of *Tg*Tic20, we complemented the conditional mutant by ectopically expressing *Tg*Tic20 from a constitutive promoter (iTic20/ΔTic20/cTic20). This restored parasite growth in the presence of ATc (Fig. A2-3*C*). We also measured growth of the parental, knockout and complemented cell lines by plaque assay (Fig. A2-S3*D*-*F*). These data supported the conclusions of the fluorescence assays that *Tg*Tic20 is essential for parasite growth.

A2.2.3 *Tg*Tic20 is essential for apicoplast protein import.

Having established that *Tg*Tic20 is an essential protein of the inner apicoplast membrane, we sought to determine its function. First, we measured the time frame for down-regulating *Tg*Tic20 expression in the mutant cell line. We harvested parasites after growing them for 0 to 4 days in

ATc and monitored protein levels by Western blot. Growth on ATc resulted in swift down-regulation of expression of the inducible *Tg*Tic20 protein (Fig. A2-4*A*, top). As a more sensitive measure for *Tg*Tic20 abundance, we immunoprecipitated *Tg*Tic20 protein from approximately 10^7 parasites. We measured immunopurified protein levels by Western blot, and found that after 2 days growth on ATc, we could no longer detect *Tg*Tic20 protein in the mutant cell line (Fig. A2-4*A*, bottom).

We hypothesised that *Tg*Tic20 may function in protein import into the apicoplast. To test this, we established several assays for successful protein import into apicoplasts (Fig. A2-4*B*). First we examined processing of the N-terminal targeting domain of apicoplast proteins, a process that likely occurs subsequent to import into the organelle stroma. Apicoplast-targeted proteins typically reveal two differently sized molecular species: a slow migrating band corresponding to the precursor protein and a faster migrating mature protein where the N-terminal targeting leader has been cleaved (Waller, R.F. et al., 1998; van Dooren G.G. et al., 2002). We asked whether leader processing was affected in the conditional *Tg*Tic20 mutant. To facilitate these studies, we generated a cell line in the conditional *Tg*Tic20 mutant background that expressed a "synthetic" apicoplast-targeted protein, consisting of the apicoplast-targeting leader of *Tg*FNR fused to mouse dihydrofolate reductase (a reporter protein typically used for organellar import assays in other systems; Eilers, M. and G. Schatz, 1986) and a C-terminal HA tag for detection. To gain a dynamic measure for the timing of defects on protein import in the *Tg*Tic20 mutant we conducted pulse-chase labelling experiments. We incubated mutant parasites growing in host cells for 0, 2, 3, 4 and 5 days on ATc, and radiolabeled proteins with ^{35}S-amino acids for one hour (pulse). We then washed out the radiolabel and incubated in medium containing an excess of unlabeled amino acids for an additional 2 hours (chase). We purified proteins of interest by

immuno- or affinity-purification, separated them by SDS-PAGE and detected by autoradiography. After 5 days growth on ATc, precursor FNR-DHFR-HA protein was made at similar levels to that formed in cells grown in the absence of ATc, indicating that knockdown of *Tg*Tic20 does not affect synthesis of apicoplast-targeted proteins (Fig. A2-4*C*). However, after two days growth in ATc there is a 35% reduction in formation of mature, processed FNR-DHFR-HA, decreasing to undetectable levels at day four (Fig. A2-4*C*; Fig. A2-4*D*, green diamonds).

We also monitored processing of the native apicoplast protein ACP in the *Tg*Tic20 mutant. Mature ACP contains only one sulfur-containing amino acid, making detection difficult. The experiment shown in Fig. A2-4*C* suggests that ACP is processed at day 2 on ATc and not beyond, but detection levels are too low to draw a definitive conclusion. As a control, we monitored processing of microneme protein MIC5, which occurs in a post-Golgi compartment of the secretory pathway (Brydges, S.D. et al., 2008). Even after 5 days incubation on ATc, MIC5 is processed (Fig. A2-4*C*), suggesting that *Tg*Tic20 knockdown does not affect other parts of the secretory pathway.

Although we suspect that precursor protein cleavage is a solid marker for whether proteins are able to traffic into the apicoplast stroma, it has not been formally shown that the processing event occurs here. Therefore, we sought to establish independent measures for successful protein targeting to the apicoplast. Several apicoplast enzymes are post-translationally modified by co-factors after import into the stroma. One such modification is the biotinylation of acetyl-CoA carboxylase (ACC; Jelenska, J. et al., 2001; Fig. A2-4*B*), a protein involved in biosynthesis of fatty acids. We purified biotinylated proteins using an immobilised streptavidin column. Radiolabeled biotinylated ACC is not yet detectable after the 1 hour pulse. In the absence of ATc, we observe robust biotinylation of ACC during the 2 hour chase (Fig. A2-4*C*). Biotinylated

ACC is reduced after 2 days incubation in ATc, and severely reduced after 3 days (Fig. A2-4*C*; Fig. A2-4*D*, red triangles), consistent with the results of the leader-processing assay. *T. gondii* contains a second major biotinylated protein, the mitochondrial pyruvate carboxylase (PC) enzyme (Jelenska, J. et al., 2001). Levels of biotinylated PC remain unchanged after incubation in ATc.

A second post-import modification is lipoylation of the E2 subunit of pyruvate dehydrogenase (PDH-E2). Lipoylation of PDH-E2 is solely mediated by apicoplast-targeted LipA and LipB and requires a substrate synthesized *de novo* within the apicoplast stroma (octanoyl-ACP; (Mazumdar, J. et al., 2006; Crawford, M.J. et al., 2001; Fig. A2-4*B*). In addition to apicoplast PDH-E2, *T. gondii* contains several lipoylated E2-subunit proteins in the mitochondrion (mito-E2; Mazumdar, J. et al., 2006; Crawford, M.J. et al., 2001). The mitochondrion contains a specific protein (LplA) that functions in the addition of the lipoyl-moiety to the E2 enzymes (Crawford, M.J. et al., 2001), suggesting that, much like the apicoplast, lipoylation can only occur after successful import into the organelle. We purified lipoylated proteins using an antibody against lipoic acid. After the 1 hour pulse, mito-E2 enzymes are labelled, consistent with rapid import into mitochondria (Fig. A2-4*C*). As with biotinylated ACC, lipoylated PDH-E2 is not observed until the 2 hour chase. Lipoylation of the apicoplast PDH-E2 is reduced after 2 days growth on ATc and severely reduced after 3 days, while modification of mitochondrial enzymes was not affected, even after 5 days incubation on ATc (Fig. A2-4*C*; Fig. A2-4*D*, blue squares).

Together, these data indicate that knockdown of *Tg*Tic20 impairs import of apicoplast-targeted proteins into the stroma of the organelle, but does not impair targeting of proteins to other destinations of the secretory pathway or to the mitochondrion. To rule out the possibility that

defects in apicoplast protein import resulted from non-specific effects of ATc, we performed pulse-chase analysis on the parental strain grown on ATc for up to 5 days. These results indicate that neither processing of the FNR-DHFR-HA leader nor biotinylation of ACC are affected by ATc alone (Fig. A2-4*E*).

Preliminary experiments revealed that *Tg*Tic20 knockdown results in defects in apicoplast segregation or biogenesis. This raised the possibility that the inhibition of apicoplast protein import may be a consequence of the loss of apicoplasts from significant numbers of parasites rather than a specific effect on apicoplast import. Alternatively, lack of protein import could lead to biogenesis defects. To visualise the apicoplast and allow us to establish the sequence of events, we targeted RFP to the apicoplast of *Tg*Tic20 mutant parasites. After prolonged incubation in ATc, we observed vacuoles where not all plastids contained visible apicoplasts (Fig. A2-5*A*). Quantification revealed that major defects in apicoplast biogenesis occurred after 5 days of incubation in ATc, subsequent to the observed defects in apicoplast protein targeting (Fig. A2-5*B*).

We were able to identify a sequential series of phenotypes in the *Tg*Tic20 mutant. Two days after initiation of the mutant phenotype, *Tg*Tic20 protein is no longer detectable. Coincident with *Tg*Tic20 knockdown is a reduced efficiency of apicoplast protein import, which increases to essentially complete inhibition four days after the addition of ATc. Five days after initiation, we observe major defects in apicoplast biogenesis, with arrest of parasite growth approximately 6 or 7 days after the addition of ATc. It is likely that death of the parasites results from the loss of apicoplasts and impairment of apicoplast functions. Apicoplasts are thought to perform several essential functions, such as the biosynthesis of fatty acids and isoprenoids (Mazumdar, J. et al., 2006; Ralph, S.A. et al., 1998). It is likely that the failure to correctly target biosynthetic proteins

(such as ACP, ACC and PDH-E2, Fig. A2-4*C*) leads to ablation of these pathways, resulting in inhibition of parasite growth. Indeed, the apparent universal defects seen in apicoplast protein import in the *Tg*Tic20 mutant suggest that we are simultaneously impairing all apicoplast proteins, making this mutant an attractive candidate to identify novel apicoplast functions (e.g. through proteomic and metabolomic approaches).

Our results indicate that *Tg*Tic20 is required for apicoplast protein import. What, then, is the function of *Tg*Tic20 in this process? The precise role of Tic20 in chloroplast import in plants has been elusive. Knockdown of plant Tic20 by antisense RNA results in a reduced efficiency of chloroplast protein import (Chen, X. et al., 2002). Based on its integral membrane localisation, it has been postulated that plant Tic20 forms part of the protein import channel of the inner chloroplast membrane (Kouranov, A. et al., 1998), although no direct experimental evidence supports this. Our results suggest that knockdown of *Tg*Tic20 protein expression does not immediately ablate import. Two days after the addition of ATc, the amount of *Tg*Tic20 is below our limits of detection. At this time point, apicoplast protein import is clearly affected (as measured by three independent assays), yet still occurs at between 65 and 77% the level of wild-type cells (Fig. A2-4*D*). This argues against *Tg*Tic20 functioning directly in an inner membrane import channel, since the lack of an import channel would likely result in immediate ablation of import into the apicoplast. We considered the possibility that *T. gondii* harbours proteins that can partly complement the function of *Tg*Tic20. Plants contain multiple Tic20 paralogues and may also have additional non-related proteins with similar functions to Tic20 (Reumann, S., K. Inoue and K. Keegstra, 2005; Teng, Y.S. et. al., 2005). However, we did not identify *Tg*Tic20 paralogues in the *T. gondii* genome. Another possibility is that *Tg*Tic20 is an accessory or regulatory component of a putative import complex in the inner membrane. In such a scenario,

*Tg*Tic20 may influence the efficiency of protein import through this complex, assembly of the complex, or be involved in a separate process that is essential for functioning of the inner membrane import complex. Identifying and characterising additional inner membrane import components should allow us to address these questions.

A2.3 Concluding Remarks.

During their intracellular development, apicomplexan parasites such as *T. gondii* must target large numbers of proteins to their apicoplast. Protein targeting occurs via the secretory pathway, and requires proteins to cross four membranes before reaching the organelle stroma (Hempel, F. et al., 2007). There has been considerable speculation about how protein targeting across these four membranes is mediated (e.g. Hempel, F. et al., 2007; McFadden, G.I. and G.G. van Dooren, 2004), but there has been a distinct lack of functional evidence for the various models. In this study we demonstrate that *Tg*Tic20 is essential for protein import across the inner apicoplast membrane. As such, *Tg*Tic20 is, to our knowledge, the first experimentally characterized apicoplast import protein identified in Apicomplexa.

Emerging evidence suggests that *T. gondii* and other Apicomplexa belong to a eukaryotic "supergroup" known as the Chromalveolata (Fast, N.M. et. al., 2001; Moore, R.B. et. al., 2008). Chromalveolates include other major eukaryotic groups such as dinoflagellates and heterokonts (including diatoms and brown algae). A distinguishing feature of chromalveolates is the presence of a plastid that was derived by secondary endosymbiosis from a red alga. Chromalveolate plastids, then, represent a cellular *ménage a trois* of three 'founder' organisms: a cyanobacterium, a red alga and a heterotrophic eukaryote. An early requirement in the acquisition of plastids is the evolution of protein import machinery. An intriguing evolutionary question is which of these founders 'donated' the import machinery and whether the origin of

individual translocons is tied to the origin of the membrane they cross. Three types of translocons of have been speculated to potentially act in apicoplast protein import: primary plastid-derived Tic and Toc complexes and, more recently, Der1-containing complexes re-tooled from their original role in protein retro-translocation across the ER membrane (Sommer, M.S. et. al., 2007). In this study we show that the innermost apicoplast membrane is crossed using machinery derived (at least in part) from the inner membrane Tic translocation complex of the red algal chloroplast, and we note that Tic homologs are present in other chromalveolates such as diatoms (Fig. A2-S1; McFadden, G.I. and G.G. van Dooren, 2004). Rather than evolving a fundamentally different means of protein import into secondary plastids, Apicomplexa and their chromalveolate cousins made use of the machinery already available from their primary plastid progenitors. It remains to be determined whether components of the Toc and Der1 complexes mediate import across other apicoplast membranes. The approaches for characterising and localising candidate apicoplast import proteins that we describe here provide an experimental framework to conclusively test these hypotheses.

A2.4 Materials and Methods

A2.4.1 Parasite culture and manipulation.

Parasites were passaged in human foreskin fibroblasts and genetically manipulated as described previously (Striepen B. and D. Soldati, 2007). GenBank accession number for TgTic20 is EU427503. Plasmid construction and flow cytometry techniques are described in detail in SI Material and Methods. All parasite strains described in this paper were cloned by either limiting dilution or flow cytometry. Where indicated, parasites were grown in anhydrotetracycline (IBA, St Louis, MO) at a final concentration of 0.5 μg/ml.

A2.4.2 Immunoprecipitation, SDS-PAGE and Immunoblotting.

For pulse-chase analyses, infected host cells were radiolabelled with 100 μCi/ml of ^{35}S methionine and cysteine (GE Healthcare) for one hour. Cells were either harvested (pulse) or washed twice and incubated in parasite growth medium lacking radioactive amino acids for 2 hours (chase) before harvesting. Proteins of interest were purified by immunoprecipitation or affinity purification and separated by SDS-PAGE using standard procedures (van Dooren G.G. et. al., 2002), and detected by autoradiography or PhosphorImaging (GE Healthcare). Immunoblotting, sodium carbonate extractions and Triton X-114 phase partitioning were performed by standard procedures. Detailed protocols are listed in SI Materials and Methods.

A2.4.3 Microscopy.

Immunofluorescence assays and light microscope imaging were performed essentially as previously described (Striepen B. and D. Soldati, 2007). Detailed methods for electron microscopy are included in SI Materials and Methods.

Supplemental Materials and Methods

A2.S1. Gene cloning and sequence analyses

We used sequence from a Tic20 homolog from the red alga *Cyanidioschyzon merolae* (CMS050C; Matsuzaki, M. et al., 2004) as a query for an iterative PSI-BLAST search (Altschul, S. F., et al.,1997) against a non-redundant protein sequence database (ncbi.nlm.nih.gov/blast/Blast.cgi). After two iterations using default parameters, we identified a hit to genes from *Theileria* species and used these as query sequences to identify Tic20 homologs in other Apicomplexa. We identified one predicted gene in *Toxoplasma gondii* with homology to the *Theileria* genes. An EST covered the 3' end of this gene. To identify the 5' end, we performed 5' RACE using the SMART RACE cDNA amplification kit (BD Biosciences), using the initial primer 5'-TTGAGATATATCATGCTCA and the nested primer 5'-AGCATAGACAGTAGCAAA. Sequencing of the entire *Tg*Tic20 gene revealed the presence of three introns, and an open reading frame of 1170 bp. GenBank accession number for *Tg*Tic20 is EU427503. To compare *Tg*Tic20 with other known Tic20 homologs we generated a multiple sequence alignment as previously described (Gubbels, M. J. et al, 2006). Other sequences included in the alignment were identified from publicly available databases.

A2.S2. Plasmid construction, parasite transfection and parasite culture

The open reading frame of *Tg*Tic20 was amplified by PCR using the primers 5'-AGATCTAAAATGGGGTTCCCTTCAGCTCTCT and 5'-CCTAGGGTACGAGTCTGACGGCTTCTCGCCGAT. The resultant PCR product was subcloned into pCR2.1 (Invitrogen) and verified by sequencing. The resulting vector was digested with *Bgl*II and *Avr*II and cloned into the equivalent sites of the pCTH vector (GvD and R. Opperman, unpublished) to generate pCTH(Tic20). This vector contains the Tic20 open

reading frame downstream of the *T. gondii* alpha-tubulin promoter, with a 3' HA-tag fusion, and a chloramphenicol-resistance marker for selection in *T. gondii*. This was transfected into RH strain *T. gondii* parasites as previously described (Striepen, B. and D. Soldati, 2007). Stable lines expressing the tub-*Tg*Tic20-HA construct were generated by chloramphenicol selection (Kim, K., D. Soldati and J.C. Boothroyd, 1993).

To generate a conditional knockout of *Tg*Tic20, we first generated a parental strain that expressed inducible copies of *Tg*Tic20. To do this, we placed the *Tg*Tic20 open reading frame into the *Bgl*II and *Avr*II cut sites of pDt7s4M vector (GvD, unpublished), which contains a mutant form of DHFR that encodes resistance to pyrimethamine, an inducible teto7/sag4 promoter, and a c-myc tag at the 3' end of the gene-of-interest. We transfected the resulting construct into TATi strain *T. gondii* parasites, containing a transactivating tetracycline repressor protein to enable inducible repression of *Tg*Tic20-c-myc gene expression (Meissner, M., D. Schluter and D. Soldati, 2002). We then proceeded to knockout endogenous *Tg*Tic20 through double homologous recombination. We amplified approximately 2 kb downstream of the *Tg*Tic20 protein coding sequences with the primers 5'-AAGCTTACAAGTTGCAGTAGGTGTTCCA and 5'-CTCGAGAAGCAGTGTGGTCGAAAGATA and placed this into the *Hin*dIII and *Xho*I cut sites of the vector pTCY (GvD, unpublished). pTCY is a modified version of the ptubCAT vector (a kind gift from Markus Meissner, U. Heidelberg), containing a chloramphenicol resistance marker driven by the tubulin promoter and containing multi-cloning sites flanking both sides of the expression cassette. This vector also contains a YFP marker for negative selection of successful homologous recombinants (Mazumdar, J. et al., 2006). We amplified approximately 2 kb upstream of the *Tg*Tic20 protein coding sequence with the primers 5'-

ACTAGTAACAGCGCTGTCTCCCCCATAA and 5'-AGATCTTTTCCTCGAGGCAGTAGTATA and ligated this into the *Spe*I and *Bgl*II cut sites of the pTCY(Tic20 3') vector. This generated the vector pTCY(Tic20 KO). We linearised this plasmid with *Not*I and transfected it into the parental strain and selected on chloramphenicol. After obtaining chloramphenicol-resistant parasites, we cloned YFP negative parasites by cell sorting as described previously (Mazumdar, J. et al., 2006). We isolated 16 clones and performed diagnostic PCR and Southern blotting (see below) to identify successful targeting of the *Tg*Tic20 locus.

To complement the *Tg*Tic20 knockout, we expressed a tub-*Tg*Tic20-HA plasmid containing a phleomycin-resistance cassette in the knockout parasite line. We generated this vector by digesting the phleomycin-resistance cassette of the pBSSK+ SAG1/Ble/SAG1 (a kind gift from David Sibley, Washington U.) with *Hin*dIII and *Spe*I and ligated this into the equivalent sites of pCTH(Tic20) to generate the vector pBTH(Tic20). We transfected this into the *Tg*Tic20 knockout cell line and selected for parasites stably expressing the complemented *Tg*Tic20-HA construct by phleomycin selection as previously described (Messina, M. et al., 1995).

To generate *Tg*Tic20 parental, mutant and complemented cell lines expressing tandem Tomato RFP parasites, we amplified tandem dimeric tomato sequence (Shaner, N. C., et al., 2004) using the primers 5'-AGTCCCTAGGGTGAGCAAGGGCGAGGAG and 5'-AGTCCCCGGGCTTGTACAGCTCGTCCATGC. We digested the resulting PCR product with *Avr*II and *Xma*I and ligated it into the equivalent sites of pCTG (G.v.D. and R. Opperman, unpublished). This generated the vector $pCTR_{2T}$, containing the tandem dimeric Tomato RFP expressed from the tubulin promoter. We transfected this construct into the various cell lines, and subjected parasites to three or four rounds of cell sorting before cloning. Cell sorting was

performed using a MoFlo sorter (Dako, Ft. Collins, CO), with an Enterprise 621 laser tuned to 488 nm. We sorted cells expressing Tomato parasites using a 570/40 nm BP filter.

To generate vectors for the split GFP system (Cabantous, S. et al., 2005), we amplified GFP 1-10 with the primers 5'-AGTCCCTAGGAGCAAAGGAGAAGAACTTTT and 5'-AGTCCCCGGGTTAGGTACCCTTTTCGTTGGGATCT and GFP-11 with the primers 5'-AGTCCCTAGGGGTTCCGATGGAGGGTCTGGTG and 5'-AGTCCCCGGGTTATGTAATCCCAGCAGCATT (parent vectors were kind gifts from Geoff Waldo, Los Alamos National Research Laboratory). We digested the resultant PCR products with *Avr*II and *Xma*I and ligated these into the equivalent sites of the pCTG vector to make the vectors $pCTG_{1\text{-}10}$ and $pCTG_{11}$. We digested the FNR leader sequence from the ptubFNR-RFP/sagCAT vector (Striepen, B., et al., 2000) and the P30 sequence from the ptubP30-GFP/sag-CAT vector (Striepen, B., et al., 1998) with *Bgl*II and *Avr*II, ligated these into equivalent sites in the $pCTG_{1\text{-}10}$ vector, and transfected RH strain *T. gondii* parasites. Parasites stably expressing FNR-GFP 1-10 and P30-GFP 1-10 were obtained through chloramphenicol selection. The entire *T. gondii* ACP coding region was digested from the vector ptubACPfull-GFP/sag-CAT (Waller, R. F., et al., 1998) with *Bgl*II and *Avr*II and ligated into equivalent sites of $pCTG_{11}$. The result vector was transfected into the cell line stably expressing FNR-GFP 1-10 and fluorescent parasites obtained through several rounds of cell sorting using a MoFlo flow cytometer (Dako, Ft. Collins, CO) with an Enterprise 621 laser tuned to 488 nm using a 530/40 nm BP filter. P30 and the complete Tic20 open reading frame were digested from the ptubP30-GFP/sag-CAT and pCTH(Tic20) vectors with *Bgl*II and *Avr*II, and ligated into the equivalent sites of $pCTG_{11}$. The chloramphenicol-resistance cassette of $pCTG_{11}$(P30) was replaced with a DHFR cassette encoding resistance to pyrimethamine by digestion of the pKOsagDHFR vector (a kind gift from

Marc-Jan Gubbels, Boston College) with *Hin*dIII and *Spe*I and ligation into the equivalent sites of $pCTG_{11}$(P30). The resultant construct was transfected into the FNR-GFP 1-10 cell line and selected on pyrimethamine as previously described (Donald, R. G. And D.S. Roos, 1993) and cloned by limiting dilution. The chloramphenicol-resistance cassette of $pCTG_{11}$(Tic20) was replaced with a BLE cassette encoding resistance to phleomycin by digestion of the pBSSK+ SAG1/Ble/SAG1 vector with *Hin*dIII and *Spe*I and ligation into equivalent sites of $pCTG_{11}$(Tic20). The resultant $pBTG_{11}$(Tic20) vector was transfected into the parasite line expressing FNR-GFP 1-10, selected on phleomycin and cloned by limiting dilution. Fluorescence intensities were quantified by flow cytometry using a MoFlo cytometer as described above, and results were analysed and graphed used FlowJo software (Tree Star, Inc., Ashland, OR).

To generate a cell line expressing both tub-*Tg*Tic20-HA and APT1-YFP, we made a plasmid expressing APT1-YFP containing a phleomycin selectable marker. We digested pBTH(Tic20) and an APT1-YFP vector (a kind gift from Manami Nishi and David Roos, U. Pennsylvania) with *Bgl*II and *Not*I, and ligated APT1-YFP into the equivalent sites of the pBT vector. We transfected this construct into the cell line expressing tub-*Tg*Tic20-HA and generated parasites stably expression APT1-YFP by phleomycin selection.

To generate a construct that targeted mouse DHFR fused to a C-terminal HA-tag to the apicoplast, we digested the FNR leader sequence from the ptubFNR-RFP/sagCAT vector (Striepen, B., et al., 2000) with *Bgl*II and *Avr*II and ligated this into the equivalent sites of pCTH. We then amplified mouse DHFR from cDNA (a kind gift from Jörn Lakowski, U. Georgia) with the primers 5'-CCTAGGGGTGGAAGCATGGTTCGACCATTGAACTGC and 5'-ACTAGTGTCTTTCTTCTCGTAGACTT. Mouse DHFR was digested with *Avr*II and *Spe*I and

ligated into the *Avr*II site of pCTH(FNR). We replaced the chloramphenicol resistance cassette with a phleomycin-resistance cassette and transfected the resultant pBTH(FNR-mDHFR) vector into the *Tg*Tic20 knockout and parental lines. Stable parasites expressing this construct were obtained through phleomycin selection.

To generate *Tg*Tic20 knockout and parental cell lines expressing apicoplast-targeted RFP, we digested the ptubFNR-RFP/sagCAT vector with *Bgl*II and *Not*I and ligated this into the equivalent sites of pBTH(Tic20). We transfected the resultant pBTR(FNR) construct into the *Tg*Tic20 knockout and parental lines and obtained stable expressors through phleomycin selection.

A2.S3. Parasite growth assays

To measure parasite growth we performed plaque assays by adding 400 parasites to a T25 flask containing a confluent monolayer of human foreskin fibroblasts. We grew parasites undisturbed for 9 days, washed once with phosphate-buffered saline (PBS; 0.8% w/v NaCl, 0.02% w/v KCl, 0.14% w/v Na_2HPO_4, 0.02% KH_2PO_4, pH 7.4), fixed the monolayer with ethanol, and stained with 2% (w/v) crystal violet in a solution of 20% (v/v) ethanol and 0.8% (w/v) ammonium oxalate. Tandem tomato RFP fluorescence plate assays were performed essentially as previously described (Gubbels, M. J., C. Li and B. Striepen, 2003). Human foreskin fibroblast cells were grown to confluency in 96-well Costar optical bottom plates (Corning Incorporated, Corning, NY). We seeded 4000 parasites per well, and measured Tomato fluorescence daily using a SpectraMax M2^e microplate reader (Molecular Devices, Sunnyvale, CA). Readings were taken from the bottom, using a 544 nm excitation and a 590 nm emission wavelength with a 570 nm cutoff. Percent positivity values were derived as previously described (Gubbels, M. J., C. Li and B. Striepen, 2003).

A2.S4. Western blotting and detection of membrane proteins

Protein samples (typically to a cell equivalent of 5 x 10^6 parasites) were loaded onto precast 12 % Bis-Tris and 3-8% Tris-Acetate NuPAGE gels (Invitrogen). After separation by electrophoresis, proteins were transferred to nitrocellulose membrane. Blots were probed with antibodies against ACP (1:1000 to 1:2000 dilution; a kind gift from Geoff McFadden, U. Melbourne, Waller, R. F., et al., 1998), GRA8 (1:200 000; a kind gift from Gary Ward, U. Vermont, (Carey, K. L., C.G. Donahue and G.E. Ward, 2000), anti-HA (1:100 to 1:500; clone 3F10, Roche Applied Science), anti c-myc (1:50 to 1:100; clone 9E10, Roche Applied Science) and anti-GFP (1:1000; Torry Pines Biolabs). HRP-conjugated anti-rat and anti-rabbit antibodies (Pierce) were used at 1:5000 to 1:10000 dilutions, while HRP-conjugated anti-mouse antibodies (TrueBlot, eBioscience) were used at 1:1000 dilution.

Membrane proteins were identified by alkaline (sodium carbonate) extractions and by Triton X-114 phase partitioning. For alkaline extractions (Fujiki, Y. et al., 1982), parasite pellets were washed once in PBS then incubated in 100 mM sodium carbonate (pH 11.5 or 12.5) for 30 minutes on ice. Samples were centrifuged at 150 000 g at 4^{o}C for 45 minutes. Pellets (containing integral membrane proteins) were resuspended in sample buffer, while supernatant proteins were precipitated by trichloroacetic acid before resuspension in sample buffer. Triton X-114 partitioning was performed as described previously (Bordier, C., 1981; Mullin, K. A., et al., 2006). Briefly, parasite pellets were resuspended in PBS containing 1 % (v/v) Triton X-114, 2 mM EDTA and supplemented with protease inhibitors (Complete protease inhibitor cocktail, Roche). Cells were incubated on ice for 30 minutes and centrifuged to remove insoluble material. The supernatant was layered onto a sucrose cushion (6% sucrose, 0.06% Triton X-114 in PBS) and incubated at 37^{o}C for three minutes. The sample was centrifuged for 2 minutes at room

temperature, and the layer above the cushion (containing soluble phase proteins) subjected to trichloroacetic acid precipitation before resuspension in sample buffer. The detergent micelles below the sucrose cushion (containing membrane proteins) were also extracted, resuspended in cold PBS and subjected to trichloracetic acid precipitation before resuspension in sample buffer. Proteins were separated by SDS-PAGE and detected by Western blotting (see above).

A2.S5. Pulse-chase and immunoprecipitation

Confluent T25 flasks were infected with approximately 2 x 10^6 parasites and allowed to grow for two days. Infected host cells were starved for one hour in cysteine and methionine-free Dulbecco's Modified Eagles medium supplemented with 1% dialysed foetal bovine serum and antibiotics. Infected host cells were radiolabelled with 100 μCi/mL of ^{35}S methionine and cysteine (GE Healthcare) for one hour. Cells were either harvested (pulse) or washed twice with 10 mL of parasite growth medium, and incubated in 10 mL parasite growth medium for 2 hours (chase) before harvesting. Proteins of interest were purified by immunoprecipitation or affinity purification (see below) and separated by SDS-PAGE as described above. Gels were dried and bands were visualised by autoradiography or using a Storm 860 PhosphorImager (GE Healthcare). Band intensities were quantified using ImageQuant TL software (GE Healthcare).

For immunoprecipitations, parasites were lysed from host cells by passage through a 26 gauge needle, and pelleted by centrifugation at 1500 g for 10 minutes. Pellets were washed in PBS then lysed for 30 minutes on ice in immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) noniodet P-40 substitute (Fluka), 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulphate, 2 mM EDTA) supplemented with protease inhibitors. Samples were centrifuged to remove insoluble material. Proteins-of-interest were purified by immunoprecipitation using either anti-HA conjugated agarose beads (Roche Applied Science),

anti-c-myc-conjugated agarose beads (Santa Cruz Biotechnology) or with antibodies bound specifically to protein A-sepharose CL-4B beads (GE Healthcare). For the latter application, samples were pre-cleared by incubation in 30 to 40 μL of a 50 % slurry of Protein A-Sepharose CL-4B beads. Antibodies including MIC5 (a kind gift from Vern Carruthers, U. Michigan, Brydges, S. D., et al. 2000), anti-lipoic acid (Calbiochem) and anti-ACP were bound to protein A-sepharose CL-4B beads for one hour at 4^{o}C before addition to pre-cleared lysates. All samples were incubated overnight at 4^{o}C then washed 4 times in immunoprecipitation wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) noniodet P-40 substitute, 0.5 % (w/v) sodium deoxycholate, 0.25 % w/v bovine serum albumin, 2 mM EDTA) and twice in PBS. Samples were eluted by boiling in reducing or non-reducing sample buffer then separated by SDS-PAGE as described above. For affinity purification of biotinylated proteins, lysates were incubated overnight with immobilised streptavidin-agarose beads (Pierce). Samples were washed 4 times in Streptavidin wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 % (v/v) noniodet P-40 substitute, 0.5 % (w/v) sodium deoxycholate, 0.1 % w/v sodium dodecyl sulfate, 2 mM EDTA) and twice in PBS, before elution by boiling in reducing sample buffer.

A2.S6. Southern blotting

Genomic DNA was extracted from parasite strains using the DNeasy kit (Qiagen). 2 μg was digested overnight with KpnI and separated by electrophoresis on a 0.8 % agarose gel. Gels were treated with 0.2 M HCl for 15 minutes, denatured in 1.5 M NaCl/0.5 M NaOH for 30 minutes and neutralised for 40 minutes in 1.5 M NaCl/1 M Tris base (pH 8.0). DNA was capillary transferred onto Nytran SuPerCarge nylon membrane (Schleicher and Schuell BioScience, Keene, NH) overnight in 20X SSPE buffer (3 M NaCl, 20 mM EDTA, 0.2 M sodium phosphate, pH 7.4), then crosslinked using a CL-1000 UV crosslinker (UVP, Upland, CA). The membrane

was prehybridised for four hours at 42°C in 40 % (v/v) formamide, 1.25 X SSPE, 0.625 % (w/v) sodium docecyl sulfate, Denhardt's solution (0.02 % w/v Ficoll, 0.02 % w/v polyvinylpyrrolidone, 0.02 % w/v bovine serum albumin) and 10 % (w/v) dextran sulfate. A probe against *Tg*Tic20 was amplified from genomic DNA using the same primers as for amplifying the entire open reading frame (above). The probe was radiolabeled with ^{32}P-dATP using the Random Primers DNA Labelling System (Invitrogen) as per the manufacturer's instructions, and hybridised to the nylon membrane overnight at 42°C. The membrane was washed three times in 2X SSPE for 15 minutes at room temperature, three times in 2X SSPE containing 1 % (w/v) sodium dodecyl sulfate for 15 minutes at 42°C, and rinsed a further two times in 2X SSPE. Bands were detected by exposure to autoradiography film.

A2.S7. Microscopy

Light microscope images were taken with a DM IRBE inverted epifluorescence microscope (Leica) fitted with a 100X oil immersion objective lens (PL APO 1.40 NA). Images were recorded using a Hamamatsu C4742-95 digital camera, and adjusted for brightness and contrast using Openlab software (Improvision). Live imaging was performed on infected coverslips in a sealed chamber at room temperature. For immunofluorescence, we fixed infected coverslips in 3 % paraformaldehyde for 15 minutes, permeabilised in 0.25 % Triton X-100 for 10 minutes, then blocked in 2 % (w/v) bovine serum albumin for 30 minutes. Samples were labelled with primary antibody for 1 hour, washed 3 times in PBS, incubated in secondary antibody for a further hour and washed 3 times in PBS. Primary antibodies used were rabbit anti-ACP (1:1000 to 1:2000 dilution) and rat anti-HA (1:100 to 1:500). Secondary antibodies used were goat anti-rabbit Alexa Fluor 546 (1:500) and goat anti-rat Alexa Fluor 488 (1:200; Molecular Probes, Eugene, OR).

For electron microscopy, flasks containing confluent human foreskin fibroblast cells were infected with the tub*Tg*Tic20-HA strain of *T. gondii*. Cryosectioning was done according to (Tokuyasu, K. T., 1973; Tokuyasu, K. T., 1978; Tokuyasu, K. T., 1980). Cells were fixed for 24 hours in 2 % formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, and subsequently embedded in 12% gelatin in phosphate buffer. The gelatin embedded cells were cut in cubes and infiltrated with 2.3 M sucrose for 10 to 20 hours at 4^{o}C. Samples were mounted on a sample holder, frozen in liquid nitrogen and cryo-sectioned using cryo-ultramicrotome UCT/FCS (Leica Microsystems, Vienna, Austria). The cryo-sections were picked up with a drop of 1% methyl cellulose and 1.15 M sucrose in PBS (Liou, W., H.J. Geuze and J.W. Slot, 1996) and transferred to Formvar-carbon-coated hexagonal 200 mesh grids. The grids with ultra-thin sections were washed for 30 min at 37 ^{o}C in phosphate buffer (pH 7.4) and blocked for 15 minutes on drops of PBS containing 1% BSA and 0.05% cold water fish skin gelatin (Sigma). The grids were incubated for 60 minutes with anti-HA antibody (12CA5, Roche), rinsed in PBS containing 0.1% BSA and 0.005% cold water fish skin gelatin, and incubated for 20 minutes with a bridging rabbit anti-mouse antibody (DakoCytomation). After the second washing step, the grids were incubated for 20 minutes with protein A coupled to gold (PAG, 10 nm; Medical School, Utrecht University, the Netherlands). The label was fixed onto the cryo-sections with 1% glutaraldehyde in PBS for 5 minutes. For double labelling, the cryo-sections were subsequently blocked as described above, then incubated for 60 minutes with anti-ACP antibody, rinsed and incubated for 20 minutes with PAG 15 nm. The grids were fixed for 5 minutes in 1% glutaraldehyde in PBS and rinsed in distilled water for 10 minutes. Cryosections were poststained with 2% uranyl acetate in 0.15 M oxalic acid (pH 7.4; Tokuyasu, K. T., 1978) and embedded in 1.8% methyl cellulose containing 0.4% aqueous uranyl acetate. Grids were

examined in a transmission electron microscope (Tecnai 12, FEI Company, Eindhoven, the Netherlands) at 120 kV. Images were recorded using a CCD camera (MegaView II, Soft Imaging Systems GmbH, Münster, Germany). Image processing was done with Analysis 3.2 (Soft Imaging Systems GmbH, Münster, Germany).

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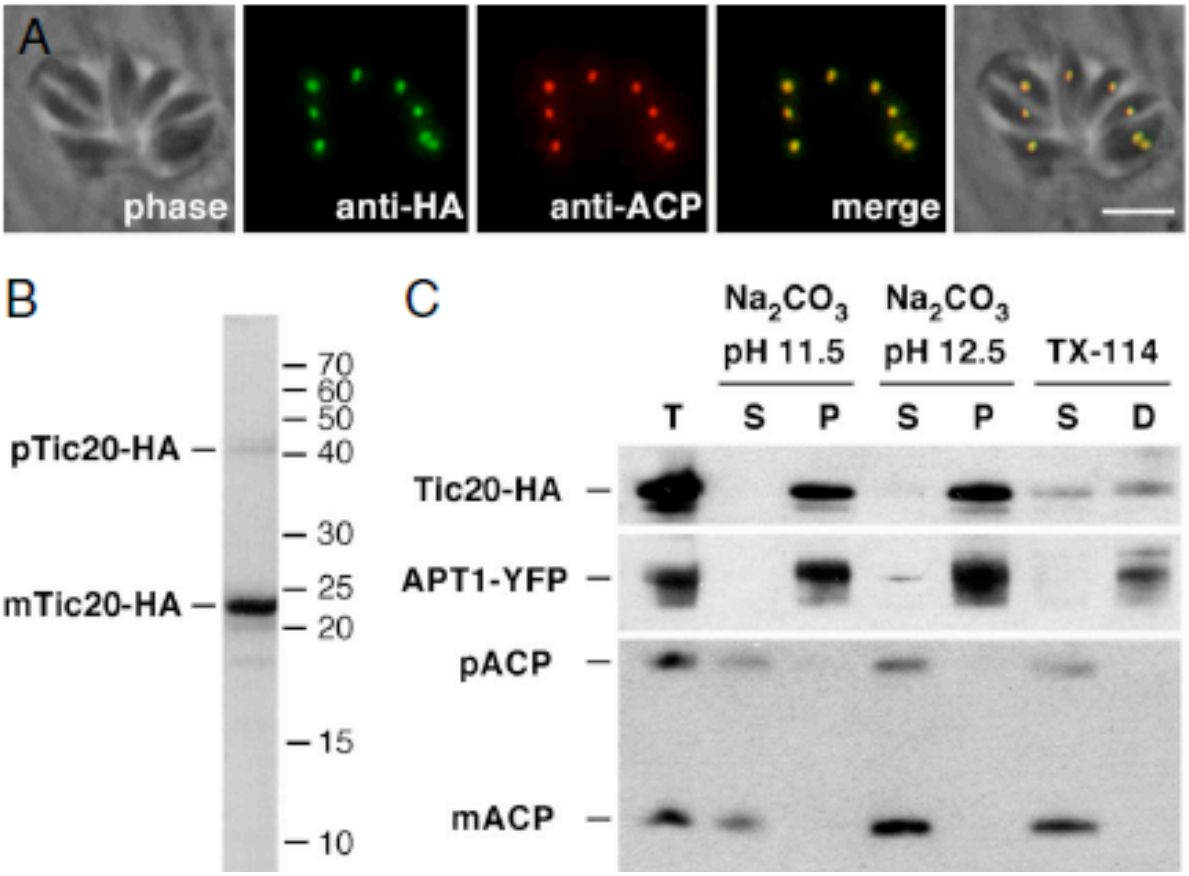
Main Figures

Figure A2.1:

*Tg*Tic20 is an apicoplast integral membrane protein. *(A)* Immuofluorescence assay depicting an eight-cell *T. gondii* vacuole. *Tg*Tic20-HA (green) co-localises with the apicoplast marker ACP (red). Scale bar is 5 μm. *(B)* A Western blot of protein extracts from the *Tg*Tic20-HA line with anti-HA antibodies. A mature *Tg*Tic20 protein species is labelled at around 23 kDa, while a weaker precursor band is labelled at around 40 kDa. *(C)* Proteins were extracted from the *Tg*Tic20-HA/APT1-YFP line and fractionated into soluble (S) and membrane pellet (P) fractions by sodium carbonate treatment, or into soluble (S) and detergent (D) phases by TX-114 phase partitioning. Total protein extracts (T) are shown in the first lane. (D) Transmission electron micrograph of the *Tg*Tic20-HA cell line, where *Tg*Tic20-HA is labelled with 10 nm gold beads and ACP with 15 nm gold beads. Arrowheads show *Tg*Tic20-HA labelling at the membranes of the organelle. (Scale bar 100 nm.)



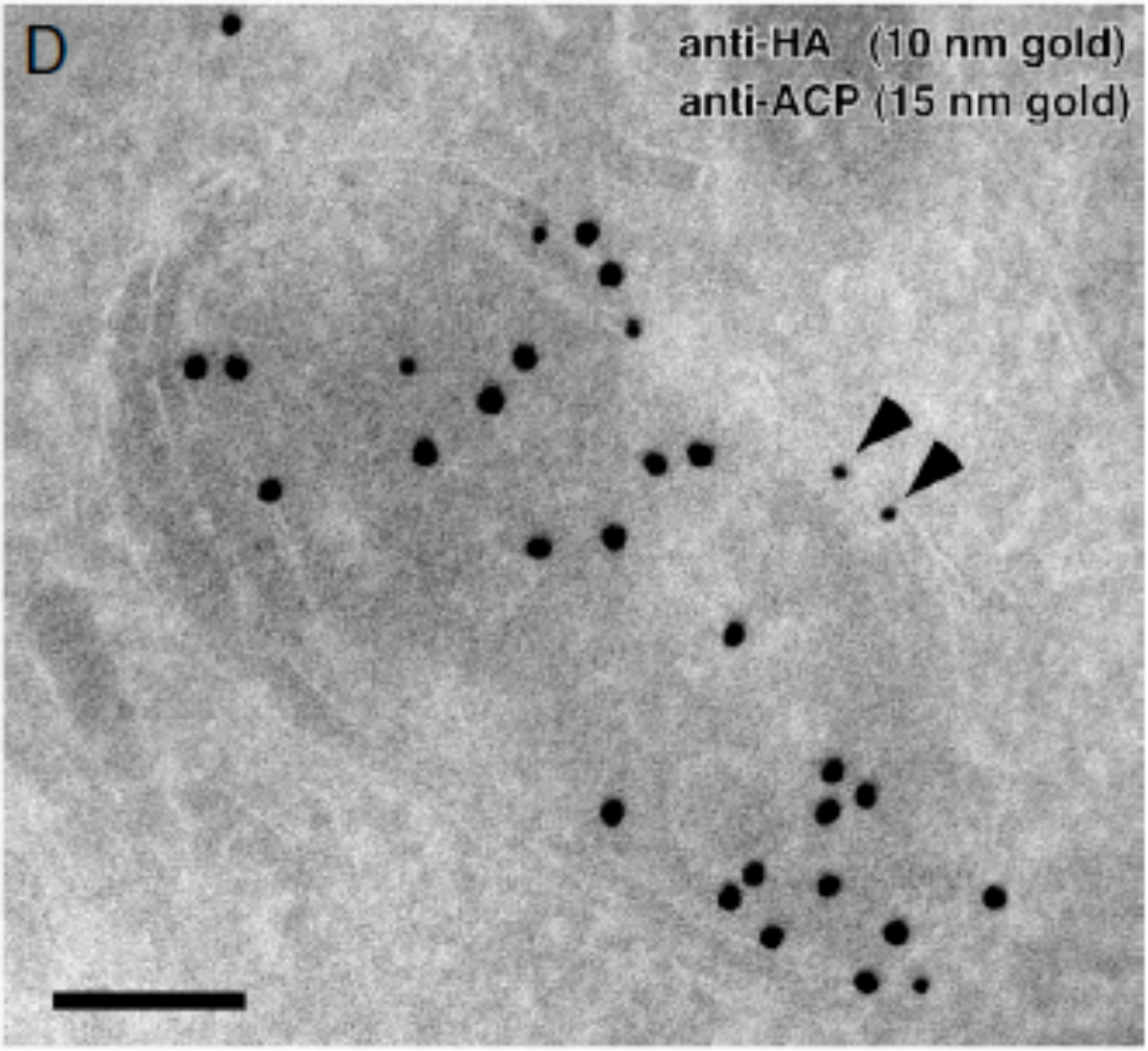

D
anti-HA (10 nm gold)
anti-ACP (15 nm gold)

Figure A2.2:

*Tg*Tic20 localises to the inner apicoplast membrane. *(A-B)* Live images of *T. gondii* cells expressing FNR leader fused to GFP 1-10, where GFP-11 was fused to the C-terminus of either ACP (A) or *Tg*Tic20 (B). In both cell lines, green fluorescence co-localises with the apicoplast stromal marker FNR-RFP. Scale bar is 2 μm. *(C)* Live image of *T. gondii* parasites expressing the secretory marker protein P30 fused to both GFP 1-10 and GFP-11. Green fluorescence localises to the parasitophorous vacuole. *(D)* Model for localisation of *Tg*Tic20. We predict that *Tg*Tic20 localises to the inner membrane of the apicoplast, with the C-terminus in the stroma. *In silico* predictions suggest the presence of four transmembrane domains (Fig. S1).

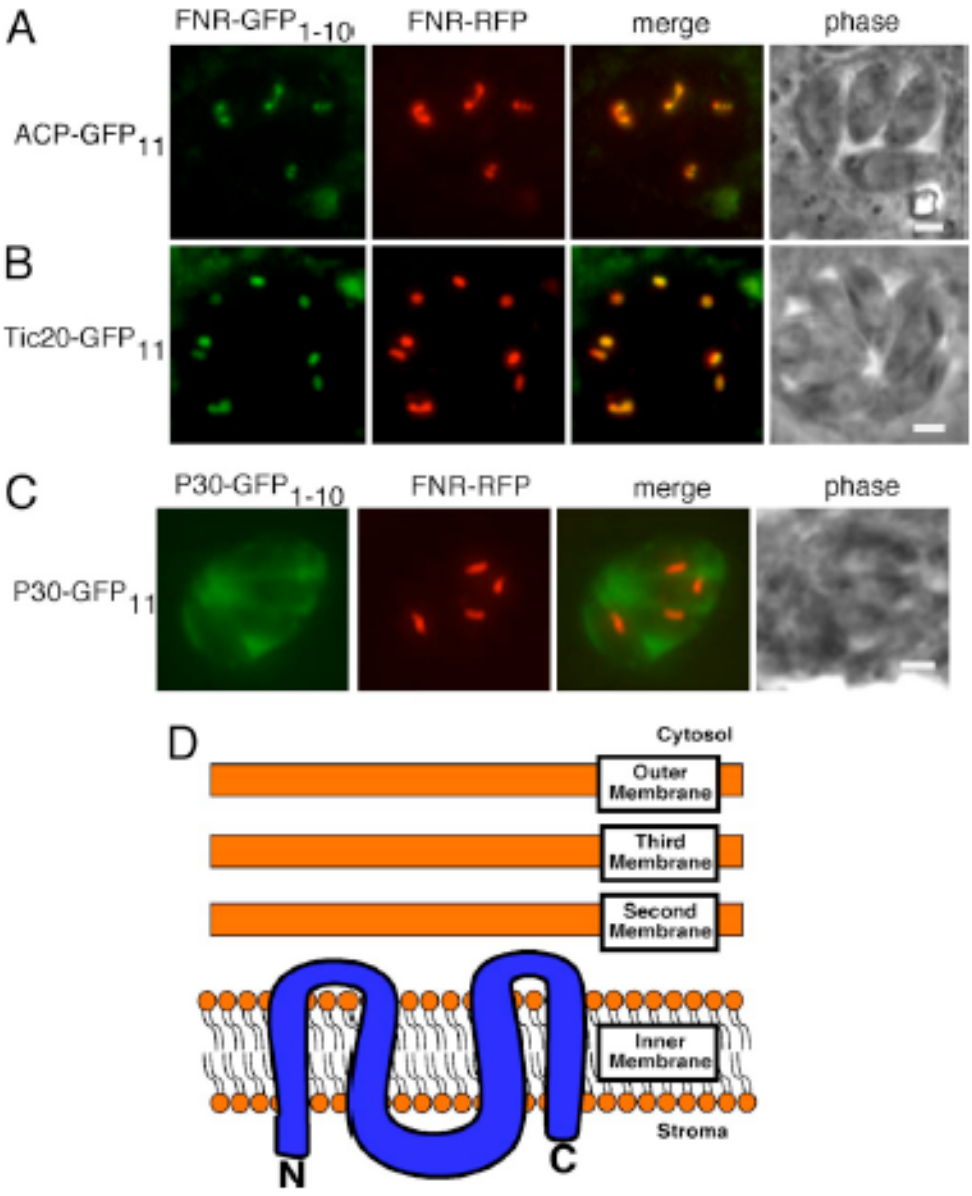


Figure A2.3:

*Tg*Tic20 is essential for growth of *T. gondii* parasites. (*A-C*) We monitored growth of parental (*A*), knockout (*B*) and complemented (*C*) parasite lines expressing tandem tomato RFP by a fluorescence growth assay. Cells were grown in the absence (green diamonds) or presence (blue squares) of ATc, or incubated for three days in ATc before beginning the growth assay in the presence of ATc (red diamonds). Values are presented as percent positivity and error bars reflect one standard deviation from the mean.

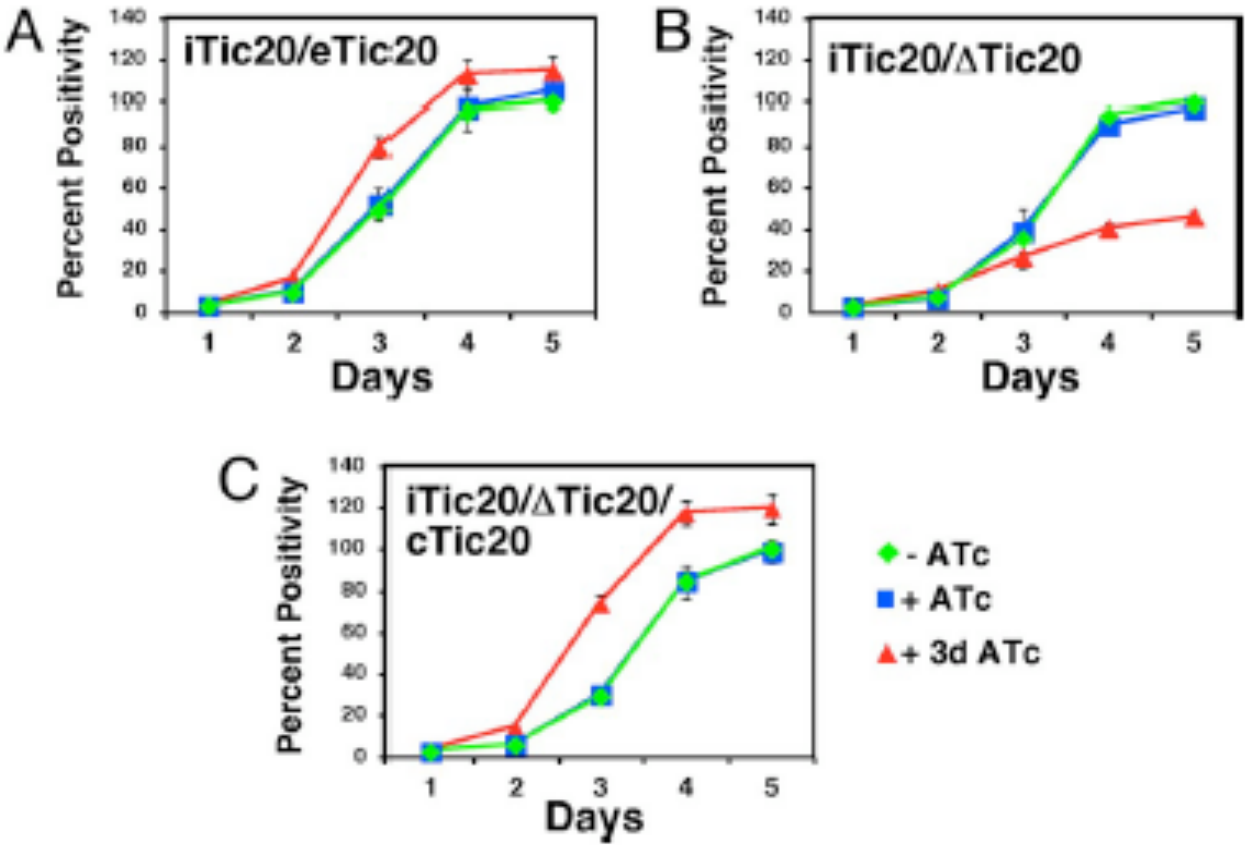
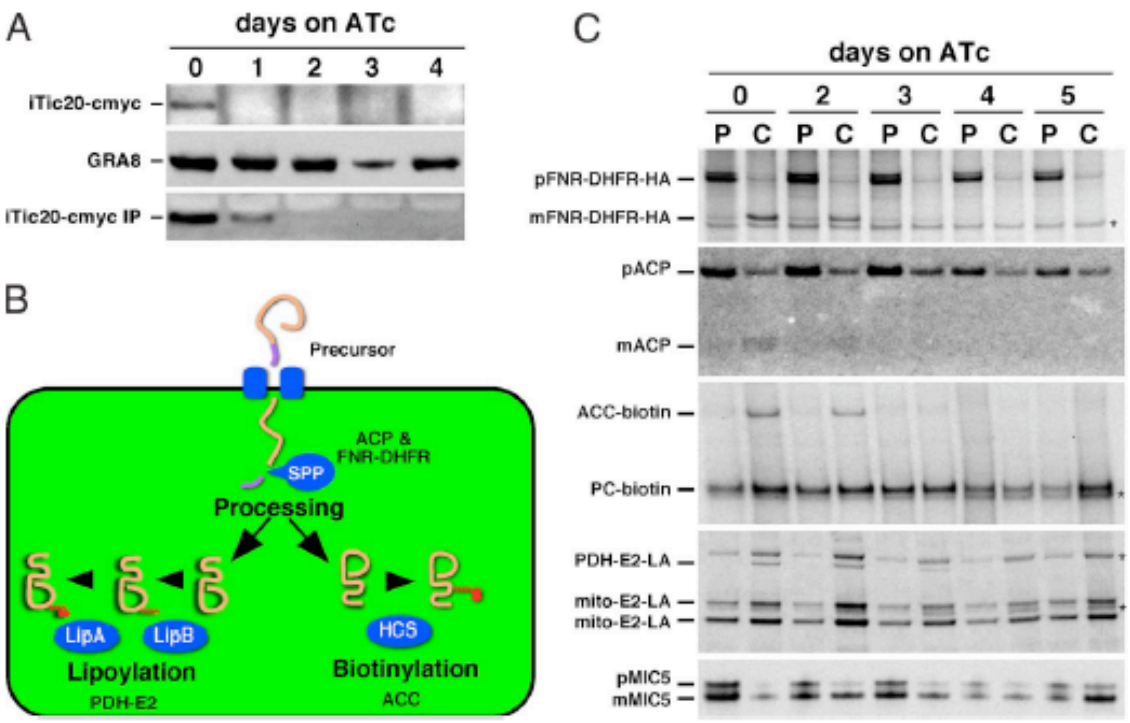


Figure A2.4:

*Tg*Tic20 is essential for apicoplast protein import. *(A)* Regulation of the inducible *Tg*Tic20-c-myc protein. iTic20/ΔTic20 parasites were grown for 0 to 4 days on ATc. Proteins were extracted and subjected to Western blotting with either anti-c-myc or anti-GRA8 antibodies (as a loading control), or subjected to immunoprecipitation of the inducible *Tg*Tic20-c-myc protein followed by Western blotting with anti-c-myc antibodies (bottom lane). *(B)* Schematic depiction of the three protein import assays used in this study. We measured cleavage of preprotein leader sequences by the stromal processing peptidase (SPP), biotinylation of ACC by a holocarboxylase synthetase (HCS), and lipoylation of PDH-E2 by LipB and LipA. All three processes are thought to occur in the apicoplast stroma. *(C)* Pulse-chase analysis of proteins from the *Tg*Tic20 knockout line grown for 0, 2, 3, 4 or 5 days on ATc. Infected host cells were incubated in medium containing ^{35}S-amino acids for one hour and either harvested (P) or further incubated in non-radioactive medium for two hours (C). After detergent solubilization, proteins were purified by immunoprecipitation or affinity purification and separated by SDS-PAGE before detection by autoradiography. Protein bands marked by an asterisk in lanes containing biotinylated and lipoylated proteins represent contaminating host cell proteins. The band marked by an asterisk in HA pulldown lanes likely results from the use of an alternative internal start codon representing a shorter cytosolic version of FNR-DHFR-HA. *(D)* Quantification of bands in *C*. FNR-DHFR-HA values (green diamonds) were quantified as the percentage of mature protein in the chase compared to the precursor protein after the pulse. ACC-biotin values (red triangles) were quantified as a percentage of the intensity of PC in the same lane. PDH-E2-LA values (blue squares) were quantified as a percentage of the intensity of the lower-most mito-E2 band in the same lane. Values for each day are expressed as a percentage of the no ATc value. *(E)* Pulse-chase analysis of proteins from the *Tg*Tic20 parental (iTic20/eTic20) line, performed in an identical manner to *C*.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GRA8
iTic20-cmyc IP
B
Precursor
ACP &
FNR-DHFR
SPP
Processing
LipA
LipB
Lipoylation
PDH-E2
HCS
Biotinylation
ACC
C
days on ATc
0 2 3 4 5
P C P C P C P C P C
pFNR-DHFR-HA
mFNR-DHFR-HA
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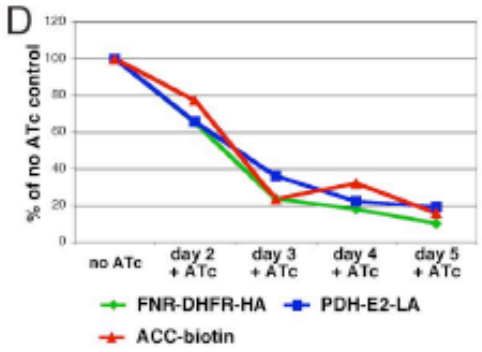
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% of no ATc control
120
100
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60
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FNR-DHFR-HA
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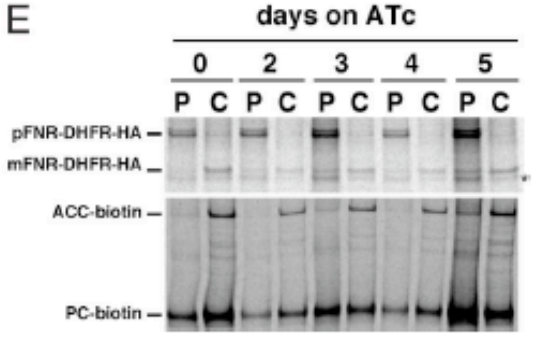
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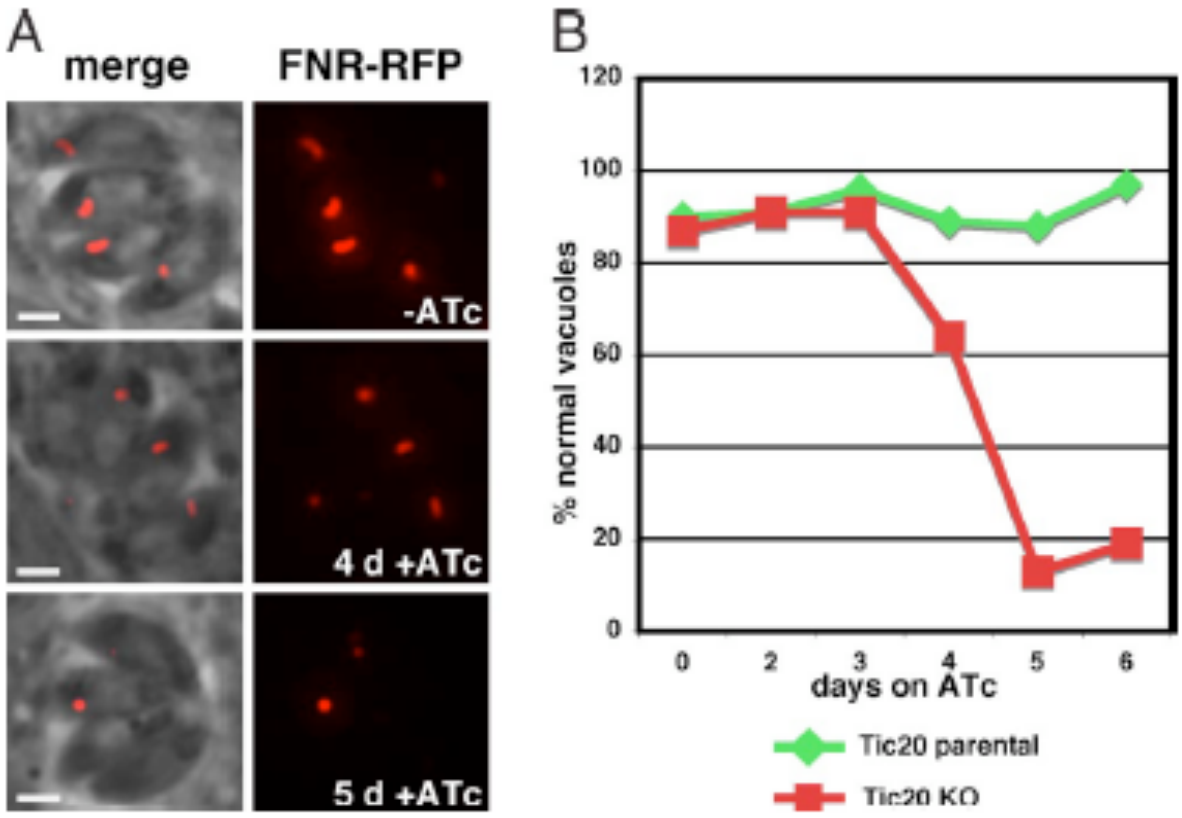
pFNR-DHFR-HA
mFNR-DHFR-HA
ACC-biotin
PC-biotin

Figure A2.5:

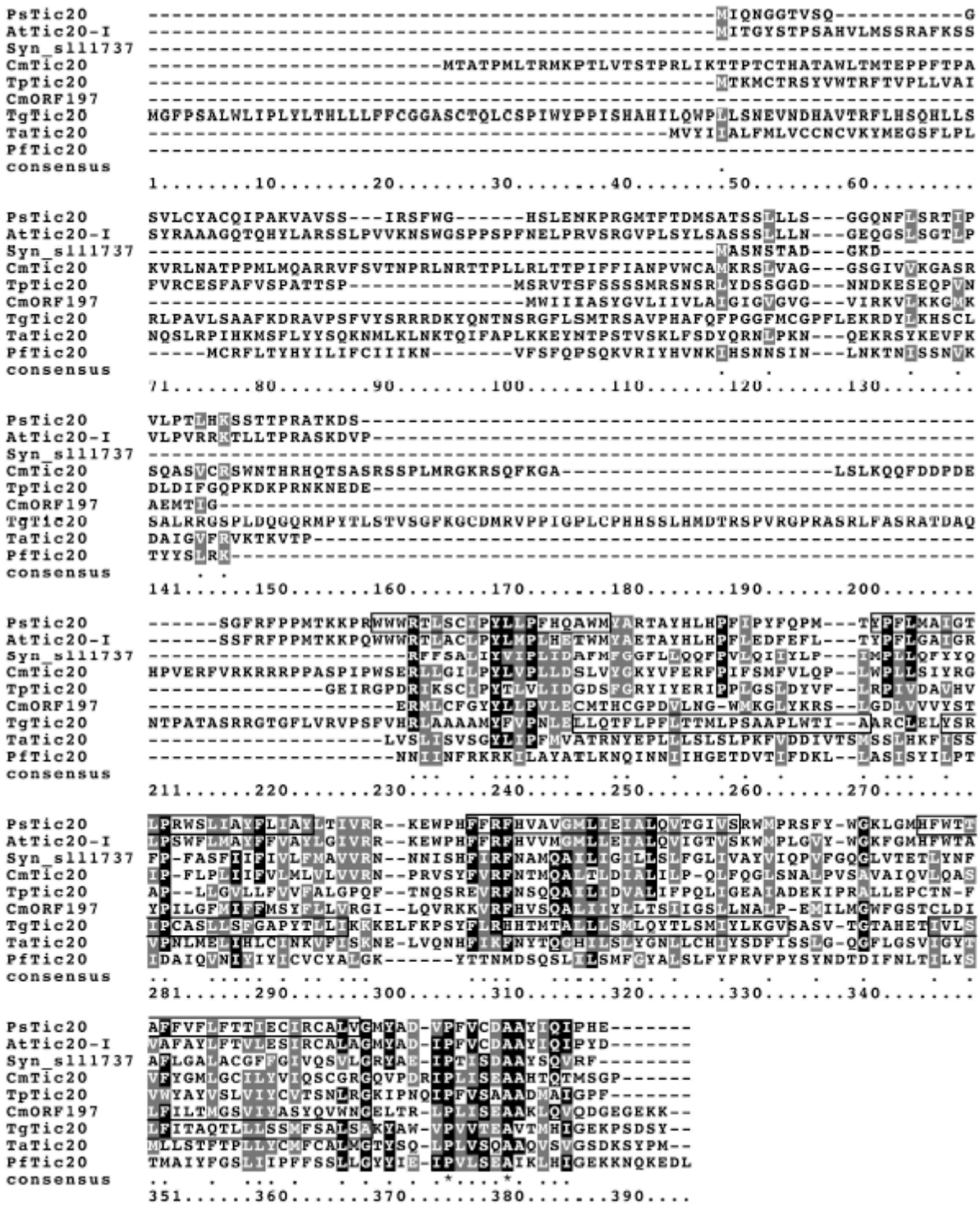
*Tg*Tic20 is required for apicoplast biogenesis. *(A) Tg*Tic20 knockout parasites containing apicoplast-targeted RFP (FNR-RFP) were grown for 0, 4 or 5 days on ATc and subjected to live cell imaging. Scale bar is 2 μm. *(B) Tg*Tic20 parental (green diamonds) or knockout (red squares) parasites containing apicoplast-targeted RFP were grown for 0 to 6 days on ATc. 100 four-cell vacuoles were imaged at each time point. We graphed the percentage of vacuoles at each time point where every parasite in that vacuole contained an apicoplast.



Supplemental Figures

Figure A2.S1:

Multiple sequence alignment of Tic20 protein homologs. The alignment includes Tic20 homologs from the plants *Pisum sativum* (PsTic20; GenBank accession number AAC64607) and *Arabidopsis thaliana* (AtTic20-I; NP_171986), the cyanobacterium *Synechocystis* species PCC 6803 (Syn_sll1737; NP_440747), the red alga *Cyanidioschyzon merolae* nuclear-encoded (CmTic20; genome accession number CMS050C, http://merolae.biol.s.u-tokyo.ac.jp/; Matsuzaki, M., et al., 2004 and *C. merolae* plastid-encoded (CmORF197; CMV078C), the diatom *Thalassiosira pseudonana* (TpTic20; gene model predicted by authors based on sequence available at genome website: http://genome.jgi-psf.org/Thaps3/Thaps3.home.html; Armbrust, E. V., et al. 2004), and the Apicomplexa *Toxoplasma gondii* (TgTic20; this study; EU427503), *Theileria annulata* (TaTic20; XP_951914) and *Plasmodium falciparum* (PfTic20; AAN36039). Conserved and similar residues with an identity threshold of 0.60 are shaded and marked by asterisks and dots on the consensus line. The boxed regions in the PsTic20 and TgTic20 sequences represent predicted transmembrane domains (based on TMHMM Server predictions; http://www.cbs.dtu.dk/services/TMHMM/).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consensus

Figure A2.S2:

Quantification of split GFP experiments. *(A)* To quantify fluorescence levels, cell strains depicted in Fig. 2, in addition to cell strains expressing FNR-GFP 1-10 alone and FNR-GFP 1-10 with P30-GFP-11 were analysed by flow cytometry. When the split GFP molecules are targeted to separate compartments of the secretory pathway (left, green line), fluorescence intensity equates to that of FNR-GFP 1-10 alone (red line on all graphs). Fluorescence intensities in strains co-expressing FNR-GFP 1-10 with either ACP-GFP-11 (centre, green line) or *Tg*Tic20-GFP-11 (right, green line) are considerably greater than in FNR-GFP 1-10 alone. *(B)* Western blot of parasite cell lines containing either FNR-GFP 1-10 alone, or co-expressed with ACP GFP-11 or *Tg*Tic20 GFP-11. The anti-GFP antibody labels GFP 1-10 alone. Each cell line contains low levels of precursor FNR-GFP 1-10, with most GFP 1-10 existing in the mature, processed form, suggesting that in each cell line, most GFP 1-10 reaches the apicoplast stroma. Asterisk labels a possible GFP 1-10 degradation product, similar to those previously observed for apicoplast-targeted GFP molecules (Donald, R. G. And D.S. Roos, 1993).

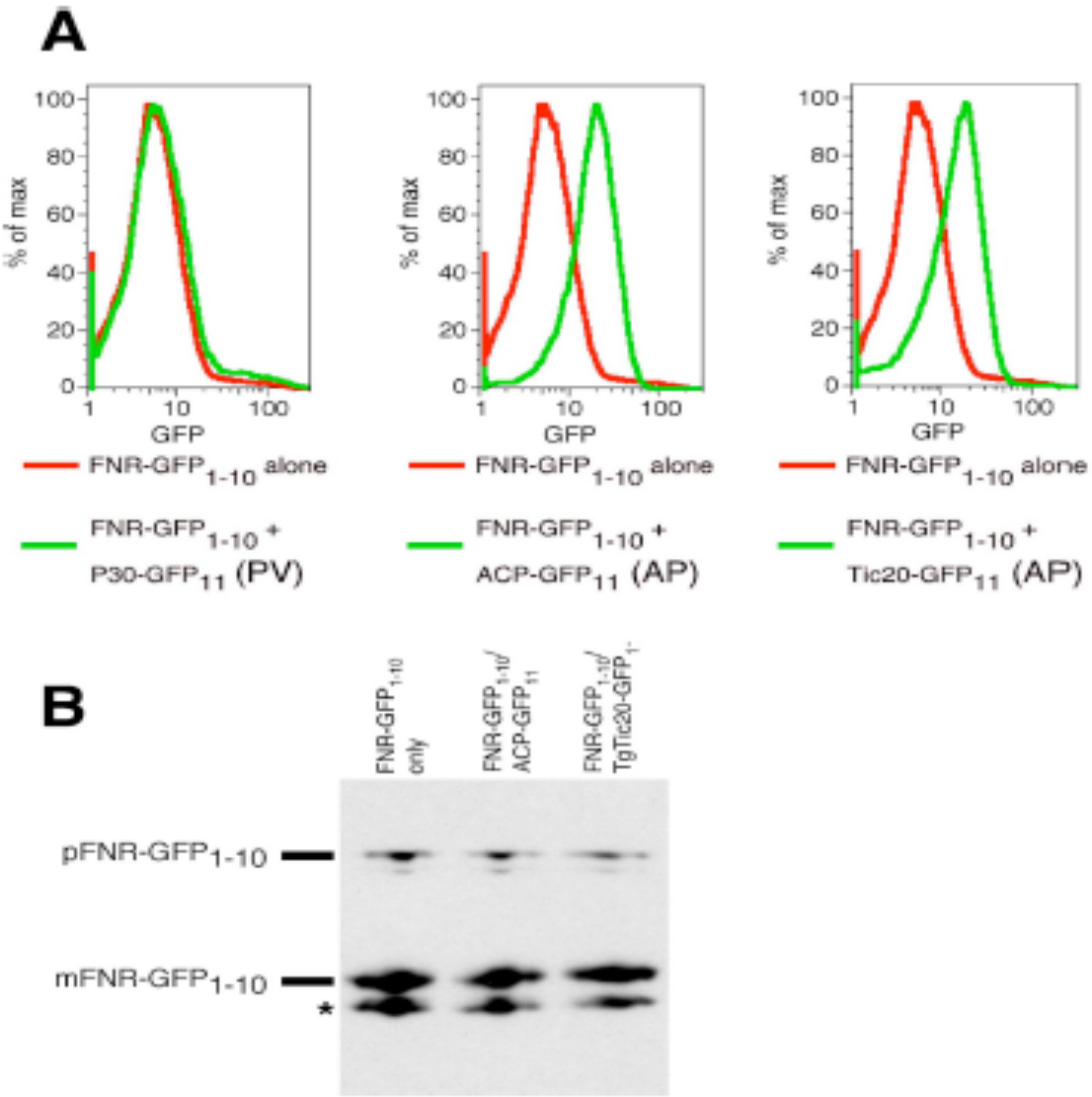
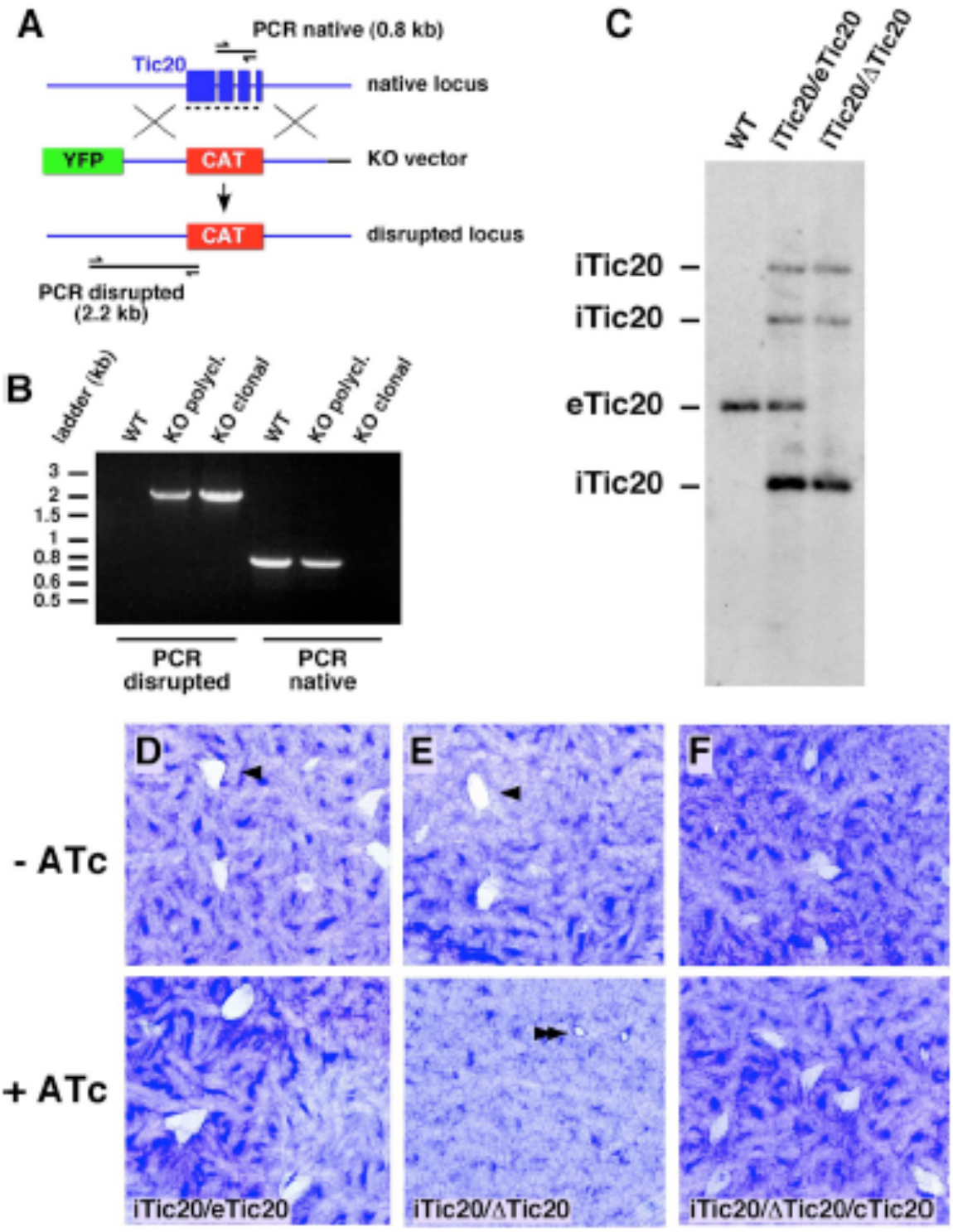


Figure A2.S3:

Genetic disruption of *Tg*Tic20. *(A)* A schematic depicting genetic disruption of the native TgTic20 locus. We generated a knockout construct (KO vector) containing a chloramphenicol acetyl transferase (CAT) gene flanked by 2 kb sequences homologous to the 5' and 3' flanks of the native *Tg*Tic20 locus. A YFP marker for negative selection is located outside this knockout cassette. We transfected linearised knockout vector into a parasite cell line expressing inducible copies of *Tg*Tic20, selected for chloramphenicol-resistant parasites, and then further selected for homologous integrants through fluorescence sorting of parasites not expressing YFP. *(B)* We performed diagnostic PCR analysis to verify disruption of the *Tg*Tic20 locus. One set of PCR primers was specific for the disrupted *Tg*Tic20 locus (PCR disrupted) and a second set was designed within introns of the *Tg*Tic20 gene and is thus specific to the native locus (PCR native). We observed a PCR product of the predicted size for the native gene in wild type (WT) parasites but not in knockout parasite clones (KO clonal). Conversely, we observed a PCR product of the predicted size for the disrupted gene in clonal knockout but not in wild-type parasites. *(C)* We performed Southern blotting on RH strain wild-type (WT), parental (iTic20/eTic20) and knockout (iTic20/ΔTic20) parasites using a probe against the entire *Tg*Tic20 gene. A band the expected size for the endogenous gene (eTic20) is expressed in both wild type and parental parasites, but not in knockout parasites. Extra bands in the parental and knockout strains represent introduced inducible copies of the *Tg*Tic20 gene (iTic20). **(***D-F***)** To further measure the effects of *Tg*Tic20 knockdown on parasite growth, we performed plaque assays on parental parasites (D), knockout parasites (E) and knockout parasites complemented with ectopically expressed *Tg*Tic20 (F) in the absence (top) or presence (bottom) of ATc. We grew parasites for 9 days in the absence or presence of ATc. During this period, parasites will go through several replication cycles, and form zones of clearance (plaques) in the host cell monolayer. We observed a severe growth defect in the presence of ATc in the knockout cell line (*E*, double arrowhead), but not in the parental or complemented lines (*D* and *F*). Arrowheads and double arrowheads depict large and small plaques, respectively.


A
PCR native (0.8 kb)
Tic20
native locus
YFP
CAT
KO vector
CAT
disrupted locus
PCR disrupted
(2.2 kb)
B
ladder (kb)
WT
KO polycl.
KO clonal
WT
KO polycl.
KO clonal
3
2
1.5
1
0.8
0.6
0.5
PCR disrupted
PCR native
C
WT
iTic20/eTic20
iTic20/ΔTic20
iTic20
iTic20
eTic20
iTic20
D
E
F
- ATc
+ ATc
iTic20/eTic20
iTic20/ΔTic20
iTic20/ΔTic20/cTic20