THE MAINTENANCE AND REPLICATION OF THE APICOPLAST GENOME IN *TOXOPLASMA GONDII*

by

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(Under the Direction of Boris Striepen)

ABSTRACT

The phylum Apicomplexa comprises a group of intracellular parasites of significant global health and economic concern. Most apicomplexan parasites possess a relict plastid organelle, which no longer performs photosynthesis but still retains important metabolic functions. This organelle, known as the apicoplast, also contains its own genome which is required for parasite viability. The apicoplast and its genome have been shown to be useful as therapeutic targets. However, limited information is available about the replication and maintenance of plastid DNA, not just in apicomplexan parasites but also in plants and algae. Here we use the genetic tools available in the model apicomplexan *Toxoplasma gondii* to examine putative apicoplast DNA replication and condensation factors, including homologs of DNA polymerase I, single-stranded DNA binding protein, DNA gyrase, and the histone-like protein HU. We confirm targeting to the apicoplast of these candidates, which are all encoded in the nucleus and must be imported. We created a genetic knockout of the *Toxoplasma* HU gene, which encodes a homolog of a bacterial protein that helps condense the bacterial nucleoid. We show that loss of HU in *Toxoplasma* results in a strong decrease in apicoplast DNA content, accompanied by biogenesis and segregation defects of the organelle. We were also interested in examining the roles of enzymes that might be more directly involved in DNA

replication. To this end we constructed conditional mutants of the *Toxoplasma* gyrase B homolog and the DNA polymerase I homolog, which appears to be the result of a gene fusion and contains multiple different catalytic domains. We find that these proteins are essential to the parasite and required for apicoplast DNA replication. Together these data highlight the importance of the apicoplast DNA in apicomplexan cell biology and increase our understanding of plastid genome biology.

INDEX WORDS: Apicoplast genome, DNA replication, HU.

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DEDICATION

This work is dedicated to my sister, Alison. Her journey has been an inspiration and shows that hard work and persistence always pay off.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The phylum Apicomplexa consists of numerous parasitic protozoa which cause a variety of diseases and have both significant health and economic impacts globally. Among these parasites, perhaps the best known are *Plasmodium* species, the causative agents of malaria. Also included are parasites such as *Cryptosporidium* species, which cause gastrointestinal disease, and *Toxoplasma gondii*, which causes Toxoplasmosis. *T. gondii* can infect mammals and birds and can invade any nucleated cell type. Most immunocompetent people can be infected without producing symptoms, but Toxoplasmic encephalitis produces disease in immunosuppressed individuals and has been associated with AIDS. *T. gondii* also causes congenital disease when a woman becomes infected for the first time during pregnancy.

The epidemiology of malaria in the past century has shown us that apicomplexan parasites are very adaptable and possess a strong ability to become resistant to commonly used drug treatments. Thus it is imperative to identify novel drug targets so that new and better treatments may be developed.

Most Apicomplexan parasites harbor a remnant chloroplast called the apicoplast. This organelle is thought to have arisen via a secondary endosymbiosis event, in which a eukaryotic cell engulfed an alga already possessing a plastid. Consistent with this hypothesis, the apicoplast is surrounded by four membranes and contains its own 35 kb genome. This plastid organelle has since lost its photosynthetic function, but it houses

various biosynthetic pathways including type II fatty acid synthesis (FASII), heme synthesis, and isoprenoid biosynthesis [1-4]. It has been shown that pathways in the apicoplast are essential for cell viability in *T. gondii* [5, 6] and *P. falciparum* [2, 7, 8]. Because this organelle is unique to these parasites, apicoplast proteins and structures become good candidates for drug targets. Loss of the apicoplast genome is also lethal to *T. gondii*. This was first observed using Ciprofloxacin, an antibiotic that kills *T. gondii* in cell culture that is known to target DNA gyrase [9].

The 35 kb apicoplast genome is well conserved throughout the Apicomplexa, suggesting that drugs which target the apicoplast genome in *Toxoplasma* may also work in the other apicoplast-bearing apicomplexans. Additionally, since the apicoplast is a unique organelle, there is great potential for drugs which are pathogen-specific. However, there is limited information about how the apicoplast genome is replicated. Even in the more widely studied plastid-bearing organisms like *Arabidopsis* and *Chlamydomonas* the information on this front is lacking. It is known that most chloroplasts possess a polymerase related to bacterial DNA polymerase I [10, 11]. Also included in chloroplast replication machinery are DNA primase, DNA helicase, and DNA gyrase [12-14]. However, although biochemical studies have been performed on these proteins, there is still a dearth of phenotypic studies to demonstrate that they are indeed required for DNA replication to proceed in the chloroplast. In addition, chloroplast DNA segregation is poorly understood and the potential roles DNA-binding proteins play in this process remain untested.

Chloroplast genomes also need proper packaging and topology in order to interact correctly with other proteins. In eukaryotic nuclei this is achieved through histones, which upon interaction with each other and with DNA result in the nucleosomal structure of eukaryotic genomes. Histones can be modified which allow the DNA topology and packaging to be regulated, which in turn affects gene expression. Histones

are unique to eukaryotic nuclear genomes and this system is not present in bacteria; rather, bacterial genomes are packaged by other DNA binding proteins such as HU, IHF, Fis, and H-NS [15]. Chloroplast genomes are even simpler than bacteria, and thus not all the DNA-binding protein machinery is conserved. Many plant and algal plastids seem to lack DNA compaction components homologous to those in bacteria and eukaryotic nuclei. An exception is HU, which is common in red algal plastids and has also been conserved in the apicoplast [16, 17].

Proteins involved in the replication and condensation of the apicoplast genome have potential as attractive drug targets; many are expected to be essential since the apicoplast genome is required for parasite survival. The focus of this work is to characterize proteins involved in the replication and maintenance of the apicoplast genome using the molecular tools available in *Toxoplasma*. Our initial efforts identified a number of homologs to bacterial DNA replication and repair machinery, including DNA polymerase I, single-stranded DNA binding protein, DNA gyrase, the recombinational helicase RecG, and HU. The DNA polymerase I homolog also contains primase, helicase, and 3'-5' exonuclease domains, and we hypothesize that this large protein, along with single-stranded DNA binding protein and DNA gyrase, make up the primary components of apicoplast DNA replication.

Furthermore, there is a need for a good apicoplast DNA marker in microscopy studies. Visualization of apicoplast DNA is valuable for addressing questions about genome biology in this organelle, particularly in phenotypic studies of DNA replication and segregation mutants. At present there is no stain specific for apicoplast DNA, and the discovery of a marker that can be used to specifically visualize apicoplast DNA would facilitate studies of the plastid genome and faster characterization of potential drug targets involved in the processes of the apicoplast genome. We have identified a homolog of the DNA-binding HU protein in the *Toxoplasma* apicoplast. HU has been

observed to serve varied functions, most notably DNA compaction and stabilization of supercoils, in bacteria and algae [18, 19]. In addition to our interest in the role of HU in apicoplast biology, if TgHU binds apicoplast DNA it has the potential to be quite useful as a DNA marker.

1.2 The Structure of this Dissertation

This dissertation is divided into five chapters. Chapter 2 presents a review of the literature relevant to apicoplast genome biology. Chapter 3 is about the role of the Histone-like HU protein in maintaining the stability and topology of the apicoplast genome. We have constructed a complete knockout of the HU gene and found that resulting mutant has a greatly decreased apicoplast genome copy number, which ultimately results in apicoplast loss in some cells and a strong growth phenotype. This work was submitted to *Eukaryotic Cell* at the end of January 2012 and is currently in revision. Chapter 4 covers our work on the apicoplast DNA replication machinery. We have localized several putative apicoplast DNA replication factors to the apicoplast. For some of these we also have obtained conditional knockouts, and experiments suggest that these components are essential for DNA replication in the apicoplast and for parasite viability. In chapter 5 I will present my conclusions from this work.

I have also included 2 appendices in this dissertation. Appendix 1 is a review about Apicomplexan cell division published in *PLoS Pathogens* [20]. Appendix 2 is an article published in *Current Biology* about a dynamin-related protein required for apicoplast fission in *Toxoplasma* [21].

Together, the work presented in this dissertation provides new insight into how the apicoplast DNA is replicated and maintained within the parasite and the ways in which the apicoplast DNA is important for parasite survival.

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CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Endosymbiosis

The evolution of chloroplasts and mitochondria is explained by endosymbiosis theory, an idea which dates back to the late 19th century but didn't gain wide acceptance until the publication of a landmark article by Lynn Margulis in the 1960s [1]. This theory posits that at some point in evolutionary history, an ancient eukaryote engulfed a bacterium – a cyanobacterium in the case of chloroplasts, and an alpha-proteobacterium in the case of mitochondria – and over time this bacterium became reduced to a point that it was no longer a separate organism but an integral and interdependent part of the host cell. The evolution of these endosymbiotic organelles leave certain traces of their origins, including two membranes rather than one, derived from the bacterial cell wall, and their own (albeit reduced) genome. Currently, the endosymbiosis which later led to mitochondria is thought to be the main evolutionary process which gave rise to the eukaryotes. The evolution of chloroplasts led to the acquisition of photosynthesis by their eukaryotic hosts and though they are not as ubiquitous as mitochondria, they certainly contribute to a great deal of the diversity among eukaryotes.

In the currently accepted evolutionary model, chloroplasts appeared in eukaryotes after the prior acquisition of mitochondria, about 1.6 billion years ago by some estimates [2]. These cells diversified into the phyla viridiplantae, rhodophyta, and glaucophyta, collectively known as supergroup archaeplastida. Later in evolution, there arose cases in which a eukaryotic host then engulfed an alga, which over time was

again reduced to a chloroplast (in most cases; note that in chlorarachniophytes and cryptophytes the endosymbiont nucleus has also been retained albeit in reduced form and is known as a nucleomorph [3]). This is known as secondary endosymbiosis, and the resulting organelles are called secondary plastids or complex plastids. These organelles are usually characterized by three to four membranes, and similar to primary plastids possess their own genomes. Secondary endosymbiosis gave rise to a great deal of today's algal diversity. The euglenids and the chlorarachniophytes possess secondary green algal plastids, and the alveolates, the stramenopiles, the haptophytes, and the cryptophytes all have members that possess secondary red algal plastids [4]. Together, the protists containing secondary red plastids are known as the chromalveolates, which in addition to many algae also contain non-photosynthetic organisms like the Apicomplexa. This phylum comprises one branch of the alveolates and consists of obligate intracellular parasites which contain a secondary plastid which has lost photosynthetic ability.

The evolution of the apicomplexan plastid, known as the apicoplast, remains an interesting question. It is now fairly well established that the apicoplast has a red algal origin [5, 6], although occasionally some studies still argue for a green origin [7]. One such study was only based on a single gene, elongation factor Tu [8]. In addition, many of the studies that argue a green ancestry do not include dinoflagellates, thought to be the sister group to apicomplexa, and this has the potential to introduce artifacts into the analysis.

When nuclear phylogenies are constructed, it is clear that dinoflagellates are the apicomplexans' closest sister group to the exclusion of ciliates [9, 10], and that the alveolates together (apicomplexans, dinoflagellates, and ciliates) are related to the stramenopiles [11]. However, creating plastid phylogenies in these groups is sometimes quite difficult. The protein coding genes encoded by the dinoflagellate plastid genomes

are almost exclusively genes involved in photosynthesis, and the apicomplexan plastid genomes no longer possess any photosynthesis genes. Recently, an alveolate *Chromera velia* was discovered which appears to be the closest photosynthetic ancestor of the apicomplexa [12]. The plastid genome of *C. velia* was found to encode not only photosynthesis genes but also the genes encoded on the apicomplexan plastid genome as well, making it easier to directly compare dinoflagellate and apicomplexan plastids [12, 13]. Trees constructed from plastid DNA sequences which include *Chromera* support a common ancestry for the plastids in Apicomplexans, dinoflagellates, and stramenopiles and provide a much stronger basis for a red algal ancestry of the apicoplast [13].

We know that secondary endosymbiotic events must have occurred at least three times in the past, and likely more. Among the green algae, it is now fairly well accepted that the secondary plastids in the euglenids and the chlorarachniophytes were obtained by independent events [14]. In the red algae, the chromalveolate theory suggests that all the secondary red plastids derive from a single secondary endosymbiotic event [15], but is controversial due to the number of organisms within these clades that lack plastids, and a number of recent studies argue against this hypothesis [16-18]. In addition, tertiary endosymbiotic events are also possible, in which a eukaryotic host engulfs an alga containing a secondary chloroplast. Tertiary plastids have been observed in dinoflagellates, a phylum of the alveolates which are notorious for their phagotrophy [4].

2.1.1 Phylum Apicomplexa

The Apicomplexa are a phylum of protists which along with dinoflagellates and ciliates comprise the Alveolates. All of its members are parasitic, and many cause globally important diseases, not just in humans but in economically important livestock as well. The *Plasmodium* species cause malaria, the most devastating of these diseases, which remains one of the top three infectious disease killers worldwide and

still kills about a million people each year [19]. Many other apicomplexan parasites including *Toxoplasma*, *Cryptosporidium*, and *Babesia* have gained attention recently as agents causing emerging infectious disease in humans.

These parasites are obligate intracellular pathogens and employ active invasion to enter the host cell. This process utilizes a number of novel secretory organelles and is also aided by a specialized structure of cytoskeletal filaments at the apical end. This structure is termed the apical complex and is where the phylum derives its name. Apicomplexan parasites, with the exception of some early branching members such as Cryptosporidium species and the gregarines [20, 21], also possess a secondary plastid-derived organelle called the apicoplast. As a rare example of a non-photosynthetic plastid, it has garnered much attention and been the focus of many biological studies since its discovery.

2.1.2 *Toxoplasma gondii* as a model Apicomplexan

With the rise of resistance to existing drugs used in treatment of diseases caused by apicomplexan parasites, it has been imperative to study the cell biology of these organisms in order to discover novel drug targets. Of all the apicomplexan parasites, *T. gondii* has proven to be a facile model organism to study questions of apicomplexan cell biology. Unlike many other apicomplexan parasites including *Toxoplasma* can be continuously cultivated in cell culture *in vitro*. Additionally, it can infect any nucleated cell type, so it can be grown in fibroblast cell lines which are easy to maintain.

In addition to the ease of culture, there are several genetic tools available in *T. gondii* that provide researchers with more options for addressing questions of parasite cell biology. One of the most valuable of these is the ability to create conditional mutants. For some time now, tetracycline-inducible systems have been used in *T. gondii* to generate conditional gene knockouts for studying mutant phenotypes. The most common of these is a “tet-off” system under which gene silencing can be induced [22]. A

"tet-on" system has also been developed that allows induction of gene expression, although this is less widely used [23]. In *Plasmodium*, regular gene knockouts can be constructed, but until recently these could not be made for essential genes. As a result, the conditional knockouts possible in *Toxoplasma* become an important tool for studying genes essential for viability of apicomplexan parasites. However, new reports establish a system of inducible gene deletion in *Plasmodium* cultures using Cre or FLP recombinases [24, 25].

Another important difference in the genetics of *Toxoplasma* versus *Plasmodium* lies in the repair of double strand breaks. *Plasmodium* mostly utilizes homologous recombination, while *Toxoplasma* has high rates of non-homologous end joining. This can create problems in generating knockouts, as it becomes difficult to knock out the endogenous locus of the target gene, and typically requires screening of many clones before the targeted knockout is found. Recently, however, researchers developed a knockout of the Ku80 gene, a non-essential gene that plays a key role in non-homologous end joining [26]. Transfectants of the ΔKu80 strain display much higher rates of homologous recombination than non-homologous end-joining. In this strain it was possible to create a system of endogenously tagging genes at the 3' end with HA and YFP tags [27]. With this method the tagged version is expressed from the native promoter, eliminating the possibility of changes or defects due to overexpression. Recently, our laboratory also introduced the tetracycline transactivator into the ΔKu80 strain. This also allows for creation of inducible mutants by promoter replacement, a one-step procedure utilizing homologous recombination to replace the native promoter with the tetracycline-regulatable promoter [28].

For the reasons stated above, studies on *Toxoplasma* are important to identify drug targets for treatment not only of Toxoplasmosis but also for other diseases caused by Apicomplexan parasites. A great deal of proteins are conserved across this phylum

and discoveries made in *Toxoplasma* have the potential to be valuable in the research of diseases like malaria.

2.2 The Apicoplast

The apicomplexan plastid no longer performs photosynthesis but still retains many of the metabolic functions of a chloroplast, including type II fatty acid synthesis [29], isoprenoid biosynthesis [30] and heme synthesis [31, 32]. The fatty acid synthesis and isoprenoid biosynthesis pathways have been shown to be essential to *T. gondii* [33, 34]. In the *Plasmodium* blood stage, which does not carry out fatty acid synthesis, plastid loss can be rescued by addition of the isoprenoid intermediate, isopentenyl pyrophoshate (IPP) [35], highlighting isoprenoid biosynthesis as a major reason for retention of the apicoplast. Heme synthesis in these parasites is divided between the apicoplast and the mitochondrion [36]. Currently there is no evidence to suggest that the apicoplast portion of heme synthesis is essential, but parasites may also have the ability to synthesize heme using host factors [32].

Cryptosporidium, which lacks a plastid, must also carry out some of these processes but it does so in the cytosol by other mechanisms not found in plastid-bearing apicomplexans. Type I fatty acid synthesis is cytosolic in *C. parvum,* and it has lost the enzymes for type II fatty acid synthesis [37]. *T. gondii* also encodes the enzyme for type I fatty acid synthesis, but so far there is not sufficient evidence that this protein is expressed.

The apicoplast is surrounded by four membranes. The two innermost membranes are likely derived from the primary plastid, while the third membrane is likely derived from the primary host's plasma membrane [38]. The outermost membrane of the apicomplexan plastid is probably derived from the host ER but is not continuous with it as in the case of diatom plastids [39, 40]. In the course of evolutionary history, many

genes from the former endosymbiont have been transferred to the host nucleus, and as a result it is important for the host to develop new mechanisms of protein import to traverse all these membranes into the plastid. In the apicoplast, as well as in other chromalveolate plastids, it was found that the machinery that carries out ER-associated degradation (ERAD) of proteins has been duplicated and co-opted to serve in plastid import across the third innermost membrane [16, 41]. Rather than transporting misfolded proteins from the ER into the cytosol to be degraded, it transports proteins through the second outermost apicoplast membrane. Protein transport through the two innermost membranes proceeds via machinery that contains components homologous to the TIC and TOC complexes which form the translocons through chloroplast membranes in other systems [42, 43].

Since the apicoplast is not present in the mammalian host, it has attracted much attention as a potential target of therapeutic interventions. Indeed, many of the drugs that are lethal to the parasite have targets in the apicoplast. Fosmidomycin, which blocks the activity of DoxP reductoisomerase in the isoprene biosynthesis pathway, is also lethal to *Plasmodium* species, but surprisingly not to *Toxoplasma*. Recently work in our laboratory revealed that *Toxoplasma* is resistant because it lacks a transporter to shuttle the drug into the apicoplast and thus the drug does not have access to its target. When a bacterial transporter is expressed in *T. gondii* and targeted to the apicoplast, the parasites become susceptible [34]. Apicomplexans are also sensitive to several other prokaryotic translation inhibitors, but it is not always clear whether these target the apicoplast or the mitochondrion.

The apicoplast genome has also been verified as a viable drug target. Ciprofloxacin and novobiocin, for example, target the apicoplast-targeted DNA gyrase [44, 45], and clindamycin targets apicoplast translation [46]. Ciprofloxacin stabilizes the gyrase-DNA complex, allowing the enzyme to cleave the DNA but preventing it from

religating, creating double strand breaks [47]. Previous studies have shown that ciprofloxacin treatment of parasites results in a rapid decrease of apicoplast DNA [44]. Novobiocin also targets DNA gyrase but in the B subunit, blocking its ATPase activity which in turn prevents the enzyme from completing the supercoiling reaction [48]. Clindamycin is a prokaryotic translation inhibitor found to also target the apicoplast. When parasites resistant to clindamycin were isolated, they were found to contain a mutation in the apicoplast ribosomal RNA [46]. In light of these findings, details of DNA replication and inheritance of the apicoplast genome will likely provide interesting new targets for therapeutic intervention.

2.3 Plastid Genomes

2.3.1 Chloroplast genomes

Most chloroplast genomes are between 100 to 200 kb in length, though length will vary with lineage. In higher plants, a typical chloroplast genome is around 150 kb but in red algae the genome size is usually a bit higher, around 180 kb [49]. Chloroplast genomes were traditionally conceived as circles, though some reports indicate that there are circular and linear forms, some of which may be oligomeric, usually head-to-tail [50, 51]. More complex branched formations are also found, which may indicate different mechanisms used for replication [50].

Chloroplast genomes usually contain inverted repeat (IR) regions, which contain rRNA genes. In addition to ribosomal RNAs, chloroplast genomes also typically contain tRNAs, ribosomal proteins and RNA polymerase subunits B, C1, and C2 [49]. RNA Polymerase subunit A is also needed to form a fully functional RNA polymerase, but this subunit is often encoded in the nucleus and targeted to the chloroplast. Also shared by most chloroplast genomes are genes encoding most of the components of photosystem

I, photosystem II, cytochrome b6f, and an ATP synthase, all of which are necessary for photosynthesis [49].

To ensure proper inheritance, chloroplast genomes must also be segregated equally to daughter organelles during chloroplast division. However, much less is known about chloroplast DNA segregation compared to its bacterial counterparts. Observations suggest that this process in most cases is mediated by association with thylakoid membranes [52], possibly via the MFP1 protein [53]. However the specific role of the membrane in the segregation process remains unclear. It is also unknown whether in non-photosynthetic plastids that lack thylakoids if DNA segregation is still membrane associated or if a novel mechanism of segregation has evolved.

It is also possible that chloroplast DNA segregation is tied to organellar division. Many chloroplasts utilize cyanobacterial division machinery in order to complete fission. The apicomplexans, however, do not encode any of the typical FtsZ or Arc5 homologs that are common in plant and algal chloroplasts. To ensure persistence of the plastid, the cell must find a way to divide and segregate the organelle properly during cell division. In *Toxoplasma*, which divides by a specialized process called endodyogeny, the plastid divides by association with the centrosomes, as does the nucleus [54]. Recently, a novel dynamin-related protein, DrpA, was discovered in apicomplexan parasites and was shown to have a role in apicoplast fission. A dominant negative DrpA mutant caused aberrations in apicoplast morphology, with some parasites failing to receive plastids in the budding process, and some parasites having connected apicoplasts [55]. This unique mode of plastid division may also suggest a mode of plastid DNA segregation specific to apicomplexans.

2.3.2 The apicoplast genome

The apicoplast harbors its own genome on a single 35 kb molecule, which is much smaller than most plant and algal chloroplast genomes. When first discovered, it

was thought that this DNA was actually the mitochondrial genome [56]. The mitochondrial genome was identified later and was also found to be greatly reduced in size (6 kb) compared to all other eukaryotes [57]. The apicoplast genome is predicted to encode 63 genes [58, 59], most of which appear to be involved in its own expression: there are 32 genes for tRNAs, 17 genes for ribosomal proteins, and a gene for elongation factor Tu. Similar to other chloroplast genomes, the ribosomal RNA genes are found on an inverted repeat region [58]. It also encodes three subunits of RNA polymerase, rpoB, rpoC1, and rpoC2. The rpoC2 gene contains two in-frame stop codons, making it appear to be a pseudogene, but it is also possible these stop codons may be suppressed or read through. In addition to several unknown ORFs, there are also homologs of ClpC, involved in chloroplast protease machinery, and sufB, involved in iron-sulfur cluster formation. The apicoplast genome utilizes an alternative genetic code, in which the codon UGA codes for tryptophan rather than a stop [58]. Additionally the genome is quite AT-rich – 79% AT content in *Toxoplasma* and *Eimeria* plastid genomes, and 85% in *Plasmodium* [58].

The topology of the apicoplast DNA appears to be different in different species. In *Plasmodium*, it was reported that the apicoplast genome exists mostly as circular molecules [60], while in *Toxoplasma* it appears to be present mostly in linear tandem arrays, with only a few circular copies [61]. The copy number of the *Plasmodium* apicoplast DNA also appears to be lower than the copy number in *Toxoplasma*. Original estimates placed the copy number of apicoplast DNA in *T. gondii* at about 6 [44], and close to 1 in *Plasmodium* [8], but more recent studies suggest the copy number in *Toxoplasma* is actually as high as 25 [62]. This discrepancy in topology between the two organisms raises the possibility that there may be differences in their apicoplast DNA replication machinery, but most of the proteins studied to date with a putative function in apicoplast DNA replication appear to be conserved.

2.4 Plastid DNA Replication

2.4.1 Mechanisms of Plastid DNA Replication

DNA replication in eukaryotic nuclei and in bacteria usually proceeds via a theta mechanism. In this model, replication initiates at a single origin, and replication proceeds bidirectionally until replication forks meet. This happens at the terminus of the circular chromosome in bacteria such as *E. coli*. In nuclei, which contain linear chromosomes, multiple origins of replication are usually present and replication forks proceeding from neighboring origins will eventually meet. Since most chloroplasts seem to have circular genomes, their DNA replication mechanisms might be expected to resemble those found in cyanobacteria, but this does not necessarily seem to be the case.

Early electron microscopic analysis and later analysis by 2D gel electrophoresis of chloroplast DNA molecules suggested both D-loop and rolling circle replication mechanisms [63, 64]. In the D-loop mechanism, replication initiates at two different origins. The two different replication forks then migrate unidirectionally towards one another until the replication bubbles overlap, at which point replication proceeds in the same fashion as the well-known theta mechanism. In conventional theta replication, replication starts at a single origin and proceeds bidirectionally away from the origin. The difference here is that in D-loop replication, replication proceeds from each origin on only a single strand, until the replication bubbles overlap, at which point replication proceeds on both strands.

In rolling circle replication, a double-stranded circular template is nicked, at which point replication can proceed, on one strand, around the circular template [65]. When the polymerase complex has proceeded around the circle once, it may also continue for additional rounds, eventually forming a linear tandem array. The opposite strand must be made as well, but in the systems studied so far this happens in a second round of replication. This is in contrast to the classical theta mechanism, where lagging strand

synthesis occurs at the same time as leading strand synthesis, utilizing a dimeric polymerase. Often, DNA molecules that utilize the rolling circle mechanism feature an inverted repeat (IR) region. For replication to begin, this IR region is rearranged so that rather than the strands binding to each other, the repeats will bind to each other in a cis fashion, making two opposing stem-loop structures. When this structure has formed, a nick will be created in one of the stem loops, allowing a polymerase complex to come in and displace one strand as it begins to polymerize DNA [65].

Rolling circle replication has been well described for phages and plasmids, but is less well characterized in other systems. Phages and plasmids that participate in rolling circle replication often encode proteins that carry out specialized roles in this process such as nickases [65]. Although rolling circle replication is posited as a possible replication mode in chloroplasts, no homologs of these proteins have been reported in chloroplasts so far. However, the polymerases found in most chloroplasts are related to DNA polymerase I and have 3'-5' exonuclease activity. Most of these enzymes (but not all) also seem to lack 5'-3' exonuclease activity [66]. It is known that the Klenow fragment of DNA polymerase I, which possesses the domain with 3'-5' but not 5'-3' exonuclease activity, has the capacity to perform strand displacement as it polymerizes the DNA. If the chloroplast enzymes possess this property as well it may be more efficient at rolling circle replication than other polymerases would be, since it would be able to replicate a circular molecule multiple times with minimal assistance from helicase and single-stranded DNA binding proteins, forming multimeric DNA molecules. Additionally, if the rolling circle mechanism turns out to be important for chloroplast genome biology, this may offer an explanation of why a DNA polymerase I-like enzyme was co-opted as the replicative enzyme of the plastid DNA in most chloroplasts studied.

The presence of the IR region in most plastid genomes lends credibility to these models, as this region can both form stem loop structures as required in the rolling circle

model, and support multiple origins as required by the D-loop model. Additionally, studies which support a D-loop mode of chloroplast replication have also found the stem-loop region to be important for replication initiation [67]. Only a few photosynthetic organisms have been described whose plastid genomes do not contain an IR. One of these is *Euglena gracilis* [68]. Notably, electron micrographs of the *E. gracilis* plastid genome do not reveal the same DNA replication intermediates as observed in other plastid genomes, and seem to suggest only a bi-directional theta mode of replication for this plastid [69], lending further support to the importance of an IR region in the D-loop and rolling circle models.

D-loop and rolling circle mechanisms have also been posited for apicoplast DNA replication. In *Toxoplasma*, electron microscopic studies on isolated apicoplast DNA have found lariat structures consistent with a rolling circle mechanism, which could also account for the linear tandem array topology [61]. These structures are also seen in isolated *Plasmodium* apicoplast DNA, but theta forms are observed much more frequently. 2-D gel electrophoresis suggests that these theta forms arise from D-loop replication rather than conventional theta replication, as replication bubbles are only found on DNA fragments containing both origins, and not on fragments containing only a single origin [60]. The increased amount of theta forms compared with isolated *Toxoplasma* DNA may also account for the differences observed in apicoplast DNA topology, and explain why circular DNA seems to be predominant in *Plasmodium*.

Note however that a recombination-mediated replication mechanism has also been put forward, challenging the accepted ideas about the mechanism of chloroplast DNA replication [70, 71]. This model is derived from the observation that when chloroplast genomes are isolated they often seem to be in complex forms and not simple circles as predicted from a D-loop model. In the recombination-dependent replication (RDR) model, the origins may fire to begin the process but most of the DNA replication

events will be initiated by strand invasion of linear molecules. Recombination events would also allow the generation of multimeric genomic units, as the rolling circle model does. Circles can also be generated by intramolecular recombination events, and this could help explain why the proportion of circles in some preparations is quite low (3-4%) [72].

2.4.2 Plastid DNA Replication Factors

The exact mechanisms underlying chloroplast DNA replication are still not thoroughly understood, but some previous studies give us an idea of the players involved and possible replication models. The basic components of DNA replication in other systems are expected to be important in organelle DNA replication as well. Enzymes involved in DNA replication are present in both eukaryotic (nuclear) and prokaryotic systems, but generally the eukaryotic enzymes and prokaryotic enzymes belong to distinct protein families. Due to the nature of endosymbiosis, although it is possible for host cell factors to be co-opted into new organellar roles, analysis suggests that basic housekeeping machinery often comes from the bacterial ancestor. In cyanobacteria, the ancestors of modern chloroplasts, DNA replication proceeds much as it does in other bacteria. Bacterial DNA is normally packaged by a set of Histone-like proteins including IHF, HU, H-NS, and Fis, and when replication is initiated, DNA helicase unwinds the DNA duplex. Meanwhile DNA gyrase relaxes positive supercoils generated ahead of the helicase activity. Single-stranded DNA binding protein (SSB) coats single stranded regions of DNA, allowing them to be stable and stay unwound, and DNA primase lays down RNA primers so that DNA replication may begin. The majority of chromosomal DNA replication is performed by DNA Polymerase (Pol) III, while DNA Pol I and II mostly function in DNA repair. Chromosomal DNA is replicated via a theta mechanism, in which a replication bubble is formed and one subunit of the dimeric Pol III

replicates the leading strand. Meanwhile, the other subunit replicates the lagging strand in shorter fragments generating Okazaki fragments, which are later ligated together.

In chloroplasts, however, the replicative enzyme appears to be related to DNA Pol I and not III. Initial biochemical experiments on polymerases derived from pea chloroplasts suggested that these Pol I-like chloroplast DNA polymerases bear more similarity to mitochondrial DNA Pol γ than to cyanobacterial or alpha-proteobacterial polymerases [73, 74]. This seemed to indicate that the replication machinery for chloroplast DNA evolved from host factors developed for the mitochondrion rather than from the endosymbiont. However, most of these early experiments were performed without phylogenetic analysis. After the plant genomes had been sequenced, no obvious homologs of DNA Pol γ could be found, even in plant mitochondria. Presently the distribution of DNA polymerase gamma seems to be limited to opisthokonts (animals and fungi) [75].

More recently, phylogenetic analyses have been performed on DNA polymerases targeted to organelles in plants and algae and compared to bacterial enzymes. The results seem to suggest a new clade of DNA polymerases, called plant organellar polymerases or POPs [66]. Many of these seem to be dually targeted to chloroplasts and mitochondria. Phylogenetic analysis seems to group the POPs from plants and green algae together with red algae. They also appear to be Pol I-like, but appear more closely related to the eukaryotic polymerases ν and θ, which are involved in translesion DNA synthesis in metazoa, to the exclusion of Pol I enzymes from alpha-proteobacteria and cyanobacteria. Again, this appears to indicate that the polymerase is host-derived rather than endosymbiont-derived. A previous study had placed the *Arabidopsis* POPs more closely related to bacterial than metazoan enzymes [76], but these results may be skewed by sparser sampling and a lack of algal sequences in the tree. Additionally in the more recent study, the presence of a *Dictyostelium* homolog in the POP clade [66],

which is phylogenetically distinct from plants and algae, could suggest that the POPs emerged after the acquisition of mitochondria and before the primary endosymbiosis of cyanobacteria. Apicomplexan parasites encode a plastid-targeted polymerase, but phylogenies suggest that it is more closely related to a polymerase from thermophilic bacteria than to other POPs [66, 77]. This might represent a horizontal gene transfer event.

In addition to polymerases, several other DNA replication factors have been found in chloroplasts, including primases, helicases, gyrases, single-stranded DNA binding protein, and HU. DNA primase and helicase activity has been isolated from chloroplasts [78-80], but most of the studies done on these proteins have been functional and biochemical, and few studies have endeavored to trace the ancestry of these proteins. However in 2005 a gene from *Plasmodium falciparum* was described that encodes primase, helicase, and polymerase domains, probably resulting from a gene fusion in the ancestor of Apicomplexa [77]. All domains showed catalytic activity *in vitro*, and the protein was shown to localize to the apicoplast. Phylogenetic analysis of each domain suggests that the helicase domain seems to cluster with Twinkle helicases (mitochondrial) from metazoans, while the primase domain seems to cluster with the T7-like primases, including one from *Arabidopsis*. Conclusions about the ancestry of these components will likely require more exhaustive phylogenetic studies which include more chloroplast and mitochondrial enzymes.

Although the enzymes mentioned above represent important groundwork in determining how plastid DNA is replicated, they are likely not enough to form a working replication fork. SSB is required for DNA replication in bacteria and also present in mitochondria, but hasn't been well described in chloroplasts. The only recent report of an SSB homolog in plastids came from *Plasmodium falciparum*. Like bacterial SSB, this homolog possesses the ability to bind single-stranded DNA, but it was unable to

complement *E. coli* SSB mutants, raising the possibility that there might be slight differences in function [81]. Although SSB seems to be important for DNA replication in bacteria, it is not clear whether this protein also exists in plastids outside of Apicomplexa.

DNA gyrase has also been found in plant and algal chloroplasts [82-84]. Gyrases are type II topoisomerases which work in tetrameric complexes consisting of 2 A and 2 B subunits. The A subunits perform the cleavage and ligation activity, while the B subunit is responsible for the ATPase activity which drives the overall reaction. Gyrase activity is typically essential, highlighted by the efficacy of antibiotics that target gyrase complexes. It serves not only to disentangle concatenenes, but also to regulate supercoiling in a DNA molecule. This is important during DNA replication and transcription, in which activity of DNA helicase generates positive supercoils upstream which need to be relaxed. Gyrase activity is also important for mitochondrial genomes, and in many plants the gyrase proteins are dually targeted to plastids and mitochondria [82]. In some chloroplasts, gyrases seem to be involved in nucleoid segregation during chloroplast division [83, 84]. This may indicate either that an optimal level of supercoiling is necessary for proper DNA segregation, or that catenenes can develop during division which must be resolved.

Both subunits of DNA gyrase have been identified in Apicomplexa and shown to target to the apicoplast [85]. In *Plasmodium*, the enzyme was shown to be sensitive to novobiocin *in vitro*, and novobiocin treatment resulted in death of the parasites which suggests that gyrase activity may be essential to the apicoplast. The *Plasmodium* GyrB subunit was found to have a 45-amino acid insertion that was shown to be important for its ATPase activity, but this insertion seems to be unique to *Plasmodium* and is not found in *Toxoplasma* [86].

The HU histone-like protein is also important for regulation of DNA topology and supercoiling, and is the only bacterial histone-like protein that has known homologs in chloroplasts. In bacteria it condenses the nucleoid and has the capacity to bend DNA in such a way that it can stabilize negative supercoils. HU also seems to play a role in initiation of replication [87, 88], transcription [89, 90], and DNA repair [91, 92]. Its role in supercoiling seems to be important, as DNA gyrase mutants have been isolated that can suppress HU mutants [93, 94]. This also highlights the importance of negative supercoiling in genome biology. Until recently the only chloroplasts known to contain HU homologs were in the red lineage [95, 96], but a homolog has now also been found in the green alga *Chlamydomonas reinhardtii*, where it appears to play an important role in DNA replication and stability [97]. In chloroplasts lacking an HU homolog, it is likely that other factors compact the chloroplast genome, as levels of DNA condensation and topology influence gene regulation and the interaction of DNA with other binding proteins. Plant chloroplasts also contain proteins that initially appeared to bear some structural similarity to HU [98], but sequence analysis has since revealed them not to be HU homologs and rather suggests a case of convergent evolution.

An apicoplast-targeted HU protein has also recently been identified in *Plasmodium* that is conserved in the other plastid-bearing apicomplexans, which has the ability to condense apicoplast DNA. However, these studies have not revealed whether it was essential for the parasite or if it might serve roles outside of condensation [99, 100]. Unlike the bacterial HU, it appears to lack DNA bending ability, but this is likely based on a change in sequence that is unique to the *Plasmodium* homolog and not present in *Toxoplasma*.

Chloroplasts might also be expected to perform DNA repair, but to date this has not been thoroughly examined. Recently, one of the POPs found in *A. thaliana* has been implicated in DNA repair [76]. Additionally, many chloroplasts contain homologs of RecA

[101, 102], which is important for recombination in bacteria. RecA binds single-stranded DNA and facilitates strand invasion into a homologous DNA duplex to initiate homologous recombination. *Arabidopsis* encodes several RecA homologs, and some studies suggest that one of these is important for plastid DNA stability [101] while another might be essential to the plant [102]. This generally isn't expected for proteins involved in DNA repair and might point to a role in replication in addition to or instead of repair. This particular homolog of RecA is dual-targeted to plastids and mitochondria, again suggesting that these two organelles may share some common replication mechanisms.

No RecA homologs have been discovered in the apicoplast, but *T. gondii* and *N. caninum* possess a homolog of the recombinational helicase RecG targeted to the apicoplast [Sheiner, L., unpublished data]. Interestingly, it appears to be absent from the genomes of the other apicomplexa, including the other coccidia like *Eimeria*. RecG in bacteria is usually involved in DNA repair and recombination, and not replication [103]. In the apicoplast it may also function in DNA repair, but another possibility is that it contributes to a recombination-dependent replication mechanism.

In summary the following bacterial-like DNA replication proteins are found in the apicoplast: a primase-helicase-polymerase [77, 104, 105], DNA gyrase [45, 85], SSB [81], and HU [99]. This provides a good start for understanding how DNA replication proceeds in the apicoplast. While colleagues have focused on studying these enzymes *in vitro* using recombinant protein, we have focused on examining their function using genetic analysis in *Toxoplasma*. Explicitly defining whether these proteins are essential will help us to distinguish whether these proteins are functioning as replicative enzymes or in repair and recombination, and will also help in identifying those enzymes that may be suitable drug targets.

The apicoplast of apicomplexans such as *Toxoplasma gondii* has a fascinating evolutionary history. While bacterial and nuclear DNA replication has been very well characterized, there is still much to be discovered about the processes underlying DNA replication and repair in plastids. Understanding the process of apicoplast DNA replication and characterization of the players involved will be an important endeavor not only to shed more light on the complex evolutionary relationships of Apicomplexa to other protist and algal groups but also for validation of potential drug targets.

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CHAPTER 3

THE HU PROTEIN IS IMPORTANT FOR APICOPLAST GENOME MAINTENANCE AND INHERITANCE IN *TOXOPLASMA GONDII*[1]

[1] Reiff, S.B., Vaishnava, S., and Striepen, B. The HU protein is important for apicoplast genome maintenance and inheritance in *Toxoplasma gondii*. Submitted to *Eukaryotic Cell*, 1/27/12 (in revision).

3.1 Abstract

The apicoplast, a chloroplast-like organelle, is an essential cellular component of most apicomplexan parasites including *Plasmodium* and *Toxoplasma*. The apicoplast maintains its own genome, a 35 kb DNA molecule that largely encodes proteins required for organellar transcription and translation. Interference with apicoplast genome maintenance and function is a validated target for drug therapy for malaria and toxoplasmosis. However, the many proteins required for genome maintenance and inheritance remain largely unstudied. Here we genetically characterize a nuclear encoded homolog to the bacterial HU protein in *T. gondii*. In bacteria HU is a DNA-binding structural protein with fundamental roles in transcription, replication initiation, and DNA repair. Immunofluorescence assays reveal that in *T. gondii* this protein localizes to the apicoplast. We have found that the HU protein from *Toxoplasma* can successfully complement bacterial HU mutants, supporting a similar function. We were able to construct a genetic knockout of HU in *Toxoplasma*. This HU mutant is barely viable and exhibits significant growth retardation. Upon further analysis of the mutant phenotype, we find that this mutant has a dramatically reduced apicoplast genome copy number, and furthermore suffers defects in the segregation of the apicoplast organelle. Our findings not only show that the HU protein is important for *Toxoplasma* cell biology, but also demonstrate the importance of the apicoplast genome in the biogenesis of the organelle.

3.2 Introduction

The phylum Apicomplexa consists of single-celled eukaryotic parasites which infect humans and many other animals. Infection with Apicomplexa causes a variety of diseases that have significant global health and economic impact. Among these parasites, perhaps the best known are *Plasmodium* species, the causative agents of

malaria. Another prominent apicomplexan is *Toxoplasma gondii*. While most people do not suffer symptoms upon infection, in immunosuppressed individuals *T. gondii* can cause severe encephalitis, an AIDS-defining opportunistic infection. *T. gondii* also causes congenital disease when a woman becomes infected for the first time during pregnancy.

Most Apicomplexans harbor a remnant chloroplast called the apicoplast. This plastid-like organelle, although no longer photosynthetic, still houses important biosynthetic pathways including type II fatty acid synthesis (FASII) [1], heme synthesis [2], and isoprenoid biosynthesis [3]. Apicoplast pathways were shown to be essential for cell viability in *T. gondii* and *Plasmodium* [1, 3]. Because this organelle is unique to these parasites and not found in the human host, apicoplast proteins and structures are considered excellent candidates as parasite specific drug targets.

Like other chloroplasts, the apicoplast has an evolutionary history that can be traced back to cyanobacteria. Chloroplasts evolved when a eukaryotic cell engulfed a cyanobacterium, which over time underwent massive gene transfer to the host nucleus. In this process of primary endosymbiosis the prokaryotic symbiont transformed into a subcellular organelle. Primary plastids are present in glaucophytes, plants, green algae, and red algae. Apicoplasts are derived in a secondary endosymbiotic event in which a second eukaryotic host engulfed a red alga and, as in the case of the cyanobacterial symbiont, over time the algal symbiont was reduced to a plastid organelle through gene transfer to the host nucleus.

Consistent with secondary endosymbiosis, the apicoplast is surrounded by four membranes and contains its own 35 kb genome that shares similarity with chloroplast genomes from red algae. Interestingly, while apicomplexan nuclear genomes display a surprising lack of synteny among more distant genera of the phylum [4], the apicoplast genome is extremely well conserved. The gene number and order is almost identical in

several species studied so far including *P. falciparum* [5], *T. gondii*, and *Eimeria tenella* [6]. The copy number of the genome differs between species, but the *T. gondii* apicoplast appears to contain around 25 copies by recent estimates [7]. Most of the proteins active in apicoplast metabolism, such as the enzymes in the FASII pathway, are encoded in the nucleus. The genes left behind in the apicoplast genome mostly function in apicoplast gene expression [5, 6]. However, it also encodes a small number of proteins not involved in gene expression, namely clpC and sufB. ClpC is a subunit of a protease known in other chloroplasts which may be involved in protein degradation or turnover, while sufB is involved in assembly of iron-sulfur clusters.

Because loss of the apicoplast genome has been shown to be lethal to the parasite [8], proteins involved in plastid DNA replication and stability are attractive as potential drug targets. Relatively few proteins involved in these processes in Apicomplexa have been studied so far. Both A and B subunits of DNA gyrase are known to be targeted to the apicoplast [9]. Their function is likely essential since the parasites are sensitive to drugs that target gyrase such as novobiocin and ciprofloxacin [8, 10]. Recently a gene has also been identified in *Plasmodium falciparum* [11, 12] and *Toxoplasma gondii* [13] which encodes an apicoplast-targeted multidomain protein possessing primase, helicase, and polymerase domains. The polymerase domain is related to the bacterial DNA Polymerase (Pol) I enzyme. Both Apicomplexan orthologs have been shown to possess polymerase activity and have been suggested to be the replicative enzyme of the apicoplast genome, but whether it is essential for the parasite growth has not yet been demonstrated. In *T. gondii* there is currently no other good candidate for the replicative complex of the apicoplast DNA, but in *Plasmodium* there appears to be another homolog of DNA Pol I which may have an organellar localization [14]. A homolog of SSB (single stranded DNA binding protein) has also been identified in Apicomplexa which localizes to the apicoplast and binds single stranded DNA [15].

We were interested in identifying DNA-binding proteins in *T. gondii* that promote apicoplast genome stability. Since plastids are the product of an endosymbiosis of bacteria, we searched the *Toxoplasma* genome database using several proteins known to associate with DNA in *E. coli*. One of the proteins that yielded a homolog was HU. Nuclear encoded in *Toxoplasma*, HU is a histone-like protein found in bacteria as well as red algal plastids [16], and more recently in the plastid of the green alga *Chlamydomonas reinhardtii* [17]. In bacteria it localizes to the nucleoid [18, 19] and exists *in vivo* as a 20 kDa dimer. A role for HU has been demonstrated in DNA compaction and stabilization of supercoils [20], as well as in transcription [21, 22], initiation of replication [23, 24], and DNA repair [25, 26]. In *E. coli*, HU mutants exhibit retarded growth [27] and produce a filamentation phenotype.

An HU homolog has also recently been described in *Plasmodium falciparum* that associates with the apicoplast and has the ability to bind and condense DNA [28]. Here we investigated the role of HU in *Toxoplasma*. We find that HU serves an important role in maintaining optimal levels of the plastid genome. In the absence of HU, apicoplast genome loss causes considerable defects in apicoplast biogenesis and a dramatic loss of fitness in the parasite.

3.3 Materials and Methods

Protein purification and Antibody Production

Part of the C-terminal domain of the HU gene encoding amino acids 120-209 was amplified from *T. gondii* RH cDNA using primers I (forward, BamHI site) and II (reverse, XbaI site) (Table 3.S1), and cloned into vector pMAL-2E (New England Biolabs) to create an N-terminal Maltose Binding Protein fusion. Recombinant protein was produced in *E. coli* and purified by affinity chromatography on crosslinked amylose resin [3]. Polyclonal antisera were raised in rabbits (Cocalico Biologicals). HU antibodies

were affinity purified from the antisera against purified HU crosslinked to activated CNBr Sepharose 4B (Sigma) as described previously [29].

***E. coli* complementation assays**

The conserved region of the HU gene, encoding amino acids 120-209, was amplified using primers I and II (Table 3.S1) and cloned into pCR2.1-TOPO (Invitrogen). HupA- *E. coli* (a kind gift from Dr. Rouviere-Yaniv, Institut de Biologie Physico-Chemique, Paris) [30] were transformed with the resulting plasmid as well as with empty vector. Transformed colonies were grown in LB medium with 50 µg/ml ampicillin until they reached an optical density of 0.4 at 600 nm, at which point IPTG was added to a final concentration of 2 mM. After IPTG incubation, 100 µl of 10^6 *E. coli*/ml were allowed to settle for 15 minutes on coverslips coated with poly-L-lysine. Cells were fixed with 3.5% paraformaldehyde in PBS and permeablized with 2.5% Triton X-100 in PBS, then stained with 2 µg/ml DAPI in PBS for 10 minutes. Cells were then imaged by fluorescence and phase contrast microscopy on a DM IRBE inverted epifluorescence microscope (Leica) as previously described [31]. The area of DAPI staining was measured in individual bacterial nucleoids. Nucleoids were defined as objects by generating binary image masks for each image using a thresholding function. Student's t-test was used to compare mean nucleoid areas.

Plasmid and cosmid construction

To create constructs for HU overexpression, the HU gene was amplified by PCR from *T. gondii* RH cDNA using primer III (forward, BglII site) and either primer IV (reverse, AvrII site), or primer V (reverse, stop codon and AvrII site) (Table 3.S1). The resulting PCR products were then cloned into the pCR-Blunt-II-TOPO vector (Invitrogen) following the manufacturer's instructions to create pTOPO-HU and pTOPO-HUstop. These plasmids, along with the pTubYFPYFP-sagCAT vector [32], were digested with

BglII and AvrII and the HU inserts were subsequently ligated into the cut YFP vector to create pTubHUYFP-sagCAT and pTubHUstopYFP-sagCAT.

To generate a construct to target and delete the TgHU gene, we modified cosmid clone TOXPJ14, which contains the TgHU locus. Briefly, primers VI and VII (Table 3.S1) were used to amplify a sequence containing the chloramphenicol acetyl transferase (CAT) cassette along with a gentamycin resistance marker from the plasmid pH3CG by PCR. Cosmid recombineering was employed as described previously [33] to generate the TOXPJ14-HUKO construct.

Parasite culture and genetic manipulation

For continuous culture, *T. gondii* tachyzoites were grown in hTERT human fibroblasts in Dulbecco's modified Eagle medium supplemented with 1% fetal bovine serum at 37°C and 5% CO_2. The parental strain of the knockout parasites, which was also used as a control in subsequent experiments, was the ΔKu80/TATi strain [34-36], a derivative of RH strain *T. gondii*. ΔHU parasites were grown in the same conditions with 3% serum, and 5 ml of a 10 ml culture was passed to a new flask at about 50% lysis which was achieved after 2 weeks in a T-25 flask.

For transfection, 10^7 parasites were filtered, pelleted, and resuspended in cytomix buffer [35]. 35 μg plasmid or cosmid DNA is electroporated into parasites (1.5 kV, 25 □F, 25 Ω) using a BTX ECM 630 electronic cell manipulator. Parasites were transferred into a fresh human foreskin fibroblast culture and allowed to grow overnight before beginning drug selection. Selection of parasites for integration of TOXPJ14-HUKO was performed with 68 ng/ml chloramphenicol. Parasites were passaged twice and then cloned in 96 well plates by limiting dilution [37].

Immunofluorescence assays and phenotype counting

Immunofluorescence assays were performed as previously described [38] in infected human foreskin fibroblasts. The rabbit anti-HU antibody was used at 1:2000,

rabbit anti-Cpn60 was used at 1:3000 [39], rabbit anti-centrin (a kind gift from Dr. Cheeseman, Whitehead Institute, Boston) was used at 1:500, rabbit anti-IMC1 was used at 1:1000 (a kind gift from Dr. Beckers, University of North Carolina, Chapel Hill), mouse anti-CAT (Abcam) was used at 1:3000, and mouse anti-PDH-E2 (a kind gift from Dr. Bohne, University of Göttingen, Göttingen) was used at 1:500. Mouse anti-GFP was used at 1:200 (Roche). Alexafluor 488- and 546-conjugated goat anti-rabbit and goat anti-rat secondary antibodies were used at 1:200 (Invitrogen). Alexafluor 488-conjugated goat anti-mouse secondary antibodies were used at 1:300 (Invitrogen). Microscopy was performed as previously described [33].

To quantify HU-YFP staining in transient transfections with pTubHUYFP-sagCAT, parasites were identified by IMC1 staining and HU-YFP was visualized in fixed cells with anti-GFP staining. Apicoplasts were visualized by FNR-RFP fluorescence. For each vacuole counted, the total number of parasites in the vacuole, total number of parasites containing apicoplasts, and total number of parasites expressing HU-YFP were recorded. Percentages of parasites displaying normal phenotypes were calculated and statistical significance was assessed using Fisher's exact test.

To quantify the number of apicoplasts per vacuole, 100 4-cell vacuoles were counted and numbers of apicoplasts per vacuole were recorded. Apicoplasts were visualized by anti-Cpn60 staining. In the comparison between ΔKu80/TATi and ΔHU, individual parasites within vacuoles were discerned by anti-IMC1 staining of the inner membrane complex. To quantify vacuole size, 100 vacuoles containing 2 or more parasites were counted and number of parasites per vacuole was recorded. The mean number of apicoplasts per 4 cell vacuole was also calculated and the statistical significance of differences was evaluated using Student's t-test. To quantify apicoplast-centrosome association, 100 duplicated centrosomes (stained with anti-centrin) were counted and scored on whether they had localizations adjacent to part of an apicoplast

(stained with anti-PDH-E2) or not. Statistical significance was assessed with Fisher's exact test.

For drug treatments, ΔKu80/TATi parasites were used to infect host cells on coverslips and treated with either 10 μM Ciprofloxacin or 40 ng/ml Clindamycin. Coverslips were fixed at various time points (24, 48, or 72 hours for apicoplast counting and 8, 16, 24, 32, 40, or 48 hours for centrosomes-apicoplast association scoring) and immunofluorescence assays and phenotype counting were performed as described above.

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization assays were performed as described [40]. Briefly, parasite-infected host cells grown on coverslips were fixed and permeablized as described above. Four portions of the apicoplast genome collectively representing the inverted rRNA repeat region were amplified using primer pairs VIII and IX, X and XI, XII and XIII, and XIV and XV (Table 3.S1). These amplicons were purified and labeled with digoxygenin-dUTP by nick translation (Roche) before hybridization to cells. To detect apicoplast DNA, cells were incubated with sheep anti-digoxygenin Fab fragments (Boehringer Mannheim) at 1:3000, followed by incubation with donkey anti-sheep antibodies conjugated to AlexaFluor 488 at 1:400 (Molecular Probes).

Southern hybridization

To confirm the replacement of the native HU locus, a 589-bp probe complementary to a region upstream of the HU gene was amplified by PCR from RH genomic DNA using the primers XVI and XVII (Table 3.S1). The PCR product was gel purified and radiolabelled with ^{32}P-dCTP in a random priming reaction (Invitrogen). 2 μg genomic DNA from parental and HU knockout parasites was digested with EcoRV and AvrII for the Southern hybridization.

To compare DNA levels between parental and ΔHU parasites, DNA fragments were amplified by PCR from RH genomic DNA from both the nuclear UPRT locus and the apicoplast genome as described previously [4]. The PCR products were then radiolabelled as above, and 2 µg genomic DNA from parental and ΔHU parasites were digested with HindIII. After hybridization, Southern blots were exposed to film for 48 hours. Densitometry with ImageJ (http://rsbweb.nih.gov/ij/) was used to quantify band intensity from the autoradiograph on film and apicoplast DNA levels were normalized to nuclear DNA levels.

Plaque Assays

Confluent hTERT T-25 cultures were infected with 1000 parasites from either the ΔHU strain or the ΔKu80/TATi parental strain. After incubation for the indicated time flasks were fixed with ethanol and stained with crystal violet as previously described [37].

Quantitative PCR

DNA was extracted from parasites upon lysis of T-25 cultures and purified via phenol/chloroform extraction with subsequent ethanol precipitation. Quantitative PCR (qPCR) was performed using primers and PCR programs as described [41]. Amplicons of the UPRT locus and the apicoplast genome were amplified using GoTaq polymerase (Promega) as described [41], and then cloned into the pCR2.1-TOPO vector (Invitrogen) to create standards. A standard curve was created for each qPCR reaction based on serial dilutions of these plasmids (10^2 copies – 10^7 copies). Amplifications were performed in triplicate on a Bio-Rad iQ5 Real-Time PCR Detection System. Each 20 µl reaction was supplied with iQ SYBR Green Supermix (Biorad) and also contained primers at 0.5 µM and 50 ng template DNA. Results were analyzed using Biorad iQ5 software. The average number of apicoplast genomes was divided by average number of nuclear genomes to calculate the apicoplast copy number per cell.

3.4 Results

Toxoplasma HU complements the *E. coli* hupA- mutant

We were interested in identifying *Toxoplasma* proteins that serve a role in DNA replication and topology of the apicoplast genome. We performed BLAST searches against the *Toxoplasma gondii* genome database (http://www.toxodb.org/) using sequences of DNA replication proteins and histone-like proteins from *E. coli* as queries. One of the proteins that we identified in these searches was TgME49_027970, a homolog of the *E. coli* HU protein, which we call TgHU. This protein was predicted to contain a signal peptide based on analysis with SignalP (http://www.cbs.dtu.dk/services/SignalP/), and TargetP also predicted a plastid-targeting transit peptide (http://www.cbs.dtu.dk/services/TargetP/). We amplified and subcloned the cDNA of TgHU and confirmed the gene model as currently annotated in the database. We constructed alignments of the predicted protein to HU proteins from bacteria, and these reveal that the putative *T. gondii* protein possesses a region of 90 amino acids which is highly conserved when compared to its bacterial orthologs, in addition to unique N- and C-terminal extensions (Figure 3.1A) [28].

The primary role of the bacterial HU is to condense the nucleoid. We wanted to investigate whether the *T. gondii* HU similarly has the ability to condense DNA. In *E. coli,* mutant bacteria lacking the hupA gene encoding the beta subunit of HU exhibit slowed growth and a filamentation phenotype characterized by large and poorly segregated nucleoids. We transformed hupA- mutant bacteria with a plasmid encoding the conserved region of the *T. gondii* HU protein (amino acids 120-209) or a control plasmid. Mutant, transgenic, and control strains were then cultured and imaged. Nucleoids were visualized by DAPI stain (Figure 3.1B) and bacterial cells were imaged using phase contrast illumination. We then compared the areas of DAPI fluorescence in individual bacteria (Figure 3.1C). Wildtype *E. coli* have a coccoid rod shape, and their nucleoids,

typically also rod shaped, reside in the middle of the cell with a mean area of 0.60 μm^2. HupA- bacteria, however, exhibited filamentous DAPI staining, with a mean area of 1.92 μm^2. This is a highly significant difference from the wildtype nucleoids ($p < 0.001$, Student's t test). When control (empty vector) DNA was transformed into these mutants, the DAPI staining pattern remained filamentous and again the difference from wildtype nucleoid size is statistically significant ($p < 0.001$, Student's t test). In contrast, upon transformation with the TgHU expression vector, the DAPI staining appeared more compact and no longer filamentous with a mean area of 0.53 μm^2, indicating a reversion in phenotype back to the wild type state. When comparing this value to the wildtype nucleoid size, the difference is no longer statistically significant ($p = 0.2723$, Student's t test). This suggests that TgHU has the ability to compact bacterial DNA and restore a normal cell cycle, implicating a conserved function in *Toxoplasma*.

The *Toxoplasma* HU protein is targeted to the apicoplast

In silico analysis predicted an apicoplast localization for HU. To test this, we generated antibodies. The conserved region of the protein, amino acids 120-209, was amplified from *T. gondii* cDNA. This amplicon was cloned into the pMAL-2e vector, introducing an N-terminal maltose binding protein fusion, and expressed in *E. coli*. Recombinant protein was purified and antibodies were raised in rabbits. After western blotting of parasite lysates, the HU anti-serum recognizes a band of about 16 kDa, consistent with the predicted size of the mature protein after the signal and transit peptides have been cleaved off (Figure 3.2A). Immunofluorescence assays using the purified anti-HU antibody show co-localization of the HU protein to the E2 subunit of pyruvate dehydrogenase (PDH-E2), an apicoplast marker, and to the DAPI stain of the apicoplast genome (Figure 3.2B-F).

Overexpression of an HU-YFP transgene results in unequal distribution and apicoplast loss

To visualize the protein in living cells, we also attempted to tag TgHU with yellow fluorescent protein (YFP) by C-terminal translational fusion using a construct that expresses the transgene from the strong *Toxoplasma* tubulin promoter. We transfected this construct into a parasite strain expressing red fluorescent protein (RFP) fused to the signal sequence of the apicoplast protein ferredoxin NADP+ reductase (FNR) [42], but stable lines could not be obtained after drug selection. Fluorescence microscopy at 48 hours post-transfection revealed robust transient expression of the transgene and localization of HU-YFP to the apicoplast, marked by FNR-RFP (Figure 3.3A). However, the distribution appeared to be uneven – in some vacuoles expressing HU-YFP, we observed apicoplasts without any HU-YFP labeling (Figure 3.3B-C). We quantified this distribution (Figure 3.3G) and found that in vacuoles where HU-YFP expression was detected, 27% of the parasites were lacking HU-YFP staining. Additionally, vacuoles expressing HU-YFP had an increased incidence of apicoplast loss (as indicated by loss of FNR-RFP labeling) in one or more of the parasites. 11% of transfected parasites were missing apicoplast staining, compared with less than 1% in untransfected parasites, representing a statistically significant difference ($p = 0.0007$, Fisher's exact test). These observations suggest the HU-YFP overexpression resulted first in unequal HU-YFP localization and then in apicoplast loss.

We noted that in transgenic parasites that display uneven distribution of HU-YFP, the DAPI staining of the apicoplast genome appeared to be similarly unequal and correlated with the HU-YFP localization: a larger more intense spot in some parasites and apparent absence in others (Figure 3.3D). Unambiguous detection of the apicoplast genome by DAPI staining is not possible in all parasite cells. When the apicoplast and nucleus are close (e.g. during mitosis) the much more pronounced DAPI signal for the

nuclear DNA makes it difficult to appreciate the apicoplast genome. We therefore performed fluorescent *in situ* hybridization to specifically examine the distribution of the plastid genome in a pool of parasites transfected with HU-YFP. While in untransfected parasites most vacuoles showed a fluorescent signal indicating a plastid genome for each parasite, transfected parasites frequently showed vacuoles with only one or few apicoplast nucleoids (Figure 3.3E-F). Overall this suggests that overexpression of HU-YFP causes not only improper segregation of HU-YFP but missegregation of the entire HU-genome apicoplast nucleoid. Loss of apicoplast genome in the progeny explains why stable transgenics were not obtained.

Transfection with similar plasmids containing the HU cDNA under control of the tubulin promoter with or without a small C-terminal myc epitope tag in place of the YFP tag also failed to generate stable lines, suggesting that overexpression of the gene may be detrimental. Selection of parasites transfected with a fusion construct utilizing an HA tag and under control of the T7S4 regulatable promoter [34], which is weaker than the tubulin promoter, resulted in a stable line characterized by mistargeting of the transgene (data not shown). Together this suggests that the level of HU protein per cell is very important and that TgHU has a very narrow expression range in which it can carry out its function optimally.

Loss of the *Toxoplasma* HU severely impairs growth

We wanted to isolate a mutant lacking HU to study its function and the consequences of its loss. To this end, we constructed a genetic knockout in *T. gondii*, utilizing a cosmid recombineering strategy which results in high frequency of gene targeting [33]. Briefly, a cosmid clone containing the full HU locus was selected, and the HU gene was replaced with the gene encoding the chloramphenicol acetyl transferase (CAT) drug marker. The resulting knockout cosmid was then transfected into ΔKu80/TATi parasites [36] which favor homologous recombination of the transfected

DNA [35, 43]. After selection of parasites with chloramphenicol, clones were isolated by limiting dilution. Clones were tested for gene replacement by Southern blot analysis using a probe to the 5' non-coding region of the HU locus. Note that the 3.3 kb EcoRV/AvrII wild type fragment is lost in the pictured clone and replaced by a 1.3 kb fragment predicted for the targeted locus (Figure 3.4A-B).

We also tested for the loss of HU protein expression in the mutant by immunofluorescence assay, staining with antibodies to HU and CAT (the gene for this drug resistance protein replaces the HU gene in the mutant). Parental strain parasites showed clear HU staining and no anti-CAT fluorescence, while ΔHU mutant parasites exhibited strong anti-CAT fluorescence but lacked HU staining (Figure 3.4C). This demonstrated that the ΔHU parasites robustly expressed the drug selectable marker and no longer expressed the HU gene consistent with ablation of the locus.

While the ability to generate a direct HU knockout suggests that it is not an absolute requirement for parasite survival, we noticed extremely slow growth in the mutant compared to the parental strain. To examine the growth phenotype more closely we performed a plaque assay directly comparing plaque formation of the HU knockout parasites to the parental strain (Figure 3.4D). In this experiment, parasites were grown in a monolayer of host cells for several days. During this time, individual parasites will invade cells in the monolayer and go through repeated cycles of invasion, cell division, and egress, creating plaques in the monolayer. By day 6 post-infection, the parental line showed detectable plaques, which proceeded to increase greatly in size through day 13. For the HU knockout parasites, on the other hand, no plaques were visible at day 6, and by day 13 plaques appeared but were extremely small.

We first considered that the small plaque size we observed for the HU mutant may be due to a reduced rate of parasite replication resulting from impaired apicoplast function. This would be consistent with predicted functions of HU in transcription control

and could for instance result from loss of transcription of critical apicoplast genome encoded proteins. To test this, we analyzed the rate of cell division in the mutant parasites. *T. gondii* replicates in a binary budding process known as endodyogeny. After host cell invasion, a single parasite inhabits each parasitophorous vacuole. Successive rounds of division will result in progressive doubling of parasite numbers with an average doubling time of 6-8 hours for RH strain [44]. We counted and recorded the numbers of parasites per vacuole at different time points post infection for mutant and parental strain parasites (Figure 3.5), and these data were then $\log_2$-transformed before analysis to reflect number of doublings. At 24 and 32 hours we see a significant difference in median parasite doublings (Table 3.1). These data suggest that ΔHU parasites are progressing more slowly through the cell and division cycle. However, this difference does not appear sufficient to account for the drastic growth retardation observed in plaque assays and cell culture.

Loss of HU in *Toxoplasma* results in decreased apicoplast genome numbers

We considered whether a significant proportion of the parasites resulting from intracellular replication might be inviable in the HU mutant and not able to initiate or complete another full cycle of infection. This has been observed previously as a consequence of poor inheritance of the apicoplast genome following drug treatment targeting the genome [8]. As HU localizes to the apicoplast and can complement the *E. coli* homolog, it likely binds apicoplast DNA as seems to be the case for its *Plasmodium* homolog [28]. HU may be important for apicoplast DNA compaction during division and/or affect interactions of the genome with other DNA binding proteins. To examine the consequences of a lack of HU on apicoplast genome inheritance, we examined apicoplast genome abundance by quantitative PCR on ΔHU and parental strain parasites (Figure 3.6A). Using one primer pair suitable to amplify a segment of the apicoplast genome and a second primer pair specific for a single locus in the nuclear

genome, we compared the copy number of apicoplast DNA to nuclear DNA. Since *Toxoplasma* tachyzoites are haploid and contain only one copy of the nuclear genome prior to S phase, this ratio should approximate the copy number of the apicoplast genome across the population. In parental strain parasites, we measured a ratio of 21 ± 3 apicoplast genomes per nuclear genome. To demonstrate the ability of this assay to detect changes in apicoplast DNA content, we also measured DNA content of parasites treated with 10 μM ciprofloxacin for 72 hours, which has been shown in previous studies to reduce apicoplast genome copy number [8]. Indeed, in ciprofloxacin treated parasites, we observed a 51% decrease in apicoplast DNA copy number, down to 10.5 ± 6.5 apicoplast genomes per nuclear genome. In the ΔHU clone, we see a ratio of 2 ± 0.5 apicoplast genomes per nuclear genomes which represents an 89.9% reduction (Table 2), indicating that HU loss results in drastic decrease in apicoplast DNA levels. To independently confirm this using an assay independent of DNA amplification, we performed a Southern blot, probing for nuclear and apicoplast DNA in parental and knockout strains (Figure 3.6B). Densitometry of the resulting autoradiograph shows that apicoplast DNA in the mutant is 2.4% of the wild type level, compared to a decrease to 38.4% in ciprofloxacin treated parasites (Figure 3.6C). We conclude that HU function is critical to maintain a full complement of multiple apicoplast genomes per organelle and parasite.

Loss of HU in *Toxoplasma* results in an apicoplast segregation defect

We demonstrated above that overexpression of a tagged HU causes unequal apicoplast segregation and we therefore hypothesized that loss of HU might also result in segregation defects. To explore this possibility we performed immunofluorescence assays on the HU knockout using an antibody to the luminal apicoplast protein Cpn60 and compared staining to parental strain parasites. Infected host cells containing 4 daughter parasites were counted 16 hours post infection. In healthy parasites we expect

to see one apicoplast in every parasite, or 4 apicoplasts total per vacuole. In the parental strain, 96% of vacuoles had one apicoplast per cell, with only 4% of the vacuoles missing one or more apicoplasts. In contrast, in the HU knockout, 53% of vacuoles were missing at least one apicoplast, and 8% were missing all 4 apicoplasts (Figure 3.7). The median number of apicoplasts per vacuole was 4 for parental strain parasites and 3 for ΔHU parasites ($p<0.001$, Kolmogorov-Smirnov test). This indicates that loss of HU results in aberrant apicoplast division creating inviable parasites.

We wanted to examine segregation of the mitochondrion to make sure that this was an apicoplast-specific effect. Some DNA replication proteins have previously been observed to target to both the mitochondrion and the plastid in plants [45, 46], and we know dual targeting can also take place in *Toxoplasma* [47]. Although we did not observe any HU localization in the mitochondrion, it is possible that the genome is too small and too distributed to be able to detect protein binding to mitochondrial DNA in immunofluorescence assays. To address this, we also counted the presence of mitochondria in 4-cell vacuoles using the mitochondrial marker. In parental strain parasites, every parasite counted contained a mitochondrion. In ΔHU parasites, 93% of vacuoles also had a normal mitochondrial distribution. 1% of vacuoles contained a parasite lacking a mitochondrion, and 6% contained “blebbing” mitochondria instead of the typical tubular mitochondrial morphology. Since this phenotype was often observed in smaller parasites or parasites with dimmer IMC1 staining, we suspect that this is a characteristic of dying parasites. As the majority of parasites contain normal mitochondria, it appears that loss of HU does not affect mitochondrial segregation and is plastid-specific.

In light of the observed plastid loss, we wanted to test if segregation of the organelle might be hampered. During parasite cell division, the centrosome duplicates and facilitates the division and segregation of the nucleus and the apicoplast. During this

time the centrosome maintains a close association with the apicoplast and the nucleus [42]. To investigate the plastid-centrosome associations in our mutants, we performed immunofluorescence assays staining the apicoplast marker PDH-E2 and the centrosome marker centrin, and examined whether duplicated centrosomes were associated with the apicoplast. In the parental strain duplicated centrosomes were closely associated with apicoplasts, but in the HU mutant we found increased numbers of parasites whose centrosomes did not seem to exhibit any apicoplast association (Figure 3.S1A). We quantified these associations, and in the HU mutant 33% of the duplicated centrosomes lacked apicoplast associations, compared with only 18% in the parental strain (Figure 3.S1B). This difference was statistically significant ($p=0.0079$, Fisher's exact test) and indicates that loss of HU decreases the efficiency of centrosome association for the apicoplast, which contributes to unequal apicoplast segregation. Note that this observation does not rule out additional defects in apicoplast biogenesis.

While HU loss appears to reduce the efficiency of apicoplast segregation and cause plastid loss, the mechanism by which this is occurs is unclear. One possibility is that the reduction in apicoplast genome copy number leads to reduced expression of apicoplast-encoded proteins important for biogenesis and division. Alternatively, the apicoplast DNA may be physically required for centrosome association e.g. as a special marker of the site of attachment. To attempt to distinguish between these two scenarios, we compared parasites in which we inhibited either apicoplast DNA replication with ciprofloxacin or apicoplast translation with clindamycin. If the genome physically required for organelle segregation, we expect to find ciprofloxacin, which effectively removes the genome, to impact segregation stronger than clindamycin which only silences its translation. We examined and quantified plastid loss using the assay performed previously on the HU mutants at 24, 48, and 72 hours of treatment with each drug (Figure 3.S2A). At 24 and 48 hours plastid loss is only minimal and median plastid count

in 4-cell vacuoles is 4 for both treatments. At 72 hours we measure pronounced plastid loss in treated parasites when compared to untreated controls, and median plastid count drops down to 3 for ciprofloxacin-treated and 2 for clindamycin-treated parasites. However, we did not observe statistically significant differences between the two drug treatments ($p = 0.667$, Kolmogorov-Smirnov test).

We also examined apicoplast-centrosome associations in drug treated parasites at several time intervals by immunofluorescence assay. We observed a gradual decrease in apicoplast-centrosome associations with prolonged treatment, and this reduction appeared greater upon ciprofloxacin than clindamycin treatment (Table 3.S2, Figure 3.S2B). However, this difference did not reach statistical significance (Fisher's exact test).

3.5 Discussion

The Histone-like HU protein serves to compact the genomes of bacteria and algal chloroplasts, mainly of those in the red lineage. In bacteria, HU has also been shown to maintain negative supercoiling of genomic DNA, which can influence a variety of processes including initiation of replication, transcription, and DNA repair [23-26]. We demonstrate here that *Toxoplasma gondii* encodes an HU homolog that is targeted to the apicoplast, and we study its molecular function analyzing bacterial and parasite mutants.

The primary function of the *E. coli* HU protein is to condense DNA and stabilize negative supercoils in the circular chromosome [48], thereby regulating DNA topology. Our experiments show that the *Toxoplasma* HU is able to complement loss of HU in bacteria. This suggests that TgHU serves a similar condensation function. This is consistent with biochemical studies, which demonstrated that recombinant HU from *Plasmodium falciparum* is capable of condensing plasmid DNA *in vitro* [28]. Like its

Toxoplasma homolog, the *Plasmodium* HU protein was shown to localize to the apicoplast. Our *in vivo* measurements further establish a dramatically reduced copy number of the apicoplast genome in the HU mutant. This argues for defects in DNA replication as a result of lacking HU activity. Taken together, this indicates that proper DNA compaction is necessary for efficient replication and inheritance of the genome. Recent studies on the HU homolog HLP in *Chlamydomonas reinhardtii* show that RNAi knockdown of HLP similarly result in reduced chloroplast nucleoid content [17] in the green chloroplast lineage. This is consistent with our results and suggestive of a common role for HU in algal plastids across the evolutionary spectrum (note that HU is not found in chloroplasts of plants).

While the low apicoplast genome copy number we observe in the *Toxoplasma* HU mutant indicates defects in DNA replication it does not rule out additional defects in DNA segregation. Such defects have been observed in bacterial HU and DNA Gyrase mutants, leading to a model in which genome segregation requires an optimal level of DNA compaction achieved through the collective and balanced activity of these proteins [49]. How exactly HU impacts segregation is not fully understood, but the level of compaction of the chromosome could impact the binding of other proteins involved in the segregation process. While DNA replication requires direct access to stretches of single-stranded DNA, many other DNA-binding proteins bind to the major or minor grooves of the DNA double helix, and these interactions may be affected by altered condensation, writhe, or bending of the DNA. In the partitioning system of the plasmid P1, for example, the efficiency of partitioning is increased when DNA is bent by IHF [50], a paralog of HU also involved in bacterial DNA condensation.

How genome segregation and overall division are coordinated during the division of bacteria and bacterial derived organelles like mitochondria and plastids remains an active area of research and debate. In apicomplexans, the apicoplast occupies a

subcellular location apical to the nucleus and close to the centrosome. Upon duplication of the centrosome, the apicoplast shows tight physical association with both daughter centrosomes and elongates as the centrosomes move apart [42]. The elongated organelle is then constricted by a cytokinetic ring unique to the apicomplexan budding process. MORN1 is a key protein of this ring and is required for optimal apicoplast division [51]. The final step of apicoplast fission depends on the dynamin-like protein DrpA [31]. Overall therefore apicoplast division is ruled by eukaryotic mechanisms and organelle fission and segregation is accomplished and positioned by factors found outside of the apicoplast in the cytoplasm, most importantly the centrosome [42].

It is not known what role, if any, the apicoplast DNA plays in this process. In bacteria, the chromosome seems to play a direct role in cell division. The genome exclusion hypothesis posits that proteins such as FtsZ that define the cellular point of fission preferentially localize to areas of the cell devoid of DNA [52, 53]. Thus, the position of the two segregated nucleoids at opposite poles of the cell may contribute to initiation of the division furrow at a mid-cell location. FtsZ and several associated factors are found in essentially all primary and secondary chloroplasts studied so far [54]. While there are some eukaryotic elements of plastid division, it appears that the event is controlled and initiated from the inside out, with the bacterial machinery in the lead to define the point of fission. In contrast, FtsZ and other bacterial division proteins are markedly absent in Apicomplexa [55]. In parasites overexpressing HU-YFP we observed individuals that possessed an apicoplast but lacked detectable nucleoid staining. This suggests that the apicoplast DNA is not a *sine qua non* requirement for organelle division, and that the nucleoid may not be as important in this process as its counterpart in bacterial division [56].

However, we note that extended periods of loss of HU not only results in loss of the apicoplast genome but also in loss of the entire organelle, possibly due to unequal

segregation. Similar to our knockout mutants, sustained overexpression of HU-YFP also resulted in apicoplast loss. This strongly suggests that while not acting in the immediate spatial control of organelle division, factors on the inside of the apicoplast are ultimately required for its biogenesis and inheritance. This is consistent with the phenotype of several mutants in apicoplast protein import and metabolism [1, 38, 39]. Loss of the organelle is a secondary long-term consequence in most of these mutants. We therefore consider loss of apicoplast genome a primary phenotype and loss of the entire organelle a secondary phenotype of loss of HU.

Overall our experiments show that the level of HU in the apicoplast is extremely important and that the parasite only tolerates changes within a very narrow range. Based on our findings and additional studies on bacterial HU, binding of HU to DNA and the resulting compaction likely has a major effect on the ability of other DNA associating proteins to interact with the genome. Ultimately, this means that HU binding impacts a variety of vital processes like DNA replication, transcription, and DNA repair. Additional studies are needed to understand whether the role of HU has a purely structural function in apicoplast DNA topology or whether, not unlike eukaryotic histone proteins, HU also has important regulatory roles in the biology of the apicoplast genome.

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Figures and Tables

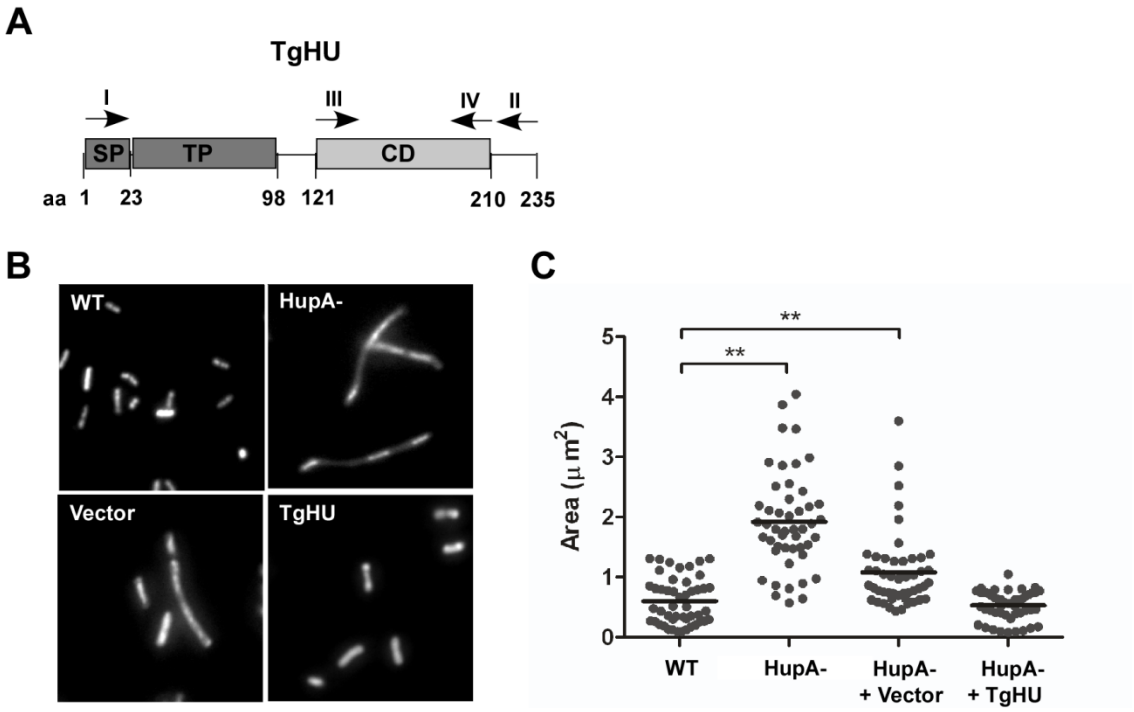

Figure 3.1: TgHU complements HupA- mutation in *E. coli*. A: TgHU encodes a 235 amino acid (aa) protein which includes a 23-aa signal peptide (SP) and a 75-aa transit peptide (TP) for targeting to the plastid, along with a conserved domain also found in bacterial HU proteins (CD). Arrows indicate positions of primers I, II, III, and IV used for plasmid construction as described in Materials and Methods. B: HupA- *E. coli* were transformed with either an expression plasmid carrying the conserved portion of the TgHU gene or a control plasmid. Nucleoid size of transformants was compared to untransformed mutant and wildtype *E. coli* by analyzing DAPI staining. HupA- *E. coli* (top right) display a filamentation phenotype and have less condensed nucleoids compared to wildtype *E. coli* (top left). Transformation with vector only (bottom left) had no impact, but transformation with TgHU (bottom right) resulted in rescue of the wild type phenotype. C: Quantification of bacterial nucleoid size for various strains displaying the contiguous area occupied by DAPI stained DNA. Differences in nucleoid size of untransformed HupA- bacteria and HupA- bacteria transformed with control DNA are statistically significant compared to wildtype *E. coli* nucleoids ($p<0.0001$ in both cases), but differences in nucleoids of HupA- bacteria transformed with TgHU are not ($p=0.2723$, Student's t-test).

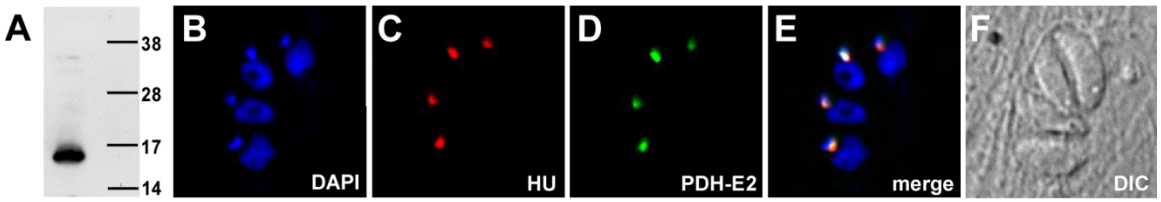

Figure 3.2: TgHU protein localizes to the apicoplast. A: Western blot of *T. gondii* lysate using rabbit serum raised against recombinant TgHU. B-F: Immunofluorescence assay of a 4 cell vacuole in RH strain *T. gondii.* Staining of an antibody raised against recombinant TgHU protein (C) colocalized with the apicoplast marker anti-PDH-E2 (D) and the DAPI stain of the apicoplast DNA (B, note that the larger nuclear genome is also stained). Merge (E) and DIC images (F) of the 4-cell vacuole are also shown.

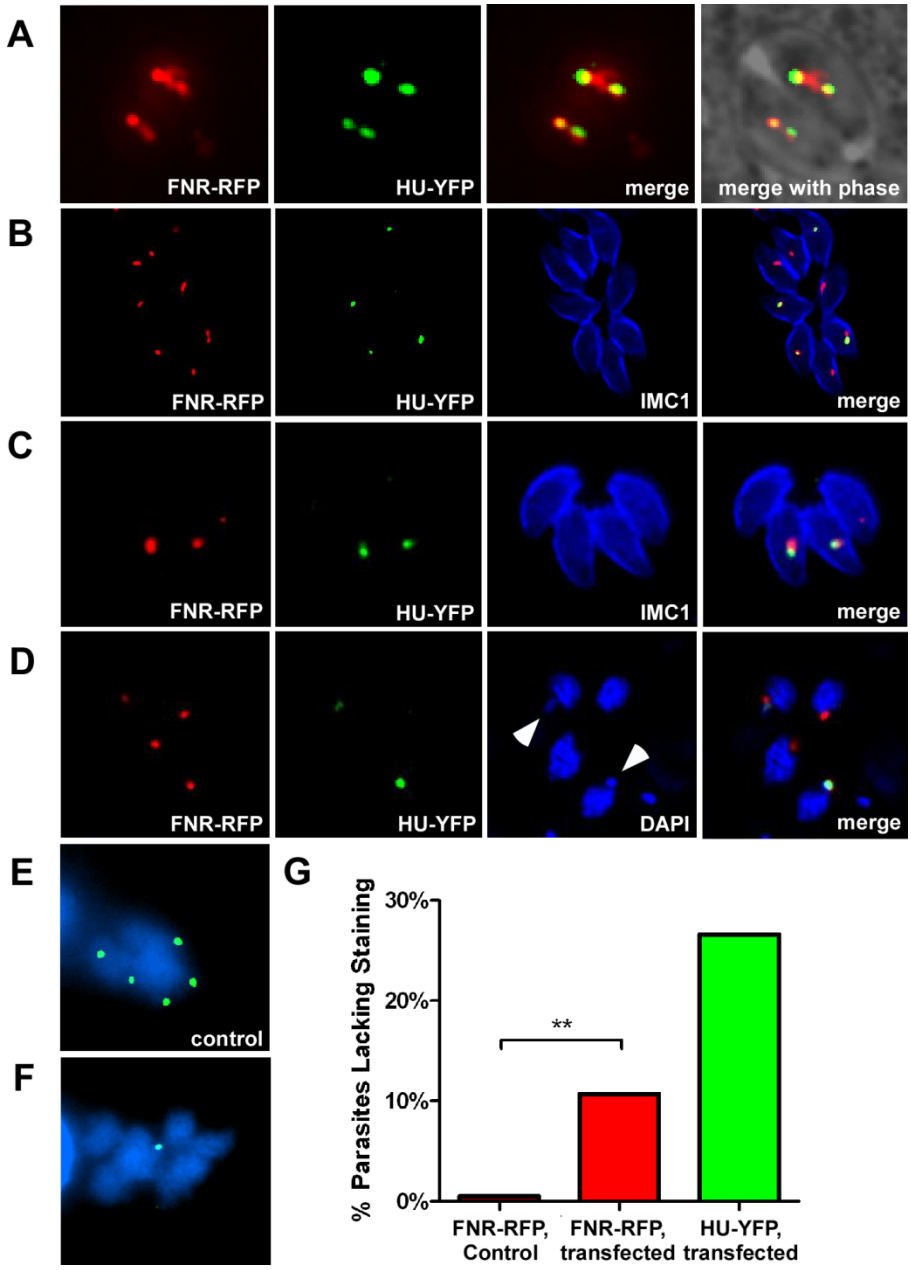

Figure 3.3: Overexpression of HU-YFP results in unequal plastid distribution. A construct encoding HU tagged with YFP at the C-terminus was transfected into parasites expressing the apicoplast marker FNR-RFP. A: Live imaging of RH strain *T. gondii.* HU-YFP (green) localized to the apicoplast, marked by FNR-RFP (red). A phase image of the 2-cell vacuole is shown in the rightmost panel. B-C: HU-YFP (green, stained with anti-GFP) shows unequal distribution in divided parasites 48 hours after infection. The luminal marker FNR-RFP (red) has a normal distribution in some vacuoles, with staining in every parasite (B), but vacuoles are also observed containing parasites that lack FNR-RFP staining (C). Parasite outlines are stained with anti-IMC1 (blue). D: HU-YFP is associated with the apicoplast nucleoid. HU-YFP staining (green) is not observed in

every apicoplast in the vacuole (red, FNR-RFP), but where present localizes to apicoplasts where apicoplast DNA (white arrowheads) is also found. Nuclear and apicoplast DNA is stained with DAPI (blue). E-F: Fluorescent *in situ* hybridization of transiently transfected parasites. Apicoplast DNA of fixed cells was hybridized with digoxygenin-labeled DNA probes and immunofluorescence assays were performed using anti-digoxygenin (green) and counter-stained with DAPI (blue). In control cells a hybridization signal is observed proximal to every nucleus (E). In parasites transiently expressing HU-YFP, vacuoles with unequal hybridization signals of apicoplast DNA are frequently observed (F). G: HU-YFP and FNR-RFP distribution in transfected parasites was quantified. By 48 hours post-transfection, 10.7% of parasites are lacking apicoplasts, compared to 0.5% in untransfected parasites. HU-YFP staining in transfected parasites is observed even less, with 26.6% of parasites in vacuoles expressing HU-YFP lacking staining. In each bar on the graph, n = 100.

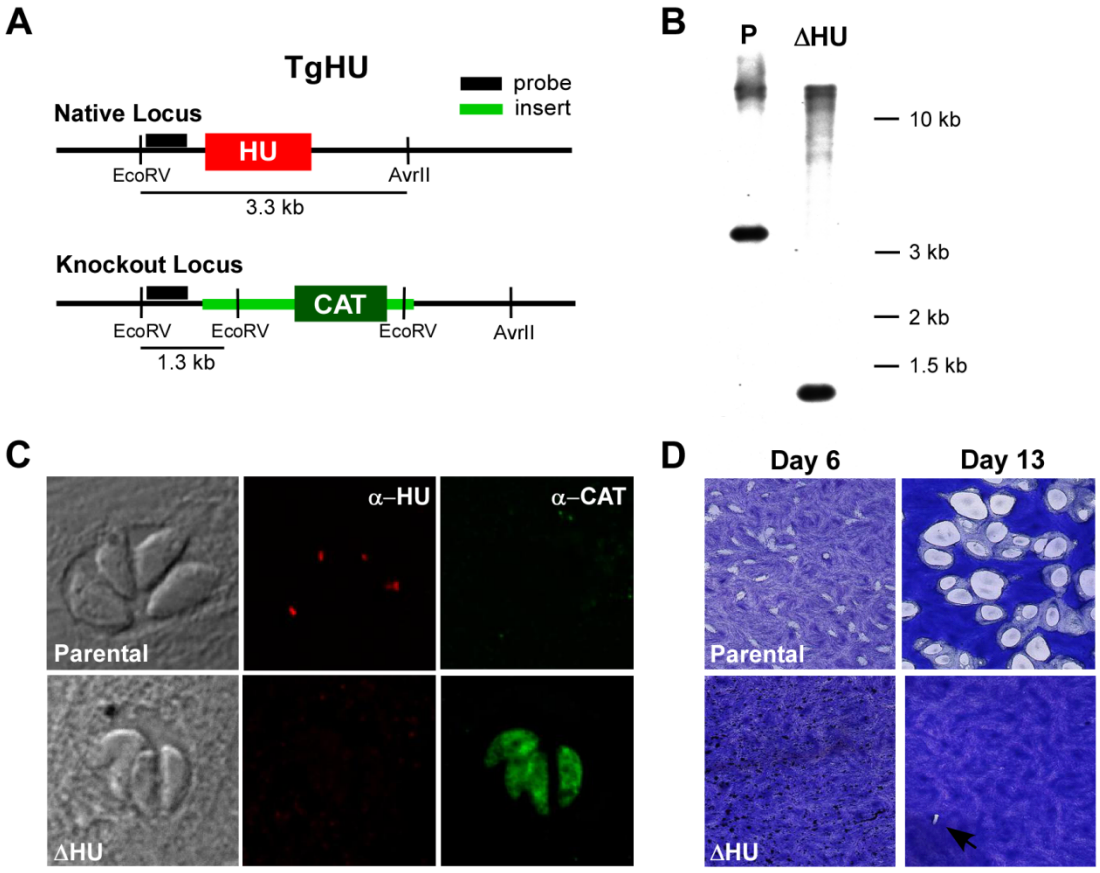

Figure 3.4: Deletion of the TgHU locus. A: Diagram of the native and modified TgHU locus. Southern blotting of genomic DNA cut with AvrII and EcoRV and hybridized with a probe specific to a region upstream of the HU locus is predicted to yield a 3.3 kb band for the native locus or a 1.3 kb band for the modified locus. B: Southern blot analysis comparing parental strain DNA and DNA isolated from the ΔHU mutant. C: Immuno-

fluorescence assays on parental strain (top) and ΔHU (bottom) parasites, stained with anti-HU (red, middle panels) and anti-CAT (green, right panels) and shown with a DIC reference image (left panels). Parental strain exhibits HU staining but no CAT staining, while ΔHU parasites exhibit CAT staining but lack HU staining. D: Plaque assays of ΔHU parasites compared to the parental strain. Parental strain parasites yield plaques by day 6 (top left) which greatly increase in size by day 13 (top right), while no plaques can be seen in ΔHU parasites on day 6 (bottom left). A few ΔHU plaques (black arrow) begin to appear by day 13 (bottom right), but these are greatly reduced in size.

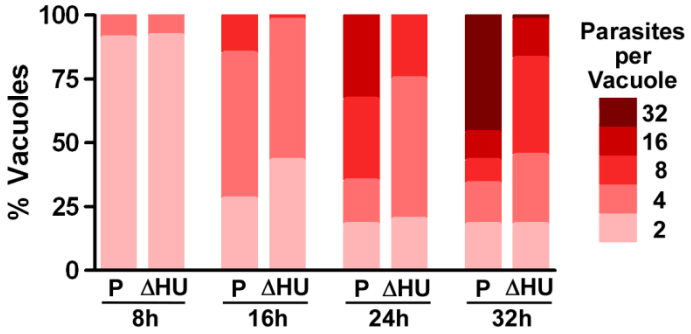

Figure 3.5: Distribution of Vacuole Sizes in parental and ΔHU parasites. Host cells were infected with parasites, and number of parasites per vacuole was counted in 100 individual vacuoles at four different time points. One-cell vacuoles were disregarded, to avoid the inclusion of parasites that were merely attached and not yet invaded. The distribution of vacuole sizes for each strain at each time point is displayed.

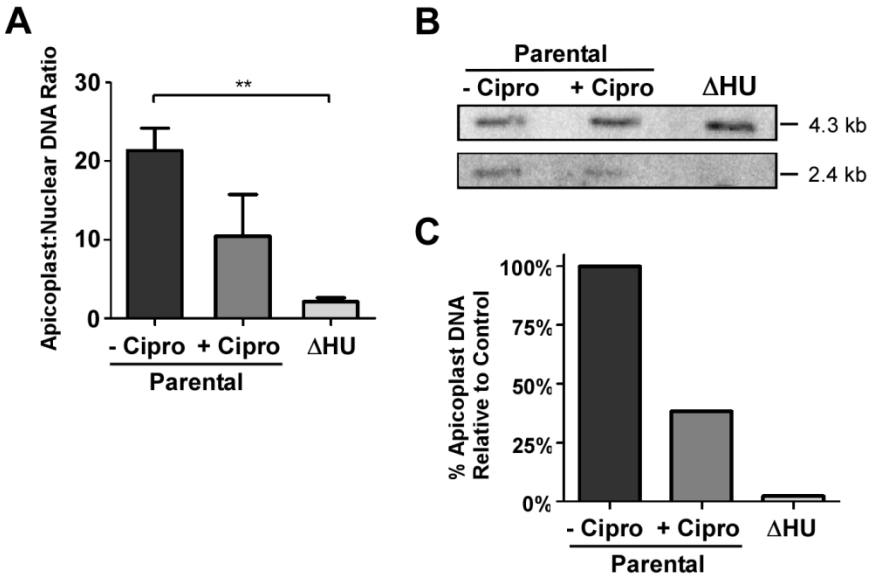

Figure 3.6: ΔHU parasites display reduced apicoplast genome copy numbers. A: Quantitative PCR was performed on mutant and parental strain parasites using separate primer pairs annealing to the apicoplast or nuclear genome. Results are presented as the ratio of apicoplast DNA copies to nuclear DNA copies. Parental strain parasites show an apicoplast DNA copy number of 21.35 ± 2.83, while ΔHU parasites show an apicoplast DNA copy number of 2.15 ± 0.51. Parental strain parasites treated for 72 hours with 10 μM Ciprofloxacin are shown for comparison, which have an apicoplast DNA copy number of 10.46 ± 6.50. Apicoplast DNA levels of the ΔHU parasites are statistically significant compared to untreated parental strain parasites (**, $p<0.005$, Student's t-test). B: Southern blot showing parasite genomic DNA products for nuclear and apicoplast DNA as independent confirmation of quantitative PCR phenotype. C: Densitometry on Southern blot after normalization of apicoplast DNA levels to nuclear DNA levels. Values are shown as percent of untreated parental apicoplast DNA content. Ciprofloxacin treatment reduces apicoplast DNA levels of the parental strain to 38.4% of untreated levels, and ΔHU parasites exhibit apicoplast DNA levels of only 2.4% of the untreated parental strain.

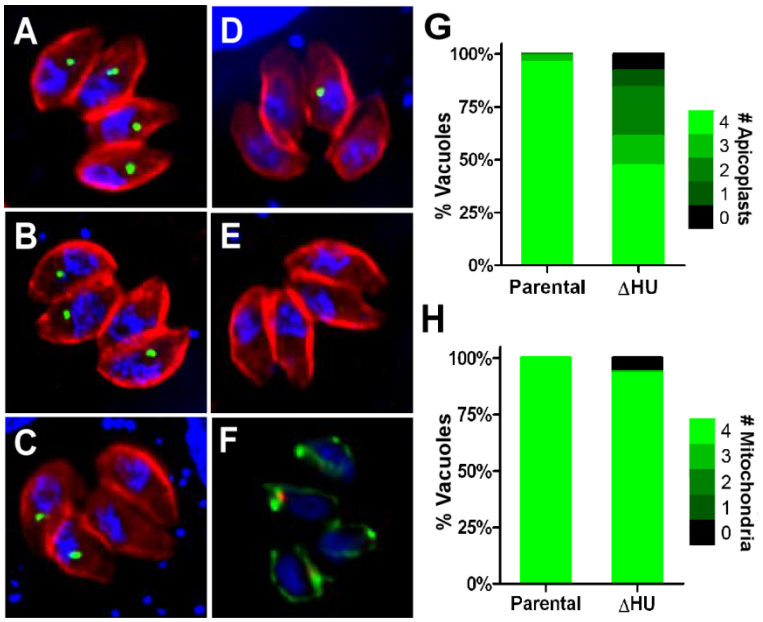

Figure 3.7: Apicoplast loss in ΔHU parasites. The number of apicoplasts in parental and ΔHU parasites at the 4-cell stage was quantified by fluorescence microscopy. A-E: Representative images from ΔHU parasites showing 4-cell vacuoles with 4, 3, 2, 1, and 0 apicoplasts, respectively. Cells are stained with rabbit anti-IMC1 (red, inner membrane complex, outline of parasite), mouse anti-PDH-E2 (green, apicoplast marker), and DAPI (blue). F: Representative images from ΔHU parasites showing 4-cell vacuoles with normal mitochondrial morphology. Cells are stained with mouse anti- (green, mitochondria), rabbit anti-Cpn60 (red, apicoplast marker), and DAPI (blue). G-H:

Apicoplast (G) and mitochondria (H) counts in parental strain and ΔHU parasites. The number of apicoplasts in 100 4-cell vacuoles of each strain were counted at 16 hours post-infection.

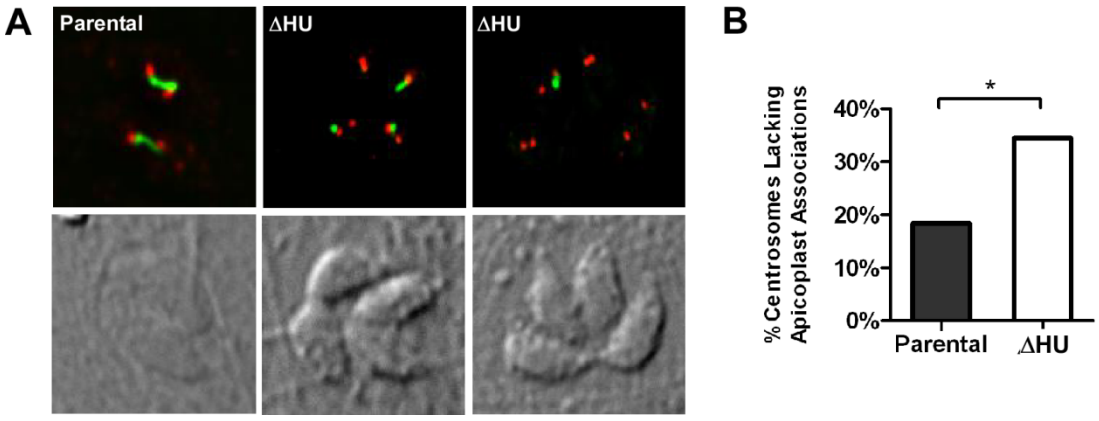

Figure 3.S1: HU loss results in reduced association with centrin during cell division. The number of duplicated centrosomes associating with an apicoplast was quantified during fluorescence microscopy. A: During cell division in *T. gondii,* the poles of the apicoplast (green, anti-PDH-E2) are associated with the centrosomes (red, anti-centrin) in the parental strain (P, left). ΔHU parasites (middle and right) exhibit reduced association of apicoplasts with centrosomes. DIC reference images are shown in bottom panels. B: Quantification of apicoplast-centrosome association in parental strain versus ΔHU parasites. Duplicated centrosomes were scored on whether they were observed to associate with an apicoplast or not, in parasites that still retained apicoplasts. 18.3% of parental strain duplicated centrosomes showed apicoplast associations (n=120), while only 34.4% of HU duplicated centrosomes did (n=96). The observed difference is statistically significant (p =0.0079, Fisher exact test).

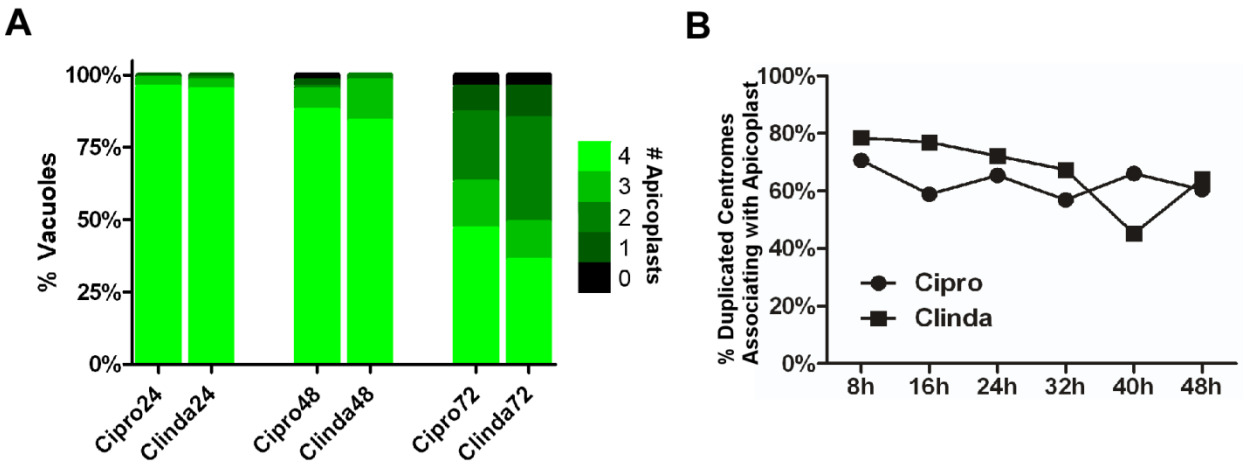

Figure 3.S2: Defective apicoplast segregation in Ciprofloxacin- and Clindamycin-treated parasites. Parasites were treated with either 10 μM Ciprofloxacin or 40 ng/ml Clindamycin at various time points. The number of apicoplasts and centrosome-apicoplast associations were counted. A: Distribution of apicoplast numbers in four cell vacuoles at 24, 48, and 72 hours post-treatment. At 24 and 48 hours, the median apicoplast number per vacuole was 4, as expected for untreated parasites. After 72 hours, the medians decreased to 3 for Ciprofloxacin treated parasites and 2 for Clindamycin treated parasites. B: Quantification of the number of duplicated centrosomes with an associated apicoplast, in parasites that still contain an apicoplast, at 8, 16, 24, 32, 40, and 48 hours post-treatment. Differences between the two drug treatments were not statistically significant.

Table 3.1: Mean vacuole sizes in parental and mutant strains.

Time	Parental Median Doublings[1]	ΔHU Median Doublings[1]	p-value[2]
8h	1	1	1.000
16h	2	2	0.193
24h	3	2	<0.001
32h	4	3	<0.001

[1]Parasites per vacuole were counted and transformed on a log2 scale to reflect number of doublings.

[2] P-values were calculated with the Kolmogorov-Smirnov test.

Table 3.2: Apicoplast DNA levels from Quantitative PCR and Southern Blot.

Strain	Mean Ratio[1]	Decrease[2]	p-value[3]	Southern Normalization[4]
Parental, untreated	21.35 ± 2.83	--	--	100.0%
Parental, treated	10.46 ± 6.50	51.0%	0.145	38.4%
ΔHU	2.15 ± 0.51	89.9%	0.003	2.4%

[1]Mean ratio of copy number of apicoplast DNA to copy number of nuclear DNA from Quantitative PCR analysis.
[2]Relative to untreated parental DNA samples.
[3]P-values were calculated using Student's t-test, comparing mean ratios to untreated parental samples.
[4]Apicoplast band intensities on Southern blot were normalized to nuclear band intensities and expressed as a percent of untreated parental levels.

Table 3.S1. List of Primers.

Primer	Sequence (5'-3')	Direction
I	ATCGGATCCGCGGTCACTCGGAAAGACCTC	Sense
II	GATTCTAGACTAGTTCCAGCCAAAGAGGCC	Anti-sense
III	ATCAGATCTTAAAATGCAGACGCTTTCGCTGTCTT	Sense
IV	GATCCTAGGGTTCCAGCCAAAGAGGCCTTTTTTCT	Anti-sense
V	CCTAGGCTAGTTCCAGCCAAAGAGGCCTTT	Anti-sense
VI	CGTTTGGAAGTCGTGCCTTTTTCGTCGGTGAAAAAGTC GTGTCTCCATCCCTCGACTACGGCTTCCATTGGCAAC	Sense
VII	AATTTCGACAGAACTCGACATCCGCACGGTGTACTTGC TTCTGTCACATGATACGACTCACTATAGGGCGAATTGG	Anti-sense
VIII	GTACAGCTACCTTGTTACGACTTC	Sense
IX	GAGTTTGATCCTRGCTCMG[1]	Anti-sense
X	CKGAGCYAGGATCAAACTC[1]	Sense
XI	CGCACTYTTTAAARGAWAACTGCTTC[1]	Anti-sense
XII	GAAGCAGTTWTCYTTTAAARAGTGCG[1]	Sense
XIII	CCCAGCTCACGTGCCGCTTTAATGGGCGAAC	Anti-sense
XIV	CCCAGCTCACGTGCCGCTTTAATGGGCGAAC	Sense
XV	CGTTTAGTTGTAAAATTATATAAACCACC	Anti-sense
XVI	GTAAACACTTCCACGCACCC	Sense
XVII	TCGGTGTGTGTGTGTGCAAC	Anti-sense

[1]These primers utilize the degenerate code.

Table 3.S2. Percentage of Duplicated Centrosomes Associating with Apicoplasts in Ciprofloxacin- or Clindamycin-treated parasites.

Time	Cipro %	Clinda %	Cipro n	Clinda n	p-value[1]
8h	70.59	78.43	102	102	0.261
16h	58.82	76.92	102	104	0.007
24h	65.38	72.12	104	104	0.370
32h	56.86	67.31	102	104	0.151
40h	66.04	45.19	106	104	0.003
48h	60.38	64.15	106	106	0.671

[1]P-values were calculated using Fisher's exact test on contingency tables of raw data.

CHAPTER 4

CHARACTERIZATION OF APICOPLAST DNA REPLICATION PROTEINS IN *TOXOPLASMA GONDII*

4.1 Introduction

Apicomplexans are a group of obligately intracellular parasites that infect humans and other animals and thus have both significant health and economic impacts globally. The diseases caused by these organisms include Toxoplasmosis, Babesiosis, and malaria, which alone kills over a million people each year [1]. Recently, *Toxoplasma gondii* has become a model system for Apicomplexa including *Plasmodium*, as a result of the simple culture conditions and the vast array of genetic tools available including conditional gene knockouts.

Most Apicomplexan parasites harbor a remnant chloroplast called the apicoplast. This plastid organelle has since lost any photosynthetic function, but retains important metabolic functions. It houses various biosynthetic pathways including type II fatty acid synthesis [2], heme synthesis [3, 4], and isoprenoid biosynthesis [5], and several of these processes are essential for the parasite [2, 5]. Because this organelle is only found in the parasites and not in the human host, apicoplast proteins and structures are enticing candidates for drug development.

Like its chloroplast ancestor, the apicoplast also contains its own genome, although at 35 kb it is quite reduced in comparison and has lost all photosynthetic genes. It has been shown that the apicoplast genome is essential for parasite viability. This was first observed using Ciprofloxacin [6], an antibiotic that targets DNA gyrase and results in

double strand breaks in DNA. Additionally, the apicoplast genome is very well conserved throughout the plastid-bearing apicomplexans. Most of the genes encoding apicoplast proteins are nuclear encoded. These were probably apicoplast-encoded originally but have been laterally transferred to the nucleus over time. The 63 genes left behind mostly function in the expression of the apicoplast genome and include genes for an RNA polymerase, ribosomal RNAs, ribosomal proteins, tRNAs, and transcription factors [7, 8]. However, it also encodes two proteins that may act beyond transcription and translation, clpC and sufB. ClpC is a homolog of a chaperone protein which may be involved in protein turnover in the apicoplast [9], while sufB is likely involved in assembly of iron-sulfur clusters [10]. In addition there are also several unidentified ORFs potentially encoding additional apicoplast proteins. It may be that some of these proteins are critical for apicoplast biosynthetic processes or biogenesis and therefore essential for parasite survival.

We know that the apicoplast genome is required for survival, but there is limited information about how it is replicated. In *Plasmodium,* plastid DNA appears to replicate primarily by a D-loop mechanism, which initiates in the inverted repeat region [11]. In *T. gondii*, however, it has been proposed that rolling circle replication (RCR) is the predominant replication method, primarily because linear tandem arrays of the *T. gondii* apicoplast genome have been observed [12]. Linear tandem arrays of the plastid genome are also thought to occur in *Eimeria tenella* [13] and *Neospora caninum* [14], so this may prove to be a mode of replication common to the coccidia. RCR is a common replication mode of plasmids, bacteriophages, and viroids, but it has also been inferred to contribute to replication of chloroplast genomes [15]. RCR often requires a special initiator protein, and none of those previously studied have obvious homologs in *Toxoplasma*. Nonetheless, the difference in apicoplast DNA topology between

Toxoplasma and *Plasmodium* may indicate differences in replication mechanisms which could involve yet to be discovered factors.

Proteins involved in apicoplast DNA replication make attractive drug targets; they are likely essential since the apicoplast genome is essential. The Prex proteins from *P. falciparum* and *T. gondii* were recently characterized biochemically and shown to have DNA polymerase activity *in vitro* [16, 17], and it has been suggested that these may be the replicative polymerases of the apicoplast genome. Prex appears to be a fusion of a primase-helicase protein and a polymerase (containing both polymerase and 3'-5' exonuclease domains), with a spacer region in between. The polymerase region seems to be most closely related to the Pol I enzyme from the bacterium *Thermus aquaticus*, while the primase domain appears to be similar to the bacteriophage T7 primase, and the helicase domain with Twinkle, the mammalian mitochondrial helicase [17]. Localization studies of PfPrex, however, confirmed the localization of the enzyme to the apicoplast and not to the mitochondrion [17]. The PfPrex preprotein is about 235 kDa but it is thought that the enzyme is subsequently cleaved at a site between the helicase domain and the polymerase domain [17, 18].

Homologs of DNA gyrase and single-stranded DNA binding protein (SSB) have also been found to localize to the apicoplast in *Plasmodium* [19-21]. SSB is required during replication to coat single-stranded sections of DNA to keep the helix unwound, and DNA gyrase is a type II topoisomerase that serves to relax positive supercoils generated ahead of the replication fork by DNA helicase. Gyrase may also help regulate the topology and segregation of the DNA [22-24]. Furthermore, it is the target of antibiotics such as novobiocin and ciprofloxacin. Since apicomplexan parasites are sensitive to these drugs [6, 20], it is likely that DNA gyrase activity is essential in the apicoplast.

To gain a better understanding of how DNA replication proceeds in the apicoplast, we were interested in further elucidating the roles these proteins play in parasite cell biology *in vivo.* In this study we created conditional knockouts of proteins involved in DNA replication in the apicoplast to determine whether the genes are essential and to investigate their involvement in apicoplast DNA replication. Here we present apicoplast localizations of several putative apicoplast DNA replication proteins in *T. gondii*, and show that TgPrex and TgGyrB are both required for the replication of apicoplast DNA.

4.2 Materials And Methods

Plasmid Construction

Plasmids for endogenous C-terminal tagging were constructed as previously described [25]. Briefly, about 1.5 kb of the 3' end of each target gene was amplified from RH genomic DNA by PCR. Primers I and II were used to amplify a fragment from Prex, primers III and IV were used to amplify a fragment from SSB, and primers V and VI were used to amplify a fragment from GyrA (Table 4.1). These fragments were then cloned into the parent vector pLIC-3xHA-CAT by a ligation independent cloning method.

To construct the plasmid expressing a tagged GyrB transgene, the cDNA of GyrB was amplified by PCR using primers VII and VIII (Table 4.1) and cloned into the pKS-UPRT-T7S4-myc vector between a BglII and an AvrII site.

Plasmids for promoter insertion were constructed as previously described [26]. Briefly, the 5' end of each target gene was amplified by PCR from RH genomic DNA. Primers IX and X were used to amplify the fragment from Prex, and primers XI and XII were used to amplify the fragment from GyrB (Table 4.1). The resulting amplicons were then cloned into the pDT7S4HA plasmid between BglII and XmaI sites in the case of Prex, or BglII and AvrII sites in the case of GyrB.

Cell Culture

T. gondii tachyzoites were passaged on Human Foreskin Fibroblasts in Dulbecco's modified Eagle medium with 2% serum. Parasites transfected with plasmids for endogenous tagging were selected with 6.8 ng/mL chloramphenicol, and parasites transfected with plasmids for promoter insertion were selected with 1 uM pyrimethamine. Drug was added to medium starting the day after transfection.

Immunofluorescence Assays and Western blotting

Immunofluorescence assays were performed as previously described. Briefly, parasites were allowed to invade host cells grown on coverslips overnight. Cells on coverslips were then fixed with 3% paraformaldehyde in PBS, permeabilized in 0.25% Triton X-100 in PBS, and then blocked with 1% BSA in PBS before being incubated with primary and secondary antibodies.

Western blotting was performed as previously described [27]. To make parasite lysates, egressed parasites were collected and centrifuged, and resuspended in reducing sample buffer (Invitrogen) at 5 x 10^5 parasites/µL. Lysates were then run by SDS-PAGE on Tris-Glycine gels (Bio-Rad) before transfer to 0.2 um nitrocellulose membranes and subsequent antibody labeling.

Rat anti-HA (Roche) was used at 1:50 and rabbit anti-myc (Pierce) was used at 1:500. Rabbit anti-HU and anti-Cpn60 [28] were used at 1:2000 and 1:3000, respectively. Alexafluor 488-conjugated goat anti-rat and alexafluor 546-conjugated goat anti-rabbit were used at 1:400 (Invitrogen). HRP-conjugated goat anti-rat and goat anti-rabbit (Bio-Rad) were used at 1:5000.

Quantitative PCR

Quantitative PCR was performed as previously described [29]. Parasite DNA was isolated using the DNeasy kit (Qiagen). To create standards, a 345-bp DNA fragment was amplified from the UPRT locus in the nuclear genome, and a 305-bp DNA fragment

was amplified from the apicoplast genome by PCR. These fragments were then cloned into the pCR2.1 TOPO vector (Invitrogen) to create template for standards. iQ SYBR Supermix (Bio-Rad) was used in 20 ul reactions containing 0.5 uM of each primer and 50 ng DNA except in the standards, which used serial dilutions of plasmid copy number (10^2-10^7 copies). Reactions were run on an iQ5 Real Time PCR detection system using iQ5 software (Bio-Rad).

4.3 Results

Apicoplast localization of putative DNA replication proteins

The *T. gondii* nuclear genome encodes homologs of DNA Polymerase I, A and B subunits of DNA Gyrase, and single-stranded DNA binding protein (SSB). We wanted to determine whether these proteins targeted to the apicoplast. To achieve this, we utilized a system of endogenous gene tagging mediated by homologous recombination [25]. In contrast to expressing a tagged transgene, endogenous tagging eliminates the possibility of negative side effects from overexpression. Using the gene models predicted on the *Toxoplasma* genome database (http://www.toxodb.org/), 3' ends of the coding sequence of target genes were cloned into a plasmid, resulting in a C-terminal fusion to a triple HA epitope, with a marker for chloramphenicol resistance downstream. The resulting plasmids were transfected into parasites. Since *Toxoplasma* typically employs a high degree of non-homologous end joining rather than homologous recombination, we used the ΔKu80/TATi strain of parasites to promote higher rates of homologous recombination [26].

Using this method we obtained stable lines expressing Prex-HA, GyrA-HA, or SSB-HA. In the case of GyrB drug selection proved difficult, and in the resulting population only a fraction of the parasites were expressing the HA tag. We considered that a C-terminal tag might be detrimental to the function of this protein or its ability to

form complexes. To remedy this, we amplified the coding sequence of GyrB from *T. gondii* mRNA and constructed a plasmid for expression that added a C-terminal myc epitope tag. This construct was then transfected into the GyrA-HA strain in order to be able to visualize both gyrase subunits in the same parasite line. Since this strain also expresses the native non-tagged GyrB, we hoped to decrease problems associated with function of the tagged version. Stable lines were obtained after transfection and selection.

We performed immunofluorescence assays on the tagged lines using an anti-HA or anti-myc antibody for the engineered tag and an anti-Cpn60 antibody to visualize the apicoplast (Figure 4.1). In all cases, we detected apicoplast localization. Interestingly, the Prex and SSB proteins did not seem to localize all throughout the apicoplast, but exhibited a punctuate pattern at the poles, and sometimes an additional spot in the middle.

TgPrex and TgGyrB are essential for parasite growth

Loss of apicoplast DNA has been shown to result in parasite death [6], and factors involved in the replication of the apicoplast genome are expected to be essential as well. To test this hypothesis we constructed conditional knockouts of both Prex and GyrB. After the correct coding sequences were confirmed by sequencing cDNA, we attempted to insert the regulatable T7S4 promoter upstream of the translation start site of each gene (Figure 4.2A). This promoter drives expression of the locus after insertion, but can be silenced by addition of anhydrous tetracycline to the culture medium. The 5' region of the target gene was cloned into the pDT7S4 plasmid, which also encodes a DHFR that provides resistance to pyrimethamine to transgenic parasites. In the case of Prex, transfection was performed in the strain expressing the triple HA tag. For GyrB, transfection was performed in the ΔKu80/TATi strain. Parasites that survived drug

selection were cloned out, and subsequent experiments were performed clones that tested positive for promoter insertion by PCR (data not shown).

To initially monitor regulation of expression in the iPrexHA$_3$ parasites, immunofluorescence assays were performed on parasites that had been preincubated for 3 days with or without the addition of anhydrous tetracycline (ATc). Parasites were stained with antibodies to the HA tag and to the apicoplast marker Cpn60. In the absence of tetracycline, PrexHA$_3$ was observed in all parasites, but in parasites pre-incubated with tetracycline the expression of PrexHA$_3$ was greatly reduced (Figure 4.2C). Protein levels were also examined in Western blots using an anti-HA antibody. After 3 days the protein level of Prex decreased by about 80% (Figure 4.2B). We also measured protein level after pre-incubation with ATc for 6 days, but no additional decrease was observed (data not shown). We also wanted to examine regulation of our GyrB mutant, but we could not measure protein levels directly since we have no gyrase antibody and there is no tag in this mutant line. Instead, we examined transcript levels. We isolated RNA and synthesized cDNA from parasites grown in the presence or absence of ATc. GyrB could be amplified by PCR from cDNA pools of parasites untreated with ATc, but not from treated parasites. In contrast, amplification of a control cDNA remains at similar levels to uninduced parasites (Figure 4.2D).

To investigate whether Prex or GyrB were essential for parasite growth, plaque assays were performed. Flasks were infected with 1000 parasites and incubated for 11 days. In the absence of tetracycline parasites produced numerous large plaques, but in parasites that had been preincubated in ATc for 3 days, only a small number of minute plaques were observed for both mutants (Figure 4.3). In contrast, ΔKu80/TATi strain parasites produced numerous plaques of similar size in both the absence and presence of ATc. This suggested that both Prex and GyrB are important for parasite survival.

Loss of TgPrex or GyrB reduces apicoplast genome copy number

Loss of either Prex or GyrB appeared to result in parasite death, and we next wanted to examine why these proteins were essential for growth. As they are expected to be involved in apicoplast DNA replication, we used a quantitative PCR assay to examine apicoplast DNA content in these mutants in the presence or absence of tetracycline. We measured the amount of both nuclear DNA and apicoplast DNA. As *Toxoplasma* tachyzoites are haploid, ratio of apicoplast genomes to nuclear genomes should approximate the copy number of the apicoplast genome per cell. For both the Prex and GyrB mutants (Figure 4.4), apicoplast genome content was highest in the absence of ATc, then started to decrease after incubation with ATc for three days, and was lower still after a six day incubation. This suggested that these proteins indeed were involved in apicoplast DNA replication. In contrast, the apicoplast DNA levels of the parental strain treated with ATc remained similar to untreated. Additionally, we also found that the apicoplast DNA content of the Prex mutant in the absence of ATc was significantly lower than both the parental strain and the GyrB mutant. It is possible that replacement of the promoter in this strain affects the efficiency of its expression and may result in decreased apicoplast DNA replication prior to ATc induced repression.

4.4 Discussion

This work describes *in vivo* phenotypic analysis of mutants for proteins that have been suggested to function in apicoplast DNA replication. Here we show that both the Prex and GyrB enzymes are essential for parasite growth. Furthermore, loss of these enzymes decreases the copy number of the apicoplast genome, strongly suggesting that these are the primary enzymes involved in apicoplast DNA replication.

Apicoplast DNA, which can be identified by both DAPI stain and antibody staining of the DNA-binding HU, typically localizes throughout the lumen of the plastid. However,

our immunofluorescence assays of the DNA replication machinery described here do not share this ubiquitous distribution in the lumen but instead localizes mainly to the poles of the apicoplast. This could potentially provide a means of segregating DNA, in which replicating DNA is drawn along with the replication factory to the poles of the plastid when the organelle begins to elongate during division. It is also possible that the DNA is segregated by proteins that are not involved in replication, but no homologs of bacterial DNA segregation proteins have been reported in the apicoplast so far. Additionally, unlike plant and algal chloroplasts, organelle division and fission is not mediated by FtsZ and other components derived from the cyanobacterial ancestor. Rather, it is mediated by a process coordinated from the outside of the apicoplast. The apicoplast divides by association with the centrosomes, and fission is completed by a novel dynamin-related protein [30, 31]. It is still unknown how spindle microtubules interact with the apicoplast, however, and whether elements exist in the apicoplast membranes that allow coordination of organellar division and DNA segregation.

Although plant and algal plastids are known to divide using bacterial-like FtsZ-driven machinery, mechanisms of DNA segregation are not fully elucidated. Many observations suggest that this process is facilitated by association of the DNA with thylakoid membranes. However as a prime example of a non-photosynthetic plastid, apicoplasts do not contain thylakoids. It is possible here that DNA becomes associated with the innermost apicoplast membrane through the positioning of the replication machinery, but whether these proteins are indeed associated with the membrane or how they form foci at the poles of the plastid remains to be elucidated.

The quantitative PCR experiments show quite convincingly that both Prex and GyrB are required for apicoplast DNA replication. However, in future studies it may also be interesting to examine mitochondrial DNA levels after loss of these proteins. Although our immunofluorescence assays only show apicoplast localization, the mitochondrial

genome is smaller and lies inside a larger organelle. It might be possible that mitochondrial localization of DNA-binding proteins would not be detectable. In plants and algae, several proteins including DNA polymerase and DNA gyrase are known to be dual-targeted to plastids and mitochondria [31, 32]. No mitochondrial DNA replication machinery has been described in apicomplexans, so it would be interesting to address this possibility.

With the studies described here, a clearer picture of the apicoplast DNA replication process is beginning to emerge. Although the existence of some of the proteins we describe had previously been reported in *Plasmodium*, this study provides direct evidence that the Prex and GyrB proteins are required for DNA replication in the apicoplast. Future studies may reveal additional proteins that are essential in this process, and may provide insight as to how the different apicoplast DNA replication mechanisms observed across Apicomplexa may be mediated.

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Figures and Tables

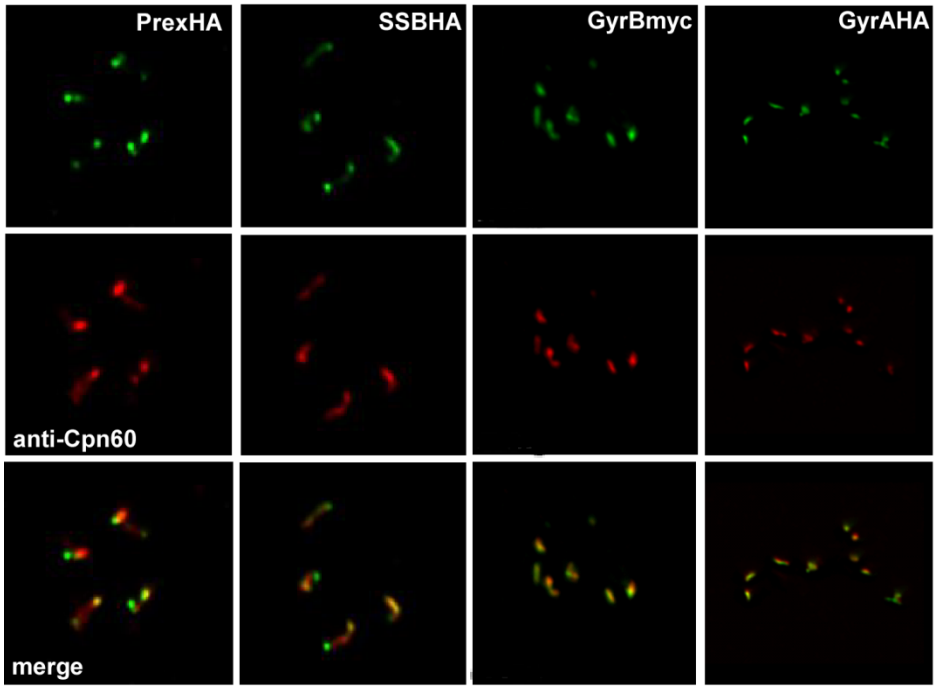

Figure 4.1: Apicoplast localizations of DNA replication proteins.
Immunofluorescence assays were performed on parasite lines expressing tagged versions of the target genes. Anti-HA or anti-myc signal (green, top) on tagged versions of Prex, SSB, GyrB, or GyrA colocalized to the apicoplast, marked by anti-Cpn60 (red, middle). Merge images shown on bottom panels.

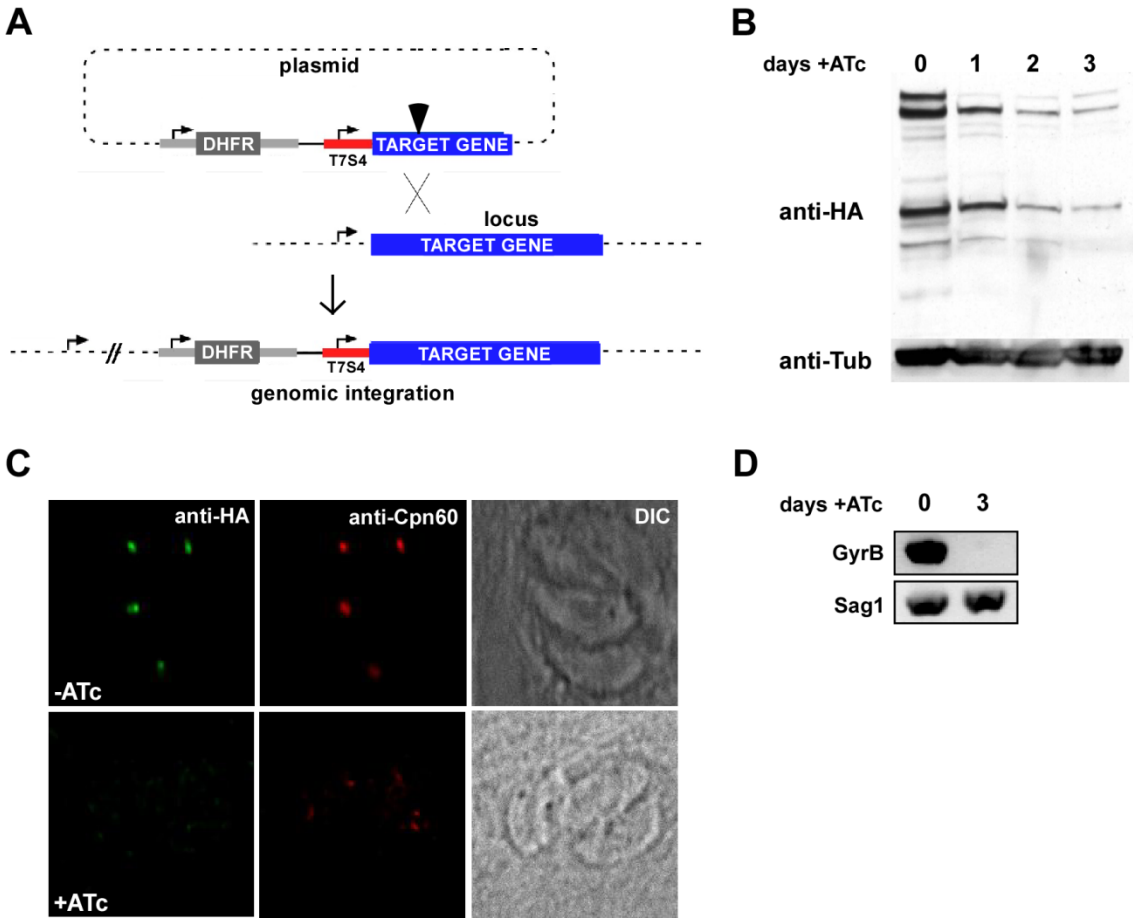

Figure 4.2: Conditional gene knockouts of Prex and GyrB. A: Diagram of promoter insertion conditional knockout strategy. A plasmid is constructed containing the 5' end of the target gene downstream of the regulatable T7S4 promoter. When this plasmid is linearized in the cloned insertion and transfected into ΔKu80/Tati strain parasites, it integrates into the genome by homologous recombination, inserting the T7S4 promoter and the DHFR drug marker upstream of the target gene in the parasite genome. B: Western blot showing decreasing levels of Prex protein after addition of anhydrous tetracycline. The multiple bands in each lane correspond to different levels of Prex protein processing. Tubulin is shown as a loading control (bottom). C: Fluorescence microscopy of iPrexHA$_3$ cells with and without addition of anhydrous tetracycline (bottom and top, respectively). Anti-HA staining (green, left panels) indicates presence or absence of Prex protein. Cpn60 (red, middle panels) is used as an apicoplast marker. DIC image in right panels. D: PCR on 1 µg of cDNA made from iGyrB parasites grown in the absence or presence of anhydrous tetracycline for 3 days. Reactions were performed with primers annealing to GyrB cDNA (XIII and XIV in Table 4.1) and primers annealing to Sag1 (XV and XVI in Table 4.1) were used in loading controls.

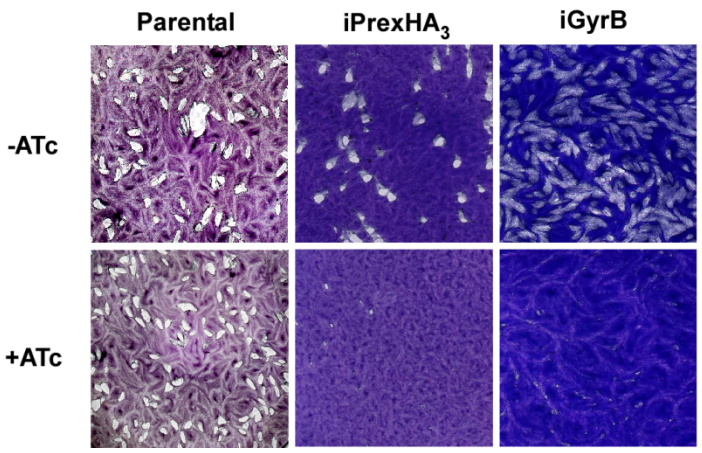

Figure 4.3: Prex and GyrB are essential for the parasite. Plaque assays of parental strain (left), iPrexHA$_3$ (middle), and iGyrB (right) parasites without anhydrous tetracycline (top) and with a 3 day preincubation of anhydrous tetracycline (bottom).

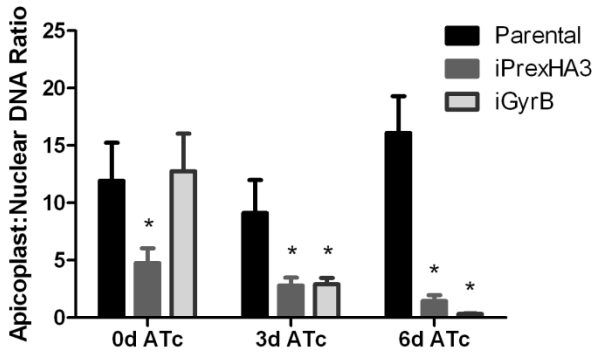

Figure 4.4: Apicoplast DNA content of Prex and GyrB mutants. Quantitative PCR was performed on parental strain, iPolAHA3 and iGyrB parasites and the ratio of apicoplast DNA to nuclear DNA copies is shown. Statistics were performed using Student's t test comparing each sample to the parental strain (*, $p < 0.05$).

Table 4.1: Primers

#	Sequence (5' to 3')	Strand	Description
I	TAC TTC CAA TCC AAT TTA ATG CAC CAC AAT AAT GCT GGT CCA CGG	sense	PolA 3' tagging
II	TCC TCC ACT TCC AAT TTT AGC CGG CTT GTC TGC CCA GCT GTC	antisense	PolA 3' tagging
III	TACTTCCAATCCAATTTAATGCACCGTT TTCTGCCCAACTCTG	sense	SSB 3' tagging
IV	TCCTCCACTTCCAATTTTAGCGCGGAA AAGGCGATACATGC	antisense	SSB 3' tagging
V	TACTTCCAATCCAATTTAATGCCGACG GAAGAAAGGGATATCG	sense	GyrA 3' tagging
VI	TCCTCCACTTCCAATTTTAGCAACTCTA AACAACCAGTCTATCTTCTTG	antisense	GyrA 3' tagging
VII	AGATCTAAAATGAAGGCCTCCTCAGAG	sense	GyrB cloning into pKS-UPRT-T7S4-myc
VIII	CCTAGGAACATCCAGCTCTTCCAC	antisense	GyrB cloning into pKS-UPRT-T7S4-myc
IX	TGATCAAAAATGCGTCCGGTTGAG	sense	PolA promoter insertion
X	AAACCCGGGGTCCGTTCAGGTAGAAAC	antisense	PolA promoter insertion
XI	AGATCTAAAATGAAGGCCTCCTCAGAG	sense	GyrB promoter insertion
XII	CCTAGGGAAAGCGATGGAGACAGAG	antisense	GyrB promoter insertion
XIII	CGTCTTCACCTCCAGTTCCATC	Sense	GyrB expression analysis
XIV	GTGAAGACTGTCTCTAACGC	antisense	GyrB expression analysis
XV	AACATTGAGCTCCTTGATTCCTG	Sense	Sag1 expression control
XVI	GGAACAGTACTGATTGTTGTCTTG	antisense	Sag1 expression control

CHAPTER 5

CONCLUSIONS

In this work we set out to gain a better understanding of how the apicoplast genome is replicated and maintained during the tachyzoite stage of *T. gondii* infection. Characterization of the proteins involved not only has the potential to help us gain insights into chloroplast DNA replication in general and how the apicoplast has diverged from its algal ancestor, but may also result in new and interesting drug targets. Currently only a limited number of chloroplast DNA replication proteins have been identified in other plants and algae, but in none of these cases has the role been investigated using genetic manipulation and as a result we still have only a limited understanding of how DNA replication proceeds in plastid organelles. This study was undertaken in two main parts: in one we sought to find proteins essential for apicoplast DNA replication, and in another we wanted to investigate the role of the histone-like HU protein in replication and other aspects of apicoplast genome biology.

HU is important for apicoplast DNA replication and inheritance

Attempts had previously been made to delete the HU locus in *Plasmodium*; when this failed, it was suggested that HU was essential for parasite survival. However, we were able to obtain a genetic knockout of HU in *Toxoplasma*, which results in a very strong growth phenotype. It could be that HU plays a more critical role in the *Plasmodium* apicoplast, but it is also highly likely that the simplicity of culture conditions and drug selection for *Toxoplasma* make null mutants of important proteins easier to

obtain. If this is the case, perhaps it may serve as a cautionary tale against assuming a gene is essential when a knockout attempt in *Plasmodium* yields no survivors.

Phenotypic analysis of our HU mutants revealed a slow growth phenotype and extremely low apicoplast DNA levels, which suggested that loss of HU decreases the efficiency of DNA replication in the apicoplast or leads to DNA loss as a result of unequal segregation. The mutants also had a fairly high frequency of apicoplast loss, likely as a downstream effect of genome loss. This could be due to problems in apicoplast biogenesis, or problems in organelle segregation. We observed a decreased number of associations between apicoplasts and centrosomes in our mutants, suggesting a segregation defect. However, this decrease was fairly modest so biogenesis defects are probably also playing a role. Ultimately this indicates that topology and organization of the apicoplast DNA is important in its interactions with other DNA-binding proteins. The HU knockout we have constructed may prove to be quite helpful in elucidating the role that genome condensation and topology have on all the functions of the DNA in the plastid, including DNA replication, transcription, and segregation during plastid division.

We were also able to create an HU-specific antibody that provides a strong and specific signal in immunofluorescence assays. As there is currently no apicoplast-specific DNA dye, and staining of the apicoplast DNA by a general DNA dye like DAPI or Hoechst is often obscured by the nuclear signal, HU may be of use as an apicoplast genome marker in microscopy studies and may prove to be helpful in further studies of apicoplast genome segregation.

An essential replication factory is located at the periphery of the apicoplast

We also were interested in examining the machinery more directly related to DNA replication in the apicoplast. Previous studies had discovered several candidates in apicomplexans: genes encoding two gyrase subunits, a primase-helicase-polymerase fusion gene known as Prex, and an SSB gene [1-4]. We created parasite strains

expressing tagged versions of these genes, and confirmed their localizations to the apicoplast in *Toxoplasma*. We also created conditional mutants of GyrB and Prex in order to study their functions *in vivo*.

The staining patterns of SSB and Prex in the apicoplast seemed to be mostly at the poles of the organelle and appeared punctate, and did not precisely co-localize with luminal apicoplast proteins. This interesting observation suggests that the replication machinery is not scattered throughout the plastid but located to discrete foci. This has previously been reported in *E. coli* [5] but remains under contension [6]. It will be interesting to assess whether the discrete localization of replication foci plays a role in DNA segregation. With the conditional mutants of these proteins, perhaps studying DNA segregation into daughter apicoplasts at different time points after tetracycline induction will provide an indication of whether the replication complex plays an important role in DNA segregation. In particular, the Prex mutant will be helpful here, since it encodes multiple protein domains. Loss of Prex will likely have a major impact of complex formation on any leftover replication proteins.

The focal localization observed also raises the possibility that the DNA replication machinery is associated with the innermost plastid membrane. In plant chloroplasts, the mechanism of DNA segregation during plastid division is not yet fully known, but observations suggest that DNA segregation is carried out in association with thylakoid membranes [7]. Apicoplasts are not photosynthetic and lack thylakoids, but over the course of evolution it is possible that segregation of the plastid genome in Apicomplexans has retained a membrane association with a different membrane.

Our conditional mutants of Prex and GyrB show that these enzymes are essential for parasite survival and for apicoplast DNA replication. Our quantitative PCR assays demonstrated that loss of these proteins results in stark decreases in apicoplast DNA levels. Furthermore, our Prex conditional mutants have a high degree of reversion

to the wildtype, possibly caused by gene duplication. Replacement of the Prex native promoter with the inducible promoter, even before induction of gene silencing, seems to result in somewhat decreased levels of plastid DNA replication, suggesting that the enzyme is no longer being produced at high enough levels. The parasites may not survive well in this state for long, which would result in high selective pressure for a reversion.

Perspective

Our conditional mutants of Prex and GyrB show that these enzymes are essential for parasite survival and for apicoplast DNA replication. With these data we are able to begin to piece together the minimum requirements for DNA replication in the apicoplast. DNA gyrase seems to be a requirement and Prex is also essential but it will be important to analyze the importance of each domain separately. If all domains prove to be essential for DNA replication, then in addition to gyrase there would be essential primase, helicase, and polymerase activities as well. Studies are ongoing to create a conditional mutant of SSB, which we expect is also essential for DNA replication. A few other proteins are likely missing from this picture: DNA ligase to ligate Okazaki fragments together, if lagging strand synthesis occurs; and RNase to remove RNA primers. A sliding clamp and clamp loader may not be necessary given the small size of the genome. Overall, it seems we will soon have an almost complete picture of the players that comprise the primary replication machinery of the apicoplast in *Toxoplasma*. However, questions remain.

How is apicoplast DNA replication initiated? In bacteria, initiation of replication is the primary means by which DNA replication is regulated. The initiator protein DnaA can bind a few dnaA boxes at the origin of replication sequence OriC during most of the cell cycle when it is in its inactive state. Then upon ATP binding, it is able to bind extra dnaA boxes. In this state the protein can homo-oligomerize, and this causes melting of the

DNA at OriC, allowing other proteins such as DNA helicase to bind a single-stranded region and begin the replication process [8]. In apicomplexans we now have good candidate genes for a primase and helicase but no DnaA homologs have been identified so far. DnaJ has previously been shown to bind the inverted repeat region of the apicoplast genome in *Plasmodium* [9], but DnaJ in other systems primarily serves as a chaperone, so what effect DnaJ binding has on apicoplast DNA replication is still unclear. Elucidating the mechanisms of replication initiation in the apicoplast will help reveal whether apicoplast DNA replication is regulated at all, and to what degree.

Why is the mechanism of apicoplast DNA replication different in *Toxoplasma* and *Plasmodium*? Previous studies have suggested that the *Toxoplasma* apicoplast genome is mostly present in linear tandem arrays and replicates by a rolling circle mechanism, while the *Plasmodium* apicoplast genome is mostly present in circular form and replicates primarily using a D-loop mechanism [10, 11]. However, not only is the apicoplast genome highly conserved between these two organisms, but this work suggests that the bulk of the replication machinery also seems to be shared. So what is responsible for the differences in topology or replication mechanisms? At this point it is still hard to say, and the difference may be due to proteins involved in replication that have not yet been discovered in the apicoplast. For instance, an undiscovered endonuclease may be important in initiating rolling circle replication in *Toxoplasma*, as it is in plasmids and phages [12]. However, at present there are a few differences we are aware of in apicoplast genome biology between the two species.

First, there are some structural differences in DNA-binding proteins in the apicoplast. Gyrase B, for example, was found to have an additional 45-amino acid toprim (topoisomerase-primase) domain in *Plasmodium* that is not present in the *Toxoplasma* homolog [13]. This domain was found to be important for its ATPase activity. In addition, the *Plasmodium* HU was found to lack a residue that is normally associated with DNA

bending [4]. This residue is present not only in bacterial versions of HU but also in the *Toxoplasma* homolog. Perhaps structural differences like these could help to account for differences in replication mechanisms, although how they would do this is still unclear.

We also know that *Toxoplasma* possesses a homolog of RecG that is not present in any of the other apicomplexans whose genomes are sequenced, except for *Neospora*. This gene was found in our laboratory to be non-essential, and the knockout line also appears not to have any altered sensitivity to Ciprofloxacin (Lilach Sheiner and Sarah Reiff, unpublished observation). RecG is involved in branch migration of holliday junctions and helps carry out DNA repair in *E. coli*. If the *Toxoplasma* homolog was similarly involved in repair of double strand breaks in the apicoplast, we would expect the mutant to exhibit increased sensitivity to ciprofloxacin [14], but this is not the case. It is possible that it is important for a different type of DNA repair, but alternatively it may have a non-essential role in DNA replication. To investigate whether it is important for rolling circle replication it would be interesting to perform electron microscopy on isolated apicoplast DNA in wildtype and RecG mutant parasites. However, the *Eimeria* genome appears not to encode a homolog, but its apicoplast genome is still present in linear tandem arrays [15], so the RecG enzyme may prove not to be a contributing factor, either.

The apicoplast genomes of *Toxoplasma* and *Plasmodium* are highly conserved and both are quite AT-rich, but to slightly different degrees – *Plasmodium* is 85% AT-rich while *Toxoplasma* and *Eimeria* are 79% AT-rich [16]. It is possible then that the differences in replication mechanisms are due to the way in which DNA replication is initiated, and this in turn would be dependent upon origin sequences. In plastid genomes, initiation of replication generally happens in the inverted repeat region. Slight sequence differences in these regions may lead to differences in the way in which DNA replication is initiated, which in turn may affect the way in which replication proceeds

across the apicoplast genome. However, there is much more to learn about how DNA replication is initiated in the apicoplast of *Toxoplasma* and *Plasmodium* before we will have a clear picture of whether this is a major factor contributing to mechanisms of replication in the apicoplast.

In conclusion, there is still much to be learned about apicoplast DNA biology, but the genetic tools available in *Toxoplasma* will be a great help in answering these questions. Conditional knockouts provide a good way to assess how essential a protein is to the parasite, and we now know that conditional mutants of plastid DNA replication proteins can be constructed and their phenotypes measured. The research presented here provides a good foundation for our understanding of how the apicoplast DNA is replicated and maintained. These studies not only highlight plastid DNA replication as an effective drug target, but may also provide insight into how DNA replication occurs in other plastids as well.

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APPENDIX A1

BUILDING THE PERFECT PARASITE: CELL DIVISION IN APICOMPLEXA[2]

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Synopsis

Apicomplexans are pathogens responsible for malaria, toxoplasmosis and crytposporidiosis in humans, and a wide range of livestock diseases. These unicellular eukaryotes are stealthy invaders, sheltering from the immune response in the cells of their hosts, while at the same time tapping into these cells as source of nutrients. The complexity and beauty of the structures formed during their intracellular development have made apicomplexans the darling of electron microscopists. Dramatic technological progress over the last decade, has transformed apicomplexans into respectable genetic model organisms. Extensive genomic resources are now available for many apicomplexan species. At the same time parasite transfection has enabled researchers to test the function of specific genes through reverse and forward genetic approaches with increasing sophistication. Transfection also introduced the use of fluorescent reporters opening the field to dynamic real time microscopic observation. Parasite cell biologists have used these tools to take a fresh look at a classic problem: how do apicomplexans build the perfect invasion machine, the zoite, and how is this process fine-tuned to fit the specific niche of each pathogen in this ancient and very diverse group. This work has unearthed a treasure trove of novel structures and mechanisms that are the focus of this review.

A lean and mean invasion machine

A wide variety of pro- and eukaryotic pathogens have evolved the ability to invade and replicate within the cells of their hosts. Few have developed the level of sophistication and control exerted by the members of the Apicomplexa [1]. Upon contact with a suitable host cell, Apicomplexans can invade within seconds, with minimal apparent disturbance of the infected cell (Figure A1.1). This process is dependent on actin and myosin and is driven by parasite and not host motility [2,3]. Tightly associated with host cell penetration, is the secretion of three distinct parasite organelles - rhoptries,

micronemes and dense granules. Secretion is timed in succession and secreted proteins play key roles in adhesion, motility and formation and elaboration of the parasitophorous vacuole, a new cellular compartment established during invasion that the parasite occupies during its intracellular development (see [4,5] for detailed reviews of this process in *Toxoplasma* and *Plasmodium* respectively).

The cellular structure of the zoite, the non-replicative extracellular stage, appears streamlined towards one goal: finding and invading the next host cell. Zoites are found at various stages of the apicomplexan life cyle and are the product of asexual as well as sexual replication processes (see Figure A1.1A for a simplified apicomplexan life cycle). The zoite is highly polarized, with the apical tip containing the organizing center for the subpellicular microtubles that run along the longitudinal axis of the parasite [6]. This axis also polarizes the cell's motility, driving the parasite into host cells with its apex first. In some species the tip is further elaborated by the conoid, a cytoskeletal structure which is built from a unique, tightly wound tubulin polymer, and that is extended during invasion and motility [7]. Importantly, the apical end is also the site for rhoptry and microneme secretion, with these organelles tightly packed into the anterior portion of the cell. While the anterior of the zoite is focused on invasion, the rest of cell carries the genetic material and tools to grow and develop once in the host cell, including a nucleus and a single mitochondrion, plastid, and Golgi.

Divide and conquer

While invasive zoites are similar across the phylum, intracellular stages differ dramatically in size, shape and architecture (see Figure A1.2 for a selection of micrographs). The basis for this diversity lies in the flexibility of the apicomplexan cell cycle. Apicomplexans are able to dissociate and variably mix and match three elements that follow each other invariably in most other cells: DNA replication and chromosome segregation, nuclear division, and lastly cytokinesis or budding (see Figure A1.3 for a

schematic). While *Toxoplasma* completes all elements of the cycle after each round of DNA replication, *Plasmodium* and *Sarcocystis* forgo cytokinesis and/or nuclear divisions for multiple cycles, forming stages that are multinucleate or contain a single polyploid nucleus (these division modes are also known as endodyogeny, schizogony and endopolyogeny [8-10]. These differences are not limited to different species but also occur between different life cycle stages in a single species, asexual stages of Toxoplasma in the cat intestine e.g. divide by endodyogeny and endopolygeny [11]. In each case, however, the development will culminate in the emergence of multiple invasive zoites, which seek new host cells to invade. Apicomplexans of the genus *Theileria* are a surprising exception to this divide and conquer scenario. *Theileria* sporozoites remain in the lymphocyte that they initially invade, where they amplify in numbers without resorting to leaving the shelter of the host cell. The key to this trick lies in this parasite's ability to transform the host cell through manipulation of the NFkB pathway. The parasite assembles and activates a mammalian IKK signalosome on its surface, promoting unchecked host cell replication [12,13]. *Theileria* also interacts with host cell microtubules, enabling these parasites to migrate to, and apparently latch onto, host cell centrosomes. This results in partitioning of parasites into forming daughter cells of the host exploiting the host's mitotic spindle (see Figure A1.2 and A1.3 [12,14] and D. Dobbelaere, personal communication).

Checkpoints and master switches

Initial work using inhibitors of DNA synthesis (e.g. aphidocolin) and microtuble disrupting agents suggested that classical cell cycle checkpoints might be lacking in apicomplexans [15,16], pointing to potentially novel mechanisms of control over their complex cell cycles. However, studies using different blocking agents (thymidine, pyrrolidine dithiocarbamate) and characterization of a series of temperature sensitive mutants have found that the *Toxoplasma* cell cycle can be halted at what appear to be

specific points, including the G1/S and S/M boundaries [17-19]. Furthermore, genomic and experimental surveys for proteins commonly associated with cell cycle checkpoints have identified numerous candidates including cyclins, and cyclin dependent kinases in *Plasmodium* and *Toxoplasma* [20-23]. An attractive model could suggest the presence of developmentally regulated sets of cell cycle factors resulting in different cell division types, which are in turn controlled by master switches. For example, we could hypothesize that *Toxoplasma* tachyzoites contain master switches to promote nuclear division following DNA synthesis, and cell division following mitosis. Down-regulation of the nuclear division master switch would result in the multiple rounds of DNA synthesis observed during *Sarcocystis* endopolygeny, while down-regulation of the cytokinesis master switch would lead to the multinucleated schizonts observed in other stages of the *Toxoplasma* life cycle, as well as in *Plasmodium* blood stages. Some initial support for this idea has begun to emerge. A series of homologues of the centrosome associated NIMA kinase (which in fungi controls entry into mitosis and spindle formation) have been shown to be essential for cell cycle progression and survival in *Plasmodium* through gene targeting studies [24-27], and in *Toxoplasma* through analysis of temperature sensitive parasite mutants (M.-J. Gubbels & B.S., unpublished). NIMA genes appear to be differentially expressed over the Plasmodium life cycle. Nek4, for example, is specifically expressed in the female gametocyte and required for the initial chromosome duplication in the ookinete (zygote) preceding meiosis [25,26], but is dispensable in other stages.

Counting chromosomes

A fascinating question when considering the various forms of apicomplexan cell division, is how do parasites keep track of their chromosomes in polyploid stages and how do they know how many zoites to make upon cytokinesis? Two observations might be important: the final budding of zoites is invariably associated with a last round of DNA

replication and nuclear division, and studies that have used high doses of microtubule disrupting agents have found this to lead to a catastrophic breakdown of the coordination of nuclear division and budding in a variety of species [15,28-30]. This suggests that the mitotic spindle, or its organizing center, controls the number of daughter cells and the site where they are to be formed. Apicomplexans use an intranuclear spindle and maintain the nuclear envelope throughout mitosis. The spindle resides in a dedicated elaboration of the nuclear envelope, the centrocone ([31]; Figure A1.4A), and interacts with the cytoplasmatic centrosome through an opening of the envelope. Interestingly, recent studies in *Toxoplasma* and *Sarcocystis* using antibodies to tubulin and MORN1 (a protein that localizes to the centrocone, see below) have shown that the centrocone is maintained throughout the cell cycle [29,32]. Persistence of the spindle, and persistent kinetochore attachment of chromosomes to the spindle microtubules, would provide a mechanism to maintain the integrity of chromosomal sets through polyploid stages [29], however this hypothesis requires experimental validation. While centrocone-like structures have been identified in *Plasmodium* during mitosis and budding [9,33], it is currently not clear if these persist (developing reagents to the *Plasmodium* homolog of the MORN1 protein should quickly resolve this question).

Building the zoite scaffold

Apicomplexans preassemble zoites as buds either internally in the cytoplasm (*Toxoplasma*) or directly under the surface membrane (*Plasmodium*). The scaffold for bud assembly and the outline of the new daughter cells is provided by the pellicle, which consists of subpellicular microtubules and the inner membrane complex (IMC). The subpellicular microtubules emerge from an apical microtubule organizing center associated with the polar rings and run along the longitudinal axis of the cell [34,35]. The IMC is a system of flattened membrane cisternae stabilized by a protein meshwork associated with its inner membrane. Several of the protein components of this meshwork

have been characterized and they share weak similarity with articulins, filament proteins found in ciliates [36-38]. Several IMC proteins show dynamic regulation, with their expression timed to coincide with budding [29]. Some IMC proteins also undergo proteolytic processing, a process suggested to confer increased rigidity to the IMC following its deposition [38,39]. More recently, proteins integral or tightly associated with the outer IMC membrane have been identified. GAP 50 together with GAP 45 serves as internal anchors of myosin A and the associated gliding motility machinery [40,41], the function of PHIL1 which forms a ring structure at the apical tip of the bud remains to be elucidated [42].

Following mitotic separation of the chromosomes, budding initiates in the direct vicinity of the centrosomes. The first identifiable sign of the bud is a flattened vesicle associated with a small number of evenly spaced microtubules [8,31,43,44]. This structure is further elaborated into a cup, with the conoid at its apex and microtubules extending from the conoid to posterior ring delimiting the bud. Genetic and proteomic studies in *Toxoplasma* have identified a number of proteins associated with these early processes, and fluorescent protein tagging and live cell microscopy has painted a highly dynamic picture of their localization and function. The *T. gondii* genome encodes several centrin genes, with centrin 1, 2 and 3 having been localized by GFP fusion [45-47]. While centrin 1 and 3 appear to be focused at the centrosome, centrin 2 additionally labels the conoid and a peculiar group of punctate structures in the apex of the cell [45,46]. Dynein light chain, a component of the dynein minus end directed microtubular motor, has been detected near the centrosome and the conoid, and may be involded in conoid and centrosomal movements. MORN1 has been particularly informative as a marker for budding, as it labels both the centrocone/spindle and the apical and posterior ends of the bud (Fig. A1.4, [32,46]). The precise chronology of assembly – especially in the very early phase of bud development – remains to be elucidated, and would benefit

from the generation of mutants for the various steps involved. Early electron microscopic studies have implicated a striated fiber as an organizing element [44], interestingly, proteins similar to algal striated fiber assemblins have been identified recently in apicomplexans and have been shown to localize to the centrosomal region during budding [48]. Once the bud is assembled it grows rapidly, most likely driven by microtubule growth. This process runs opposite to spindle extension and effectively partitions the nucleus and much of the cytoplasm. Toward the end of bud development, the MORN1 ring at the posterior end of the bud shows pronounced contraction (see Figure A1.4 J-K), which likely aids in organellar division (see below) and cytokinesis. Several observations are consistent with an association of this ring with myosin B/C [32,49], however the actin destabilizing drug cytochalasin D does not interfere with parasite division [15].

Completing parasite assembly

A fully-formed Apicomplexan parasite requires a multitude of organelles and intracellular structures that will enable it to carry out the next task of its life cycle – to egress from the host cell and invade a new one. Rhoptries, micronemes and dense granules form *de novo* during budding, anterior to the nucleus, endowing each daughter cell with the apical secretory organelles necessary for invasion. Expression of rhoptry and microneme proteins is regulated at the transcriptional level and timed to conincide with budding [50-53]. The apicomplexan secretory pathway is highly polarized, with an ER exit site localized on the apical face of the nucleus adjacent to the centrocone [43,54,55]. Here proteins are loaded into coated vesicles that travel to the Golgi and on to several (still poorly characterized) trans-Golgi, pre-rhoptry and pre-miconeme compartments [54,56,57]. The Golgi is associated with the centrosome(s), which play an important role in its duplication [58]. Golgi duplication is among the earliest events of budding [47,59]. In *Plasmodium*, the Golgi divides multiple times during intracellular

development, and upon zoite formation a single Golgi is associated with each bud [60]. The spatially fixed line-up of ER exit site and Golgi and their association with the nucleus and centrosome likely acts as a highly effective cellular 'funnel', directing the flow of proteins and membranes into the growing buds. IMC proteins, including the N-glycosylated GAP50 [40], probably derive from the Golgi, suggesting that membranes of the IMC form from Golgi-derived vesicles. This would explain the necessity for early division of the Golgi during budding, and suggests that Golgi positioning by the centrosome is critical in mediating deposition of the IMC.

Apicomplexans harbor two endosymbiont-derived organelles, the mitochondrion and the apicoplast, both of which perform a broad array of metabolic functions and are essential for intracellular parasite development [61-64]. These organelles carry their own genomes [65-69] and therefore cannot be formed *de novo*, but must undergo division followed by segregation into buds. Genomic analyses in apicomplexans have identified proteins commonly involved in mitochondrial division, like dynamin related proteins [62]. However, the FtsZ based division machine found in a wide variety of chloroplasts has been lost in apicomplexans [70,71]. Instead of relying on their ancestral prokaryotic division ring, it would appear that apicoplasts have developed novel means of division. One model suggests that the force for apicoplast division is provided by association of the apicoplast with the mitotic spindle [72]. Dynamic association between the centrosome(s) and the apicoplast has been demonstrated in *Toxoplasma* and *Sarcocystis* and provides a likely means by which these organelles are properly segregated into forming buds [29,72]. In both organisms fission of the organelle into daughter plastids is tightly associated with budding, and the constrictive MORN1 ring found at the posterior end of each bud provides an attractive candidiate for a fission mechanism (see Figure A1.4C, [32,71]). A second model suggests that apicoplast fission is independent of cytokinesis and relies on a medial division ring formed by yet to

be identified components [73]. The development of the plastid in organisms dividing by schizogony, like *Plasmodium* and *Eimeria*, is not fully understood [74,75]. While centrosome association is likely to be involved in the segregation into daughters, it is unclear if such association occurs in earlier stages. In *Plasmodium*, mitochondria and apicoplasts form a physical association shortly before budding [74], suggesting that segregation of these organelles into daughter buds is tightly linked. Nevertheless, better *in vivo* markers (especially for the centrosome) are needed to identify mechanisms of organellar division and segregation in these organisms.

Outlook

The advent of reverse genetics for a variety of apicomplexans has led to a renaissance in studying the cell biology of these parasites. A number of exciting new structures and mechanisms have been discovered in this process. Not unlike the study of host cell invasion, exploring the intracellular development of apicomplexans has brought out conserved themes at the mechanistic level, suggesting significant similarity between different species within the phylum. The ‘post-genomic’ era of apicomplexan cell biology offers powerful experimental avenues that will undoubtedly drive our understanding of cell division and zoite formation. Gene expression profiling using microarrays, now available for several systems, has identified large groups of candidate genes that are expressed during budding. Comparative genomic analysis can be used to further narrow the list of candidates. The ever improving forward and reverse genetics tool box offers robust experimental avenues to test the function of essential genes, and genetic analysis will be critical to establish the sequence of events during budding [76]. The coming years will likely reveal an increasingly detailed and mechanistic picture of these tiny diabolical, yet fascinating, invasion machines.

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Tables and Figures

Pathogen	Disease	Host	Transmission
Plasmodium falciparum	Malaria	Human	Mosquito bite
Toxoplasma gondii	Encephalitis	Human, variety of animals	Meat, water
Cryptosporidium parvum	Enteritis	Human, cattle	Water
Theileria parva	Lymphoproliferation	Cattle	Tick bite
Eimeria tenella	Enteritis	Chicken	Fecal-oral
Sarcocystis neurona	Myeloencephalitis	Horse, variety of animals	Water, fecal-oral

doi:10.1371/journal.ppat.0030078.t001

Table A1.1: Apicomplexan Parasites.

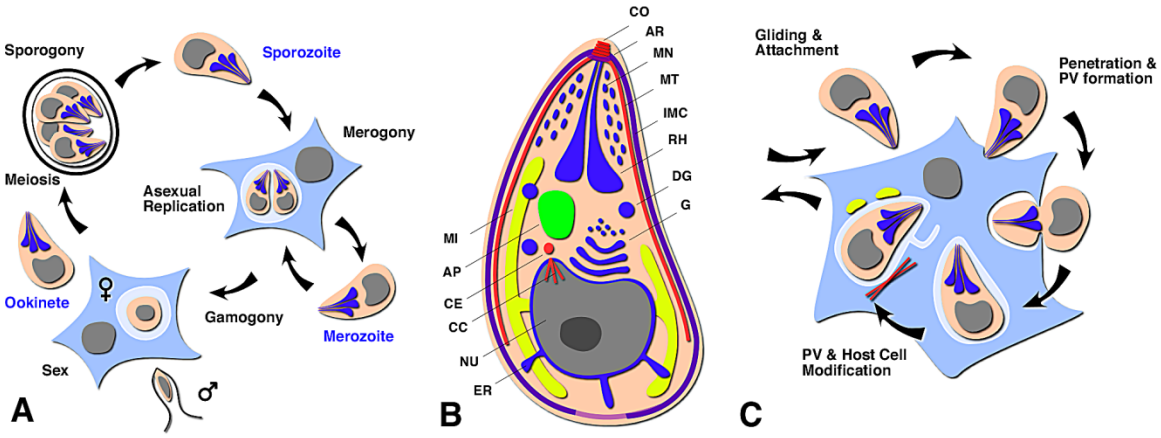

Figure A1.1: Apicomplexa are intracellular parasites. (**A**) Highly simplified apicomplexan life cycle. Apicomplexans are haplonts and meiosis (sporogony) immediately follows fertilization. Fertilization occurs within a host cell or less frequently extracellulary giving rise to an invasive stage zygote (ookinete). (**B**) Schematic representation of a zoite (not all structures are present in all apicomplexans). AP, apicoplast; AR, apical rings; CC, centrocone; CE, centrosome; CO, conoid; DG, dense granule; ER, endoplasmic reticulum; GO, Golgi; IMC, inner membrane complex; MI, mitochondrion; MN, microneme; MT, subpellicular microtubule; RH, rhoptry. (**C)** Zoites actively invade the cells of their hosts establishing a specialized parasitophorous vacuole (in some species the parasite lyses the vacuole and develops free in the cytoplasm).

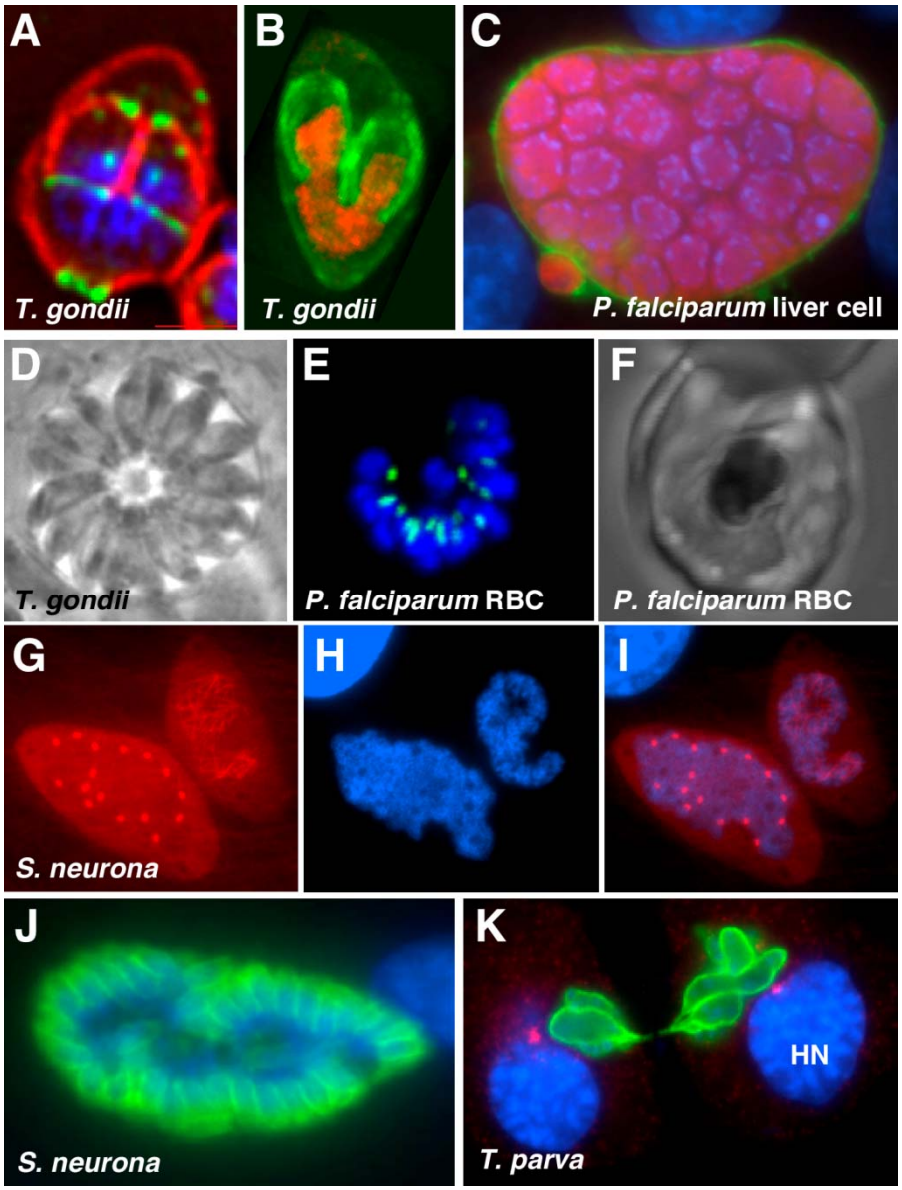

Figure A1.2: The diversity of intracellular development in apicomplexans. (A) *T. gondii*, two daughters are formed during budding, IMC1, red; MORN1, green (reproduced with permission from [32]). (B) *T. gondii,* Histone H2, red; IMC3, green (reproduced from [71]). (C) *P. falciparum* liver schizont, budding results in massive numbers of zoites (courtesy to Volker Heussler). (D) *T. gondii*, phase. (E-F) *P. falciparum* late erythrocyte schizont, ACP (plastid) green. (G-I) *S. neurona* two intracellular stages with polyploid nuclei, one in interphase one during mitosis; red, tubulin. (J) *S. neurona* budding, green, IMC3. (K) A *Theileria* schizont divideds in associations with its host cell; PIM (parasite surface), green; g-tubulin (host centrosomes), red (courtesy to Dirk Dobbelaere). The DNA dye DAPI is shown in blue throughout. Not to scale.

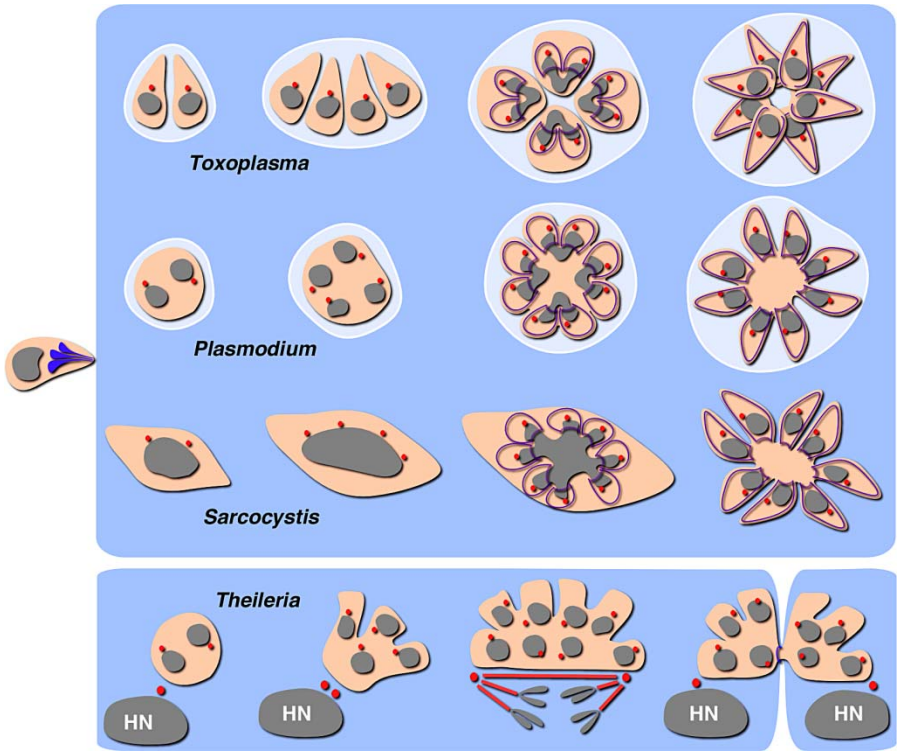

Figure A1.3: The flexibility of apicomplexan cell division. Schematic outline of cell division by *Toxoplasma* (endodyogeny), *Plasmodium* (schizogony) and *Sarcocystis* (endopolygeny). The *Theileria* schizont is divided in association with host cell division (HN, host nucleus). DNA, grey; IMC, purple, centrosome, red. Note that a centriole as center of the spindle plaque body has not been clearly demonstrated in the *P. falciparum*. Both *Sarcocystis* and *Theileria* develop directly in the host cell cytoplasm while *Toxoplasma* and *Plasmodium* are contained within a parasitophorous vacuole (light blue).

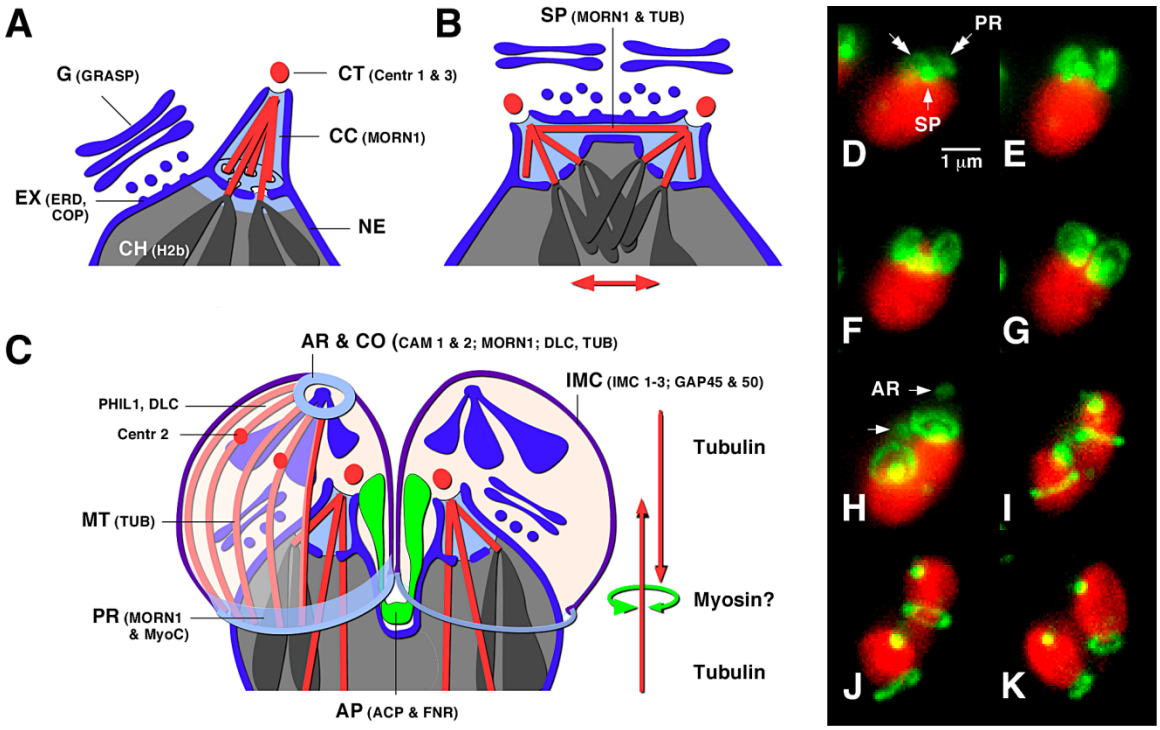

Figure A1.4: The mechanics of apicomplexan mitosis and budding. Schematic representation of the nucleus during interphase (**A**), mitosis (**B**), and mid-stage budding (**C**). EX, ER exit site; PR, posterior ring (see Fig. 2 for additional organelle abbreviations). Smaller type abbreviations refer to organelle specific marker proteins in *T. gondii* (most are available as flurescent protein *in vivo* tags, see text for further detail and reference). (D-K) Time lapse series of nuclear division in *T. gondii* reproduced from [32]. The nucleus is labeled in red (Histone H2b-RFP) MORN1 in green (MORN1-YFP).

APPENDIX A2

A NOVEL DYNAMIN-RELATED PROTEIN HAS BEEN RECRUITED FOR APICOPLAST FISSION IN *TOXOPLASMA GONDII*[1]

[1] Van Dooren, G.G., Reiff, S.B., Tomova, C., Meissner, M., Humbel, B.M., Striepen, B. 2009. A novel dynamin-related protein has been recruited for apicoplast fission in *Toxoplasma gondii*. *Curr Biol.* 19(4): 267-276.

A2.1 Summary

Background

Apicomplexan parasites cause numerous important human diseases including malaria and toxoplasmosis. Apicomplexa belong to the Alveolata, a group that also includes ciliates and dinoflagellates. Apicomplexa retain a plastid organelle (the apicoplast) that was derived from an endosymbiotic relationship between the alveolate ancestor and a red alga. Apicoplasts are essential for parasite growth and must correctly divide and segregate into daughter cells upon cytokinesis. Apicoplast division depends on association with the mitotic spindle, although little is known about the molecular machinery involved in this process. Apicoplasts lack the conserved machinery that divides chloroplasts in plants and red algae, suggesting that these mechanisms are unique.

Results

Here we demonstrate that a dynamin-related protein in *Toxoplasma gondii* (*Tg*DrpA) localizes to punctate regions on the apicoplast surface. We generate a conditional dominant-negative *Tg*DrpA cell line to ablate *Tg*DrpA function and demonstrate that *Tg*DrpA is essential for parasite growth and apicoplast biogenesis. Fluorescence recovery after photobleaching and time-lapse imaging studies provide evidence for a direct role for *Tg*DrpA in apicoplast fission.

Conclusions

Our data suggests DrpA was likely recruited from the alveolate ancestor to function in fission of the symbiont, and ultimately replaced the conserved division machinery of that symbiont.

A2.2 Introduction

Plastid organelles trace their evolutionary origins to cyanobacteria that were incorporated into eukaryotic cells by a process of endosymbiosis. This evolutionary history dictates that they cannot be formed *de novo*. Instead, existing plastids divide to give rise to daughter organelles that partition into daughter cells upon cell division. Previously studied plastids contain an FtsZ-based division apparatus, retained from the cyanobacterial endosymbiont [1]. In addition, plant and red algal plastid division involves a dynamin-like protein called ARC5 [2, 3].

Apicoplasts, the non-photosynthetic plastids of apicomplexan parasites, must correctly divide and segregate into daughter cells for parasites to remain viable [4]. Surprisingly, apicomplexan genomes lack homologues to both ARC5 and FtsZ [5], suggesting that apicoplast division is mechanistically different to that in previously studied plastids. One striking difference is the association of the apicoplast with the centrosomes of the mitotic spindle [6, 7]. This association is thought to ensure proper segregation during cytokinesis, parcelling out apicoplasts to a highly variable number of daughter cells formed in the complex apicomplexan budding process [8]. While centrosome association provides a unifying model for segregation, it remains unclear how apicoplast fission occurs. One model suggests that fission depends on force generated by daughter cell budding [6], while electron microscopic studies identify apparent plastid division rings [9, 10], suggesting that protein components may mediate fission.

Apicomplexan genomes encode three dynamin-related proteins that are phylogenetically distinct from ARC5. Dynamins are large GTPase proteins that function in a range of contractile processes, including the scission of endocytic vesicles in metazoan cells and the fission of mitochondria and peroxisome organelles [11], and we were interested in whether these had a role in apicoplast division. In this study, we

characterise dynamin-related protein A (DrpA) in the apicomplexan *T. gondii*. We demonstrate that *Tg*DrpA is required for apicoplast fission, and we present a detailed model for how *Tg*DrpA functions in this process.

A2.3 Results

T. gondii contains three dynamin-like proteins that are phylogenetically distinct from ARC5 dynamins.

Using previously characterised proteins from yeast, plants and red algae, we performed homology searching for dynamin-related proteins in apicomplexan parasites. We examined the genomes of *T. gondii*, *Plasmodium yoelii*, *P. falciparum, Theileria parva* and *Cryptosporidium parvum*. In each organism, we identified two characteristic dynamin-related proteins. To ascertain the evolutionary history of apicomplexan dynamins, we performed phylogenetic analyses on a multiple sequence alignment of a broad spectrum of dynamin-related proteins. These indicated that ARC5/Drp5B dynamins from plants, diatoms, green and red algae cluster together with strong bootstrap support, and are sister to a group of dynamins involved in cytokinesis {Miyagishima, 2008 #850}. These ARC5 and related proteins are clearly distinct from apicomplexan dynamins (Figure A2.1). One apicomplexan dynamin group (that we term Dynamin-related protein B or DrpB) forms a well-supported clade that includes dynamins from ciliates, a phylum of alveolates related to Apicomplexa (Figure A2.1; ref. [12], Breinich et al, this issue). The other apicomplexan dynamin (which we term DrpA) forms a clade with ciliate dynamins that is not well supported by bootstrap analysis. Removing ARC5 and related sequences enables us to incorporate more characters in our analysis and this additional data suggests that DrpA also forms an alveolate-specific clade (Figure A2.S1A). A third protein with some similarity to dynamins is also present in apicomplexan genomes. These so-called DrpC proteins match only to the GTPase

domain of dynamins and phylogenetic analyses indicate that DrpCs are very divergent from other dynamin proteins (Figure A2.S1B). We conclude that ARC5 is not a conserved component in apicoplast division.

_Tg_DrpA localises to the periphery of the apicoplast.

To characterise the function of DrpA in *Toxoplasma gondii* we examined its localisation. We generated parasites expressing the entire open reading frame of *Tg*DrpA fused to an N-terminal HA tag, expressed from the native *Tg*DrpA promoter, and performed immunofluorescence assays. *Tg*DrpA localises in many small patches throughout the cytosol of *T. gondii*, while a major component of *Tg*DrpA fluorescence clusters at the apical end of the cell (Figure A2.2A). Co-localisation with the apicoplast stromal marker acyl carrier protein (ACP) indicates that this cluster occurs around the periphery of the apicoplast (Figure A2.2A). During division, apicoplasts form a distinctive U-shape, with the base of the "U" being the point of organelle fission [6, 10]. In dividing apicoplasts, we typically observed *Tg*DrpA localising to this point of fission, as well as to the ends of the organelle (Figure A2.2B-C, arrows). Later in apicoplast fission, when the base of the apicoplast becomes more constricted, the punctate dot of *Tg*DrpA observed early in the process appears to disperse into a more tubule-like structure between the dividing apicoplasts (Figure A2.2C, arrowheads). We found that *Tg*DrpA does not localise to the Golgi (Figure A2.S2A), and only occasionally to the mitochondrion (Figure A2.S2B).

We performed an anti-HA Western blot on proteins extracted from the HA-DrpA cell line. This revealed the presence of a band of approximately 90 kDa, conforming to the expected size of HA-tagged DrpA (Figure A2.2D). We next performed protease protection assays in conditions where cytosolic but not apicoplast stromal markers were accessible to thermolysin. We found that *Tg*DrpA was sensitive to thermolysin (Figure A2.S2C), consistent with *Tg*DrpA localising to the cytosol, although in the absence of

clear markers for the four membranes that surround the apicoplast, our data cannot rule out the possibility that DrpA might localise to one or more of these intermembrane spaces.

***Tg*DrpA is essential for parasite growth.**

Having established the localisation of *Tg*DrpA, we next wanted to determine its function. Dynamin proteins are self-assembling GTPases containing an N-terminal GTPase domain, a middle domain and a C-terminal GTPase effector domain (GED; Figure A2.3A, top). Expression of dynamins with mutations in the GTP-binding site has been shown in other systems to specifically disrupt dynamin function in a dominant-negative fashion (eg. [16, 17]). We generated a dominant-negative DrpA where a lysine in the GTP-binding site was changed to an alanine ($DrpA^{K42A}$). To generate stable cell lines inducibly expressing dominant-negative DrpA, we fused a destablisation domain (DD) tag to the N-terminus of $DrpA^{K42A}$ (Figure A2.3A, bottom). DD-tagging promotes proteosomal degradation of the protein, with degradation prevented by the small molecule Shield-1 [18, 19]. To determine whether we could regulate expression of DD-$DrpA^{K42A}$, we grew parasites for 0 to 20 hours on 0.1 µM Shield-1, extracted proteins and performed Western blotting. In the absence of Shield-1, we detected low levels of DD-$DrpA^{K42A}$ (Figure A2.3B). Levels increased 3 hours after the addition of Shield-1, and were maximal after about 9 hours (Figure A2.3B).

To determine whether DrpA is essential for parasite growth, we expressed tandem-yellow fluorescent protein (YFP) in DD-$DrpA^{K42A}$ mutant parasites, and monitored growth using a fluorescence growth assay [20]. DD-$DrpA^{K42A}$ mutant parasites grew robustly in the absence of Shield-1 (Figure A2.3C, bottom). However, compared to wild-type parasites (Figure A2.3C, top), growth of DD-$DrpA^{K42A}$ parasites in 0.1 µM Shield-1 was slowed after about 5 days. Preincubation of DD-$DrpA^{K42A}$ mutant parasites in Shield-1 for three days resulted in negligible growth of the parasites.

As a second measure for parasite growth, we performed plaque assays. *T. gondii* parasites egress from host cells and invade nearby cells. Over time this parasite growth forms zones of clearance (plaques) in the host cell monolayer. Plaque size is therefore an indication of growth, and plaque number an indication of parasite viability. We grew *Tg*DrpA^{K42A} mutant parasites in the absence or presence of Shield-1 for 9 days. In the presence of Shield-1 we saw a severe reduction in plaque size compared to the no-Shield-1 control (Figure A2.S3A), consistent with the importance of *Tg*DrpA for parasite growth. We also preincubated parasites for 12 hours in the presence of Shield-1, washed out the drug for a further 12 hours, and set up plaque assays in fresh flasks in the absence or presence of Shield-1. As expected, parasites grown in the presence of Shield-1 exhibited severe defects in growth. Interestingly, parasites preincubated with Shield-1 for 12 hours then grown in the absence of Shield-1 had plaques of a similar size to parasites grown entirely in the absence of Shield-1, but contained approximately 63% the number of plaques observed in the no Shield-1 control. This suggests that approximately 40% of parasites are no longer viable after a 12 hour incubation in Shield-ion, HU.%□□□□□Ő□□□Ő□ ▪ ▪笮鈌▪Ű□Ā

function results in a delayed death effect that is typical of processes affecting the apicoplast [4, 21].

***Tg*DrpA is essential for normal apicoplast morphology and biogenesis.**

To directly test the impact of loss of DrpA function on the apicoplast, we generated a DD-DrpA^{K42A} mutant cell line that targeted red fluorescent protein (RFP) to the apicoplast. In the absence of Shield-1, apicoplast morphology appeared normal, with a single apicoplast organelle localising to the apical end of each parasite (Figure A2.4A). Upon incubation in Shield-1, we observed severe defects in apicoplast biogenesis (Figure A2.4B-D). Apicoplasts frequently occurred as branched tubules that appeared to connect several cells within a vacuole (Figure A2.4C-D). We also observed apicoplasts

mis-localised to the basal end of parasites (Figure A2.4B) or entirely missing from one or more parasites within a vacuole (Figure A2.4C-D). To quantify these defects, we grew DD-DrpA^{K42A} mutant parasites for 0, 3, 6, 9, 12 and 20 hours in Shield-1. We imaged 100 four cell vacuoles and scored apicoplast morphologies into four categories: normal apicoplasts (as in Figure A2.4A), basal stunted (Figure A2.4B), basal elongated (Figure A2.4C-D), and cells where apicoplasts were absent (Figure A2.4C-D). In the absence of Shield-1, most apicoplasts appeared normal (Figure A2.4E). After six hours of growth on Shield-1, most apicoplasts localised to the basal end of the cell and were stunted in appearance. After around 12 hours an increasing number of the basally-localised apicoplasts were elongated, while approximately 40% of parasites had lost their apicoplast (Figure A2.4E). This value correlates to the loss of viability in 40% of parasites after 12 hour incubation in Shield-1 (Figure A2.S3A), and we hypothesise that the growth defects we observe in the *Tg*DrpA^{K42A} mutant results from loss of the apicoplast.

To gain a dynamic understanding of the observed phenotypes, we performed time-lapse imaging of mutant parasites expressing cytosolic YFP and apicoplast-targeted RFP. We added Shield-1 to parasites 6 hours before commencing imaging. Initially there were no obvious defects in apicoplast morphology, with both cells in the two-cell vacuole containing a single, apically-localised apicoplast (Figure A2.4F; Supplemental Movie 1). After approximately 150 minutes, apicoplasts from both cells formed a “U” shape, typical of apicoplast immediately preceding fission [6, 10]. Subsequent imaging revealed that apicoplasts are unable to divide. Approximately 20-30 minutes later, cytokinesis commenced, with apicoplasts not dividing and becoming localised to the basal end of the cell (Figure A2.4F). We followed this vacuole for a further 6 hours. Apicoplasts remained localised to the basal end of each cell in the vacuole, remaining in a small “stumpy” form. After about 5 hours, apicoplast began

elongating from the basal end of the cell. These data suggest that apicoplast morphology is normal up to the point of apicoplast division. Unable to divide, apicoplasts localise to the basal end of the cell and elongate before the next round of cell division.

We sought to quantify the basal localisation of apicoplasts in the DD-DrpA^{K42A} mutant. We generated a DD-DrpA^{K42A} mutant cell line expressing YFP-MORN1 and apicoplast RFP. MORN1 forms a contractile, basal complex in parasites, in addition to labelling the centrocone and growing daughter bud (Figure A2.5A; [22, 23]). In the presence of Shield-1, apicoplasts exit parasites through the YFP-MORN1 labelled basal complex (Figure A2.5B, arrows), with the apicoplast constricting at the basal complex. We grew parasites for 24 hours on Shield-1 and measured the distance between the basal complex to the apicoplast of the same cell. In the absence of Shield-1, the average distance of the apicoplast from the basal complex is 2.710 μm (standard deviation 0.546), while the value drops to 0.535 μm (standard deviation 0.672) in the presence of Shield-1 (Figure A2.5C). We next examined the DrpA mutant phenotype by electron microscopy. We observed apicoplasts localising to the basal end of the parasite, and in some cases exiting the parasite (Figure A2.5D-E, pink arrows).

We conclude that incubation of the DD-DrpA^{K42A} mutant in Shield-1 results in rapid and severe defects in apicoplast biogenesis and division. The DrpA^{K42A} mutant is predicted to act in a dominant-negative way to ablate DrpA function. However, the data presented in Figures A2.4 and A2.5 do not rule out the possibility that the observed defects in apicoplast fission are a consequence of DrpA overexpression. To test this, we overexpressed wild-type DrpA and DrpA where the entire GTPase domain was deleted. We observed no effects on apicoplast biogenesis in cells overexpressing wild-type DrpA, while deletion of the entire GTPase domain showed a phenotype identical to the DrpA^{K42A} point mutant (Figure A2.S3D-E). We conclude that the DrpA^{K42A} mutant acts in a domainant-negative way to disrupt native *Tg*DrpA functions. We examined the effects

of ablating *Tg*DrpA function on other cellular functions. We found that DrpA has no role in protein targeting to the apicoplast or secretory pathways, or in biogenesis of micronemes and rhoptries, specialised secretory organelles in Apicomplexa (Figure A2.S4A-C). We found that although there were no consistent defects, we could not entirely rule out a minor role for DrpA in mitochondrial biogenesis (Figure A2.S4D-E).

***Tg*DrpA mutants are incapable of apicoplast fission.**

Our data indicate that *Tg*DrpA is essential for apicoplast biogenesis. To elucidate the mechanism for *Tg*DrpA function in this process we examined whether *Tg*DrpA has a role in apicoplast fission. In the absence of apicoplast fission, we predict that apicoplasts from adjoining cells would remain connected. To experimentally test this, we performed fluorescence recovery after photobleaching (FRAP). We imaged $DrpA^{K42A}$ parasites expressing apicoplast-targeted RFP, grown in the absence or presence of Shield-1. We laser bleached apicoplast fluorescence from one parasite within a vacuole and measured recovery over two minutes. In the absence of Shield-1, we observed no fluorescence recovery of apicoplast fluorescence (Figure A2.6A-B; Supplemental Movie 2). Average recovery in fluorescence after two minutes was 0.8 % of relative fluorescence units with a standard deviation of 0.9%. For parasites grown in Shield-1, we saw a consistent and significant recovery of fluorescence (Figure A2.6C-D; Supplemental Movie 3). Average recovery in fluorescence after two minutes was 22.3% with a standard deviation of 6.2%. Our data suggest that apicoplasts of adjoining cells in a single vacuole maintain a physical connection in the DrpA mutant, consistent with a defect in apicoplast fission. We performed similar FRAP analysis on mitochondrial fluorescence and found negligible recovery (Figure A2.S4F-I).

Having established that *Tg*DrpA is required for apicoplast fission, we sought to elucidate the mechanistic role of *Tg*DrpA in this process. *T. gondii* daughter cells form within mother cells by internal budding (Figure 7A, [8]). The scaffold of the daughter

buds consists of subpellicular microtubules and an inner membrane complex (IMC), flattened membrane sacs that are stabilised by a network of IMC proteins. Daughter buds form near the centrosomes and extend towards the basal end of the mother cell, incorporating the nucleus and various organelles, before contracting at the base to enclose the newly formed daughter (Figure A2.7A; [23, 24]). MORN1 is a recently identified protein that localises to a ring at the growing end of the daughter bud and ultimately forms the basal complex (Figure A2.7A; [8, 22, 23, 25]). In addition, MORN1 localises to the centrocone, an elaboration of the nuclear envelope that contains the mitotic spindle and localises adjacent to the centrosome. Immediately before apicoplast fission, the apicoplast typically adopts a “U”-shape, with the ends of the daughter bud localising to the base of the “U” (Figure A2.7B, Figure A2.2C; [6]). We have previously hypothesised that the growing daughter bud functions in apicoplast division, possibly generating the force necessary for fission [5, 6]. To examine this, we performed time-lapse imaging on the apicoplast RFP/DrpA^{K42A} mutant cell line grown in the absence of Shield-1 using YFP-MORN1 as a dynamic marker for the growth of the daughter bud (Figure A2.7C). After 35 minutes of imaging, each cell contains a single apicoplast and two MORN1 rings (Figure A2.7C; Supplemental Movie 4). After 70 minutes, the MORN1 rings have moved towards the basal ends of the parasites. The apicoplast in the bottom parasite has adopted a “U” shape, with the two MORN1 rings localised at the base of the “U”. As daughter budding proceeds, the “U”-shape of the apicoplast elongates, and after 90 minutes, the MORN1 rings have moved further towards the basal end of the mother cell, and the apicoplast in this parasite has divided. After 100 minutes, the MORN1 ring has extended further still towards the basal end of the cell, while the apicoplasts remain at the apical end of the forming daughters, associated with the centrosomes/ centrocones. Soon after, the cells undergo cytokinesis, with the MORN1 rings becoming the basal complex of the newly formed daughter cells (Supplemental Movie 4).

These data suggest that growth of the daughter bud is involved in generating the "U"-shaped apicoplast, and that apicoplast division occurs when the MORN1 ring localises to the base of the "U". This raises two hypotheses for the role of DrpA in apicoplast fission: DrpA may function in formation of the daughter bud, which in turn is necessary for apicoplast fission, or DrpA functions directly in apicoplast fission and has no effect on daughter bud formation. To test this, we visualised the daughter bud of $DrpA^{K42A}$ mutants co-expressing apicoplast RFP by immunofluorescence assays with an anti-IMC antibody, growing parasites in the presence of Shield-1. In parasites where apicoplasts are unable to divide, the daughter bud appears normal (Figure A2.7D). To examine daughter bud formation and apicoplast fission in a more dynamic way we performed time-lapse imaging on the previously described YFP-MORN1 and apicoplast-targeted RFP cell line grown in the presence of Shield-1 (Figure A2.7E). We examined a four-cell vacuole grown for 6 hours in Shield-1 where apicoplast morphology initially appeared normal. After 40 minutes, we see the development of MORN1 rings at the apical end of the parasites, near the apicoplast (Figure A2.7E; Supplemental Movie 5). After 90 minutes we see the formation of "U"-shaped apicoplasts in each parasite, with the two MORN1 rings for each parasite localising at the base of the "U". Purple arrows indicate the direction of daughter cell budding. Twenty minutes later, the "U"-shaped apicoplasts have stretched out further, but have not divided. After 125 minutes, cytokinesis has begun. In the top cell, the MORN1 rings have contracted to close off the newly formed daughter cells (arrowheads), but the apicoplast is clearly not divided. The apicoplast ends remain attached to centrosomes/centrocones (white arrow). Five minutes later, one apicoplast branch has released from the centrosome/centrocone, and appears to localise to the basal end of the daughter cell (white arrow). After 195 minutes of imaging, the apicoplasts of all 8 newly formed daughters localise to the basal end of the cell, and are no longer connected to the centrosomes. We conclude that after

ablation of DrpA, daughter cell budding is normal. Furthermore, apicoplast division appears normal up to the point of organellar fission. Together with the localisation of native DrpA to the base of the “U”-shaped apicoplast (Figure A2.2B-C), these data suggest a direct role for DrpA in apicoplast fission. Although daughter budding has a role in extension of the apicoplast into an elongated “U” shape[5, 6], this extension is not sufficient to mediate apicoplast fission.

A2.4 Discussion

The data presented in this study indicate that *Tg*DrpA functions in apicoplast fission. Curiously, apicomplexan DrpA proteins are phylogenetically distinct from ARC5 dynamins that play a similar role in chloroplast division in plants [2]. This suggests that DrpA evolved from a host cytoplasmic dynamin that was recruited to endosymbiont division independently of ARC5, a remarkable example of convergent evolution. Dynamins appear to be promiscuous membrane-modifying enzymes, whose cellular function is largely dependent on the membranes to which dynamin is recruited. It is therefore of considerable interest to identify the mechanisms of *Tg*DrpA recruitment to the apicoplast. The apicoplast progenitor was a red alga [26, 27] that likely had an ARC5 and FtsZ-based chloroplast division apparatus. Why was it necessary to evolve a second dynamin to replace the function of these ubiquitous plastid division proteins? A crucial step in the establishment of a successful endosymbiotic organelle is a mechanism to correctly divide and partition within the host cell [28]. Compared to their red algal precursors, apicoplasts are surrounded by two additional membranes, the outermost of which is an endosomal membrane. During the early phase in the endosymbiotic relationship, ARC5 and FtsZ were encoded by the red algal genome and functioned in division of the two innermost membranes. A separate mechanism was required to divide the outer membranes, and our data suggests that DrpA may have

been recruited for this role (possibly from an original role in the endosomal pathway). It is not clear why ARC5 and FtsZ were subsequently lost, but we speculate that the general reduction in size of the apicoplast that occurred upon loss of photosynthesis may have simplified this division process to the extent where DrpA alone was sufficient to mediate fission. It is noteworthy that DrpA homologues are present in *Cryptosporidium* species (Figure A2.1), Apicomplexa that have lost their apicoplast. It is conceivable that *Cryptosporidium* DrpA has acquired a novel function, but equally possible that the role of DrpA in apicoplast fission evolved more recently. Examining the role of DrpA homologues in *Cryptosporidium* and other alveolates should provide clues to how and when its role in apicoplast fission evolved.We demonstrate that ablation of DrpA function results in specific defects in apicoplast fission. We also demonstrate a role for the extension of daughter buds in generating the “U”-shape of apicoplasts that immediately precedes fission. Likely this process is mediated by growth of subpellicular microtubules, and we have previously shown that treatment of *T. gondii* with the microtubule disrupting agent oryzalin, which disrupts subpellicular microtubules, inhibits apicoplast fission [6]. Based on these observations, we have argued that the force generated by daughter budding is required for apicoplast fission [5, 6]. We now extend this model to include a role for DrpA. Although strong DrpA labelling is apparent at the site of apicoplast fission, DrpA associates with the apicoplast at all points in the cell cycle (Figure A2.2A-C). Why, then, does DrpA-mediated fission only occur during daughter-cell budding? In Figure 8 we present a model for how fission and budding are mechanistically coordinated. Apicoplast ends become associated with centrosomes, which anchors them to the apical end of the cell. Soon after, daughter budding commences, with the ends of the forming daughter cells (as marked by the MORN-1 ring) stretching and consequently constricting the apicoplast. In yeast, dynamin-mediated mitochondrial fission requires the assembly of dynamin spirals around the organelle at the site of fission [29]. It is thought that these

spirals form at sites where mitochondria are already constricted [29, 30]. A recent study of the DrpA homologue in the apicomplexan *Plasmodium falciparum* (PfDYN2) demonstrated that PfDYN2 is capable of self-association and GTP hydrolysis [31], suggesting that DrpA likely functions in a similar way to other characterised dynamin-related proteins. We propose that stretching of "U"-shaped apicoplasts by forming daughter cells constricts the organelle to the extent where DrpA can assemble in spirals. As in other dynamin-based constriction models, GTP hydrolysis causes extension of the DrpA spiral (Figure A2.2C, arrowheads) and results in further constriction of the apicoplast, until organellar fission is complete. In such a way, parasites can coordinate apicoplast fission with cytokinesis. Under this model, daughter cell budding is responsible for initial constriction of the apicoplast, DrpA functions in the actual fission process, and centrosome attachment mediates correct segregation of the apicoplast into daughter cells. Ongoing studies seek to experimentally test this model.

A2.5 Experimental Procedures

Parasite culture and manipulation

Parasites were grown in human foreskin fibroblasts as previously described [32]. We grew DrpA^{K42A} mutant parasites in 0.1 – 0.2 μM Shield-1 (a kind gift from Tom Wandless, Stanford U.) where applicable. Fluorescence growth assays were performed as previously described [20]. Cloning and plasmid construction are described in the Supplemental Data.

Phylogenetic analyses

We generated multiple sequence alignments of dynamin homologues from a range of organisms using ClustalX. Sequences used for alignments were identified on publicly available databases. Phylogenetic analyses were performed using PHYLIP as previously described [33]. The GenBank accession number for *Tg*DrpA is FJ264918.

Accession numbers for other proteins used in the alignment are listed in the Supplemental Data. Alignments are available from the authors upon request.

Protein analyses

Western blotting and pulse-chase analyses were performed as described previously [34]. For Western blotting, we used anti-HA antibodies (Roche) at a dilution of 1:100 and anti-GRA8 (a kind gift from Gary Ward, U. Vermont) at 1:200,000.

Microscopy

Fluorescence and live cell images were acquired using both a DM IRBE inverted epifluorescence microscope (Leica) fitted with a 100X oil immersion objective lens (PL APO 1.40 NA) and an IX71 inverted epifluorescence microscope (Olympus) with a 100X oil immersion lens (UPlanApo 1.35 NA). Images on the Leica microscope were recorded using a Hamamatsu C4742-95 digital camera, and adjusted for brightness and contrast using Openlab software (Improvision). Images on the Olympus microscope were recorded using a Photometrics Coolsnap HQ camera and processed using SoftWoRx software (Applied Precision). Time-lapse imaging was performed in a humidified chamber heated to 37^{o}C with 5% CO_2, with cells grown in Mattek glass-bottom culture dishes. Images were processed to account for cell drifting. Photobleaching of RFP in the apicoplast was performed on the Olympus microscope, using ten 300 ms pulses with a 488 nm laser on a specified, diffraction-limited region. Conditions for bleaching of RFP in the mitochondrion was identical, except that we used 1 second pulses. Immunofluorescence assays were performed as previously described [34]. We used anti-ACP antibodies at a dilution of 1:2000, anti-HA at 1:50 to 1:100, anti c-myc (Roche) at 1:500, anti-MIC5 at 1:500, anti-ROP4 (a kind gift from Gary Ward, U. Vermont) at 1:500 and anti-IMC (Mab 45.36; a kind gift from Gary Ward, U. Vermont) at 1:500 to 1:2000. For electron microscopy, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at room temperature, followed by fixation with 1%

osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 hours on ice. Afterwards the samples were brought through a graded series of ethanol and subsequently infiltrated with increasing concentrations of Epon : ethanol (3:1; 1:1; 1:3 for 2 hours each and finally pure Epon overnight). Following change for fresh Epon, samples were polymerized at 60°C for 48 hours and sectioned as monolayers. Sections (60 nm) were collected on Formvar-coated, carbon-stabilized hexagonal 100 mesh copper grids and post-stained for 4 minutes with 20% (w/v) uranyl acetate in 70% (v/v) methanol/water followed by 2 minutes Reynolds's lead citrate staining[39]. The grids were examined in a transmission electron microscope Tecnai 12 (FEI Company, Eindhoven, The Netherlands) at 120kV. Images were recorded using a CCD camera (MegaView II, Olympus Soft Imaging Solutions GmbH, Münster, Germany). Image processing was done with Analysis 3.2 (Soft Imaging Systems GmbH, Münster, Germany).

Acknowledgments

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science and education (BMBF) to MM, and a grant from the National Institutes of Health to BS (AI 64671).

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Figures

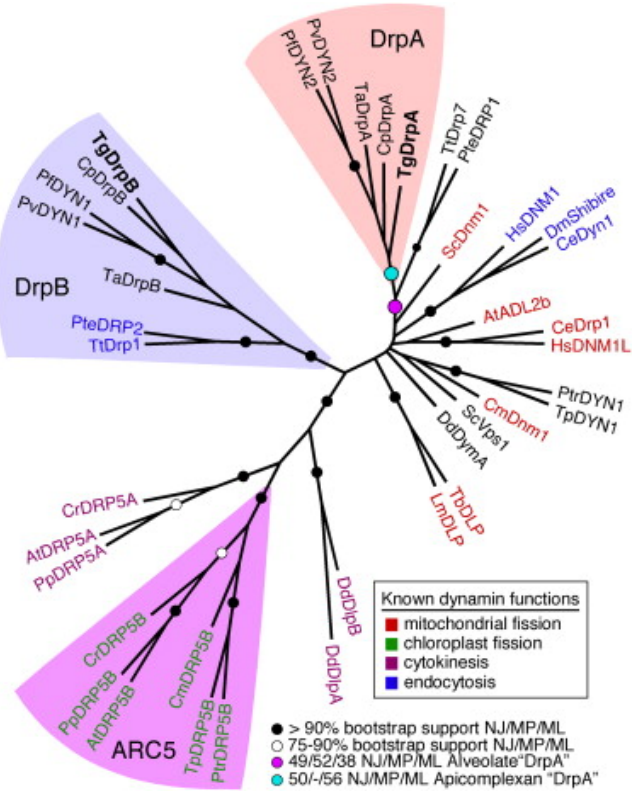


Figure A2.1: Phylogenetic analyses of dynamins. We generated a multiple sequence alignment of the conserved region of a range of dynamin related proteins. The analysis included 449 residues and 39 taxa. We generated phylogenetic trees using PHYLIP, performing bootstrapping with 400 replicates. Bootstrap values based on Neighbour-joining (NJ), Maximum Parsimony (MP) and Maximum-Likelihood (ML) analyses were determined. In this figure we depict the consensus Maximum likelihood tree. Dynamin homologues included in the analysis are from *Drosophila melanogaster* (DmShibire), human (HsDNM1 and HsDNM1L), *Caenorhabditis elegans* (CeDyn1 and CeDrp1), *Cyanidioschyzon merolae* (CmDnm1 and CmDRP5B), *Dictyostelium discoideum* (DdDymA, DdDlpA and DdDlpB), the diatoms *Thalassiosira pseudonana* (TpDYN1 and TpDRP5B) and *Phaeodactylum tricornutum* (PtrDYN1 and PtrDRP5B), *Saccharomyces cerevisiae* (ScVps1 and ScDnm1), *Arabidopsis thaliana* (AtADL2b, AtDRP5A and AtDRP5B), *Physcomitrella patens* (PpDRP5A and PpDRP5B), *Chlamydomonas reinhardtii* (CrDRP5A and CrDRP5B), the ciliates *Tetrahymena thermophila* (TtDrp1 and TtDrp7) and *Paramecium tetraurelia* (PteDRP1 and PteDRP2), the apicomplexans *Plasmodium falciparum* (PfDYN2 and PfDYN1), *Plasmodium vivax* (PvDYN2 and PvDYN1), *Toxoplasma gondii* (TgDrpA and TgDrpB), *Theileria annulata* (TaDrpA and TaDrpB) and *Cryptosporidium parvum* (CpDrpA and CpDrpB), and the trypanosomatids *Trypanosoma brucei* (TbDLP) and *Leishmania mexicana* (LmDLP).

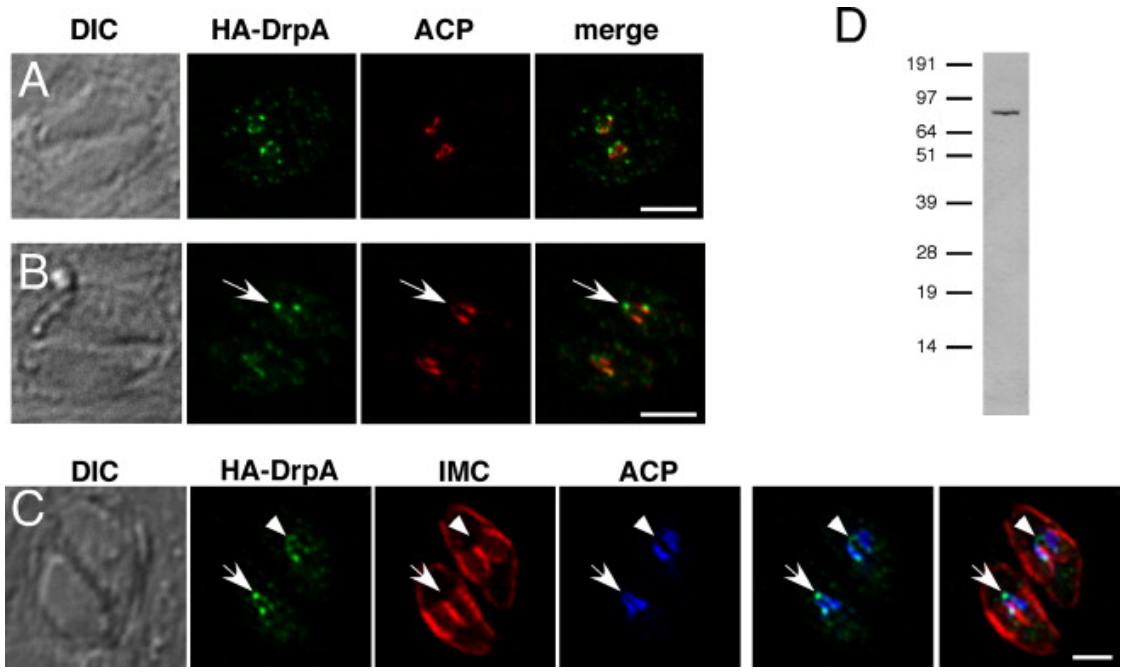


Figure A2.2: *Tg*DrpA localises to the apicoplast. (A-B) Immuofluorescence assays of a cell line expressing HA-tagged *Tg*DrpA, labelled with anti-HA (green) and the apicoplast stromal marker anti-acyl carrier protein (ACP; red) antibodies. Arrows point to sites of apicoplast fission. Scale bars are 2 μm. (C) Immunofluorescence assay of the HA-*Tg*DrpA cell line, labelled with anti-HA (green), anti-IMC (red) and anti-ACP (blue) antibodies. Arrows point to sites of apicoplast fission, where HA-*Tg*DrpA forms a punctate dot. Arrowheads point to the tubule-like structure adopted by HA-*Tg*DrpA later in apicoplast fission, when the apicoplast has become further constricted. (D) Anti-HA Western blot of the HA-*Tg*DrpA cell line, labelling a protein band at approximately 90 kDa.

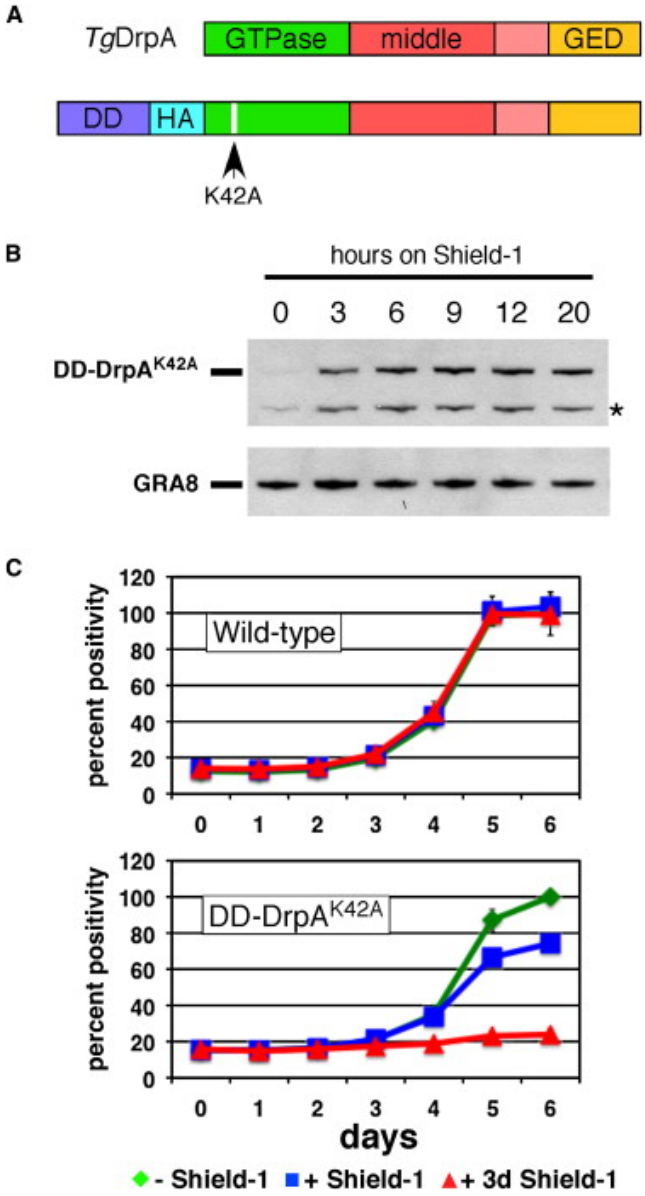


Figure A2.3: *Tg*DrpA is essential for parasite growth. (A) Dynamin-related proteins such as *Tg*DrpA consist of three conserved domains: an N-terminal GTPase domain, a middle domain and a C-terminal GTPase effector domain (GED). To generate dominant-negative *Tg*DrpA mutants, we mutated the lysine in the GTP-binding motif of the GTPase domain to alanine (K42A). We fused an N-terminal destabilisation domain (DD) and HA-tag to this construct and generated clonal cell lines. (B) To demonstrate regulated expression of DD-DrpA^{K42A}, we performed Western blotting of cells grown for 0 to 20 hours in 0.1 µM Shield-1, probing blots with anti-HA antibodies and anti-GRA8 antibodies as a loading control. Asterisk represents a probable degradation product of DD-DrpA^{K42A}. (C**)** We performed fluorescence growth assays on wild-type (top) and DD-DrpA^{K42A} (bottom) parasites expressing tandem-YFP. Parasites were grown in the absence of Shield-1 (green diamonds), in the presence of Shield-1 (blue squares) or pre-incubated for three days in the presence of Shield-1 before the assay, and continued to grow in Shield-1 (red triangles). Error bars represent one standard deviation from the mean.

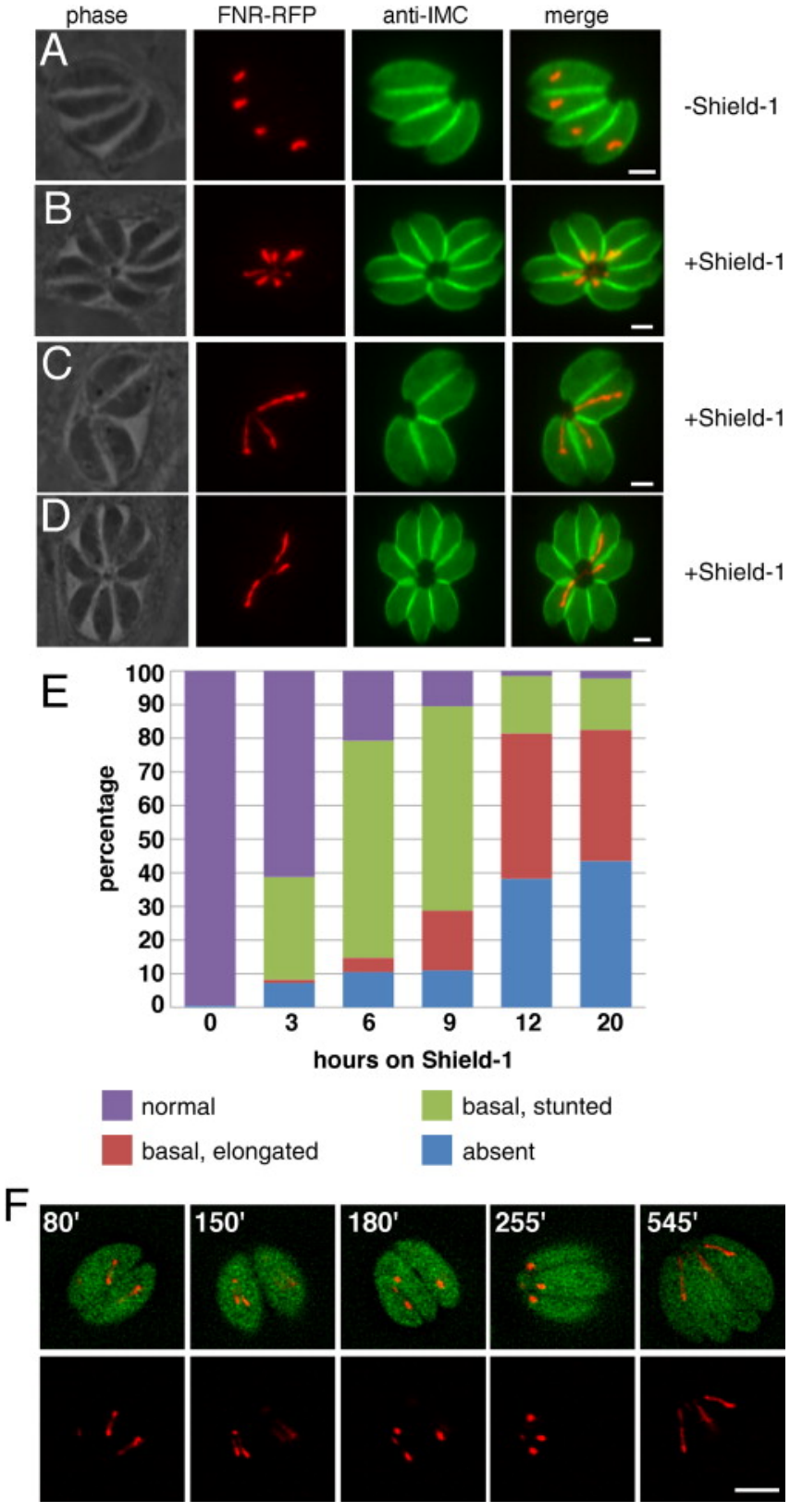


Figure A2.4: *Tg*DrpA is essential for apicoplast biogenesis. (A-D) Immuno-fluoresence assays of DD-DrpA^{K42A} parasites co-expressing apicoplast-targeted RFP

(red), co-labelled with anti-IMC antibodies (green). In the absence of Shield-1, every parasite contains a single apically-localised apicoplast (A). In the presence of Shield-1, apicoplasts localise to the basal end of the parasite (B), frequently elongating towards the apical end of the cell and being absent in some cells (C-D). Scale bars are 2 µm. (E) Quantification of the apicoplast defect in DD-DrpA^{K42A} parasites. We grew parasites for 0-20 hours on Shield-1 and imaged 100 four-cell parasite vacuoles. We classified apicoplasts into four catagories: normal apicoplasts (purple), basal stunted apicoplasts (green), basal elongated apicoplasts (orange), and cells lacking apicoplasts (blue). (F) Time-lapse imaging of apicoplasts in DD-DrpA^{K42A} parasites co-expressing cytosolic YFP and apicoplast-targeted RFP, grown in the presence of Shield-1. Scale bar is 2 µm.

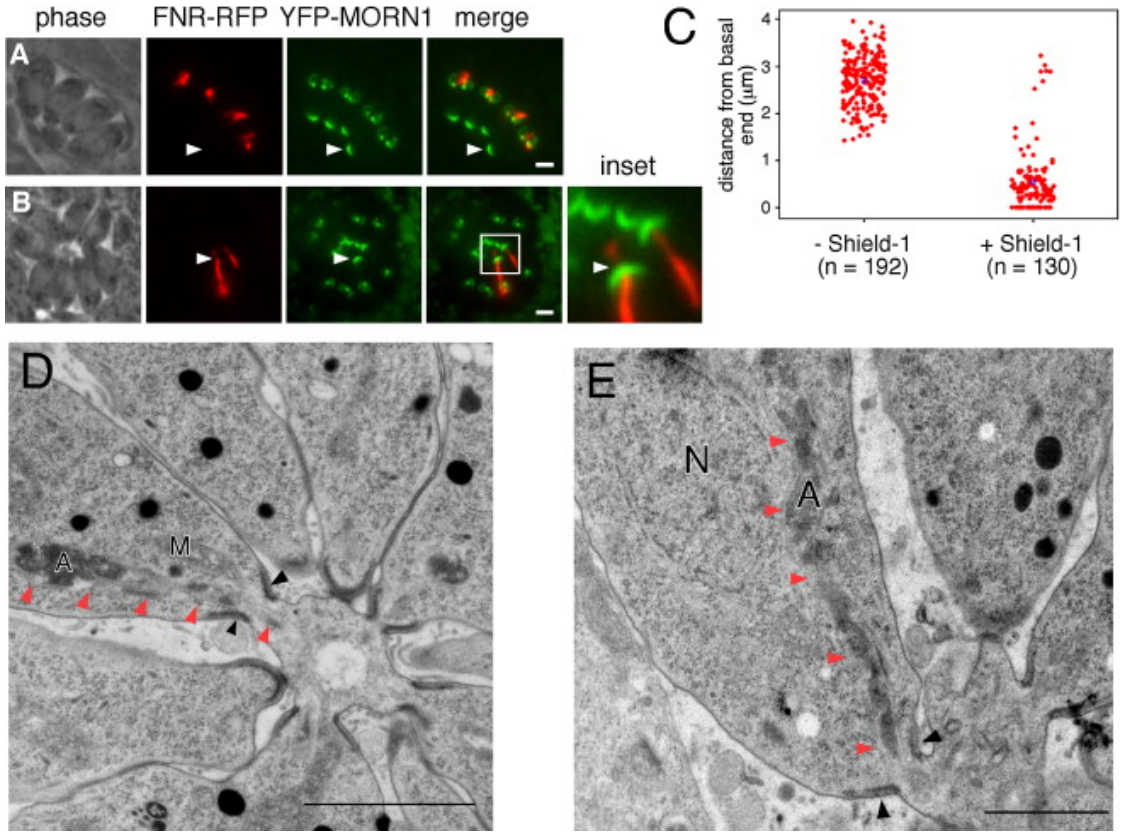


Figure A2.5: Apicoplasts localise to the basal end of parasites upon over-expression of dominant-negative *Tg*DrpA. (A-B) Live cell imaging of DD-DrpA^{K42A} parasites grown in the absence (A) or presence (B) of Shield-1, co-expressing FNR-RFP and YFP-MORN1. Arrow depicts the basal complex of a parasite, co-localising in (B) with a point of constriction in the apicoplast. Scale bars are 2 µm. (C) Quantification of the distance between the YFP-MORN1 labelled basal complex and the nearest point of the apicoplast. DD-DrpA^{K42A} parasites were grown in the absence (left) or presence (right) of Shield-1, with distance in µm shown on the y-axis. The blue circle represents the mean value for each data set. (D-E) Electron micrograph images of DD-DrpA^{K42A} parasites grown on Shield-1. The basal end of parasites is marked by an electron dense area that likely corresponds to the contractile, MORN1-containing basal complex (black arrowheads). Apicoplasts (A) localise to the basal end of the parasites (pink arrow-

heads). The mitochondrion (M) and nucleus (N) are also shown. Scale bar is 1500 nm in (D) and 1000 nm in (E).

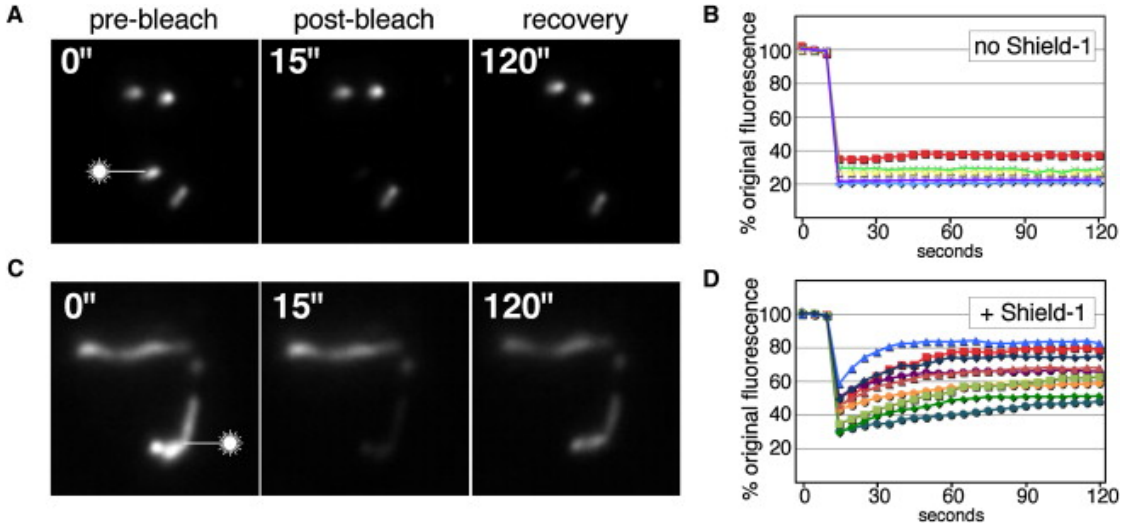


Figure A2.6: Disruption of *Tg*DrpA function results in defects in apicoplast fission. (A-D) Fluorescence recovery after photobleaching in apicoplasts of DD-DrpA^{K42A} parasites grown in the presence or absence of Shield-1. We imaged parasites at 5 second intervals over 2 minutes, bleaching a small region of the field-of-view (at the position indicated by the laser symbol) after 10 seconds. (A) and (C) show images from single experiments, imaged before (left, 0”), directly after (middle, 15”) and at the end of the experiment (right, 120”). (B) and (D) show quantifications of fluorescence recovery over time in five (B) or ten (D) independent experiments. DD-DrpA^{K42A} parasites co-expressing apicoplast-targeted RFP were grown in the absence (A-B) or presence (C-D) of Shield-1.

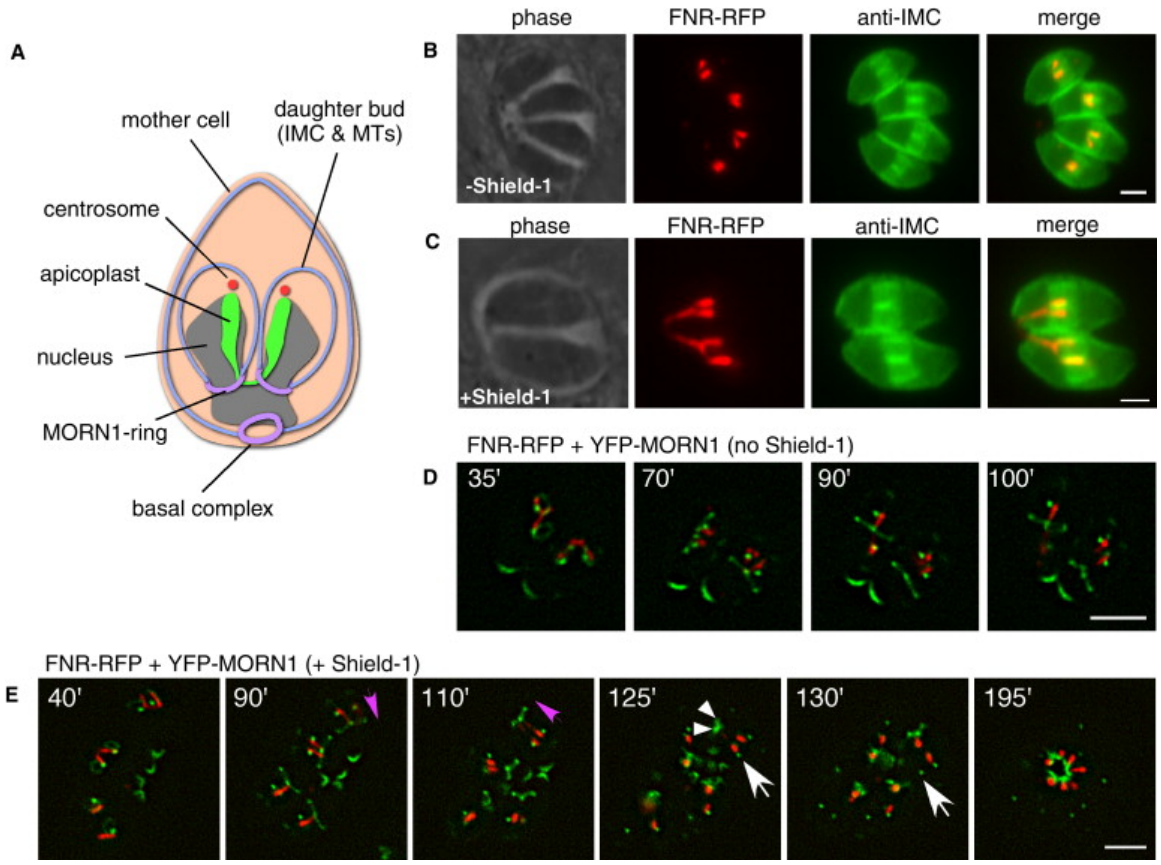


Figure A2.7: *Tg*DrpA has a direct role in apicoplast fission. (A) Schematic of a dividing *T. gondii* parasite. The daughter bud (blue) comprises of subpellicular microtubules and the inner membrane complex (IMC). Subpellicular microtubules and an IMC are also present in the mother cell. MORN1 (lilac) labels the basal end of the mother cell (basal complex), the growing ends of the daughter buds (MORN1-ring), and the centrocone, an extension of the nuclear envelope that localises near the centrosomes (red). A pre-divided, "U"-shaped apicoplast (green) and a pre-divided nucleus (grey) are also depicted. (B) Immunofluorescence assay of DD-DrpA^{K42A} parasites grown in the absence of Shield-1, co-expressing apicoplast RFP (red) and labelled with an anti-IMC antibody (green). The inner membrane complex of daughter cell buds can be seen inside the mother cells, with the ends of the daughter IMC localising at the base of the dividing, "U"-shaped apicoplasts. (C) Time-lapse imaging of DD-DrpA^{K42A} parasites grown in the absence of Shield-1, co-expressing FNR-RFP and YFP-MORN1. YFP-MORN1 labels the basal end of the parasites, the centrocone (an extension of the nuclear envelope that localises adjacent to the centrosome) and the growing bud of the daughter cell. (D) Immunofluorescence assay of DD-DrpA^{K42A} parasites grown in the presence of Shield-1, co-expressing apicoplast RFP (red) and labelled with an anti-IMC antibody (green). (E) Time-lapse imaging of DD-DrpA^{K42A} parasites grown in the presence of Shield-1, co-expressing FNR-RFP and YFP-MORN1. Purple arrows at 90 and 110 minute samples represent the direction of daughter cell budding. Note that between the 90 and 110 minute time points, this cell rotates almost 180^{o}. Arrowheads at 125 minutes depict the MORN1-labelled basal complex, and white arrows at 125 and 130 minutes label a point of attachment of the apicoplast to the centrosome/centrocone that is released 5 minutes later. Scale bars are 2 μm.

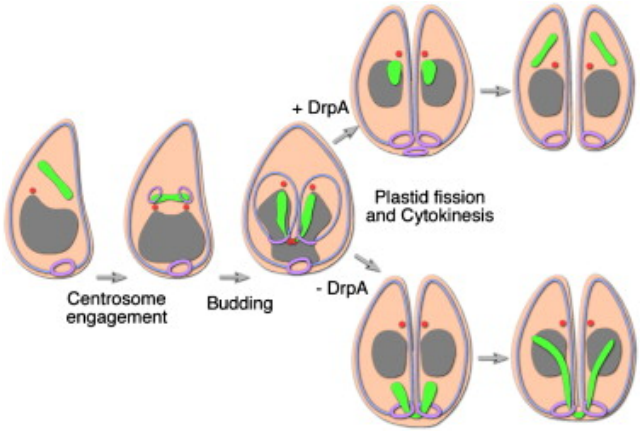
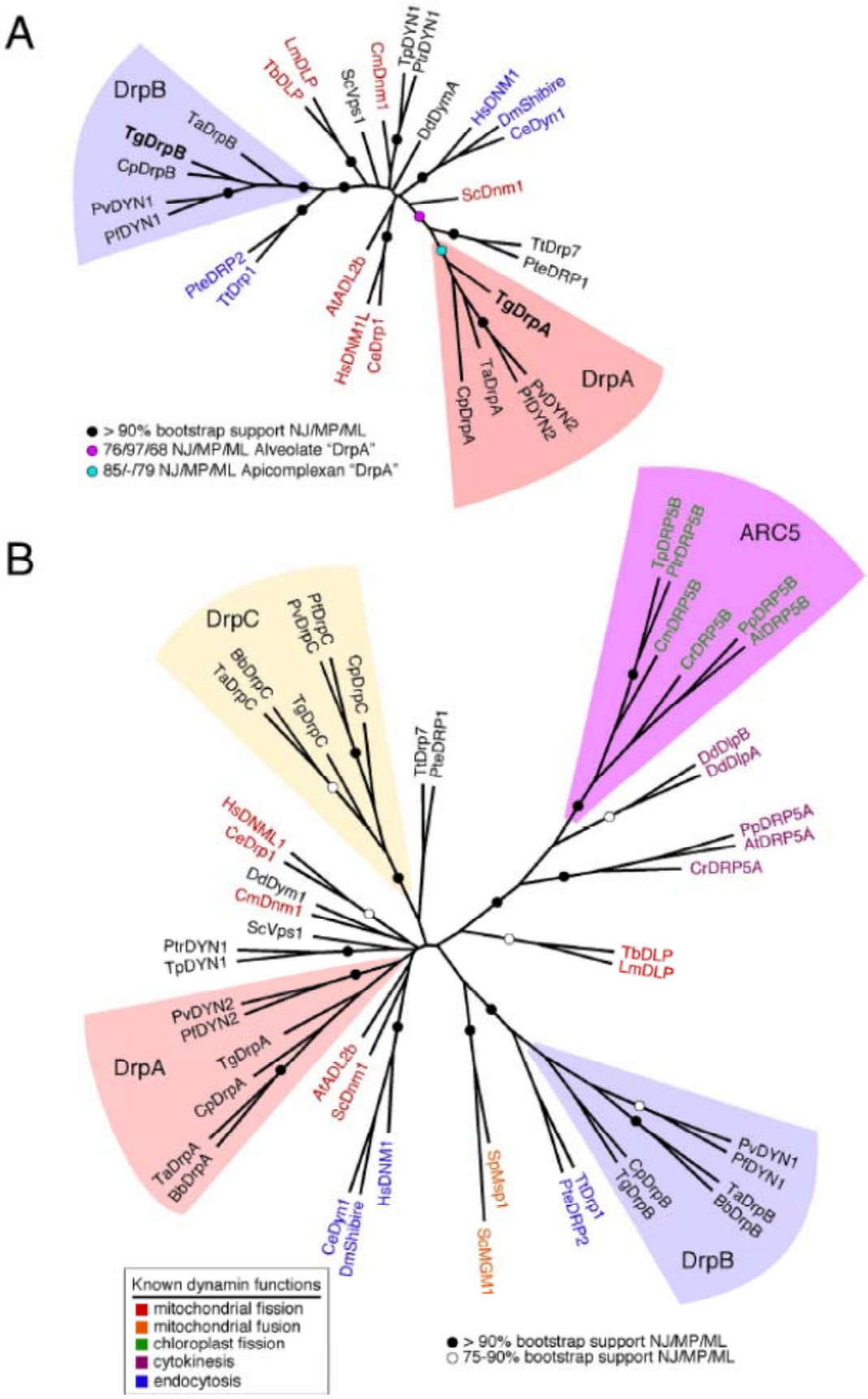


Figure A2.8: Model for the role of *Tg*DrpA in apicoplast fission. Before daughter cell budding, centrosomes (red) anchor the apicoplast (green) to the apical end of the cell. Extension of the daughter bud (lilac) results in formation of a “U”-shaped apicoplast, constricting the apicoplast at the base of the “U”. DrpA is then able to assemble into functional, multimeric spirals (red) that hydrolyse GTP and function in the actual fission of the apicoplast. When functional DrpA is present (top right), apicoplasts divide and properly segregate with centrosomes into daughter parasites. In the absence of functional DrpA, apicoplast fission is unable to occur. Extension of the daughter bud generates the force necessary to release apicoplasts from centrosomes, with apicoplasts then localising to the basal end of the newly formed daughter parasites (bottom right). Apicoplasts in the two newly formed daughter cells remain connected and elongate before the next round of cell division.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PteDRP2
TtDrp1
LmDLP
TbDLP
ScVps1
CmDnm1
TpDYN1
PtrDYN1
DdDymA
HsDNM1
DmShibire
CeDyn1
ScDnm1
AtADL2b
HsDNM1L
CeDrp1
TtDrp7
PteDRP1
TgDrpA
TaDrpA
PvDYN2
PfDYN2
CpDrpA
DrpA
● > 90% bootstrap support NJ/MP/ML
● 76/97/68 NJ/MP/ML Alveolate "DrpA"
● 85/-/79 NJ/MP/ML Apicomplexan "DrpA"
B
ARC5
TpDRP5B
PtrDRP5B
CmDRP5B
CrDRP5B
PpDRP5B
AtDRP5B
DrpC
PtrDrpC
PvDrpC
BbDrpC
TaDrpC
TgDrpC
CpDrpC
TtDrp7
PteDRP1
DdDlpB
DdDlpA
PpDRP5A
AtDRP5A
CrDRP5A
HsDNML1
CeDrp1
DdDym1
CmDnm1
ScVps1
PtrDYN1
TpDYN1
TbDLP
LmDLP
PvDYN2
PfDYN2
TgDrpA
CpDrpA
TaDrpA
BbDrpA
DrpA
AtADL2b
ScDnm1
HsDNM1
CeDyn1
DmShibire
SpMsp1
ScMGM1
TtDrp1
PteDRP2
PvDYN1
PfDYN1
CpDrpB
TgDrpB
TaDrpB
BbDrpB
DrpB
Known dynamin functions
mitochondrial fission
mitochondrial fusion
chloroplast fission
cytokinesis
endocytosis
● > 90% bootstrap support NJ/MP/ML
○ 75-90% bootstrap support NJ/MP/ML

Figure A2.S1: Phylogenetic analysis of *Tg*DrpA. We generated a multiple sequence alignment of the entire dynamin protein (587 characters in total) in the species listed in Figure 1, but excluding dynamin homologues that did not align throughout the entire protein (ARC5/DRP5B, DRP5A and DdDlpA/B homologues). We generated trees as described in Figure 1, performing bootstrapping with 500 replicates. These data indicate that DrpA from apicomplexa form an alveolate-specific clade that includes dynamin-related proteins from ciliates.

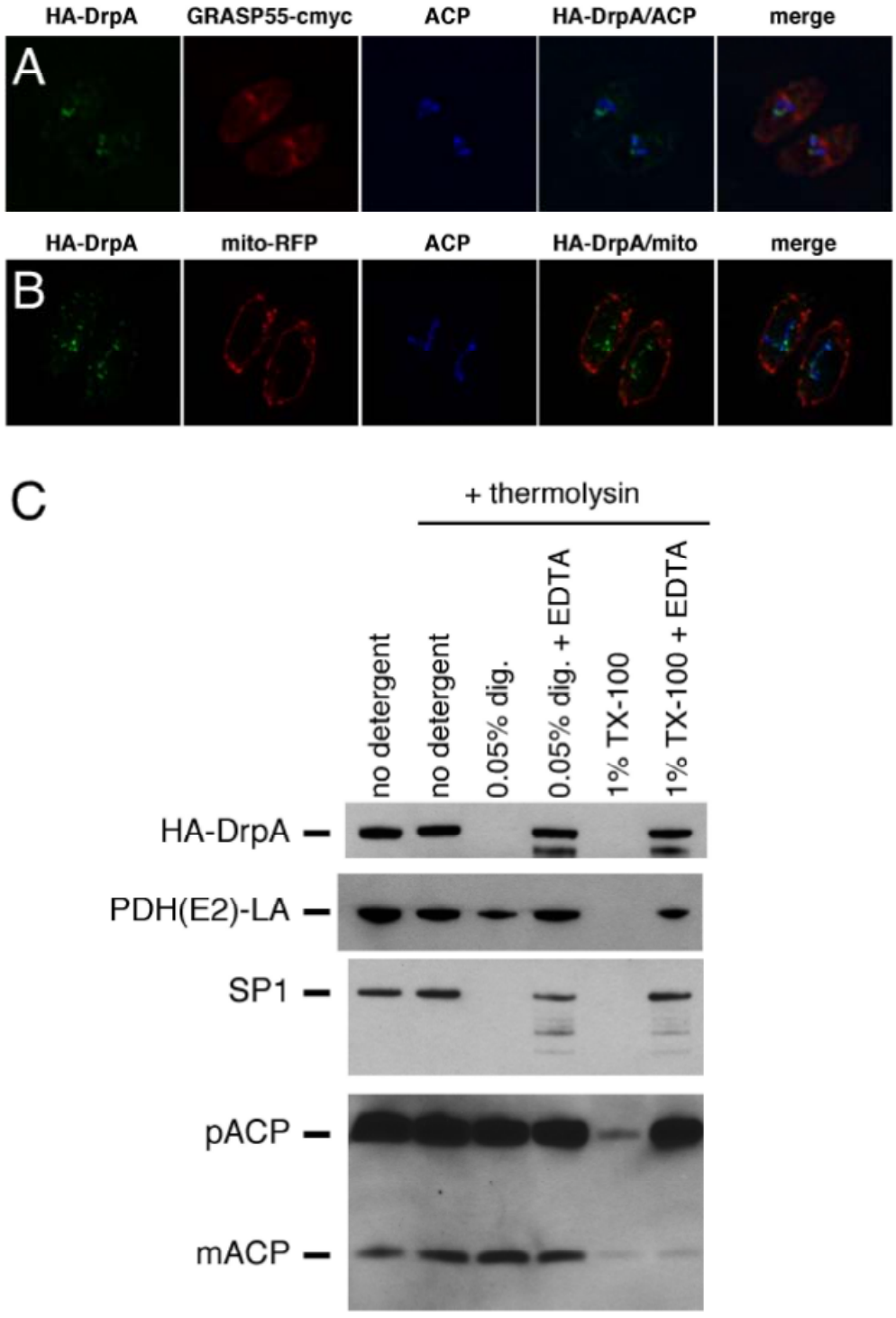

Figure A2.S2: Localisation of *Tg*DrpA. (A) Immunofluorescence assay of a cell line co-expressing HA-*Tg*DrpA and the Golgi marker GRASP55-c-myc, labelled with anti-HA (green), anti-c-myc (red) and anti-ACP (blue). *Tg*DrpA does not co-localise with the Golgi. (B) Immunofluorescence assay of the HA-*Tg*DrpA cell line transiently expressing mitochondrially-targeted RFP, labelled with anti-HA (green), RFP (red) and anti-ACP (blue). We see occasional regions of co-localisation between *Tg*DrpA and the mitochondrion, but the majority of HA-*Tg*DrpA label does not co-localise with the

mitochondrion. (C) Thermolysin protection assays on the HA-*Tg*DrpA cell line. Characterized components of the chloroplast fission machinery in plants localize to the stromal as well as the cytoplasmic face of the chloroplast envelope. We therefore wanted to determine to which sub-compartment of the apicoplast *Tg*DrpA localises. To do this, we performed thermolysin protection assays in the presence or absence of the detergent digitonin. Previous studies have demonstrated that low concentrations of digitonin solubilize the plasma membrane of *T. gondii* but not organellar membranes such as those of the apicoplast. We incubated HA-DrpA-expressing parasites in the absence of detergent, with 0.05% digitonin (0.05% dig.), or with 1% Triton X-100 (1% TX-100), added thermolysin and probed by Western blot with antibodies against the apicoplast stromal proteins acyl carrier protein (ACP)and lipoylated pyruvate dehydrogenase E2 subunit (PDH-E2-LA), the cytosolic protein soluble pyrophosphatase (SP1) and against HA-*Tg*DrpA. In the absence of thermolysin or detergent, no proteins were degraded. In the presence of 0.05% digitonin (0.05% dig), SP1 and HA-DrpA were degraded, whereas ACP and PDH-E2-LA were unaffected. Degradation could be inhibited with the addition of EDTA, an inhibitor of thermolysin, suggesting that the loss of protein we observed was specifically due to thermolysin degradation. Solubilization of all membranes with 1% Triton X-100 resulted in the loss of PDH-E2-LA and ACP protein, indicating that these proteins are sensitive to thermolysin degradation.

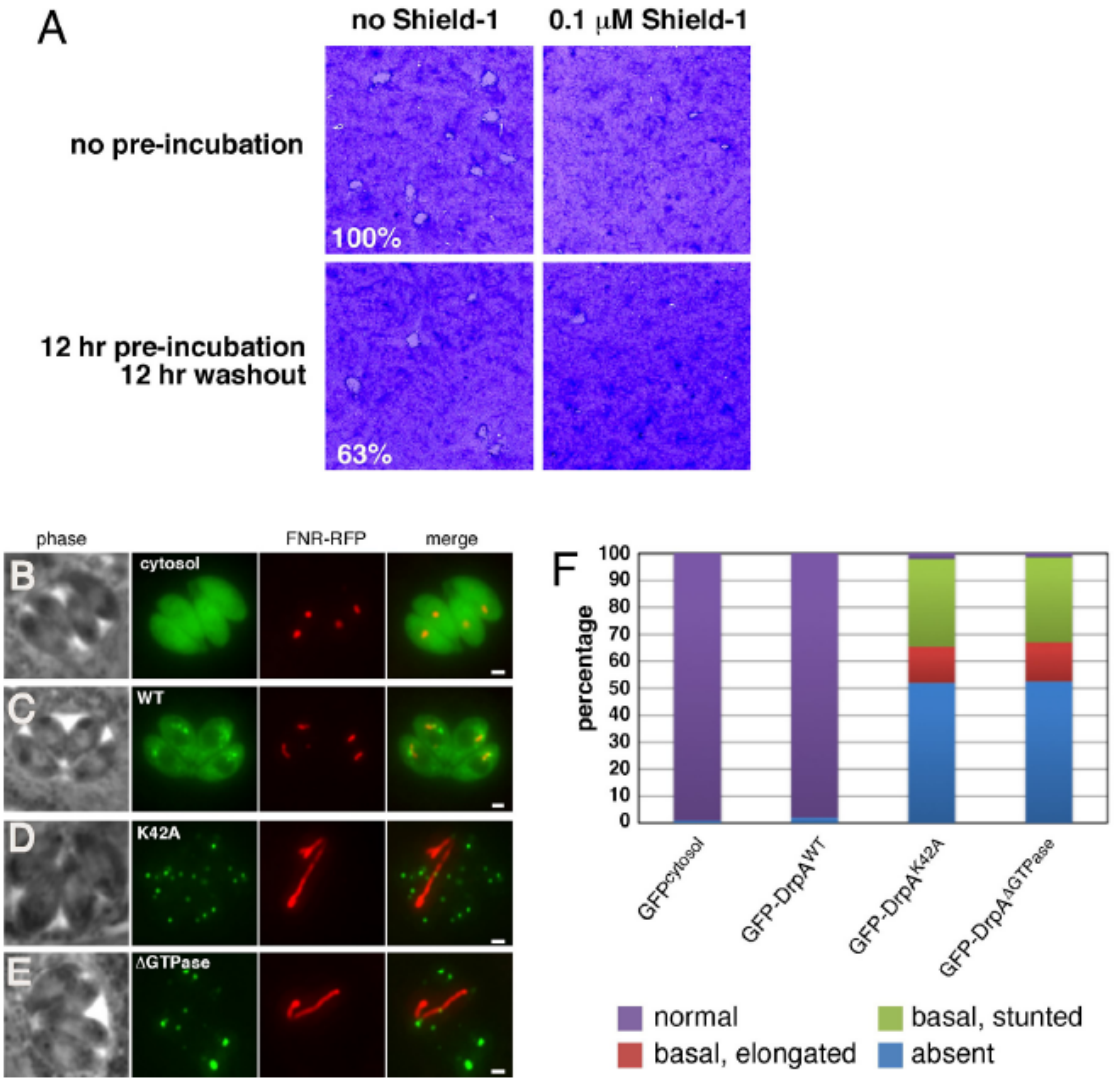

Figure A2.S3: Effects of dominant-negative *Tg*DrpA expression on parasite growth and apicoplast morphology. (A) We performed plaque assays on DD-DrpA^{K42A}-expressing parasites in the absence (left, top) or presence (right, top) of 0.1 μM Shield-1. We also pretreated DD-DrpA^{K42A}-expressing parasites in 0.1 μM Shield-1 for 12 hours, then washed out Shield-1 for 12 hours prior to setting up the plaque assay. These pre-treated parasites were grown in the absence (left, bottom) or presence (right, bottom) of 0.1 μM Shield-1. There is no difference in plaque size between parasites grown in the absence Shield 1 compared parasites pre-treated with Shield-1 for 12 hours then grown in the absence of Shield-1. However, plaque number in the wash-out experiment (bottom, left) decreases to 63% of the no-Shield-1 control. (B-E) To test whether DrpA^{K42A} mutants have a dominant-negative effect on *Tg*DrpA function or whether the observed phenotype is simply a consequence of *Tg*DrpA overexpression, we transiently overexpressed cytosolic GFP (B; cytosol), wild-type *Tg*DrpA fused to GFP (C; WT), the *Tg*DrpA^{K42A} construct fused to GFP (D; K42A), and *Tg*DrpA lacking the entire GTPase domain (E; ΔGTPase) in *T. gondii* parasites expressing apicoplast-targeted RFP. Parasites overexpressing cytosolic GFP and wild-type *Tg*DrpA show no obvious defects in apicoplast morphology (B-C). Parasites overexpressing both the *Tg*DrpA^{K42A} point mutant and the GTPase deletion show severe defects in apicoplast morphology (D-E),

indistinguishable from the phenotypes observed in the DD-regulated DrpA^{K42A} mutant shown in Figure 4. We performed these experiments with GFP fusions in order to identify transfected parasites. Parasites were imaged between 20 and 26 hours after transfection. Scale bars are 2 μm. (F) Quantification of the effects of overexpresion of the constructs described in (B-E). Fifty four-cell vacuoles (200 total parasites) were imaged for each experiment, and apicoplast morphology was categorised as described in Figure 4. Apicoplast morphology is normal in cells overexpressing cytosolic GFP and wild-type *Tg*DrpA fused to GFP. Apicoplast morphology is abnormal in cells over-expressing the *Tg*DrpA^{K42A} point mutation or overexpressing DrpA lacking the entire GTPase domain.

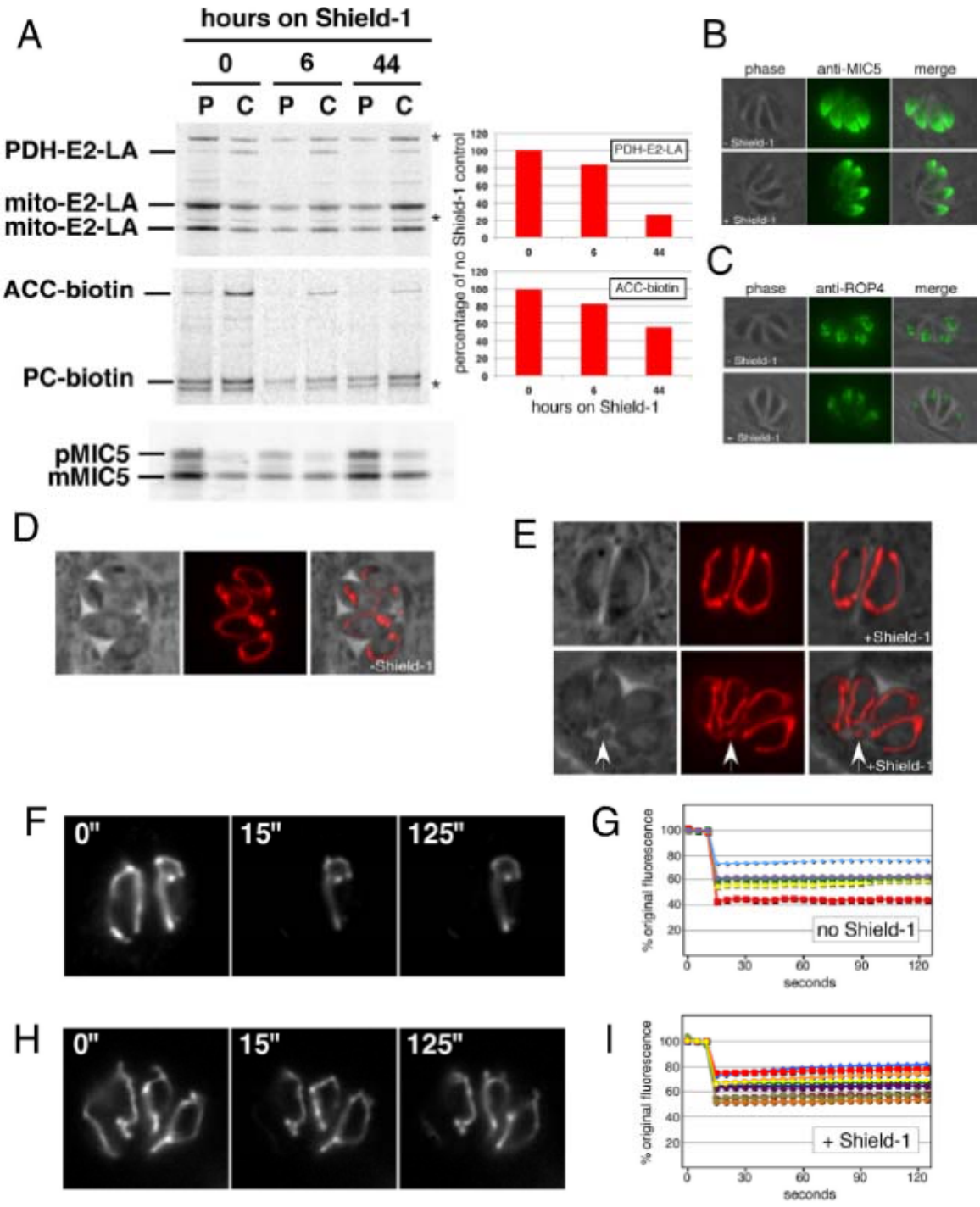

Figure A2.S4: The effects of DD-DrpA^{K42A} expression on apicoplast and endosomal protein trafficking, and microneme, rhoptry and mitochondrial biogenesis. (A) We performed pulse-chase analyses on cells grown for 0, 6 or 44 hours in Shield-1. We grew cells in radiolabelled amino acids for 1 hour, and either harvested cells (pulse; P) or washed out radiolabel and incubated in cold amino acids for a further two hours (chase; C). We purified lipolyated proteins including the apicoplast stromal pyruvate dehydrogenase E2 subunit (PDH-E2-LA) and mitochondrial lipoylated proteins (mito-E2-LA), the biotinylated apicoplast stroma-localised acetyl-CoA carboxylase (ACC-biotin)

and mitochondrial localised pyruvate decarboxylase (PC-biotin), and the processed micronemal protein MIC5 as previously described. Band intensities for PDH-E2-LA and ACC-biotin were quantified as a percentage of the no Shield-1 experiment, normalised to the mitochondrion bands as previously described (right). We conclude that ablation of *Tg*DrpA has little if any effect on protein targeting to the apicoplast, mitochondrion or pre-micronemal endosomal compartments. The decrease in apicoplast targeting observed after 44 hours is likely due to loss of apicoplasts in the majority of cells after prolonged incubation in Shield-1 (Figure 4E). (B) Immunofluorescence assays with antibodies against the micronemal protein MIC5 (green) on DD-DrpA^{K42A} parasites grown in the absence (top) or presence (bottom) of Shield-1. We conclude that ablation of *Tg*DrpA has no effect on microneme biogenesis. (C) Immunofluorescence assays with antibodies against the rhoptry protein ROP4 (green) on DD-DrpA^{K42A} parasites grown in the absence (top) or presence (bottom) of Shield-1. We conclude that ablation of *Tg*DrpA has no effect on rhoptry biogenesis.(D-E) Live cell imaging of DD-DrpA^{K42A} parasites co-expressing mitochondrially-targeted RFP (red), grown in the absence (D) or presence (E) of Shield-1. Arrow shows apparent connection of mitochondria from adjacent parasites. We conclude that in the presence of Shield-1, each parasite contains a mitochondrion, suggesting that there are no defects in mitochondrial segregation. In some vacuoles, mitochondria from adjacent parasites appeared to be connected (E, bottom, arrows), but in others they seemed to be unconnected (E, top). It is unclear whether connected mitochondria are a consequence of DrpA mutation, or simply natural variation in mitochondrial morphology. (F-I) Fluorescence recovery after photobleaching in mitochondria of DD-DrpA^{K42A} parasites grown in the presence or absence of Shield-1. We imaged parasites at 5 second intervals over 125 seconds, bleaching a small region of the field-of-view (at the position indicated by the laser symbol) after 10 seconds. (F) and (H) show images from single experiments, imaged before (left), directly after (middle) and at the end of the experiment (right). (G) and (I) show quantification of fluorescence recovery over time in five (G) or ten (I) independent experiments. DD-DrpA^{K42A} parasites co-expressing mitochondrially-targeted RFP were grown in the absence (F-G) or presence (H-I) of Shield-1. There is little fluorescence recovery in cells grown in the presence of Shield-1 and we conclude that after ablation of *Tg*DrpA function, mitochondria in adjacent cells generally do not remain connected. This is consistent with *Tg*DrpA not being involved in mitochondrial fission.

Supplementary Movies available online at:
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