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Elucidation of the Biological role of ApiAP2 Transcription factors in the cell cycle of Toxoplasma *gondii*

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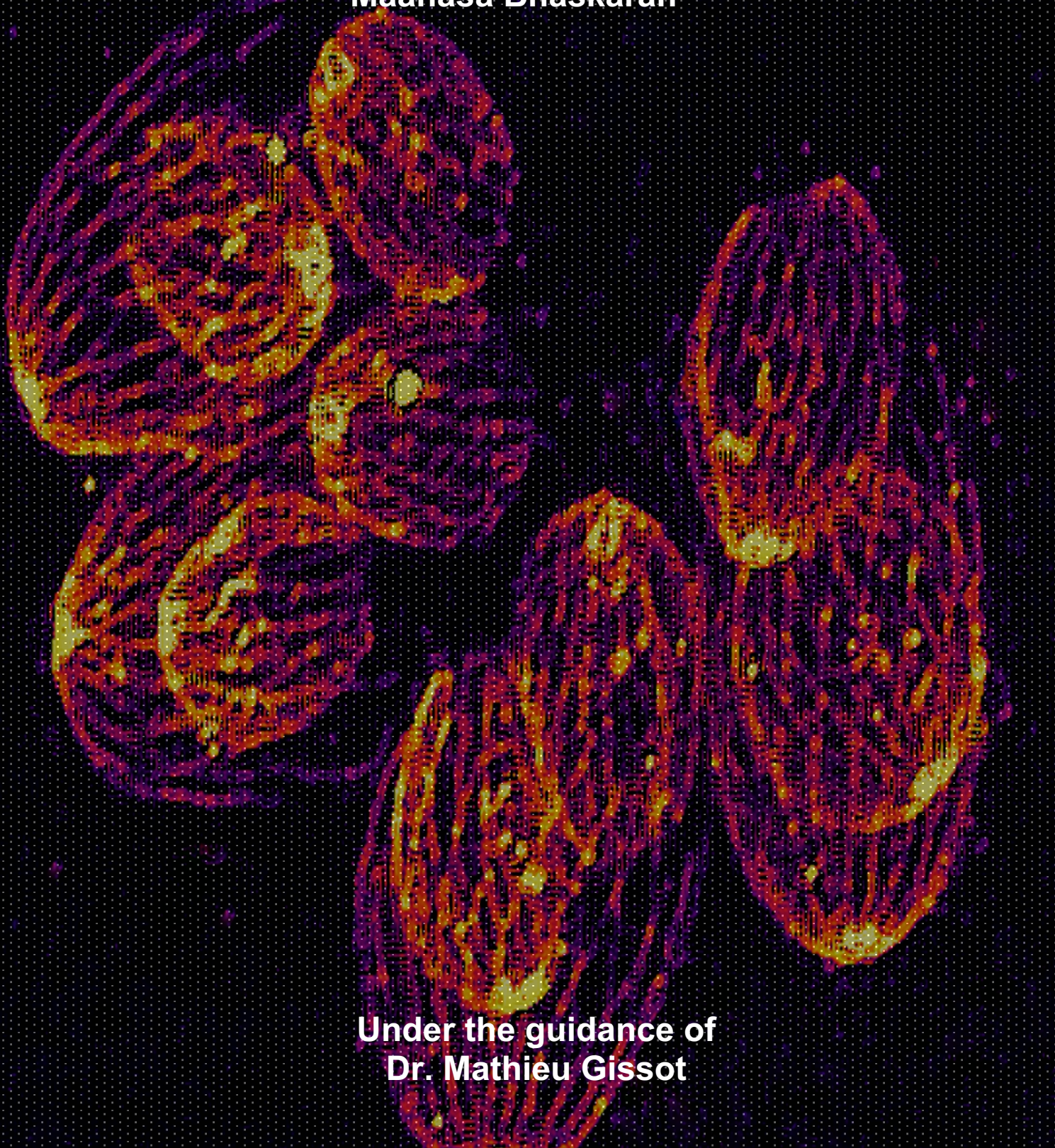

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“I am among those who think that science has great beauty.” - Marie Curie

To my grandparents

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ABSTRACT

The intracellular protozoan parasite *Toxoplasma gondii* undergoes rapid asexual replication through a process known as endodyogeny, which involves the formation of two daughter cells within a mother cell. This tightly regulated process depends on cell cycle-specific gene expression and post-translational modifications. This study builds upon previous findings that identified TgAP2IX-5 as a key transcription factor required for daughter cell budding. While TgAP2IX-5 regulates the expression of TgAP2XII-9 and TgAP2III-2, our focus is on the distinct roles of these two ApiAP2 transcription factors and the phosphatase TgPP1 in orchestrating cell cycle progression and daughter cell formation in *T. gondii*.

TgAP2XII-9 emerges as a key transcriptional regulator, orchestrating various stages of the cell cycle. Using a combination of immunofluorescence, RNA sequencing, and CUT&Tag assays, we demonstrate that TgAP2XII-9 acts as both a repressor and activator of gene expression, ensuring the proper timing and organization of daughter cell formation. TgAP2XII-9 represses genes associated with early stages of daughter budding, including those encoding apical cap and basal complex proteins, while simultaneously activating genes responsible for the elongation and maturation of the inner membrane complex (IMC), a critical structure that provides the framework for daughter cells. Depletion of TgAP2XII-9 leads to catastrophic defects in IMC scaffold formation, resulting in disorganized daughter cells and improper nuclear division. Moreover, TgAP2XII-9 is also involved in regulating the biogenesis of key virulence organelles. It activates genes associated with the formation of micronemes and dense granules while repressing rhoptry-associated genes, thus ensuring the precise timing of virulence factor production during the parasite's cell cycle. These findings highlight TgAP2XII-9's essential role in synchronizing cellular and organelle replication during *T. gondii* division.

In contrast, TgAP2III-2 does not exhibit the same transcriptional regulatory effects. While it binds extensively to the promoters of ribosomal RNA (rRNA) genes, depletion of TgAP2III-2 does not result in significant changes in transcript levels of these genes. This lack of transcriptomic alterations, despite its chromatin occupancy, suggests that TgAP2III-2 may play a role in epigenetic or chromatin-level regulation rather than direct

transcriptional control. The precise biological role of TgAP2III-2 remains elusive but is distinct from the role played by TgAP2XII-9, underscoring the functional diversity within the ApiAP2 family.

In parallel, we investigated the role of TgPP1, a serine/threonine phosphatase during the cell cycle. TgPP1 is crucial for regulating post-translational processes, including IMC assembly, organelle segregation, and nuclear division. Upon TgPP1 depletion, parasites exhibited defects similar to those seen with TgAP2XII-9 depletion, including aberrant IMC structure and failed daughter cell formation. Phosphoproteomic analyses revealed that TgPP1 dephosphorylates a wide array of proteins, including IMC components. Strikingly, TgPP1 depletion leads to the accumulation of amylopectin, a storage polysaccharide typically associated with the bradyzoite stage, linking PP1 to metabolic regulation in addition to its role in cell division.

This study expands our understanding of the molecular mechanisms driving *T. gondii* replication, from the transcriptional regulation initiated by TgAP2IX-5 to the downstream effects on cellular and metabolic processes.

Résumé

Le parasite protozoaire *Toxoplasma gondii* se réplique rapidement de manière asexuée par un processus appelé endodyogénie, qui résulte à la formation de deux cellules filles à l'intérieur d'une cellule mère. Ce processus, étroitement régulé, dépend de l'expression spécifique de gènes au cours du cycle cellulaire et de modifications post-traductionnelles. Les travaux réalisés au cours de cette thèse s'appuient sur des résultats antérieurs qui ont identifié TgAP2IX-5 comme un facteur de transcription clé, nécessaire à l'initiation des mécanismes conduisant à la création des cellules filles. TgAP2IX-5 régule l'expression de TgAP2XII-9 et TgAP2III-2, notre attention s'est porté sur les rôles distincts de ces deux facteurs de transcription ApiAP2 et aussi de la phosphatase TgPP1 dans la progression du cycle cellulaire et la formation des cellules filles chez *T. gondii*.

TgAP2XII-9 émerge comme un régulateur transcriptionnel clé, orchestrant différentes étapes du cycle cellulaire. En utilisant une combinaison d'analyses d'immunofluorescence, de séquençage ARN, et de CUT&Tag, nous démontrons que TgAP2XII-9 agit à la fois comme un répresseur et un activateur de l'expression génique, assurant la synchronisation et l'organisation de certaines étapes post-initiation dans la formation des cellules filles. TgAP2XII-9 réprime les gènes associés l'initiation de la formation des cellules fillestout en activant simultanément les gènes responsables de l'élongation et de la maturation du complexe de la membrane interne (IMC) des cellules filles. La déplétion de TgAP2XII-9 conduit à des défauts dans la formation de l'armature de l'IMC, entraînant la formation de cellules filles désorganisées. De plus, TgAP2XII-9 régule la biogenèse des organites de virulence clés. Il active l'explression d'une partie des gènes produisant les protéines destinées aux micronèmes et des granules denses tout en réprimant l'expression d'une partie desgènes produisant les protéines destinées aux rhoptries, assurant ainsi un contrôle temporal précis de l'expression des facteurs de virulence au cours du cycle cellulaire du parasite. Ces résultats mettent en évidence le rôle essentiel de TgAP2XII-9 dans la synchronisation de la réplication cellulaire chez le tachyzoite de *T. gondii*.

En revanche, TgAP2III-2 n'exerce pas les mêmes effets régulateurs. Bien qu'il se lie de manière extensive aux promoteurs des gènes de l'ARN ribosomique (ARNr), la déplétion de TgAP2III-2 n'entraîne pas de modifications significatives des niveaux de transcription de ces gènes. Cette absence d'altérations transcriptomiques, malgré son occupation de la chromatine, suggère que TgAP2III-2 pourrait jouer un rôle redondant dans la régulation transcriptionelle plutôt qu'un contrôle transcriptionnel direct. Le rôle biologique précis de TgAP2III-2 reste encore à définir, mais il est distinct de celui joué par TgAP2XII-9, soulignant la diversité fonctionnelle au sein de la famille ApiAP2.

Parallèlement, nous avons étudié le rôle de TgPP1, une phosphatase sérine/thréonine. TgPP1 est crucial pour la régulation des processus post-traductionnels, y compris l'assemblage de l'IMC, la ségrégation des organites et la division nucléaire. Suite à la déplétion de TgPP1, les parasites présentent des défauts similaires à ceux observés lors de la déplétion de TgAP2XII-9, notamment une structure IMC aberrante. Les analyses phosphoprotéomiques ont révélé que TgPP1 déphosphoryle un large éventail de protéines, y compris des composants de l'IMC. De manière frappante, la déplétion de TgPP1 entraîne l'accumulation d'amylopectine, un polysaccharide de stockage typiquement associé au stade bradyzoïte, liant ainsi PP1 à la régulation métabolique en plus de son rôle dans la division cellulaire.

Cette étude enrichit notre compréhension des mécanismes moléculaires qui sous-tendent la réplication de *T. gondii*, de la régulation transcriptionnelle initiée par TgAP2IX-5 aux effets en aval sur les processus cellulaires et métaboliques.

List of Abbreviations

ABA	Abscissic Acid
AAP	Apical Annuli Protein
AC	Apical Cap
AID	Auxin Inducible Degradation
AIP	ARO-Interacting Protein
AMA1	Apical Membrane Antigen 1
AP2	Apetala-2
APH	Acylated Pleckstrin Homology domain-containing protein
APR	Apical Polar Ring
ARO	Armadillo Repeats Only protein
ATFs	Activating Transcription Factors
BFD1	Bradyzoite Formation Deficient 1
BIC	Basal Inner Collar
BIR	Basal Inner Ring
BLAST	Basic Local Alignment Search Tool
CAM	Calmodulin-like
CDPK	Calcium Dependent Protein Kinase
CORVET	Class C Core Vacuole/Endosome Transport
CPE	Core Promoter Elements
CRISPR/Cas9	Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR associated protein 9
CRC	Corepressor Complex
CrK	Cyclin related Kinase
CT	Congenital Transmission
CTD	Carboxyl-Terminal Domain
DAG	Diacylglycerol
DAPI	Di Aminido Phenyl Indol
DBA	Dolichos biflorus Agglutinin
DCX	Doublecortin protein
DGK1	Daicylglycerol Kinase 1
DHFR	Dihydrofolate Reductase-thymidine synthase

DLC	Dynein Light Chain
DLC8a	Dynein Light Chain 8a
DMEM	Dulbecco's Modified Eagle's Medium
DPE	Downstream Promoter Elements
DrpA	Dynamin-related Protein A
DT	Dye Test
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENO1	Enolase-1
ENO2	Enolase-2
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
ERK7	Extracellular Signal-Regulated Kinase 7
ERF	Ethylene Response Factor
FBS	Fetal Bovine Serum
FCP	FIIF-associating carboxyl-terminal domain protein
FLU	Fluoridone
GAC	Glideosome Associated Connector
GAP	Glideosome Activating Protein
GAPM	Glideosome associated protein with transmembrane domain
GC	Guanylate Cyclase
GCN5	lysine acetyltransferase
cGMP	Cyclic Guanosine Monophosphate
GPI	Glycosyl-phosphatidyl-inositol
GRA	Dense GRAnules Proteins
GTFs	General Transcription Factors
GTP	Guanosine Triphosphate
GVBD	Germinal Vesicle Breakdown
HAD	Haloacid Dehalogenase
HAT	Histone Acetylase enzyme
HDAC	Histone Deacetylase enzyme
HFF	Human Foreskin Fibroblasts
HOPS	Homotypic Vacuole Fusion and Protein Sorting
HXGPRT	Hypohanthine Xanthine Guanine PhosphRibosylTransferase
hyperLOPIT	hyperplexed Localization of Organelle Proteins by Isotope Tagging
IAA	Indole-3-Acetic Acid
IECs	Intestinal Epithelial Cells
IFA	Immunofluorescence Assay

IFAT	Indirect Fluorescent Antibody Test
IFN-γ	Interferon γ
Ig	Immunoglobulin
IHA	Indirect Hemaglutination Assay
IMC	Inner Membrane Complex
IMP	Inner Membranous Particle
IP	Immunoprecipitation
IP3	Inositol-1,4,5-triphosphate
IPP	Isopentenyl diphosphate
IRG	Immune-related GTPases
ISAGA	Immunosorbent Agglutination Assay
ISC	IMC suture component
ISP	IMC Sub-compartment Proteins
IST	Inhibitor of STAT1 Transcriptional activity
ITS-1	Internal Transcribed Spacer
IVN	Intravacuolar Network
KAT	Lysine Acetyltransferase
KD	Knock-down
KDAC	Lysine Deacetyltransferase enzyme
KI	Knock-in
KMT	Lysine methyltransferase
KO	Knock-out
M	Mitosis
MAF1b	Mitochondrial Association Factor 1b
M2AP	MIC2-associated protein
MAF1	Mitochondrial Associating Factor1
MAPK	Mitogen Activated Protein Kinase
MAP	Microtubule associated Protein
MIC	Microneme proteins
MJ	Moving Junction
MLC	Myosin Light Chain
MORC	Microchidia MORN1 Membrane Occupation and Recognition Nexus1 protein
mRNA	Messenger Ribonucleic Acid

MSC1a	Mature Soluble Cytoskeletal 1a protein
MT	Microtubules
MTOC	Microtubule Organizing Center
Myo	Myosin
NFAT4	Nuclear Factor of Activated T cell 4
OA	Okadaic Acid
PA	Phosphatidic Acid
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCRs	Pre-conoidal Rings
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pI	Isoelectric point
PIC	Pre-initiation Complex
PI-PLC	Phosphinositide Phospholipase C
PIPs	Phosphatase Type 1-Interacting proteins
PKG	Protein Kinase G/cGMP-dependent Protein Kinase
PPKL	Protein Phosphatase with characteristic Kelch-like domain
PLP1	Perforin-like Protein 1
PM	Plasma membrane
PMSF	Phenylmethyl Sulfonyl Fluoride
PP2C	Protein Phosphatase 2
PPM	Periplastid membrane
PPMs	Protein Phosphatases that are Mg2+/Mn2+- dependent
PPPs	Phospho-Protein Phosphatases
PRMT	Protein Arginine Methyltransferase
PRP1	Parafusin Related Protein 1
PSPs	Protein Serine/Threonine Phosphatases
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuole Membrane
PYR	Pyremethamine
RNG1	Ring-1

RNG-2	Ring-2
RON	Rhoptry Neck Protein
ROP	Rhoptry Bulb Protein
rRNA	Ribosomal Ribonumcleic Acid
SAG	Surface Antigen Glycoprotein
SAGE	Serial Analysis of Gene Expression
SAS6L	SAS6 centriole associated-like protein
SFA	Striated-Fiber Assemblin
SLP	Shewanella-Like Phosphatase
SPM1	Subpellicular Microtubule Protein 1
SPMTs	Sub-Pellicular Microtubules
SPN	Subpellicular Network SRS
SR-SIM	Super-resolution Structured Illumination Microscopy
STAT	Signal Transducer and Activator of Transcription
TAFs	TBP Associated Factors
TBP	TATA Binding Proteins
TFA	Trifluoracetic acid
TMP	Trimethoprime
TRANSFAC	Transcription Factor Database
tRNA	Transfer Ribonucleic Acid
TSC	Transverse suture component
TSSs	Transcription Start Sites
TVN	Tubulo-Vesicular Network
U-ExM	Ultrastructure Expansion Microscopy
WB	Western Blot

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INTRODUCTION

Chapter 1 – Introduction

1. The Apicomplexan Phylum

The phylum Apicomplexa comprises a vast group of single-celled, obligate intracellular protozoan organisms, all characterized by a parasitic lifestyle. Among the over 6,000 named species (Adl et al., 2007), and likely more than one million unnamed species (Seeber & Steinfelder, 2016), some hold significant public health and economic relevance, causing severe diseases in humans and livestock that impact millions annually (Battle et al., 2012; Checkley et al., 2015; Torgerson & Mastroiacovo, 2013). Given their profound impact on global health, there is a pressing need for increased knowledge about their biology. Understanding their general biology can help exploit vulnerabilities, while insights into their interactions with host organisms can inform strategies to stimulate the immune system. This knowledge is crucial for developing effective interventions to combat the diseases they cause. The phylum Apicomplexa includes several genera of significant medical and veterinary importance (Figure 1):

1. **Plasmodium**: This genus is the etiological agent of malaria, responsible for an estimated 249 million malaria cases and 608,000 deaths were reported across 85 countries worldwide in 2022 (World Health Organization, 2023).
2. **Toxoplasma gondii**: The causative agent of toxoplasmosis, *T. gondii* infects nearly one in three adults worldwide. It causes severe threat to individuals with compromised immunity (like cancer and organ transplant patients on immunosuppressive drugs) and pregnant women. It poses severe health risks, particularly for individuals living with HIV/AIDS (Belanger et al., 1999). Cerebral toxoplasmosis remains a major concern for individuals with HIV, particularly those experiencing advanced immune system suppression.The condition typically arises from the reactivation of dormant *T. gondii* cysts in the brain, causing neurological issues such as headaches, confusion, and localized deficits. Diagnosis usually involves evaluating clinical symptoms, neuroimaging results, and serological tests, while confirmation requires detecting the parasite in cerebrospinal fluid or brain tissue (Dian et al., 2023). Although the incidence

has declined in areas with widespread antiretroviral therapy, cerebral toxoplasmosis remains a leading cause of central nervous system complications among HIV patients, particularly in regions with limited healthcare resources.

3. **Cryptosporidium**: A waterborne pathogen, *Cryptosporidium* has significant implications for immune-compromised individuals (P. R. Hunter & Nichols, 2002)
4. **Gregarina**: An invertebrate parasite, *Gregarina* serves as a useful model for studying apicomplexan motility.
5. **Eimeria:** is an important genus of apicomplexan parasites that causes coccidiosis, a significant enteric disease affecting various animals, particularly poultry and cattle, with a complex life cycle involving both sexual and asexual reproduction stages in a single host.
6. **Neospora:** parasite of significant veterinary importance, known for causing neosporosis, a leading cause of abortion in cattle and neurological disease in dogs.
7. **Sarcocystis**: Affects livestock and wildlife (birds and reptiles), causing sarcocystosis, which can lead to muscle cyst formation, abortion in animals, and foodborne infections in humans.
8. **Theileria**: Another genus of parasite that infects livestock and cattle, leading to severe economic losses due to anemia, fever, and death in affected animals.

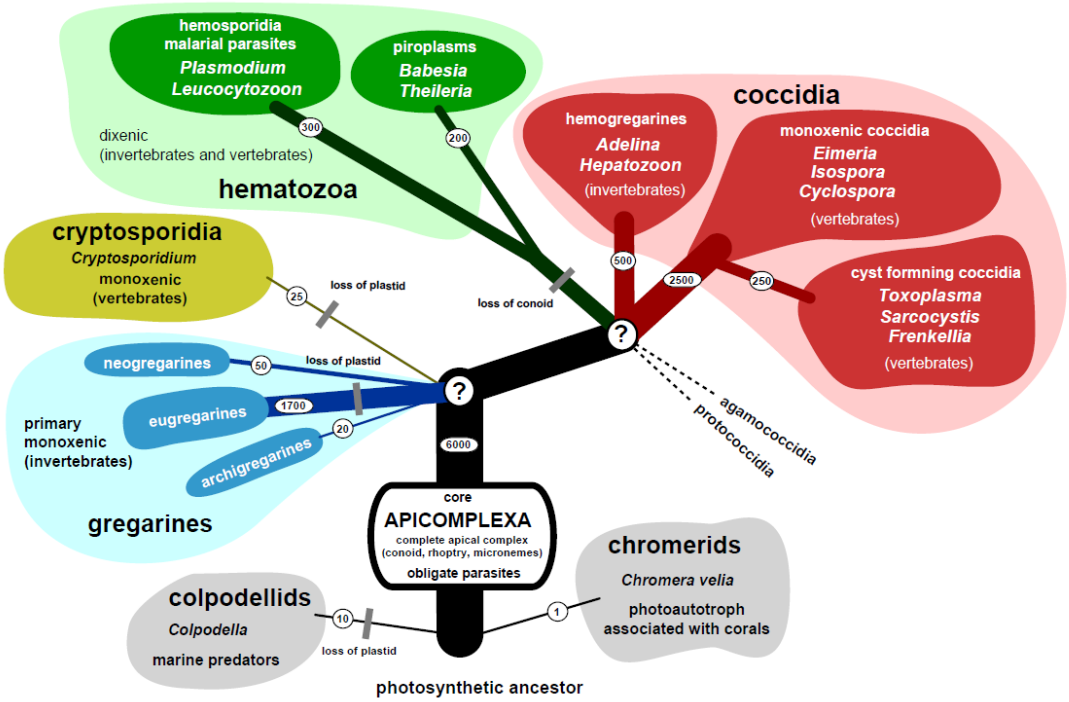

Figure 1- A schematic representation of the various Apicomplexa taxonomic subclasses (Portman & Šlapeta, 2014). The phylogenetic tree illustrates members of the Apicomplexa phylum and their closest related relatives. The four main sub-groups within the Apicomplexa phylum are: Cryptosporidia, Gregarines, Hematozoa, and Coccidia.

This extensive and diverse phylum is part of the higher-order group of protozoans known as Alveolata. The Alveolata group also includes ciliates, which are small predators, and Dinoflagellates, which are marine phytoplankton. A defining feature of Alveolata is the presence of flattened vesicle-like structures beneath the plasma membrane, known as cortical alveoli. In Apicomplexa, these structures are specifically referred to as the Inner Membrane Complex (IMC). The name Apicomplexa derives from “apex," meaning "tip" or "peak," and "complexus," meaning "interwoven" or "network." It refers to the apical complex, a specialized structure located at the tip of these parasites, which is crucial for host cell invasion (Adl et al., 2005). This structure, characteristic of the group, includes organelles like the conoid, micronemes, rhoptries, and polar rings, all of which play key roles in parasite biology (Barta, 1989). Another common feature among Apicomplexan species is their parasitic lifestyle; they have evolved to depend entirely on host cells for reproduction (Dubey, 1998).

The life cycle of apicomplexan parasites is complex, encompassing three broad stages: sporozoite, merozoite, and gametocyte (Figure 2). Despite a common general life cycle within the phylum, there are notable differences between species. Some, like *Cryptosporidium*, require a single host, while others, such as *Theileria* and *Plasmodium*, involve more intricate cycles that necessitate sexual reproduction in a vector species for transmission. Apicomplexans are distinguished by specific organelles that facilitate host cell attachment, invasion, and the formation of an intracellular parasitophorous vacuole. This organelle arsenal typically includes rhoptries, micronemes, and dense granules, with proteins stored in these vesicles released through the apical complex at the cell's anterior (Sibley, 2004). Additionally, all examined apicomplexans, except for *Cryptosporidium* and *Gregarina*, possess an apicoplast organelle, believed to be the result of an ancient secondary endosymbiotic event with an algal cell (Fast et al., 2001; Toso & Omoto, 2007; Zhu et al., 2000). The genome of this plastid has significantly reduced, retaining genes primarily involved in organelle replication (Wilson et al., 1996).

Of these parasites, *Toxoplasma gondii* is often referred to as the model apicomplexan parasite due to the ease and abundance of genetic engineering tools available for manipulating its genome. This highly successful parasite can invade any nucleated cells in warm-blooded animals and birds (V. B. Carruthers, 2002).

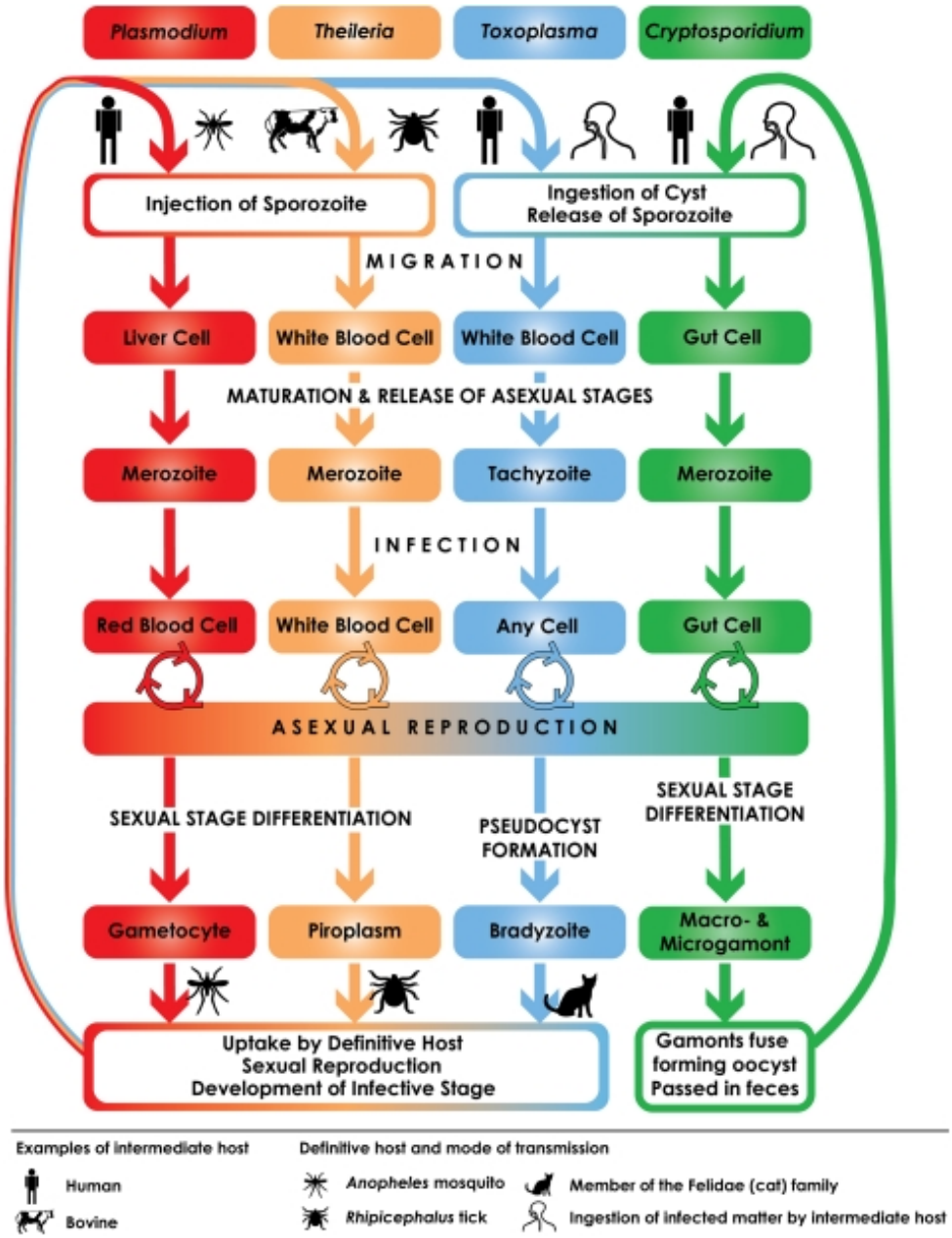

Figure 2- The lifecycle of Apicomplexans (Wasmuth et al., 2009)**.** Apicomplexan parasites share a generalized life cycle with species-specific specializations. *Plasmodium* spp. and *Theileria* spp. are transmitted and undergo sexual recombination in insect vectors, specifically the *Anopheles* mosquito and *Rhipicephalus* tick, respectively. *Cryptosporidium* can autoinfect its host; its oocysts sporulate and excyst within the same host, sustaining infection for months to years. Representing the coccidian parasites, *Toxoplasma gondii* can infect most warm-blooded animals. Differentiation of *Toxoplasma* bradyzoites into gametocytes occurs exclusively in felids (cats), although the molecular mechanisms regulating this process remain unknown. The tachyzoites undergo differentiation to form a latent and dormant form of tissue cyst called the bradyzoites, which are again taken up by the definitive host to carry on with the cycle again.

2. *Toxoplasma gondii*, a model Apicomplexan parasite

Toxoplasma gondii is a member of the Sarcocystidae family within the class Coccidia, and it stands as the sole species in the Toxoplasma genus. Coccidia are parasites that are obligate, intracellular, and capable of forming cysts, entering their host via the gastrointestinal tract. According to NCBI (Taxonomy ID: 5811), *T. gondii* is classified as follows:

Domain	**Eukaryota**
Kingdom	Alveolata
Phylum	Apicomplexa
Class	Conoidasida
Sub-class	Coccidia
Order	Eucoccidiorida
Sub-Order	Eimeriorina
Family	Sarcocystidae
Genus	*Toxoplasma*
Species	*gondii*

Table 1 - *Toxoplasma gondii* - Taxonomic Classification

Among the identified members of the Apicomplexa phylum, *T. gondii* stands out as one of the most successful parasites. This is due to its extraordinary ability to infect nearly all warm-blooded wild and domestic animals across diverse terrestrial ecosystems. *T. gondii* exhibits a cosmopolitan distribution and can infect a wide range of hosts. Additionally, it multiplies in various cell types *in vivo* and can parasitize all nucleated cells in vitro. Consistent with these characteristics, serological surveys indicate that approximately one-third of the human population has been exposed to *T. gondii* (Halonen & Weiss, 2013) with even higher prevalence rates in parts of Europe and South America. The serological markers used in these surveys identify individuals who have encountered the fast-replicating tachyzoite stage during the acute phase of infection. Presence of IgG antibodies usually reflects past infection and indicates immunity, which often correlates with the presence of the slow-replicating bradyzoite stage in tissue cysts during the chronic phase. Bradyzoites settle in tissue cells away

from the initial intestinal site of colonization, particularly in cells of the central and peripheral nervous systems, as well as in skeletal and cardiac muscles.
Reflecting the diverse host range of *T. gondii*, multiple genotypes are found globally. Predominantly, the parasite strains fall into three clonal lineages - type I, II, and III—extensively studied in laboratory mice (Khan et al., 2009). These lineages are characterized by distinct virulence profiles and cyst-forming abilities (Howe & Sibley, 1995). Type I strains, highly virulent in mice, induce hyper-inflammation and lethal parasite dissemination, failing to establish latent infections even with minimal inoculation (Boothroyd & Grigg, 2002). In contrast, type II and III strains exhibit lower virulence during acute infection phases, necessitating higher parasite doses for lethality in mice (Saeij et al., 2005). They also demonstrate slower growth rates compared to type I strains and possess a robust capacity for cystogenesis (Boothroyd & Grigg, 2002; Fuentes et al., 2001; Grigg et al., 2001; Saeij et al., 2005). Despite the focus on these main clonal lines in laboratory settings, genotyping techniques have identified *T. gondii* isolates that diverge from these established categories (Dardé, 2008; Khan et al., 2007; Robert-Gangneux & Dardé, 2012a).

T. gondii is widely regarded as a model apicomplexan due to its unique biological attributes, which facilitate comprehensive research and understanding of apicomplexan parasites. It exhibits a complex life cycle, capable of infecting a broad range of warm-blooded hosts, including humans and animals, thereby offering extensive opportunities to study host-pathogen interactions (Blader & Saeij, 2009a). Its genome has been fully sequenced, and it is highly amenable to genetic manipulation using advanced tools like CRISPR/Cas9, enabling precise functional genomics studies (Shen et al., 2014). Furthermore, *T. gondii's* ability to switch between sexual and asexual reproduction stages provides a model for investigating parasite development and differentiation (White et al., 2014a). The medical relevance of *T. gondii*, as it causes toxoplasmosis, further emphasizes its importance as a research model, offering insights into disease mechanisms and potential therapeutic targets (Webster, 2010). These characteristics collectively make *T. gondii* an exemplary model for studying the biology and pathogenicity of apicomplexan parasites.

2.1. Life cycle of *Toxoplasma gondii*

T.gondii is known to infect a wide range of hosts, from terrestrial and aquatic mammals to birds. This renders these animals as intermediate hosts because they undergo the asexual stages of the parasite (Figure 3). The sexual stages of *T. gondii* have only been observed in members of the Felidae family, including domestic cats, so they are regarded as definitive hosts (Dubey, 2009; Dubey et al., 1998; Speer & Dubey, 2001). Interestingly, new evidence has shown that inhibition of the activity of delta-6-desaturase in murine, when supplemented with linoleic acid in the diet, allows *T. gondii* to complete its sexual development within the cells of the intestines (Di Genova et al., 2019).

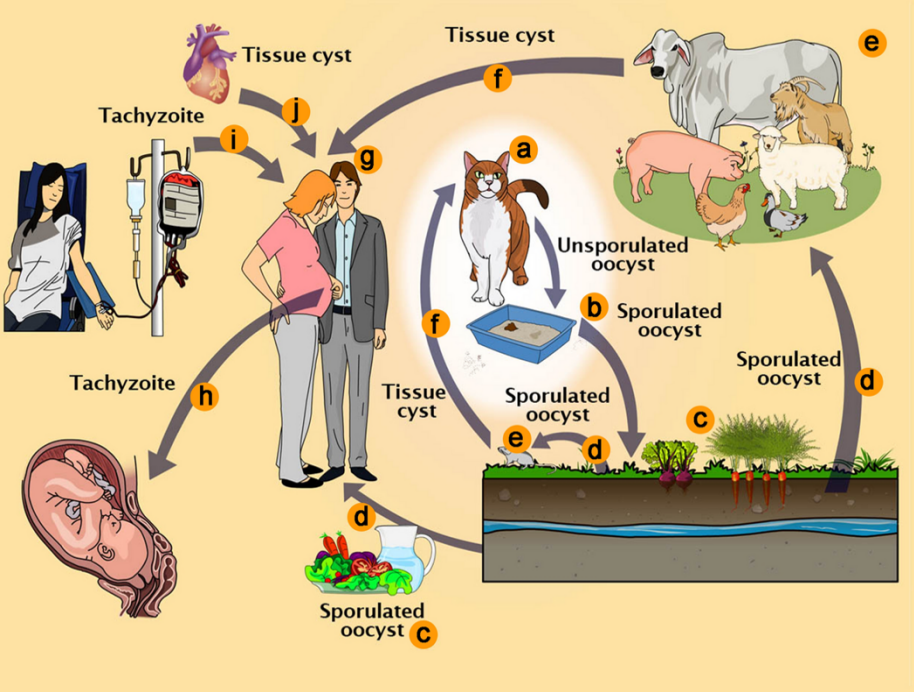

Figure 3 - Toxoplasma gondii pathways of transmission (Attias et al., 2020). Members of the Felidae family are the only known definitive hosts for Toxoplasma gondii (domestic cats and their relatives). **(a, b)** Unsporulated oocysts are excreted by cats. Large numbers of oocysts may be shed, despite the fact that they typically only shed for 1-3 weeks. **(c)** Oocysts sporulate in the environment for 1-5 days before becoming infective. **(d, e)** The consumption of oocyst-contaminated soil, water, or plant matter by intermediate hosts in nature (such as birds and rodents, cattle) results in infection. Tachyzoites form quickly after ingesting oocysts. These tachyzoites evolve into tissue cyst bradyzoites. **(f)** Ingestion of tissue cysts in uncooked meat by Intermediate hosts (humans, **g**) **(h)** Tachyzoites are transmitted through the placenta to the foetus and also through **(i)** transmission by blood transfusion and organ transplant **(j).** These tachyzoites evolve into tissue cyst bradyzoites after settling in neural and muscular tissue

(i, j). Cats become infected after ingesting intermediate hosts that contain tissue cysts. Cats can also become infected by consuming sporulated oocysts.

2.1.1. The Developmental Stages of *Toxoplasma gondii* in Its Definitive Host: Schizonts, Gametes, and Gametogenesis

Cats become infected by ingesting tissues containing bradyzoites or oocysts from the environment. Once inside the cat's digestive system, the walls of the cysts in the stomach are likely disrupted by the low pH and the action of proteolytic enzymes, leading to the release of bradyzoites or sporozoites respectively. These parasites in either case, invade the intestinal epithelial cells of the cat, and become schizonts.

At the time of schizogony, successive nuclear division takes place several times before the cells individualize. After some cycles of nuclear division, development of the inner membrane complex for the individualization of each merozoite begins concomitantly with the growth of the apical complex, a set of micronemes and rhoptries - typical Apicomplexa structures. These cytoplasmic structures align around each nucleus until they result in individual daughter cells merozoites inside the host cell. When these enterocytes burst, numerous merozoites are liberated that subsequently infect new enterocytes and multiply by schizogony (Dubey & Frenkel, 1972). By the end of each cycle, several merozoites are liberated to invade new enterocytes and thus the number of parasites multiplies exponentially (Figure 4).

Three to fifteen days after primary infection, some of the merozoites differentiate into gametocytes, the sexual forms of the parasite (Ferguson, 2002). There are two types of gametocytes: macrogametocytes (female) and microgametocytes (male). This differentiation is a critical step for the continuation of the parasite’s life cycle, allowing for sexual reproduction to occur. Microgametes, which are derived from the microgametocytes, exhibit sperm-like motility and fertilize the stationary macrogametes produced by the macrogametocytes (Speer & Dubey, 2005). The fusion of these gametes results in the formation of zygotes within the epithelial cells of the cat's intestine. These zygotes subsequently develop into oocysts. The mature oocysts are then shed in the cat's feces, entering the environment. Upon exposure to oxygen, these oocysts undergo sporulation, a process that takes one to five days,

depending on environmental conditions, and become infective by containing sporozoites (Dubey & Frenkel, 1972; Ferguson et al., 1979). These sporulated oocysts are highly resistant and can survive in various environmental conditions for extended periods, facilitating widespread transmission (Frenkel et al., 1975; C. A. Hunter & Sibley, 2012; Montoya & Liesenfeld, 2004; Robert-Gangneux & Dardé, 2012a). Sporulated oocysts can contaminate soil, water, and food, leading to transmission to intermediate hosts when ingested. In the intermediate hosts, the oocysts release sporozoites that invade host cells and transform into tachyzoites, perpetuating the asexual phase of the life cycle. This ability to infect a wide range of intermediate hosts and survive in diverse environmental conditions contribute significantly to the epidemiology of toxoplasmosis.

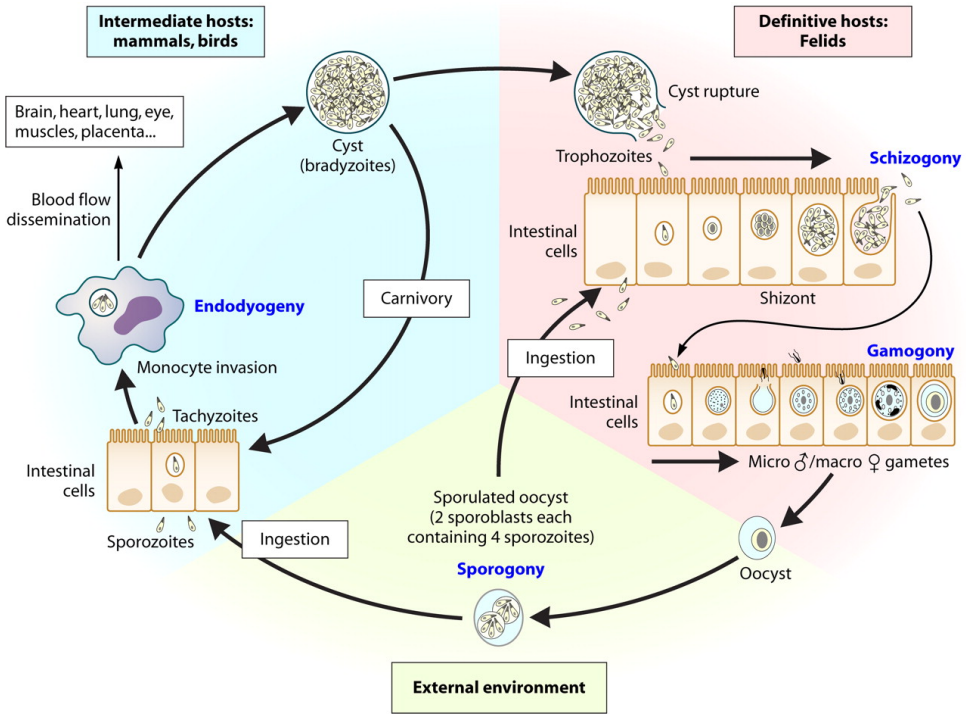

Figure 4 - Schematic representation of the three main stages of development of *Toxoplasma gondii* (Robert-Gangneux & Dardé, 2012a)**.** Sexual Phase in the **Definitive Host:** Upon ingestion of tissue cysts, the cyst wall is degraded by gastric enzymes, releasing bradyzoites that infect enterocytes. After several stages of asexual multiplication, schizonts form, where merozoites develop during schizogony. This is followed by sexual multiplication or gamogony, where merozoites differentiate into male and female gametes. Fusion of these gametes results in the formation of oocysts in the enterocytes, which are subsequently released into the environment via the cat's feces. **Environmental Phase:** Once released, oocysts undergo sporulation influenced by environmental factors such as temperature, pressure, and pH. This phase, known as sporogony, produces mature infectious oocysts, each consisting of two

sporocysts containing four sporozoites. **Asexual Phase in the Intermediate Host:** In homeotherms, ingestion of sporulated oocysts leads to the release of sporozoites that infect enterocytes and differentiate into tachyzoites. Tachyzoites replicate by endodyogeny and disseminate throughout the host's body, eventually converting into bradyzoites to form latent tissue cysts within the intermediate or final hosts.

2.1.2. The asexual stages of *T.gondii* lifecycle in intermediate Hosts

The asexual development cycle of *T.gondii* involves several stages, beginning with the ingestion of sporulated oocysts or tissue cysts by an intermediate host. Upon ingestion, the oocysts release sporozoites that invade the intestinal epithelium and differentiate into tachyzoites (Figure 4), the rapidly dividing form of the parasite. Tachyzoites replicate through a process known as endodyogeny (Figure 5), where two daughter cells are formed within the mother cell and subsequently released, allowing the infection to disseminate throughout the host's body via the bloodstream (Dubey et al., 1998). These tachyzoites can invade various tissues, including the brain, heart, and muscles, where they eventually convert into bradyzoites, forming tissue cysts. Bradyzoites are the slow-growing form of the parasite and can persist for the lifetime of the host, serving as a reservoir for transmission to definitive hosts or other intermediate hosts through predation or consumption of contaminated food (Weiss & Kim, 2000). This conversion from tachyzoites to bradyzoites is a crucial aspect of the parasite's ability to evade the host's immune response and maintain chronic infection (Pittman et al., 2014a)

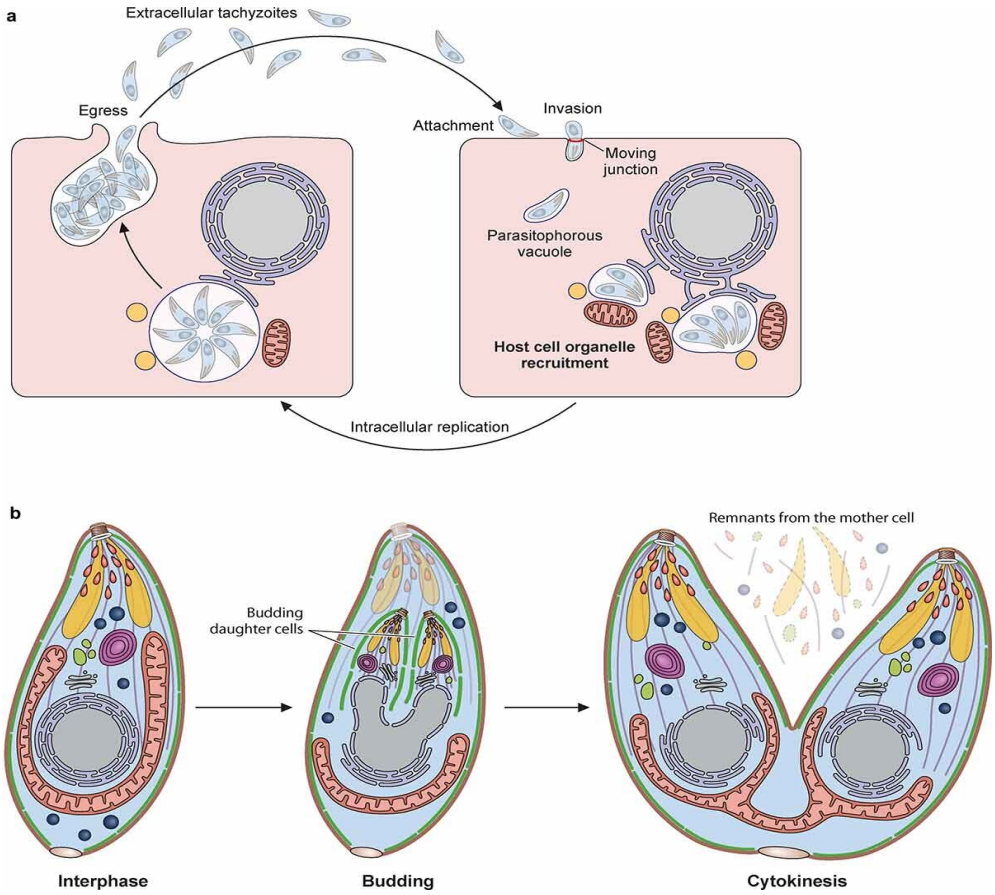

Figure 5 – Illustration of the asexual repliaction and endodyogeny (Sanchez & Besteiro, 2021)**. (a)** The lytic cycle of tachyzoites include invasion of host cell, replication within the host and egress of newly formed tachyzoites. **(b)** The process of Endodyogeny involves the organized formation and internal budding of two daughter cells within a single mother cell.

2.1.3. Differentiation to Bradyzoites from tachyzoites – A survival strategy?

The differentiation of *T.gondii* from tachyzoites to bradyzoites is a key survival mechanism that enables the parasite to persist in the host for long periods. This process is critical for the transmission and pathogenesis of *T. gondii* and involves several complex regulatory mechanisms. The transition from tachyzoites, the rapidly replicating form responsible for acute infection, to bradyzoites, the slow-growing form within tissue cysts, is often triggered by environmental stress conditions (Weiss et al., 1998a). Factors such as immune response pressure, nutrient deprivation, and changes in the host's hormonal levels can induce this differentiation. For instance, arginine deprivation has been shown to trigger cyst formation, highlighting the parasite's sensitivity to nutrient availability as a cue for stage conversion (Fox et al., 2004)

Bradyzoites are also structurally distinct from tachyzoites. They accumulate high levels of amylopectin granules in their cytoplasm, which may serve as energy reserves (Uboldi et al., 2015). The cyst wall, which encapsulates bradyzoites, is less permeable and provides a protective environment (Tomita et al., 2017), allowing the parasite to evade the host's immune system. At the molecular level, the differentiation process involves significant changes in gene expression. Stage-specific proteins, such as heat shock proteins (HSPs) and surface antigens (SAGs), play crucial roles. HSPs, for example, are involved in protein homeostasis and DNA repair, and their expression is developmentally regulated to support the transition and maintenance of the bradyzoite form (Bohne et al., 1995; Weiss et al., 1998b).

Once differentiated, bradyzoites can remain dormant within tissue cysts for the host's lifetime. These cysts are primarily found in neural and muscular tissues, where they can evade immune detection (Dubey et al., 1997). Under certain conditions, such as immunosuppression, bradyzoites can reactivate and convert back into tachyzoites, leading to re-infection and acute disease manifestations (Pan et al., 2017; Wang et al., 2017).

3. Toxoplasmosis: From Symptoms to Treatment

It is generally estimated that approximately 25 to 30% of the global human population is infected with *Toxoplasma gondii* (Montoya & Liesenfeld, 2004). However, the prevalence of infection varies widely between countries and even within different communities in the same region (Pappas et al., 2009). In North America, Southeast Asia, Northern Europe, and Sahelian countries of Africa, low seroprevalence rates of 10 to 30% have been observed. In contrast, countries in Central and Southern Europe exhibit moderate prevalence rates of 30 to 50%. High prevalence rates are found in Latin America and tropical African countries, where the infection rates can reach up to 80%. These variations can be attributed to differences in dietary habits, climate, socioeconomic conditions, and public health practices across different regions (Robert-Gangneux & Dardé, 2012b)

3.1. Modes of transmission to Humans

Until 1970, the mechanisms of transmission of *T.gondii* had not been established until its life cycle was discovered. Two basic routes of transmission take place in human beings (Montoya & Liesenfeld, 2004; Robert-Gangneux & Dardé, 2012b) The first is a **vertical transmission route**, which means the passage of tachyzoites into the foetus from an infected pregnant woman, through placentas—that is, transplacental usually after primary infection in pregnancy. Congenital toxoplasmosis is reported to have a prevalence range of 1-10 per 10,000 live births (Guerina et al., 1994; Montoya & Liesenfeld, 2004). The third trimester carries a higher probability of transmission compared to the first and second trimester, at 65%, 25% and 54%, respectively (McAuley, 2014). Though, infection acquired earlier in gestation is related to more severe pathological outcomes. **Horizontal transmission** occurs in most cases through the intake of food or water contaminated with sporulated oocysts that are released with cat feces or tissue cysts within inadequately cooked meat. Also, though less frequently, transmission may come from blood transfusions or organ transplants containing cysts. One of the causes for a rather frequent disease in transplant patients is the reactivation of latent infection, mainly as a result of the immunosuppressive therapy they receive (Roth et al., 1971). Occupational transmission has also been reported to occur in cases where there has been exposure to contaminated needles, labware, or animal models during investigation work (Kayhoe et al., 1957; Remington & Gentry, 1970).

3.2. Dissemination within the intermediate host

The invasion of host cells by *T. gondii* is a complex process mediated by secretory organelles, namely the micronemes, rhoptries, and dense granules (Dubey & Beattie, 1988). These organelles secrete proteins that facilitate the attachment, penetration, and formation of the PV within the host cell (V. B. Carruthers & Sibley, 1997). Microneme proteins (MICs) are released first and play a crucial role in gliding motility and initial host cell attachment (Sibley, 2004). Following attachment, rhoptry proteins (ROPs) are secreted and are essential for the formation of the moving junction, a

structure that enables the parasite to penetrate the host cell membrane without disrupting it completely (Soldati & Meissner, 2004).

Once inside the host cell, *T. gondii* resides within the PV, a specialized compartment that protects it from the host cell's lysosomal degradation pathways (Boothroyd & Dubremetz, 2008). The PV membrane (PVM) is extensively modified by dense granule proteins (GRAs), which are secreted by the parasite and alter the host cell environment to favour parasite survival and replication (Mordue & Sibley, 1997). For instance, GRA proteins facilitate the recruitment of host mitochondria and endoplasmic reticulum to the PVM, providing the parasite with nutrients and other essential molecules (Mercier et al., 2005).

T. gondii employs a variety of strategies to manipulate host cellular mechanisms to its advantage. One key aspect is the modulation of the host immune response. *T. gondii* can interfere with the host's innate and adaptive immune responses by secreting effectors that inhibit pro-inflammatory cytokine production and promote an anti-inflammatory environment (C. A. Hunter & Sibley, 2012). This immune modulation allows the parasite to establish a chronic infection within the host (Dupont et al., 2012). In addition, *T. gondii* influences host cell signaling pathways to promote its own survival and replication. For example, the parasite can modulate the host cell cycle and apoptosis pathways, ensuring that infected cells remain viable for the duration of the parasite's replication cycle (Carmen & Sinai, 2007). Moreover, *T. gondii* infection can lead to alterations in host cell metabolism, redirecting nutrients and energy sources to support the parasite's growth (Blader & Saeij, 2009b).

After replication within the PV, *T. gondii* tachyzoites are released and can infect neighboring cells, facilitating local dissemination (Lambert & Barragan, 2010). For systemic spread, the parasite can infect immune cells, such as macrophages and dendritic cells, which act as "Trojan horses" by transporting the parasite to distant tissues (Courret et al., 2006; Zenner et al., 1998). Once in a new tissue, *T. gondii* can differentiate into bradyzoites, the slow-growing form that resides within tissue cysts.These cysts can persist for the lifetime of the host, providing a reservoir for potential reactivation and transmission (Dubey, 1998).

3.3. Clinical Manifestations of Toxoplasmosis in humans

Congenital Toxoplasmosis

Congenital toxoplasmosis occurs when the *Toxoplasma gondii* parasite crosses the placental barrier during the primary infection of a pregnant woman. As previously noted, there is an inverse relationship between the transmission rate and the severity of the infection (Dunn et al., 1999). Consequently, the clinical manifestations of congenital toxoplasmosis can vary widely. The most severe outcomes include fetal death in utero or the development of mental and psychomotor retardation at birth. However, in 85% of cases, congenital toxoplasmosis is either asymptomatic or results in retinochoroiditis, which can cause visual impairment or blindness in its most severe forms (McAuley, 2014).

Toxoplasmosis in Immunocompromised Individuals

Toxoplasmosis poses a significant risk to immunocompromised individuals due to the reactivation of a chronic infection, characterized by the transformation of bradyzoite-containing cysts into highly replicative tachyzoites. This leads to inflammation and tissue destruction. Reactivation of latent cysts can result in disseminated toxoplasmosis, affecting multiple organs. The primary pathologies observed are:

a) **Cerebral Toxoplasmosis**

Cerebral toxoplasmosis is the most prevalent clinical manifestation in immunocompromised individuals (Lee & Lee, 2017; Luft et al., 1993). This condition typically presents with fever and symptoms such as headaches, motor or sensory deficits, or psychiatric disturbances(Montoya & Liesenfeld, 2004). It is particularly common among HIV-positive patients (Suzuki et al., 1988) and was a major cause of mortality prior to the advent of antiretroviral therapies.

b) **Ocular Toxoplasmosis**

Ocular toxoplasmosis, commonly seen in individuals infected in utero, also affects 10 to 20% of postnatally infected patients (McAuley, 2014). This condition manifests as visual disturbances, which can be irreversible, and the presence of floaters caused by the destruction of the posterior parts of the eye, particularly the retina (Montoya and

Liesenfeld, 2004). The primary cause is often the reactivation of cysts in the retinal area (Montoya and Liesenfeld, 2004).

Retinochoroiditis is the most frequent ocular manifestation of congenital toxoplasmosis (McAuley, 2014). Irreversible central vision loss can occur if the macula and/or optic nerve are affected, or due to complications such as retinal detachment (McAuley, 2014). Approximately 25% of patients with ocular toxoplasmosis experience a measurable decrease in vision (McAuley, 2014). Visual field impairment is even more common, occurring in about two-thirds of cases (McAuley, 2014).

c) Pulmonary Toxoplasmosis

In cases of severe immunosuppression, rare but potentially fatal pulmonary complications can occur in the form of pneumonia (Rabaud et al., 1996). With the success of antiretroviral therapy in HIV patients, pulmonary toxoplasmosis is now more commonly observed in patients with other types of immunodeficiencies, such as transplant recipients, elderly individuals with weakened immune systems, and infants whose immunity is still immature (Rabaud et al., 1996; Montoya & Liesenfeld, 2004). The development of early diagnostic methods for these infections is a major challenge, as it can help reduce medical costs, morbidity, and the unfortunately high mortality associated with this disease (Montoya & Liesenfeld, 2004).
New atypical genotypes from South America (such as ToxoDB genotype #13 from the Caribbean and ToxoDB genotype #6 from Brazil) have been identified, showing increased virulence in mice. These genotypes are more likely to cause pneumonia with significantly higher parasite loads in the lungs and eyes, affecting both immunocompromised and immunocompetent individuals (Hamilton et al., 2019).

3.4. Diagonosis

Recent advances in the diagnosis of toxoplasmosis have tremendously increased accuracy and speed in the detection of *T.gondii* infections. Advances in serological testing have been critical. Advanced techniques in ELISA through recombinant antigens have improved the specificity and sensitivity to increase diagnostic accuracy for both acute and chronic infections of ocular toxoplasmosis, which is crucial for physicians in proper clinical management (Fadel et al., 2024).

Molecular techniques also play a major role in diagnosing the disease, especially with the availability of real-time PCR (qPCR), which has become a gold standard because of its high sensitivity and specificity. This method can detect low concentrations of *T. gondii* DNA in clinical samples, such as blood, cerebrospinal fluid, and amniotic fluid, targeting gene regions comprising the B1 gene and the 529-bp repeat element (Edvinsson et al., 2006; Q. Liu et al., 2015). Dual-target real-time PCR hydrolysis probes labeled with fluorescence have also been resourceful as they enhance the performance for *T. gondii* DNA detection accuracy (Gomez et al., 2019). Other promising molecular techniques include Loop-Mediated Isothermal Amplification (LAMP), which has been shown to be simple and cost-effective and, therefore, is of particular value in resource-limited settings. LAMP assays have shown very high sensitivity and specificity to allow early diagnosis and large-scale screening (Cao et al., 2022; Mirahmadi et al., 2020). In addition to the advances in molecular and serological diagnosis, imaging techniques have significantly contributed to the diagnosis and monitoring of ocular toxoplasmosis. Optical Coherence Tomography (OCT) outlines high-resolution images of retinal lesions. Fundus photography and angiography techniques, fluorescein, and indocyanine green angiography play a pivotal role in delineating the severity and prognosis of ocular toxoplasmosis cases (Gomez et al., 2019). All these collective improvements in molecular, serological, and imaging methods have greatly advanced in diagnostics of toxoplasmosis, leading to the era of more accurate, rapid, and reliable detection of T. gondii infections will be crucially important for improved patient outcomes and possible management strategies.

3.5 Treatment

Toxoplasmosis treatment has evolved significantly over the years, with various therapeutic strategies being employed to manage the infection effectively. The standard treatment for toxoplasmosis typically involves a combination of pyrimethamine and sulfadiazine, accompanied by folinic acid to mitigate bone marrow suppression caused by pyrimethamine (Montoya & Liesenfeld, 2004; CDC, 2023). This regimen is particularly effective for acute infections and is widely used for treating severe cases, including those in immunocompromised patients and in congenital toxoplasmosis. Spiramycin is another antibiotic used, especially in pregnant women

during the first trimester to prevent fetal transmission, as it does not cross the placenta as readily as pyrimethamine-sulfadiazine (Toxoplasmosis: Pediatric OIs | NIH) In cases where patients exhibit intolerance to the standard regimen, alternatives such as trimethoprim-sulfamethoxazole (TMP-SMX) and clindamycin are employed. TMP-SMX has shown comparable efficacy with a better tolerability profile (Dunay et al., 2018).

Recent advancements have identified several drugs that can be repurposed for treating toxoplasmosis. A study highlighted six such drugs that show potential against *T.gondii*, offering new avenues for treatment, especially for drug-resistant strains (Santos et al., 2023). Additionally, antibiotic treatments for ocular toxoplasmosis, which is the most common cause of posterior uveitis, have been extensively reviewed. Antibiotics and corticosteroids are used to reduce the size of the retinochoroidal scar, decrease the risk of recurrence, and alleviate acute symptoms, although the treatment remains controversial and requires further evidence-based studies to optimize protocols (Feliciano-Alfonso et al., 2021).

Moreover, a multidisciplinary group working in France has provided recommendations for managing maternal and congenital toxoplasmosis, emphasizing the need for tailored treatment strategies based on the stage of pregnancy and the severity of the infection (Peyron et al., 2019). Despite these advancements, there is still a need for new therapeutic agents that can effectively target the tissue cyst stage of the parasite, as current treatments are unable to eliminate encysted bradyzoites, which can lead to chronic infection and reactivation.

4. The Intricate Architecture of *Toxoplasma gondii*: Unveiling the Ultrastructure

The tachyzoite stage of *T.gondii*, known for its rapid proliferation, exhibits a highly specialized and intricate ultrastructure that is crucial for its pathogenicity and survival. Tachyzoites are crescent-shaped cells, typically measuring 4-7 micrometers in length and 2-4 micrometers in width, with distinct subcellular features that include the apicoplast, rhoptries, micronemes, and dense granules (Dubey, Lindsay, & Speer, 1998; Black & Boothroyd, 2000). These organelles play pivotal roles in the parasite's invasion, replication, and evasion of the host immune system. The pellicle, consisting

of an inner membrane complex and subpellicular microtubules (Figure 6), provides structural support and enables the gliding motility essential for host cell invasion (Nishi et al., 2008). The apical complex, featuring the conoid, rhoptries, and micronemes, is vital for the parasite's ability to penetrate host cells and establish infection, as it secretes proteins that facilitate attachment and entry (Carruthers & Sibley, 1997).

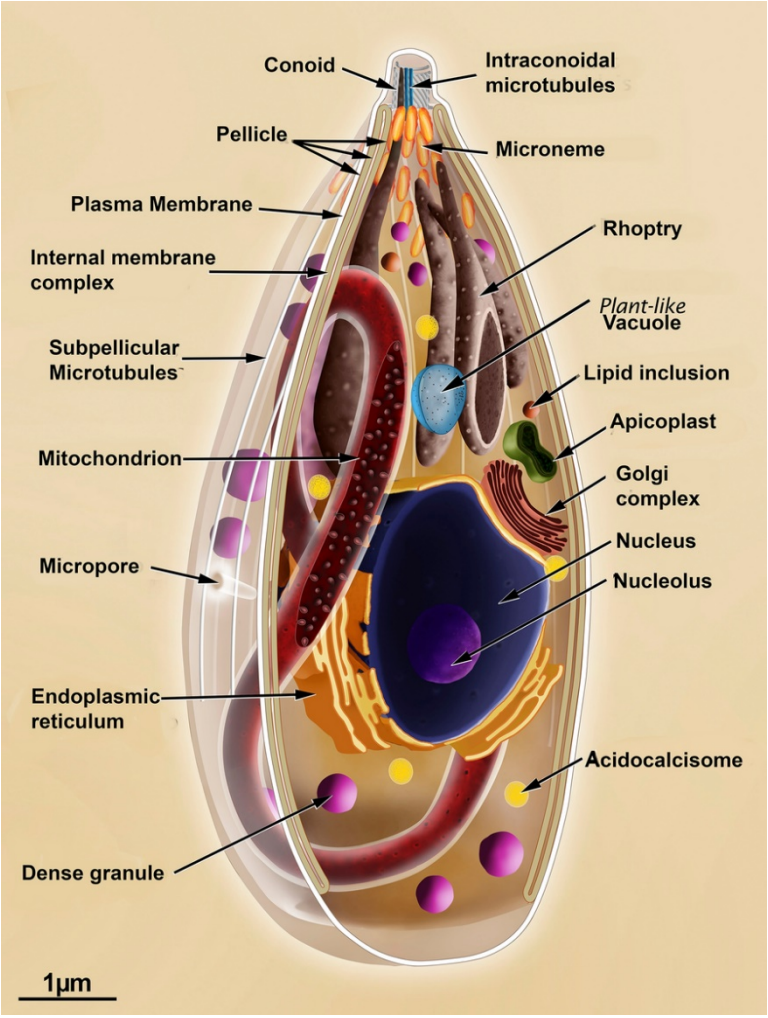

Figure 6 - Schematic longitudinal section view of a *T. gondii* tachyzoite (Attias et al., 2020).Tachyzoite is a crescent-shaped parasite stage that has rounded posterior ends and an anterior end with a conical point. Its membrane structure is intricate and consists of a bilayer inner membrane complex and an outer plasma membrane (PM) (IMC). The secretory organelles (micronemes and rhoptries) and microtubules coupled to a polar ring make up the spirally arranged conoid that is part of the apical complex structure. Furthermore, tachyzoites possess an apicoplast, a four-membranous plastid-like organelle that plays an important role in carbon metabolism. Acidic compartments known as acidocalcisomes are in charge of calcium storage and ion flux. The whole

complement of eukaryotic cell organelles, such as the nucleus, mitochondrion, endoplasmic reticulum, and Golgi complex, are also present in tachyzoites.

4.1 The Cytoskeleton

The cytoskeleton of Toxoplasma gondii is a complex and highly organized structure (Figure 7) that plays crucial roles in the parasite's biology, including invasion, motility, and cell division. The major components of the cytoskeleton include

- Subpellicular microtubules (SPMTs)
- Inner membrane complex (IMC)
- Apical complex
- Basal complex
- Centrosome and spindle

4.1.1. Inner Membrane Complex and Pellicle Architecture in *Toxoplasma gondii*

4.1.1.1. Composition of the IMC

Apicomplexans, ciliates, and dinoflagellates are categorized as alveolate organisms (Cavalier-Smith & Chao, 2004; Goodenough et al., 2018; Leander & Keeling, 2003; Saldarriaga et al., 2004). Alveolates feature a system of flattened vesicles called alveoli located just beneath the plasma membrane, forming a pellicle structure consisting of three-unit membranes. In apicomplexans, this network of alveoli associated with the plasma membrane is known as the inner membrane complex (IMC). The IMC is essential for the replication, motility, and host cell invasion of *Toxoplasma*. The IMC begins at the apical polar ring (APR), leaving the parasite's extreme apical region covered only by the plasma membrane, possibly aiding secretion. In the posterior of the tachyzoite, the IMC plates merge into a turbine-shaped structure (N. S. Morrissette et al., 1997; Porchet & Torpier, 1977). The *Toxoplasma* zoites is maintained by 22 evenly spaced subpellicular microtubules that interact with the pellicle's cytosolic face (Nichols & Chiappino, 1987). These microtubules spiral gently from the APR to a point behind the nucleus, giving the parasite its elongated, serpentine shape and apical polarity (N. S. Morrissette & Sibley, 2002; Stokkermans et al., 1996). The subpellicular

microtubule's minus ends are anchored in the APR, with support provided by APR projections resembling a cogwheel in transverse sections (Leung et al., 2017; Russell & Burns, 1984). The pellicles are divided into apical, central, and basal subdomains, each possessing unique characteristics provided by different cytoskeletal components. Fourier analysis of isolated subpellicular microtubules has shown that they are coated with a microtubule-associated protein (MAP) that binds at regular intervals of 32 nm (N. S. Morrissette et al., 1997). The IMC vesicles are interconnected by suture proteins that link transverse segments or associate with both the lateral and longitudinal edges of individual plates (A. L. Chen et al., 2015, 2017; Lentini et al., 2015; Tilley et al., 2014). These IMC plates, being distinct vesicles, determine the polarity and pellicle subdomains through the specific occupancy of proteins in various subcompartments, defining the apical cap (AC), lateral, and basal zones (Beck et al., 2010a; Fung et al., 2012) (Figure 7 (A)). During replication by endodyogeny, it is crucial to distinguish the maternal plasma membrane-associated IMC from the unassociated bud IMC that encloses developing daughters. Many IMC-located proteins selectively or predominantly associate with either maternal or daughter IMCs (Figure 7), directing the development, maturation, and emergence of the daughter cells. The IMC plates are connected by junctions forming transverse and longitudinal sutures (Beck et al., 2010b).

The AC is composed of a single ring-shaped Inner Membrane Complex (IMC) compartment marked by TgISP1 and nine AC proteins (Beck et al., 2010b; A. L. Chen et al., 2015, 2017; Fung et al., 2012). Beneath this cap, flattened IMC vesicles form spiraled strips around the body of the zoite, aligning with the underlying microtubules. The AC network extends about 900 nm from the apical ring and is composed of a dense fibrous net that appears to closely interact with the subpellicular microtubules. This region matches the apical cap previously described (B. Anderson-White et al., 2012a; B. R. Anderson-White et al., 2011; A. L. Chen et al., 2015). In this arrangement, the apical cap is connected to the region of the subpellicular microtubules anchored to the apical polar ring, indicating that the subpellicular network and the apical cap are distinct entities. In thin sections of both intact extracellular tachyzoites and during invasion, a dense apical zone can be observed beneath the IMC and on the cytoplasmic face. Another AC protein, TgAC10, was identified using the BIOID technique (Tosetti et al., 2020). TgAC9 and TgAC10 are recruited early during

endodyogeny to the apical caps of forming daughter parasites, even before TgISP1 recruitment. Super-resolution microscopy (STED) showed that TgAC9 and TgAC10 belong to the alveolin cytoskeleton and are organized in regular periodic rows. TgAC9 colocalizes with TgGAP45 and TgISP1, indicating its localization at the IMC and its confinement to the subpellicular microtubules (SPMTs) side. Ultrastructure expansion microscopy (U-ExM) demonstrated that TgAC9 and TgAC10 colocalize between the SPMTs below the conoid and the APR (Tosetti et al., 2020). Mutant parasites conditionally depleted of TgAC9 and TgAC10 show severe defects in microneme secretion, invasion, and egress, as well as significant morphological abnormalities in the apical complex region, lacking the conoid and the APR during the final stages of division or daughter parasite emergence from the mother cell (Tosetti et al., 2020). U-ExM microscopy also revealed that TgAC9 and TgAC10 depletion leads to highly disorganized SPMTs (Tosetti et al., 2020). Additionally, TgAC9 forms a complex with TgERK7, a conserved MAP kinase essential for proper conoid formation, with its localization at the apical cap region depending on TgAC9 (Back et al., 2020; O'Shaughnessy et al., 2020). The kinase ERK7 is crucial for the maturation of the apical complex, with its loss leading to the degradation of the complex. Another study by (O'shaughnessy et al., 2023) identified a E3 ligase, CSAR1 which counteracts the premature degradation of the apical complex when ERK7 is inactivated, highlighting its protective role. Genetic disruption of CSAR1 suppresses the loss of the apical complex associated with ERK7 knockdown, demonstrating a novel survival mechanism for the parasite.

The lateral IMC compartment is identified by TgISP2 and TgISP4, while TgISP3 is found here as well but also localizes to the basal IMC compartment, where individual plates merge to close the posterior of mature parasites (Figure 7(B)). The localization of ISPs to specific compartments requires palmitoylation and/or myristoylation. TgISP1's presence in the AC prevents other ISPs from occupying this compartment (Beck et al., 2010b). The single apical IMC plate connects to the lateral and posterior plates via IMC suture components (ISCs) at both lateral and longitudinal boundaries. TgISC1, TgISC4, and TgISC5 are detergent-insoluble, and TgISC3 is abundant in daughter buds. Additionally, a set of proteins is specifically linked to transverse sutures, including Transverse Suture Component (TSC) proteins like TgTSC1 (also known as CBAP and SIP), TgTSC2, TgTSC3, and TgTSC4.

Five to six apical annuli, identified by the markers TgCen2 and TgAAP1 (also referred to as TgPAP1), are situated at the interface between the Apical Complex (AC) and the lateral seams of the IMC plates (Figure 7 (A)). Recently discovered apical annuli proteins (AAPs) are organized into four concentric rings, with diameters ranging between 200 and 400 nm (Engelberg et al., 2020; Hu et al., 2006; Suvorova et al., 2015a).These AAPs are unique to coccidians and contain protein signatures typical of centrosomal proteins, indicating that the annuli may originate from centrosomes. The annuli form a pore-like structure that is likely involved in signaling and the exchange of materials across the IMC.

The apical annuli of *T.gondii* are specialized ring-like structures within the parasite's cytoskeleton that play critical roles in its biology. These structures, located near the apical end of the parasite within the Inner Membrane Complex (IMC), are composed of several key proteins, including five AAPs are designated AAP1-5, which are characterized by coiled-coil domains common in structural proteins (Engelberg et al., 2020). Additionally, Centrin2, typically associated with centrosomes, is also a component of the apical annuli, along with an apical annuli methyltransferase (AAMT) that likely modifies other annuli proteins (Engelberg et al., 2020). The apical annuli proteins interact with IMC suture proteins, suggesting that they are embedded within the IMC sutures, which connect alveolar vesicles (Ouologuem & Roos, 2014a). Functionally, the apical annuli may provide pores in the mother IMC to allow the exchange of building blocks and waste products during daughter cell formation, and they have been identified as specialized sites for post-invasion secretion (B. Anderson-White et al., 2012b). Studies have also identified an unconventional SNARE complex that mediates exocytosis at the plasma membrane and vesicular fusion at the apical annuli, highlighting their role in secretion (Chelaghma et al., 2024a) . AAPs facilitate the docking and fusion of dense granules with the plasma membrane. The depletion of these proteins impairs dense granule secretion, indicating their essential function in vesicle trafficking and fusion at these specialized cellular structures (Chelaghma et al., 2024a) . Phylogenetic analysis suggests that AAPs are conserved primarily in coccidian apicomplexan parasites, which multiply by internal budding, although some components like TgLMBD3 are universally present in apicomplexans and their close relatives (Chelaghma et al., 2024a). This conservation underscores the importance of the apical annuli in the unique biology of these parasites.

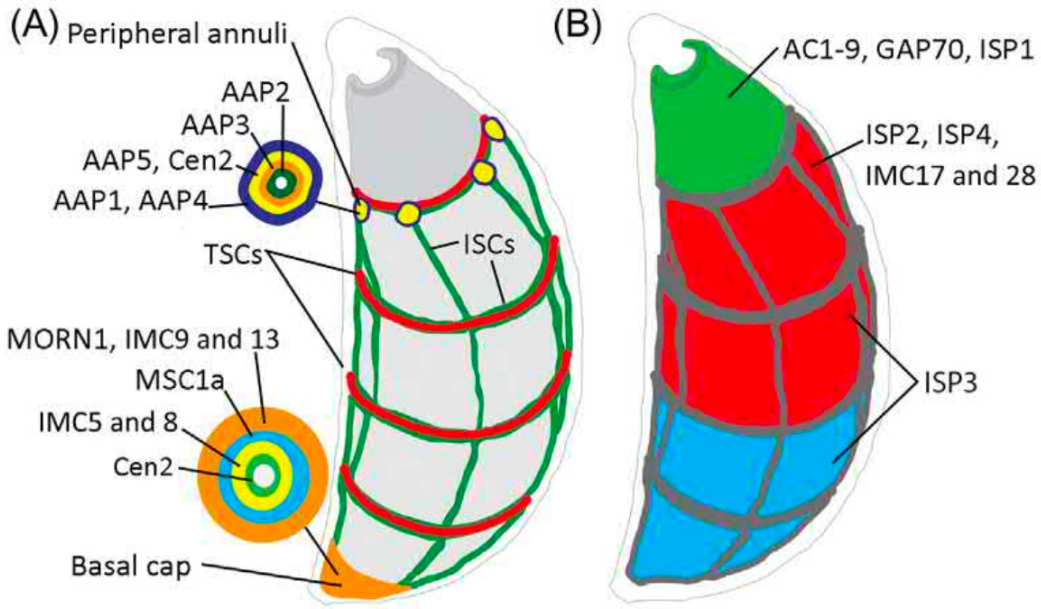

Figure 7 - **Schematic representation of Toxoplasma gondii IMC subdomains and associated proteins** (N. Morrissette & Gubbels, 2014)**. (A)** The IMC is partitioned into distinct apical, lateral, and basal subdomains, each characterized by specific protein markers. The apical annuli are marked by AAP1, AAP2, AAP3, AAP4, and AAP5, along with Cen2. TSCs indicate the transverse suture components, while ISCs (IMC suture components) join individual IMC plates. MORN1, IMC9, IMC13, and MSC1a are localized in the basal cap, along with IMC5, IMC8, and Cen2. **(B)** The ring-shaped apical complex (AC) is defined by AC1-9, GAP70, and ISP1. The lateral IMC compartment features ISP2, ISP4, IMC17, and IMC28. ISP3 localizes both to the lateral and basal compartments, where individual IMC plates merge to form the posterior end of mature parasites. The differential localization of these proteins is crucial for defining the polarity and functional compartmentalization of the IMC, with specific roles in the development, maturation, and emergence of daughter cells.

4.1.1.2. Daughter IMC formation

Before the onset of S phase, the earliest sign that tachyzoites have committed to division is the duplication of the Golgi apparatus (Nishi et al., 2008). Daughter cells develop through a series of coordinated steps closely linked to the cytoskeleton (Figure 8)

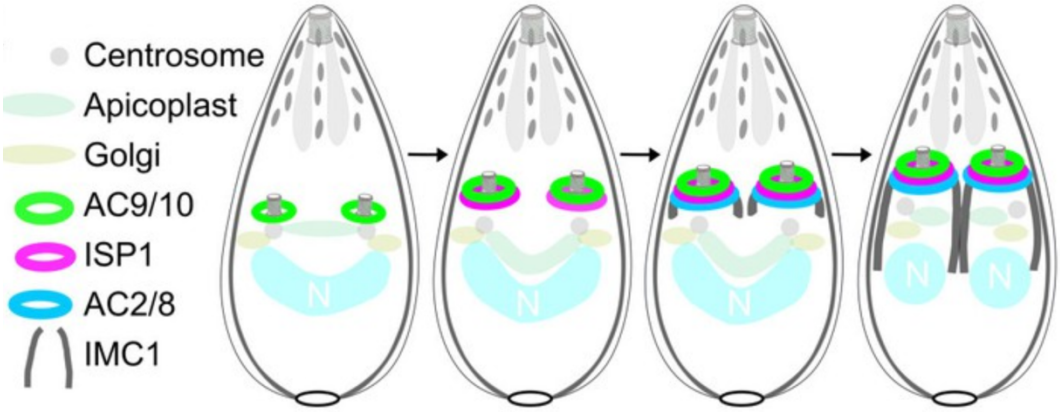

Figure 8 - Schematic representation of endodyogeny events (Tosetti et al., 2020), illustrating the sequential incorporation of various components into the inner membrane complex (IMC), subpellicular network (SPN), and cytoskeleton. The diagram highlights the step-by-step insertion of centrosomes, apicoplast, Golgi apparatus, AC9/10, ISP1, AC2/8, and IMC1, detailing their relative positions and interactions during the process.

The assembly of the daughter cells revolves around the formation of the cytoskeletal scaffold, which begins at the centrosome's outer-core (B. R. Anderson-White et al., 2011; C. T. Chen & Gubbels, 2019; Suvorova et al., 2015a). BCC0, along with IMC32 and IMC43, forms an essential daughter bud assembly complex that is crucial for the early stages of daughter cell formation (Pasquarelli et al., 2023, 2024). Another earliest indication of daughter scaffold formation is the localization of TgIMC15 on newly duplicated centrosomes (Anderson-White et al., 2011). TgIMC15 is then transferred to the developing daughter scaffolds alongside Rab11b (B. Anderson-White et al., 2012a), indicating that Rab11b likely facilitates the membrane-based delivery of TgIMC15

(Harding & MeissFollowing this, SFA fibers emerge to link each centrosome to a daughter bud. Subsequently, the subpellicular microtubules of the budding structure are established, with nascent daughter buds marked by a unique arrangement of tubulin into five spots encircling a central spot, resembling flower petals (Nagayasu et al., 2017). Although the timing of microtubule assembly is not fully understood, TgALP1 appears at the bud before TgMORN1 or other IMC family proteins, suggesting a role in early daughter cell formation (Gordon et al., 2010). TgMORN1 initially appears as a diffuse structure, later forming a ring that signifies the development of the basal

complex (Ferguson et al., 2008). ISP proteins are subsequently integrated into the IMC, starting with TgISP1, which occupies the cap region and prevents the localization of other ISPs (Beck et al., 2010). Several members of the alveolin family, including TgIMC1, 3, 6, 8-11, and 13, are then incorporated into the structure (Anderson-White et al., 2011). There are 14 alveolin IMC proteins that are essential for providing tensile strength to the parasite's pellicle. They are characterized by conserved repeats such as “EKIVEVP”, “EVVR”, or “VPV” within domains rich in valine and proline residue (Gould et al., 2008; Mann & Beckers, 2001). These proteins localize to both the mother and daughter cytoskeletons. TgIMC1 and TgIMC4 are evenly distributed between the mother and nascent daughter IMCs, while TgIMC3, TgIMC6, and TgIMC10 are predominantly found in forming daughters but disappear upon maturation (B. R. Anderson-White et al., 2011). In contrast, TgIMC7, TgIMC12, and TgIMC14 are exclusively localized to the mature parasite's cortical cytoskeleton during the G1 phase of the cell cycle and are absent in developing daughters. These three proteins are crucial for maintaining structural stability in both mature and daughter parasites. TgIMC11 is localized to the apical and basal poles of the parasite (B. R. Anderson-White et al., 2011). Once the cap alveolus is fully formed, the apical annuli are constructed at the junctions between the cap and the median alveoli (Hu et al., 2006; Engelberg et al., 2020; Suvorova et al., 2015). An IMC-associated palmitoyl transferase, TgDHHC14, modifies several proteins to anchor them to the IMC membrane (Frenal et al., 2010, 2013). As the daughter buds grow and envelop the nucleus, forming a horseshoe shape, subpellicular microtubules and their associated MAPs extend along the expanding daughter IMC (N. S. Morrissette & Sibley, 2002; Tran et al., 2012). Throughout this growth phase, both acyl-anchored and integral membrane proteins, such as TgGAP40, TgGAP50, and TgGAPMs, are continuously incorporated into the developing buds (Gaskins et al., 2004; Harding et al., 2019; Harding & Meissner, 2014; Johnson et al., 2007). When the buds reach their maximum width, several key events signify a major transition in their maturation. TgCaM1, TgCaM2, and TgDLC are deposited onto the conoid (Anderson-White et al., 2011; Hu et al., 2006). At the posterior end of the bud, TgIMC5, 8, 9, and 13 are recruited to the basal complex, where TgMORN1 has been present since the beginning of bud formation (Anderson-White et al., 2011). At this stage, proteins such as TgSSNA1/DIP13 (Leveque et al., 2016), TgMyoJ (Frenal et al., 2017b), and TgCen2 (Hu, 2008), along with the phosphatase TgHAD2a (Engelberg et al., 2016), are

integrated into the basal complex. The tapering of the posterior ends of the daughter buds is driven by contractile forces involving actin (Periz et al., 2017), TgMyoJ (Frenal et al., 2017b), and TgCen2 (Hu et al., 2006).

Certain IMC proteins, like TgIMC19, TgIMC21, and TgIMC25, are found in both mature parasites and nascent developing buds. Others, such as TgIMC17, TgIMC18, and TgIMC20, are specific to mature parasites, while TgIMC16 and TgIMC29 are exclusive to developing daughter buds (A. L. Chen et al., 2015, 2017). Disruption of TgIMC29 results in severe replication defects, highlighting its critical function in the early stages of daughter bud formation (A. L. Chen et al., 2015). A study by (Back et al., 2023), utilized proximity labeling to identify new IMC proteins specific to the daughter IMC with IMC29 as bait and identified proteins—IMC30, IMC31, IMC35, and IMC36 that localize to the body of the daughter IMC, showing similarities in positioning with IMC29. Additionally, BCC0 and BCC3, previously known for dynamic localization, were found to partially colocalize with IMC29, aligning with earlier findings (Engelberg et al., 2022). Two proteins, AC12 and AC13, were localized to the apical cap of daughter buds, suggesting a potential role alongside FBXO1 in apical cap formation, a topic for future research. The dispensable GWCS scores assigned to several of these proteins imply potential functional redundancy in the daughter sub compartment (Sidik et al., 2016). These findings open new avenues for investigating mechanisms involved in daughter cell scaffold construction. Alongside the daughter IMC proteins, several maternal IMC proteins and previously known BCC proteins were also identified. Interestingly, four proteins showed unique IMC-associated localizations at the interface between parasites or outward-facing regions, with IAP2 and IAP3 partially colocalizing with the apicoplast during cell division, suggesting a role in connecting the dividing apicoplasts to the IMC (Back et al., 2023). IAP2, identified as a possible myosin heavy chain, may play a part in the cytokinesis phase of division, consistent with myosin's known roles in parasite motility and cell segregation(Back et al., 2023).

Additionally, TgIMC32, a conserved protein across the Apicomplexa phylum, is essential for parasite survival. It localizes to the IMC's body portion and is deposited on nascent daughter buds early during internal budding. Conditional depletion of IMC32 in mutant parasites results in a collapsed IMC, highlighting its critical role (Torres et al., 2021). Another crucial set of proteins includes IMC43, and IMC44. IMC43

is a novel daughter IMC protein that is recruited at the earliest stages of daughter bud initiation. It regulates the localization of IMC32 and IMC44, forming a bud assembly complex essential for the proper formation of the daughter IMC during endodyogeny (Pasquarelli et al., 2023). IMC43 and IMC32 collaborate to form a critical complex necessary for organizing the inner membrane complex (IMC) during the earliest stages of daughter bud formation. IMC43 recruits and maintains the proper localization of IMC32, while IMC32 independently localizes to the early daughter cell scaffold but relies on IMC43 for organization during the later stages of budding (Pasquarelli et al., 2023). Similarly, BCC0, in conjunction with IMC32 and IMC43, is also required for daughter bud assembly, further emphasizing the importance of these proteins in establishing the structural integrity of the IMC(Pasquarelli et al., 2024). Depletion of any of these proteins results in severe morphological defects, disrupted endodyogeny, and impaired replication.

The IMC plays a crucial role not only in daughter cell development during endodyogeny but also in motility and invasion. In many apicomplexans, IMC-tethered myosin moves adhesin-associated F-actin to the apical pole of zoites, facilitating motility. To effectively move adhesins rearward, IMC-tethered myosins must resist displacement. This resistance is provided by the subpellicular microtubules and alveolin network, which stiffen the IMC. Integral membrane proteins called GAPMs link these filaments with glideosome components. GAPMs are a conserved family of apicomplexan proteins divided into three orthologous subsets (GAPM1, GAPM2, and GAPM3) (Bullen et al., 2009; Harding et al., 2019). These proteins have five or six membrane-spanning domains and are localized to the IMC in both mature parasites and developing daughter buds. The second and fourth loops between transmembrane domains are highly conserved across apicomplexan species, suggesting essential interactions with other proteins. Depletion of GAPM1a causes significant replication defects, leading to disorganization and disassembly of subpellicular microtubules. GAPM1a stabilizes cortical microtubules, which are critical for the parasite's structural integrity. GAPM proteins are strong candidates for the IMC-associated integral membrane proteins (IMPs) that link the subpellicular microtubules and alveolin network to the IMC, providing shape, tensile strength, and rigidity.

4.1.2. The architecture and functional elements of the Apical complex

At the core of the parasite's structural integrity and its ability to move and invade host cells lies a complex network of microtubules and molecular structures closely associated with the pellicle, situated at both the apex and base of the organism (Frixione E et al., 1996). The apical tip features a distinctive tube-like structure known as the conoid. The conoid is capped by two preconoidal rings and contains two microtubules, approximately 400 nm in length, extending through its center and terminating within the parasite's cytoplasm (N. S. Morrissette et al., 1997) (Figure 9). The extension and retraction of the conoid during host cell invasion suggests a mechanical role for the apical complex in attachment or penetration (Mondragon & Frixione, 1996).

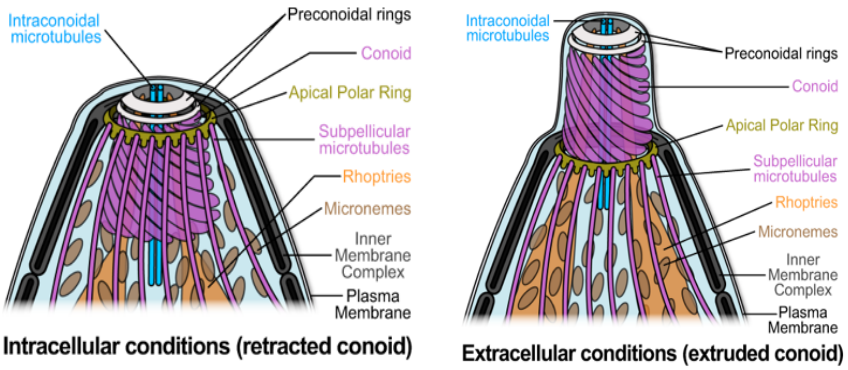

Figure 9 – Illustration of the Apical complex with its components (Dos Santos Pacheco et al., 2024)**.** The apical complex comprises several key structural and functional components, including the cytoskeleton made of microtubules, the alveolin network, the inner membrane complex (IMC), the 'conoid complex', and the secretory organelles. The apical complex features three main tubulin-based structures: two intraconoidal microtubules, the conoid fibers of the conoid, and the subpellicular microtubules. The two intraconoidal microtubules and subpellicular microtubules consist of 13 protofilaments, similar to those found in other organisms. In contrast, the tubulin fibers of the conoid are unique, composed of only nine protofilaments, forming a distinctive 'comma' shape in cross-section.

The conoid measures 380 nm in diameter and consists of 10-14 filaments, each approximately 430 nm long. These filaments are arranged in a left-handed spiral, forming a funnel-like shape (Hu et al., 2002; N. S. Morrissette et al., 1997; Nichols & Chiappino, 1987). The conoid's distinct feature is the spiral arrangement of its α-tubulin

microtubule filaments, resembling a compacted spring. The conoid in *T. gondii* is characterized by the presence of the apicortin/doublecortin protein known as DCX, which has two tubulin-binding domains: P25-α and DCX (Nagayasu et al., 2017; Orosz, 2009). TgDCX is exclusively localized to the conoid and plays a crucial role in its stability and the overall fitness of the parasite (Nagayasu et al., 2017). Loss of TgDCX leads to morphological defects in the conoid, causing it to become shorter and disordered, which in turn impairs host cell invasion (Nagayasu et al., 2017). TgDCX significantly stabilizes the curvature of the conoid-forming microtubules, facilitating effective invasion of the host cell. (Leung et al., 2020).

The conoid also contains several proteins essential for parasite motility, such as TgMyoH, which interacts with myosin light chain proteins (MLCs) including TgMLC3, TgMLC5, and TgMLC7 (Graindorge et al., 2016). Additionally, TgMyoH likely interacts with calmodulin-like proteins TgCAM1, TgCAM2, and TgCAM3 within the conoid, which probably regulate TgMyoH activity in a calcium-dependent manner (Long et al., 2017). A study by Dos Santos Pacheco et al., 2024 identified and characterized three intraconoidal microtubule-associated proteins - TgICMAP1, TgICMAP2, TgICMAP3. Knockout and knockdown experiments demonstrated that these proteins are essential for proper rhoptry docking and discharge. Ultrastructure expansion microscopy (U-ExM) and cryo-electron tomography (cryo-ET) revealed that the loss of ICMAP proteins disrupted the architecture of the conoid and intraconoidal microtubules. In summary, the conoid is a crucial structure for *T. gondii* invasion, acting as a scaffold for the coordinated release of rhoptry proteins. Unlike TgDCX and ICMAPs, TgMyoH is not critical for conoid integrity or rhoptry discharge. However, it plays a crucial role in facilitating the translocation of actin filaments that polymerize at the parasite's apical tip, making it indispensable for motility, host cell invasion, and egress. Proteins associated with the conoid, such as TgDCX, TgMyoH, and ICMAPs, collaborate to maintain conoid structure, ensuring efficient rhoptry docking and secretion during host invasion (Dos Santos Pacheco et al., 2024; Nagayasu et al., 2017).

A calcium-binding protein, TgCentrin2, localizes to the anterior of the preconoidal rings at the apex of *T. gondii*. It is also found in the annuli at the posterior edge of the apical region, within the basal complex, and in the centrioles (Hu et al., 2006; Lentini et al., 2019; Leung et al., 2019). TgCentrin2 is crucial for the lytic cycle of tachyzoites (Lentini

et al., 2019; Leung et al., 2019). Parasites depleted of TgCentrin2 exhibit defects in motility, adhesion, invasion, and egress, and their ability to secrete micronemes is severely compromised. Nonetheless, the overall structure and positioning of micronemes and rhoptries are not impacted (Lentini et al., 2019). TgCentrin2 is also crucial for maintaining the structure of the peripheral annuli. In parasites conditionally depleted of TgCentrin2, approximately 70% show a faint signal for peripheral annuli protein 2 (TgPAP2), improper PAP2 localization, or a significantly reduced number of parasites with PAP2 at the annuli compared to wildtype parasites (Lentini et al., 2019). These findings were corroborated by a similar study by (Leung et al., 2019), which confirmed the essential role of TgCentrin2 in the lytic cycle stages and microneme exocytosis. TgCentrin2 in the centrioles likely plays a crucial role in regulating parasite replication (Leung et al., 2019).

The apical polar ring (APR) in *T. gondii* is marked by the presence of the Ring 1 protein (TgRNG1), which localizes to the APR in the late stages of daughter parasite formation and appears within daughter cells before the mother cell disassembles (Tran et al., 2010). Attempts to create a TgRNG1 knock-out strain have been unsuccessful, indicating its essential nature. Another APR protein, TgRNG2, connects the APR to the conoid base, forming a ring with its amino-terminal associated with the conoid and carboxy-terminal anchored to the APR. During conoid extrusion, TgRNG2's terminal orientations flip as the conoid passes through the APR (Katris et al., 2014). Unlike TgRNG1, TgRNG2 associates with the apical complex early in endodyogeny, and its inducible knock-down mutant reveals that TgRNG2 is crucial for parasite growth, affecting motility, invasion, and secretion of microneme and rhoptry contents without altering pellicle structure (Katris et al., 2014).

A recent study using the hyperLOPIT method identified 63 apical proteins, with 41 previously known and 22 newly identified and predicted to localize to the apical region (Barylyuk et al., 2020). These proteins included apical cap proteins, apical annuli proteins, conoid-associated proteins, APR proteins, and invasion-associated components. They resolved into two clusters, apical 1 and apical 2, differentiated by their isoelectric points (pI); apical 1 protein have a basic pI, while apical 2 proteins have an acidic pI (Barylyuk et al., 2020). A follow-up study expanded the number of putative apical proteins to 95, verifying the localization of 13 proteins to the apical extremity, 23 to the same apical extremity, and 21 to the apical cap or other IMC sub-compartments,

leaving 38 novel predicted apical proteins with unverified localization (Koreny et al., 2021). Super-resolution microscopy confirmed the localization of these novel apical proteins to various parts of the conoid and APR (Koreny et al., 2021).

The intricate architecture of *T. gondii's* apical complex, centered around the conoid, plays a vital role in the parasite's motility, invasion, and overall fitness. Advanced techniques such as BIOID proximity labeling, super-resolution microscopy, and ultrastructure expansion microscopy have been instrumental in mapping these proteins' locations and interactions, revealing the complexity of their roles in maintaining the parasite's invasive machinery. The continued exploration of these proteins and their interactions is essential for understanding *T. gondii's* pathogenesis and could offer new avenues for therapeutic intervention. These findings underscore the importance of the apical complex in the parasite's lifecycle and highlight potential targets for disrupting its ability to infect host cells.

4.1.3. The architecture and functional elements of the Basal complex

The basal complex (BC) is a ring-like structure located at the posterior end of daughter cell scaffolds, essential for completing cell division; (Gubbels et al., 2006; Heaslip et al., 2010; Hu et al., 2006; Lorestani et al., 2010). It functions as a contractile ring that separates daughter parasites at the end of the division process (Gubbels et al., 2006, 2020a; Lorestani et al., 2010). Positioned at the most basal part of the inner membrane complex (IMC), the BC plays a crucial role in the apicomplexan membrane skeleton, which guides cell division (Gubbels et al., 2020).This complex structure is composed of a series of proteins that assemble and disassemble in a regulated manner throughout the parasite's life cycle, highlighting its dynamic nature (Gubbels et al., 2022a; Roumégous et al., 2022).

BCC0 has been identified as a precursor to BC formation, exhibiting a five-fold symmetry that suggests a role in organizing the apical annuli and alveolar suture formation (Engelber et al.,2022). As the basal complex develops, additional components such as BCC1, BCC2, BCC3, and BCC4 are incorporated into the structure. The underlying mechanism of BC constriction remained elusive for some time. This was due to the surprising finding that removing the MyoJ motor complex,

which is crucial for BC contraction, led to only minor growth issues (Frénal et al., 2017a). In contrast, the depletion of the scaffolding protein MORN1 proved fatal, resulting in daughter cells that remain conjoined and unable to separate (Heaslip et al., 2010; Lorestani et al., 2010). This discrepancy suggests there might be another contractile process that occurs before the MyoJ/Cen2/BCC1 complex is recruited during the budding phase or it points to the idea that MyoJ may not be the sole driver of BC constriction. Instead, other proteins or mechanisms might compensate for the loss of MyoJ, thus exhibiting functional redundancy. Recently, Gubbels et al. (2022 proposed a model where the BC operates like an elastic band, highlighting the significance of the expandable BC ring's stability, with MORN1 and BCC4 playing essential roles during cell division. When either component is absent, the BC initially forms but then abruptly disintegrates at the midpoint, leading to the fraying of the extending subpellicular microtubules (Gubbels et al., 2021).

Various IMC proteins are known to localize to the basal complex, including TgIMC5, TgIMC8, TgIMC9, TgIMC13, and TgIMC15 (Figure 10) (B. R. Anderson-White et al., 2011). Additional proteins that localize to the basal complex include TgCentrin2, TgHAD2a, TgDLC, TgMyoC, and TgMyoJ (Delbac et al., 2001; Hu, 2008; Lorestani et al., 2010).

TgCentrin2 becomes incorporated into the basal complex during the late stage of interphase and occupies a more distal region compared to TgMORN1 (Hu, 2008). TgCentrin2 is recruited to the basal ends of the daughter parasites prior to the onset of cytokinesis, aligning with the constriction of the basal complex. The protein contains a calcium-binding EF-hand domain, which may contribute to the contractile force of the basal complex, as artificial Ca2+ ion treatment triggers the constriction of the TgCentrin2 basal ring in daughter parasites (Hu, 2008).

TgMyoJ is also located at the basal complex and colocalizes with TgCentrin2(Frénal et al., 2017a). Parasites lacking TgMyoJ experience failed basal complex constriction, and TgCentrin2 localization is not detectable in these depleted parasites (Frénal et al., 2017a). Similarly, inducible knockdown of TgCentrin2 leads to unsuccessful basal complex constriction, indicating that both TgMyoJ and TgCentrin2 are crucial for this process (Frénal et al., 2017a). Furthermore, treating parasites with cytochalasin D, which destabilizes actin, disrupts the localization of TgCentrin2 and the constriction of

the basal complex, suggesting that actin plays a role in the assembly and constriction of the basal complex (Frénal et al., 2017a). To investigate the function of actin in *T. gondii*, a conditional knockout strain of TgACT1, the sole gene encoding actin in *T. gondii*, was developed (Periz et al., 2017). Parasites lacking TgACT1 exhibit abnormal basal complex morphology, appearing flattened (Periz et al., 2017). Using an actin chromobody, it was confirmed that actin does not form a ring structure at the basal end of developing daughter parasites but instead targets the IMC and forms a ring-like structure at the residual body (Periz et al., 2017).

TgHAD2a is an enzyme featuring a conserved haloacid dehalogenase (HAD) phosphotransferase domain. Initially, it localizes to the cytoskeleton of developing daughter parasites, eventually moving to the basal complex during contraction. At the basal complex, TgHAD2a colocalizes with TgMORN1. However, studies in mutant parasites lacking TgMORN1 revealed that TgHAD2a remains in the cortex of daughter cells but is not detected at the basal complex, indicating that while TgHAD2a localization in the cortex does not depend on TgMORN1, its presence at the basal complex does require TgMORN1 (Engelber g et al., 2016). TgHAD2a plays a role in basal complex assembly, as its conditional knockout leads to conjoined daughter parasites due to defective basal constriction. These findings highlight the crucial role of TgHAD2a in the contractile function of the basal complex (Engelberg et al., 2016).

During the late stages of cell division, as daughter cells separate from the mother, leftover materials from the mother are deposited into a structure called the residual body, located at the basal end of the daughter cells. This residual body quickly disappears after division is complete, functioning as a temporary recycling bin. Two possible models describe the fate of the residual body and its contents: (1) it is gradually degraded within the vacuole and integrated into the surrounding intravacuolar network (IVN) tubules, or (2) the digested contents are reabsorbed by the daughter cells still connected to the residual body. The first model applies if the residual body detaches from the daughter cells. However, it is often observed that the cytoplasmic bridge, which connects the daughter parasites, persists after the residual body is emptied. This bridge facilitates communication between cells and ensures synchronized cell division cycles within the vacuole (Frénal et al., 2017a; Hunt et al., 2019; Periz et al., 2017). Over multiple division rounds, the cytoplasmic bridge links

numerous parasites and helps maintain the rosette organization of parasites (Muiz-Hernández et al., 2011). The BC is strategically positioned to interact with the residual body and cytoplasmic bridge. MyoI plays a critical role in establishing and maintaining the bridge, with MyoJ supporting this function (Periz et al., 2017). The reason for maintaining the cytoplasmic bridge is unclear, as removing MyoI does not impact parasite fitness (Periz et al., 2017). While the bridge supports synchronized division, the necessity for synchronized cell cycles is not apparent. In fact, the bridge is lost during interactions with activated macrophages and during bradyzoite differentiation, leading to unsynchronized, slower divisions within tissue cysts (Frénal et al., 2017a).

(Gubbels et al., 2022a) suggested that, in addition to MyoI, proteins recruited to the BC at the end of cell division might help stabilize the cytoplasmic bridge. These proteins include FIKK kinase, CaM, MSC1a, and two newly identified proteins, BCC6 (a phosphatase) and BCC7 (a hypothetical protein) (Gubbels et al., 2021a; Lorestani et al., 2012; Skariah et al., 2016).

The link between BC and the cytoplasmic bridge has been analysed using mutant affecting rosette organization in the vacuole. While 70% of wild-type vacuoles displayed rosettes, BTP1 and CaM knockouts showed rosettes in only 15-20% of vacuoles, and MyoI, BCC7, and FIKK knockouts exhibited rosettes in 35-45%. BCC6 and MSC1a-depleted parasites showed no significant difference from wild-type. This suggests that most mature BC proteins play a role in forming and maintaining the cytoplasmic bridge. Further ultrastructural analysis of representative knockout strains confirmed that BTP1-KO parasites lost the cytoplasmic bridge, resulting in vacuoles with asynchronously dividing parasites. Conversely, BCC6-KO parasites showed synchronous division and connections to the residual body, aligning with high rosette incidence. FIKK-KO parasites displayed random vacuole organization and abnormal IVN membrane structures, indicating that proteins associated with the mature BC might also affect IVN formation and function.

The basal complex of *T. gondii* is thus a multifunctional structure integral to the parasite's biology. Its roles in cell division, motility, and possibly nutrient acquisition and cyst formation highlight its importance and make it a compelling target for future research.

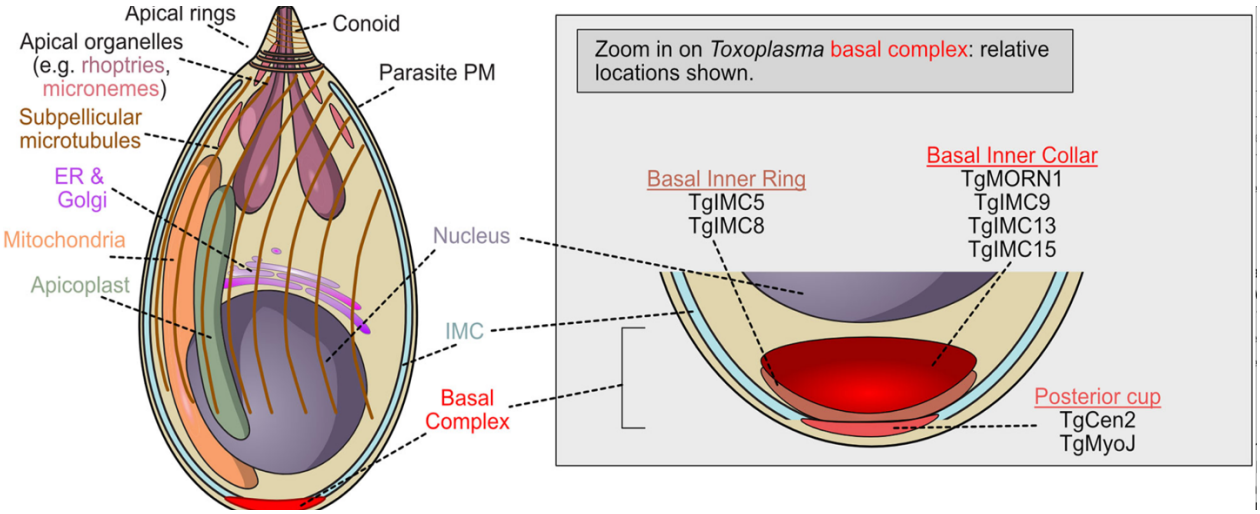

Figure 10 - Diagram of *T. gondii* tachyzoite highlighting the distribution of proteins within the basal complex (Morano & Dvorin, 2021)**.** The zoomed-in section illustrates the basal complex at the completion (or near completion) of cytokinesis. At this point, TgMORN1, an early recruit to the complex, is found alongside TgIMC9, TgIMC13, and TgIMC15 in the basal inner collar. The posterior cup, potentially providing contractile force, contains TgMyoJ and TgCentrin2. TgIMC5 and TgIMC8 are situated within the basal inner ring, positioned between the other protein groups.

4.2 Specialized Organelles of *Toxoplasma gondii*

4.2.1. The Apicoplast

The apicoplast is a non-photosynthetic plastid found in the protozoan parasite *T. gondii*. It plays a crucial role in the parasite's unique form of cell division known as endodyogeny, a process in which two daughter cells are formed within a single mother cell. This organelle is involved in several essential biosynthetic pathways, including fatty acid synthesis, isoprenoid precursor synthesis, and heme biosynthesis, which are vital for the survival and proliferation of the parasite (Lim & McFadden, 2010; Ralph et al., 2004).

During endodyogeny, the apicoplast must replicate and be correctly segregated into each daughter cell to ensure their viability. The mechanism of apicoplast division and segregation is intricately linked with the cell division machinery of *T. gondii*. The apicoplast is physically associated with the centrosomes, which serve as

organizational hubs during cell division, anchoring the apicoplast within the developing daughter cells and ensuring its inheritance (Striepen et al., 2000b; Vaishnava et al., 2005). As endodyogeny progresses, the elongating apicoplast is guided to the basal ends of the daughter cells. A sharp bend in the apicoplast occurs where the basal complexes of the daughter cells meet, suggesting a tight regulation of its positioning and segregation (Gubbels et al., 2006). The successful division of the apicoplast relies on MORN1 (Lorestani et al., 2010). During this process, the elongated organelle is anchored within the daughter buds through its connection to the centrosomes, while the unseparated organelle extends to the basal ends of the daughter cytoskeleton buds, creating a sharp bend at the meeting point of the basal complexes (Gubbels et al., 2006; Vaishnava et al., 2005). The dynamin-related protein DrpA is enriched at the apicoplast's constriction point, facilitating its fission and ensuring that each daughter cell inherits an apicoplast (van Dooren et al., 2009). Although DrpA is known to localize to areas of high mechanical stress, it is not yet clear whether it is specifically recruited to the basal complex during this process. The completion of apicoplast division aligns with the onset of BC constriction, highlighting the synchronized nature of these events (Hu, 2008) and coinciding with the formation of the electron-dense bulb that contains IMC proteins, MyoJ, and Cen2.

This coordination is essential for the successful division and viability of the daughter cells, as the apicoplast provides critical metabolic functions that the parasite cannot obtain from its host (Lim & McFadden, 2010). The proper inheritance and functionality of the apicoplast are therefore fundamental to the lifecycle of *T. gondii* and its ability to propagate within its host. The apicoplast is involved in several biosynthetic pathways that are vital for parasite survival. It is responsible for the synthesis of fatty acids (Lim & McFadden, 2010; Ralph et al., 2004) .The fatty acids synthesized in the apicoplast are critical for the formation of cellular membranes, including those of the parasite's own organelles and the parasitophorous vacuole membrane that surrounds the parasite within host cells (Mazumdar et al., 2006). This synthesis is vital for maintaining the structural integrity and function of these membranes, contributing to the parasite's ability to invade and replicate within host cells. The apicoplast also participates in the synthesis of isoprenoid precursors, which are crucial for numerous cellular processes, including the formation of important biomolecules such as hormones and vitamin (Ralph et al., 2004). Additionally, the apicoplast contributes to the biosynthesis of

heme, a key component in electron transport and redox reactions (Lim & McFadden, 2010; Ralph et al., 2004). These unique functions make the apicoplast an attractive target for drug development, as its pathways are distinct from those found in the host, offering a means to selectively disrupt parasite metabolism without affecting the host cells (Lim & McFadden, 2010).

4.2.2. The Micronemes

Micronemes are specialized secretory organelles in *T.gondii* that play a pivotal role in the parasite's invasion of host cells (Carruthers & Tomley, 2008). These organelles are located at the apical end of the parasite and are responsible for the release of microneme proteins (MICs), which are essential for host cell attachment, gliding motility, and the formation of the moving junction (MJ) necessary for invasion (Soldati-Favre, 2008). Microneme proteins are typically released in a stepwise manner upon contact with the host cell. This secretion is triggered by an increase in intracellular calcium concentration (Lourido & Moreno, 2015). The MICs are composed of both transmembrane and soluble proteins that contain various adhesive domains such as thrombospondin type I-like repeat (TSR), apple-like, epidermal growth factor-like (EGF), and chitin binding-like (CBL) domains (Sheiner et al., 2010). These domains facilitate the interaction with host cell receptors, thereby mediating the attachment and invasion processes. Micronemes are categorized into two distinct populations based on their biogenesis and dependence on specific Rab GTPases. The Rab5A/C-dependent micronemes include proteins such as MIC3, MIC5, and MIC8, which are essential for effective host cell invasion and egress, as they undergo calcium-dependent exocytosis at the apical tip of the parasite (Kremer et al., 2013). In contrast, the Rab5A/C-independent micronemes comprise proteins like MIC2 and M2AP, which utilize alternative pathways for their secretion (Kremer et al., 2013). This differentiation in populations allows *T. gondii* to fine-tune its secretory mechanisms during the invasion process, ensuring that it can effectively attach to and penetrate host cells under varying conditions.

Among the key microneme proteins, the TgMIC1/4/6 complex is one of the most extensively studied in *T.gondii* (Cérède et al., 2005). It includes TgMIC1, TgMIC4, and TgMIC6, which work together to mediate host cell adhesion and invasion. TgMIC1 and TgMIC4 function as soluble adhesins, while TgMIC6 is a transmembrane protein that

anchors the complex to the parasite's surface and interacts with the actomyosin system via aldolase binding (Blumenschein et al., 2007). Another important complex is TgMIC2/M2AP, where TgMIC2 is a transmembrane protein crucial for gliding motility and host cell attachment (Huynh & Carruthers, 2006). It was previously proposed that the connection between the parasite and TgMIC2 occurs through the aldolase enzyme (TgALD), which can bind to F-actin (Jewett & Sibley, 2003). Transgenic parasites lacking TgALD showed reduced motility and a compromised ability to invade host cells (Starnes et al., 2009). However, more recent studies have identified that the *T.gondii* glideosome-associated connector (TgGAC) is responsible for binding F-actin and moving along the parasite, linking to the TgMIC2 adhesin during motility and invasion (Jacot et al., 2016). The TgMIC3/8 complex is also significant, with TgMIC3 being a soluble adhesin and TgMIC8 a transmembrane protein essential for the formation of the moving junction (Kessler et al., 2008). The absence of MIC8 results in a failure to form the MJ, thereby blocking the invasion process. MIC8's function is unique and cannot be complemented by other microneme proteins, highlighting its distinct role in the invasion process.

The secretion of rhoptries is a tightly regulated process that begins with the discharge of rhoptries upon host cell contact. This is followed by the release of rhoptries, which form the moving junction through which the parasite invades the host cell (V. Carruthers & Boothroyd, 2007). The MICs bind to receptors on the host cell surface, forming a molecular bridge that connects the parasite to the host cell. This interaction is crucial for the parasite's gliding motility and subsequent invasion. The MICs are often processed by proteolytic cleavage during their transport through the secretory pathway or after exocytosis (Dowse & Soldati, 2004). This processing is important for their function and may contribute to the disassembly of complexes after invasion. For instance, TgMIC2 is cleaved by a rhomboid protease, which is essential for its function during invasion (Buguliskis et al., 2010).

In conclusion, micronemes and their associated proteins are essential for the invasion and virulence of *T.gondii*. The precise regulation of MIC secretion and function ensures the parasite's ability to effectively invade host cells and establish infection.

4..2.3. The Rhoptries

Each tachyzoite contains between 8 and 12 rhoptries, which are approximately 2-3 µm in length (Boothroyd & Dubremetz, 2008; Dubey et al., 1998). These organelles have a club-like shape and are enclosed by a membrane. They feature a uniform composition at the anterior part, known as the neck, and a varied composition at the base, known as the bulb. Consequently, the proteins in these regions are referred to as rhoptry neck proteins (RONs) and rhoptry bulb proteins (ROPs), respectively (Bradley et al., 2005; Dubremetz, 2007). Studies have demonstrated that rhoptries are actively anchored at the parasite's apex by the Armadillo Repeats Only Protein (TgARO) through a process dependent on actomyosin (Mueller et al., 2013, 2016). Subcellular fractionation studies have identified over 30 rhoptry proteins (Bradley et al., 2005; Leriche & Dubremetz, 1991) and this number is steadily increasing. Additionally, the rhoptry content includes membranous structures that are high in (Foussard et al., 1991a). RONs are crucial for forming the moving junction (MJ), a temporary ring-like adhesive structure that helps the parasite penetrate the host cell during invasion (Alexander et al., 2005; D. Bargieri et al., 2014; Lebrun et al., 2005a). While RONs facilitate invasion, ROPs are released after the MJ is established and are directed to the developing parasitophorous vacuole, the parasitophorous vacuole membrane (PVM), or the host cell cytoplasm (Figure 11).

The rhoptry neck proteins (RONs) are secreted first during the invasion process and are essential for forming the moving junction (MJ), a structure that forms a tight connection between the parasite and the host cell membrane. This moving junction is critical for the parasite to propel itself into the host cell. Key RONs include RON2, RON4, and RON5, which form a complex that interacts with the microneme protein AMA1. RON2 spans the host cell membrane and serves as a receptor for AMA1, facilitating the formation of the moving junction (Besteiro et al., 2011; M. H. Lamarque et al., 2014). Previous studies have further elucidated the mechanisms by which RONs contribute to invasion. For instance, RON2's interaction with AMA1 has been shown to trigger conformational changes essential for anchoring the MJ, which is crucial for the parasite's entry into the host cell (M. Lamarque et al., 2011; Tyler & Boothroyd, 2011).

Following the release of RONs, the rhoptry bulb proteins (ROPs) are secreted into the host cell cytoplasm and the forming PV. These proteins play diverse roles in

modulating host cell functions and establishing a favorable environment for parasite replication. For example, ROP16 is a kinase that phosphorylates host STAT3 and STAT6 transcription factors, thereby suppressing the host immune response (Saeij et al., 2007). ROP18, another key virulence factor, phosphorylates immunity-related GTPases (IRGs) to prevent their accumulation on the PV membrane, thus protecting the parasite from host cell defenses (Fentress et al., 2010).

The ROP2 family of proteins, also called the ROPK superfamily which includes ROP2, ROP3, and ROP4, is also notable. The ROP2 family was first identified as a group of rhoptry proteins made up of three proteins, each recognized by a single monoclonal antibody (Sadak et al., 1988). ROP2 inserts into the PVM and mediates an association between the PV and host cell mitochondria, which is important for nutrient acquisition and parasite survival (Bradley et al., 2005). ROP4, a member of this family, is secreted during or shortly after invasion and associates with the PVM through its transmembrane domain. It becomes phosphorylated in the infected cell, either by host cell kinases or parasite kinases activated by host cell factors (Carey et al., 2004). Recent research has expanded our understanding of these interactions, highlighting how the ROP2 family proteins modify host cell metabolic pathways to benefit parasite growth (Carey et al., 2004; El Hajj et al., 2007; Li et al., 2020). Proteins in the ROP2 family share certain distinctive features. They have a kinase-fold at the C-terminus and a region rich in basic amino acids at the N-terminus. The N-terminal part of the kinase domain contains an activation loop and a substrate-binding site, which is the most conserved part of the ROPK proteins. Several ROPK proteins are predicted to be enzymatically active due to the presence of a complete catalytic triad (Peixoto et al., 2010). However, not all ROPK family members are active enzymes because some lack a glycine loop necessary for ATP stabilization and a conserved aspartate in the catalytic loop, which is crucial for phosphotransferase activity. These inactive ROP proteins are called "pseudokinases" and include ROP2, ROP4, ROP5, ROP7, ROP8, and ROP54. X-ray crystallography has revealed that these pseudokinases retain a 'kinase-fold,' although it differs from the conventional type and is characterized by Toxoplasma-specific modifications. These pseudokinases are thought to play roles in scaffolding and substrate sequestration (Labesse et al., 2009; Qiu et al., 2009; Reese et al., 2011). Some polymorphic ROP proteins are critical virulence factors for the parasite in type I strains. The complex formed by ROP5, ROP17, and ROP18, which

localizes at the parasitophorous vacuole membrane (PVM), is responsible for phosphorylating immunity-related GTPases (IRGs). This phosphorylation prevents IRG accumulation at the PV, thereby preventing PV lysis in mice (Etheridge et al., 2014; Fleckenstein et al., 2012). In Type II strains, the ROP5 gene has a polymorphism that makes it inactive (Behnke et al., 2011), leading to a different outcome where the PVM integrity is compromised and PV lysis occurs. TgROP18 is also involved in modulating the function of the transcription factor ATF6-β (Yamamoto et al., 2011). TgROP17 influences the host cell transcriptome (Li et al., 2019) and also plays a role in effectively translocating dense granule proteins across the PVM (Panas et al., 2019)

Other rhoptry proteins play a crucial role in the differentiation of the *T. gondii* parasite. Studies have shown that the ROP28 protein is expressed five times more during chronic infection than during acute infection (Pittman et al., 2014b). In another study, the deletion of 26 ROPK gene loci encoding 31 unique Type II ROPK proteins revealed that several of these proteins moderately affect cyst burden (Fox et al., 2016). However, certain ROPK proteins, such as ROP5, ROP17, ROP18, ROP35, and ROP38/29/19, are critical for establishing chronic infection in vivo (Fox et al., 2016). Additionally, research showed that deleting ROP21, ROP27, ROP28, and ROP30 did not significantly impact bradyzoite differentiation in vitro, although a combined knock-out of ROP21 and ROP17 led to a 50% reduction in cyst burden in vivo (Jones et al., 2017). The bradyzoite rhoptry protein TgBRP1 was also studied, revealing that its absence does not significantly affect tissue cyst formation but likely plays a crucial role in the sexual stages of merozoites in felines (Schwarz et al., 2005). Subcellular fractionation of rhoptries has shown a high concentration of lipids, particularly cholesterol (Foussard et al., 1991b). However, experimental evidence indicates that cholesterol is not necessary for invasion (Coppens & Joiner, 2003). Rhoptry proteins and lipids are released through rhoptry exocytosis and contribute to modifying the parasitophorous vacuole membrane (PVM) (Coppens & Vielemeyer, 2005).

Studies have revealed that rhoptry proteins can be delivered to uninfected host cells through structures called evacuoles, allowing the parasite to manipulate host cell functions even before invasion (Koshy et al., 2012). This pre-emptive strike mechanism further highlights the sophisticated strategies employed by *T.gondii* to ensure successful infection and survival within the host.

The tethering of rhoptries to the apical vesicle is critical for the regulated discharge of these organelles during host cell invasion. A key player in this process is the Armadillo Repeats Only Protein (ARO), which localizes to the rhoptry membrane and is anchored to the cytosolic face via myristoylation and palmitoylation. ARO interacts with Myosin F (MyoF) and ARO-Interacting Protein (AIP), forming a complex that stabilizes the rhoptries at the apical pole (Beck et al., 2013). Additionally, Rhoptry Apical Surface Proteins (RASPs), such as RASP1, RASP2, and RASP3, coat the rhoptry neck and facilitate the docking and fusion of rhoptries with the apical vesicle. RASP2, in particular, binds phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PIP2), which are crucial for membrane fusion during exocytosis (Suarez et al., 2019). Another essential component is the TgNd6 and TgNd9 complex, which assembles the apical rosette, a structure that bridges the rhoptries with the plasma membrane and the apical vesicle, ensuring proper secretion during invasion (Aquilini et al., 2021). These interactions collectively ensure that rhoptries are properly positioned and primed for secretion, playing a vital role in the parasite's ability to invade and establish infection in the host cell.

In conclusion, the rhoptries are integral to the parasite's life cycle, housing a variety of proteins and lipids vital for both invasion and the formation of the parasitophorous vacuole. These components are crucial for sustaining the tachyzoite during the acute phase of infection, ensuring its survival and proliferation. Furthermore, the proteins within the rhoptries are instrumental in the development of tissue cysts, a key feature of chronic infection. This dual role highlights the rhoptries' importance in both immediate and long-term parasitic strategies.

4.2.3. The Dense granules

These spherical structures, approximately 200 nm in diameter, are distributed throughout the parasite's cytoplasm and are the last of the three secretory organelles (along with micronemes and rhoptries) to discharge their contents (Mercier & Cesbron-Delauw, 2015)(Mercier & Cesbron-Delauw, 2015). The contents of dense granules, known as dense granule proteins (GRAs), are continuously secreted throughout the intracellular cycle of *T. gondii*. These proteins are essential for the modification of the parasitophorous vacuole (PV) and the host cell, creating an environment conducive to parasite replication and survival (Bai et al., 2018). To date, over 40 GRA proteins have

been identified, with their functions ranging from structural roles in the PV to manipulation of host cell processes (Nadipuram et al., 2020). Dense granule biogenesis and trafficking within the parasite are complex processes that are not fully understood. Studies have shed light on some aspects of these mechanisms. Dense granules are formed at the Golgi apparatus and then transported to the parasite periphery by a MyoF motor, which moves them along actin filaments (Heaslip et al., 2016). Additionally, TgRab11A plays a crucial role in facilitating the transport of dense granules (Venugopal et al., 2020). This transport involves both microtubule-dependent and actin-dependent mechanisms (Heaslip et al., 2016). Interestingly, dense granule motions are highly dynamic, with granules demonstrating either directed or random, diffusive-like motion. Some granules have been observed to switch between these two modes of motion, and some even move bidirectionally (Heaslip et al., 2016).The secretion of dense granules occurs through direct fusion with the parasite plasma membrane. However, the exact location of this fusion is still debated. Some studies suggest that secretion may occur through gaps in the inner membrane complex (IMC), possibly at structures called the apical annuli (Dubremetz et al., 1993; Paredes-Santos et al., 2013). However, direct evidence for this is still lacking, and the mechanism by which dense granules traverse the IMC to reach the plasma membrane remains an open question in the field (Chelaghma et al., 2024b; Griffith et al., 2022).

Once secreted, GRA proteins play diverse roles in modifying the PV and host cell environment. Recent proteomic and transcriptomic approaches, coupled with CRISPR-Cas9 technology, have accelerated the identification and characterization of GRA proteins (Mayoral et al., 2022; Nadipuram et al., 2020). These studies have revealed that some GRAs are processed by the Golgi-resident aspartyl protease ASP5, which cleaves many GRAs at a conserved TEXEL motif. This processing is crucial for the correct trafficking and function of several GRAs (Coffey et al.,2015; Hammoudi et al., 2015).

One of the primary functions of certain GRAs is the formation and maintenance of structural components within the parasitophorous vacuole (PV). GRA2, GRA4, and GRA6 are involved in the formation of the intravacuolar network (IVN) (Figure 11), a tubular membrane structure within the PV (Mercier et al., 2002). GRA2, in particular,

plays a crucial role in inducing and stabilizing this tubular network structure (Travier et al., 2008).

Several GRAs are associated with the parasitophorous vacuole membrane (PVM), where they likely play roles in modifying the PVM and mediating interactions with the host cell. GRA3, GRA5, GRA7, GRA8, and GRA10 are preferentially detected as PVM-associated proteins (Nam, 2009) These proteins are important for maintaining the integrity of the PVM and facilitating the parasite's interactions with the host cell environment.

Nutrient acquisition is another critical function of certain GRAs. GRA17 and GRA23 are involved in small molecule transport across the PVM (Figure 11) (Gold et al., 2015). Deletion of GRA17 leads to severe growth defects. Current research has identified GRA47 and GRA72 as pore-forming proteins on the PVM, involved in nutrient flux across the membrane (Bitew et al., 2023). These proteins are essential for the parasite's ability to acquire nutrients from the host cell.

Host cell manipulation is a key aspect of *T. gondii's* survival strategy, and several GRAs play important roles in this process. GRA16 and GRA24 are exported beyond the PVM into the host cell nucleus, where they modulate host gene expression (Bougdour et al., 2013; Braun et al., 2013). GRA15 activates the host NF-κB pathway, modulating the immune response (Figure 11) (Rosowski et al., 2011). GRA18 is localized in the host cell cytosol and is involved in host-parasite interactions(He et al., 2018). Immune modulation is another crucial function of certain GRAs. GRA25 modulates cytokine production in infected macrophages (Shastri et al., 2014), while GRA7 contributes to acute virulence by increasing the turnover of immunity-related GTPases (IRGs) in the host cell (Alaganan et al., 2014). Some GRAs are involved in protein trafficking within the parasite. GRA44 and GRA45 are involved in the trafficking and translocation of other GRAs (Nadipuram et al., 2020). This function is critical for the proper localization and function of other GRA proteins.

Certain GRA proteins function as virulence factors. Specifically, GRA1, GRA2, GRA6, and GRA7 are crucial for parasite virulence and triggering immune responses in the host (Fox et al., 2019; Guevara et al., 2021a). Recent in vivo CRISPR screens from Torelli et al., 2024 have revealed that several dense granule proteins (GRAs) are

critical for virulence and survival of *T.gondii* in vivo. Among them, GRA12 was identified as the most important, functioning across various parasite strains and mouse subspecies. GRA12 is crucial for maintaining the parasitophorous vacuole (PV) integrity and preventing immune clearance, particularly in interferon-gamma (IFNγ)-activated macrophages. Its deletion leads to collapsed PVs, increased necrotic host cell death, and early parasite egress, highlighting its essential role in sustaining the parasite's replicative niche during infection. Additionally, other GRAs, such as GRA45 and GRA23, also contribute to parasite virulence by supporting the correct localization and function of key proteins within the PV membrane (Gold et al., 2015; Wang et al., 2020). GRA60 has been identified as a key virulence factor that influences the host's cell-autonomous immunity and also plays a significant role in the elimination of immunity-related GTPases (IRGs) (Nyonda et al., 2021). Type I parasites that lack the TgGRA60 gene exhibit reduced virulence and a lower cyst burden, along with the recruitment of IRG proteins Irgb10 and Irga6. Additionally, TgGRA60 was found to interact with the TgROP18 and TgROP5 proteins (Nyonda et al., 2021).

Several GRA proteins play a role in the formation of tissue cysts, including TgGRA4, TgGRA6, TgGRA3, TgGRA7, TgGRA8, TgGRA14, TgGRA2, TgGRA9, and TgGRA12 (Fox et al., 2011, 2019; Guevara et al., 2021a). The removal of TgGRA4 and TgGRA6 from Type II parasites led to a significant reduction in cyst burden compared to the wildtype strains (Fox et al., 2011). Moreover, eliminating both TgGRA4 and TgGRA6 from the same strain resulted in an even more pronounced defect in tissue cyst formation (Fox et al., 2011). In a follow-up study, the depletion of PVM-associated GRAs, including TgGRA3, TgGRA7, TgGRA8, and TgGRA14, along with IVN-associated GRAs such as TgGRA2, TgGRA9, and TgGRA12, in Type II strains resulted in substantial reductions in cyst burden in vivo (Fox et al., 2019). During tissue cyst development, GRA proteins exhibit dynamic behavior, as demonstrated by research on the localization of IVN-associated GRA proteins (TgGRA1, TgGRA4, TgGRA6, TgGRA9, and TgGRA12) in developing in vitro tissue cysts at different time points (Guevara et al., 2019). A recent study explored four genes related to GRA12 (GRA12A, GRA12B, GRA12C, and GRA12D), revealing that the GRA-12 gene family is involved in chronic infection (Guevara et al., 2021a). Like TgGRA12, the genes associated with it are linked to the intravacuolar network (IVN) in tachyzoites. In immature cysts induced by a high pH shift, TgGRA12A, TgGRA12B, and TgGRA12D

are found localized to the cyst wall, suggesting these proteins play a role in the transformation of the parasitophorous vacuole into a tissue cyst. Similarly, in mature cysts, TgGRA12A, TgGRA12B, and TgGRA12D are present on the cyst wall as well as within the cyst matrix (Guevara et al., 2021a). Parasites lacking TgGRA12A showed a marked reduction in cyst burden in vivo, whereas those without TgGRA12B displayed a significant increase in cyst burden in vivo (Guevara et al., 2021b).

In conclusion, the diverse functions of GRA proteins underscore their importance in *T. gondii* biology and host-parasite interactions. From structural roles within the PV to manipulation of host cell processes, these proteins are critical for the parasite's survival and virulence. As research continues, our understanding of GRA functions continues to expand, revealing the complex interplay between these proteins and their roles in *T. gondii* pathogenesis.

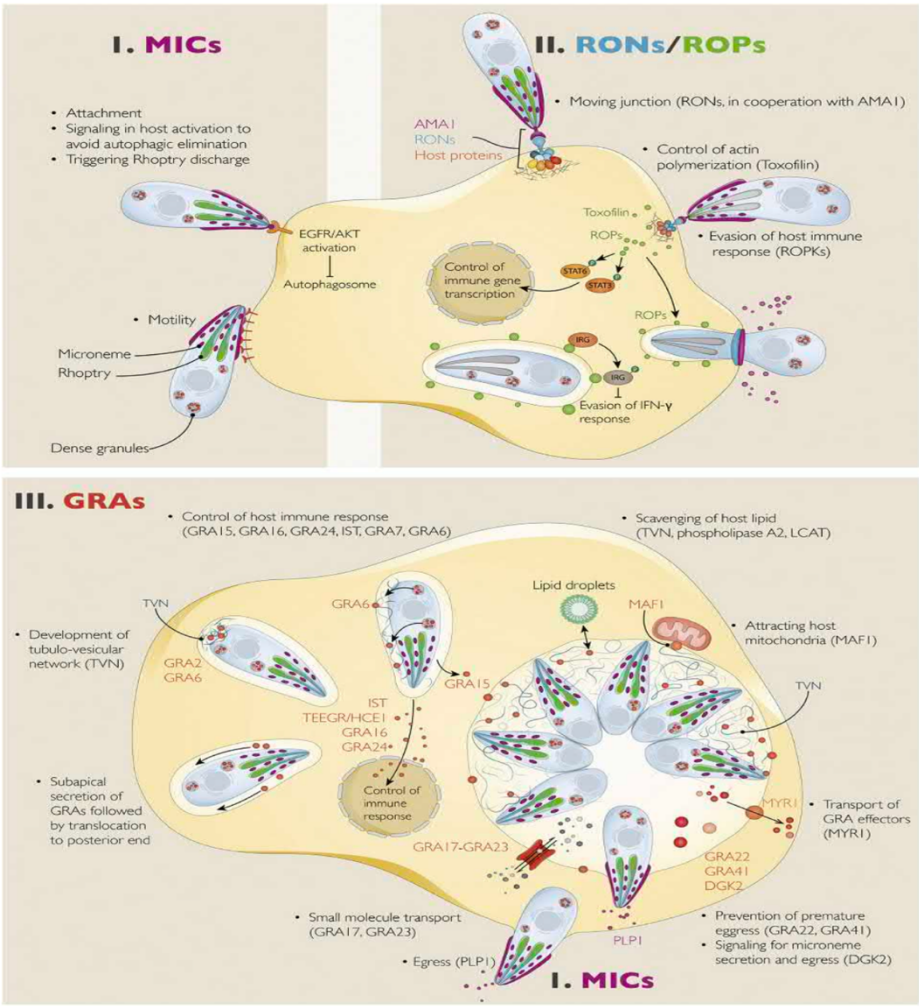

Figure 11- Illustration of the roles of secretory organelle proteins in *Toxoplasma gondii* host cell interactions (Lebrun et al., 2020)**.** The diagram highlights the specific contributions of microneme proteins (I), rhoptry proteins (II), and dense granule proteins (III). While most functions are attributed to individual proteins, a notable exception is the cooperation between AMA1 and RON proteins in facilitating the moving junction process.

5. The Lytic cycle of *Toxoplasma gondii*

The lytic cycle of *T.gondii* involves several key steps that enable the parasite to replicate and spread within its host. Initially, the tachyzoites, which are the rapidly dividing form of the parasite, attach to and invade host cells. This invasion is facilitated by the parasite's surface proteins, such as microneme and rhoptry proteins (V. B. Carruthers & Sibley, 1997). Once inside the host cell, *T. gondii* creates a parasitophorous vacuole, which protects it from the host's immune system (Sibley, 2004). Inside this vacuole, the parasite replicates asexually through a process called endodyogeny, where it produces daughter cells (Mital et al., 2005). As the number of tachyzoites increases, the host cell eventually ruptures, releasing the parasites into the surrounding tissue (Black & Boothroyd, 2000). This release allows the tachyzoites to infect new cells, thus spreading the infection throughout the host. The ability of *T. gondii* to evade immune responses and replicate quickly within host cells contributes to its effectiveness as a pathogen and its widespread prevalence (Dubey, 2008).

5.1 Motility, Adhesion, Invasion

5.1.1. The Glideosome complex

The motility of *T. gondii* is a highly specialized process that enables the parasite to invade host cells and disseminate within the host organism. This process involves a complex array of components and mechanisms, primarily centered around the glideosome motor complex, actin-myosin interactions, and regulated secretion of adhesive proteins.

Motility in *T.gondii* is characterized by various gliding movements. On a two-dimensional coated surface, the parasite exhibits three distinct types of gliding: circular

gliding, helical rotation gliding, and upright twirling gliding (Håkansson et al., 1999). In contrast, when observed in a three-dimensional motility assay, the parasite moves in a clockwise corkscrew trajectory. This pattern is attributed to the shape of the tachyzoite and the left-handed microtubules present in its cytoskeleton (Leung et al., 2014). The apparatus responsible for this movement is known as the glideosome, which powers the parasite's motility, migration, invasion, and egress from the host cell. Located between the plasma membrane and the inner membrane complex (IMC), the glideosome is conserved among Apicomplexa members (Opitz & Soldati, 2002). The key components of the glideosome include the actin-myosin motor, specifically Myosin A (TgMyoA), and several associated proteins such as TgGAP40, TgGAP45, and TgGAP50, which anchor the motor complex to the IMC (Figure 12, (Frénal et al., 2010)). TgMLC1 interacts with TgGAP45 to anchor TgMyoA to the IMC, positioning it correctly within the parasite's tachyzoite pellicle. TgGAP45, a key element of the glideosome, is synthesized in the cytosol. The protein is directed to the plasma membrane via acylation at its N-terminus, while its association with the IMC is sustained by its C-terminal end (Frénal et al., 2010). TgGAP45 has two orthologues: TgGAP70, which is part of the apical glideosome containing TgMyoA, and TgGAP80, which is found in the basal glideosome with TgMyoC (Frénal et al., 2014).

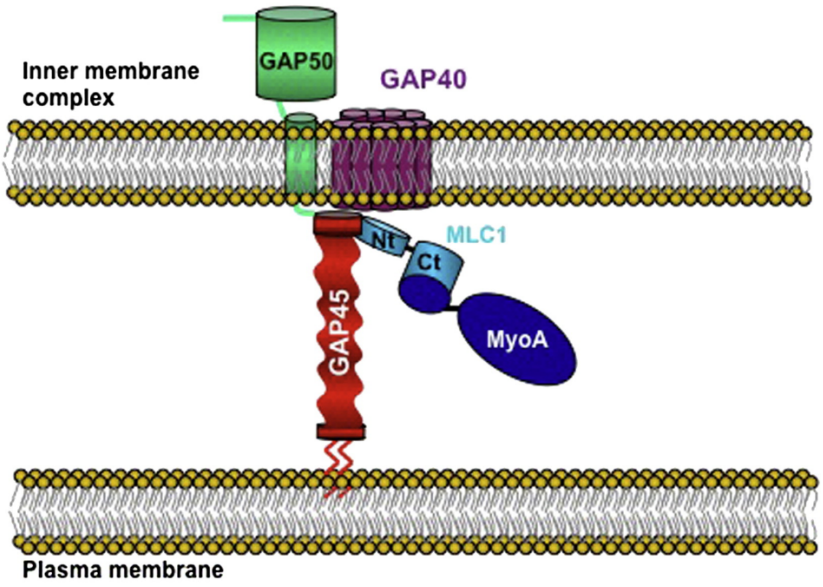

Figure 12 – Illusration of the glideosome complex (Frénal et al., 2014)**.** GAP45 spans between the inner membrane complex (IMC) and the plasma membrane, where its N-terminal is

acylated for membrane targeting. GAP50, GAP40, and MLC1 are associated with the IMC, with MLC1 anchoring TgMyoA to the complex. The interactions of these components are crucial for the gliding motility of the parasite.

TgMyoA serves as the driving motor for gliding motility in the parasite, exhibiting mechanical properties similar to skeletal muscle myosin with a movement speed of approximately 3 μm per second. It converts the chemical energy from ATP hydrolysis into mechanical energy, enabling movement along polymerized actin filaments (Herm-Götz et al., 2002). The stable attachment of TgMyoA to the parasite's cytoskeleton is essential for generating traction. This attachment is mediated by TgGAP40 and TgGAP50 on the IMC side, while TgMyoA must also anchor to host cell substrates in the extracellular space. Mutant parasites with conditional depletion of glideosome components (such as TgMyoA, TgMLC1, TgELC1, and TgGAP45) exhibit significantly impaired motility, which adversely affects their survival (Frénal et al., 2017b). Although *T. gondii* tachyzoites lacking TgMyoA can still replicate within host cells, their lytic cycle is severely disrupted (Andenmatten et al., 2013). This compensation is likely due to the presence of another myosin heavy chain, TgMyoC, which can function with TgGAP45 in the absence of TgMyoA (Frénal et al., 2014). One study explored the role of palmitoylation in the interaction between MyoA's light chain, TgMLC1, and the IMC-anchored protein TgGAP45. It was found that mutations blocking TgMLC1 palmitoylation disrupted its binding to TgGAP45, yet surprisingly, this had little effect on the parasite's motility. This finding challenges the existing model of apicomplexan motility, suggesting that the binding of TgMLC1 to TgGAP45 may not be as critical as previously thought . The glideosome complex is an intricate and adaptable system that empowers *T. gondii* to effectively move and infiltrate host cells, a process that is crucial for its ability to cause disease.

5.1.2. Adhesion to host cell

T.gondii adheres to host cells through a complex process involving multiple steps and molecular interactions. The initial adhesion is mediated by the apical end of the parasite by its surface proteins, which establishes a tight junction with the host cell surface. This interaction is facilitated by the sequential secretion of proteins from the parasite's secretory organelles.

Microneme proteins (MICs) play a crucial role in adhesion. These proteins contain adhesive domains, such as EGF-like and thrombospondin type I repeats, which interact with host cell surface molecules. Although the specific host cell receptors are not well-defined, interactions with glycosaminoglycans have been implicated. These interactions are generally low affinity but multivalent, allowing for repeated rounds of attachment and release, which supports the parasite's gliding motility. *T. gondii*, three primary adhesion complexes are exocytosed from micronemes: TgMIC6-TgMIC1-TgMIC4, TgMIC2-TgM2AP, and TgMIC8-TgMIC3 (V. B. Carruthers & Tomley, n.d.). Among these, TgMIC2 plays a significant role in the parasite's gliding motility, as mutations in the TgMIC2 tail region impair tachyzoite movement, although its complete absence does not entirely inhibit motility (Andenmatten et al., 2013; Kappe et al., 1999; Rugarabamu et al., 2015).This suggests that other microneme proteins likely contribute to the parasite's motility.

The secretion of microneme proteins is triggered by a reduction in potassium levels, which leads to an increase in intracellular calcium concentrations. This calcium surge activates specific kinases and coordinates microneme secretion (Lourido & Moreno, 2015). Calcium-dependent protein kinases (CDPKs), which are typically found in plants, are crucial for this process. Specifically, TgCDPK1 is essential for microneme exocytosis and protein secretion (Lourido et al., 2010). Another kinase, TgCDPK3, is involved in egress and plays a key role in initiating motility through the phosphorylation of the myosin motor protein, TgMyoA (Garrison et al., 2012; Lourido et al., 2012; McCoy et al., 2012).

Beyond CDPKs, other proteins and molecules are involved in the signaling pathways that regulate *T. gondii* motility and the glideosome. For instance, cGMP-dependent protein kinase (PKG) facilitates microneme secretion and egress from the host cell independently of calcium ions (Lourido et al., 2012). TgGC, a purine nucleotide cyclase enzyme responsible for converting GTP into cGMP, is critical for tachyzoite cell-to-cell transmission by regulating invasion, migration, and egress (Bisio et al., 2019; K. M. Brown & Sibley, 2018; Yang et al., 2019a). TgGC's role in cGMP production is essential for activating TgPKG.

Additionally, phosphatidic acid (PA) is a lipid that plays a key role in regulating microneme secretion (Bullen et al., 2016). Phosphatidylinositol phospholipase C (PI-PLC) activates a signaling cascade in response to increased extracellular potassium levels, producing second messengers like diacylglycerol (DAG) and inositol-1,4,5-

triphosphate (IP3). DAG is converted into PA by diacylglycerol kinase 1 (DGK1) (Bullen et al., 2016). PA is recognized by an acylated pleckstrin homology domain-containing protein (APH) located on the surface of micronemal proteins. In *T. gondii*, TgAPH is essential for microneme secretion; depletion of TgAPH results in a significant block in microneme protein release, severely impairing glideosome function (Bullen et al., 2016).

5.1.3. Host Cell Invasion and the Establishment of the Moving Junction (MJ)

T.gondii displays an exceptionally broad host range, capable of invading almost any cell type, whether from mammals, fish, or even insects. The only known exception to this are plant protoplasts, which resist invasion (Werk & Fischer, 1982).During the process of host cell invasion, tachyzoites makes initial contact with the host cell using the apical tip of the parasite. This invasion process involves the sequential release of three types of secretory organelles: first, the micronemes, followed by the rhoptries, and finally, the dense granules. The parasite infiltrates the host cell within 15 to 30 seconds. This invasion is driven by actomyosin motors, with micronemes and rhoptries playing key roles in the formation of the moving junction (MJ), a temporary structure that secures the parasite to the host cell's plasma membrane. Initially, after the parasite attaches to the host cell surface, the invasion begins with the secretion of Rhoptry Neck proteins (RONs) into the host cell membrane. These proteins interact with the microneme protein AMA1, which is located on the parasite's plasma membrane, leading to the formation of the moving junction. Once the moving junction is established, the parasite's acto-myosin cytoskeleton facilitates its movement forward. Subsequently, the rhoptry bulb proteins (ROPs) are released into the host cell, and the parasite becomes encapsulated within a parasitophorous vacuole.

TgAMA1 is a type I transmembrane protein characterized by a short C-terminal cytoplasmic tail and a large N-terminal extracellular ectodomain that contains approximately 16 conserved cysteine residues (Donahue et al., 2000; Hehl et al., 2000). After being secreted onto the parasite's surface, TgAMA1 undergoes cleavage of its N-terminal ectodomain, which is then shed. TgRON2, TgRON4, TgRON5, and TgRON8 have been identified as components of the TgAMA1-associated MJ complex (Alexander et al., 2005; Besteiro et al., 2009; Lebrun et al., 2005b). The MJ acts as a flexible, ring-like structure that maintains close proximity between the parasite and the

host's plasma membrane, facilitating the flow of membranes around this connection. TgAMA1 was initially thought to be essential for parasite survival, as no inducible mutants could be created (Mital et al., 2005). However, advancements in genetic tools for *T. gondii* allowed the creation of a knockout (KO) strain for the TgAMA1 gene (D. Y. Bargieri et al., 2013). This study demonstrated that AMA1 is not required for the formation of a fully functional tight junction (TJ), as the RON proteins operate independently of AMA1. Later research by (M. H. Lamarque et al., 2014) showed that in the absence of TgAMA1, the parasite can adapt by overexpressing homologous proteins such as TgAMA2, which interacts with TgRON2 to facilitate invasion. A double KO of both TgAMA1 and TgAMA2 resulted in a further reduction in invasion efficiency, though it remained partially effective. This led to the discovery of another TgAMA1 homolog, TgAMA4, which can bind to TgRON2L1, a homolog of TgRON2 expressed in the sporozoite stage. Another complex, TgAMA3/TgRON2L1, specific to the sporozoite stage, was also identified. In summary, these findings underscore the critical role of TgAMA/TgRON complexes in the invasion process and demonstrate the parasite's adaptive capacity to ensure successful entry into host cells and continuation of its lytic cycle (Figure 13).

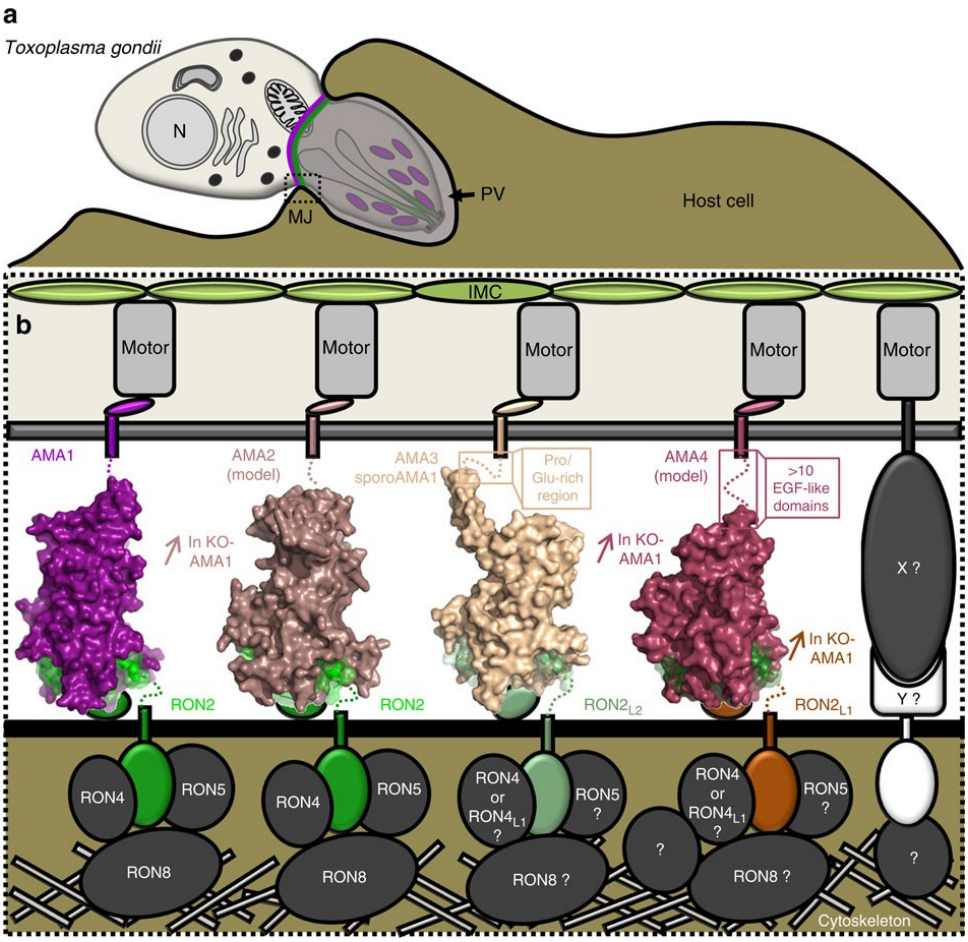

Figure 13- Schematic Overview of TgAMA/TgRON Complexes in the Moving Junction (MJ) (Lamarque, M.H. et al., 2014). This illustration depicts (a) a tachyzoite partially invading a host cell and (b) various proposed models of the *T. gondii* MJ. The figure highlights four different AMA/RON interactions that facilitate the parasite-host interface, with the cytoplasmic tail of AMA linking the MJ to the gliding motor located in the inner membrane complex (IMC). The RON complex, consisting of RON4, RON5, and RON8 tethered to RON2, is positioned beneath the host plasma membrane, potentially interacting with the host cytoskeleton. The primary invasion pathway for *Toxoplasma* tachyzoites and *Plasmodium* merozoites is mediated by the AMA1/RON2 complex. Additionally, three other pairs, homologous to AMA1 and RON2, are suggested, with AMA3/RON2L2 and AMA4/RON2L2 predominantly expressed during the sporozoite stage. AMA2/RON2 and AMA4/RON2L1 illustrate the MJ's molecular adaptability by compensating for the loss of AMA1. X and Y represent unidentified, divergent components that may contribute to MJ architecture.

5.1.4. Formation of Parasitophorous vacuole

The formation of the parasitophorous vacuole membrane (PVM) in *Toxoplasma gondii* is a sophisticated process that involves the secretion of various proteins from

the parasite's dense granules and rhoptries. These proteins are crucial for manipulating the host cell environment, allowing the parasite to establish a niche conducive to its survival and replication. Upon invasion, *T. gondii* actively remodels the host cell membrane to form the PVM, a specialized compartment that protects the parasite from host cell defenses and provides a stable environment for its growth. The lipid bilayer that forms the PVM is majorly derived from the plasma membrane of the host, this has been confirmed by electrophysiological study of tachyzoites entering the host cell (Suss-Toby et al., 1996).

Dense granule proteins (GRAs) play a significant role in the formation and maintenance of the PVM. GRA64 is another dense granule protein that interacts with host cell ESCRT (Endosomal Sorting Complexes Required for Transport) components. This interaction suggests a role in organizing the recruitment of ESCRT proteins to the PVM, which may influence the formation of vesicular structures within the vacuole and contribute to the structural organization of the PV (Coppens et al., 2006). The involvement of ESCRT components highlights the parasite's ability to co-opt host cell machinery to maintain its intracellular niche. Rhoptry proteins are known to be secreted during the invasion process and contribute to the initial formation of the PVM by remodelling the host cell membrane. These proteins, in conjunction with GRAs, ensure the establishment of a protective and nutrient-rich environment, enabling *T. gondii* to evade host immune responses and thrive within host cells. The coordinated action of these proteins underscores the complexity of *T. gondii*'s intracellular lifestyle and its ability to manipulate host cell processes for its benefit.

5.1.5. Parasite egress and host cell lysis

After the intracellular replication of tachyzoites, they actively exit the host cell by rupturing its membrane. The precise signals that trigger this egress are not fully understood. Egress begins when the parasites detach from the intravacuolar network (IVN), disrupting the rosette structure, and the parasitophorous vacuole membrane (PVM) shifts towards the host cell’s nucleus (Caldas & de Souza, 2018). The PVM is then destroyed, allowing the parasites to glide freely within the host cell cytoplasm before eventually breaking through the host cell’s plasma membrane. Known signals that prompt egress include a drop in pH levels, a decrease in potassium ion

concentration, and the accumulation of phosphatidic acid within the parasitophorous vacuole (PV) (Bisio et al., 2019; Roiko et al., 2014; Yang et al., 2019).

The accumulation of abscisic acid (ABA) during parasite development acts as a quorum sensing mechanism, with ABA levels rising just before tachyzoites exit the host cell and remaining low during replication. ABA triggers parasite egress by causing the release of intracellular calcium, which aligns with microneme secretion and the parasite's motility, facilitating its escape from the host cell (Nagamune et al., 2008). Previous studies have linked host cell membrane permeability to a rapid decrease in potassium levels, occurring 2-3 minutes before egress. It was found that this drop in cytoplasmic potassium ions initiates parasite release (Moudy et al., 2001). Additionally, nucleoside triphosphate hydrolases (NTPases) promote egress by depleting ATP in the PV, which is necessary for the calcium-dependent release (Stommel et al., 1997). Cysteine proteases, such as calpain, are believed to play a crucial role in disrupting the PVM and host cell membrane, contributing to parasite egress. Parasites' perforin function might inadvertently activate Calpain (Chandramohanadas et al., 2009).

Acidification of the PV stimulates microneme secretion and activates perforin-like protein 1 (TgPLP-1), which relies on calcium release and is essential for permeabilizing the PVM. TgPLP-1 forms pores approximately 100 Å in diameter within the host cell's membrane, leading to its disruption and facilitating parasite egress. Studies on TgPLP-1 deficient parasites showed that these mutants were unable to exit the host cell and remained trapped (Giudice et al., 2010; Kafsack et al., 2009). Moreover, the host's immune response can also induce parasite egress. T-cells targeting infected *T. gondii* cells through death ligand or perforin/granzyme-dependent cytotoxicity can destroy the host cell, triggering the release of the parasites, which can then infect neighboring cells (Persson et al., 2007).

Another unsuspecting protein that is involved in regulation of egress is Protein Phosphatase 1 (PP1)**.** In a 2022 study by (Herneisen et al., 2022), PP1was identified as a Ca^{2+}-responsive enzyme that relocalizes to the parasite's apex upon Ca^{2+} store release, a key event triggering motility and invasion. This relocalization highlights PP1's involvement in modulating downstream pathways critical for parasite motility, which is essential for successful egress. Furthermore, conditional depletion of PP1 impairs the parasite's ability to regulate Ca^{2+} uptake, thereby inhibiting motility and blocking egress from the host cell. These findings underscore PP1's significant role as

a regulator of Ca^{2+} dependent signaling networks, particularly in orchestrating the cytoskeletal changes necessary for egress.

5.2. Cell Cycle Dynamics and Intracellular Replication

Toxoplasma gondii exhibits several modes of division throughout its complex life cycle (Figure 14), adapting its replication strategy to different host environments and life cycle stages. The primary modes of division observed in *T. gondii* are endodyogeny, endopolygeny, and schizogony (Gubbels et al., 2021a).

Endodyogeny is the predominant form of division for tachyzoites and mature bradyzoites in the intermediate host. In this process, two daughter cells are formed internally within an intact mother cell, which maintains its shape and apical polarity throughout the division (Nishi et al., 2008). The daughter cells consume the mother cell as they develop, eventually emerging to continue the infection cycle. Endodyogeny involves a single nuclear division that results in the formation of two daughter parasites per replication cycle. This process involves a complex reorganization of cellular structures, including the Golgi complex, centrosome, and other organelles (Nishi et al., 2008).

Endopolygeny is observed in immature bradyzoites and some asexual stages. This mode of division involves multiple rounds of nuclear division followed by the formation of multiple daughter cells within the mother cell (Ferguson, 2002). Unlike endodyogeny, which typically produces only two daughter cells, endopolygeny involves multiple rounds of S-phase and mitosis (S/M) without cytokinesis, leading to the creation of a multinucleated mother cell, followed by the synchronous formation of numerous daughter parasites from a single mother cell.

In both processes, mitosis occurs without the breakdown of the nuclear membrane (a process known as closed mitosis) and involves the formation of internal buds, which organize a full complement of organelles within the IMC cytoskeletal scaffolds for each daughter parasite. The maternal IMC-plasma membrane structure is preserved until the buds are fully formed, at which point the plasma membrane detaches from the maternal IMC and associates with the daughter IMC buds, forming mature daughter pellicles.

In contrast, some apicomplexans replicate through schizogony, a process that requires the disassembly of the mother pellicle before multiple rounds of nuclear division, with

daughter parasites subsequently budding from the plasma membrane (Francia and Striepen, 2014).

Interestingly, in immature bradyzoites, endodyogeny generally occurred asynchronously, and parasites were frequently seen dividing through cycles of schizogony or endopolygeny (Dzierszinski et al., 2004). This adaptability in division strategies likely contributes to the parasite's success in various host environments. The choice of division mode in *T. gondii* is influenced by factors such as life cycle stage and host environment (Gubbels et al., 2021b) .

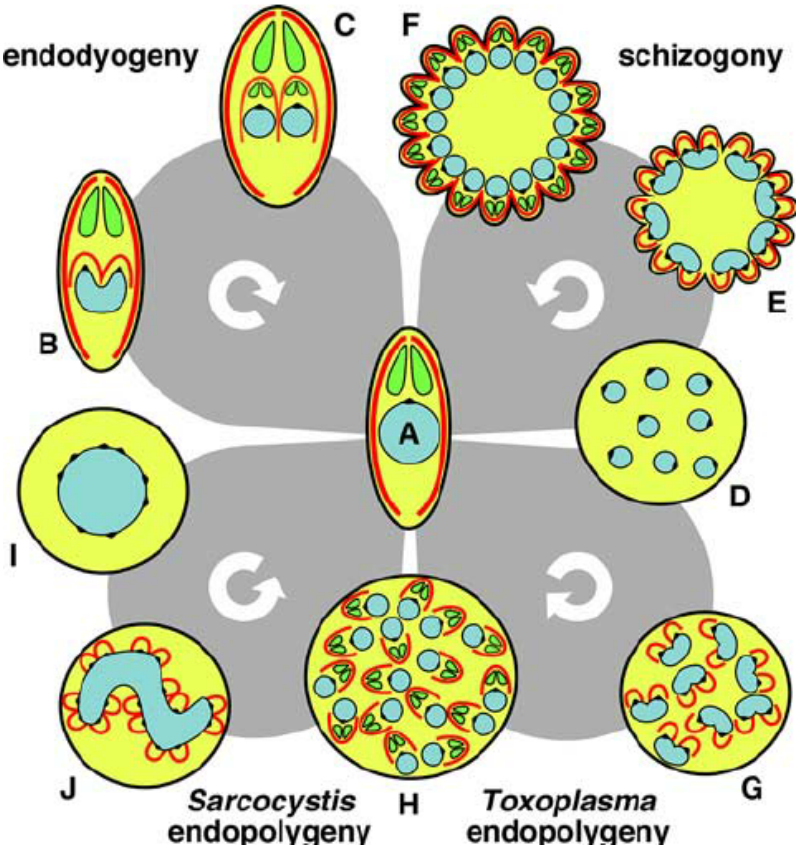

Figure 14 - Illustration of three distinct modes of intracellular replication in Apicomplexan parasites(Ferguson et al., 2008)**. Endodyogeny** (A-C): Seen in *Toxoplasma gondii* tachyzoites, involves a single nuclear division resulting in two daughter cells forming within the mother cell. **Schizogony** (D-F): Common in parasites like *Plasmodium*, where multiple rounds of nuclear division occur before many daughter cells form simultaneously around the periphery of the mother cell. **Endopolygeny** (G-J): Observed in *Toxoplasma* merozoites and *Sarcocystis*, involves several rounds of nuclear division without cytokinesis, leading to the formation of a multinucleated cell that eventually produces multiple daughter cells. In *Sarcocystis* we observe multiple rounds of synchronous DNA replication and segregation in the polyploid nucleus.

5.2.1. Unconventional Cell Cycle in *Toxoplasma gondi*

Tachyzoites have an unusual cell cycle with a closed mitosis divided into three phases (G1, S, and M), until recently the G2 phase was assumed to be absent (J. Radke, 2001). A recent study by Hawkins et. (2024) revealed that a short G2 phase maybe be present that might overlap with the S/M phase. In *T. gondii* tachyzoite replication, there does not appear to be a significant pause before mitotic initiation. Rather, the tachyzoite cell cycle has a major G1 that lasts more than half of the division cycle.

The timing of the *T. gondii* cell cycle is distinctive, with G1 phase comprising approximately 60% of the cycle, S phase about 30%, and mitosis following immediately after DNA replication. Cytokinesis begins in late S phase and overlaps with mitosis (Gubbels et al., 2008). An unusual feature of the S phase is the bimodal distribution of DNA content, with a smaller population of parasites containing 1-1.7N DNA and a larger population with nearly diploid (~1.8N) DNA content, possibly representing a distinct premitotic checkpoint(Radke et al., 2001; White et al., 2014b). Like in mammals, cell division appears to be coordinated by the cyclin/Cdk (Cyclin-dependent kinase) control system (Gubbels et al., 2008; Kvaal et al., 2002).

Phosphorylation and dephosphorylation are pivotal processes in the regulation of the cell cycle in *Toxoplasma gondii*. Reversible phosphorylation, a key regulatory mechanism, involves specific protein kinases and phosphatases working in opposition to control phosphorylation processes. Phospho-proteomics studies in eukaryotes have shown that around 50% of proteins in humans, mice, and yeast are phosphorylated, classifying them as phosphoproteins (Vlastaridis et al., 2017).

Kinases transfer a phosphoryl group from ATP to hydroxyl groups on serine, threonine, or tyrosine residues in substrates, which range from small molecules to proteins. This phosphorylation, a post-translational modification, can trigger various downstream effects like activation, repression, and signaling cascades (Manning, 2002). Toxoplasma's genome includes 159 kinase-related genes, representing about 2% of its total genes, encoding 108 true kinases and 51 pseudokinases (Gaji et al., 2021; Lorenzi et al., 2016). Cyclin-dependent kinases (CDKs) are crucial for cell cycle regulation in higher eukaryotes, activated by binding to cyclins, which provide substrate specificity. CDKs belong to the serine/threonine kinase family and are fully active only as heterodimers (L. Liu et al., 2019). The *Toxoplasma* genome encodes 10 CDK-related kinases (Crks), with eight expressed in tachyzoites and bradyzoites, and the

other two in merozoites and sporozoites (Alvarez & Suvorova, 2017). Unlike conventional eukaryotic cell cycles, *Toxoplasma* has atypical cyclins, mostly constitutively expressed (White & Suvorova, 2018).

Phosphatases, alongside kinases, play a critical role in cell cycle regulation by removing phosphate groups from proteins, thereby reversing the effects of phosphorylation. In *T. gondii*, specific phosphatases are involved in dephosphorylating key substrates, ensuring that cell cycle progression is tightly controlled and that transitions between different phases are appropriately regulated. For instance, phosphatases counterbalance the action of TgCrks by deactivating phosphorylated proteins at various cell cycle checkpoints, preventing aberrant cell division and ensuring the parasite's successful replication and invasion processes.

Details regarding which specific cyclin/Cdk complexes govern each phase of the cycle were previously unknown. It is only recently, through the functional characterization of Crks (Cdk-related kinases), that cyclin/Crk complexes have been linked to specific cell cycle phases. Among the seven atypical cyclins of types P, H, L, and Y, and the ten Cdks, five have been shown to play roles in cell division processes (Alvarez et al., 2017). For example, the TgCrk1/TgCycL complex is crucial for the formation of daughter cells, TgCrk2 interacts with TgPHO80 (a type P cyclin) to prevent cell cycle arrest in G1 phase, TgCrk5 regulates an S phase checkpoint (Figure 15). TgCrk6 plays a vital role in enabling the parasite to progress from the metaphase stage to the anaphase stage during cell division. In an attempt to characterize the role of TgCrk4, Hawkins et al. (2024) identified the unrecognized G2 phase in Apicomplexa cell cycle and established that TgCrk4-TgCyc4 complex plays a pivotal role in the unconventional cell cycle of the tachyzoite by regulating the G2/M transition, which is crucial for maintaining the fidelity of centrosome and chromosome replication.

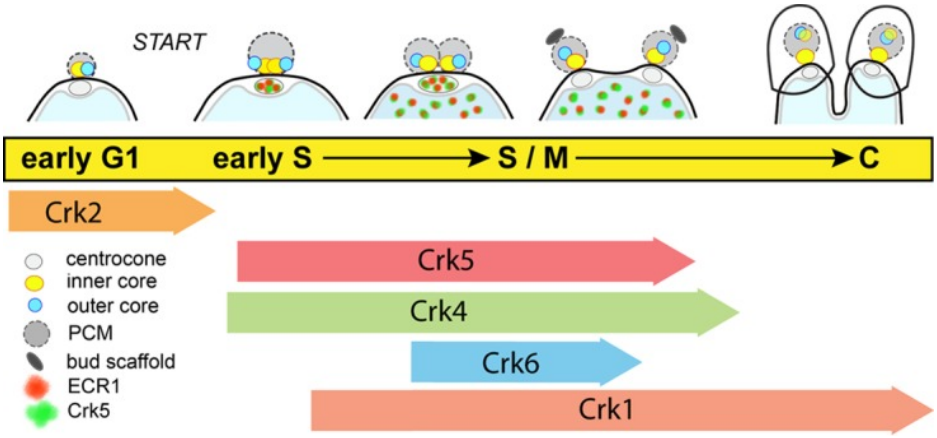

Figure 15 - Regulatory mechanisms in the cell cycle of Toxoplasma gondii tachyzoites (Naumov et al., 2017). The figure delineates the key phases of the T. gondii cell cycle, focusing on the timing of centrosome and centrocone duplication during early G1, S, and M phases. The upper section illustrates the sequential mitotic events, while the lower section outlines the involvement of specific TgCrk kinases (Crk1, Crk2, Crk4, Crk5, and Crk6) in regulating cell cycle transitions.

5.2.2. Cell Division and Daughter Cell Formation

Apicomplexan genomes vary significantly in size, ranging from 1 to 100 Mb, and are structured into 3 to 14 chromosomes typical of eukaryotes, each featuring telomeric repeats at the ends and a centromere defined by chromatin. In species like *T.gondii* (Gissot et al., 2012) and *Plasmodium falciparum* (O'Donnell et al., 2002), the telomeres cluster together and anchor to the nuclear envelope. During cell division, the centromeres are also clustered and attached to the nuclear periphery (Brooks et al., 2011; Hoeijmakers et al., 2012). However, in non-dividing *P. falciparum* sporozoites, centromeres are not clustered (Bunnik et al., 2019). Additionally, during interphase in the schizogony of *Plasmodium* within erythrocytes, some centromere dissociation occurs (Arnot et al., 2011; Gerald et al., 2011; Roques et al., 2019; Zeeshan et al., 2020). This behavior appears to be species-specific, as unclustered centromeres have not been observed in *T. gondii* tachyzoites (Brooks et al., 2011; C. T. Chen & Gubbels, 2015; Farrell & Gubbels, 2014). Nonetheless, there is a high degree of chromosomal organization that helps maintain heterochromatin structure during DNA synthesis and segregation (Bunnik et al., 2019; Fraschka et al., 2018).

Key Events in G1 Phase through S Phase of Tachyzoite Cell Cycle

During this phase, the parasite performs canonical housekeeping tasks to prepare for S phase commitment, similar to other eukaryotes (Courjol et al., 2017). During G1, the cell undergoes growth and protein synthesis, with key processes such as organelle biogenesis (notably the apicoplast and mitochondria) and chromatin licensing for replication initiation (Nishi et al., 2008). Regulatory proteins, including the origin recognition complex (ORC) and minichromosome maintenance (MCM) proteins, ensure the cell is primed for DNA replication (White et al., 2014b). Additionally, metabolic pathways, especially those involved in nucleotide biosynthesis, are activated to ensure sufficient resources for DNA synthesis (Radke & White, 1998). Checkpoints involving cyclins and CDKs regulate the transition into S-phase, ensuring the cell is ready for replication (White et al., 2014). This phase also involves early steps in apicoplast DNA replication, crucial for the parasite's survival (Nishi et al., 2008). One of the earliest observable events is Golgi replication, which begins in G1 and progresses through late G1 (Nishi et al., 2008). As G1 advances, the centrosome migrates to the basal side of the nucleus in late G1 (Hartmann et al., 2006). This repositioning of the centrosome is an important precursor to subsequent cell cycle events. The centrosome functions as a centriole-associated microtubule organizing center (MTOC). During the G1 phase, the centrosome associates with the Golgi and undergoes division via lateral elongation (Hartmann et al., 2006; Nishi et al., 2008; Pelletier et al., 2002). The centrosome duplicates at the basal end of the nucleus and migrates toward the apical end during the S phase (Hartmann et al., 2006). Structurally, the centrosome is bipartite, comprising an inner core and an outer core (Suvorova et al., 2015). The inner core of the centrosome contains proteins such as TgCEP250 and TgCEP250-L1, which are present throughout the cell cycle, including G1 (Suvorova et al., 2015b). These proteins are crucial for maintaining centrosome integrity and coordinating nuclear events. The inner core is tightly aligned with the centrocone, a mitotic structure embedded in the nuclear envelope that mediates the connection between kinetochores and the centrosome inner core. The outer core of the centrosome contains proteins like TgCentrin1, TgSfi1, and TgSAS-6. TgCentrin1 serves as a marker for the outer core and is involved in centrosome duplication, which begins in late G1 (Suvorova et al., 2015). TgSfi1, a centrin-binding protein, localizes near TgCentrin1 and is involved in centrosome replication at the G1/S transition.

Depletion of TgSfi1 led to a drastic reduction in the cores associated with TgCentrin1 (Suvorova et al.,2015).

The centrosome is condensed and gradually enlarges as G1 progresses, remaining associated with the nuclear envelope. During late G1, the outer core expands and replicates, while the inner core separates from the outer core but stays connected to the centrocone, a conical structure crucial for mitosis in Apicomplexans (Ferguson et al., 2008; Suvorova et al., 2015). The centrocones are located near the chromosome tethering sites during interphase (B. Anderson-White et al., 2012a; Brooks et al., 2011; Francia & Striepen, 2014) and allow spindle fibers to enter the nucleus for chromosome separation during mitosis (Francia et al., 2014). After the outer core replicates, the inner core also duplicates, maintaining its proximity to the nucleus while the outer core stays distal. Super-resolution microscopy has revealed a linear alignment of outer cores, inner cores, and centromeres (Suvorova et al., 2015).

As the parasite approaches the late G1 phase, proteins like TgMAPK-L1 temporarily encircle the centrosome, ensuring that the centrosome core duplicates only once per cell cycle and facilitating the linkage between the nuclear centrocone and the daughter basal complex (Suvorova et al., 2015). Another kinase crucial for regulating centrosome replication is TgNek1 (C. T. Chen & Gubbels, 2013). Parasites harboring the temperature-sensitive V-A15 mutant, which involves a point mutation in TgNek1, exhibited defects in parasite budding. Additionally, TgArk3, which is localized at the centrosome and partially associated with TgCentrin1 at the outer core, has been shown to play a significant role in controlling centrosome division (Berry et al., 2016). Parasites with depleted TgArk3 displayed normal nuclear cycles but experienced defects in daughter cell formation, specifically in budding and cytokinesis (Berry et al., 2016). In contrast, parasites lacking TgArk2 did not exhibit any discernible phenotype (Berry et al., 2016). Calcium-dependent protein kinase 7 (TgCDPK7) also plays a vital role in maintaining centrosome integrity by ensuring proper positioning and segregation of the centrosomes.

Key Events through S Phase and Mitosis of Tachyzoite Cell Cycle

During the S/M phase of the cell cycle, key processes such as mitosis, chromosome replication, and budding take place. This phase is essential for maintaining parasite

integrity and ensuring the proper execution of mitosis. A checkpoint exists toward the end of the S phase, where chromosome replication slows down or pauses just before the onset of mitosis, with the DNA content reaching approximately 1.8 (Radke et al., 2001; Alvarez and Suvorova, 2017).

Spindle pole duplication occurs within the nuclear envelope fold, followed by migration, likely driven by growing microtubules between the poles, forming an expanded centrocone-tunnel (Gubbels et al., 2006). The separation of spindle poles splits the tunnel, resulting in two centrocones with spindle microtubules extending into the nucleoplasm (J. M. Brown et al., 1999). The spindle poles do not fully migrate to opposite sides of the nucleus but instead align at an angle, a process known as pleuromitosis. Spindle assembly begins in late S phase, coinciding with a near-diploid sub-population. It is unclear whether this population reflects a pause in chromosome replication, but it is likely that chromosome replication is rapidly completed around the time of spindle assembly. This might be associated with closed mitosis, where kinetochores of duplicated chromosomes are transferred to the new spindle pole, a critical step to ensure each nucleus receives one chromosome copy. This transition could represent a modified G2 phase, during which components necessary for cytokinesis and mitosis accumulate.

Budding and Cytokinesis

The internal budding process of *T. gondii* tachyzoites is driven by the formation of the daughter parasites' cytoskeleton, initiated at the centrosomes (Hu, 2008; Striepen et al., 2007). As mitosis and cytokinesis proceed simultaneously, the cytoskeleton grows from the apical to the basal end (Figure 16). After the centrosomes migrate to the apical side of the nucleus and align with the centrocone, the first signs of the daughter cytoskeleton appear as small hazy structures near the centrosomes, which can be labeled with TgMORN1 (Gubbels et al., 2006; Hu, 2008). These structures develop into basal complexes, and TgMORN1 rings are found at both ends of mature parasites. As division progresses, these rings move towards the basal end of the developing parasites, driven by microtubule polymerization (Gubbels et al., 2006). The formation of the apical complex, including the conoid, was thought to follow the creation of MORN1 rings, with the conoid then localizing to the apex and the basal ring migrating across the nucleus (Hu, 2008) until recently but some research has introduced

nuanced details that expand or refine this understanding. For instance, while the MORN1 rings are crucial for apical complex assembly, recent findings suggest that the assembly of these structures may be more modular and dynamic than previously thought. Some studies have shown that components of the apical complex can form simultaneously or in parallel, rather than in a strict sequential manner (Engelberg et al., 2020). Additionally, the conoid itself has been revealed to be a more dynamic structure, capable of transient disassembly and reassembly, depending on the developmental stage or conditions, which challenges the idea of a fixed localization process (Back et al., 2020). Furthermore, high-resolution imaging studies have revealed more complex interactions between MORN1 and other structural components like Centrin and SAS6L, suggesting that the basal ring's migration may involve multiple regulatory steps beyond simple physical movement (Francia et al., 2012).

The formation of the inner membrane complex (IMC) occurs in two stages: first, during the elongation of the daughter cells' IMC within the mother cell, and second, through the recycling of the mother cell's IMC membranes after the daughter cells emerge (Ouologuem & Roos, 2014b). The assembly of IMC proteins follows a specific temporal and spatial pattern, with proteins being expressed precisely when needed during the cell cycle (Anderson-White et al., 2011).

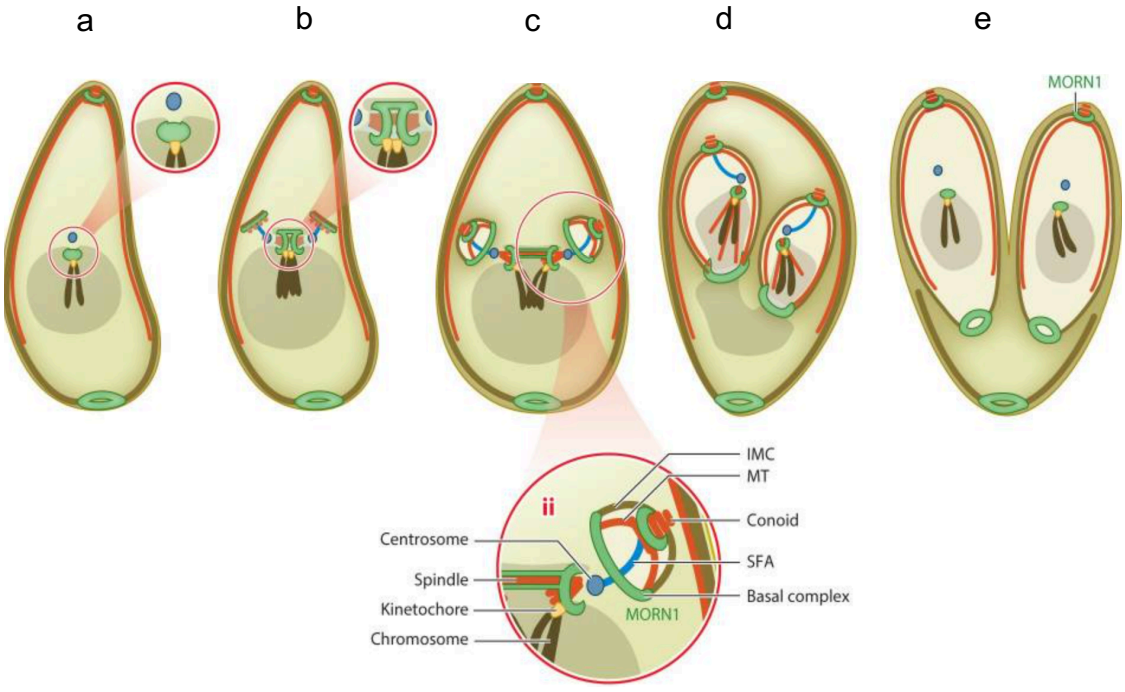

Figure 16 - Schematic Overview of Tachyzoite Cortical Cytoskeleton Dynamics During Cell Division (Blader et al., 2015). **(a, b)** During closed mitosis, chromosomes remain uncondensed, clustering at the centrosomes and anchored to the centrocone via the kinetochore. **(c)** The daughter cells' cortical cytoskeleton is constructed, starting from the apical pole and extending towards the basal end. **(d)** The mother cell's nucleus remains connected to the developing daughter cells through the centrosome, which links to the conoid via SFA. **(e)** The daughter cells are separated as the basal complex tightens in the later stages of the cell cycle.

The formation of daughter cells in *T.gondii* is a complex, multi-step process requiring the coordinated assembly of cytoskeletal components, establishment of cell polarity, and the activity of the basal complex (BC). The early stages involve the assembly of key cytoskeletal structures, specifically the cortical microtubules (MTs) and the conoid, which develop simultaneously. According to Arias Padilla, Murray, et al.(2024), these structures begin forming near the centrioles in distinct patterns. The cortical MTs are anchored in the apical polar ring, while the conoid forms from spirals of tubulin polymers. Both structures, although originating from separate precursor components, assemble in a synchronized manner.

The cortical MTs, which provide structural integrity to the daughter cells, nucleate from the apical polar ring with an initial five-fold rotational symmetry that later expands into a 22-fold array as the cells mature. Disruption of certain apical polar ring proteins, such as kinesinA and APR1, can result in abnormal numbers of MTs, suggesting these proteins play a crucial role in regulating proper MT nucleation. Meanwhile, the conoid, made of tubulin polymers arranged in spirals, develops alongside the MTs and contributes to the scaffold that supports the forming daughter cells. Preconoidal rings, which form shortly after the conoid, are thought to act as organizing centers for the conoid fibers.

Apical-basal polarity is crucial for cellular organization during daughter cell formation. Arias Padilla, Lopez, et al. (2024) demonstrated that this polarity is established early, during the nucleation of cortical MTs. The apical polar ring (APR), which functions as a microtubule-organizing center (MTOC), assembles before the cortical MT array and directs the growth of MTs toward the centrioles. The APR also plays a vital role in organizing the cell’s architecture by anchoring the minus ends of MTs while working in

coordination with the basal complex to establish the cell's overall polarity. Proteins like APR2 and kinesinA are essential for maintaining MT array structure and enhancing the efficiency of cell invasion, underscoring the importance of the apical polar ring in stabilizing the daughter cell cytoskeleton.

The basal complex, which forms independently of the MT array, develops alongside the apical polar ring, and both structures play complementary roles in organizing MTs and maintaining structural integrity. The BC assembles before MT nucleation, suggesting a temporal relationship in their development. This structure is crucial for the final stages of daughter cell division, as it ensures proper contraction and segregation of the cells. Proteins like MORN1 and Centrin are involved early in the formation of the BC, and their recruitment occurs in tandem with the growth of the daughter cytoskeleton. Intermediate filament-like proteins such as IMC5, IMC8, IMC9, and IMC13 reinforce the structural integrity of the developing cell (J. Liu et al., 2016).
A key player, BCC0, establishes the five-fold symmetry observed in both the apical annuli and the IMC sutures, guiding the bidirectional growth of the daughter cell cytoskeleton. BCC0 is recruited to the apical region and extends toward the basal direction, laying the foundation for the daughter cell's structural framework (Engelberg et al., 2022). As a contractile ring, the BC plays a crucial role during the final stages of daughter cell budding, ensuring proper segregation from the mother cell. Proteins such as MORN1 and BCC4 are essential for assembling the BC, which uses Myosin J (MyoJ) rather than the typical eukaryotic actin-myosin system to constrict the BC, completing cell division.

In summary, the formation of daughter cells in *T. gondii* is a highly regulated process dependent on the coordinated interplay of cytoskeletal components, apical-basal polarity, and the basal complex. The assembly of the MT array and the conoid lays the structural foundation of the daughter cells, while the apical polar ring organizes this framework. The basal complex, supported by proteins like MORN1 and BCC4, ensures the final stages of cytokinesis by constricting and separating the daughter cells from the mother.

Organelle Segregation

The partitioning and segregation of various organelles in the developing daughter parasites are highly coordinated (Nishi et al., 2008). The process begins with the division of the Golgi and centrosome, followed by the apicoplast, which divides concurrently with the parasite's nuclear division (Striepen et al., 2000a) (Figure 17). During this process, the apicoplast remains connected to the centrosomes of the mitotic spindle. Time-lapse video microscopy has revealed that apicoplast division occurs at the apical end of the nucleus, starting with an ovoid shape that elongates into a U-shaped intermediate before undergoing fission, resulting in two daughter plastids. This fission happens just before the daughter parasites emerge from the mother cell (Striepen et al., 2000). Additionally, during endodyogeny, the plastid associates not only with the centrosomes but also with the spindle and the inner membrane complex (Striepen et al., 2000).

. Following the apicoplast division, the endoplasmic reticulum and mitochondria divide next (Gubbels, White et al., 2008; Nishi et al., 2008). The parasite's secretory organelles, rhoptries, and micronemes, are synthesized anew within each daughter cell rather than being inherited from the mother cell (Nishi et al., 2008). Once formed, these organelles move to the apical complex at the apex of the daughter parasites, a process that likely depends on cytoskeletal components acting as pathways for molecular motors (Francia & Striepen, 2014). The mitochondrion divides late in the cell cycle and is incorporated into the daughter cells just before their complete emergence from the mother parasite (Nishi et al., 2008). The daughter cells then acquire their plasma membrane from the mother cell during cytokinesis (Gubbels et al., 2008). After all the organelles are assembled within the daughter cells, they begin to emerge from the mother cell, leaving behind residual bodies composed of degraded secretory organelles and parts of the mother cell's mitochondrion (Attias et al., 2019; Muñiz-Hernández et al., 2011; Nishi et al., 2008). The entire process of daughter parasite formation takes approximately 6-7 hours in a type I strain, after which the daughter cells are capable of initiating another division cycle (Figure 17).

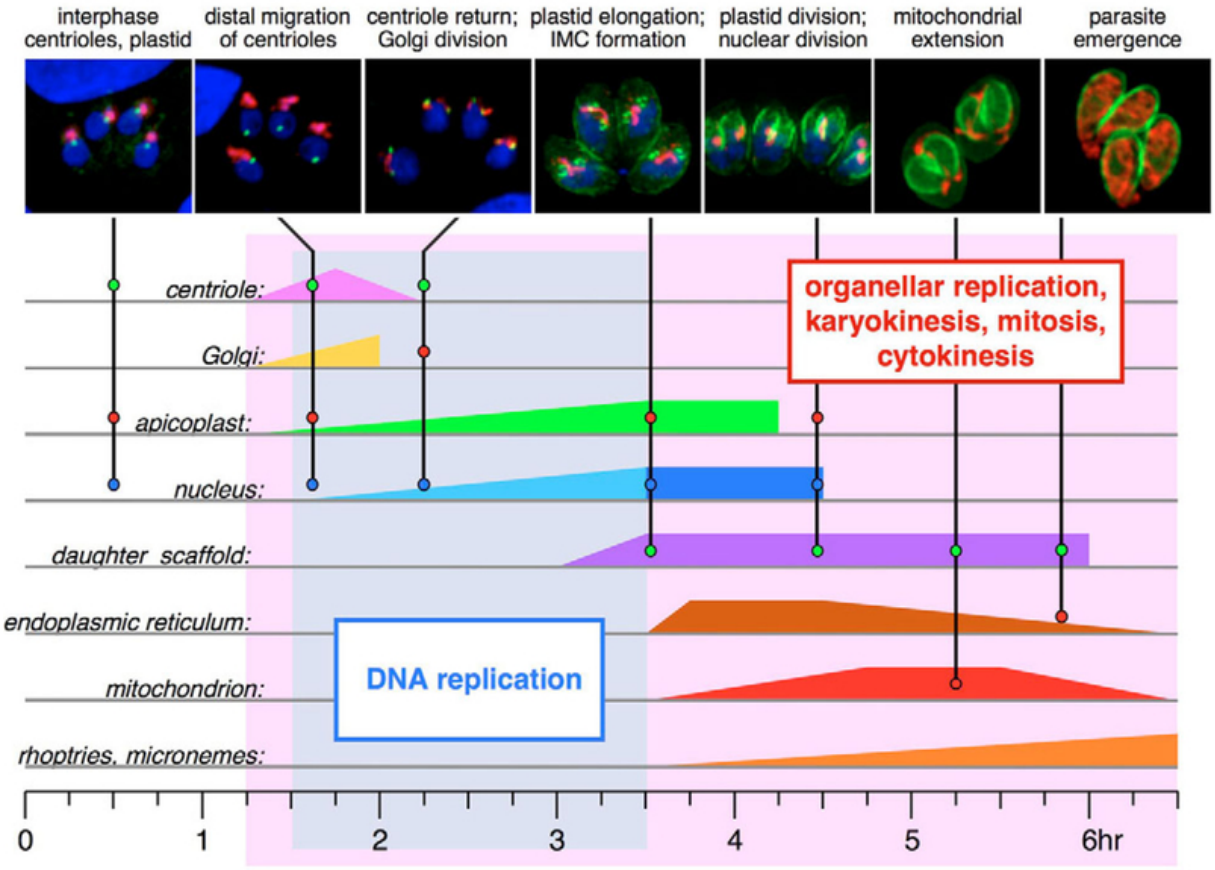

Figure 17 - Timeline of Organelle Replication During the Cell Cycle (Nishi et al., 2008). A detailed schematic of the timeline for organelle replication during the cell cycle. It highlights the coordinated sequence in which each organelle undergoes replication, with the duration of each replication phase shown along the timeline. The uppermost panel features images illustrating the key morphological events associated with organelle replication, capturing the exact timepoints at which each organelle divides.

Maturation of the tachyzoites

The later stages of endodyogeny, remain largely unclear. After organelles are incorporated into the forming daughter cells, the basal end of the developing cytoskeleton contracts, pinching off the daughters. This contraction was initially observed using TgMORN1, a marker that highlights the basal ring of the cytoskeleton (Gubbels et al., 2006). While MORN1 itself lacks motor domains, centrin2, which does have contractile properties, accumulates at the basal ring during the final stages of cytoskeleton assembly (Hu, 2008).

Once the inner membrane complex (IMC) and microtubule skeleton reach maturity, a series of modifications occurs. The first involves cross-linking the IMC filaments, resulting in a rigid structure through proteolytic cleavage and cross-linking (Mann et

al., 2002). Meanwhile, the mother cell's filaments destabilize and disassemble, requiring precise local control of stabilization and destabilization. Another key modification is the insertion of the preassembled "glideosome" into the IMC-membrane alveoli (Gaskins et al., 2004). As the daughter cells emerge, the mother cell's plasma membrane is recycled and immediately associates with the daughter cells where the IMC disassembles. In the furrow between the emerging daughters, new plasma membrane is added via vesicle fusion (Morrissette and Sibley, 2002).

The daughter cells typically remain connected by a cytoplasmic bridge, which likely closes due to mechanical forces from host cell pressure and/or the parasites' motility upon egress from the host cell, following a principle similar to the 'rotokinetic' mechanism described by (J. M. Brown et al., 1999) . The only remnant of the mother cell is a small residual body containing leftover organelle fragments and cytoskeleton, which are digested and recycled.

6. Gene expression and its regulation in *Toxoplasma gondii*

6.1. Transcription and the parasite transcriptome

In Apicomplexa, the first transcriptomic studies using microarrays conducted on *P. falciparum* revealed that over 80% of transcripts are regulated during the sexual phases or the intraerythrocytic cycle. The fluctuations in transcript levels throughout the cycle suggest the adoption of a "just-in-time" regulatory mechanism, which aligns with the low proportion of constitutively expressed mRNA (Llinás & DeRisi, 2004). Similar observations were made in *T. gondii*, where transcription is highly dynamic, with many transcripts expressed at specific parasitic stages. Microarray experiments on the development of the bradyzoite stage showed that mRNA levels directly correlate with the expression of known bradyzoite-specific proteins, suggesting a hierarchical activation of genes governing the transition from the tachyzoite to the bradyzoite stage (Cleary et al., 2002) Furthermore, transcriptome analysis of *T. gondii* at different developmental stages using SAGE (Serial Analysis of Gene Expression) indicates that transcriptional mechanisms play a key role in parasite development (J. R. Radke et al. 2005).

Additionally, it has been shown that the expression of tachyzoite genes is finely regulated, with about one-third of the genes being controlled in a cell cycle-dependent manner (Behnke et al. 2010). In synchronized tachyzoite cultures, researchers observed that the parasite's transcriptome can be divided into two distinct waves. Genes involved in biosynthetic and metabolic functions are expressed during the G1 phase, while those involved in the formation of daughter cells and specific Apicomplexan organelles (rhoptries and micronemes) are expressed during the S and M phases. The transcripts necessary for the production of ROPs and MICs are expressed in a precise biosynthetic order (rhoptry proteins encoding trancripts before microneme proteins encoding trancripts) and are therefore highly regulated, correlating with the previously described "just-in-time" mode (Behnke et al. 2010).

In *T. gondii* tachyzoites, more than a third of the genes, amounting to 2833, are tightly regulated, with their expression peaking at specific stages of the cell cycle (Behnke et al., 2010).

Single-cell RNA sequencing (scRNA-seq) of tachyzoites and tachyzoites grown under alkaline conditions that induce bradyzoite formation has validated the findings reported by Behnke et al. (2010). These scRNA-seq studies identified distinct clusters of gene expression corresponding to various cell cycle phases, including G1a, G1b, S, M, and C (Waldman et al., 2020; Xue et al., 2020). It was observed that bradyzoite formation predominantly occurred during the G1b phase (Xue et al., 2020). Additionally, a strong correlation was found between the expression of specific genes and their corresponding cell cycle stages (Waldman et al., 2020; Xue et al., 2020). When scRNA-seq was performed on tachyzoites, tachyzoite-induced cells, and TgBFD1 knockout cells, the differences in cell populations were primarily linked to either the cell cycle phase or the state of differentiation (Waldman et al., 2020). These findings underscore the value of scRNA-seq in providing a detailed understanding of the relationship between the cell cycle and bradyzoite differentiation.

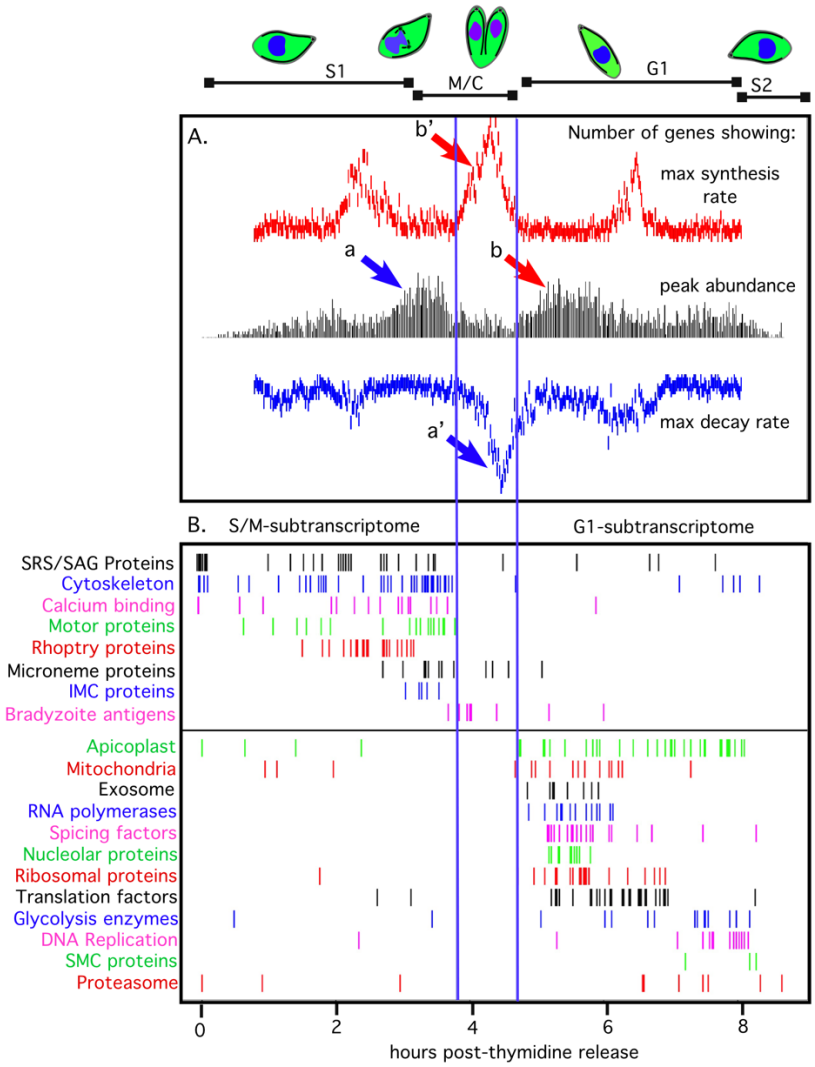

Figure 18 - The illustration of the two functional sub-transcriptomes observed during the *Toxoplasma gondii* tachyzoite cell cycle, as described by Behnke et al. (2010). **(A)** The RNA abundance profile throughout the cell cycle reveals two significant peaks corresponding to the S/M and G1 sub-transcriptomes, separated by a transition phase characterized by simultaneous RNA synthesis and degradation. The histograms represent the number of genes, with the red curve indicating the maximum net increase in mRNA synthesis, the black curve showing peak RNA abundance, and the blue curve (inverted scale) displaying the maximum net rate of RNA decay. During the 8.7-hour replication cycle following thymidine release, the peaks of RNA abundance mark the presence of two waves within the sub-transcriptomes. Genes labeled 'a' (black peak in the S/M phase) show a maximum net decrease in mRNA levels during the later transition phase, corresponding to peak 'a' (blue). Conversely, genes labeled 'b' (black peak in the G1 phase) primarily originate from the earlier transition phase marked by a peak 'b' (red), indicating maximum net mRNA synthesis. The transition phase is associated with rapid fluctuations in transcript levels, coinciding with cell budding, the initiation of the cell cycle, and the decay of the mother cell. **(B)** The expression peaks of mRNAs, categorized by their functional groups, are distributed between the two sub-transcriptomes. Genes essential for the basal

development of the parasite peak during the G1 wave, while those associated with Apicomplexan-specific functions peak during the S/M wave.

6.1.1. Transcriptional regulation

In eukaryotes, successful transcription depends on the coordinated action of several coregulatory complexes that ensure the accurate transcription of RNA from specific genomic loci. *Toxoplasma gondii* contains homologs of RNA polymerases found in other well-studied eukaryotes, including RNA polymerase I, which synthesizes ribosomal RNA (rRNA); RNA polymerase II, which transcribes mRNA for protein synthesis; and RNA polymerase III, which produces small RNAs like tRNA (Meissner & Soldati, 2005). In other eucaryotes, activating transcription factors (ATFs) bind to cis-acting promoter elements and play a crucial role in recruiting chromatin remodeling enzymes that relax chromatin near these elements. This relaxation allows the recruitment of a multi-subunit Mediator complex, which in turn activates the RNA polymerase II preinitiation complex (PIC) (Blazek et al., 2005). The accessibility of cis-elements to ATFs is influenced by factors such as chromatin state, interactions with remodeling enzymes, and the cell cycle stage (Fry, 2002). Once chromatin is relaxed, the PIC assembles at core promoter elements with the help of Mediator, RNA polymerase II, and general transcription factors (GTFs).

The largest subunit of RNA polymerase II in *T. gondii* (TgRPB1) is marked by a carboxy-terminal domain (CTD), which undergoes progressive phosphorylation during transcription (Figure 21 B). This phosphorylation occurs at two serine residues, serine 5 and serine 2 (Deshmukh et al., 2016, 2018). The CTD of TgRPB1 contains nine heptapeptide repeats (YSPxSPx) that show high conservation at serine 5 and serine 2 (Deshmukh et al., 2016). Phosphorylation of serine 5 is mediated by the cyclin-dependent kinase TgCdk7, while TgCrk9, a Cdk-related kinase, phosphorylates serine 2 (Deshmukh et al., 2016,2018).

In other eukaryotes, promoters of class II genes, which encode proteins, typically contain core promoter elements (CPEs) such as the TATA box, initiator (INR), and downstream promoter elements (DPE) (Lemon & Tjian, 2000; Shandilya & Roberts, 2012). Certain general cis-regulatory elements, such as TATA or CAAT boxes and associated factors, which facilitate the recruitment of the RNA polymerase II complex to promoters, have been recently characterized (He et al., n.d.; Huang et al., 2022;

Markus et al., 2021). Downstream promoter elements are recognized by several GTFs, including TATA binding proteins (TBPs) and others like TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, which facilitate the binding of RNA polymerase II to DNA (Blazek et al., 2005; Featherstone, 2002; Ranish & Hahn, 1996). Although some GTFs and TAFs (TBP-associated factors) were not identified in *T. gondii* through homology searches, two TBP homologs have been identified, and their roles have been studied through chromatin immunoprecipitation experiments (Hakimi et al., data on toxodb.org).

A recent study by the Lourido lab explored transcription initiation at *T. gondii* promoters during both acute and chronic stages at nucleotide resolution (Markus et al., 2021). Using 5' end RNA-sequencing, they generated a genome-wide map of transcription initiation for 7,603 protein-coding genes (~91%) during tachyzoite and bradyzoite stages. They found that 66% of the transcription start site (TSS) predictions differed from existing gene models in ToxoDB by more than 40 nucleotides. Additionally, this study revealed the presence of stage-specific alternative TSSs, leading to mRNA isoforms with distinct 5' ends (Markus et al., 2021). Upon manual inspection, 26 genes were confirmed to have alternative TSSs, with 16 exhibiting stage-specific usage shifts, while the remaining 10 showed alternative TSSs used similarly across different stages. For instance, TGME49_200250 displayed a stage-dependent shift resulting in a 649-nucleotide extension (Markus et al., 2021).

In *T. gondii*, the TSS is located deep within the +1 nucleosome, approximately 41 bp from its upstream end, a model that parallels the nucleosome-internal TSS observed in yeast (Albert et al., 2007). *T. gondii* 5' leaders, which are crucial for ribosome entry during cap-dependent translation, are among the longest in eukaryotes, with lengths reaching up to ~800 nucleotides (Markus et al., 2021).

6.1.2. Promoter motifs and cis-elements

Toxoplasma gondii is distinguished by the presence of specific regulatory elements situated upstream of its target promoters. For example, the promoter of the gene encoding the main surface antigen, SAG1, contains a cis-element composed of six tandem repeats of 27 base pairs each. These repeats play a crucial role in initiating transcription (Soldati & Boothroyd, 1995) and are marked by a central heptamer motif (A/TGAGAGC) (Bohne & Roos, 1997; Matrajt et al., 2004; Mercier et al., 1996; Nakaar

et al., 1998). When analyzing promoters that regulate the expression of MIC proteins, two conserved sequences were identified: 5'GCGTCDCW, known as the MICA motif, and 5'SMTGCAGY, referred to as the MICB motif. Notably, the MICA motif is similar, in reverse orientation, to the conserved cis-acting element found in the TgSAG1 promoter (Mullapudi et al., 2009).

Moreover, specific regulatory cis-elements are also dependent on the parasite's developmental stage. Research on bradyzoite-specific gene promoters revealed that these cis-regulatory elements are essential for gene transcription (Behnke et al., 2008). For instance, the promoter of the bradyzoite-specific gene TgB-NTPase includes two regulatory motifs: TGTGTG and CAGC. Similarly, the bradyzoite-specific gene TgBAG1 has a promoter with a regulatory motif TACTGG (Behnke et al., 2008). In another study focusing on the binding profile of a Myb-like transcription factor, TgBFD1, which acts as a master regulator of bradyzoite differentiation (Waldman et al., 2020), the CUT & RUN method (Skene & Henikoff, 2017) demonstrated that TgBFD1 binds to 509 genes, with binding sites preferentially located near transcription start sites (TSSs) (Waldman et al., 2020). Motif enrichment analysis identified analogous motifs (CACTGG) upstream of bradyzoite-specific genes, TgBFD1's promoter, and the early bradyzoite marker AP2 transcription factor, TgAP2IX-9 (Waldman et al., 2020).

An analysis using FIRE (Finding Important Regulatory Elements) was conducted on the proximal promoter regions of all cyclical mRNAs to identify potential DNA regulatory elements. This analysis revealed nine distinct DNA motifs (Behnke et al., 2010) distributed across genes that peak in transcription throughout the tachyzoite cell cycle. The DNA motifs highly represented in the promoters of G1 phase genes are generally less common in S/M phase promoters, and vice versa. Notably, one of the DNA motifs enriched in G1 promoters, 5'-TGCATGC-3', is identical to the TgTRP2 cis-element, which is essential for the transcription of ribosomal proteins (Mullapudi et al., 2009; van Poppel et al., 2006). This motif is also the 6 bp core DNA binding site identified by PBM for the transcription factor AP2XI-3, whose mRNA levels peak during the G1 phase (Behnke et al., 2010). Another motif, 5'-CACACAC-3', is enriched in the promoter of the AP2 gene AP2XI-4, a nuclear factor involved in regulating bradyzoite-specific gene expression during parasite differentiation and cyst formation (Walker et al., 2012).

TgAP2XI-5, another transcription factor, binds specifically to the GCTAGC motif, and this interaction is crucial for the full activation of the *ROP18* promoter during the S/M phase (Walker et al., 2013). A recent study by Lou et al. (2024) identified distinct DNA motifs in the promoters of AP2-targeted genes, some of which overlap with known binding sites for other AP2 family members. For instance, the "GCTAGC" motif, previously associated with AP2XI-5, was identified as a target of AP2XII-8. Additionally, motifs such as "CAAGACA" were highlighted, supporting the regulatory role of AP2XII-8 in coordinating gene expression during the G1 phase (Lou et al., 2024).

Moreover, nucleotide-resolution TSS analysis revealed a novel motif, gCATGCa, present in 44% of *T. gondii* promoters, typically located about 82 nucleotides upstream of the TSS (Markus et al., 2021).

6.2 Transcription factors and their classification

Gene expression is primarily regulated at the level of transcription. This regulation is carried out by a diverse group of transcription factors. These proteins interact with DNA at promoters and enhancers, often recognizing specific sequences or structural characteristics.

In model eukaryotes, transcription is primarily regulated through the sequence-specific binding of transcription factors (TFs) to DNA elements that are approximately 5-25 base pairs long (Wingender, 1993). These transcription factors can either activate or repress gene expression. The Transcription Factor Database, known as TRANSFAC, provides detailed information on gene expression at the transcriptional level, including data on various TFs, their target genes, and the specific sequences they bind to.

Research on Apicomplexan genomes, such as those of *Plasmodium* and *Cryptosporidium*, has shown that these species have significantly fewer transcription factors compared to yeast or other eukaryotes (Templeton, 2004). This suggests that Apicomplexans may use different mechanisms for gene regulation. It has been demonstrated that ApiAP2 TFs in Apicomplexans bind to specific DNA sequences to

control gene expression (Balaji et al., 2005; Silva et al., 2008). Furthermore, a detailed examination of the *Plasmodium* genome revealed the absence of specific transcription factors with homeodomains, basic domains, and FKH domains (Aravind et al., 2003). A more recent bioinformatics study indicates that *Plasmodium* species lack basic domain and winged-helix TFs (Sardar et al., 2019). This analysis also suggests the presence of new TF families in Apicomplexans, including TUB, NAC, BSD, HTH, Cupin/Jumonji, winged-helix, and FHA families (Sardar et al., 2019). In *T. gondii*, no TUB proteins have been identified, but the TGME49 strain has been found to contain two NAC proteins, one BSD protein, three HTH proteins, five Cupin/Jumonji proteins, two winged-helix proteins, and nine FHA proteins (Sardar et al., 2019).

6.2.1. Superclass 1 – Basic domains

Class I TFs are involved in various biological processes, including cell growth, differentiation, and responses to environmental stimuli (Suzuki & Yagi, 1995). A well-known subgroup within Class I TFs is the bZIP (basic leucine zipper) transcription factors, which feature a basic region for DNA binding and a leucine zipper motif that facilitates dimerization. This structural combination allows them to regulate gene expression effectively (Coulson, 2003). Research on the enhancer binding protein α (C/EBP α) has shown that the DNA-binding domain of bZIP transcription factors (TFs) features a positively charged segment known as the basic region, which is linked to a 'leucine zipper' made up of a heptad repeat of leucine amino acids (Landschulz et al., 1988). These bZIP proteins bind to DNA through the formation of dimers, where uninterrupted α-helices create a structure similar to chopsticks. The leucine zipper regions play a crucial role in promoting dimerization, resulting in the formation of two parallel, coiled α-helices that align perpendicularly to the DNA helix. The basic regions of the bZIP TFs each interact with one half of the palindromic sequence within the major groove of the DNA. This interaction stabilizes the dimer and induces the protein to fold into a helical structure (Miller, 2009). In *Toxoplasma gondii*, one potential bZIP TF (TGME49_305220) has been identified through recent in silico analysis by Sardar et al. (2019), but there is currently limited information available regarding the function of this protein.

6.2.2. Superclass 2 - Zinc-coordinating DNA-binding domains

Zinc-coordinating DNA-binding domains are a hallmark of Superclass 2 transcription factors (TFs), which play essential roles in gene regulation across various organisms. These transcription factors are characterized by the presence of zinc ions that are coordinated by cysteine or histidine residues within the protein, which helps to stabilize their DNA-binding structures (Landschulz et al., 1988). This structural stability allows these TFs to interact specifically with DNA, enabling them to regulate the expression of target genes crucial for cellular processes.

In *T. gondii*, zinc finger proteins, a major family within this superclass, utilize zinc ions to form finger-like protrusions that can insert into the DNA helix, allowing them to recognize and bind specific DNA sequences. This binding is critical for the regulation of genes involved in the parasite's development and differentiation (Miller, 2009). Additionally, zinc-coordinating TFs in *T. gondii* include other important factors like GATA factors, which similarly rely on zinc ions for structural integrity and are involved in regulating genes essential for the organism's life cycle (Sardar et al., 2019).

One of the most common motifs is the C2H2 (Cys2His2) zinc finger, which is composed of a β-sheet and an α-helix that together coordinate a zinc ion (Zn2+). This zinc ion, which is crucial for stabilizing the protein structure, is chelated by two cysteines and two histidines (Lee et al., 1989). The C2H2 zinc finger motif is the most frequently found DNA-binding motif among eukaryotic transcription factors. Among the 37-zinc finger coordinating proteins identified by Bischoff et al. (2010) in *Plasmodium falciparum*, 27 belong to the C2H2 family. The remaining proteins contain zinc finger motifs of the B-box or MYND types. Zinc finger proteins have also been identified in *Toxoplasma gondii*, including 12 C2H2-type proteins. Although their precise roles remain unclear, a study on the cellular response to alkaline stress showed an increase in the transcription of genes encoding zinc finger proteins (Naguleswaran et al., 2010). This finding aligns with previous observations in yeast, where proteins like Rim101, NRG2, and TIS11 were implicated in similar stress responses (Lamb et al., 2001). However, it has not been proven that the proteins in *T. gondii* are orthologous to those in yeast. The general principle governing the function of zinc-finger proteins is that an increased number of zinc fingers corresponds to a greater diversity of ligands to which the fingers can specifically bind (Iuchi, 2001).

Disruption of the TgZFP1 zinc finger protein locus, which contains a CCHC motif, resulted in an impaired bradyzoite differentiation phenotype (Vanchinathan et al., 2005). Moreover, several zinc finger proteins with different domains (including C2H2, MYND, and B-box) were found to be upregulated during alkaline stress in *T. gondii* (Naguleswaran et al., 2010). Another zinc finger protein, TgZNF2, which is conserved across eukaryotes, has been shown to play a crucial role in the nuclear translocation of polyadenylated mRNA from the nucleus to the cytoplasm. Inducible knockdown mutants of TgZNF2 exhibited cell cycle arrest during the G1 phase, indicating that TgZNF2 is vital for the parasite's survival (Gissot et al., 2017).

More recently, it was discovered that the zinc-finger protein TgZFP2 also causes cell cycle arrest. Conditional knockout studies of TgZFP2 revealed that, although the nuclear cycle remained unaffected, the budding cycle was significantly impaired (Semenovskaya et al., 2020). Interestingly, neither TgZNF2 nor TgZFP2 was found to bind DNA, suggesting that their primary binding target is likely RNA.

6.2.3. Superclass 3 - Helix-turn-helix domains

Helix-Turn-Helix (HTH) superclass transcription factors represent a large and diverse group of proteins. This group can be categorized into five subclasses based on the domains they contain: homeo, Forkhead, Heat Shock, Tryptophan, or TEA. These transcription factors are widely distributed, occurring in both eukaryotic and prokaryotic organisms (Harrison et al., 1990). The HTH motif comes in various forms but typically includes two α-helices, each composed of approximately 20 amino acids, connected by a short turn. The second helix interacts with the DNA's major groove, while the first helix provides structural stability. However, certain HTH transcription factors, like MYB proteins (part of the tryptophan group subclass), consist of three repeats of around 50 amino acids, known as R1, R2, and R3, forming three helices that constitute the DNA-binding domain. MYB proteins, which include three members (A, B, and C), are crucial for cell survival and play a key role in regulating the cell cycle and differentiation. These MYB proteins can also interact with other transcription factors to modulate gene transcription.

As noted earlier, Apicomplexans have a relatively small number of HTH domain transcription factors. According to research by Bischoff et al. (2010), only eight of these transcription factors are found in the *Plasmodium falciparum* genome. Among them,

the PfMyb1 protein has been identified as a regulator of key genes during the parasite's pre-erythrocytic stage through direct DNA binding (Gissot et al., 2005). The presence of HTH transcription factors, particularly MYB proteins, in *P. falciparum* suggests some level of conservation within Apicomplexans. Data from ToxoDB indicate that *Toxoplasma gondii* encodes seven Myb proteins, though their functions have not yet been fully explored.

In a more recent study, a Myb-like transcription factor called TgBFD1 was identified and characterized (Waldman et al., 2020). This transcription factor was found to play a crucial role in regulating differentiation. Parasites lacking TgBFD1 were unable to differentiate in vitro, and these mutants also lost the ability to form brain tissue cysts in mice (Waldman et al., 2020). Additionally, an in-silico study by Sardar et al. (2019) identified three novel putative HTH proteins in the *Toxoplasma gondii* Me49 strain, which are predicted to localize to nuclear chromatin based on recent hyperLOPIT data (Barylyuk et al., 2020).

The discovery of these new HTH transcription factors in the *T. gondii* genome could provide valuable insights into their potential role in gene regulation. However, their exact functions and mechanisms of action are yet to be fully understood.

6.2.4. Superclass 4 - β-Scaffold Factors with Minor Groove Contacts

These are unique class of transcription factors that interact with DNA primarily through the minor groove, utilizing a β-sheet scaffold for binding. These transcription factors play a critical role in the regulation of gene expression by altering DNA structure without unwinding it, which can influence the binding of other transcriptional machinery (Harrison et al., 1990).

A well-known example of this class is the TATA-binding protein (TBP), which is essential for the initiation of transcription in eukaryotes. TBP binds to the TATA box in the promoter region of many genes, inducing a bend in the DNA that is necessary for the assembly of the transcriptional pre-initiation complex (Roeder, 1996). This bending is crucial as it facilitates the recruitment of RNA polymerase II and other factors required for the start of transcription (Kim et al., 1993).

Another important member of this superclass is the High Mobility Group (HMG) proteins, which also bind to the minor groove of DNA. HMG proteins are known for

their ability to induce significant bends in the DNA, thereby influencing the accessibility of chromatin and regulating the expression of genes. This structural modulation of DNA by HMG proteins is essential for various cellular processes, including differentiation and response to stress (Bustin, 2001). The interaction of β-scaffold factors with the DNA minor groove is generally more subtle compared to major groove interactions, but it is critical for the fine-tuning of gene expression and the structural organization of chromatin. These factors are essential for maintaining the integrity and functionality of the transcriptional regulation mechanisms in eukaryotic cells (Luger et al., 1997).

In *Toxoplasma gondii*, three orthologs of the HMGB1 (High Mobility Group Box-containing) protein have been identified. A phylogenetic and bioinformatics analysis showed that all three orthologs (HMGB1a, HMGB1b, HMGB1c) possess an HMG box domain. One of these orthologs, named TgHMGB1a, was found to play a role in the regulation of gene expression. Specifically, a transgenic strain that overexpressed TgHMGB1a exhibited increased expression of virulence factors (H. Wang et al., 2014).

6.2.5. Superclass 5 – Other Super classes

This category includes various transcription factors that do not fall into the other major superclasses, such as those with specific DNA-binding domains that are less common or structurally unique. Due to the limited number of transcription factors (TFs) found in Apicomplexans, several theories have been proposed to explain this phenomenon. One theory suggests that the genomes of these parasites may encode TFs with DNA-binding domains that are either only distantly related or completely unrelated to those already known. Another theory proposes that the scarcity of TFs might indicate an alternative transcriptional regulation mechanism in Apicomplexans, potentially involving chromatin remodeling factors working alongside other DNA-binding proteins. To investigate these possibilities, Balaji et al. (2005) analyzed predicted nuclear proteins in Apicomplexans and discovered TFs containing an AP2 (Apetala-2) domain. These proteins have been shown to bind specific DNA sequences (Balaji et al., 2005; De Silva et al., 2008) and to play a role in regulating gene expression (J. Wang et al., 2014), suggesting they are strong candidates for gene transcription regulation.

6.3 AP2 Transcription Factors: Key Regulators of Gene expression

6.3.1. Discovery & Evolution if AP2 transcription factors in plants

In *Arabidopsis thaliana*, AP2 transcription factors were initially identified as crucial regulators of floral development. This discovery highlighted the AP2 gene's role in determining floral organ identity, leading to extensive research on this transcription factor family (Jofuku et al., 1994). Subsequent studies revealed that AP2 transcription factors are part of a larger superfamily known as AP2/ERF (Ethylene Response Factor), which is essential for regulating plant growth, development, and responses to environmental stresses (Sakuma et al., 2002).

The AP2/ERF family is characterized by the presence of one or more AP2 DNA-binding domains. Comprehensive studies have identified a vast number of AP2/ERF genes across various plant species. For example, a genome-wide analysis in sunflower (*Helianthus annuus*) identified 288 AP2/ERF genes, which were categorized into four subfamilies: ERF, AP2, RAV, and Soloist, illustrating the diversity and complexity of this transcription factor family in plants (Bahrami et al., 2021).

In *Arabidopsis thaliana*, the AP2/ERF transcription factor family, also referred to as AP2/EREBP, consists of 145 distinct proteins, which are grouped into five subfamilies: 1) The DREB subfamily (Dehydration Responsive Element Binding) with 56 proteins, 2) The ERF subfamily with 65 proteins, each containing one AP2 domain and a WLG domain, 3) The AP2 subfamily, which includes 14 proteins with two AP2 domains, 4) The RAV subfamily with 6 proteins that have two AP2 domains and a B3 binding domain, and 5) A subfamily of other proteins, comprising 4 proteins with one AP2 domain and a WLG domain (Sakuma et al., 2002).

Structural analyses of AP2 proteins in *Arabidopsis* and *Plasmodium* have shed light on the mechanism by which the AP2 domain binds specific DNA motifs. Using nuclear magnetic resonance (NMR), structural studies of AtERF1 showed that the AP2 domain's secondary structure consists of a three-stranded antiparallel β-sheet and an α-helix aligned parallel to the β-sheet. This structure revealed that DNA interacts with 11 highly conserved residues, 7 of which specifically bind to the consensus DNA sequence AGCCGCC, known as the GCC box.

This study identified key residues within the AP2 domain that directly interact with DNA, primarily located in the β-sheet. These include four arginine residues (R150, R152, R162, R170) whose guanidyl groups directly contact the DNA, along with two tryptophan residues (W154 and W172). These six residues interact specifically with the cytosine and guanine bases within the GCC box, facilitating the AP2 domain's binding to the major groove of the DNA (Allen et al., 1998).

The evolutionary path of AP2 transcription factors is intricate, involving gene duplication, divergence, and loss. Research indicates that the AP2/ERF gene family has undergone significant expansion through tandem and segmental duplications, particularly in flowering plants (Liu et al., 2015). In gymnosperms like *Taxus chinensis*, the evolutionary patterns are more complex, with some AP2/ERF genes displaying irregular domain structures compared to their counterparts in angiosperms (Zhang et al., 2021).

AP2 domains are highly conserved across different species, as shown by the presence of AP2 homologs in non-plant organisms like cyanobacteria, ciliates, and viruses (Magnani et al., 2004; Wuitschick et al., 2004). These homologs also possess a domain encoding a homing endonuclease, a mobile genetic element that can induce single or double-strand breaks in DNA, facilitating the transposition of DNA elements from one location to another. This process, known as 'homing,' involves repairing the DNA break (Chevalier et al., 2001; Koufopanou et al., 2002). It has been proposed that plant AP2/ERF transcription factors may have evolved from homing endonucleases of the HNH-AP2 family found in bacteria and viruses. This evolutionary process likely occurred through horizontal gene transfer or incorporation during an endosymbiotic event with a cyanobacterium. Over time, the homing endonuclease's original function in plants appears to have been lost, with the AP2 DNA-binding domain taking over the role of regulating gene expression to ensure proper plant development (Magnani et al., 2004).

Phylogenetic studies suggest that AP2/ERF transcription factors have evolved differently across various plant lineages. For example, the AP2/ERF family in *Arabidopsis thaliana* and *Oryza sativa* shows a high degree of conservation, while other species like *Taxus* exhibit unique evolutionary adaptations (Zong et al., 2021). Similarly, ApiAP2 transcription factors found in apicomplexan genomes may have arisen from a comparable endosymbiotic event (Balaji et al., 2005).

6.3.2. Discovery & Evolution if AP2 transcription factors in Apicomplexa

Unlike in plants, where AP2 transcription factors are well-characterized, the discovery of the ApiAP2 family within the Apicomplexa phylum, which includes parasites such as Plasmodium and Toxoplasma gondii, has highlighted a unique evolutionary trajectory. These proteins, while maintaining the characteristic AP2 DNA-binding domain, have developed functions specific to the complex life cycles of these parasites (Ferguson et al., 2008). For instance, in Theileria and Cryptosporidium, 19 ApiAP2 transcription factors have been identified, whereas Plasmodium boasts 27. Notably, Toxoplasma gondii possesses an even larger repertoire, with 67 ApiAP2 transcription factors (toxodb.org). In Apicomplexans, these transcription factors often feature up to four repeats of the AP2 domain (Balaji et al., 2005). Their expression across various life stages of the parasites implies a critical role in regulating gene expression necessary for development and interaction with the host (Painter et al., 2011). For example, specific ApiAP2 proteins in *Plasmodium falciparum* are indispensable for the transition between gametocyte and sporozoite stages, highlighting their importance in the parasite's life cycle (Yuda et al., 2009). Among the 27 ApiAP2 genes in P. falciparum, 22 are expressed during different phases of the intraerythrocytic developmental cycle, with each gene named according to the phase of its activity. Moreover, nine ApiAP2 transcription factors are conserved across various Apicomplexan species, suggesting that their common ancestor likely had at least nine such proteins (Balaji et al., 2005). Comparative genomic studies suggest that these factors have undergone lineage-specific expansions and adaptations, likely due to selective pressures in host environments (Bahl et al., 2003). The evolution of these transcription factors may have involved the recruitment of ancestral DNA-binding domains associated with mobile genetic elements, which later adapted to serve regulatory roles in transcription (Kloetgen et al., 2016). The distinction between plant AP2/ERF and ApiAP2 transcription factors exemplifies convergent evolution, where similar DNA-binding domains evolved independently to fulfill different biological needs.

6.3.3. Structure of AP2 domain and proteins

The AP2 domain in Apicomplexa, spanning roughly 60 amino acids, typically comprises three β-strands and a C-terminal α-helix (Figure 21) that stabilizes these strands (Balaji et al., 2005). Notably, between the second and third β-strands, there's an insertion that forms a positively charged hairpin structure, likely enhancing DNA affinity through nonspecific interactions with the DNA backbone (Campbell et al., 2010). Despite sharing similarities with plant AP2 domains, Apicomplexan AP2 domains show distinct variations in conserved residues. For example, the R150 residue is frequently replaced by Y or S, and R152 is often D or N in Apicomplexa, potentially reflecting adaptations to their AT-rich genomes (De Silva et al., 2008). The crystal structure of the PfAP2-Sp/Exp (PF3D7_1466400) AP2 domain in Plasmodium falciparum has revealed its binding mechanism to double-stranded DNA in the major groove and showed that two AP2 domains can dimerize via a domain-swapping mechanism involving their α-helices (Lindner et al., 2010).

In addition to the AP2 domain, some ApiAP2 proteins also feature the ACDC domain, which forms a non-canonical four-helix bundle, despite low sequence conservation across different proteins (Oehring et al., 2012). The ACDC domain's role is not entirely understood, but it may be involved in protein-protein interactions or dimerization, as suggested by its strong crystal packing contacts (Oehring et al., 2012). In P. falciparum, 8 out of the 27 ApiAP2 proteins contain this domain (Campbell et al., 2010).

When analyzing 211 ApiAP2 domains from Apicomplexans like Plasmodium, Theileria, and Cryptosporidium, along with those from plants and bacteria, 12 residues were found to be highly conserved among 241 of the 285 domains studied. These conserved residues play a crucial role in stabilizing hydrophobic interactions within the domain (Balaji et al., 2005). While most ApiAP2 family members contain a single globular AP2 domain, some in Apicomplexa possess up to four AP2 domains. Additionally, the AT-hook motif, another DNA-binding motif, is associated with some AP2 domains in Apicomplexans (Aravind, 1998).

Specific DNA binding studies of two P. falciparum ApiAP2 proteins, PF14_0633 and PFF0200c, revealed that these proteins specifically bind to the DNA sequences TGCATGCA and GTGCAC, respectively (Silva et al., 2008). Further structural studies of the ApiAP2 domain from PF14_0633 have shown that while it retains several canonical features found in plant AP2 domains, key differences exist. For instance,

unlike the plant AP2 domains that function as monomers, P. falciparum AP2 domains dimerize through a domain-swapping mechanism, where α-helices are exchanged between monomers to form a dimer (Lindner et al., 2010). This dimerization may play a significant role in bringing distant DNA loci together, potentially regulating sporozoite-specific gene expression (Lindner et al., 2010).

6.3.4. The Multifaceted Role of AP2 Transcription in the regulation of gene expression in *T.gondii*

. The ApiAP2 transcription factors (TFs) play a pivotal role in modulating stage-specific gene expression in tachyzoites (Figure 19), with distinct ApiAP2 TFs regulating the expression of genes critical for both the tachyzoite and bradyzoite stages.

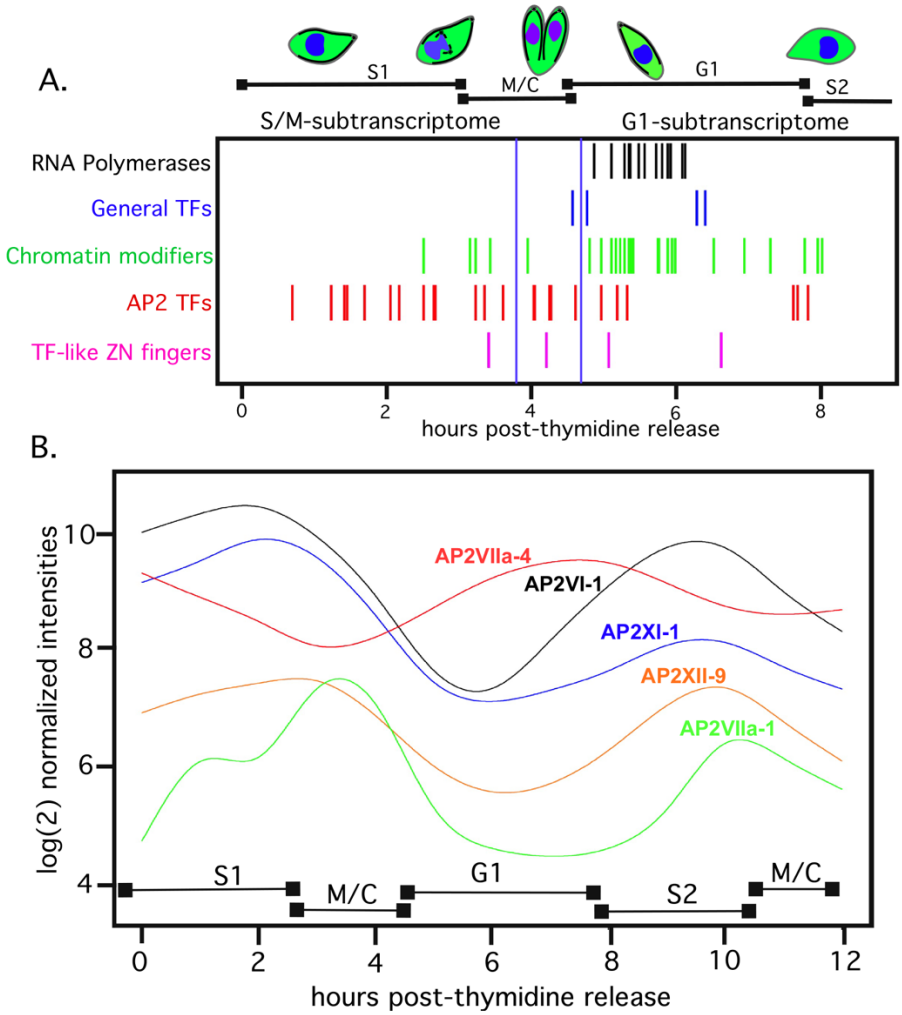

Figure 19 - Illustration of the putative transcription factors in *Toxoplasma gondii* that are active during the cell cycle (Behnke et al., 2010). **(A)** the mRNA levels of RNA polymerases, general transcription factors, chromatin modifiers, AP2 transcription factors, and TF-like Zn fingers throughout the cell cycle in a synchronized tachyzoite culture. **(B)** depicts spline model curves for specific cell cycle-regulated AP2 mRNAs, indicating their relative mRNA abundance and timing. The expression of these selected factors occurs sequentially, with peaks at various stages of the cell cycle.

6.3.4.1. AP2 Transcription factors in bradyzoites and other life stages

T. gondii encodes 67 putative ApiAP2 TFs, with 11 specific to the bradyzoite stage, which is essential for establishing chronic infection in the host (Behnke et al., 2010). The transition from tachyzoites to bradyzoites is triggered by a Myb family transcription factor, bradyzoite formation-deficient 1 (BFD1), which acts as a master regulator (Waldman et al.,2020). Subsequently, AP2 factors play sequential roles in this process (Figure 20). One of the earliest characterized ApiAP2 TFs in bradyzoites is TgAP2XI-4, which peaks during the G1/S phase and is crucial for regulating bradyzoite-specific genes. Knockout studies have shown that TgAP2XI-4 deficiency leads to significant downregulation of bradyzoite-specific genes and a reduced cyst burden in mice, highlighting its importance in the bradyzoite stage (Walker et al., 2013). Another significant ApiAP2 TF, TgAP2IX-9, acts as a repressor of bradyzoite differentiation. Overexpression of TgAP2IX-9 reduces cyst formation under stress conditions, while its depletion promotes cyst formation, indicating its role in preventing premature bradyzoite development. TgAP2IX-9 binds to promoters of key bradyzoite-specific genes such as TgBAG1 and TgB-NTPase, further underscoring its regulatory role (Radke et al., 2013). Conversely, TgAP2IV-3 functions as an activator of bradyzoite differentiation, directly binding to and upregulating the expression of bradyzoite-specific genes like TgBAG1. This interplay between TgAP2IV-3 and TgAP2IX-9 illustrates the complex regulatory network governing bradyzoite differentiation (Hong et al., 2017). The upregulation of BFD1, BFD2/ROCY1, and certain AP2 factors is associated with increased permissiveness for bradyzoite formation. This suggests these factors work together to drive the tachyzoite-to-bradyzoite transition.(Xia et al., 2024)

Recent studies have expanded our understanding of these transcription factors. For instance, the repression of bradyzoite differentiation by TgAP2IV-4 and TgAP2IX-4, both expressed during the S/M phase of the tachyzoite cell cycle, has been linked to their role in maintaining tachyzoite identity. The loss of these factors leads to the inappropriate expression of bradyzoite-specific genes and a failure to establish chronic infection in mice (Radke et al., 2018; Huang et al., 2017). Moreover, single-cell RNA sequencing (scRNA-seq) analyses have identified several other ApiAP2 TFs, such as TgAP2Ib-1 and TgAP2IX-1, which are overexpressed in clusters enriched for bradyzoite-specific genes, suggesting their involvement in the transition to the bradyzoite stage (Xue et al., 2020).
Further research using RNA sequencing (RNA-seq) on in vitro brain cell cultures infected with *T. gondii* has revealed that the expression profiles of these TFs vary significantly during bradyzoite differentiation. Notably, two distinct clusters of bradyzoite genes have been identified: one that includes TgAP2IX-9, expressed early in differentiation, and another that includes TgAP2XI-4, expressed later, indicating a sequential regulation of bradyzoite gene expression (Mouveaux et al., 2021).

Interestingly, TgAP2IX-4, while not essential for tachyzoite growth, plays a role in regulating bradyzoite-specific genes under stress conditions. The knockout of TgAP2IX-4 leads to enhanced expression of bradyzoite genes and a decrease in tissue cyst formation, indicating its role in preventing premature differentiation into bradyzoites (Huang et al., 2017). Recent findings have shown that TgAP2IX-4 interacts with other AP2 TFs, such as TgAP2XII-2, and components of the MORC complex, suggesting its involvement in transcriptional repression through the recruitment of MORC (Farhat et al., 2020; Srivastava et al., 2020).

A significant breakthrough came in 2024 when Wang et al. and Antunes et al. demonstrated that AP2XI-2 and AP2XII-1 act as negative regulators, suppressing merozoite-primed pre-sexual commitment during asexual development. They showed that depletion of AP2XI-2 in the type II Pru strain induced merogony and the production of mature merozoites in an alkaline medium. AP2XII-1 depletion led to multiple rounds of merogony and merozoite production, with stronger effects in an alkaline environment. This study sheds light on the molecular mechanisms controlling sexual commitment in *T. gondii*, a key factor in the parasite's transmission.

This builds on earlier research, including a 2022 study by Farhat et al., which highlighted the role of AP2 factors in the epigenetic rewiring of *T. gondii* during its developmental transitions. AP2 factors were shown to coordinate with epigenetic regulators to control gene expression during stage changes. In 2023, Kourosh et al. underscored how AP2 factors act as both activators and repressors within the parasite's cell cycle, often working in complex with other proteins for precise gene regulation. Another 2023 study by Srivastava et al. identified AP2XII-2 as crucial for repressing sexual stage genes. Through CUT&Tag analysis, they revealed that AP2XII-2, along with epigenetic regulators HDAC3 and MORC, targets genomic loci linked to sexually committed parasites. AP2X-10 and AAH1 were identified as key genes under AP2XII-2 control.

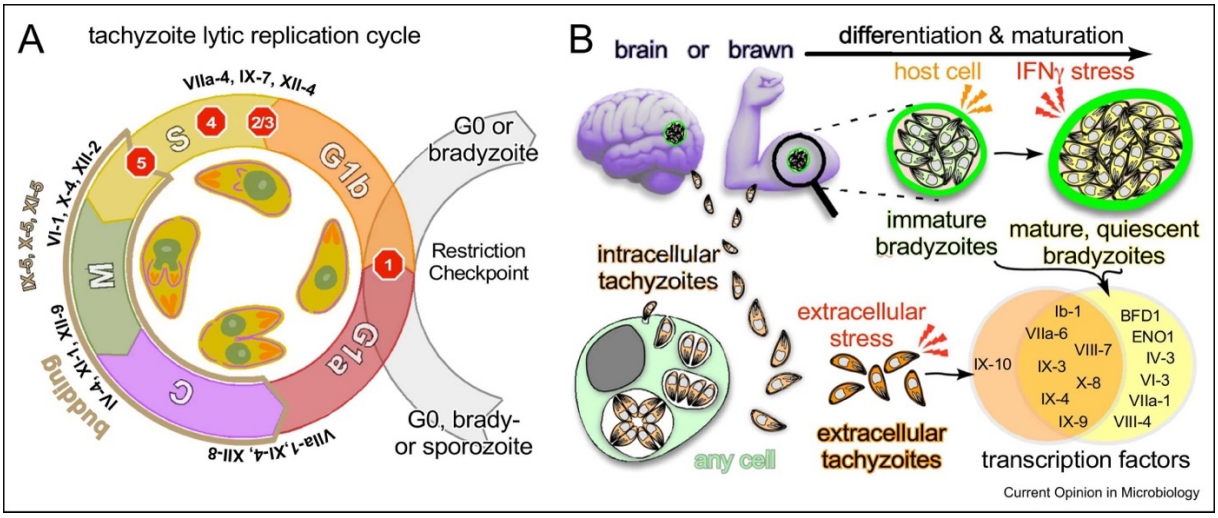

Figure 20 - Schematic representation of the AP2 transcription factors involved in the lytic replication cycle of *Toxoplasma gondii* tachyzoites and their role in stress responses and stage differentiation (Zarringhalam et al., 2023)**. (A)** Depicts the tachyzoite cell cycle with key checkpoints: 1) restriction, 2) DNA licensing, 3) centrosome duplication, 4) spindle assembly, and 5) daughter cell assembly. The restriction checkpoint is crucial as it determines whether the tachyzoites proceed to the extracellular G0 stage or differentiate into other stages. The specific AP2 factors that have been experimentally identified or validated are indicated at the corresponding stages where they function. **(B)** Illustrates the role of AP2 transcription factors in the shared stress response between extracellular tachyzoites and differentiating bradyzoites, highlighted by the lightning bolts. Additional transcription factors, such as BFD1 and enolase 1 (ENO1), are also present in bradyzoites, indicating their unique roles in this stage.

6.3.4.2. AP2 Transcription factors in Tachyzoites

Eleven ApiAP2 transcription factors (TFs) are known to function in *Toxoplasma gondii* tachyzoites, with three shown to form complexes with chromatin remodeling enzymes. TgAP2IX-7 and TgAP2X-8 interact with TgGCN5b, a lysine acetyltransferase (KAT) responsible for acetylating histone H3 at specific lysine residues (J. Wang et al., 2014). A dominant-negative mutant of TgGCN5b disrupts parasite replication and histone acetylation, decreasing the expression of target genes. These findings suggest that ApiAP2 TFs help recruit TgGCN5b to promoters, regulating gene expression since TgGCN5b lacks DNA-binding motifs (J. Wang et al., 2014).

In a more detailed investigation, several ApiAP2 TFs were confirmed to interact with TgGCN5b (Harris et al., 2019). TgAP2IX-7 was shown to associate with TgGCN5b, TgADA2a, TgAP2X-8, and a few other proteins. Similar interactions were also demonstrated for TgAP2XII-4 and TgAP2VIIa-5 (Harris et al., 2019). Interestingly, the association between TgGCN5b and AP2 TFs diminishes under alkaline stress, suggesting that specific ApiAP2 TFs likely help recruit TgGCN5b to gene regulatory complexes (Harris et al., 2019). Additionally, TgAP2VIII-4, originally identified as part of the *T. gondii* repressor complex (TgCRC), interacts with TgHDAC3 to repress transcription by activating the histone deacetylase (Saksouk et al., 2005).

TgAP2XI-5, an ApiAP2 transcription factor that is continuously expressed during the tachyzoite stage, plays a significant role in controlling virulence genes, including those associated with rhoptry and microneme functions (Walker et al., 2013). Through ChIP-on-chip assays, TgAP2XI-5 was shown to bind to hundreds of gene promoters, many of which are linked to parasite virulence and host invasion. RSAT computational analysis identified GCTAGC as the DNA motif to which TgAP2XI-5 binds (Walker et al., 2013).

Moreover, co-immunoprecipitation experiments confirmed that TgAP2XI-5 forms a complex with TgAP2X-5, a transcription factor predominantly expressed during the S/M phases of the cell cycle (Lesage et al., 2018). Depleting TgAP2X-5 caused downregulation of 153 genes and upregulation of 70 genes, with many of the differentially expressed genes peaking in the S/M phases. These included several

virulence genes, and depletion of TgAP2X-5 resulted in a marked reduction in virulence in vivo. However, ChIP-chip analysis of TgAP2X-5-tagged strains showed that TgAP2X-5 does not bind directly to promoters. In contrast, experiments assessing TgAP2XI-5's binding to promoters in the absence of TgAP2X-5 showed reduced binding at 92 promoters, suggesting that TgAP2XI-5's association with some promoters depends on TgAP2X-5 (Lesage et al., 2018). In the presence of TgAP2X-5, TgAP2XI-5 binds to promoters of virulence genes that are downregulated when TgAP2X-5 is depleted. These findings demonstrate that TgAP2XI-5 and TgAP2X-5 cooperate in regulating the expression of virulence factors in *T.gondii* (Lesage et al., 2018). Further, recent research has also highlighted the role of TgAP2IX-1 in modulating the surface antigens of tachyzoites, a process crucial for immune evasion. This TF was shown to control the switch of surface antigens, including SAG1, and alter gene expression in a manner reminiscent of the sexual stage, pointing to its significant role in the parasite's adaptive strategies (Xue et al., 2020).

One of the most significant advances in the study of ApiAP2 TFs came with the identification of TgAP2IX-5, which has been characterized as a key regulator of the asexual cell cycle in tachyzoites. The study identifies TgAP2IX-5, a transcription factor in *T.gondii*, as essential for the parasite's growth, cell cycle regulation, and daughter cell formation (Khelifa et al., 2021). TgAP2IX-5 is dynamically expressed during the G1/S transition and early S phase, and its depletion through inducible knockdown (iKD) results in a severe defect in parasite proliferation, with an accumulation of multinucleated parasites and impaired daughter cell formation. The knockdown also disrupts organelle replication, particularly plastid division. RNA-seq analysis reveals that TgAP2IX-5 regulates the expression of over 900 genes, with many involved in cell-cycle-dependent processes and daughter cell formation, particularly those encoding components of the Inner Membrane Complex (IMC) and apical complex. ChIP-seq analysis shows that TgAP2IX-5 directly binds to the promoters of key genes for parasite replication. Furthermore, TgAP2IX-5 may regulate its own expression, indicating a potential feedback loop, and its depletion triggers the upregulation of genes related to bradyzoite development, suggesting a role in developmental choices. These findings highlight the critical function of TgAP2IX-5 in coordinating cell cycle progression and proliferation in *T. gondii*. (Khelifa et al., 2021; Wang et al., 2021).

TgAP2XII-8 was also described as another key player in tachyzoite gene regulation. This study from (Lou et al., 2024) combines single-cell transcriptomics (scRNA) and chromatin accessibility (scATAC) analyses to create detailed pseudo-timelines for gene regulation during the *T.gondii* lytic cycle. TgAP2XII-8 is expressed during the C/G1 phase. Mapping DNA motifs under ATAC peaks revealed novel and known patterns, such as the AP2XII-8 motif, which plays a key role in regulating G1 progression. It binds to a specific DNA motif ([T/C]GCATGCA) in the promoter regions of its target genes and acts an as activator for genes expressed in the G1 phase, with a particular focus on ribosomal protein genes. Additional proteins might interact with the AP2XII-8 motif in complex ways, affecting gene regulation. Moreover, some AP2XII-8 target genes remain inactive, suggesting co-regulation by other factors. The study also points to potential epigenetic mechanisms involving factors like MORC and HDAC3. Depletion of AP2XII-8 blocks G1-S progression and suggests other factors, such as AP2X-7, may contribute to this regulation.

AP2XII-9 is the most recent transcription factor to be characterized. TgAP2XII-9 plays a crucial role during the S/M phase of the parasite's cell cycle, regulating the expression of key genes involved in daughter cell formation and invasion machinery. The knockdown of TgAP2XII-9 results in significant defects in parasite growth, replication. Additionally, it controls the transcription of genes related to the inner membrane complex (IMC) and rhoptry proteins and micronemes, essential for cell division and host cell invasion. (Manuscript from this thesis is under revision; Shi et al., 2024)

In conclusion, AP2 TFs in *T. gondii* form a sophisticated regulatory network that controls gene expression throughout the parasite's complex life cycle. Their ability to interact with epigenetic modifiers and other regulatory proteins allows for precise control of developmental transitions and responses to environmental cues. Ongoing research continues to uncover the intricate mechanisms by which these factors orchestrate the parasite's gene expression programs.

6.4. Epigenetic mechanisms in *Toxoplasma*

T. gondii possesses a wealth of epigenetic machinery, including histone-modifying enzymes and unusual histone variants. There are various mechanisms involved in epigenetic regulation: 1) mechanisms that affect the accessibility of chromatin to regulatory factors, which include DNA methylation, histone modifications, and nucleosome positioning; 2) mechanisms that involve noncoding RNA to influence several nuclear or cytoplasmic processes. Recent models of transcriptional activation have identified a greater number of cofactors than were recognized two decades ago, with chromatin remodelers playing a crucial role in enhancing gene transcription, thereby placing chromatin at the forefront of studies on epigenetic control of gene expression.

Toxoplasma gondii expresses four canonical histones (H2A, H2B, H3, and H4), each encoded by a single-copy gene, except for H2B, which is encoded by two distinct genes located on different chromosomes. However, these two genes do not produce identical proteins. H2Ba is predominantly expressed in the tachyzoite form, while H2Bb is associated with the sexual stages. The histones H2A and H2B in *T. gondii* share 79% and 75% similarity with their human counterparts, respectively. Like H4, histone H3 has a high sequence similarity to human histone 3, differing by eight amino acids in the N-terminal and globular domains, and it is one residue longer at the C-terminal end. The differences are mainly concentrated in the N-terminal and C-terminal regions for H2A and in the N-terminal region for H2B. These histones share a common structure, consisting of a globular body and an N-terminal tail for H2B, H3, and H4, and a C-terminal tail for H2A, which is exposed outside the nucleosome and subject to numerous post-translational modifications (PTMs), including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination, isomerization, crotonylation, and O-GlcNAcylation. Additionally, *T. gondii* possesses an H1 histone, a small basic protein corresponding only to the C-terminal part of the human H1 histone, recently identified as TgH1-like. This protein appears to bind to other histones and is involved in cell division. These post-translational modifications are highly dynamic and occur on various histones within a nucleosome in an interdependent manner, contributing to what Strahl and Allis termed the "histone code" in 2000.

6.4.1. Chromatin remodeling and modifications

Two primary mechanisms are employed to regulate chromatin structure by altering its level of compaction. One method involves chemical alterations through covalent modifications of histones, while the other utilizes ATP to reposition nucleosomes. Both of these chromatin-modifying strategies are conserved across eukaryotes and are also present in *Toxoplasma gondii*.wo primary mechanisms are employed to regulate chromatin structure by altering its level of compaction. One method involves chemical alterations through covalent modifications of histones, while the other utilizes ATP to reposition nucleosomes. Both of these chromatin-modifying strategies are conserved across eukaryotes and are also present in *Toxoplasma gondii*.

6.4.1.1. Enzymatic modifications of chromatin

In *Toxoplasma gondii*, the N-terminal tails of apicomplexan histones are subject to a variety of covalent modifications, including acetylation, methylation, succinylation, phosphorylation, ubiquitination, and SUMOylation (Braun et al., 2009; Dixon et al., 2010; El Bissati et al., 2016; Jeffers & Sullivan, 2012; Nardelli et al., 2013; Silmon de Monerri et al., 2015). These post-translational modifications form a "histone code" that plays a critical role in the activation and repression of specific genes (Jenuwein et al., 2001; Nardelli et al., 2013). Unlike other eukaryotes, *Toxoplasma* and *Cryptosporidium* lack DNA cytosine methylation (Gissot et al., 2008).

Acetylation of Histones

Histone acetylation in *Toxoplasma gondii* involves the addition of acetyl groups to lysine residues on the N-terminal tails of histones, which neutralizes their positive charge, leading to a more relaxed chromatin structure and activation of gene transcription. Conversely, the removal of acetyl groups, associated with transcriptional repression, is carried out by histone deacetylases (HDACs). In eukaryotes, various histone acetyltransferases (HATs) and HDACs regulate the acetylation status of histones within nucleosomes (Sterner & Berger, 2000; Thiagalingam et al., 2003). In *T. gondii*, there are about seven HATs and seven HDACs, now referred to as lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) due to their ability to modify non-histone substrates as

well (Allis et al., 2007; Jeffers & Sullivan, 2012). The KATs in *T. gondii* belong to two families: the GCN5 family and the MYST family. TgMYST-A and TgMYST-B are essential members of the MYST family, crucial for parasite survival (Smith et al., 2005), while TgGCN5a and TgGCN5b, members of the GCN5 family, preferentially acetylate specific lysine residues on histone H3 (Bhatti et al., 2006; Saksouk et al., 2005).

TgGCN5a plays a significant role in gene regulation, particularly during stress-induced bradyzoite development, as knockout studies show its absence prevents the upregulation of approximately 75% of bradyzoite-specific genes (Naguleswaran et al., 2010). Attempts to knock out TgGCN5b have been unsuccessful, indicating its essential role in the tachyzoite stage (Wang et al., 2014). These GCN5 KATs are potential drug targets, as demonstrated by the inhibitory effects of garcinol on TgGCN5b, which severely impacts parasite survival (Jeffers et al., 2016).

T. gondii also harbors around seven KDACs categorized into three classes, with TgHDAC3 being the most studied. TgHDAC3, part of the TgCRC complex, is involved in histone deacetylation and can be inhibited by specific HDAC inhibitors (Saksouk et al., 2005). Treatment with the FR235222 inhibitor leads to hyperacetylation of histone H4, promoting bradyzoite differentiation (Bougdour et al., 2009). TgHDAC3, along with TgMORC, plays a critical role in gene silencing during the tachyzoite stage, as shown by their co-purification and overlapping binding sites in the genome, particularly in hypoacetylated regions (Farhat et al., 2020).

Besides TgHDAC3, other type I HDACs are essential for tachyzoite replication, unlike type III HDACs (TgSIR2 and TgSIR2b), as revealed by genome-wide CRISPR/Cas9 screening (Sidik et al., 2016). Histone acetylation, particularly of H3 and H4, serves as a marker for gene activation, with these modifications being enriched at the promoter regions of actively transcribed genes (Gissot et al., 2007). Notably, tachyzoite-specific promoters are acetylated in tachyzoite-stage parasites, while bradyzoite-specific promoters become acetylated under bradyzoite-inducing conditions (Saksouk et al., 2005). The differential association of TgGCN5 and TgHDAC3 with stage-specific promoters further

highlights the role of chromatin-modifying enzymes in gene regulation through acetylation and deacetylation processes.

Methylation of Histones

Histone methylation involves adding methyl groups to lysine (K) and arginine (R) residues, which can either activate or repress gene transcription (Zhang et al., 2001). Lysine and arginine can be mono-, di-, or trimethylated, with methylation carried out by protein arginine methyltransferases (PRMTs) and histone lysine methyltransferases (KMTs). *Toxoplasma gondii* has five PRMT homologs, known as TgPRMT1-5. Two of these, TgPRMT1 and TgCARM1 (TgPRMT4), have been shown to methylate specific residues: TgPRMT1 targets H4R3, while TgCARM1 methylates H3R17 (Saksouk et al., 2005). TgPRMT1 also interacts with 68 potential substrates, including AP2 transcription factors and regulatory RNA-binding proteins (Yakubu et al., 2017).

TgSET8, a histone lysine methyltransferase related to human SET8, can mono-, di-, and tri-methylate H4 at lysine 20 (H4K20) and is primarily found in intergenic regions (Sautel et al., 2007). ChIP-on-chip data revealed TgSET8's binding to rRNA gene loci, where H4K20 is methylated, and its association with DNA repeats near telomeres. Additionally, H4K20me1 and H4K20me3 were enriched in heterochromatic regions, indicating that H3K9 and H4K20 methylation work together in chromatin silencing.

Another modification site, H4K31, located on the lateral surface of histone 4 at the nucleosome's dyad axis, has been studied in *T. gondii*. H4K31me1 is enriched in large gene bodies, while H4K31ac is found in intergenic regions, suggesting that H4K31me1 is primarily associated with gene bodies (Sindikubwabo et al., 2017).

The *T. gondii* genome also encodes seven demethylases from the JmjC family, though further research is needed to fully characterize these enzymes. A genome-wide CRISPR screen has indicated that two homologs of lysine dimethyl-transferases are important for tachyzoite fitness (Bougdour et al., 2014; Chang et al., 2007; Sidik et al., 2016).

6.4.1.2. ATPase-dependent chromatin remodeling

Chromatin remodeling through DNA-dependent ATPases is another key mechanism by which chromatin structure is altered. SWI/SNF2 family DNA-dependent ATPases form complexes that play crucial roles in gene activation or repression (Mohrmann & Verrijzer, 2005). The energy from ATP hydrolysis drives changes in nucleosome positioning and conformation by altering the interactions between histones and DNA (Lusser & Kadonaga, 2003). These ATPase-dependent remodeling complexes include SWI/SNF2 family members, which are defined by a unique ATPase domain featuring a DEXDc region at the N-terminal end and a HELICc region at the C-terminal end.

The SWI/SNF2 family is divided into four classes based on sequence homology and structural features: Snf2, which contains bromodomains; ISWI, characterized by a SANT domain; Mi-2, which includes a chromodomain; and the Ino80/SRCAP/p400 class, distinguished by a long insert between the N-terminal and C-terminal regions (Kingston et al., 1999). In Apicomplexans, including *Toxoplasma gondii*, SWI/SNF2 family members are present, such as the ISWI homolog SNF2L in *Plasmodium* (Ji & Arnot, 1997). Additionally, a SCRAP homolog exists in the genomes of *Toxoplasma*, *Plasmodium*, and *Cryptosporidium* (Sullivan et al., 2003).

A yeast two-hybrid screen using TgSRCAP as "bait" in *T. gondii* identified interacting proteins, most of which are parasite-specific with no eukaryotic homologs, likely involved in DNA processes like transcription (Nallani & Sullivan, 2005). *Toxoplasma* also has 15 other SWI2/SNF2 homologs, with at least one member representing each of the four classes. Among them is a predicted SWI2/SNF2 family member with a chromodomain, part of the TgCRC complex, likely an ortholog of Mi-2 (Saksouk et al., 2005). Notably, *T. gondii* has two SWI2/SNF2 homologs related to Snf2, one of which contains a bromodomain and has unique features specific to the parasite, making it a potential drug target (Jeffers et al., 2017).

7. Other mechanisms in cell cycle regulation

7.1. Roles of different Phosphatases

Phosphorylation is a key regulatory mechanism in *T.gondii* cell cycle control, influencing various proteins involved in crucial processes like DNA replication, mitosis, and organelle division. Cyclin-dependent kinases (CDKs), transcription factors, and chromatin modifiers rely on phosphorylation to regulate gene expression and ensure proper progression through cell cycle phases as discussed in previous chapters. Additionally, phosphorylation controls the function of unique *T. gondii* structures like the centrocone, as well as signal transduction pathways that respond to environmental cues. These phosphorylation events are finely tuned by the counteracting action of protein phosphatases, which remove phosphate groups, restoring proteins to their inactive or altered states. Protein phosphatases play a critical role in balancing phosphorylation-dependent processes, ensuring precise regulation of the cell cycle, stress responses, and parasite development.

Protein phosphatases are categorized based on the amino acid they target into three main types: serine/threonine phosphatases, tyrosine phosphatases, and dual-specificity phosphatases, which target all three residues. Protein serine/threonine phosphatases (PSPs) are further divided into three families: phosphoprotein phosphatases (PPPs), protein phosphatases that depend on Mg2+/Mn2+ ions (PPMs), and aspartate-based phosphatases (Shi et al, 2009). The PPP and PPM families have been widely researched in eukaryotic systems. In apicomplexans, the organisms *Plasmodium falciparum* and *Toxoplasma gondii* have been found to possess PPP family members like PP1 and PP2A, as well as PPM members such as PPM2 and PPM5, which play significant roles in the sexual stages of *P. falciparum* and ookinete development, respectively (Guttery et al., 2014). However, there is limited information on aspartate-based phosphatases, which include FIIF-associating carboxyl-terminal domain (FCP) phosphatases and small CTD phosphatases, collectively referred to as the FCP/SCP family (C. Yang & Arrizabalaga, 2017). Additionally, apicomplexans contain phosphatases with distinct Kelch-like domains (PPKL), which are also present

in plants and alveolates, and Shewanella-like phosphatases (SLP), a group found exclusively in bacteria (Kutuzov & Andreeva, 2008).

The PPP family includes PP1, PP2A, PP2B (calcineurin), PP4, PP5, PP6, and PP7, with most members having a catalytic subunit that interacts with various substrates. In contrast, PPM family members lack regulatory subunits and rely on conserved motifs for specificity. In *P. falciparum* and *T. gondii*, all PPP subfamilies are present, except for PP2A, which has two isoforms in *T. gondii* (Yang & Arrizabalaga, 2017). However, *C. parvum* lacks PP6 and PP7, while *B. bovis* lacks PP2B and PP6. PPP proteins are conserved across species, with overlapping functions preventing lethality in eukaryotes (Brautigan & Shenolikar, 2018).

PP2A is critical in cell cycle regulation, controlling transitions such as G2/M and G1/S, and disruption leads to cell cycle delays (Jiang, 2006). Although PP2A plays key roles in mammals, its specific function in *T. gondii* remains unclear, and *T. gondii* also lacks a Cdc25 phosphatase ortholog, essential for CDK regulation (Shen & Huang, 2012).

The PPM family of metal-dependent protein phosphatases, which bind either Mn2+ or Mg2+, is highly conserved across both eukaryotes and prokaryotes (Chen et al., 2017; Kamada et al., 2020). PPM phosphatases have diversified evolutionarily, leading to species-specific functional specialization. For instance, yeast has eight PPM isoforms, while mammals have 20. Phylogenetic studies show that most conserved residues critical for phosphatase function are located in the catalytic core (Kamada et al., 2020).

In apicomplexans, PPM family members are present in varying numbers: 13 in *P. falciparum*, 33 in *T. gondii*, 14 in *C. parvum*, and 4 in *B. bovis*. These PPMs cluster into 10 main groups, with Groups II and X being particularly significant. Group II likely includes PDPs, while Group X contains PfPPM3 and homologs of TgPPM3, which may be targeted to the parasitophorous vacuole (PV) or host cell (Yang & Arrizabalaga, 2017).

In *P. falciparum*, PPM2 and PPM5 have regulatory roles in translation and developmental processes. Knockout studies of PfPPM2 showed defects in gamete formation and ookinete development, while PfPPM5 knockouts resulted in abnormal ookinete size and number (Guttery et al., 2014). In *T. gondii*, TgPPM13 regulates actin

dynamics through its interaction with CKII, influencing the parasite's motility (Delorme et al., 2003; Jan et al., 2007). TgPPM20, involved in parasite growth, and TgPPM3C, which aids in GRA16 export, are essential for parasite virulence (Gilbert et al., 2007; Mayoral et al., 2020).

Aspartate-based phosphatases differ from the PPP and PPM families by their use of aspartate for catalysis. Most aspartate-based phosphatases contain a conserved catalytic motif, DxDT/V. Humans have eight of these phosphatases, while yeast has five. In apicomplexans, *C. parvum* has five putative aspartate-based phosphatases, *T. gondii* has eight, *P. falciparum* has four, and *B. bovis* has six. The most studied member of this family is FCP1 (also known as CTDP1), which plays a key role in dephosphorylating the CTD of RNA polymerase II's large subunit. The CTD region, with a consensus sequence of YSPTSPS, has residues that can be phosphorylated, except for the two prolines (Stiller & Cook, 2004; Yang & Stiller, 2014). In yeast, CTD has been linked to gene expression and serves as a docking platform, though its role in RNA polymerase activity in apicomplexans is still unclear (Corden, 2013).

Apicomplexans have two orthologs of FCP1, unlike humans and yeast, which have only one. In *T. gondii*, the CTD region is unusual, with only two tandemly repeated heptapeptides, while the remaining eight are scattered. This suggests that the scattered heptapeptides might not perform the typical CTD functions, which are usually based on heptapeptide pairs (Stiller & Cook, 2004).

7.2.1. Protein phosphatase-1 (PP1) in Eukaryotic cell & *Toxoplasma*

Protein phosphatase 1 (PP1), a key member of the PPP family, was first identified for its role in dephosphorylating glycogen phosphorylase (Cori & Cori, 1945). Since then, PP1 has been shown to regulate crucial cellular processes such as division, metabolism, transcription, translation, and apoptosis (Ceulemans & Bollen, 2004). Structurally, PP1 is a holoenzyme consisting of a catalytic and regulatory subunit. The catalytic subunit is highly conserved and coordinates metal ions like Mn2+ and Fe2+ to function (Shi, 2009). Despite the existence of multiple PP1 isoforms in eukaryotes, humans possess three distinct PP1 isoforms (PP1-A, PP1-B, and PP1-C) coded by different genes, while yeast has only one isoform (Rebelo et al., 2015).

PP1 is essential for cell viability, and disruptions in its interactions with regulatory proteins (PIPs) can lead to various diseases (Ferreira et al., 2019). In *Toxoplasma gondii*, PP1 plays an important role during the parasite's invasion of host cells. Inhibitors like okadaic acid (OA) and tautomycin (TAU) reduce *T. gondii* invasion by 50%, highlighting PP1's involvement in this process (Delorme et al., 2002). Phosphatase assays confirmed PP1 activity in *T. gondii*, and immunoprecipitation studies showed PP1 interacting with other proteins during dephosphorylation events (Delorme et al., 2002).

Localization studies of TgPP1 in *T. gondii* revealed that it is found in the nucleus of tachyzoites and in the cytoplasm of bradyzoites. Overexpression of TgPP1 appears to impact parasite growth, as stable transgenic lines could not be maintained (Daher et al., 2007). TgPP1 forms a complex with TgLRR1, a homolog of the yeast sds22 protein, which likely inhibits PP1 activity. This interaction was confirmed through pull-down and immunoprecipitation assays (Daher et al., 2007).

Further studies on TgPP1 regulation demonstrated that Inhibitor-2 (I2) in *T. gondii* acts similarly to its homolog in *Plasmodium falciparum*, inhibiting PP1 activity via specific binding motifs (Deveuve et al., 2017).

A recent study by Khelifa and Bhaskaran et al. (2024) sheds light on the roles of PP1 in *Toxoplasma gondii*. The research revealed that TgPP1 primarily functions through post-translational modifications, with phosphorylation and dephosphorylation events governing key processes such as cell division and amylopectin regulation. TgPP1 is crucial for regulating the tachyzoite cell cycle, affecting the formation of the inner membrane complex (IMC), organelle segregation, and nuclear division. Depleting TgPP1 results in abnormal IMC formation, missegregation of organelles like the Golgi and plastid, and an unexpected accumulation of amylopectin, a polysaccharide typically found in the latent bradyzoite stage. The study attributes this amylopectin build-up to the dephosphorylation of proteins involved in its metabolism. Overall, the research underscores TgPP1's pleiotropic role in regulating both intracellular development and metabolic processes in *T. gondii*, with significant influence on cell division and amylopectin accumulation.

OBJECTIVES

Chapter 2 – Objectives

The cell cycle of *T. gondii* is tightly controlled and relies on both transcriptional and post-transcriptional regulatory mechanisms. The dynamic nature of transcriptional regulation throughout the tachyzoite cell cycle was demonstrated by analyzing the transcriptome of synchronized parasites expressing thymidine kinase (Behnke et al., 2010). This extensive study revealed that over 2,000 transcripts were regulated in a dynamic manner during the cell cycle, suggesting precise control over gene expression exerced at the transcriptional level.

Transcription factors (TFs) of the ApiAP2 family are essential regulators of gene expression *T. gondii*. These TFs coordinate complex processes such as cell division, development, and host-parasite interactions by binding to specific DNA sequences and controlling gene transcription. Among these, the role of TgAP2IX-5 has been of particular interest due to its critical function in regulating the parasite's cell cycle and proliferation.

Recent studies have demonstrated that TgAP2IX-5 is a cell-cycle-regulated transcription factor, expressed predominantly during the early S phase, which is essential for the growth and proliferation of *T. gondii*. Functional knockdown of TgAP2IX-5 results in severe defects in parasite daughter cell formation, impaired organelle division, and ultimately, a complete block in parasite replication. Through comprehensive RNA-seq and ChIP-seq analyses, it has been shown that TgAP2IX-5 directly regulates over 600 genes, many of which are crucial for the cell cycle, specifically those involved in the late S and M phases, including the Inner Membrane Complex (IMC) proteins and genes essential for daughter cell formation.

Notably, this study revealed that TgAP2IX-5 also exerts control over several other ApiAP2 TFs, including TgAP2XII-9 and TgAP2III-2, both of which are directly bound by TgAP2IX-5 at their promoters and downregulated upon TgAP2IX-5 depletion. TgAP2XII-9 arnd TgAP2III-2 are highly expressed during the late S phase. These findings suggest that TgAP2IX-5 acts as a master regulator, orchestrating a transcriptional network that includes other ApiAP2 TFs, thereby ensuring proper

progression through the cell cycle and controlling key developmental processes in *T. gondii*.

This thesis explored the functional roles of TgAP2XII-9 and TgAP2III-2, focusing on their regulation by TgAP2IX-5 and their contributions to cell cycle control. By investigating these transcription factors, we aim to shed light on the intricate regulatory networks that govern parasite development.

RESULTS

Chapter 3 – Results

3.1. Introduction

This chapter presents the findings from our study on the role of ApiAP2 transcription factors TgAP2XII-9 and TgAP2III-2 in the cell cycle regulation of the tachyzoite of *T. gondii*. The work focuses on how these factors influence daughter cell assembly and the expression of virulence genes during the S/M phase of the tachyzoite stage. We demonstrate the crucial role of TgAP2XII-9 in coordinating the proper formation of the inner membrane complex and organelle development, while TgAP2III-2 shows non-essential functions in this process.

Our data, which were obtained through transcriptomic analysis, phenotypic assays, and CUT&Tag experiments, underscore TgAP2XII-9's pivotal role in activating and repressing a set of genes that are vital for the completion of the daughter cell formation and microneme biogenesis. The results reveal the dual function of TgAP2XII-9 in the transcriptional regulation of genes necessary for cell cycle progression and its downstream impacts on parasite proliferation.

This work has been submitted to *PLOS Pathogens* and is currently under revision, with the final submission expected by mid-November 2024. To maintain consistency and reflect the precise structure of the submitted work, we have opted to present this chapter in the format of the manuscript as submitted.

Cascading expression of ApiAP2 transcription factors control daughter cell assembly in Toxoplasma gondii.

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ABSTRACT

Pathogenesis of Toxoplasma gondii in the intermediate host is based on the tachyzoite ability to divide rapidly to produce significant amount of daughter cells in a reduce time frame. The regulation of the cell-cycle specific expression program is therefore key to their proliferation. Transcriptional regulation has a crucial role in establishing this expression program and transcription factors regulate many aspects of tachyzoite cell cycle. We explored the role of two ApiAP2 transcription factors, TgAP2XII-9 and TgAP2III-2, during the cell cycle of the tachyzoite form. While TgAP2III-2 has only a minor impact on the tachyzoite proliferation, we show that TgAP2XII-9 regulates many aspects of the cell cycle including the proper assembly of the daughter cells inner membrane complex and temporal expression of many virulence genes. Creation of a double mutant strain for TgAP2XII-9 and TgAP2III-2 shows that TgAP2XII-9 had a prominent role during daughter cell assembly. Using transcriptomics and CUT&TAG, we demonstrate that TgAP2XII-9 mainly acts through the transcriptional control of at least 300 genes promoters. Interestingly, TgAP2XII-9 plays a crucial role repressing the expression of genes necessary for budding initiation and activating genes necessary for microneme de novo formation. We also explored the importance of the AP2 domain of TgAP2XII-9 demonstrating its critical role to exert its function. Therefore, we showed that TgAP2XII-9 is a crucial transcription factor which is key to daughter cell assembly post budding initiation.

INTRODUCTION

Toxoplasma gondii is a eukaryotic pathogen classified within the phylum Apicomplexa, which encompasses many protozoan parasites of significant medical and veterinary concern, including *Plasmodium* (the causative agent of malaria) and *Cryptosporidium* (responsible for cryptosporidiosis). *T.gondii* has garnered considerable attention as an opportunistic pathogen linked to encephalitis and systemic infections in immunocompromised individuals, particularly those with HIV/AIDS [1] . Approximately one-third of the world's population is estimated to be infected by Toxoplasma gondii. This parasite has the ability to cross the blood-brain barrier and establish a chronic infection by differentiating into a dormant, drug-resistant bradyzoite stage [2]. The rapidly growing tachyzoite form of the parasite is responsible for the clinical manifestations of the disease in humans. The tachyzoite's ability to proliferate is key to its pathogenesis. *Toxoplasma gondii* tachyzoites exhibit an unusual cell cycle characterized by three phases: G1, S, and closed Mitosis. Up until recently the G2 phase was thought to be notably short or absent [3].

(Hawkins, Wang, et al., 2024b) identified a G2 phase that is short and maybe overlapping with the S/M phase.Tachyzoites multiply asexually within the host cell through a process called endodyogeny where two daughter parasites form within the parent parasite. The G1 phase of *T.gondii* endodyogeny, when canonical housekeeping tasks preparing for the S phase occur, comprises about half of the division cycle. During the S phase, organelle duplication is coordinated with the formation of daughter cells in a process called budding [3]. Budding initiates early in the S-phase, marked by the formation of the initial cytoskeletal components, including the apical and basal poles, above the newly duplicated centrosomes [4–11]. The inner membrane complex (IMC) of the daughter cells is generated from the apical pole, beginning with the formation of the apical cap of the IMC, followed by the central IMC and basal IMC sub-compartments [12]. This process first encapsulates the divided Golgi [13] , followed by the apicoplast [14] and subsequently nucleus and endoplasmic reticulum [15]. As the daughter parasites mature, the maternal cytoskeleton disintegrates, and the maternal plasma membrane is repurposed onto the emerging daughters [9].

The tachyzoite cell cycle is regulated by both transcriptional and post-translational mechanisms. For instance, transcription factors (TFs) such as AP2X-5 and AP2XI-5 have been demonstrated to control the cell-cycle-dependent expression of virulence factors [16]. Additionally, cyclin-dependent kinases (Cdks) and cyclins play crucial roles in regulating budding and the overall cell cycle of tachyzoites [17–20]. The majority of transcriptional regulators of the tachyzoite cell cycle belong to the plant-like Arabidopsis APETALA-2 (AP-2) family [21,22]. The AP2 domain functions as a DNA-binding domain and can act cooperatively in regulating cell-cycle-dependent expression profiles [16]. The roles of some of the AP2 TFs have been uncovered. For instance, TgAP2XII-8 regulates ribosomal RNA production during the early G1 phase [23]. TgAP2IX-5 controls the initial steps of budding [24] by regulating the expression of hundreds of genes, including those coding for elements destined for early incorporation into the developing daughter buds, such as apical cap proteins TgAC2, TgAC7, and TgISP1 and budding markers such as TgIMC1, TgIMC3, TgIMC4, and TgIMC10. Additionally, TgAP2IX-5 directly controls the expression of other TFs. Notably TgAP2III-2, TgAP2XII-2, and TgAP2XII-9. TgAP2XII-2 is essential for the proper progression through S-phase [25] indicating that TgAP2IX-5 controls the expression of TFs that may be essential for the continuation of the cell cycle. The roles of TgAP2III-2 and TgAP2XII-9 remain unexplored.

In this study, we functionally characterized two cell-cycle-dependent ApiAP2 TFs, TgAP2XII-9 and TgAP2III-2, whose expression is directly regulated by TgAP2IX-5. While TgAP2III-2 had non-essential roles during the tachyzoite cell cycle, we demonstrate that TgAP2XII-9 is crucial for proper formation of daughter parasites after budding initiation. TgAP2XII-9 acts as a repressor of a subset rhoptries, rhoptry neck proteins, and IMC apical cap genes. Conversely, it activates the expression of a set of genes encoding for micronemes and dense granule proteins. Thus, TgAP2XII-9 is a critical TF that controls gene expression during the daughter cell completion and the de novo formation of virulence organelles.

RESULTS

TgAP2XII-9 and TgAP2III-2 are cell-cycle regulated and expressed during the S/M phase

Based on their CRISPR phenotype scores of -4.32 and -3.22 respectively, TgAP2XII-9 (TGME49_251740) and TgAP2III-2 (TGME49_253380) genes may contribute to fitness. To elucidate their role in regulating the cell cycle expression program of tachyzoites, we used an auxin-inducible degron (AID) system. This involved fusing the AID sequence and an HA-tag to the C-terminus of TgAP2XII-9 and TgAP2III-2 at their respective endogenous locus via a CRISPR/Cas9 strategy (supplementary fig. 1A). PCR confirmed the correct integration of the tag (Supplementary Fig. 1B(i) and 1B(ii)). The AID system allows conditional depletion of the protein upon adding Auxin to the parasite's growth medium. Western Blot analysis validated the system's functionality, showing a distinct band at the expected protein's size (218 kDa and 185 kDa) in the absence of auxin (Fig. 1A and 1B), with protein depletion occurring within 30 minutes of auxin addition.

Subsequently, immunofluorescence assays using cell cycle markers such as Centrin1 (marking the outer core of the centrosome) revealed that TgAP2XII-9 and TgAP2III-2 localize to the nucleus and are expressed during the late S and early M phase (Fig. 1C and 1D). Their expression is absent during the G1 phase before centrosome division and early S phase when centrosomes have divided but remain close to each other (Fig. 1C and D, first two panels). During the late S and M phases, when centrosomes migrate and when daughter cell formation (indicated by the budding marker IMC1) occurs, TgAP2XII-9 and TgAP2III-2 proteins are expressed (Fig. 1C and 1D, last two panels), indicating that their expression peaks after TgAP2IX-5 expression in good concordance with their transcript expression profiles [26].

TgAP2XII-9 is crucial for the growth and proliferation of tachyzoites *in vitro*

To functionally characterize TgAP2XII-9 and TgAP2III-2, we performed a standard growth assay to assess parasite replication within host cells 24 hours after adding auxin. We found that for TgAP2XII-9, the mean number of parasites per vacuole decreased to 2.8, while in the controls, the mean number exceeded 5 (Fig. 1E). This indicates a clear defect in parasite proliferation. Further phenotypic analyses revealed that the degradation of TgAP2XII-9 significantly impairs replication capacity in vitro. In a plaque assay, mutant parasites exposed to auxin failed to form any lysis plaques on a monolayer of host cells 7 days post-infection, unlike the parental strain under the same conditions (Fig. 1F). Quantifying plaque numbers in each strain (Fig. 1G), with and without auxin, also demonstrates that TgAP2XII-9 expression is crucial for the parasite's growth and proliferation *in vitro*. In contrast, the TgAP2III-2 iKD strain did not show any replication defects in the presence or absence of auxin and plaque assays showed a normal capacity to form plaques in the presence of auxin and after TgAP2III-2 depletion (Supplementary Fig 1C(i) and 1C(ii)). This indicates that TgAP2XII-9 is crucial for tachyzoite proliferation while TgAP2III-2 is dispensable.

TgAP2XII-9 is crucial for the proper formation of daughter parasites

To further explore the biological function of TgAP2XII-9, we conducted immunofluorescence assays (IFAs) to inspect the IMC formation (Fig. 2A) after a short 6-hour auxin treatment (representing the time needed for the parasite to complete one cell cycle). In the absence of TgAP2XII-9, the parasites displayed defects in IMC scaffold organization, resulting in the formation of disordered IMCs (Fig. 2A, lower panel). Conversely, TgAP2XII-9 parasites formed well-structured vacuoles without auxin (Fig. 2A, upper panel). Quantitative analysis of this phenotype revealed that approximately 80% of vacuoles contained disorganized IMCs after 6 hours of auxin treatment (Fig. 2B). Given the crucial role of the IMC in daughter cell formation, proper cellular content segregation, and daughter cell scaffold formation, we aimed to investigate the impact of TgAP2XII-9 on daughter cell development. Using TgIMC1 as a marker, we assessed the proportion of vacuoles undergoing budding following a 6-hour auxin exposure. It became evident that there was a notable decrease in the percentage of budding vacuoles, indicating the parasites' compromised ability to generate daughter cells correctly compared to mutant parasites cultured without auxin (see Fig. 2C and D(i)).

Additionally, the number of nuclei per parasite increased as a consequence of the inability of the daughter parasites to correctly bud, as depicted in Fig. 2C (lower panel, iKD TgAP2XII-9 + Auxin, within the enclosed circle). Quantitative analysis revealed a significant proportion of vacuoles with two or more nuclei per parasite in the presence of auxin (Fig. 2D(ii)), while this phenotype was nearly absent in the parental strain and the mutant strain in the absence of auxin. Taken together, these results suggest that TgAP2XII-9 is crucial for the proper formation and completion of the daughter cell scaffold.

TgAP2XII-9 exhibits no discernible effect on the initiation of daughter parasite budding.

To investigate the role of TgAP2XII-9 in the budding process, we used an early budding marker (TgISP1, apical cap) (Fig. 2E) as a proxy for the budding initiation. Quantification of budding vacuoles after 6 hours of auxin treatment revealed no significant difference in the proportion of vacuoles undergoing budding (Fig. 2F(i)). However, after overnight (~18 hours) auxin treatment, there was a slight decrease in budding vacuoles compared to mutant parasites without auxin (Fig. 2F(ii)). This reduction could be due to prolonged auxin exposure causing indirect effects rather than a direct result of TgAP2XII-9 depletion. Additionally, since TgIMC3 is an earlier budding marker than TgIMC1 but a later marker than TgISP1, we quantified budding parasites using TgIMC3. We observed a significant decrease in budding vacuoles after 6 hours of auxin treatment compared to parental and mutant strains without auxin (Fig. 2F(iii)) indicating that TgAP2XII-9 depletion does not affect budding initiation but rather elongation of IMC as early as the appearance of TgIMC3 on daughter buds.

To better visualize the defects in the daughter cell formation, we performed expansion microscopy (Fig. 2G). Although the parasite cytoskeleton is formed (as marked by acetylated-α-Tubulin), we confirmed the deformation of the IMC (Circled, Fig. 2G(ii)). As expected, we could identify parasites with an accumulation of multiple nuclei (Circled, Fig. 2G(ii)). We also noticed that there were daughter parasites that were formed without a nucleus (circled, Fig. 2G(iii)) and parasites that had no IMC or nucleus but with a well-formed cytoskeleton (arrowed, Fig 2G(iii)). There were also vacuoles with unorganized IMC and unsegregated nuclear material and abnormally formed

cytoskeleton (arrowed, Fig. 2G(iv)). These data indicate that TgAP2XII-9 has a profound influence on daughter cell formation and the coordination of this process.
We also examined the effect of TgAP2XII-9 depletion on organelle duplication and segregation. The Golgi complex and plastid were labelled in parasites (Supplementary Fig. 2A), and the Golgi-to-nucleus and plastid-to-nucleus ratios were calculated (Supplementary Fig. 2B(i) and 2B(ii)). We found no significant difference in these ratios between the presence and absence of auxin, as expected since TgAP2XII-9 expression peaks after both organelle divisions. Overall, these findings suggest that TgAP2XII-9 is not involved in initiating daughter parasite budding but exerts its effects later in the budding cycle.

TgAP2XII-9 and TgAP2III-2 have no combinatorial effect on the parasite biology *in vitro*

To explore the combinatorial effects of TgAP2XII-9 and TgAP2III-2 depletion, we generated a double mutant strain by knocking out TgAP2III-2 in our inducible knockdown (iKD) TgAP2XII-9 mutant strain. In this strain, in the absence of auxin, only TgAP2III-2 is depleted, while in the presence of auxin, both TgAP2XII-9 and TgAP2III-2 proteins are depleted. This strain is hereafter referred to as the Double Mutant. As anticipated, no plaques were observed in the Double Mutant strain in the presence of auxin (Supplementary Fig. 3A(i) and 3A(ii)), consistent with the essential nature of TgAP2XII-9 for the parasite.

Quantitative growth assays revealed no additive impact on parasite proliferation due to the simultaneous depletion of TgAP2III-2 and TgAP2XII-9 (Supplementary Fig. 3B). Moreover, the presence of auxin led to the accumulation of multiple nuclei in the Double Mutant, mirroring the phenotype observed in the iKD TgAP2XII-9 strain (Supplementary Fig. 3C and 3D). Further examination of the IMC defect phenotype, for which TgAP2XII-9 is critical, indicated no additional defects in the Double Mutant compared to the iKD TgAP2XII-9 strain (Supplementary Fig.3C). This was confirmed by the quantification of the percentage of vacuoles with IMC defects (Supplementary Fig.3E) which remained similar to that of iKD TgAP2XII-9 single mutant. These findings collectively indicate that depleting both TgAP2III-2 and TgAP2XII-9 does not result in an additive effect on parasite biology.

TgAP2XII-9 regulates the expression of various cell cycle-regulated genes

Given the potential role of TgAP2XII-9 as a transcription factor, we aimed to identify its regulated genes using RNA sequencing analysis. RNA sequencing was conducted at two time points: 2 hours post TgAP2XII-9 depletion to capture the genes immediately affected, and 6 hours post-depletion, corresponding to the completion of one tachyzoite cell cycle. Data analysis was performed using DESeq2 with an adjusted p-value cutoff of 0.01 and a minimum fold change of 2 (Fig. 3A, B). Significant transcriptomic changes were observed in the iKD TgAP2XII-9 mutant, revealing 1569 (Supplementary Table 1) and 1398 (Supplementary Table 2) differentially expressed genes (DEGs) after 2 and 6 hours of auxin treatment, respectively. A substantial number of genes were common between both datasets. Overlapping these datasets resulted in a final set of 1329 DEGs, with 567 transcripts upregulated (Fig. 3C, Supplementary Table 3) and 762 transcripts downregulated (Fig. 3D, Supplementary Table 4) following TgAP2XII-9 depletion. We analysed the cell cycle expression of the upregulated and downregulated genes, displaying their expression profiles using a heatmap (supplementary Fig. 4A and 4B). Most upregulated genes showed a peak expression during the late C and G1 phases, with a few peaking during the S phase (Supplementary Fig. 4A). The downregulated genes exhibited a heterogeneous expression pattern throughout the cell cycle, with peaks during the late S, M, and cytokinesis phases (supplementary Fig. 3B).

Upon analyzing the differential enrichment between upregulated and downregulated transcripts encoding proteins localized to specific organelles in the parasites, we identified a notable trend: a significant proportion of the upregulated transcripts encode proteins that localize to the Rhoptries (5.7% of upregulated transcripts in the HyperLOPIT dataset; Fig. 3E) compared to 0.9% of downregulated transcripts (Fig. 3E and 3G), the apical compartment (4.2% of upregulated transcripts in the HyperLOPIT dataset; Fig. 3E) compared to 1.8% of downregulated transcripts (Fig. 3G), and the IMCs (4.2% of upregulated transcripts in the HyperLOPIT dataset; Fig. 3E) compared to 1.8% of downregulated transcripts (Fig. 3G). Additionally, we observed that a significant proportion of downregulated transcripts encode proteins that localize to the dense granules (17.9% of downregulated transcripts in the HyperLOPIT dataset; Fig.

3G) compared to 4.5% of upregulated transcripts, and to the micronemes (8% of downregulated transcripts in the HyperLOPIT dataset; Fig. 3G) compared to 0.8% of upregulated transcripts. According to the HyperLOPIT dataset, the upregulated genes comprise 17% of the apical proteome (11/63), 14% of the rhoptry proteome (15/106), and 14% of the IMC proteome (11/81). In contrast, the downregulated genes represent 15% of the dense granule proteome (19/124) and 18% of the microneme proteome (9/51).

Examining the cell cycle expression of the upregulated transcripts associated with Rhoptries, Apical Caps, and IMCs (where data is available) revealed that most of these transcripts peak concurrently with TgAP2XII-9 expression (Fig. 3F). Conversely, the cell cycle expression of most of the downregulated transcripts associated with dense granules and micronemes peaked immediately after the expression of TgAP2XII-9 (Fig. 3H).

Collectively, these findings suggest that TgAP2XII-9 may function as an activator of the expression of a subset of dense granules and microneme genes, which exhibit a cell cycle-regulated expression peaking immediately after TgAP2XII-9 (Fig. 3F), and as a repressor of the expression of a subset of rhoptries, IMC, and apical genes (Fig. 3G), whose expression peaks earlier than that of TgAP2XII-9.

TgAP2XII-9 is enriched at the promoters of important *T.gondii genes*

Using RNA-seq, we identified that TgAP2XII-9 regulates gene expression either directly or indirectly. To pinpoint the specific promoters targeted by TgAP2XII-9, we conducted CUT & Tag analysis. Biological triplicates of endogenous HA-tagged TgAP2XII-9 were generated, along with a replicate of the RH-Tir1ΔKU80 strain, and subjected to sequencing. Significant peaks (p-value < 0.05) were identified using MACS2 software. Subsequently, ChIPSeeker annotated these peaks, revealing that 95% were located at authentic promoter sites for TgAP2XII-9 (Fig. 4A(i)) and 49% for RH-Tir1ΔKU80 (Fig. 4A(ii)). Across the three replicates, 2088 (Supplementary Table 5) peaks were exclusive to TgAP2XII-9 and absent in RH-Tir1ΔKU80. In line with its hypothesized function as a transcriptional regulator, TgAP2XII-9 is found proximal to the transcription start sites of protein-coding genes (Fig. 4B).

Analysis of cell cycle expression patterns revealed that genes with TgAP2XII-9-bound promoters predominantly exhibited peak expression during S and M phases (Fig. 4D) in line with its expression pattern. CUT & Tag data demonstrated that TgAP2XII-9 binds to promoters of key genes encoding proteins crucial of the IMC, apical complex, rhoptries, micronemes, and dense granules (Figure 4D). Specifically, promoters of genes bound by TgAP2XII-9 include genes encoding for IMC proteins (e.g., TgISP3, TgIMC42; Fig. 4D(i)), ROP proteins (e.g., TgRON5, TgRON8; Fig. 4D(ii)), and MIC proteins (e.g., TgMIC9, TgMIC3; Fig. 4D(iii)). These findings underscore TgAP2XII-9's role as a genuine TF by directly interacting with promoters of genes essential for daughter cell formation.

Since RNA-seq alone cannot identify genes directly regulated by TgAP2IX-5, we integrated RNA-seq with CUT & Tag datasets. We identified differentially expressed genes (both upregulated and downregulated) from the RNA-seq data and compared them with genes targeted by TgAP2XII-9 based on CUT & Tag analysis. This comparison revealed an overlap of 300 genes (Fig. 5A, Supplementary Table 6). Detailed analysis showed that 31% of the upregulated genes and 16% of the downregulated genes are directly targeted by TgAP2XII-9, indicating that TgAP2XII-9 regulates genes mostly expressed during the S and M phases and some expressed during the cytokinesis phase (Fig. 5B). Given TgAP2IX-5's role in activating genes involved in daughter parasite formation, we examined genes regulated by both TgAP2XII-9 and TgAP2IX-5. Out of the 300 genes directly targeted and regulated by TgAP2XII-9, only 18 were also targeted by TgAP2IX-5 (Fig. 5C). Nearly all these genes are initially activated by TgAP2IX-5 and later repressed by TgAP2XII-9, including TgAP2XII-9 itself. These findings suggest that TgAP2XII-9 regulates a distinct set of genes and acts downstream of TgAP2IX-5.

We also looked at genes that are coregulated by TgAP2XI-5 and TgAP2XII-9. We overlapped the ChIP-on-chip data of TgAP2XI-5 [27] and identified 80 genes that were also bound by TgAP2XI-5 at their promoters (Fig. 5D), in line with previous results showing that TgAP2XI-5 binds to promoter of genes preferentially expressed during the S and M phase.

Since TgAP2XII-9 and TgAP2XII-2 share a similar transcriptomic profile and are activated by TgAP2IX-5, we investigated whether TgAP2XII-2 also targets the direct targets of TgAP2XII-9. To this end, we compared our data with the CUT&Tag data of

TgAP2XII-2 [28]. Remarkably, out of the 300 direct targets of TgAP2XII-9, 242 were also bound by TgAP2XII-2 at their promoters (Fig. 5E). However, the expression of most of these genes did not vary after TgAP2XII-2 depletion [28]. Given that TgAP2XII-2 is known to interact with the MORC/HDAC3 complex [28], we examined whether these 242 genes are also targets of MORC. We compared this data with the ChIP-seq data of MORC [29]. Only 25 genes were bound by MORC at their promoters (Fig. 5E), and these genes did not belong to any specific gene set. Notably, two AP2 transcription factors (AP2Ib-1 and AP2IV-3), involved in bradyzoite-specific gene expression, were present. This data indicate that TgAP2XII-2 has a different biological function than AP2XII-9 although they bind to similar promoters.

When examining the 25 genes whose promoter is bound by TgAP2XII-2, TgAP2XII-9 and MORC, we found that most of them had a peak expression during the M and Cytokinesis phases (supplementary Fig. 5A). All these 25 genes were highly expressed throughout the sexual stages with their expression peaking at EES5 and tissue cysts (Supplementary Fig.5B).

We also investigated other ApiAP2 TFs potentially regulated by TgAP2XII-9 and found that 7 ApiAP2 TFs were bound and regulated by TgAP2XII-9. Of these, four AP2s (TgAP2IV-4, TgAP2IX-8, TgAP2IX-9, TgAP2XI-2) were upregulated when TgAP2XII-9 was depleted, and 2 AP2s (TgAP2IV-3, TgAP2Ib-1) were downregulated. Analysis of the cell cycle expression of these AP2s suggests that most peak during the late C and early G1 phases, except for TgAP2IV-4, which peaks during the S/M phase along with TgAP2XII-9 (Fig. 6A). To investigate the link between TgAP2XII-9 and differentiation, we examined the expression profile of upregulated and downregulated genes during the parasite life cycle (Fig. 6B). Interestingly, downregulated genes in the absence of TgAP2XII-9 are preferentially expressed in tachyzoite but also bradyzoites and sexual stages (Fig. 6C), while TgAP2XII-9 depletion induced the overexpression of mostly tachyzoite-specific genes (Fig. 6B). These data indicate that AP2XII-9 may produce a permissive environment for expression of genes that preferentially expressed in bradyzoites and sexual stages.

Surprisingly, TgAP2XII-9 was enriched at its own promoter (Supplementary Fig.6A), and the TgAP2XII-9 transcript was upregulated in the presence of auxin based on RNA-seq. These data suggest that TgAP2XII-9 may directly regulate its own transcript expression, indicating a possible negative feedback loop.

Complementation restores TgAP2XII-9 phenotypes observed.

We created a complemented strain (iKD-C TgAP2XII-9) by inserting a myc-tagged version of the TgAP2XII-9 gene, driven by its own promoter, into an exogenous locus (*uprt*; Fig. 7A). The expression and localization of the exogenous TgAP2XII-9-myc in this strain were confirmed through IFA (Supplementary Fig 6B). We compared the percentage of parasites expressing the myc-tagged copy with those expressing the endogenous HA-tagged version. About 30% of the asynchronous parasite population expressed the myc-tagged gene in the complemented strain, similar to the parental iKD TgAP2XII-9 strain (Supplementary Fig. 6C).

To assess if the iKD TgAP2XII-9 strain phenotype could be rescued by ectopic expression of TgAP2XII-9, we measured the percentage of vacuoles with IMC defects in the iKD-C TgAP2XII-9 strain both in the absence and presence of auxin (Supplementary Fig. 6D). We found very few to no vacuoles with IMC defects, indicating that the IMC defect phenotype observed in the iKD TgAP2XII-9 strain was due to the lack of TgAP2XII-9 protein. Furthermore, plaque assays showed that the parasites could form lysis plaques even in the presence of auxin, similar to their behaviour in the absence of auxin (Fig. 7B). These results demonstrate that the TgAP2XII-9 protein is responsible for the phenotypes observed.

The AP2 domain of TgAP2XII-9 is crucial for its function.

We investigated the role of the AP2 domain in TgAP2XII-9 function. To this end, we used the complementation plasmid previously employed to create the iKD-C TgAP2XII-9 strain, and deleted the AP2 domain (Fig. 7A). This complemented strain was designated as iKD-C TgAP2XII-9 ΔAP2. The expression and the localization of the exogenous copy of iKD-C TgAP2XII-9 ΔAP2 was verified by immunofluorescence (Supplementary Fig. 6E). To examine the impact of deleting the AP2 domain, a plaque assay was conducted. The monolayer of HFF cells inoculated with iKD-C TgAP2XII-9 ΔAP2 parasites in the presence of auxin showed little to no lysis plaques, in contrast to the presence of lysis plaques in wells infected with iKD-C TgAP2XII-9 parasites (Fig. 7B and 7C). However, the co-expression of the endogenous TgAP2XII-9 protein and the exogenous AP2 deleted copy of the protein seems to have a deleterious effect on

parasite growth (Fig. 7C) since the number of lysis plaque was reduced in the iKD-C TgAP2XII-9 ΔAP2 strain in absence of auxin. We then focused on the notable phenotype of abnormal IMC defects and recorded the proportion of vacuoles exhibiting this phenotype (Fig. 7D). Surprisingly, only a small proportion of vacuoles exhibited the phenotype, showing that the TgAP2XII-9 ΔAP2 protein could partially complement the phenotype observed (Fig. 7E). Overall, these results indicate that the AP2 domain is crucial for the function of TgAP2XII-9 but not for the disordered IMC phenotype observed.

DISCUSSION

Proliferation is key to *T.gondii* pathogenesis in the intermediate hosts. The tachyzoite employs a unique, rapid replication method where daughter parasites are formed within a single mother cell (endodyogeny). However, how gene expression is regulated during this process is only partially understood.

Our study corroborates the findings of Shi et al. (2024) [30] regarding the essential role of TgAP2XII-9. However, since the data generated by Shi *et al.* is produced after 24-hour auxin treatment, a direct comparison between the phenotypes observed in their study or RNA-seq data is not feasible. By focusing on short auxin treatments (2h or 6h), our approach aimed to identify the direct, early consequences of TgAP2XII-9 depletion. When we compared our CUT & Tag data with the corresponding dataset from Shi et al., we found a significant overlap, with more than 50% of their identified targets also appearing in our data. This overlap reinforces the validity of our findings and underscores the critical role of TgAP2XII-9 in the parasite's biology.

In our study, we have characterized two cell cycle-regulated ApiAP2 TFs (TgAP2XII-9 and TgAP2III-2) that are predominantly expressed during the S/M phase of the tachyzoite cell cycle. While TgAP2III-2 has no measurable impact on the ability of the tachyzoite to grow, TgAP2XII-9 depletion resulted in significant defects in daughter bud formation and disorganization of the IMC. Both TFs were shown to be regulated by TgAP2IX-5 [24] and we hypothesized that they may be important for the continuation of the cell cycle. TgAP2IX-5 is a crucial TF regulating the initiation of budding and we expected both TgAP2XII-9 and TgAP2III-2 to regulate subsequent steps of the cell cycle. Depletion of TgAP2III-2 did not cause a defect in parasite proliferation. In

contrast, we show that TgAP2XII-9 is important for the formation of daughter cells but does not prevent the initiation of budding and the IMC apical cap formation (Figure 2). Instead, TgAP2XII-9 is important for IMC elongation and proper formation of the buds. Much like the erythrocytic cycle of *Plasmodium* [31] the identification of tachyzoite cell cycle-regulated expression profiles [26] suggests the presence of a cascade of TFs regulating this process.

This is the first evidence that the tachyzoite cell cycle-regulated expression program is controlled by a series of ApiAP2 TFs, cascading to implement the specific expression programs at each phase of the tachyzoite cell cycle. TgAP2IX-5 controls the expression of TgAP2XII-9 (also TgAP2XII-2 and TgAP2III-2) which in turn establishes the crucial expression profiles required for the progression of the cell cycle by coordinating the temporal expression of many transcripts.

TgAP2IX-5 controls genes that are essential for budding initiation, while TgAP2XII-9 seems important for the subsequent phases of daughter cell construction. When examining the genes that are directly controlled by TgAP2XII-9 (genes both present in the RNA-seq and CUT&Tag dataset), we noticed that genes encoding for ISP3 and Apical Annuli proteins AAP5 and AAMT were downregulated. These proteins are expressed after the IMC apical cap deposition and are present at the central IMC sub compartment (ISP3) or the apical annuli (AAP5 and AAMT). Interestingly, ISP3 maternal staining dissipates as daughter parasites form, indicating that ISP3 may be synthesized in daughters and degraded in mothers [32]. Our data confirm this hypothesis and suggest an active role of TgAP2XII-9 in this process. Apical annuli proteins may be inserted in the suture of the IMC plaques and therefore are needed once the apical and central IMC plaques are formed [33]. While TgAP2XII-9 seems to activate the expression of some IMC proteins, our analysis also detected IMC and basal complex genes that were directly repressed by TgAP2XII-9. Notably, the expression of transcripts encoding AC1 and IAP2, two apical cap proteins, and BCC3 and BCC7, two early markers of the basal complex [34,35] are directly repressed by AP2XII-9. These data indicate that TgAP2XII-9 seems to exert a dual activity of repressing the early budding markers (e.g apical cap and early basal complex component) and activating the expression of transcript encoding for proteins needed during IMC elongation. Overall, our data indicate that the production and assembly of each daughter cell IMC subcompartments correspond to a strictly controlled process

that involves the timely expression of IMC encoded transcripts and proteins in different temporal waves that are controlled at least by TgAP2IX-5 and TgAP2XII-9. Indeed, it has been hypothesized that the apical cap of the IMC is assembled in the apical direction while the central and basal compartments are in the basal direction [36].

Defects in daughter cell formation have downstream effects on other phenotypes such as nuclear segregation. For example, ILP1 overexpression, which predominantly localizes to budding daughters, leads to severely deformed cytoskeletons and abnormally large nuclei, suggesting a disruption in mitotic coordination similar to the phenotype that we see in AP2XII-9 depleted parasites [37]. Interestingly, ILP1 transcript is overexpressed in AP2XII-9 depleted parasites, recapitulating some of the phenotypes we observed.

Much like daughter cell IMC formation, the *de novo* production of rhoptries and micronemes are tightly regulated. Cell-cycle transcript expression profiles show that the temporal expression of rhoptries (early S phase) and micronemes (early M phase) are different [34]. Consistent with these gene expression patterns, we showed that transcripts encoding rhoptry proteins, which peak prior to TgAP2XII-9 expression are repressed by this TF, whereas expression of transcripts encoding microneme proteins is activated by TgAP2XII-9. TgAP2XI-5 and TgAP2X-5 were already shown to be important in regulating the expression of virulence factor genes, specifically as activators of rhoptry-encoded transcripts [16]. Our findings indicate that transcript expression profiles linked to the cell cycle, similar to those observed during differentiation, must be kept repressed until their expression becomes necessary. This regulatory pattern is particularly evident for transcripts encoding rhoptry and microneme proteins, which need to be expressed precisely when these organelles are formed *de novo*. Thus, TgAP2XII-9 is positioned as a repressor of a subset of rhoptry protein encoded transcripts and an activator of a subset of microneme protein encoded transcripts, contrasting with the roles of TgAP2XI-5 and TgAP2X-5, which primarily activate transcripts encoding rhoptry proteins.

Surprisingly, many downregulated genes coded for dense granule proteins, including MYR2, MYR3, GRA12B, GRA12D, GRA7, GRA8, GRA37, and GRA49. These GRAs, directly or indirectly regulated by TgAP2XII-9, are associated with the parasitophorous vacuole (PV) or the intravacuolar network (IVN) [38–42]. Although the expression of

GRA proteins is thought to be constitutive, the GRA protein-encoded transcripts that are regulated by TgAP2XII-9 seem to be cell-cycle regulated. This indicates that part of the PV and IVN formation is dependent on the cell cycle, suggesting a cross-talk between the tachyzoite cell cycle and the PV and IVN formation. Whether TgAP2XII-9 depletion affects the formation and stability of the IVN remains to be explored.

Upon TgAP2XII-9 depletion, some kinase transcripts were upregulated. Cyclin-related kinases like TgCRK1 and TgCRK6 are significantly dysregulated after TgAP2XII-9 depletion as suggested by our RNAseq data. Notably, the TgCRK6 promoter is also directly bound by TgAP2XII-9 and its transcript expression is being repressed. It is interesting to note that TgCRK6 interacts with TgAP2IX-5 and has similar temporal expression and localization to that of TgAP2IX-5 [43] It was also speculated that the role of TgCRK6 might be to inactivate TgAP2IX-5 just after budding has been initiated [43]. If in fact that is the case, TgAP2XII-9 (activated by AP2IX-5) might be repressing the gene expression of TgCrk6 when it is no longer needed, for example, after budding initiation. Overall, our data show that TgAP2XII-9 is acting as a crucial transcription factor at a turning point during the cell cycle when daughter cell buds are formed and microneme biogenesis must occur.

We identified that a majority of the genes that are downregulated in response to *TgAP2XII-9* knockdown are preferentially expressed in bradyzoites or sexual stages compared to tachyzoites (Fig. 6C). This is probably linked to the list of ApiAP2 TFs that are dysregulated after TgAP2XII-9 depletion. Nine AP2 transcription factors are significantly upregulated (AP2IV-4, AP2IX-8, AP2XI-2, AP2IX-9, AP2XII-9, AP2IV-2, AP2XI-3, AP2XI-4 and AP2III-2) and 4 are significantly downregulated (AP2IV-3, AP2Ib-1, AP2IX-3 and AP2IV-1). Of these TgAP2XII-9 binds to the promoters of AP2IV-4, AP2IX-8, AP2XI-2, AP2IX-9, AP2XII-9, AP2IV-3, and AP2Ib-1. TgAP2IV-4 and TgAP2IX-9 are known repressors of bradyzoite-specific genes [44,45], whereas TgAP2IV-3, TgAP2Ib-1 and TgAP2XI-4 are activators [46,47]. These data suggest that TgAP2XII-9 directly represses the transcripts of other AP2s, such as AP2IV-4 and AP2IX-9, which in turn may repress bradyzoite-specific gene expression, while it activates the expression of AP2IV-3 and AP2Ib-1, which stimulate the bradyzoite-specific gene expression. This is reminiscent of the data published on TgAP2IX-5 [24], which was shown to activate the expression of AP2IV-4 (a repressor of bradyzoite differentiation). These data reinforce the link between the cell cycle and differentiation

that was previously shown [48,49] where AP2 TFs important for differentiation are expressed at a specific point of the cell cycle (early M phase). At this specific point, by controlling the expression of a subset of AP2 TFs, TgAP2XII-9 might create a more permissive environment for the bradyzoite expression program and offer a possible getaway toward bradyzoite differentiation. In contrast, TgAP2IX-5, which acts to initiate budding, promotes the repression of the bradyzoite-specific expression program.

By the use of different complementation constructs, we examined the role of the AP2 domain in the function of TgAP2XII-9. As expected, the AP2 domain is critical for the essential function of TgAP2XII-9. However, when complementing the iKD strain using a construct deleted for the AP2 domain, some of the phenotypes, such as the disordered IMC phenotype, were partially complemented. This indicates that TgAP2XII-9 may exert some of this function through other parts of the proteins. ApiAP2 TFs are known to heterodimerize and cooperate to exert their function [16,50]. TgAP2XII-9 might therefore interact and cooperate with other proteins to regulate this phenotype independently from the presence of the AP2 domain.

Finally, we observed that TgAP2XII-9 binds to its own promoter and represses it which seems to be a typical characteristic of other AP2s. This feature was also shown for TgAP2IX-5 [24], TgAP2XI-5 [27], and TgAP2XII-2 [28]. This indicates that negative feedback loops are a common regulatory mechanism for these TFs and during the tachyzoite cell cycle, adding another layer of complexity to gene regulation in *T.gondii*.

In conclusion, we showed that TgAP2XII-9 plays a crucial role as a TF during daughter cell formation by activating genes that are required during the process of daughter cell IMC elongation and microneme *de novo* synthesis and repressing the expression of genes necessary during budding initiation.

MATERIALS & METHODS

Parasite culture, transfection, and purification

The tachyzoites from the RH ∆ku80 Tir1 strain of *Toxoplasma gondii* were grown in human foreskin fibroblasts (HFF) under controlled laboratory conditions, using Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1% penicillin-streptomycin. This particular strain, RH ∆ku80 Tir1, is recognized for its rapid proliferation due to the deletion of the ku80 gene, which promotes successful homologous recombination and transfection. Moreover, this strain produces the Tir1 protein, facilitating the regulated breakdown of labelled proteins upon the introduction of auxin to the culture medium. The cultivation process occurred in ventilated tissue culture flasks within a HERA cell VLOS 160i CO2 incubator (Thermo Scientific) maintained at 37 °C and 5% CO2. Transgenes were delivered through electroporation utilizing a BTX Harvard apparatus electroporator (ECM 630), and stable transformants were identified by growing them in media containing specific concentrations of mycophenolic acid (MPA)- 25 µg/ml, xanthine (50 µg/ml), pyrimethamine (2 µM), or FUDR (5 µg/ml). Clonal lines were isolated through a process of limited dilution. Before extracting total RNA, genomic DNA, or protein, intracellular parasites underwent purification via sequential syringe passage, first through a 17 gauge and then 26-gauge needles (Terumo AGANI needles) and filtration of the parasite through 3-µm polycarbonate membrane filter (Whatman).

Generation of transgenic *T.gondii* strains

The iKD TgAP2XII-9 strain was developed by utilizing the RH ∆ku80 Tir1 strain, in conjunction with a Cas9 plasmid engineered to target the gene's 3' end post the stop codon, and a PCR product containing the HA-AID cassette flanked by homology regions. The primer sequences utilized in this experiment are detailed in the Supplementary Table 7. To produce the iKD and ΔAP2 complementation line, a plasmid containing 3-kb upstream of the predicted ATG of the TgAP2XII-9 gene and the full-length or AP2 domain-deleted c-myc-tagged TgAP2XII-9 gene flanked by 2 kb homology fragments for the uprt gene was co-transfected with the pSAG1::Cas9-U6::sgUPRT plasmid in the iKD TgAP2XII-9 strain to ensure insertion into the UPRT locus. The parasites were then selected using 5 µM 5-fluoro-2'-deoxyruridine (FUDR).

To produce the double mutant strain, specifically a clean knockout of the TgAP2III-2 gene in the inducible knockdown (iKD) TgAP2XII-9 background, two gRNAs were designed to target the 5' and 3' ends of the TgAPIII-2 gene. This strategy facilitated the insertion of a DHFR selection cassette flanked by 35 base pair (bp) homology regions at both the 5' and 3' ends. The procedure involved transfecting the iKD TgAP2XII-9 parasite with two Cas9 plasmids, each targeting one end of the TgAPIII-2 gene.

Growth Assays

To assess growth, we introduced 8 x 10^4 parasites of both parental Tir1 and iKD AP2XII-9 mutant strains onto HFF cell monolayers cultivated on coverslips in a 24-well plate. This setup was maintained for 24 hours under conditions with and without 0.5mM auxin (AID/indoleacetic acid) in the medium. The purpose of incorporating auxin was to trigger the degradation of TgAP2XII-9 protein. Following 24-hour duration, infected coverslips were treated with 4% paraformaldehyde (PFA) for fixation. The fixed parasites were then subjected to staining using anti-TgEno2 to visualize parasite nuclei and anti-TgIMC1 antibodies to visualize the Inner Membrane Complex (IMC). The quantification involved counting the number of parasites per vacuole, with 100-200 vacuoles analyzed per biological replicate. Each growth assay experiment comprised three biological replicates.

Plaque Assay

Plaque assays were conducted by inoculating either 500 parasites of the Parental Tir1 strain or the iKD TgAP2XII-9/ TgAP2III-2 or the Double Mutant strain onto a monolayer of HFF cells cultivated in a 6-well plate, with the choice of normal media or media supplemented with 0.5mM auxin. The parasites were allowed to proliferate for 7 days before fixation with 100% ethanol. Plaques were visualized by staining with Crystal Violet. To assess plaque size under each experimental condition, an Excel macro was utilized for quantification.

Organelle labelling

The Parental Tir1 and iKD TgAP2XII-9, TgAP2III-2 parasites were cultured on HFF cell monolayers on coverslips within 24-well plates. They were grown in both regular media and media supplemented with auxin for either overnight or 6 hours. Subsequently, the parasites were fixed using 4% PFA and stained with antibodies. The nucleus was marked using anti-TgEno2, and the count of nuclei per parasite was conducted. For the labeling of the Inner Membrane Complex, both the parental and iKD TgAP2XII-9 strains were allowed to grow on HFF cells for 18-20 hours, followed by a 6- or 18-hour treatment with auxin. Intracellular parasites were then labelled using anti-TgISP1 and anti-TgIMC1 antibodies. The components of the centrosome were labelled after overnight growth of both the parental and iKD TgAP2XII-9 strains, followed by a 6-hour auxin treatment, using anti-TgCentrin1 and anti-TgChromo1 antibodies. Golgi and plastid labelling were performed after overnight growth of both the parental and iKD TgAP2XII-9 strains in auxin-containing media, using anti-TgSortilin and anti-TgACP antibodies, respectively.

Immunofluorescence assays (IFA)

Immunofluorescence experiments were conducted following the fixation of intracellular parasites cultivated on coverslips using 4% PFA for 30 minutes. Subsequently, the coverslips were washed three times with 1X PBS buffer. Permeabilization was achieved by incubating the samples for 30 minutes in a buffer composed of 1X PBS, 0.1% Triton 100X, 0.1% glycine, and 5% FBS. Following permeabilization, primary antibody incubation was performed for 1 hour, with the antibodies diluted in the same buffer used for permeabilization. Afterward, the coverslips containing the fixed intracellular parasites were washed three times with 1X PBS and incubated for 1 hour with DAPI and secondary antibodies conjugated to either Alexa-594 or Alexa-488. Following another three washes with 1X PBS buffer, the coverslips were mounted onto microscope slides using Moviol. Primary antibodies used included anti-TgIMC1 (a gift from Prof. Ward, University of Vermont), anti-TgEno2, anti-TgISP1, anti-TgCentrin1 (a gift from Prof. Gubbels, College of Boston), anti-TgACP (a gift from Pr. Striepen, U. Penn), anti-TgSortilin, and anti-HA (Sigma Aldrich), anti-myc (abcam), anti-TgIMC3(a gift from Prof. Gubbels, College of Boston) were used at the following dilutions: 1:500, 1:1000, 1:500, 1:500, 1:500, 1:500, 1:1000, 1:200 and 1:2000 respectively. Signal visualization involved manually counting 100-300 parasites for each replicate, with a

total of three replicates carried out for each experiment. Immunofluorescence assay experiments were visualized using the ZEISS LSM880 confocal microscope at 63X magnification, and image processing was conducted using CARL Zeiss Zen software.

Ultrastructure Expansion Microscopy (ExM) Procedure

Coverslips with HFF monolayers were inoculated with the iKD TgAP2XII-9 strain. The iKD strain was cultured either in normal media or media treated with auxin for 6 hours. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) and prepared for ultrastructure expansion microscopy (U-ExM) following previously described protocol [51]. Briefly, the coverslips were incubated for 5 hours in a 2× 1.4% acrylamide (AA)/2% formaldehyde (FA) mix at 37°C. Gelation was performed by incubating in a solution containing ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and a monomer mixture (19% sodium acrylate, 10% AA, and 0.1% bis-acrylamide in 10× PBS) for 1 hour at 37°C. The gels were then denatured at 95°C for 1.5 hours. Following denaturation, gels were incubated in double-distilled H2O (ddH2O) overnight to allow for expansion. The next day, gels were washed three times in PBS (10 minutes each) before incubation with primary antibodies for 3 hours at 37°C. After primary antibody incubation, gels were washed three times in PBS-Tween 0.1%, followed by incubation with secondary antibodies for 3 hours at 37°C. The gels were washed again three times in PBS-Tween 0.1% and then incubated in ddH2O for a second round of expansion before imaging. Confocal imaging was conducted using a ZEISS LSM880 Confocal Microscope at 63x magnification. Primary antibodies used were anti-TgIMC3 (a gift from Prof. Gubbels, Boston College) at a dilution of 1:1000, and acetylated α-tubulin (Santa Cruz Biotechnology) at a dilution of 1:200.

RNA sample preparation and extraction

RNA samples were prepared by infecting HFF cell monolayers in T175 flasks with iKD TgAP2XII-9 parasites for 24 hours, followed by a 2 or 6-hour treatment with auxin before collecting the samples and adding Trizol (Invitrogen). Control samples were cultured in regular media. RNA extraction was conducted according to the manufacturer's instructions, followed by removal of genomic DNA and purification using the RNase-free DNase I Amplification Grade Kit (Sigma). The quality of all RNA samples was assessed using an Agilent 2100 Bioanalyzer, with only samples having an integrity score of 8 or higher included in the RNA library preparation. 5 biological

replicates were generated for Auxin treated conditions and 3 biological replicates were generated for control conditions.

RNA Library Preparation and Validation

RNA libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina), following the manufacturer's protocol. Validation of the libraries was performed using DNA high-sensitivity chips on an Agilent 2100 Bioanalyzer. Quantification of the libraries was conducted using quantitative PCR (12K QuantStudio).

RNA-sequencing analysis

Bcl2fastq 2.17 (Illumina) was utilized for demultiplexing. The quality of the dataset was assessed using FastQC v0.11.8-0, while adapter treatment for sequencing was performed using Cutadapt v1.18. Trimmomatic v0.39 was employed to filter out reads shorter than 30 bp and those with low-quality bases. Following data cleaning, alignment against the *T.gondii* ME49 genome from ToxoDB was carried out using HiSAT2 v2.2.1. Gene expression quantification was performed on annotated genes using htseq-count from the HTseq suite v1.99.2. Differential gene expression analysis was conducted using DeSeq2 v1.22.1, with P-values adjusted using the Benjamin-Hochberg method. Gene expression exhibiting a fold change >2 or < -2 and an adjusted P value < 0.01 was deemed significantly differentially expressed.

Western Blotting

Western blot analysis was conducted by cultivating 2x10^6 parasites of the iKD TgAP2XII-9 strain in regular media overnight, followed by the addition of auxin for durations of 30 minutes, 1, 2, and 6 hours. Control samples were left to grow in normal media. Parasite samples were harvested by filtration and subsequent centrifugation. The resulting pellet was re-suspended in a loading buffer composed of 240 mM Tris-HCl pH 6.8, 8% SDS, 40% sucrose, 0.04% bromophenol blue, and 400 mM DTT. This was followed by denaturation through incubation of the parasite samples at 95°C for 10 minutes. Protein extracts were separated by electrophoresis on an 8% polyacrylamide gel and then transferred onto a nitrocellulose membrane (GE

Healthcare) for 90 minutes at 100V. To block the membrane, a blocking buffer containing 5% milk in TBS buffer comprising 100 mM Tris pH 8, 150 mM NaCl, and 0.1% Tween was employed. The Western blot membranes were then subjected to incubation with primary antibodies for 1 hour, followed by four washes and an additional hour of incubation with secondary antibodies. Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was utilized to visualize protein bands, with ChemiDocTM XRS+ (Biorad) employed for band visualization. The antibodies used included anti-HA, anti-Ty, anti-Myc, and anti-TgMIC3, each at a dilution of 1:1000, 1:500, 1:500, and 1:400, respectively. The secondary antibody utilized was species-specific and conjugated to HRP.

Cleavage under targets and tagmentation (CUT & Tag)

CUT&Tag was employed to identify the genomic localization of TgAP2XII-9. For each sample, intracellular parasites cultured for 24 hours were harvested from a T-175 flask, lysed using a syringe, filtered through a 3 µm filter, and quantified. A total of 20 million (2 × 10^7) parasites were centrifuged at 2,000 × g for 10 minutes, and the resulting pellets were directly processed using the CUT&Tag-IT Assay Kit (Active Motif 53160).

Indexed libraries for each sample were evaluated using Agilent Bioanalyzer, pooled, and sequenced on a NovaSeq6000 to generate paired-end reads. The reads were demultiplexed using bcl2fastq version 2.20.0 and processed with cutadapt v3.4 to eliminate sequencing adapters from the 3' end of reads, discarding any reads with less than 30 base pairs. The remaining reads were aligned to version 64 of the *Toxoplasma gondi*i ME49 reference obtained from ToxoDB using HISAT2 v2.2.1. For each sample, peaks were called using the callpeak command within MACS2. Overlapped peaks across the three biological replicates were determined using the bedtools overlap function. Peak annotation was conducted with CHIPSeeker (an R package) employing a 2-kb cutoff distance, and the peaks were finally annotated against version 64 of the *T.gondii* reference genome in ToxoDB.

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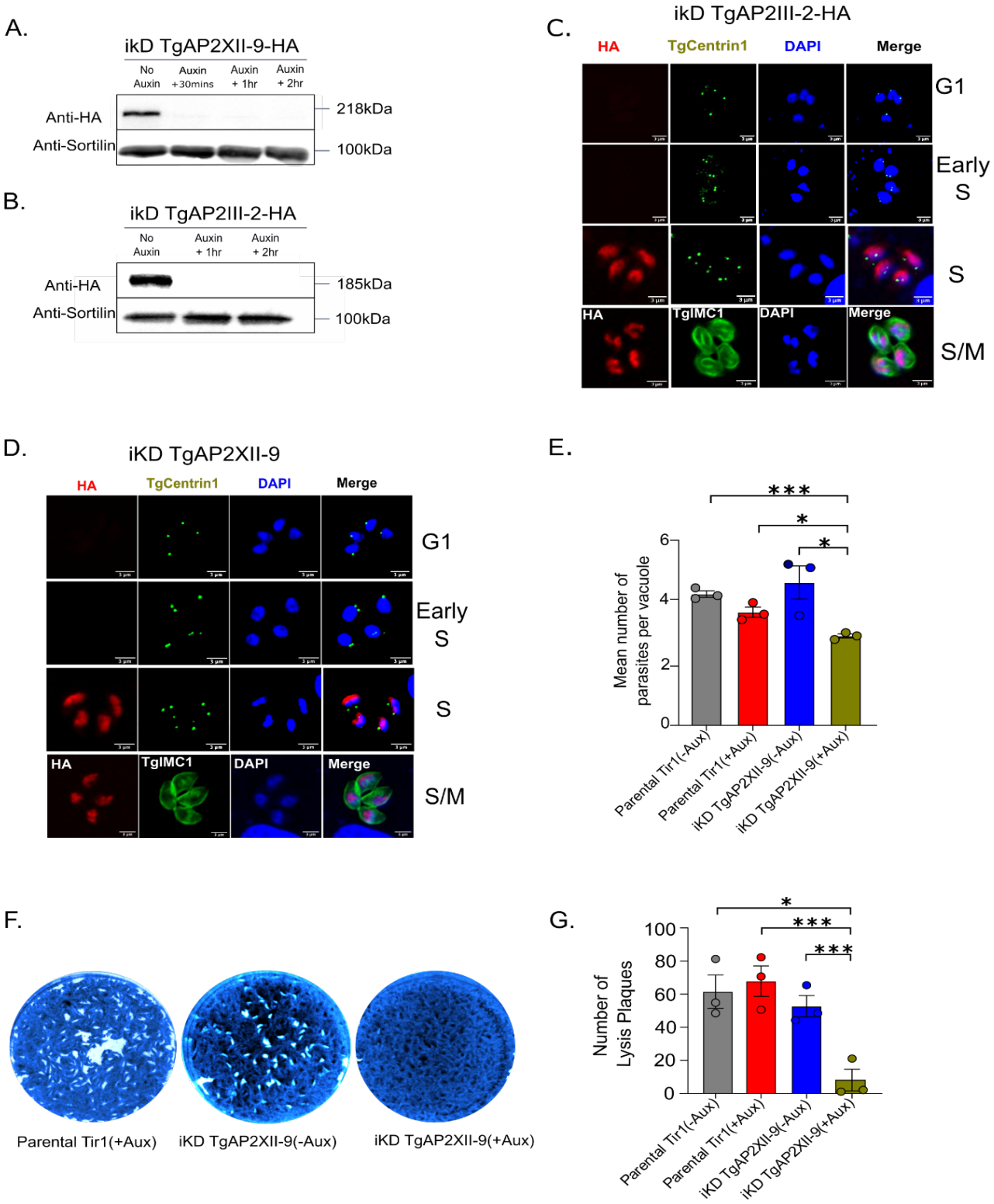
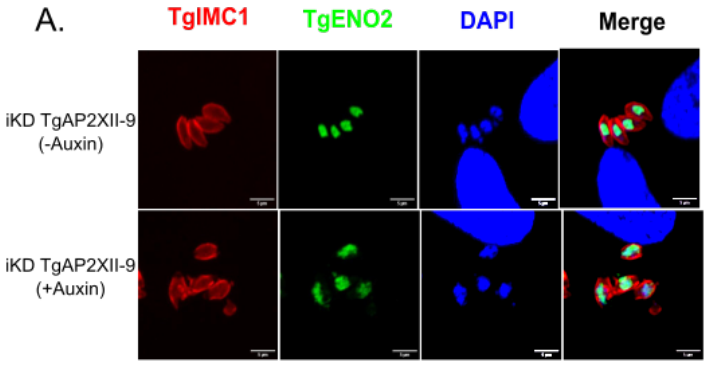
Data Availability Statement

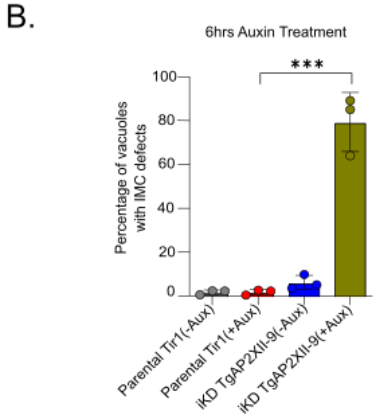
The RNA-seq data and CUT & Tag data was deposited to the SRA database under the identifier PRJNA1134678

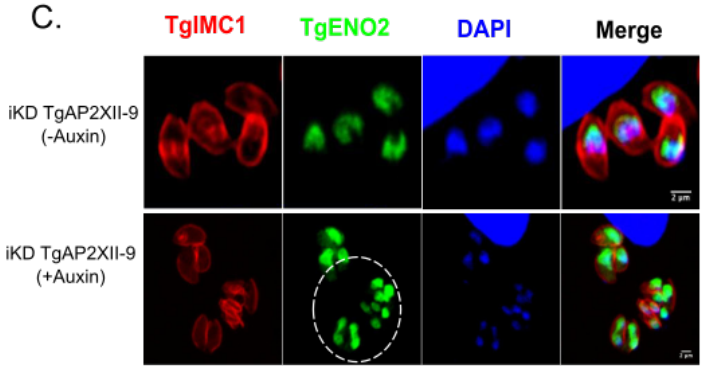


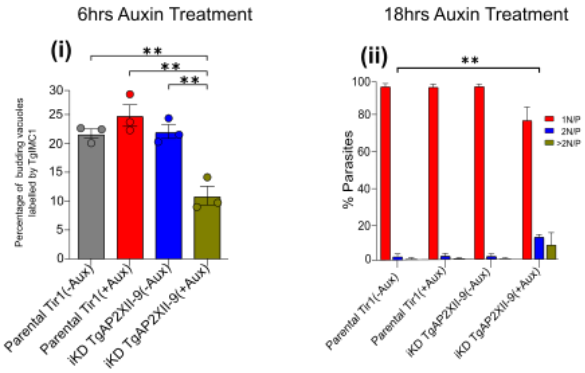
Figures

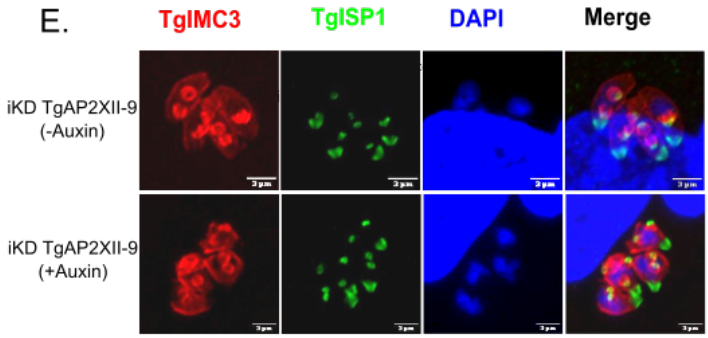
Figure 1: TgAP2XII-9 and TgAP2III-2 are cell-cycle regulated and expressed during the S/M phase. TgAP2XII-9 is essential for growth and proliferation *in vitro* (A, B) Western blot analysis of total protein extracts from parental and iKD TgAP2XII-9 and TgAP2III-2 strains treated with Auxin for varying durations. The blots were probed with anti-HA to detect TgAP2XII-9 and TgAP2III-2 protein levels (upper panel) and with anti-TgSortilin as a normalization control (lower panel), validating the AID system. **(C, D)** Cell cycle expression of TgAP2III-2 and AP2XII-9 as realized by IFA using anti-HA antibody and cell cycle markers TgCentrin1 and TgIMC1. Scale bar =3 μm **(E) Growth assay for parental and iKD TgAP2XII-9 strains with and without 24-hour auxin treatment.** Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ***$p < 0.001$, *$p<0.05$, Data are presented as mean ± s.d. ($n = 3$). **(F)** Plaque assay depicting proliferation and growth of Parental and iKD AP2XII-9 strains with and without auxin for 7 days. **(G)** Quantification of the no.of lysis plaques for parental and iKD TgAP2XII-9 strains. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by **$p < 0.01$, *$p<0.05$, Data are presented as mean ± s.d. ($n = 3$).

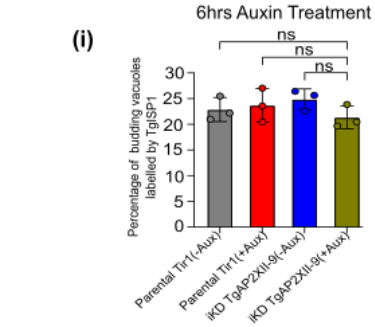

A.
TgIMC1
TgENO2
DAPI
Merge
iKD TgAP2XII-9
(-Auxin)
iKD TgAP2XII-9
(+Auxin)

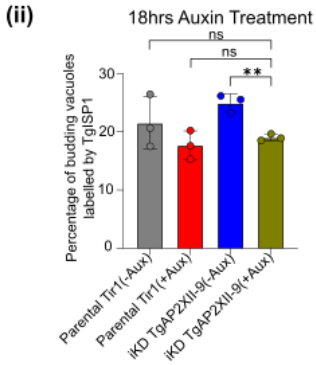

B.
6hrs Auxin Treatment
***
Percentage of vacuoles with IMC defects
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)

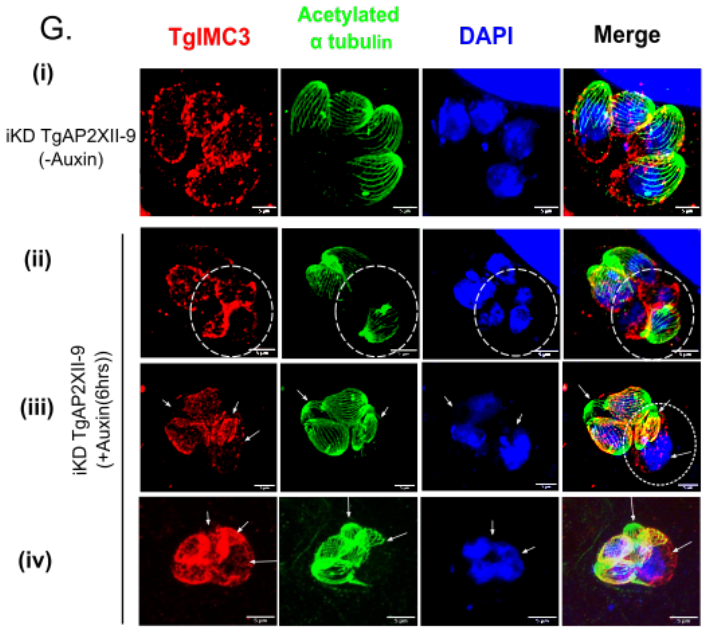

C.
TgIMC1
TgENO2
DAPI
Merge
iKD TgAP2XII-9
(-Auxin)
iKD TgAP2XII-9
(+Auxin)


D.
6hrs Auxin Treatment
18hrs Auxin Treatment
(i)
(ii)
Percentage of budding vacuoles labelled by TgIMC1
% Parasites
1N/P
2N/P
>2N/P
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)


E.
TgIMC3
TgISP1
DAPI
Merge
iKD TgAP2XII-9
(-Auxin)
iKD TgAP2XII-9
(+Auxin)


F. .
(i)
6hrs Auxin Treatment
Percentage of budding vacuoles labelled by TgISP1
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)


(ii)
18hrs Auxin Treatment
Percentage of budding vacuoles labelled by TgISP1
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)


G.
TgIMC3
Acetylated
α tubulin
DAPI
Merge
(i)
iKD TgAP2XII-9
(-Auxin)
(ii)
(iii)
(iv)
iKD TgAP2XII-9
(+Auxin(6hrs))

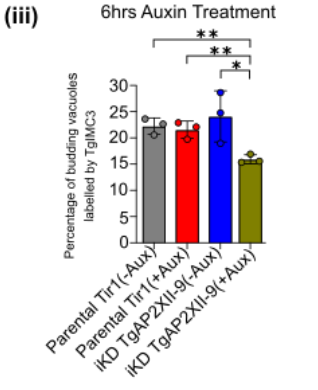

(iii)
6hrs Auxin Treatment
Percentage of budding vacuoles labelled by TgIMC3
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)

Figure 2: TgAP2XII-9 is crucial for the proper formation of daughter parasites (A) Defects in the IMC formation or organization observed after 6 hours Auxin treatment as realized by IFA and confocal imaging with TgIMC1 and TgENO2; Scale bar=5µm **(B)** Quantification of the IMC defect phenotype between the parental and iKD TgAP2XII-9 strains on 6hrs of auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ***$p < 0.001$, data are presented as mean ± s.d. (n = 3) **(C)** IFA and confocal imaging depicting the budding of iKD TgAP2XII-9 parasites and accumulation of multiple nuclei after the addition of Auxin using anti-TgIMC1 and anti-TgENO2 antibodies; scale bar = 2µm **(D)(i)** Quantification of percentage of vacuoles undergoing budding after 6hrs auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by **$p < 0.01$, data are presented as mean ± s.d. (n = 3). **(D)(ii)** Quantification of percentage of vacuoles having multiple nuclei. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by **$p < 0.01$, data are presented as mean ± s.d. (n = 3), N represents nuclei and P is parasites. **(E)** IFA and confocal imaging depicting the budding of parasites labelled by TgIMC3 and TgISP1; scale bar = 3 µm. **(F) (i)** Quantification of budding vacuoles using anti-TgIMC3 after 6hrs of Auxin treatment, Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by **$p < 0.01$, *$p<0.05$, data are presented as mean ± s.d. (n = 3). **(F) (ii)** Quantification of budding vacuoles using anti-TgISP1 after 6hrs of Auxin treatment, a two tailed Student's Statistical analysis was performed using a two-tailed Student's T-test, with significance indicated by ns>0.05, data are presented as mean ± s.d. (n = 3). **(F) (iii)** Quantification of budding vacuoles using anti-TgISP1 after 18hrs of Auxin treatment, a two tailed Student's Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns>0.05, **$p < 0.01$ data are presented as mean ± s.d. (n = 3). **(G)** Expansion microscopy depicting defects in the IMC (circled) and deformations in the cytoskeleton (panel (iv)). The parasite IMC (IMC3, red) and cytoskeleton (acetylated tubulin, green) were labelled as well as the nucleus by DAPI (blue). Parasite without nucleus(panel(iii)) and a parasite bearing multiple nuclei(panel(iii)) are indicated by white arrows. Scale bar = 5 µm

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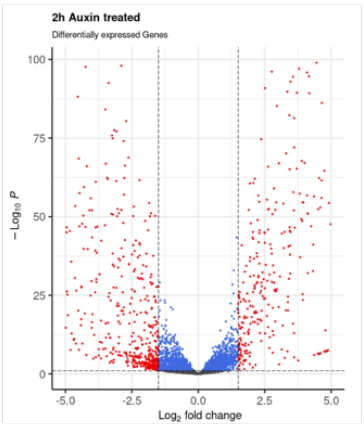
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Log2 fold change

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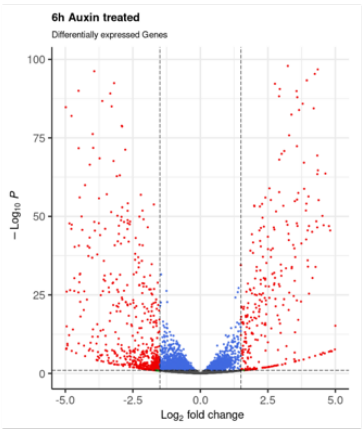
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Differentially expressed Genes
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5.0
Log2 fold change

C.


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6h Up regulated
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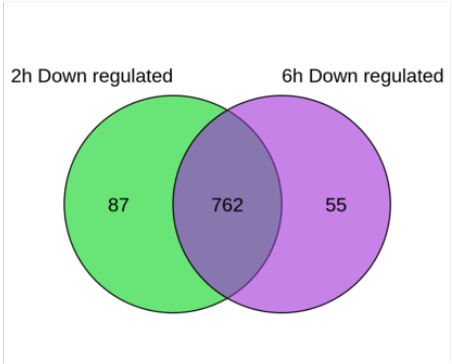
567
14

D.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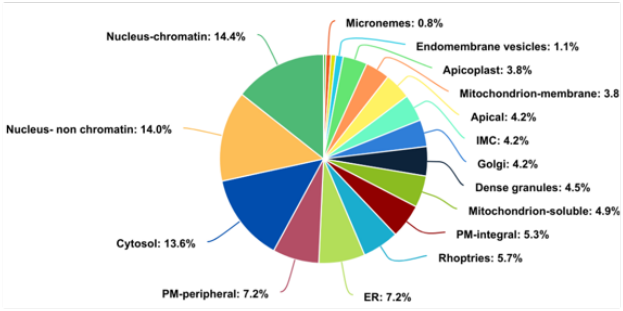
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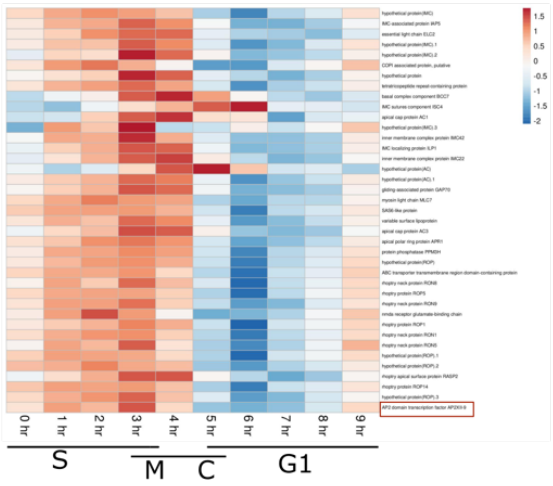
IMC: 4.2%
Golgi: 4.2%
Dense granules: 4.5%
Mitochondrion-soluble: 4.9%
PM-integral: 5.3%
Rhoptries: 5.7%
ER: 7.2%
PM-peripheral: 7.2%
Cytosol: 13.6%
Nucleus- non chromatin: 14.0%
Nucleus-chromatin: 14.4%

F.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S
M
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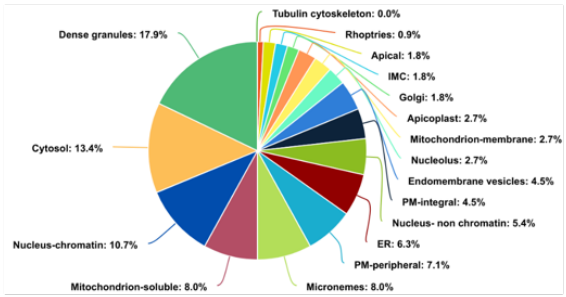
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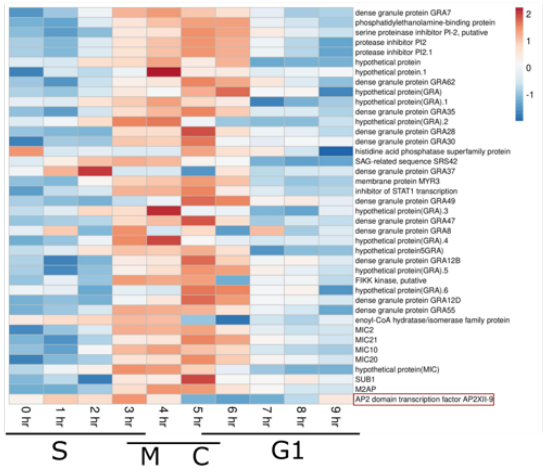
Rhoptries: 0.9%
Apical: 1.8%
IMC: 1.8%
Golgi: 1.8%
Apicoplast: 2.7%
Mitochondrion-membrane: 2.7%
Nucleolus: 2.7%
Endomembrane vesicles: 4.5%
PM-integral: 4.5%
Nucleus- non chromatin: 5.4%
ER: 6.3%
PM-peripheral: 7.1%
Micronemes: 8.0%
Mitochondrion-soluble: 8.0%
Nucleus-chromatin: 10.7%
Cytosol: 13.4%
Dense granules: 17.9%

H.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phosphatidylethanolamine-binding protein
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protease inhibitor PI2
protease inhibitor PI2.1
hypothetical protein
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hypothetical protein(GRA)
hypothetical protein(GRA).1
dense granule protein GRA35
hypothetical protein(GRA).2
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dense granule protein GRA30
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dense granule protein GRA12B
hypothetical protein(GRA).5
FIKK kinase, putative
hypothetical protein(GRA).6
dense granule protein GRA12D
dense granule protein GRA55
enoyl-CoA hydratase/isomerase family protein
MIC2
MIC21
MIC10
MIC20
hypothetical protein(MIC)
SUB1
M2AP
AP2 domain transcription factor AP2XII-9
0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
7 hr
8 hr
9 hr
S
M
C
G1

Figure 3: TgAP2XII-9 regulates the expression of various cell cycle-regulated genes. (A) Volcano plot of differentially expressed genes from RNA-sequencing analysis of TgAP2XII-9 parasites treated with auxin for 2 hours. **(B)** Volcano plot of differentially expressed genes from RNA-sequencing analysis of TgAP2XII-9 parasites treated with auxin for 6 hours. **(C)** Venn diagram showing the overlap of upregulated genes from 2h and 6h RNA-Seq data. **(D)** Venn diagram showing the overlap of downregulated genes from 2h and 6h RNA-Seq data. **(E)** Pie chart showing the percentage and localisation of upregulated transcripts from RNA-Seq data. **(F)** Heatmap showing the cell cycle expression of Rhoptry, IMC and Apical complex proteins encoding upregulated transcripts peaking simultaneously with TgAP2XII-9 expression (denoted in a box). The phases of the cell cycle are depicted at the bottom. **(G)** Pie chart showing the percentage and localisation of downregulated transcripts from RNA-Seq data. **(H)** Heatmap showing the cell cycle expression of micronemes and Dense granule proteins encoding downregulated transcripts. Expression of TgAP2XII-9 is denoted. The phases of the cell cycle are depicted at the bottom.

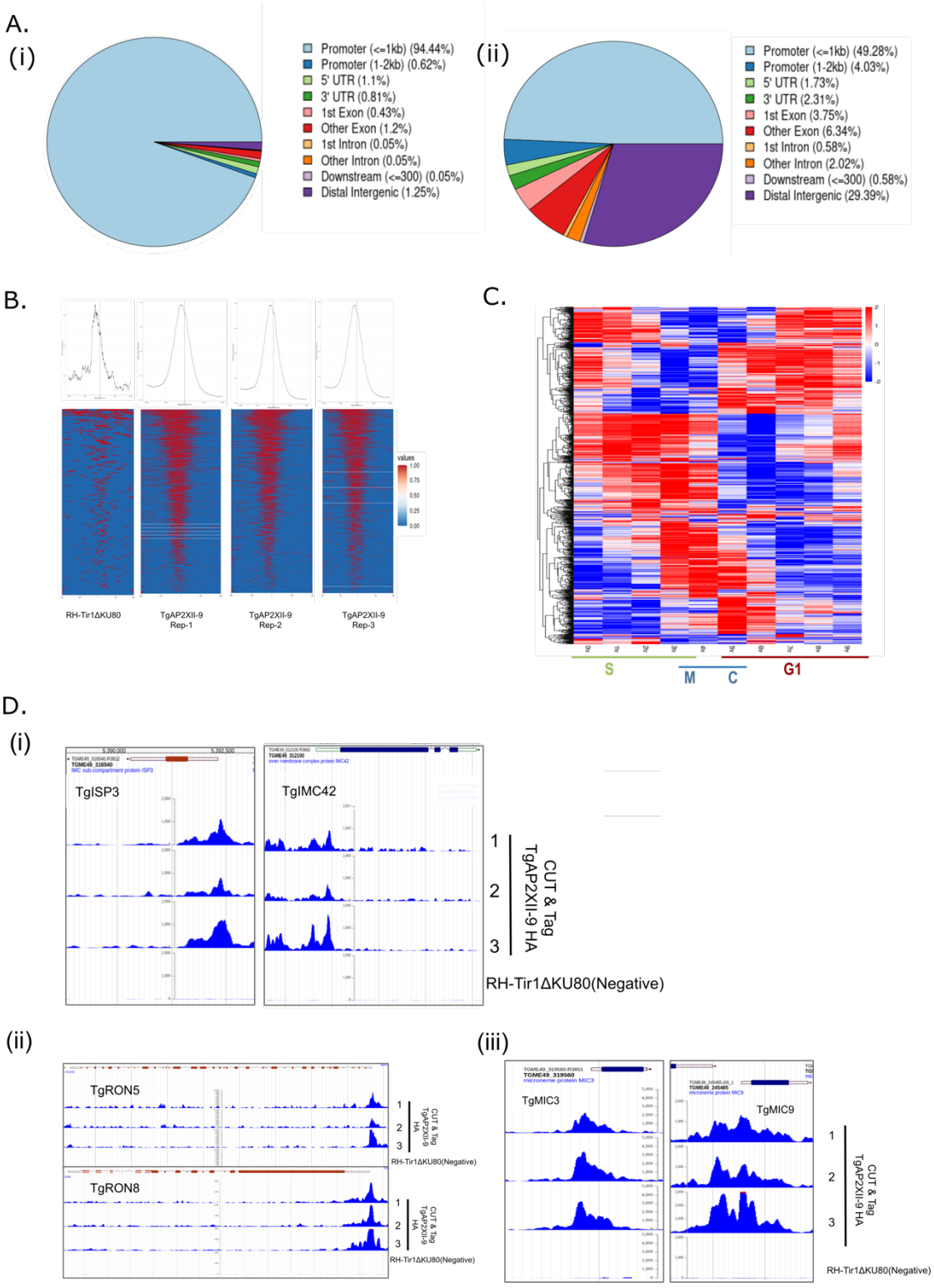


Figure 4: TgAP2XII-9 is enriched at the promoters of important *T.gondii genes* **(A) (i)** Pie chart showing the percentage of peaks (from 3 replicates) annotated in the different regions in the iKD TgAP2XII-9-HA strain. **(A) (ii)** Pie chart showing the percentage of peaks annotated in the different regions in the Parental Tir1 strain. (B) Density graphs and Heatmaps of the parental and TgAP2XII-9-HA (3 replicates) peaks located -2kb and +2kb from TSS. **(C)** Heatmap of cell cycle expression of all the genes

that are bound by TgAP2XII-9 at their promoter. **(D)** CUT & Tag representing the direct targeting of TgAP2XII-9 to the promoters of TgISP3, TgIMC42 **(i)**, TgRON5, TgRON8**(ii)**, TgMIC3, TgMIC9**(iii).** Peak tracks for the 3 replicates of TgAP2XII-9-HA along with parental Tir1 are shown.

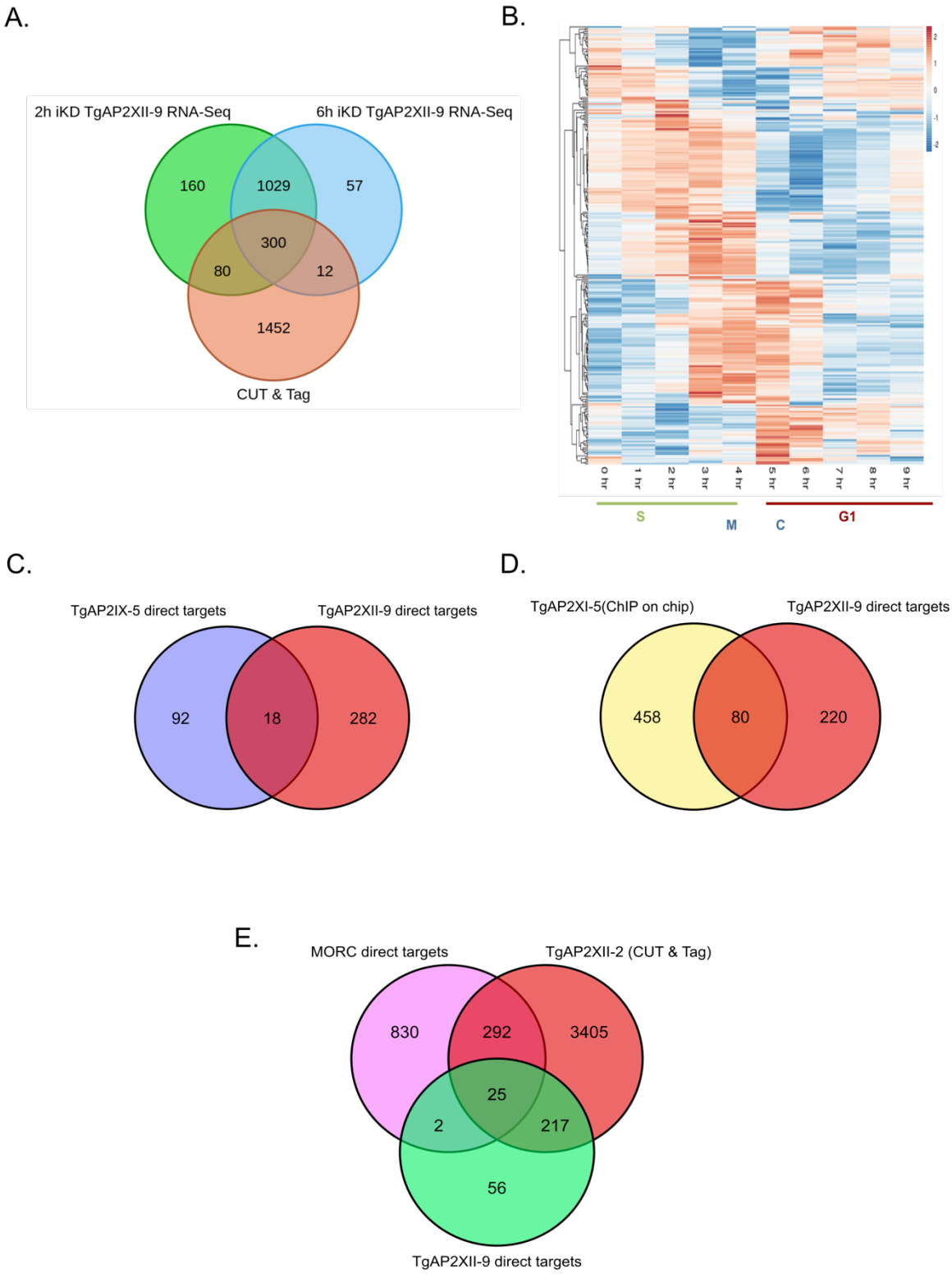


Figure 5: TgAP2XII-9 directly regulates key genes involved in the daughter parasite formation. (A) Venn diagram showing the overlap of genes from 2h RNA-Seq, 6h RNA-Seq and CUT & Tag data. **(B)** Heatmap of cell cycle expression of genes directly regulated and targeted by TgAP2XII-9. **(C)** Venn diagram indicating that majority of genes targeted by TgAP2XII-9 are different to that targeted by TgAP2IX-5. **(D)** Venn diagram indicating that majority of genes targeted by TgAP2XII-9 are different

to that targeted by TgAP2XI-5. **(E)** Venn diagram indicating the overlap of genes bound by both TgAP2XII-9 and TgAP2XII-2 and direct targets of MORC.

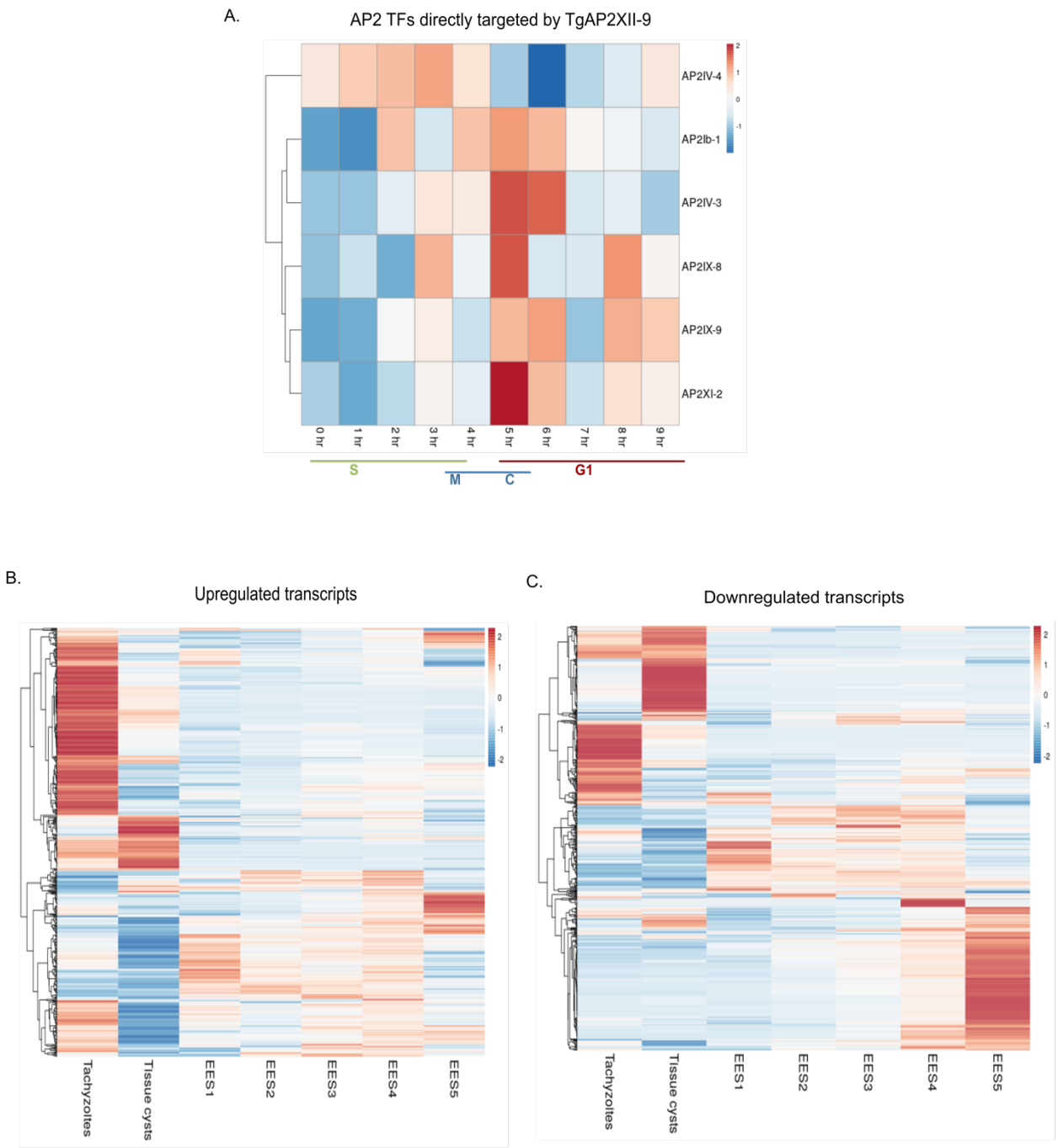


Figure 6: TgAP2XII-9 directly regulates other AP2 transcription factors and might provide a conducive environment for the expression of genes preferentially expressed in bradyzoites. (A) Heatmap of cell cycle expression of the AP2 TFs directly regulated by TgAP2XII-9**. (B)** Heatmap of expression of upregulated transcripts through the tachyzoite, bradyzoite and sexual stages. Most of the overexpressed transcripts upon TgAP2XII-9 depletion are preferentially expressed at the tachyzoite stage of the parasite **(C)** Heatmap of expression of downregulated transcripts through the tachyzoite, bradyzoite and sexual stages. Many of the downregulated transcripts upon TgAP2XII-9 depletion are preferentially expressed at bradyzoite and throughout

the sexual stages especially during EES5. EES stands for enteroepithelial developmental stages.

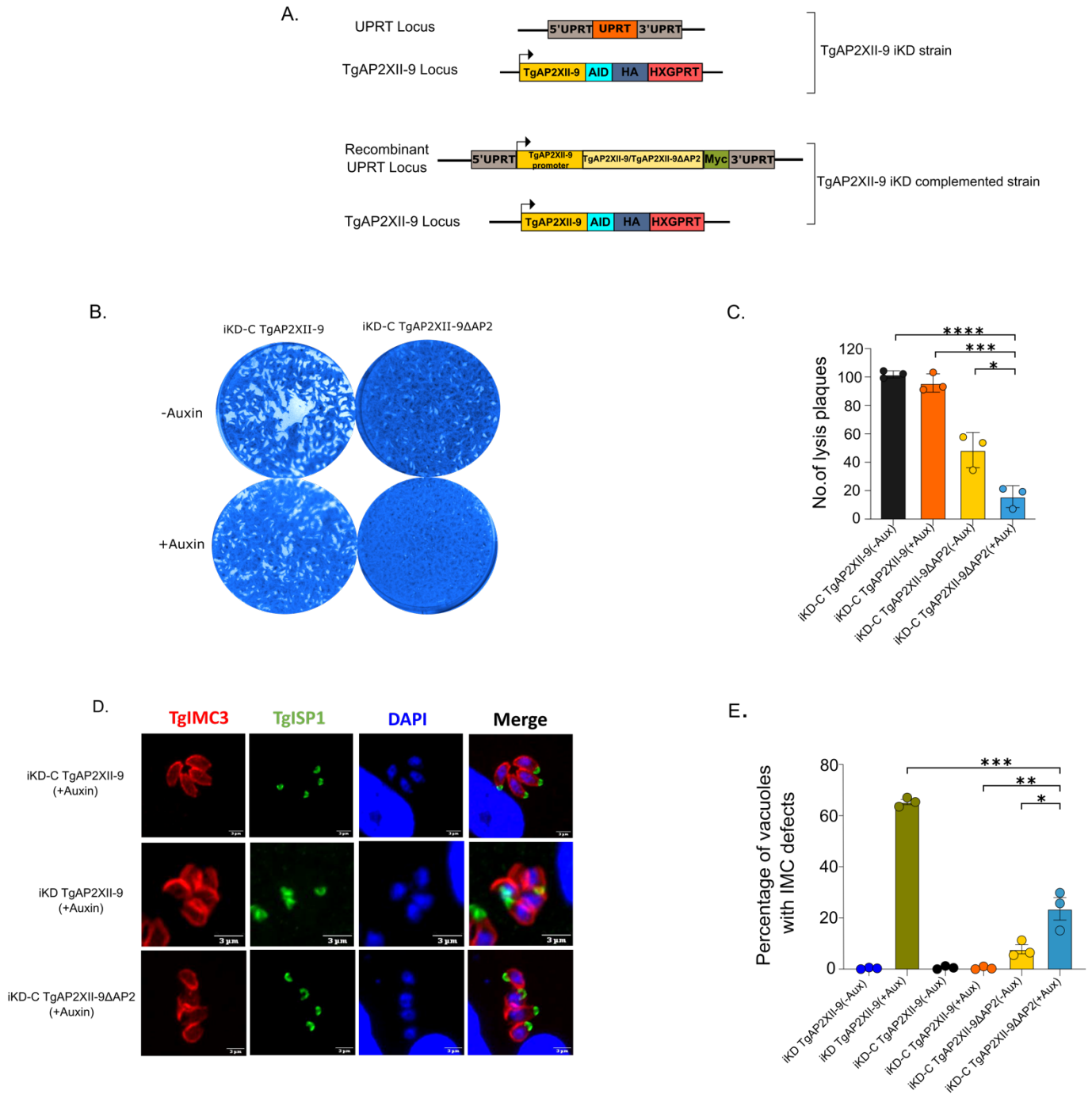
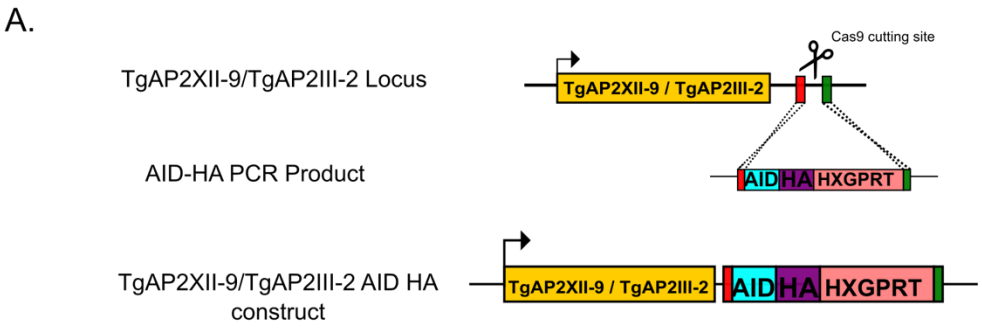
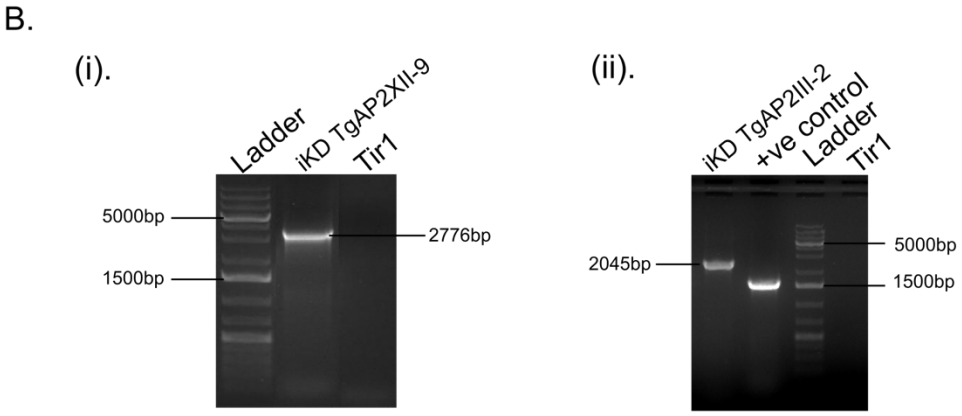


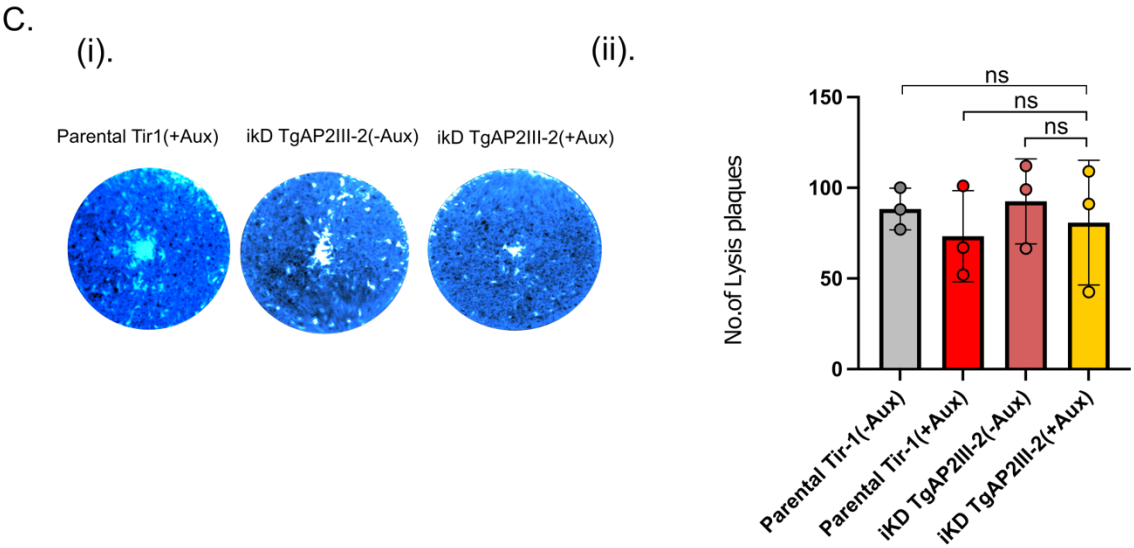
Figure 7: TgAP2XII-9 is responsible for the phenotypes observed and the AP2 domain is crucial for its function. (A) Schematic representation of the iKD TgAP2XII-9 complementation strategy. The UPRT locus was targeted for the insertion of exogenous myc-tagged TgAP2XII-9/TgAP2XII-9ΔAP2, driven by its native promoter, to generate the complemented TgAP2XII-9 iKD strain. **(B)** Plaque assay showing the proliferation of the iKD-C TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 strains in the presence and the absence of Auxin. **(C)** Quantification of the number of plaques in the iKD-C TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 strains. Statistical analysis was

performed using a two-tailed Student's t-test, with significance indicated by ****p<0.0001, ***p < 0.001, *p<0.05. Data are presented as mean ± s.d. (n = 3). **(D)** IFA and confocal imaging illustrate the IMC defect phenotype seen by using TgIMC3 and TgISP1 in the iKD TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 in presence of Auxin but not in the iKD-C TgAP2XII-9, scale bar = 3 μm**. (E)** Quantification of the IMC defect phenotype using TgIMC3 between iKD TgAP2XII-9, iKD-C TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 strains. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by *p<0.05, **p<0.01, ***p < 0.001, Data are presented as mean ± s.d. (n = 3).

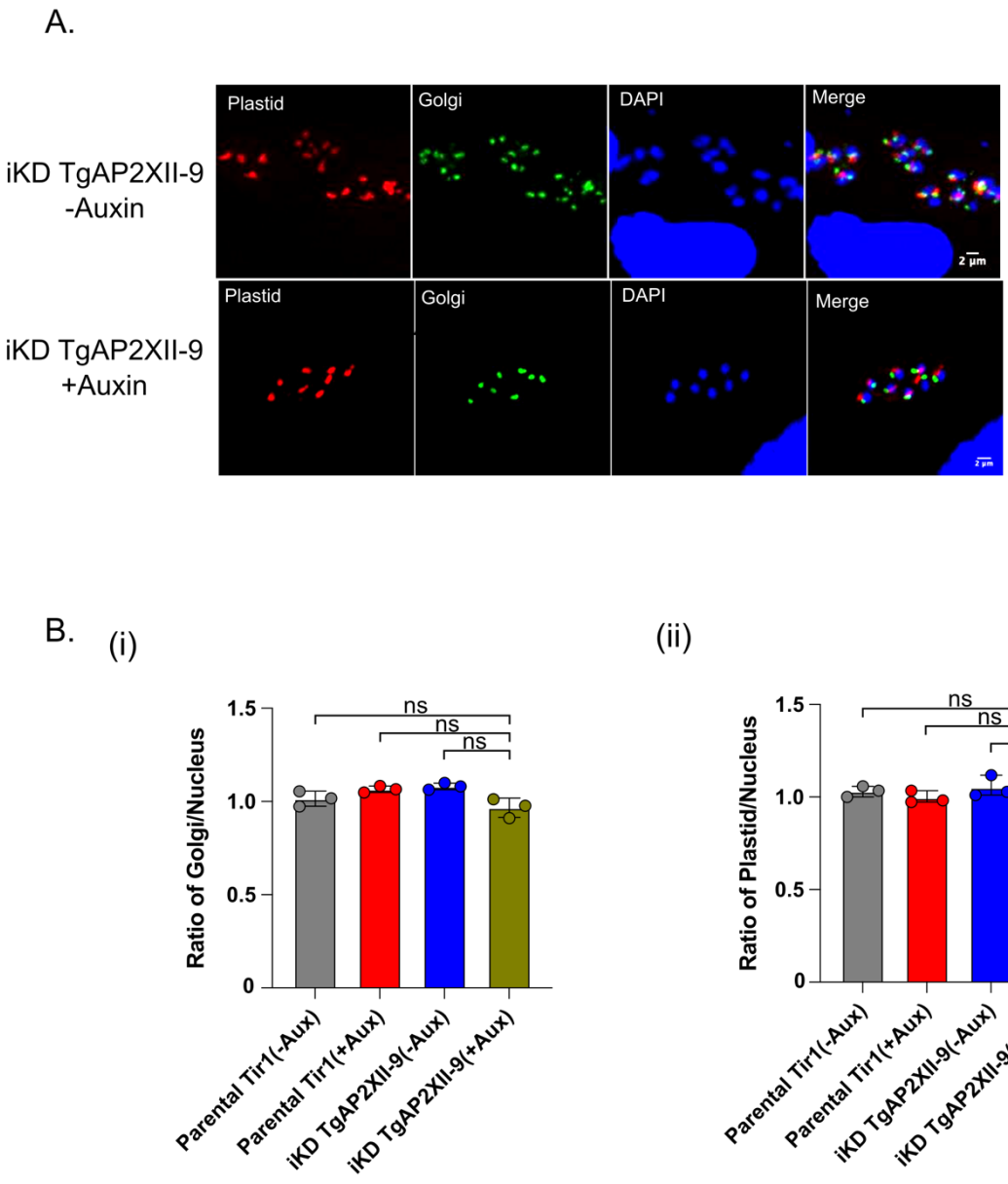
Supplementary Figures



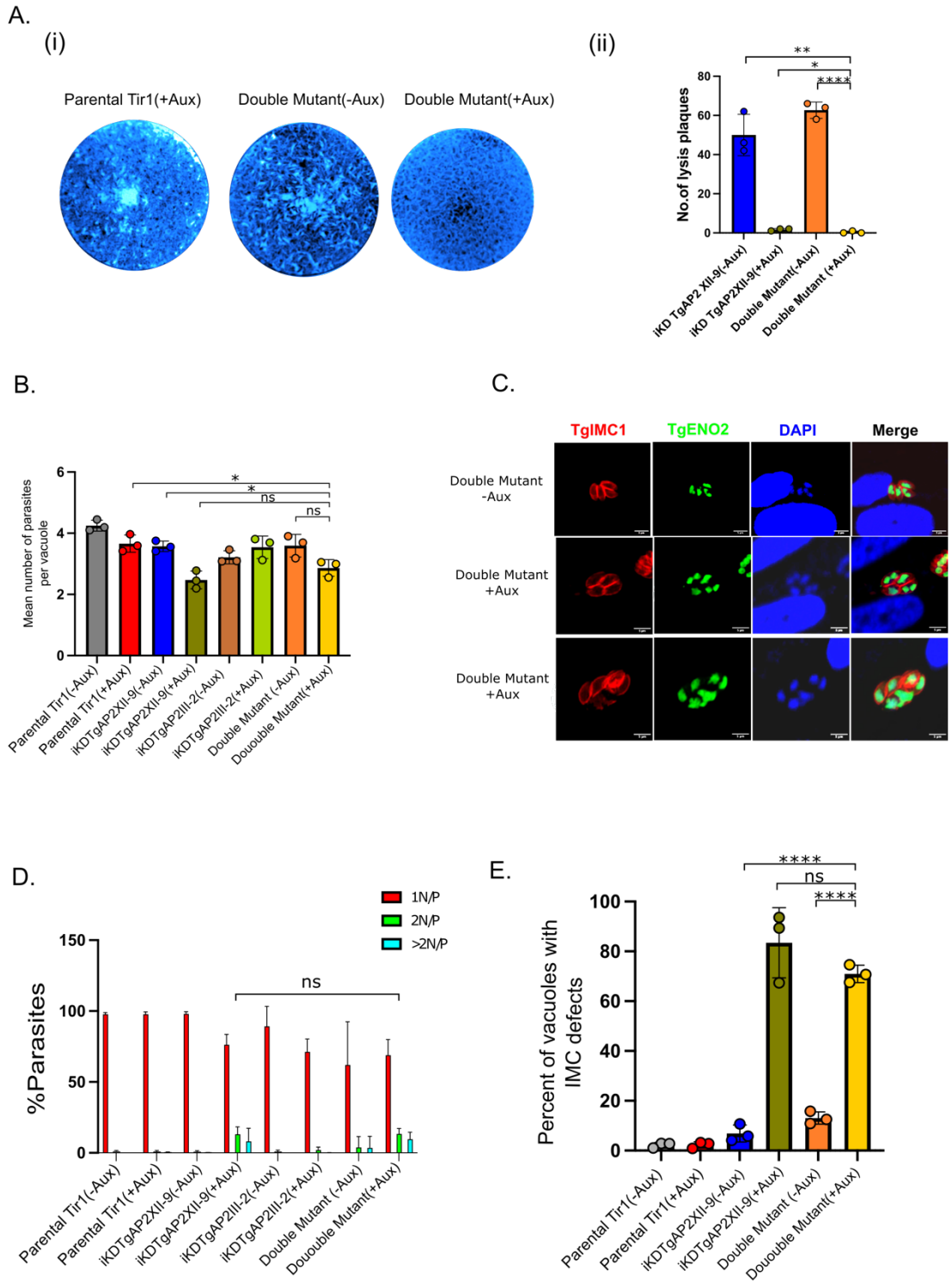




Supplementary Figure 1: iKD TgAP2XII-9 and iKD TgAP2III-2 mutant construction and TgAP2III-2 is dispensable for parasite proliferation *in vitro.* **(A)** Illustration of strategy used to construct the iKD mutants of TgAP2XII-9 and TgAP2III-2. A CRISPR/Cas9-assisted homologous recombination was used to generate the iKD strains, in which the endogenous TgAP2XII-9 and tgAP2III-2 is tagged with an AID domain, HA tag and HXGPRT selection cassette. **(B)** PCR verification of the integration of the HXGPRT-HA-AID cassette at the correct genomic locus of the iKD TgAP2XII-9 **(i)** and TgAP2III-2 **(ii)** mutant. A band corresponding to 2776bp and 2045bp using iKD TgAP2XII-9 and iKD TgAP2III-2 genomic DNA respectively, confirms cassette integration, compared to the absence of this band using Tir1(WT)genomic DNA. A positive control was used to confirm the presence of the genomic DNA. **(C)(i)** Plaque assay depicting the proliferation and growth of the iKD AP2III-2 and Parental strains in presence and absence of Auxin. **(C)(ii)** Quantification of the number of lysis plaques in the iKD TgAP2III-2 and parental strain reveals the non-essentiality of TgAP2III-2. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns>0.05. Data are presented as mean ± s.d. (n = 3).

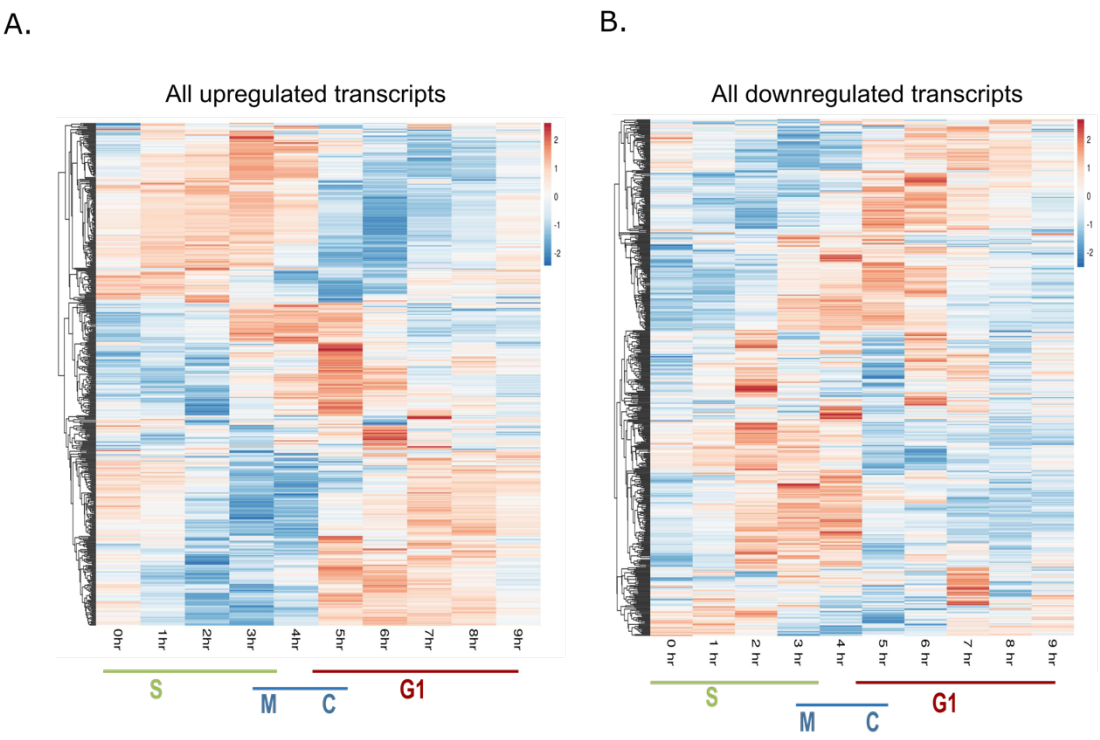


Supplementary Figure 2: Plastid and Golgi segregation in the iKD AP2XII-9 throughout the tachyzoite stage. (A) IFA and confocal imaging depicting iKD TgAP2XII-9 parasites labelled plastid (red) and Golgi (green) in the presence and absence of overnight Auxin treatment. The IFA revealed no segregation defects in Golgi and plastid. **(B)(i)** Bar graph representing the ratio of Golgi: nucleus using the parental and iKD TgAP2XII-9 strains in the absence and presence of overnight auxin treatment. A Student's *t*-test was performed, significance denoted by ns>0.05; mean ± s.d. ($n = 3$). **(B)(ii)** Bar graph representing the ratio of Plastid: nucleus using the parental and iKD TgAP2XII-9 strains in the absence and presence of overnight auxin treatment. A Student's *t*-test was performed, significance denoted by ns>0.05; mean ± s.d. ($n = 3$)



Supplementary Figure 3: TgAP2XII-9 and TgAP2III-2 have no combinatorial effect on the parasite biology *in vitro*. (A) (i) Plaque assay depicting the proliferation of the double mutant in presence and absence of auxin. **(A)(ii)** Quantification of the number of plaques in the iKD TgAP2XII-9 and Double mutant strains. A two tailed Student's *t*-test was performed, significance denoted by *p<0.05, **p<0.01, ****p<0.0001;

mean ± s.d. (*n* = 3). **(B) Growth assay for parental and iKD TgAP2XII-9, iKD TgAP2III-2 and the Double mutant strains with and without 24-hour auxin treatment.** Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by *p<0.05, ns>0.05. Data are presented as mean ± s.d. (n = 3). **(C)** IFA and confocal imaging illustrating the multiple nuclei and IMC defect phenotype labelled by TgIMC3(red) and TgENO2(green) in the Double mutant strain in presence and absence of auxin, scale bar = 5 µm. **(D)** Quantification of the multiple nuclei phenotype in the **parental and iKD TgAP2XII-9, iKD TgAP2III-2 and the Double mutant strains with and without overnight auxin treatment.** Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns>0.05. Data are presented as mean ± s.d. (n = 3). **(E)** Quantification of the IMC defect phenotype in the **parental and iKD TgAP2XII-9 and the Double mutant strains after 6 hrs. auxin treatment.** Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ****p<0.0001, ns>0.05. Data are presented as mean ± s.d. (n = 3).



Supplementary Figure 4: TgAP2XII-9 regulates key genes important for daughter parasite formation. (A) Heatmap showing the cell cycle expression all the upregulated transcripts upon the depletion of TgAP2XII-9. Majority if the upregulated transcripts show peak expression across the cell cycle. (B) Heatmap showing the cell cycle expression all the downregulated transcripts upon the depletion of TgAP2XII-9. Majority if the downregulated transcripts show peak expression during the late S, M,C and G1 phases of the cell cycle.

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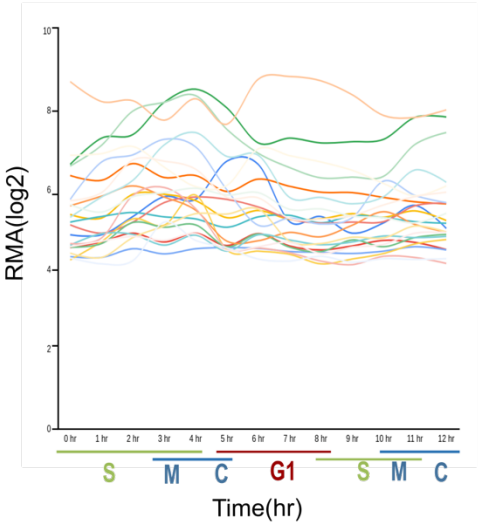

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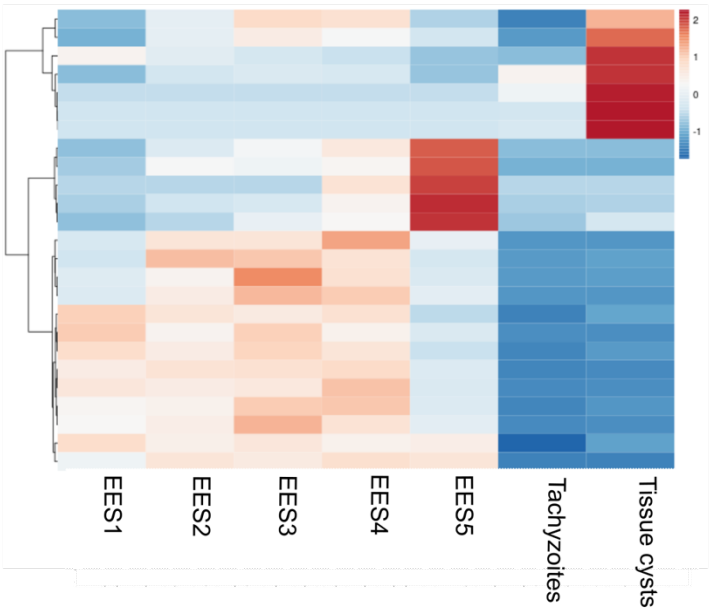
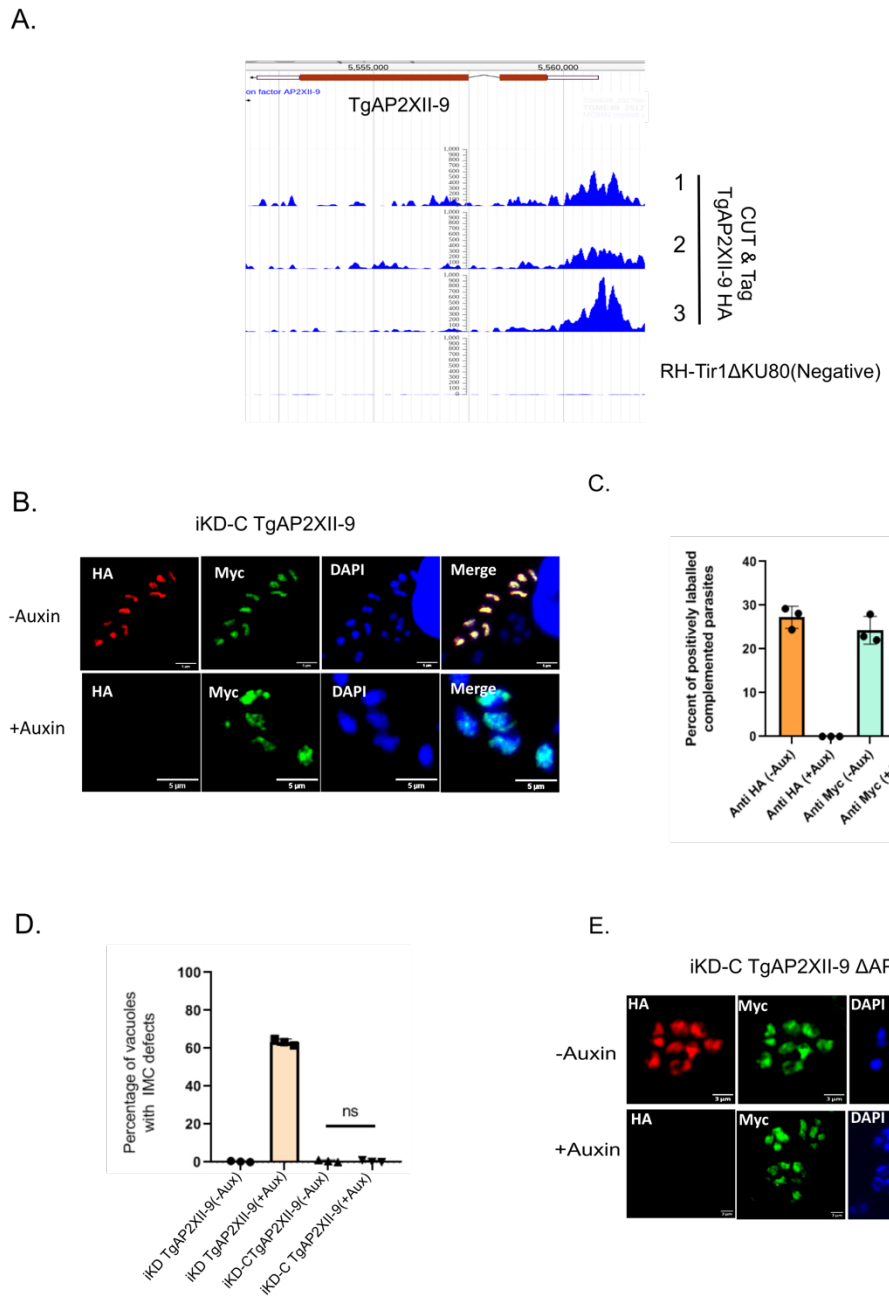


Supplementary Figure 5: TgAP2XII-9 has a different biological role from TgAP2XII-2 and MORC. (A) Cell cycle expression of the 25 genes that are directly regulated and targeted by MORC and TgAP2XII-9 and bound by TgAP2XII-2 at their promoters. Most of them show basal level of expression throughout the cell cycle, while some of them show expression peaks during the M, C and the G1 phase. **(B)** Heatmap of the 25 genes during the different life stages of the parasite show that these genes are preferentially expressed in the bradyzoite and sexual stages of the life cycle.



Supplementary Figure 6: Complementation of the iKD TgAP2XII-9 demonstrate that the TgAP2XII-9 protein is responsible for the phenotypes observed in the mutant and the AP2 domain is crucial for its function. (A) CUT & Tag tracks of 3 replicates of TgAP2XII-9-HA and Tir1 strains showing the targeting of TgAP2XII-9 to its own promoter suggesting a negative feedback loop. **(B)** IFA and confocal imaging illustrating the localisation of the complemented TgAP2XII-9 to the nucleus in presence of auxin when the native TgAP2XII-9-HA is depleted. Endogenous TgAP2XII-9 tagged with HA is represented in red while exogenous TgAP2XII-9 tagged with myc is represented in green. Scale bar = 5 µm. **(C)** Bar graph representing the expression of TgAP2XII-9 using anti-HA and anti-myc antibodies in the complemented strain. mean ± s.d. (n=3 independent experiments). **(D)** Quantification of the IMC defect phenotype in the iKD-C TgAP2XII-9 and iKD TgAP2XII-9 strains. Statistical analysis was

performed using a two-tailed Student's t-test, with significance indicated ns>0.05. Data are presented as mean ± s.d. (n = 3). **(E)** IFA and confocal imaging illustrating the localisation of the complemented TgAP2XII-9 to the nucleus in presence of auxin when the native TgAP2XII-9-HA is depleted. Endogenous TgAP2XII-9 tagged with HA is represented in red while exogenous TgAP2XII-9 tagged with myc is represented in green. Scale bar = 5 µm. **(D)** IFA and confocal imaging illustrating the localisation of the complemented TgAP2XII-9ΔAP2 to the nucleus in presence of auxin when the native TgAP2XII-9-HA is depleted. Endogenous TgAP2XII-9 tagged with HA is represented in red while exogenous TgAP2XII-9ΔAP2 tagged with myc is represented in green. Scale bar = 3 µm

3.2. Complementary Results

3.2.1. Functional Characterization of TgAP2III-2

To investigate the role of TgAP2III-2, growth assays were performed using both wildtype (Tir1) parasites and iKD mutants for TgAP2III-2. Parasites were allowed to grow for 24 hours, after which the number of parasites per vacuole was quantified. The results showed no significant differences in the growth rates between the wildtype and iKD TgAP2III-2 mutants, indicating that depletion of TgAP2III-2 does not lead to an apparent growth defect (Figure 24A).

Additionally, budding assays were conducted to assess the potential role of TgAP2III-2 in parasite replication. The percentage of budding parasites in both wildtype and iKD TgAP2III-2 mutants, with (18hrs) or without auxin-induced depletion, remained comparable. These findings suggest that TgAP2III-2 is not essential for the budding process in *T. gondii*. (Figure 24 B).

Furthermore, no defects were observed in nuclear segregation, as iKD TgAP2III-2 mutants did not exhibit the accumulation of multiple nuclei even after 24 hrs of Auxin treatment, a phenotype previously noted in iKD TgAP2XII-9 mutants. This suggests that, unlike TgAP2XII-9, TgAP2III-2 does not play a critical role in the regulation of nuclear division (Figure 24 C(i) and (ii)).

Further investigations were carried out to examine the role of TgAP2III-2 in organelle inheritance. The Golgi complex and plastid were labelled in both wildtype and iKD parasites (Figure 24 D(i)), and the Golgi-to-nucleus and plastid-to-nucleus ratios were calculated after overnight (~18hrs) auxin treatment (Figure 24 D(ii) and (iii)). No significant differences were found in these ratios between the presence and absence of auxin, as expected, given that TgAP2III-2 expression peaks after both organelle divisions.

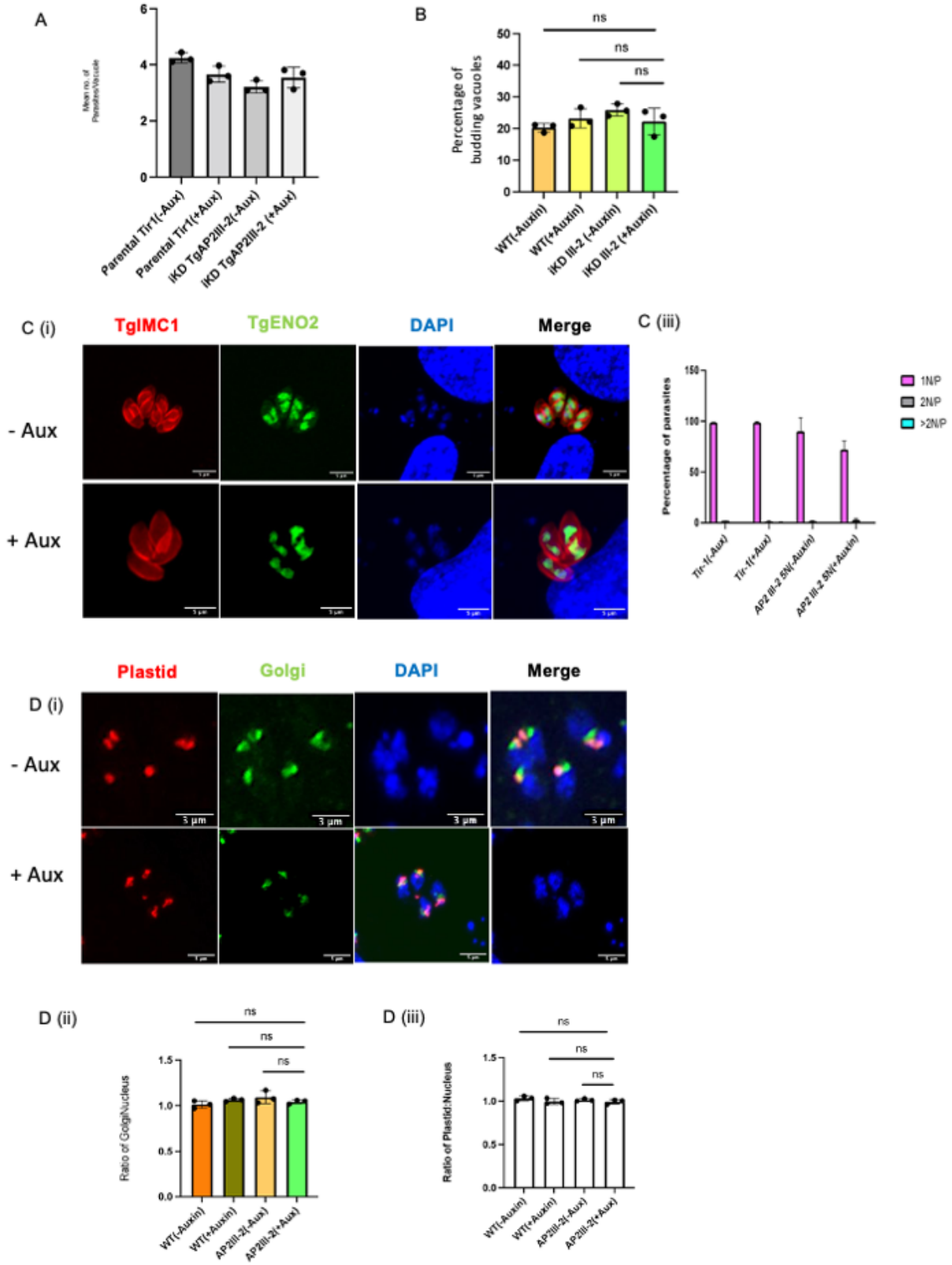


Figure 21 – TgAP2III-2 Is not essential for the proliferation nd formation of daughter cells. (A) Growth assay for parental and iKD TgAP2III-2 strains with and without 24-hour auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, there

was no significance observed. Data are presented as mean ± s.d. (n = 3). **(B)** Quantification of percentage of vacuoles undergoing budding after 18hrs auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns – not significant, data are presented as mean ± s.d. (n = 3). **(C)(i)** IFA and confocal imaging of iKD TgAP2III-2 parasites undergoing budding with and without auxin.Scale bare is indicated at the bottom right corner of each image. **(C) (iii)** Quantification of percentage of vacuoles having multiple nuclei. Statistical analysis was performed using a two-tailed Student's t-test, there was no significance observed, data are presented as mean ± s.d. (n = 3), N represents nuclei and P is parasites. **(D)(i)** IFA and confocal imaging depicting iKD III-2 parasites labelled plastid (red) and Golgi (green) in the presence and absence of overnight Auxin treatment. The IFA revealed no segregation defects in Golgi and plastid. **(D)(ii)** Bar graph representing the ratio of Golgi: nucleus using the parental and iKD TgAP2III-2 strains in the absence and presence of overnight auxin treatment. A Student's *t*-test was performed, significance denoted by ns>0.05; mean ± s.d. (*n* = 3). **(B)(iii)** Bar graph representing the ratio of Plastid: nucleus using the parental and iKD TgAP2XII-9 strains in the absence and presence of overnight auxin treatment. A Student's *t*-test was performed, significance denoted by ns>0.05; mean ± s.d. (*n* = 3)

3.2.2. The Depletion of TgAP2III-2 Does Not Significantly Impact the Overall Transcriptomic Profile

Given the potential role of TgAP2III-2 as a transcription factor, we conducted RNA sequencing analysis to identify its regulated genes. The sequencing was performed 6 hours post-depletion, coinciding with the completion of one tachyzoite cell cycle. Data analysis, using DESeq2 with an adjusted p-value cutoff of 0.05 and a minimum fold change of 2, revealed no significant transcriptomic alterations in the inducible knockdown (iKD) TgAP2III-2 mutant. Only 67 genes were differentially expressed (DEGs), with a modest log2 fold change ranging between -0.4 and 1.2.

Of the 67 DEGs, 64 were downregulated, while only 3 were upregulated. Notably, 16 of the downregulated genes encoded proteins localized to the dense granules. These downregulated genes did not exhibit a strong correlation with cell cycle regulation; most had low expression levels throughout the cell cycle, with a subset showing consistent expression across various phases (Figure 22A). The 3 upregulated genes, however, peaked in expression during the M, C, and G1 phases (Figure 22B).

We also examined the expression of these downregulated genes across different stages of the *T.gondii* life cycle following TgAP2III-2 depletion. Interestingly, only a few

genes were highly expressed throughout all stages, while the majority exhibited minimal expression across the life stages (Figure 22C).

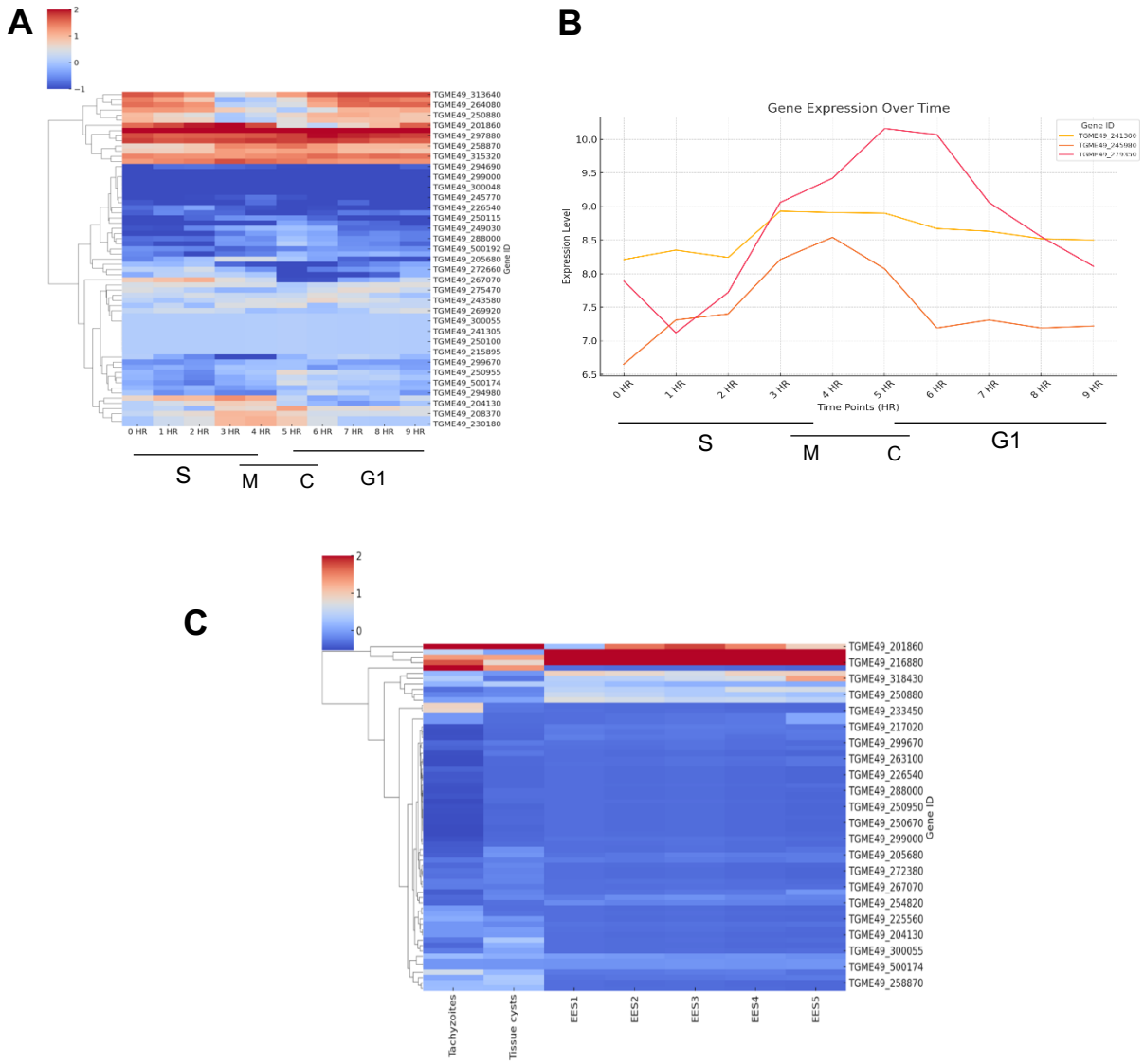


Figure 22 – Deletion of TgAP2III-2 does not cause changes in the transcriptome. **(A)** Heat map depicting the cell cycle expression of down regulated genes after depletion of TgAP2III-2. Phases are indicated at the bottom. **(B)** Graph showing the cell cycle expression of genes upregulated after TgAP2III-2 depletion. **(C)** Heatmap showing the expression of downregulated genes at various life cycle stages.

3.2.3. Genome-wide Binding of TgAP2III-2 Revealed by CUT&Tag

CUT&Tag analysis was performed to investigate the genome-wide binding profile of AP2III-2 in *Toxoplasma gondii*. The results revealed that AP2III-2 binds to the promoters of numerous genes, with a striking enrichment at ribosomal RNA (rRNA) gene promoters (For example, Figure 23B). Approximately **74.5%** of the identified binding sites were associated with rRNA genes (Table 2), indicating a potential role for AP2III-2 in regulating ribosome biogenesis.

Gene ID	Product Description	Gene ID	Product Description
TGME49_220170	hypothetical protein	TGME49_457940	18S ribosomal RNA
TGME49_220740	hypothetical protein	TGME49_457950	5.8S ribosomal RNA
TGME49_221350	Ctr copper transporter family protein	TGME49_458000	5.8S ribosomal RNA
TGME49_224180	hypothetical protein	TGME49_458010	28S ribosomal RNA
TGME49_228160	acid phosphatase	TGME49_458030	28S ribosomal RNA
TGME49_233460	SAG-related sequence SRS29B	TGME49_458040	28S ribosomal RNA
TGME49_237140	ethylene inducible protein, putative	TGME49_458100	18S ribosomal RNA
TGME49_239300	intra-conoid microtubule associated protein ICMAP1	TGME49_458140	5.8S ribosomal RNA
TGME49_248450	zinc finger, C3HC4 type (RING finger) domain-containing protein	TGME49_458320	18S ribosomal RNA
TGME49_249900	adenine nucleotide translocator	TGME49_458330	5.8S ribosomal RNA
TGME49_255060	cytochrome b(N-terminal)/b6/petB subfamily protein	TGME49_458420	28S ribosomal RNA
TGME49_264420	lipoprotein, putative	TGME49_458490	5.8S ribosomal RNA
TGME49_286050	apicoplast TIC22 protein	TGME49_458500	28S ribosomal RNA
TGME49_300340	CAF1 family ribonuclease	TGME49_458530	28S ribosomal RNA
TGME49_300618	ribosomal protein S19	TGME49_458540	28S ribosomal RNA
TGME49_300690	RNA polymerase C2, putative	TGME49_458650	18S ribosomal RNA
TGME49_302009	ORF B	TGME49_458670	28S ribosomal RNA
TGME49_302057	ribosomal protein S7	TGME49_458760	18S ribosomal RNA
TGME49_302060	elongation factor tu, apicoplast, putative	TGME49_458800	28S ribosomal RNA
TGME49_322210	apocytochrome b, putative	TGME49_458830	28S ribosomal RNA
TGME49_323500	hypothetical protein	TGME49_458840	18S ribosomal RNA
TGME49_328900	hypothetical protein	TGME49_458880	28S ribosomal RNA
TGME49_329000	hypothetical protein	TGME49_458890	5.8S ribosomal RNA
TGME49_329300	hypothetical protein	TGME49_459050	5.8S ribosomal RNA
TGME49_355100	tRNA-Ile	TGME49_459100	28S ribosomal RNA
TGME49_355120	tRNA-Ala	TGME49_459160	18S ribosomal RNA
TGME49_355190	tRNA-Thr	TGME49_459280	18S ribosomal RNA

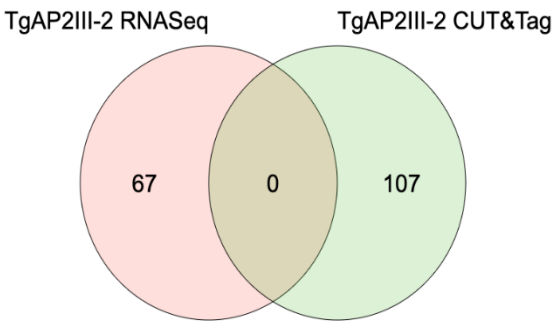
TGME49_355220	tRNA-Gly	TGME49_459310	28S ribosomal RNA
TGME49_457340	5.8S ribosomal RNA	TGME49_459320	5.8S ribosomal RNA
TGME49_457390	28S ribosomal RNA	TGME49_459350	28S ribosomal RNA
TGME49_457400	18S ribosomal RNA	TGME49_459440	5.8S ribosomal RNA
TGME49_457450	28S ribosomal RNA	TGME49_459520	28S ribosomal RNA
TGME49_457460	5.8S ribosomal RNA	TGME49_459530	5.8S ribosomal RNA
TGME49_457480	28S ribosomal RNA	TGME49_459660	18S ribosomal RNA
TGME49_457520	18S ribosomal RNA	TGME49_459790	18S ribosomal RNA
TGME49_457580	28S ribosomal RNA	TGME49_457710	5.8S ribosomal RNA
TGME49_457630	5.8S ribosomal RNA	TGME49_457730	28S ribosomal RNA
TGME49_457640	28S ribosomal RNA	TGME49_457810	5.8S ribosomal RNA
TGME49_457670	18S ribosomal RNA	TGME49_457840	5.8S ribosomal RNA

Table 2 – List of Gene promoters to which TgAP2III-2 binds.

Despite this strong promoter binding, RNA-seq analysis following AP2III-2 depletion showed no overlap between genes bound by TgAP2III-2 and those exhibiting changes in expression (Figure 23 A). This overlap analysis was performed to identify potential direct targets of TgAP2III-2 that are both bound and transcriptionally regulated by the factor. However, the lack of overlap suggests that TgAP2III-2 may not function as a conventional transcriptional regulator under the conditions tested, or that its regulatory effects are subtle or context-dependent. Additionally, as the RNA-seq relied on poly-A tail capture, any regulation of non-polyadenylated transcripts, such as rRNA, would not have been detected.

In addition to rRNA genes, TgAP2III-2 was found to bind to a variety of other promoters across the genome, including those associated with genes encoding hypothetical proteins and conserved sequences. The widespread promoter binding highlights the potential for TgAP2III-2 to play a broader regulatory role in *T. gondii*, though its precise function remains to be fully elucidated.

A



B

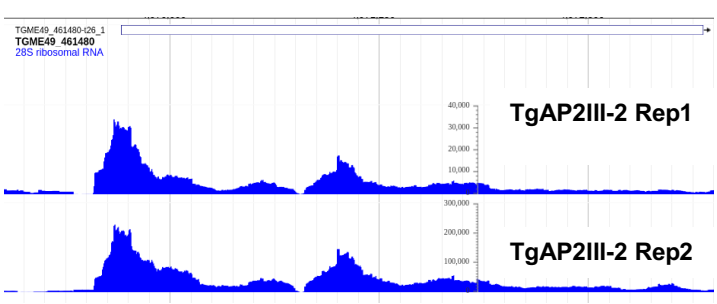


Figure 23 – TgAP2III-2 binds to many rRNA gene promoters. **(A)** Venn diagram depicting the overlap of RNA seq and CUT&Tag analysis showing there are no direct targets of TgAP2III-2. **(B)** CUT & Tag tracks of 2 replicates of TgAP2III-2-HA representing the direct targeting of TgAP2III-2 to the promoter 28S ribosomal RNA(TGME49_461480) as an example.

DISCUSSIONS AND PERSPECTIVES

Chapter 4 – Discussions and Perspectives

Proliferation is a key aspect of *T. gondii* pathogenesis, especially in the intermediate host, where tachyzoites rapidly replicate through endodyogeny, a unique form of asexual reproduction. The precise control of gene expression during the tachyzoite cell cycle, which governs various processes including daughter cell formation and virulence, is critical for the parasite's successful proliferation.

Temporal regulation of gene expression during daughter Cell Formation and Virulence Organelle Biogenesis

TgAP2XII-9 plays a crucial role in the formation of daughter cells after the initiation of budding. Our immunofluorescence assays (IFAs) showed that TgAP2XII-9 depletion causes severe defects in the inner membrane complex (IMC) scaffold organization, leading to disorganized IMC formation in the majority of vacuoles. These IMC defects are directly linked to improper cellular content segregation and failed daughter cell development. Interestingly, although TgAP2XII-9 depletion does not impact the early stages of budding, it appears to disrupt the later stages of IMC elongation, highlighting its specific role in these later phases of the cell cycle. This defect in IMC formation correlates with previously described roles of ApiAP2 transcription factors in the regulation of structural elements of the parasite's cytoskeleton and IMC. Specifically, we observed that TgAP2XII-9 represses early markers of budding, such as apical cap proteins (AC1 and IAP2)(Back et al., 2023) and basal complex proteins (BCC3 and BCC7)(Gubbels et al., 2022a), which are critical for daughter cell formation. This suggests that TgAP2XII-9 ensures the proper timing of IMC elongation and avoids prolonged expression of early bud markers, reinforcing the importance of temporal regulation during the cell cycle.

Our RNA sequencing (RNA-seq) and CUT & Tag data reveal that TgAP2XII-9 directly regulates a broad set of genes, including those encoding for proteins that are essential for daughter cell formation and virulence organelles. Intriguingly, TgAP2XII-9 acts as a dual regulator by repressing the expression of subset of genes encoding rhoptry

proteins, while simultaneously activating subset of genes involved in the biogenesis of micronemes and dense granules.

Rhoptries, which are secretory organelles that play a critical role in host cell invasion, are typically formed early in the S-phase, and our findings demonstrate that TgAP2XII-9 represses the expression of rhoptry-associated genes at this stage. In contrast, genes encoding for microneme proteins, which are also secreted during invasion but are formed later in the cell cycle, are activated by TgAP2XII-9 during the M-phase. This regulatory pattern highlights TgAP2XII-9's essential role in synchronizing the formation of virulence organelles with the overall progression of the cell cycle, ensuring that their biogenesis occurs at the appropriate time.

TgAP2XII-9 may play a critical role in regulating vesicular trafficking in the parasites by differentially controlling the expression of Rab11B and Rab18, two key Rab GTPases that are involved in the transport of materials essential for organelle formation and function. TgAP2XII-9 represses Rab11B, which is typically involved in endosomal recycling and membrane transport and IMC biogenesis (Agop-Nersesian et al., 2010). This repression early in the cell cycle may prevent premature recycling of vesicles that could misdirect or disrupt the proper trafficking of vesicles. Since rhoptries need to be precisely localized to the apical complex for host cell invasion, any premature trafficking or recycling driven by Rab11B could lead to incorrect organelle positioning and functional deficiencies. By repressing Rab11B, TgAP2XII-9 may ensure that rhoptry trafficking is delayed until the appropriate stage of the cell cycle when the apical complex is fully formed, safeguarding the parasite's invasive capabilities. On the other hand, TgAP2XII-9 activates Rab18, which is crucial for lipid droplet and membrane trafficking (Dejgaard et al., 2008), during the later stages of the cell cycle. TgAP2XII-9's activation of Rab18 likely facilitates lipid storage and mobilization, ensuring that the parasite has sufficient resources for membrane biogenesis. Rab18's involvement in lipid droplet metabolism is crucial for maintaining the proper lipid balance within the cell (Romano et al., 2017), which may indirectly support membrane formation required for organelles such as rhoptries and micronemes. The coordinated regulation of Rab18 may ensure that lipid droplets are available for the parasite's metabolic needs, and without this, membrane supply and lipid homeostasis might be disrupted, potentially compromising processes that depend on membrane integrity, such as organelle formation and host cell invasion.

ApiAP2 as repressors and activators

TgAP2XII-9 exibits a dual regulatory role that is reminiscent of the previously described activities of other ApiAP2 transcription factors, such as TgAP2IX-5, which also controls cell cycle progression and organelle formation. However, while TgAP2IX-5 primarily activates rhoptry genes, TgAP2XII-9 appears to act later in the cell cycle to repress these same genes and activate microneme genes. Previous studies have shown cooperativity between ApiAP2 TFs(Lesage et al., 2018). This suggests that ApiAP2s may form different complexes that are able to either activate or repress gene expression. TgAP2XI-5 cooperates with TgAP2X-5 to regulate a specific subset of virulence genes during the S and M phases of the cell cycle (Lesage et al.,2018). This indicates that multiple actors are required for the correct temporal expression of these virulence genes.

TgAP2XII-9's function in repressing a subset of ROPs and activating a subset of MICs can be understood as part of a larger regulatory network involving cooperative interactions between transcription factors like TgAP2XI-5 and TgAP2X-5. The cooperative binding suggests that these factors work in unison to regulate gene expression during specific stages of the cell cycle. TgAP2XII-9 integrates into this network by controlling the timing of rhoptry and microneme gene expression. This highlights the complex coordination among ApiAP2 transcription factors in regulating the parasite's developmental and invasive processes. To further concretize the attempt to decipher this regulatory network further investigations into the interacting partners of TgAP2XII-9 is required. Proximity labelling could be used to decipher the interactome.

Interconnection between transcriptional and post-translational regulation of the cell cycle.

TgAP2XII-9 also plays a critical role in coordinating the duplication and segregation of cellular organelles during the cell cycle. While our data indicate that TgAP2XII-9 is not directly involved in the early stages of organelle division (such as plastid and Golgi segregation), its depletion leads to the accumulation of multiple nuclei per parasite.

These findings align with previous studies demonstrating that transcriptional regulation of cell cycle-associated genes is essential for the proper temporal control of organelle duplication (Khelifa et al., 2021, Khelifa & Bhaskaran et al., 2024).

TgAP2XII-9 may also regulate the expression of several kinases involved in cell cycle progression, including cyclin-dependent kinases like TgCRK1 and TgCRK6. These kinases are crucial for regulating transitions between different phases of the cell cycle. TgCRK6, an essential cyclin-dependent kinase (Cdk)-related kinase, is pivotal for proper progression through mitosis. In conjunction with TgCyc1, TgCRK6 regulates key processes such as the spindle assembly checkpoint and the centromere-associated network during metaphase, ensuring accurate chromosome segregation and successful cell division (Hawkins et al., 2022). Interestingly, the TgCRK6 promoter is directly bound by TgAP2XII-9, and its transcript is repressed in TgAP2XII-9-depleted parasites. This interaction could represent a sophisticated mechanism by which TgAP2XII-9 fine-tunes cell cycle progression, preventing premature mitotic events until other critical processes, such as endodyogeny and apicoplast division (Shi et al.,2024), are completed. By modulating TgCRK6 expression, TgAP2XII-9 may ensure that mitosis does not proceed prematurely, acting like a checkpoint that coordinates the timing of cell cycle events with the broader requirements of the cell.

Notably, the role of TgCRK6 may extend to interacting with TgAP2IX-5 (Hawkins et al.,2022), which is important for the initiation of budding. Both TgCRK6 and TgAP2IX-5 share similar temporal expression patterns and localization, and it has been speculated that TgCRK6 may serve to inactivate TgAP2IX-5 (Hawkins et al.,2022) after budding initiation. This suggests a regulatory loop where TgAP2XII-9, which is activated by TgAP2IX-5, represses TgCRK6 expression once its role in inactivating TgAP2IX-5 is no longer required (i.e after budding initiation). This temporal control might ensure that cell cycle progression is tightly regulated, balancing the needs of daughter cell budding, organelle biogenesis, and mitotic events.

Additionally, TgAP2XII-9 represses Protein Phosphatase 1 (PP1), which is critical for daughter cell formation PP1 is a key regulator of multiple processes during cell division, including the proper assembly of the inner membrane complex (IMC) and the correct segregation of nuclei (Khelifa & Bhaskaran et al., 2024). PP1's involvement in dephosphorylating critical cell cycle proteins might be essential for maintaining proper

mitotic checkpoints and ensuring the successful transition from one phase of the cell cycle to the next (Khelifa & Bhaskaran et al.,2024). This highlighs the possible interconnection of transcriptional regulation and post-transcriptional regulation of the cell cycle.

AP2 domain function

The AP2 domain is typically considered the hallmark of the ApiAP2 family, responsible for DNA binding and transcriptional regulation. However, the discovery that in TgAP2IX-5, the AP2 domain is not essential for DNA binding (Sarah Khelifa, 2021) challenges this assumption and opens new avenues for understanding the versatility of this family of transcription factors. In the case of TgAP2IX-5, another domain appears to compensate for the AP2 domain's traditional DNA-binding role. This functional redundancy suggests that the DNA-binding activity within ApiAP2 transcription factors may be more flexible than previously thought. In some ApiAP2 members, the AP2 domain may serve other functions, such as protein-protein interactions or chromatin targeting, while other domains take on the role of DNA binding. This versatility could be an evolutionary adaptation allowing *T. gondii* to regulate diverse gene sets in different life cycle stages.

While the AP2 domain is crucial for TgAP2XII-9's function in directly binding DNA and regulating gene expression, its diminished importance in TgAP2IX-5 (Sarah Khelifa, 2021.) suggests that AP2 domains may also have evolved non-canonical roles in *T. gondii*. These roles might include recruiting co-factors, interacting with chromatin remodelers, or facilitating the formation of multi-protein transcriptional complexes. The diversity of functions observed across the ApiAP2 family highlights the adaptability of these transcription factors in responding to different cellular and environmental conditions.

TgAP2III-2 regulatory role

One of the key distinctions between TgAP2XII-9 and TgAP2III-2 is their effect on gene expression. While TgAP2XII-9 serves as both an activator and repressor of gene expression during specific stages of the cell cycle, TgAP2III-2's transcriptional effects are less pronounced. TgAP2III-2 binds extensively to the promoters of ribosomal RNA

(rRNA) genes, but its depletion does not result in significant changes in transcript levels of these genes as measured by RNA-seq.

The lack of significant transcriptomic changes upon TgAP2III-2 depletion, despite its extensive chromatin binding, implies that TgAP2III-2 could play a redundant role in regulating these promoters. Alternatively, the preparation of the libraries for RNA-seq involves a poly-A capture that may reduce our ability to identify rRNA variations after TgAP2III-2 depletion. Nevertheless, the lack of overlap between the RNA-seq and Cut and Tag datasets suggests that TgAP2III-2 is not the main drivers of gene expression.

The tight regulation of TgAP2III-2 gene and protein expression remain puzzling. Why would the expression TgAP2III-2 be restricted to particular cell cycle stage, when its depletion does not cause abnormal phenotypes? One way to answer this question would be to force the expression of TgAP2III-2 in the stages of the cell cycle where it is not normally expressed using an overexpression construct (promoter change or DD domain).

Alternatively, TgAP2III-2 may have other role during the life cycle of the parasite. For instance, during the switch from tachyzoite to bradyzoite stages, which involves significant changes in gene expression, TgAP2III-2 may help coordinate the epigenetic landscape that enables this differentiation.

Further investigation is needed to fully elucidate TgAP2III-2's role in chromatin regulation and its relationship with TgAP2XII-9. Future experiments could focus on identifying the chromatin modifications associated with TgAP2III-2 binding sites, such as through ATACseq analysis of histone marks or the use of chromatin accessibility assays. Additionally, exploring the potential interactions between TgAP2III-2 and known chromatin remodelers, such as the MORC complex, could provide insights into how TgAP2III-2 contributes to gene repression and the maintenance of chromatin architecture. Moreover, since TgAP2XII-9 and TgAP2III-2 both bind to the promoters of numerous target genes, it would be valuable to investigate whether these transcription factors collaborate in a sequential or combinatorial manner to regulate gene expression. For example, temporal analysis of gene expression during the cell cycle could reveal whether TgAP2III-2 acts to prime genes for later activation by

TgAP2XII-9 or if it helps to shut down transcription once TgAP2XII-9 has completed its regulatory role.

Coordination of Developmental Pathways and Chromatin Regulation by TgAP2XII-9 and TgAP2XII-2

Our data also suggest that TgAP2XII-9 may play a role in modulating the parasite's differentiation into bradyzoites, the dormant stage of *T. gondii* that is crucial for chronic infection. Interestingly, many of the genes downregulated in response to TgAP2XII-9 knockdown are preferentially expressed in bradyzoites, and several bradyzoite-specific ApiAP2 transcription factors (such as TgAP2IV-4 and TgAP2IX-9) are regulated by TgAP2XII-9. This suggests that TgAP2XII-9 may create a permissive environment for the initiation of bradyzoite differentiation by modulating the expression of other key transcription factors involved in this process during the S/M phase of the cell cycle.

Our data also suggests that AP2XII-9 likely plays a role in modulating sexual development in *T.gondii* by repressing AP2XI-2, which in turn represses genes involved in merozoite and EES (enteroepithelial stage) development. When AP2XII-9 is depleted, AP2XI-2 expression increases, which could explain the observed downregulation of EES genes after AP2XII-9 depletion, as AP2XI-2 likely enhances repression of these genes.

The observation that out of 300 direct target genes of TgAP2XII-9, 242 are also bound by TgAP2XII-2 at their promoters, yet no transcriptomic changes occur upon TgAP2XII-2 depletion, provides an intriguing window into the functional dynamics between these two transcription factors. It suggests that while TgAP2XII-2 binds to many of the same genes as TgAP2XII-9, its regulatory role may be fundamentally different or even more subtle than that of TgAP2XII-9. This divergence in functionality could be explained by several hypotheses that reflect the complexities of gene regulation in this parasite. One explanation is that TgAP2XII-2's binding to these promoters is functionally redundant with TgAP2XII-9. In this scenario, TgAP2XII-9 may serve as the primary transcription factor driving changes in gene expression, while TgAP2XII-2 acts as a backup regulator. When TgAP2XII-2 is depleted, TgAP2XII-9 might compensate for its absence, thereby preventing significant transcriptomic changes. Redundancy of this kind is not uncommon in gene regulatory networks,

where multiple transcription factors can bind to the same promoters to ensure that gene regulation is robust and resilient under various conditions.

TgAP2XII-2 interacts with the HDAC3/MORC complex to silence developmentally regulated genes, including those involved in sexual commitment (Srivastava et al., 2023). In this model, TgAP2XII-2 may be required for maintaining a poised or repressed chromatin state at specific promoters, but its depletion does not cause immediate transcriptomic changes because TgAP2XII-9 dominates transcriptional control. This could also explain why the depletion of TgAP2XII-2 has minimal effects on gene expression.

It is also possible that TgAP2XII-2's role in gene regulation is context- or stage-specific. While TgAP2XII-2 binds to many of the same promoters as TgAP2XII-9, its regulatory effect may only become significant in response to specific environmental cues. This idea is supported by findings that TgAP2XII-2 is involved in the repression of specific genes such as AP2X-10 and AAH1 (Srivastava et al., 2023) involved in sexual development of *T. gondii*.

Additionally, TgAP2XII-2 might have functionally distinct roles despite sharing target genes with TgAP2XII-9. TgAP2XII-2 could be contributing to the fine-tuning of gene expression or chromatin structure. This could explain why transcriptomic changes are not immediately observed following TgAP2XII-2 depletion, as its primary function may not involve driving transcription but rather modulating the chromatin landscape or other regulatory pathways.

Moreover, the overlap in binding sites between TgAP2XII-2 and TgAP2XII-9 raises the possibility of cross-talk between these transcription factors. TgAP2XII-2 may modulate or enhance TgAP2XII-9’s regulatory activity, perhaps by influencing its binding affinity to promoters or facilitating the recruitment of co-factors such as chromatin remodelers. This cross-regulation could result in TgAP2XII-2 playing a supportive role in TgAP2XII-9-mediated gene regulation, with its depletion having little immediate impact because TgAP2XII-9 remains active.

Negative expression feedback loops.

One of the more surprising findings of our study is that TgAP2XII-9 appears to regulate its own expression. Our CUT & Tag data indicate that TgAP2XII-9 is enriched at its own promoter, suggesting that it may exert autoregulatory control over its transcription. This autoregulation is likely part of a negative feedback loop, which serves to fine-tune the temporal expression of TgAP2XII-9 during the cell cycle. Autoregulation of ApiAP2 transcription factors has been previously described, most notably in TgAP2IX-5 (Khelifa et al.,2021) and TgAP2XII-2 (Srivastava et al., 2020), and our data suggest that TgAP2XII-9 operates under a similar mechanism. This self-regulatory mechanism adds another layer of complexity to the transcriptional networks governing the tachyzoite cell cycle, ensuring that TgAP2XII-9 expression is tightly controlled and rapidly downregulated once its function is no longer required.

Stitching it together

The intricate regulatory network involving the ApiAP2 transcription factors in *T. gondii* is a highly coordinated system that controls gene expression in a stage- and developmentally-dependent manner. The relationships between TgAP2XI-5, TgAP2X-5, TgAP2IX-5, TgAP2XII-9, and TgAP2III-2 reveal an elaborate regulatory network, ensuring that critical transcriptional programs are activated or repressed at the correct times during the parasite's life cycle. Understanding this regulatory network is essential for deciphering how *T. gondii* transitions through its life stages, coordinates daughter cell formation, and prepares for invasion.

AP2IX-5 plays a crucial role during the early stages of the cell cycle, especially during the S phase, where it regulates genes required for the early stages of daughter cell budding (Khelifa et al.,2021). AP2IX-5 also controls the expression of downstream transcription factors, such as AP2XII-9 and AP2III-2, indicating its importance as an upstream regulator in the gene network (Khelifa et al.,2021).

AP2XI-5 binds to the promoters of more than 300 genes, including many involved in invasion and virulence, such as those encoding rhoptries and micronemes (Walker et al.,2013). However, its regulatory role as either an activator or repressor remains unclear due to the lack of RNA-seq data in absence of AP2XI-5. Despite this, AP2XI-

5's binding to active promoters during the S and M phases suggests that it likely plays a role in gene activation. Notably, AP2XI-5 also binds to the promoter of AP2IX-5, indicating a potential feedback loop that may modulate the expression of this master regulator. This feedback mechanism highlights a significant interaction in the regulatory hierarchy, where AP2XI-5 could either reinforce or modulate AP2IX-5's activity, influencing downstream transcriptional programs. The exact nature of this regulation—whether activating or repressing—requires further investigation, but the potential for such interactions underscores the complexity of the gene regulatory network in *T. gondii*.

AP2XI-5's binding to the promoters of genes regulated by AP2XII-9 introduces a cooperative layer of regulation. There are 80 genes whose promoters are bound by both AP2XI-5 and AP2XII-9, but without RNA-seq data for AP2XI-5, it remains uncertain whether AP2XI-5 acts to activate or repress these genes. The dual binding suggests that AP2XI-5 may work in conjunction with AP2XII-9 to fine-tune the expression of these genes, potentially modulating their transcription based on cell cycle cues.AP2XII-9's role as both an activator and repressor at different stages of the cell cycle raises the possibility that AP2XI-5 could assist in these regulatory switches.

Together, these transcription factors form a sophisticated, multilayered network that ensures the precise timing of gene expression necessary for *T. gondii's* replication and virulence. Further studies, including RNA-seq data for AP2XI-5, will be essential to fully understand the interactions and regulatory roles of these transcription factors.

This PhD study highlights the critical interplay between ApiAP2 transcription factors in regulating the expression of genes essential for *T. gondii* development. The precise control over these transcriptional programs ensures that daughter cell formation, organelle biogenesis, and host cell invasion are tightly coordinated, allowing the parasite to successfully proliferate and adapt to different environmental conditions. Further research into the interactions between these transcription factors and their co-regulators will deepen our understanding of the regulatory networks that underpin *T. gondii's* pathogenesis

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ANNEXE-1

PLOS PATHOGENS

RESEARCH ARTICLE

Cascading expression of ApiAP2 transcription factors controls daughter cell assembly in *Toxoplasma gondii*

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Abstract

Pathogenesis of *Toxoplasma gondii* in the intermediate host is based on the tachyzoite ability to divide rapidly to produce significant amount of daughter cells in a reduce time frame. The regulation of the cell-cycle specific expression program is therefore key to their proliferation. Transcriptional regulation has a crucial role in establishing this expression program and transcription factors regulates many aspects of tachyzoite cell cycle. We explored the role of two ApiAP2 transcription factors, TgAP2XII-9 and TgAP2III-2, during the cell cycle of the tachyzoite form. While TgAP2III-2 has only a minor impact on the tachyzoite proliferation, we show that TgAP2XII-9 regulates many aspects of the cell cycle including the proper assembly of the daughter cells inner membrane complex and temporal expression of many virulence genes. Creation of a double mutant strain for TgAP2XII-9 and TgAP2III-2 shows that TgAP2XII-9 had a prominent role during daughter cell assembly. Using transcriptomics and Cut&Tag, we demonstrate that TgAP2XII-9 mainly acts through the transcriptional control of at least 300 genes promoters. Interestingly, TgAP2XII-9 plays a crucial role repressing the expression of genes necessary for budding initiation and activating genes necessary for microneme *de novo* formation. We also explored the importance of the AP2 domain of TgAP2XII-9 demonstrating its critical role to exert its function. Therefore, we showed that TgAP2XII-9 is a crucial transcription factor which is key to daughter cell assembly post budding initiation.

Author summary

Toxoplasma gondii is a is a unicellular eukaryotic pathogen that infects a wide range of animals. Pathogenesis of this parasite relies on its ability to rapidly proliferate and disseminate in the host. The *T. gondii* tachyzoite is the fast replicating form of the parasite that causes all the symptoms of toxoplasmosis in humans. Replication of this parasite form relies on a simplified and fast cell-cycle that is mainly controlled at the transcriptional level. We studied two transcription factors that bear differential role in regulating the establishment of the cell-cycle dependent expression profiles after initiation of daughter

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cell formation. We used trancriptomics, CUT&Tag and phenotypic assays to decipher the roles of these ApiAP2 transcription factors. Our data highlight the crucial role of TgAP2-XII-9 both as a repressor and activator of subset of genes. This transcription factor activates the expression of virulence factor genes such as micronemes encoding transcripts therefore regulating the biogenesis of these organelles. This study shows that ApiAP2 transcription factors are timely expressed to activate the expression profile needed at each phase of the cell cycle.

Introduction

Toxoplasma gondii is a eukaryotic pathogen classified within the phylum Apicomplexa, which encompasses many protozoan parasites of significant medical and veterinary concern, including *Plasmodium* (the causative agent of malaria) and *Cryptosporidium* (responsible for cryptosporidiosis). *T. gondii* has garnered considerable attention as an opportunistic pathogen linked to encephalitis and systemic infections in immunocompromised individuals, particularly those with HIV/AIDS [1]. Approximately one-third of the world's population is estimated to be infected by *T. gondii*. This parasite has the ability to cross the blood-brain barrier and establish a chronic infection by differentiating into a dormant, drug-resistant bradyzoite stage [2]. The rapidly growing tachyzoite form of the parasite is responsible for the clinical manifestations of the disease in humans. The tachyzoite's ability to proliferate is crucial to its pathogenesis. *T. gondii* tachyzoites exhibit an unusual cell cycle characterized by closed mitosis. The cell cycle phases include: G1, S, and M. Up until recently the G2 phase was thought to be short or absent [3]. A recent study by Hawkins et al.,2024 [4] strongly suggested the presence of a short G2 phase that overlaps with S/M phase. Tachyzoites multiply asexually within the host cell through a process called endodyogeny where two daughter parasites form within the parent parasite. The G1 phase of *T. gondii* endodyogeny, when canonical housekeeping tasks preparing for the S phase occur, comprises about half of the division cycle. During the S phase, chromosome replication and organelle duplication is coordinated with the initiation of the budding in a process called budding [3]. Centriole duplication occurs early in the S-phase, and budding begins late in the S-phase of the cell cycle. Early on, cortical microtubules and the conoid form near the centrioles, anchored by the apical polar ring (APR), which acts as a microtubule-organizing center [5]. The APR organizes microtubules (MT) growth and establishes apical-basal polarity, essential for structural organization [6]. The basal complex is formed concomitantly to the APR, as TgMORN1 and TgBCC0 are among the initial components deposited within the budding daughter cells, positioned in alignment with the duplicated centrosomes [7–14]. These proteins coordinate cytoskeletal development, with TgBCC0 and other basal complex proteins guiding the five-fold structural symmetry of the daughter cells [15]. The inner membrane complex (IMC) of the daughter cells is generated from the apical pole, beginning with the formation of the apical cap of the IMC, followed by the central IMC and basal IMC sub-compartments [16]. This process first encapsulates the divided Golgi [17], followed by the apicoplast [18] and subsequently nucleus and endoplasmic reticulum [19]. As the daughter parasites mature, the maternal cytoskeleton disintegrates, and the maternal plasma membrane is repurposed onto the emerging daughters [12]. The tachyzoite cell cycle is regulated by both transcriptional and post-translational mechanisms. For instance, transcription factors (TFs) such as TgAP2X-5 and TgAP2XI-5 have been demonstrated to control the cell-cycle-dependent expression of many virulence factors [20]. Cyclin dependent kinases (cdks) and cyclins serve as the primary regulators of the cell cycle, orchestrating progression

through various phases. Transcription factors, acting downstream of these regulators, execute specific transcriptional programs in response to Cdk and cyclin signalling [21–23]. AP2 transcription factors function as critical downstream effectors, controlling essential processes like replication and daughter cell budding in response to cell cycle cues. The majority of transcriptional regulators of the tachyzoite cell cycle belong to the plant-like Arabidopsis APETALA-2 (AP-2) family [24,25]. The AP2 domain functions as a DNA-binding domain within AP2 transcription factors, allowing each factor to bind specific promoter regions and regulate gene expression. In *T. gondii*, cell cycle-dependent expression profiles can be regulated by the cooperative action multiple AP2 transcription factors. These TFs may act in concert or sequentially at different stages of the cell cycle to fine-tune the expression of target genes essential for cell division and development [20]. The roles of some of the AP2 TFs have been uncovered. For instance, TgAP2XII-8 regulates ribosomal protein production during the early G1 phase [26]. TgAP2IX-5 controls the initial steps of budding [27] by regulating the expression of hundreds of genes, including those coding for elements destined for early incorporation into the developing daughter buds, such as apical cap proteins TgAC2, TgAC7, and TgISP1 and budding markers such as TgIMC1, TgIMC3, TgIMC4, and TgIMC10. Additionally, TgAP2IX-5 directly controls the expression of other TFs, notably TgAP2III-2, TgAP2XII-2, and TgAP2XII-9. TgAP2XII-2 is essential for the proper progression through S-phase [28] indicating that TgAP2IX-5 controls the expression of TFs that may be essential for the continuation of the cell cycle. The roles of TgAP2III-2 and TgAP2XII-9 remain unexplored.

In this study, we functionally characterized two cell-cycle-dependent ApiAP2 TFs, TgAP2XII-9 and TgAP2III-2, whose expression is directly regulated by TgAP2IX-5. While TgAP2III-2 had non-essential roles during the tachyzoite cell cycle, we demonstrate that TgAP2XII-9 is crucial for proper formation of daughter parasites after budding initiation. TgAP2XII-9 acts as a repressor of a subset rhoptries, rhoptry neck proteins, and IMC apical cap genes. Conversely, it activates the expression of a set of genes encoding for micronemes and dense granule proteins. Thus, TgAP2XII-9 is a critical TF that controls gene expression during the daughter cell completion and the de novo formation of virulence organelles.

Results

TgAP2XII-9 and TgAP2III-2 are cell-cycle regulated and expressed during the S/M phase

The genome wide CRISPR screening performed by Sidik *et al.* in 2016 [29] allows the identification of genes essential for tachyzoite growth in the *T. gondii* genome. Genes with negative fitness scores are considered essential for the parasite's fitness [29]. The CRISPR phenotype scores for TgAP2XII-9 (TGME49_251740) and TgAP2III-2 (TGME49_253380) were -4.32 and -3.22, respectively, suggesting that both genes likely contribute to the parasite's fitness. To elucidate their role in regulating the cell cycle expression program of tachyzoites, we used an auxin-inducible degron (AID) system. This involved fusing the AID sequence and an HA-tag to the C-terminus of TgAP2XII-9 and TgAP2III-2 at their respective endogenous locus via a CRISPR/Cas9 strategy (S1A Fig). PCR confirmed the correct integration of the tag (S1Bi and S1Bii Fig). The AID system allows conditional depletion of the protein upon adding Auxin to the parasite's growth medium. Western Blot analysis validated the system's functionality, showing a distinct band at the expected protein's size (218 kDa and 185 kDa) in the absence of auxin (Fig 1A and 1B), with protein depletion occurring within 30 minutes of auxin addition for iKD TgAP2XII-9 and about 1 hour for iKD TgAP2III-2.

Subsequently, immunofluorescence assays using cell cycle markers such as TgCentrin1 (marking the outer core of the centrosome) revealed that TgAP2XII-9 and TgAP2III-2 localize

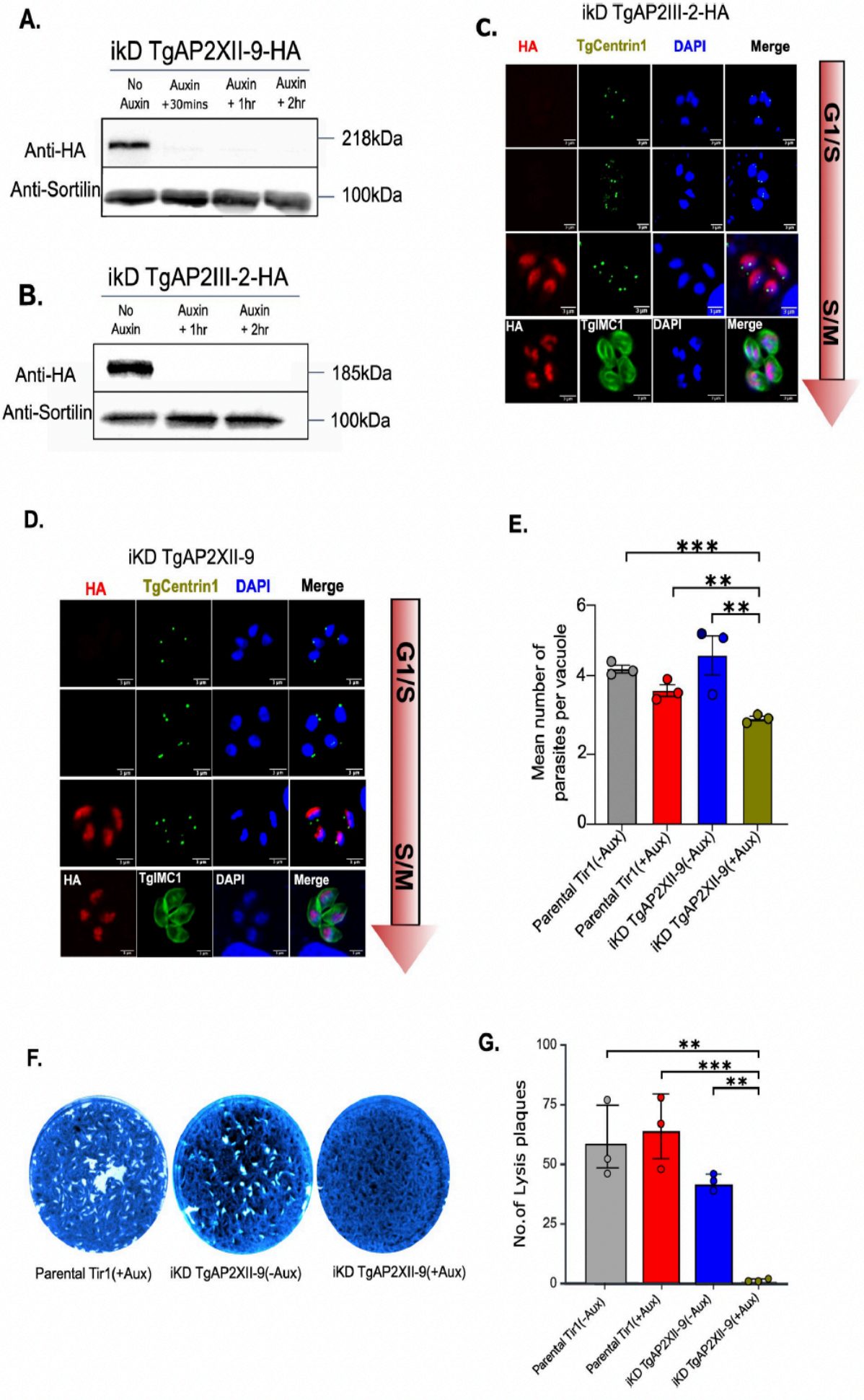
A.
ikD TgAP2XII-9-HA
No Auxin
Auxin +30mins
Auxin + 1hr
Auxin + 2hr
Anti-HA
Anti-Sortilin
218kDa
100kDa
B.
ikD TgAP2III-2-HA
No Auxin
Auxin + 1hr
Auxin + 2hr
Anti-HA
Anti-Sortilin
185kDa
100kDa
C.
ikD TgAP2III-2-HA
HA
TgCentrin1
DAPI
Merge
G1/S
S/M
HA
TgIMC1
DAPI
Merge
D.
iKD TgAP2XII-9
HA
TgCentrin1
DAPI
Merge
G1/S
S/M
HA
TgIMC1
DAPI
Merge
E.
Mean number of parasites per vacuole
0
2
4
6
***
**
**
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)
F.
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)
G.
No.of Lysis plaques
0
25
50
75
100
**
***
**
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)

Fig 1. TgAP2XII-9 and TgAP2III-2 are cell-cycle regulated and expressed during the S/M phase. TgAP2XII-9 is essential for growth and proliferation *in vitro* (A, B) Western blot analysis of total protein extracts from parental and iKD TgAP2XII-9 and TgAP2III-2 strains treated with Auxin for varying durations. The blots were probed with anti-HA to detect TgAP2XII-9 and TgAP2III-2 protein levels (upper panel) and with anti-TgSortilin as a normalization control (lower panel), validating the AID system. **(C, D)** Cell cycle expression of TgAP2III-2 and AP2XII-9 as realized by IFA using anti-HA antibody and cell cycle markers TgCentrin1 and TgIMC1. Scale bar = 3 μm **(E)** Growth assay for parental and iKD TgAP2XII-9 strains with and without 24-hour auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{***}p < 0.001$, $^{*}p<0.05$, Data are presented as mean ± s.d. (n = 3). **(F)** Plaque assay depicting proliferation and growth of Parental and iKD AP2XII-9 strains with and without auxin for 7 days. **(G)** Quantification of the no.of lysis plaques for parental and iKD TgAP2XII-9 strains. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{**}p < 0.01$, $^{*}p<0.05$, Data are presented as mean ± s.d. (n = 3).

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to the nucleus and are expressed during the late S and early M phase (Fig 1C and 1D). Their expression is absent during the G1 phase before centrosome duplication and early S phase when centrosomes have duplicated but remain in close proximity to each other (Fig 1C and 1D, first two panels). During the late S and M phases, when centrosomes are segregated and when daughter cell formation (indicated by the budding marker TgIMC1) occurs, TgAP2XII-9 and TgAP2III-2 proteins are expressed (Fig 1C and 1D, last two panels), indicating that their expression peaks after that of TgAP2IX-5. Previous studies by Behnke et al. (2010) [30] and Khelifa et al. (2021) [27] revealed that TgAP2IX-5 expression peaks earlier in the cell cycle. TgAP2IX-5 controls the expression of TgAP2XII-9 and TgAP2III-2, which is consistent with their sequential roles in coordinating cell cycle progression and daughter cell formation.

TgAP2XII-9 is crucial for the growth and proliferation of tachyzoites in vitro

To functionally characterize TgAP2XII-9 and TgAP2III-2, we performed a standard growth assay to assess parasite replication within host cells 24 hours after adding auxin. We found that for TgAP2XII-9, the mean number of parasites per vacuole decreased to 2.8, while in the controls, the mean number was ~4.4 (Fig 1E). This indicates a clear defect in parasite proliferation. Further phenotypic analyses revealed that the degradation of TgAP2XII-9 significantly impairs replication capacity *in vitro*. In a plaque assay, mutant parasites exposed to auxin failed to form any lysis plaques on a monolayer of host cells 7 days post-infection, unlike the parental strain under the same conditions (Fig 1F). Quantifying plaque numbers in each strain (Fig 1G), with and without auxin, also demonstrates that TgAP2XII-9 expression is crucial for the parasite's growth and proliferation *in vitro*. In contrast, the TgAP2III-2 iKD strain did not show any replication defects in the presence or absence of auxin and plaque assays showed a normal capacity to form plaques in the presence of auxin and after TgAP2III-2 depletion (S1Ci and S1Cii Fig). This indicates that TgAP2XII-9 is crucial for tachyzoite proliferation while TgAP2III-2 is dispensable.

TgAP2XII-9 is crucial for the proper formation of daughter parasites

To further explore the biological function of TgAP2XII-9, we conducted immunofluorescence assays (IFAs) to inspect the IMC formation (Fig 2A) after a short 6-hour auxin treatment (representing the time needed for the parasite to complete one cell cycle). In the absence of TgAP2XII-9, the parasites displayed defects in IMC scaffold organization, resulting in the formation of disordered IMCs (Fig 2A, lower panel). Conversely, TgAP2XII-9 parasites formed well-structured vacuoles without auxin (Fig 2A, upper panel). Quantitative analysis of this phenotype revealed that approximately 80% of vacuoles contained disorganized IMCs after 6 hours of auxin treatment (Fig 2B). Given the crucial role of the IMC in daughter cell formation, proper cellular content segregation, and daughter cell scaffold formation, we aimed to

Figure 2

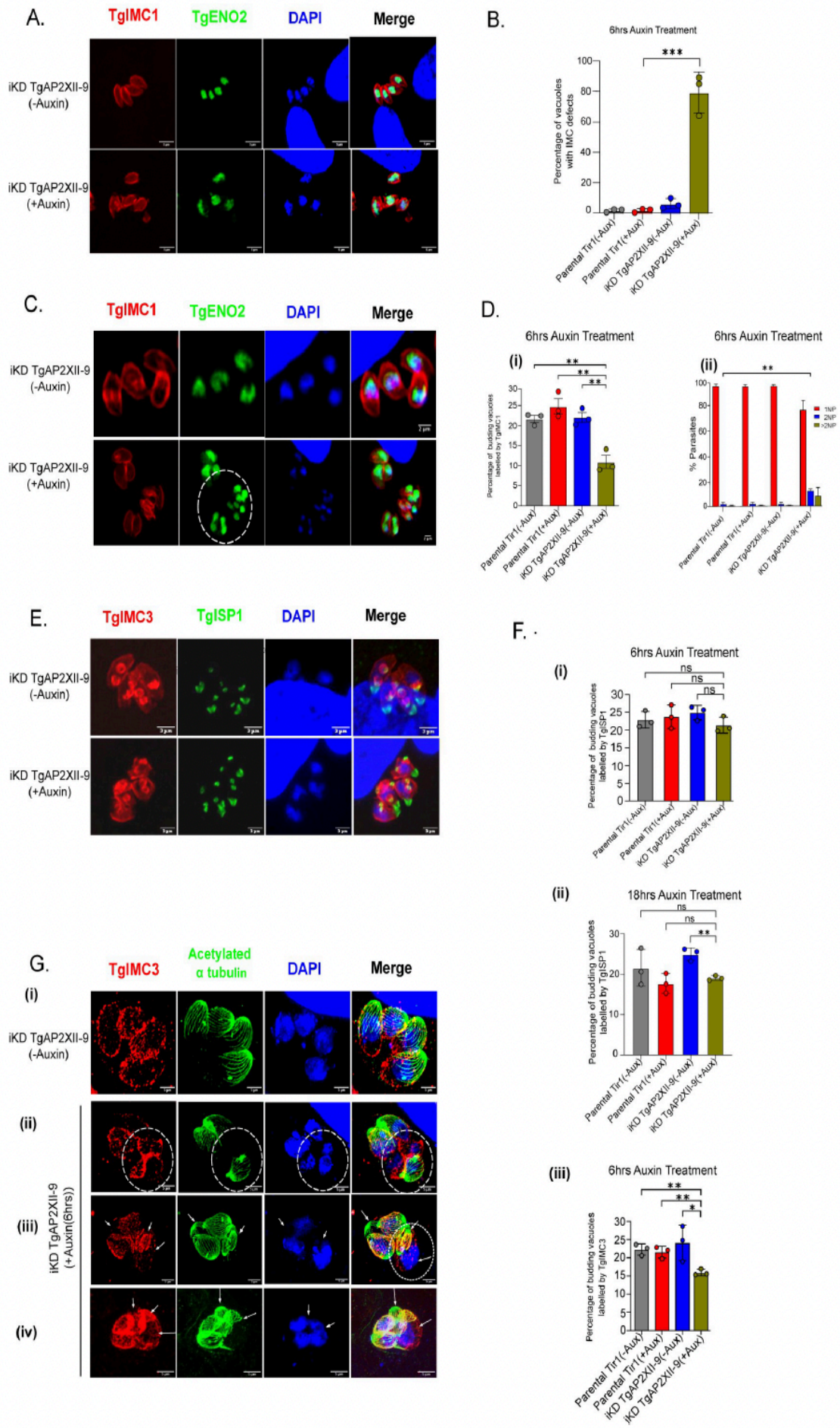
A.
TgIMC1
TgENO2
DAPI
Merge
iKD TgAP2XII-9 (-Auxin)
iKD TgAP2XII-9 (+Auxin)
B.
6hrs Auxin Treatment
***
Percentage of vacuoles with IMC defects
Parental Tir1(-Aux)
Parental Tir1(+Aux)
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)
C.
TgIMC1
TgENO2
DAPI
Merge
iKD TgAP2XII-9 (-Auxin)
iKD TgAP2XII-9 (+Auxin)
D.
6hrs Auxin Treatment
(i)
Percentage of budding vacuoles labelled by TgIMC1
6hrs Auxin Treatment
(ii)
% Parasites
1NP
2NP
>2NP
E.
TgIMC3
TgISP1
DAPI
Merge
iKD TgAP2XII-9 (-Auxin)
iKD TgAP2XII-9 (+Auxin)
F.
6hrs Auxin Treatment
(i)
Percentage of budding vacuoles labelled by TgISP1
ns
18hrs Auxin Treatment
(ii)
6hrs Auxin Treatment
(iii)
Percentage of budding vacuoles labelled by TgIMC3
G.
TgIMC3
Acetylated α tubulin
DAPI
Merge
(i)
iKD TgAP2XII-9 (-Auxin)
(ii)
(iii)
(iv)
iKD TgAP2XII-9 (+Auxin(6hrs))

Fig 2. TgAP2XII-9 is crucial for the proper formation of daughter parasites. (A) Defects in the IMC formation or organization observed after 6 hours Auxin treatment as realized by IFA and confocal imaging with TgIMC1 and TgENO2; Scale bar = 5μm (B) Quantification of the IMC defect phenotype between the parental and iKD TgAP2XII-9 strains on 6hrs of auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{***}p < 0.001$, data are presented as mean ± s.d. (n = 3) (C) IFA and confocal imaging depicting the budding of iKD TgAP2XII-9 parasites and accumulation of multiple nuclei after the addition of Auxin using anti-TgIMC1 and anti-TgENO2 antibodies; scale bar = 2μm (D)(i) Quantification of percentage of vacuoles undergoing budding after 6hrs auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{**}p < 0.01$, data are presented as mean ± s.d. (n = 3). (D)(ii) Quantification of percentage of vacuoles having multiple nuclei. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{**}p < 0.01$, data are presented as mean ± s.d. (n = 3), N represents nuclei and P is parasites. (E) IFA and confocal imaging depicting the budding of parasites labelled by TgIMC3 and TgISP1; scale bar = 3 μm. (F) (i) Quantification of budding vacuoles using anti-TgISP1 after 6hrs of Auxin treatment, a two tailed Student's Statistical analysis was performed using a two-tailed Student's T-test, with significance indicated by ns>0.05, data are presented as mean ± s.d. (n = 3). (F) (ii) Quantification of budding vacuoles using anti-TgISP1 after 18hrs of Auxin treatment, a two tailed Student's Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns>0.05, $^{**}p < 0.01$ data are presented as mean ± s.d. (n = 3). (F) (iii) Quantification of budding vacuoles using anti-TgIMC3 after 6hrs of Auxin treatment, Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{**}p < 0.01$, $^{*}p<0.05$, data are presented as mean ± s.d. (n = 3). (G) Expansion microscopy depicting defects in the IMC (circled) and deformations in the cytoskeleton (panel (iv)). The parasite IMC (IMC3, red) and cytoskeleton (acetylated tubulin, green) were labelled as well as the nucleus by DAPI (blue). Parasite without nucleus(panel(iii)) and a parasite bearing multiple nuclei(panel (iii)) are indicated by white arrows. Scale bar = 5 μm.

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investigate the impact of TgAP2XII-9 on daughter cell development. Using TgIMC1 as a marker, we assessed the proportion of vacuoles undergoing budding following a 6-hour auxin exposure. It became evident that there was a notable decrease in the percentage of budding vacuoles, indicating the parasites' compromised ability to generate daughter cells correctly compared to mutant parasites cultured without auxin (see Fig 2C and 2Di).

Additionally, the number of nuclei per parasite increased as a consequence of the inability of the daughter parasites to correctly bud, as depicted in Fig 2C (lower panel, iKD TgAP2XII-9 + Auxin, within the enclosed circle). Quantitative analysis revealed a significant proportion of vacuoles with two or more nuclei per parasite in the presence of auxin (Fig 2Dii), while this phenotype was nearly absent in the parental strain and the mutant strain in the absence of auxin. Taken together, these results suggest that TgAP2XII-9 is crucial for the proper formation and completion of the daughter cell scaffold.

TgAP2XII-9 exhibits no discernible effect on the initiation of daughter parasite budding

To investigate the role of TgAP2XII-9 in the budding process, we used an early budding marker (TgISP1, apical cap) (Fig 2E) as a proxy for the budding initiation. Quantification of budding vacuoles after 6 hours of auxin treatment revealed no significant difference in the proportion of vacuoles undergoing budding (Fig 2Fi). However, after overnight (~18 hours) auxin treatment, there was a slight decrease in budding vacuoles compared to mutant parasites without auxin (Fig 2Fii). This reduction could be due to prolonged auxin exposure causing indirect effects rather than a direct result of TgAP2XII-9 depletion. Additionally, since TgIMC3 is strongly enriched in the daughter buds and is a later marker than TgISP1, we quantified budding parasites using TgIMC3. We observed a significant decrease in budding vacuoles after 6 hours of auxin treatment compared to parental and mutant strains without auxin (Fig 2Fiii) indicating that TgAP2XII-9 depletion does not affect budding initiation but rather elongation of IMC as early as the appearance of TgIMC3 on daughter buds.

To better visualize the defects in the daughter cell formation, we performed expansion microscopy (Fig 2G). Although the parasite cytoskeleton is formed (as marked by acetylated-α-Tubulin), we confirmed the deformation of the IMC (Circled, Fig 2Gii). As expected, we could identify parasites with an accumulation of multiple nuclei (Circled, Fig 2Gii). We also

noticed that there were daughter parasites that were formed without a nucleus (circled, Fig 2Giii) and parasites that had no IMC or nucleus but with a well-formed cytoskeleton (arrowed, Fig 2Giii). There were also vacuoles with unorganized IMC and unsegregated nuclear material and abnormally formed cytoskeleton (arrowed, Fig 2Giv). These data indicate that TgAP2XII-9 has a profound influence on daughter cell formation and the coordination of this process.

We also examined the effect of TgAP2XII-9 depletion on organelle duplication and segregation. The Golgi complex and plastid were labelled in parasites (S2A Fig), and the Golgi-to-nucleus and plastid-to-nucleus ratios were calculated (S2Bi and S2Bii Fig). We found no significant difference in these ratios between the presence and absence of auxin, as expected since TgAP2XII-9 expression peaks after both organelle divisions. Overall, these findings suggest that TgAP2XII-9 is not involved in initiating daughter parasite budding but exerts its effects later in the budding cycle.

TgAP2XII-9 and TgAP2III-2 have no combinatorial effect on the parasite biology *in vitro*

To explore the combinatorial effects of TgAP2XII-9 and TgAP2III-2 depletion, we generated a double mutant strain by knocking out TgAP2III-2 in our inducible knockdown (iKD) TgAP2XII-9 mutant strain. In this strain, in the absence of auxin, only TgAP2III-2 is depleted, while in the presence of auxin, both TgAP2XII-9 and TgAP2III-2 proteins are depleted. This strain is hereafter referred to as the Double Mutant. As anticipated, no plaques were observed in the Double Mutant strain in the presence of auxin (S3Ai and S3Aii Fig), consistent with the essential nature of TgAP2XII-9 for the parasite.

Quantitative growth assays revealed no additive impact on parasite proliferation due to the simultaneous depletion of TgAP2III-2 and TgAP2XII-9 (S3B Fig). Moreover, the presence of auxin led to the accumulation of multiple nuclei in the Double Mutant, mirroring the phenotype observed in the iKD TgAP2XII-9 strain (S3C and S3D Fig). Further examination of the IMC defect phenotype, for which TgAP2XII-9 is critical, indicated no additional defects in the Double Mutant compared to the iKD TgAP2XII-9 strain (S3C Fig). This was confirmed by the quantification of the percentage of vacuoles with IMC defects (S3E Fig) which remained similar to that of iKD TgAP2XII-9 single mutant. These findings collectively indicate that depleting both TgAP2III-2 and TgAP2XII-9 does not result in an additive effect on parasite biology.

TgAP2XII-9 regulates the expression of various cell cycle-regulated genes

Given the potential role of TgAP2XII-9 as a transcription factor, we aimed to identify its regulated genes using RNA sequencing analysis. RNA sequencing was conducted at two time points: 2 hours post TgAP2XII-9 depletion to capture the genes immediately affected, and 6 hours post-depletion, corresponding to the completion of one tachyzoite cell cycle. Data analysis was performed using DESeq2 with an adjusted p-value cutoff of 0.01 and a minimum fold change of 2 (Fig 3A and 3B). Significant transcriptomic changes were observed in the iKD TgAP2XII-9 mutant, revealing 1569 (S1 Table) and 1398 (S2 Table) differentially expressed genes (DEGs) after 2 and 6 hours of auxin treatment, respectively. A substantial number of genes were common between both datasets. Overlapping these datasets resulted in a final set of 1329 DEGs, with 567 transcripts upregulated (Fig 3C and S3 Table) and 762 transcripts downregulated (Fig 3D and S4 Table) following TgAP2XII-9 depletion. We analysed the cell cycle expression of the upregulated and downregulated genes, displaying their expression profiles using a heatmap (S4A and S4B Fig). Majority of genes that were upregulated showed a peak expression during the late C and G1 phases, with a few peaking during the S phase (S4A Fig).

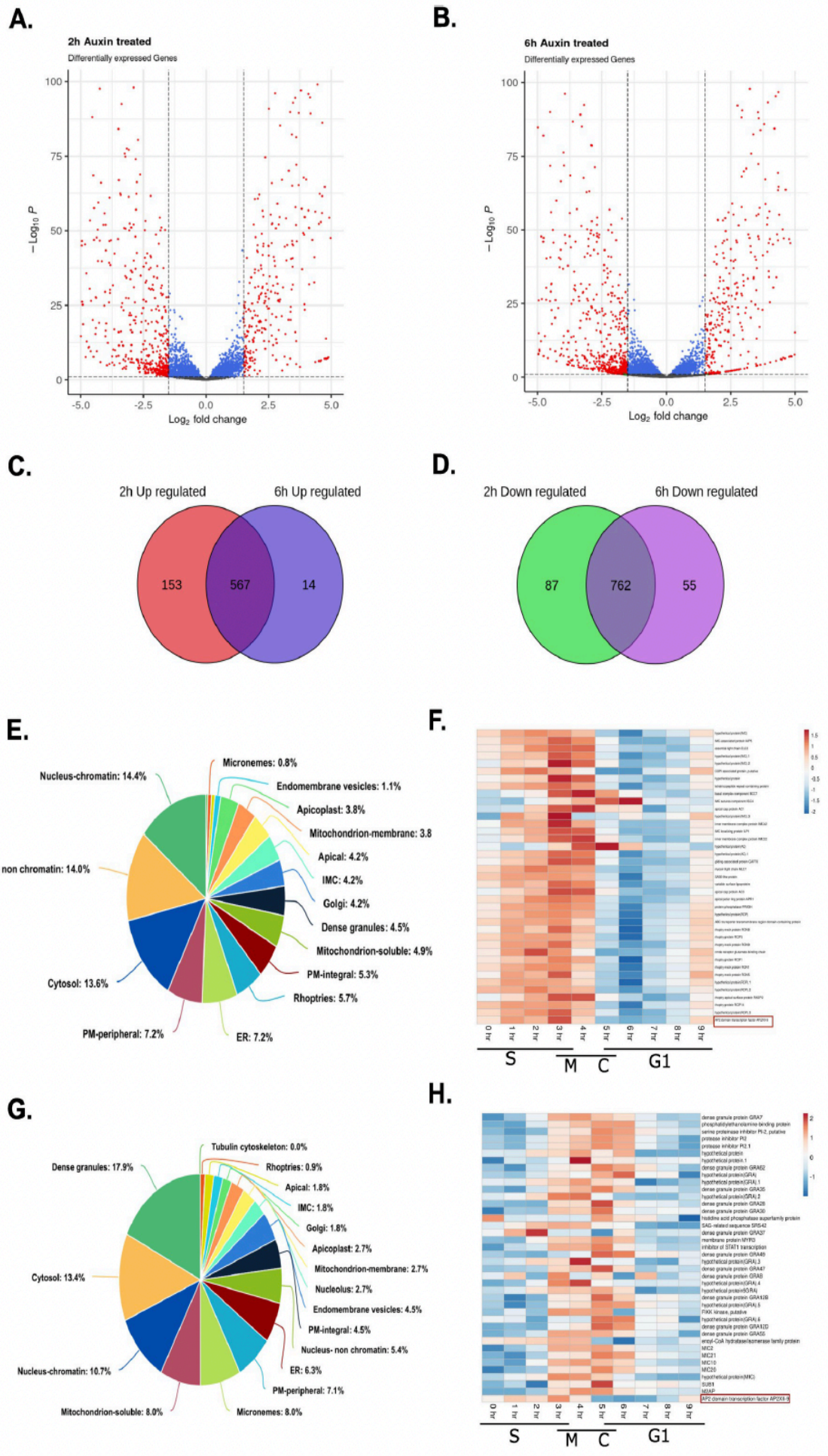
A.
2h Auxin treated
Differentially expressed Genes
−Log10 P
Log2 fold change
B.
6h Auxin treated
Differentially expressed Genes
−Log10 P
Log2 fold change
C.
2h Up regulated
6h Up regulated
153
567
14
D.
2h Down regulated
6h Down regulated
87
762
55
E.
Micronemes: 0.8%
Endomembrane vesicles: 1.1%
Apicoplast: 3.8%
Mitochondrion-membrane: 3.8
Apical: 4.2%
IMC: 4.2%
Golgi: 4.2%
Dense granules: 4.5%
Mitochondrion-soluble: 4.9%
PM-integral: 5.3%
Rhoptries: 5.7%
ER: 7.2%
PM-peripheral: 7.2%
Cytosol: 13.6%
Nucleus- non chromatin: 14.0%
Nucleus-chromatin: 14.4%
F.
S
M
C
G1
G.
Tubulin cytoskeleton: 0.0%
Rhoptries: 0.9%
Apical: 1.8%
IMC: 1.8%
Golgi: 1.8%
Apicoplast: 2.7%
Mitochondrion-membrane: 2.7%
Nucleolus: 2.7%
Endomembrane vesicles: 4.5%
PM-integral: 4.5%
Nucleus- non chromatin: 5.4%
ER: 6.3%
PM-peripheral: 7.1%
Micronemes: 8.0%
Mitochondrion-soluble: 8.0%
Nucleus-chromatin: 10.7%
Cytosol: 13.4%
Dense granules: 17.9%
H.
S
M
C
G1

Fig 3. TgAP2XII-9 regulates the expression of various cell cycle-regulated genes. (**A**) Volcano plot of differentially expressed genes from RNA-sequencing analysis of TgAP2XII-9 parasites treated with auxin for 2 hours. (**B**) Volcano plot of differentially expressed genes from RNA-sequencing analysis of TgAP2XII-9 parasites treated with auxin for 6 hours. (**C**) Venn diagram showing the overlap of upregulated genes from 2h and 6h RNA-Seq data. (**D**) Venn diagram showing the overlap of downregulated genes from 2h and 6h RNA-Seq data. (**E**) Pie chart showing the percentage and localisation of upregulated transcripts from RNA-Seq data. (**F**) Heatmap showing the cell cycle expression of Rhoptry, IMC and Apical complex proteins encoding upregulated transcripts peaking simultaneously with TgAP2XII-9 expression (denoted in a box). The phases of the cell cycle are depicted at the bottom. (**G**) Pie chart showing the percentage and localisation of downregulated transcripts from RNA-Seq data. (**H**) Heatmap showing the cell cycle expression of micronemes and Dense granule proteins encoding downregulated transcripts. Expression of TgAP2XII-9 is denoted. The phases of the cell cycle are depicted at the bottom.

https://doi.org/10.1371/journal.ppat.1012810.g003

The downregulated genes exhibited a heterogeneous expression pattern throughout the cell cycle, with peaks during the late S, M, and cytokinesis phases (S3B Fig).

Upon analyzing the differential enrichment between upregulated and downregulated transcripts encoding proteins localized to specific organelles in the parasites using the HyperLOPIT dataset from Barylyuk et al (2020) [31], we identified a notable trend: a significant proportion of the upregulated transcripts encode proteins that localize to the Rhoptries (5.7% of upregulated transcripts in the HyperLOPIT dataset; Fig 3E) compared to 0.9% of downregulated transcripts (Fig 3E and 3G), the apical compartment (4.2% of upregulated transcripts in the HyperLOPIT dataset; Fig 3E) compared to 1.8% of downregulated transcripts (Fig 3G), and the IMCs (4.2% of upregulated transcripts in the HyperLOPIT dataset; Fig 3E) compared to 1.8% of downregulated transcripts (Fig 3G). Additionally, we observed that a significant proportion of downregulated transcripts encode proteins that localize to the dense granules (17.9% of downregulated transcripts in the HyperLOPIT dataset; Fig 3G) compared to 4.5% of upregulated transcripts, and to the micronemes (8% of downregulated transcripts in the HyperLOPIT dataset; Fig 3G) compared to 0.8% of upregulated transcripts. According to the HyperLOPIT dataset, the upregulated genes comprise 17% of the apical proteome (11/63), 14% of the rhoptry proteome (15/106), and 14% of the IMC proteome (11/81). In contrast, the downregulated genes represent 15% of the dense granule proteome (19/124) and 18% of the microneme proteome (9/51).

Examining the cell cycle expression of the upregulated transcripts associated with Rhoptries, Apical Caps, and IMCs (where data is available) revealed that most of these transcripts peak concurrently with TgAP2XII-9 expression (Fig 3F). Conversely, the cell cycle expression of most of the downregulated transcripts associated with dense granules and micronemes peaked immediately after the expression of TgAP2XII-9 (Fig 3H).

TgAP2XII-9 is enriched at the promoters of *T. gondii genes*

Using RNA-seq, we identified that TgAP2XII-9 regulates gene expression either directly or indirectly. To pinpoint the specific promoters targeted by TgAP2XII-9, we conducted CUT & Tag analysis. Biological triplicates of endogenous HA-tagged TgAP2XII-9 were generated, along with a replicate of the RH-Tir1ΔKU80 strain, and subjected to sequencing. Significant peaks (p-value < 0.05) were identified using MACS2 software. Subsequently, ChIPSeeker annotated these peaks, revealing that 95% were located at promoter sites for TgAP2XII-9 (Fig 4Ai) and 49% for RH-Tir1ΔKU80 (Fig 4Aii). Across the three replicates, 2088 (S5 Table) peaks were exclusive to TgAP2XII-9 and absent in RH-Tir1ΔKU80. In line with its hypothesized function as a transcriptional regulator, TgAP2XII-9 is found proximal to the transcription start sites of protein-coding genes (Fig 4B).

Analysis of cell cycle expression patterns revealed that genes with TgAP2XII-9-bound promoters predominantly exhibited peak expression during S and M phases (Fig 4D) in line with its expression pattern. CUT & Tag data demonstrated that TgAP2XII-9 binds to promoters of

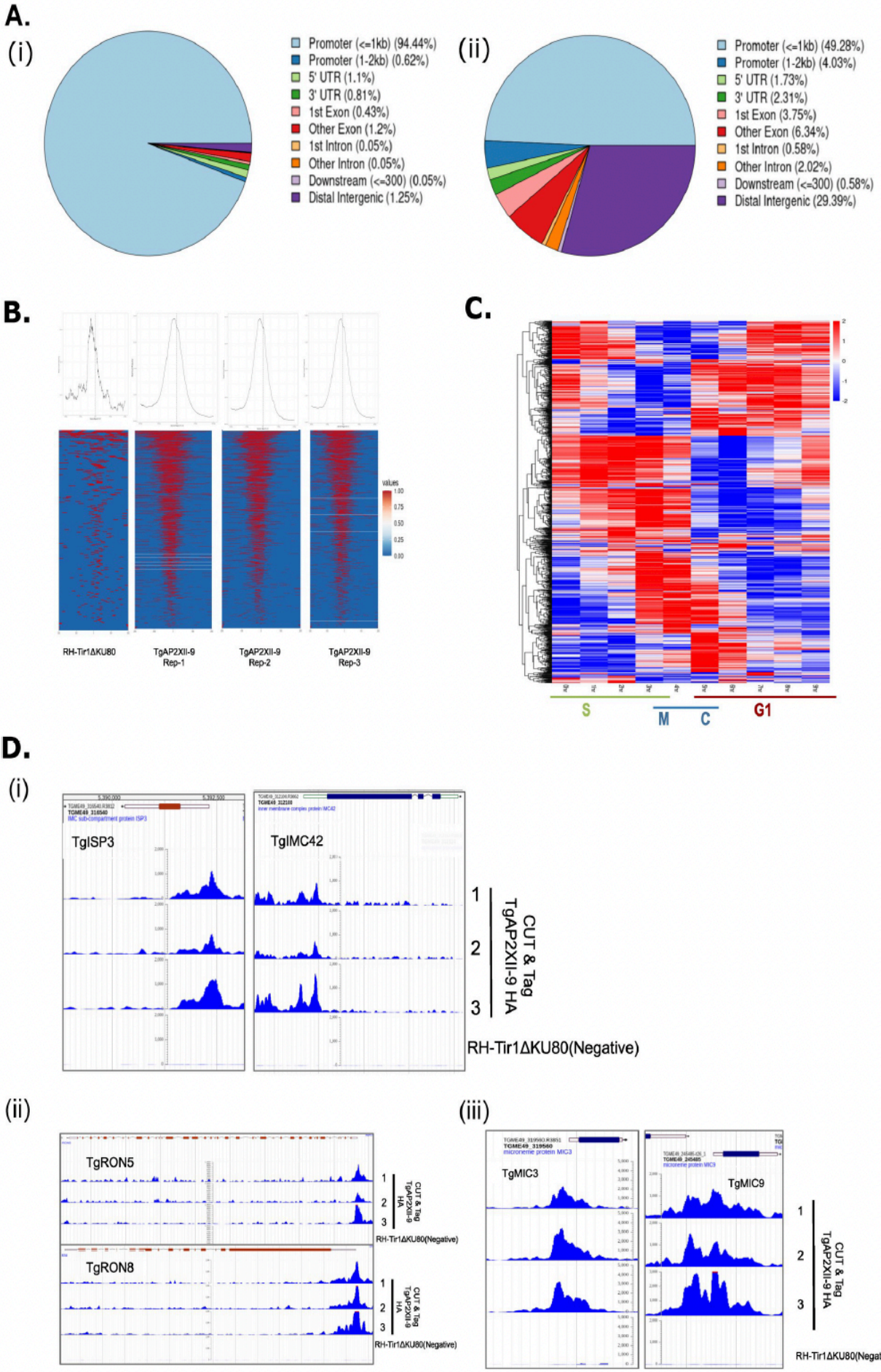
A.
(i)
Promoter (<=1kb) (94.44%)
Promoter (1-2kb) (0.62%)
5' UTR (1.1%)
3' UTR (0.81%)
1st Exon (0.43%)
Other Exon (1.2%)
1st Intron (0.05%)
Other Intron (0.05%)
Downstream (<=300) (0.05%)
Distal Intergenic (1.25%)
(ii)
Promoter (<=1kb) (49.28%)
Promoter (1-2kb) (4.03%)
5' UTR (1.73%)
3' UTR (2.31%)
1st Exon (3.75%)
Other Exon (6.34%)
1st Intron (0.58%)
Other Intron (2.02%)
Downstream (<=300) (0.58%)
Distal Intergenic (29.39%)
B.
values
1.00
0.75
0.50
0.25
0.00
RH-Tir1ΔKU80
TgAP2XII-9 Rep-1
TgAP2XII-9 Rep-2
TgAP2XII-9 Rep-3
C.
S
M
C
G1
D.
(i)
TgISP3
TgIMC42
1
2
3
CUT & Tag TgAP2XII-9 HA
RH-Tir1ΔKU80(Negative)
(ii)
TgRON5
TgRON8
CUT & Tag TgAP2XII-9 HA
RH-Tir1ΔKU80(Negative)
(iii)
TgMIC3
TgMIC9
CUT & Tag TgAP2XII-9 HA
RH-Tir1ΔKU80(Negative)

Fig 4. TgAP2XII-9 is enriched at the promoters of *T. gondii* genes. (A) (i) Pie chart showing the percentage of peaks (from 3 replicates) annotated in the different regions in the iKD TgAP2XII-9-HA strain. (A) (ii) Pie chart showing the percentage of peaks annotated in the different regions in the Parental Tir1 strain. (B) Density graphs and Heatmaps of the parental and TgAP2XII-9-HA (3 replicates) peaks located -2kb and +2kb from TSS. (C) Heatmap of cell cycle expression of all the genes that are bound by TgAP2XII-9 at their promoter. (D) CUT & Tag representing the direct targeting of TgAP2XII-9 to the promoters of TgISP3, TgIMC42 (i), TgRON5, TgRON8(ii), TgMIC3, TgMIC9(iii). Peak tracks for the 3 replicates of TgAP2XII-9-HA along with parental Tir1 are shown.

https://doi.org/10.1371/journal.ppat.1012810.g004

key genes encoding proteins crucial of the IMC, apical complex, rhoptries, micronemes, and dense granules (Fig 4D). Specifically, promoters of genes bound by TgAP2XII-9 include genes encoding for IMC proteins (e.g., TgISP3, TgIMC42; Fig 4Di), ROP proteins (e.g., TgRON5, TgRON8; Fig 4Dii), and MIC proteins (e.g., TgMIC9, TgMIC3; Fig 4Diii). These findings underscore TgAP2XII-9's role as a genuine TF by directly interacting with promoters of genes essential for daughter cell formation.

Since RNA-seq alone cannot identify genes directly regulated by TgAP2XII-9, we integrated RNA-seq with CUT & Tag datasets. We identified differentially expressed genes (both upregulated and downregulated) from the RNA-seq data and compared them with genes targeted by TgAP2XII-9 based on CUT & Tag analysis. This comparison revealed an overlap of 300 genes (Fig 5A and S6 Table). Detailed analysis showed that 31% of the upregulated genes and 16% of the downregulated genes are directly targeted by TgAP2XII-9, indicating that TgAP2XII-9 regulates genes mostly expressed during the S and M phases and some expressed during the cytokinesis phase (Fig 5B). Our results reveal that TgAP2XII-9 serves as a versatile transcriptional regulator with dual functions: it represses specific rhoptry and IMC genes while activating a subset of microneme and dense granule genes. In the light of these results, we wanted to verify if TgAP2XII-9 depletion had a direct effect on rhoptry or micronemes biogenesis. Therefore, we performed IFA using TgMIC8 (microneme marker) and TgROP17 (rhoptry marker) (Fig 5C). Upon auxin-induced depletion of TgAP2XII-9, we observed a significant disruption in microneme formation while rhoptry formation was unaffected. Specifically, in Fig 5Ciii, the micronemes are absent in budding daughter cells, although rhoptries appear unaffected. Furthermore, in Fig 5Civ, parasites with multiple nuclei are observed without a corresponding increase in micronemes, indicating a defect in organelle formation. Additionally, Fig 5Cv shows distorted and improperly localized microneme signals. Quantification of vacuoles with abnormal microneme signals upon TgAP2XII-9 depletion (Fig 5D) confirmed a statistically significant increase in abnormal localization patterns, supporting its function in microneme biogenesis.

Conversely, TgAP2XII-9 appears to act as a repressor of certain IMC genes. To confirm this, we investigated TgIMC42, whose promoter is bound by TgAP2XII-9 and is upregulated upon depletion of the transcription factor. In an iKD TgAP2XII-9 strain with Ty-tagged TgIMC42, Western blot analysis showed an increase in TgIMC42 protein levels following auxin treatment (Fig 5E), supporting the repressive role of TgAP2XII-9 on TgIMC42 expression. IFA of TgIMC42-Ty1 (red) and TgIMC3 (green) further confirmed TgAP2XII-9's role in maintaining IMC organization (Fig 5F).

These findings collectively support a model in which TgAP2XII-9 functions as an activator for microneme genes leading to the proper microneme formation. Conversely, it represses certain rhoptry, IMC, and apical genes, whose expression peaks earlier in the cycle than TgAP2XII-9.

Interplay of ApiAP2 transcription factors in regulating cell cycle and differentiation in *T. gondii*

Given TgAP2IX-5's role in activating genes involved in daughter parasite formation, we examined genes regulated by both TgAP2XII-9 and TgAP2IX-5. Out of the 300 genes directly

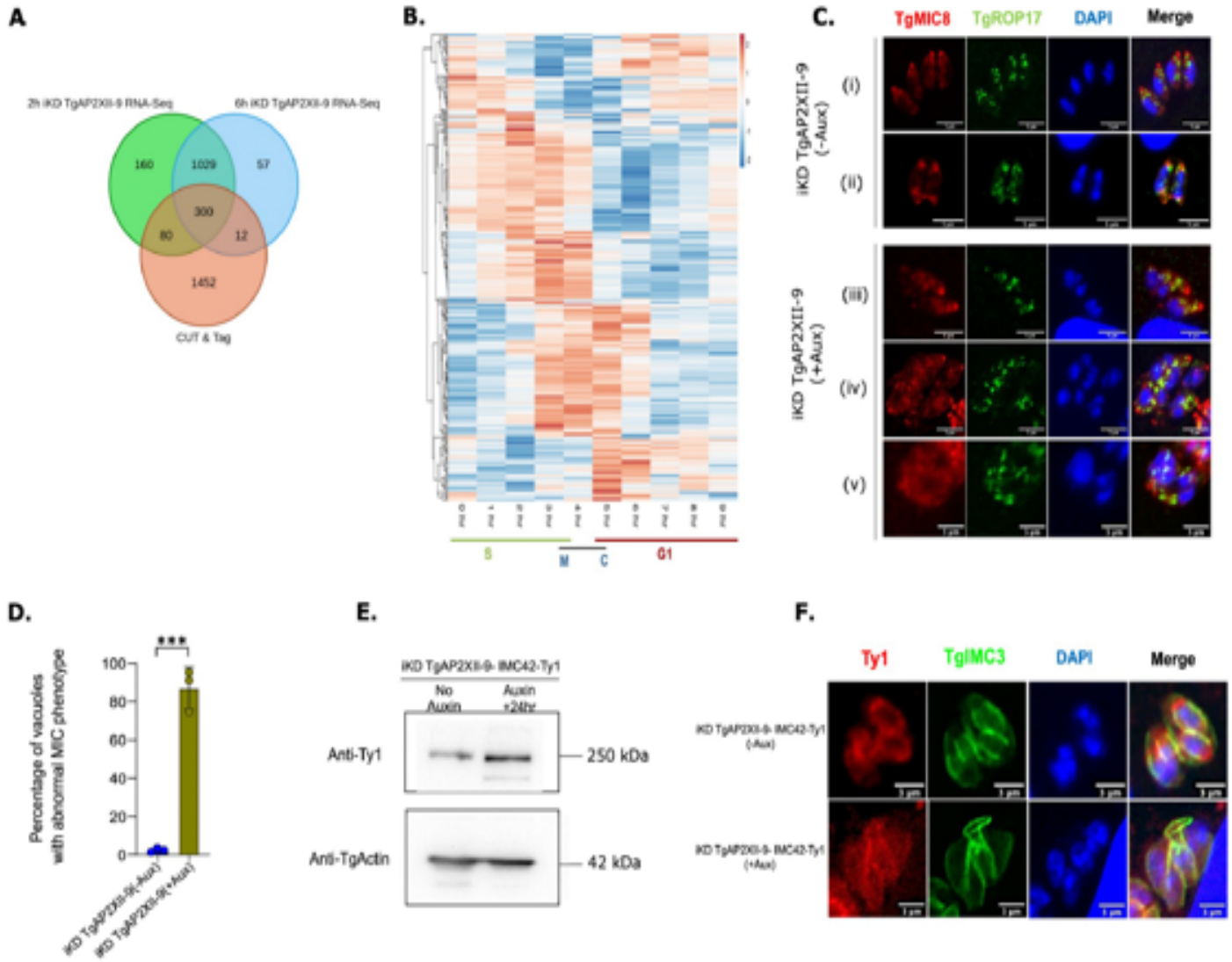

Fig 5. TgAP2XII-9 acts as a dual regulator regulating subset of MIC, ROP,GRA and IMC genes. (A) Venn diagram showing the overlap of genes from 2h RNA-Seq, 6h RNA-Seq and CUT & Tag data. (B) Heatmap of cell cycle expression of genes directly regulated and targeted by TgAP2XII-9. (C) Defects in the MIC organization observed after 24 hours Auxin treatment while ROPs remain unaffected as realized by IFA and confocal imaging with TgMIC8 and TgROP17; Scale bar indicated at the bottom right corner of each image (D) Quantification of the MIC abnormal phenotype between the parental and iKD TgAP2XII-9 strains on 24hrs of auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{***}p < 0.001$, data are presented as mean ± s.d. (n = 3) (E) Western blot analysis of total protein extracts from iKD TgAP2XII-9-IMC42-Ty1 strain treated with Auxin for 24hrs. The blots were probed with anti-Ty1 to detect TgIMC42 protein levels (upper panel) and with anti-TgSortilin as a normalization control (lower panel). (F) Defects in the IMC42 organization observed after 24 hours Auxin treatment as realized by IFA and confocal imaging with Ty1 and TgIMC3; Scale bar indicated at the bottom right corner of each image.

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targeted and regulated by TgAP2XII-9, only 18 were also targeted by TgAP2IX-5 (Fig 6A). Nearly all these genes are initially activated by TgAP2IX-5 and later repressed by TgAP2XII-9, including TgAP2XII-9 itself. These findings suggest that TgAP2XII-9 regulates a distinct set of genes and acts downstream of TgAP2IX-5.

We also looked at genes that are coregulated by TgAP2XI-5 and TgAP2XII-9. We overlapped the ChIP-on-chip data of TgAP2XI-5 [32] and identified 80 genes that were also bound by TgAP2XI-5 at their promoters (Fig 6B), in line with previous results showing that TgAP2XI-5 binds to promoter of genes preferentially expressed during the S and M phase.

Since TgAP2XII-9 and TgAP2XII-2 share a similar transcriptomic profile and are activated by TgAP2IX-5, we investigated whether TgAP2XII-2 also targets the direct targets of TgAP2-XII-9. To this end, we compared our data with the CUT&Tag data of TgAP2XII-2 [33].

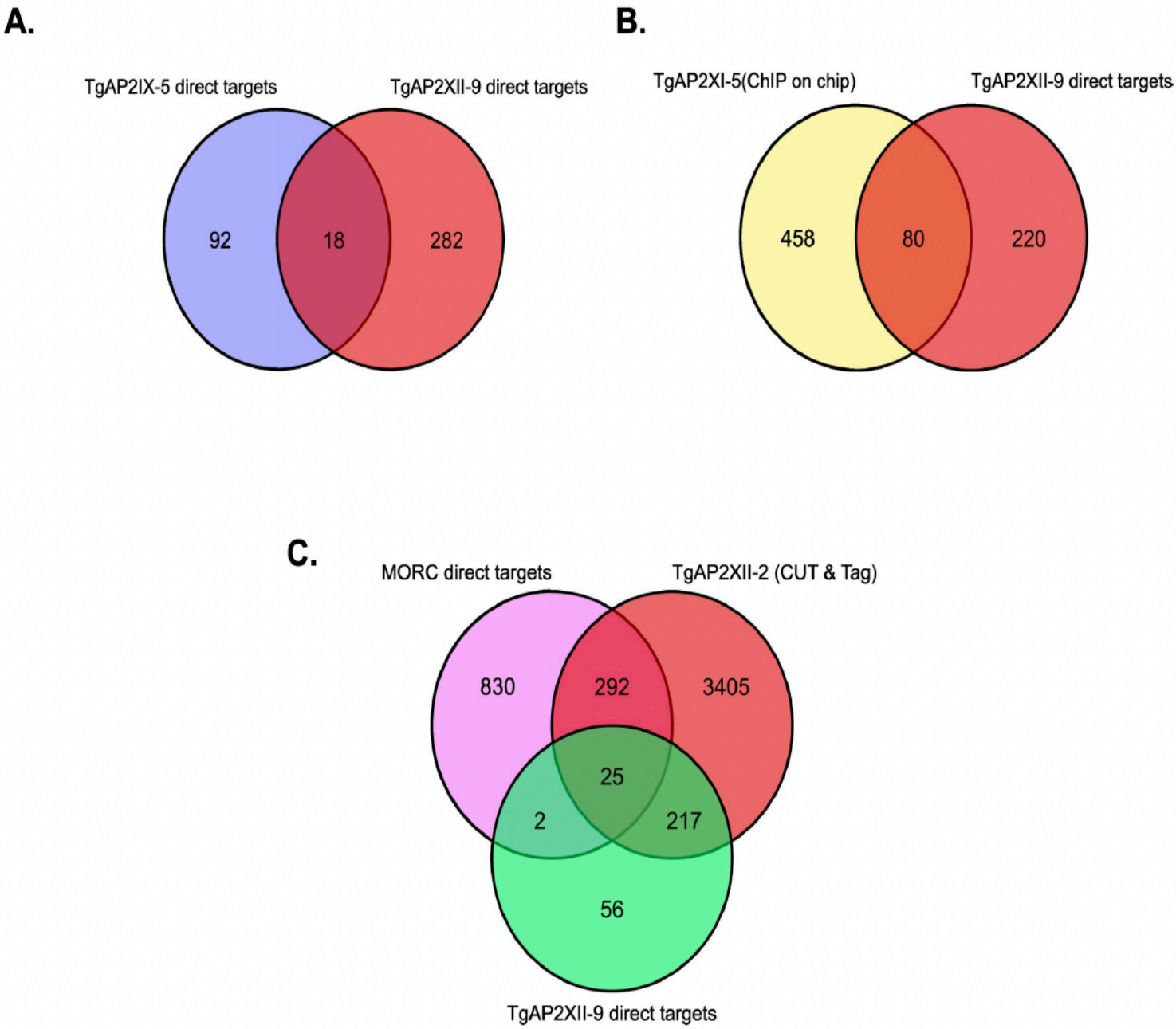

Fig 6. TgAP2XII-9 directly regulates key genes involved in the daughter parasite formation. (**A**) Venn diagram indicating that majority of genes targeted by TgAP2XII-9 are different to that targeted by TgAP2IX-5. (**B**) Venn diagram indicating that majority of genes targeted by TgAP2XII-9 are different to that targeted by TgAP2XI-5. (**C**) Venn diagram indicating the overlap of genes bound by both TgAP2XII-9 and TgAP2XII-2 and direct targets of MORC.

https://doi.org/10.1371/journal.ppat.1012810.g006

Remarkably, out of the 300 direct targets of TgAP2XII-9, 242 were also bound by TgAP2XII-2 at their promoters (Fig 6C). However, the expression of most of these genes did not vary after TgAP2XII-2 depletion [33]. Given that TgAP2XII-2 is known to interact with the TgMORC/TgHDAC3 complex [33], we examined whether these 242 genes are also targets of TgMORC. We compared this data with the ChIP-seq data of MORC [34]. Only 25 genes were bound by TgMORC at their promoters (Fig 6C), and these genes did not belong to any specific gene set. Notably, two AP2 transcription factors (TgAP2Ib-1 and TgAP2IV-3), involved in bradyzoite-specific gene expression, were present. This data indicate that TgAP2XII-2 has a different biological function than TgAP2XII-9 although they bind to similar promoters.When examining the 25 genes whose promoter is bound by TgAP2XII-2, TgAP2XII-9 and TgMORC, we found that most of them had a peak expression during the M and Cytokinesis phases (S5A Fig). All

these 25 genes were highly expressed throughout the sexual stages with their expression peaking at EES5 and tissue cysts (S5B Fig).

We also investigated other ApiAP2 TFs potentially regulated by TgAP2XII-9 and found that 7 ApiAP2 TFs were bound and regulated by TgAP2XII-9. Of these, four AP2s (TgAP2IV-4, TgAP2IX-8, TgAP2IX-9, TgAP2XI-2) were upregulated when TgAP2XII-9 was depleted, and 2 AP2s (TgAP2IV-3, TgAP2Ib-1) were downregulated. Analysis of the cell cycle expression of these AP2s suggests that most peak during the late C and early G1 phases, except for TgAP2IV-4, which peaks during the S/M phase along with TgAP2XII-9 (Fig 7A). To investigate the link between TgAP2XII-9 and differentiation, we examined the expression profile of upregulated and downregulated genes during the parasite life cycle (Fig 7B) using the data from Ramakrishnan *et al.* (2019) [35]. Interestingly, downregulated genes in the absence of TgAP2XII-9 are preferentially expressed in tachyzoite but also bradyzoites and sexual stages (Fig 7C), while TgAP2XII-9 depletion induced the overexpression of mostly tachyzoite-specific genes (Fig 7B). These data indicate that AP2XII-9 may produce a permissive environment for expression of genes that preferentially expressed in bradyzoites and sexual stages.

Surprisingly, TgAP2XII-9 was enriched at its own promoter (S6A Fig), and the TgAP2XII-9 transcript was upregulated in the presence of auxin based on RNA-seq. These data suggest that TgAP2XII-9 may directly regulate its own transcript expression, indicating a possible negative feedback loop.

Complementation restores TgAP2XII-9 phenotypes observed

We created a complemented strain (iKD-C TgAP2XII-9) by inserting a myc-tagged version of the TgAP2XII-9 gene, driven by its own promoter, into an exogenous locus (*uprt*; Fig 8A). The expression and localization of the exogenous TgAP2XII-9-myc in this strain were confirmed through IFA (S6B Fig). We compared the percentage of parasites expressing the myc-tagged copy with those expressing the endogenous HA-tagged version. About 30% of the asynchronous parasite population expressed the myc-tagged gene in the complemented strain, similar to the parental iKD TgAP2XII-9 strain (S6C Fig).

To assess if the iKD TgAP2XII-9 strain phenotype could be rescued by ectopic expression of TgAP2XII-9, we measured the percentage of vacuoles with IMC defects in the iKD-C TgAP2XII-9 strain both in the absence and presence of auxin (S6D Fig). We found very few to no vacuoles with IMC defects, indicating that the IMC defect phenotype observed in the iKD TgAP2XII-9 strain was due to the lack of TgAP2XII-9 protein. Furthermore, plaque assays showed that the parasites could form lysis plaques even in the presence of auxin, similar to their behaviour in the absence of auxin (Fig 8B). These results demonstrate that the TgAP2XII-9 protein is responsible for the phenotypes observed.

The TgAP2XII-9 AP2 domain is crucial for its function

We investigated the role of the AP2 domain in TgAP2XII-9 function by modifying the complementation plasmid previously used to create the iKD-C TgAP2XII-9 strain, deleting the AP2 domain (Fig 8A). This complemented strain, designated iKD-C TgAP2XII-9 ΔAP2, was verified for expression and localization of the exogenous iKD-C TgAP2XII-9 ΔAP2 protein via immunofluorescence (S6E Fig).

To assess the impact of AP2 domain deletion, we conducted a plaque assay. HFF cell monolayers inoculated with iKD-C TgAP2XII-9 ΔAP2 parasites in the presence of auxin showed little to no lysis plaques, unlike the lysis plaques observed in wells infected with iKD-C TgAP2XII-9 parasites (Fig 8B and 8C). Interestingly, co-expression of the endogenous TgAP2XII-9 protein and the AP2-deleted exogenous protein had a deleterious effect on parasite

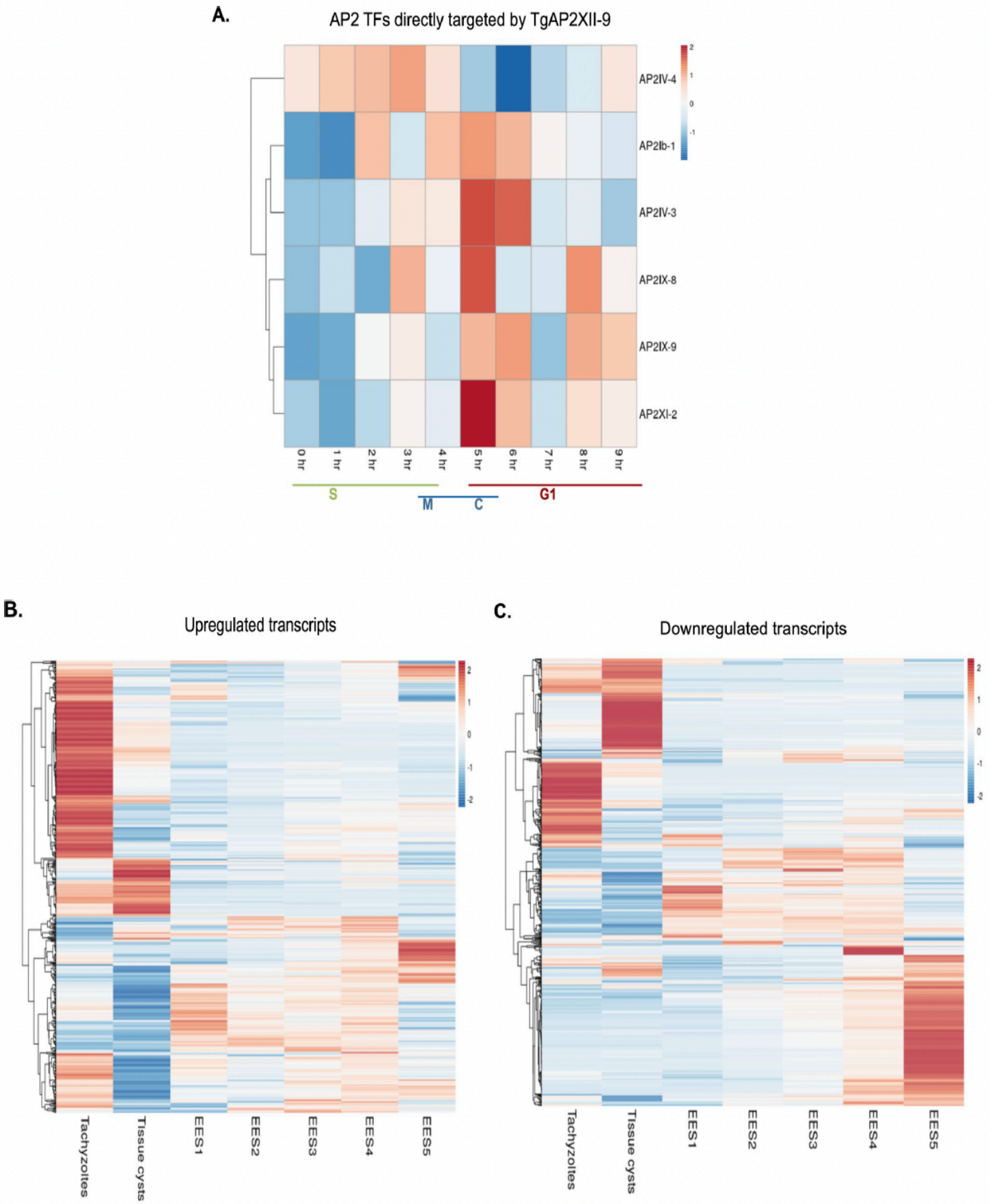

Fig 7. TgAP2XII-9 directly regulates other AP2 transcription factors and might provide a conducive environment for the expression of genes preferentially expressed in bradyzoites. (A) Heatmap of cell cycle expression of the AP2 TFs directly regulated by TgAP2XII-9. (B) Heatmap of expression of upregulated transcripts through the tachyzoite, bradyzoite and sexual stages. Most of the overexpressed transcripts upon TgAP2XII-9 depletion are preferentially expressed at the tachyzoite stage of the parasite (C) Heatmap of expression of downregulated transcripts through the tachyzoite, bradyzoite and sexual stages. Many of the downregulated transcripts upon

TgAP2XII-9 depletion are preferentially expressed at bradyzoite and throughout the sexual stages especially during EES5. EES stands for enteroepithelial developmental stages. Source data for B&C–Ramakrishnan et al.,2019 [35].

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growth, as seen by the reduced plaque formation in the iKD-C TgAP2XII-9 ΔAP2 strain even in the absence of auxin (Fig 8C).

We next examined the phenotype of abnormal IMC defects, recording the proportion of vacuoles exhibiting this feature (Fig 8D). Only a small percentage of vacuoles displayed this phenotype, suggesting that TgAP2XII-9 ΔAP2 could partially complement the IMC defect (Fig 8E). We also analyzed the microneme biogenesis phenotype across the iKD, complement, and ΔAP2 strains, quantifying the results (Fig 8F and 8H). Approximately 40% of vacuoles in the TgAP2XII-9 ΔAP2 strain exhibited an abnormal MIC phenotype in the presence of Auxin (Fig 8G). Overall, these findings indicate that the AP2 domain is crucial for TgAP2XII-9's function, though it does not fully account for the phenotypes observed.

Discussion

Proliferation is key to *T. gondii* pathogenesis in the intermediate hosts. The tachyzoite employs a unique, rapid replication method where daughter parasites are formed within a single mother cell (endodyogeny). However, how gene expression is regulated during this process is only partially understood.

Our study corroborates the findings of Shi et al. (2024) [36] regarding the essential role of TgAP2XII-9. However, since the data generated by Shi *et al.* [36] is produced after 24-hour auxin treatment, a direct comparison between the phenotypes observed in their study or RNA-seq data is not feasible. By focusing on short auxin treatments (2h or 6h), our approach aimed to identify the direct, early consequences of TgAP2XII-9 depletion. When we compared our CUT & Tag data with the corresponding dataset from Shi et al. [36], we found a significant overlap, with more than 50% of their identified targets also appearing in our data. This overlap reinforces the validity of our findings and underscores the critical role of TgAP2XII-9 in the parasite's biology.

In our study, we have characterized two cell cycle-regulated ApiAP2 TFs (TgAP2XII-9 and TgAP2III-2) that are predominantly expressed during the S/M phase of the tachyzoite cell cycle. While TgAP2III-2 has no measurable impact on the ability of the tachyzoite to grow, TgAP2XII-9 depletion resulted in significant defects in daughter bud formation and disorganization of the IMC. Both TFs were shown to be regulated by TgAP2IX-5 [27] and we hypothesized that they may be important for the continuation of the cell cycle. TgAP2IX-5 is a crucial TF regulating the initiation of budding and we expected both TgAP2XII-9 and TgAP2III-2 to regulate subsequent steps of the cell cycle. Depletion of TgAP2III-2 did not cause a defect in parasite proliferation. In contrast, we show that TgAP2XII-9 is important for the formation of daughter cells but does not prevent the initiation of budding and the IMC apical cap formation (Fig 2). Instead, TgAP2XII-9 is important for IMC elongation and proper formation of the buds. Much like the erythrocytic cycle of *Plasmodium* [37] the identification of tachyzoite cell cycle-regulated expression profiles [30] suggests the presence of a cascade of TFs regulating this process.

This is the first evidence that the tachyzoite cell cycle-regulated expression program is controlled by a series of ApiAP2 TFs, cascading to implement the specific expression programs at each phase of the tachyzoite cell cycle. TgAP2IX-5 controls the expression of TgAP2XII-9 (also TgAP2XII-2 and TgAP2III-2) which in turn establishes the crucial expression profiles required for the progression of the cell cycle by coordinating the temporal expression of many transcripts.

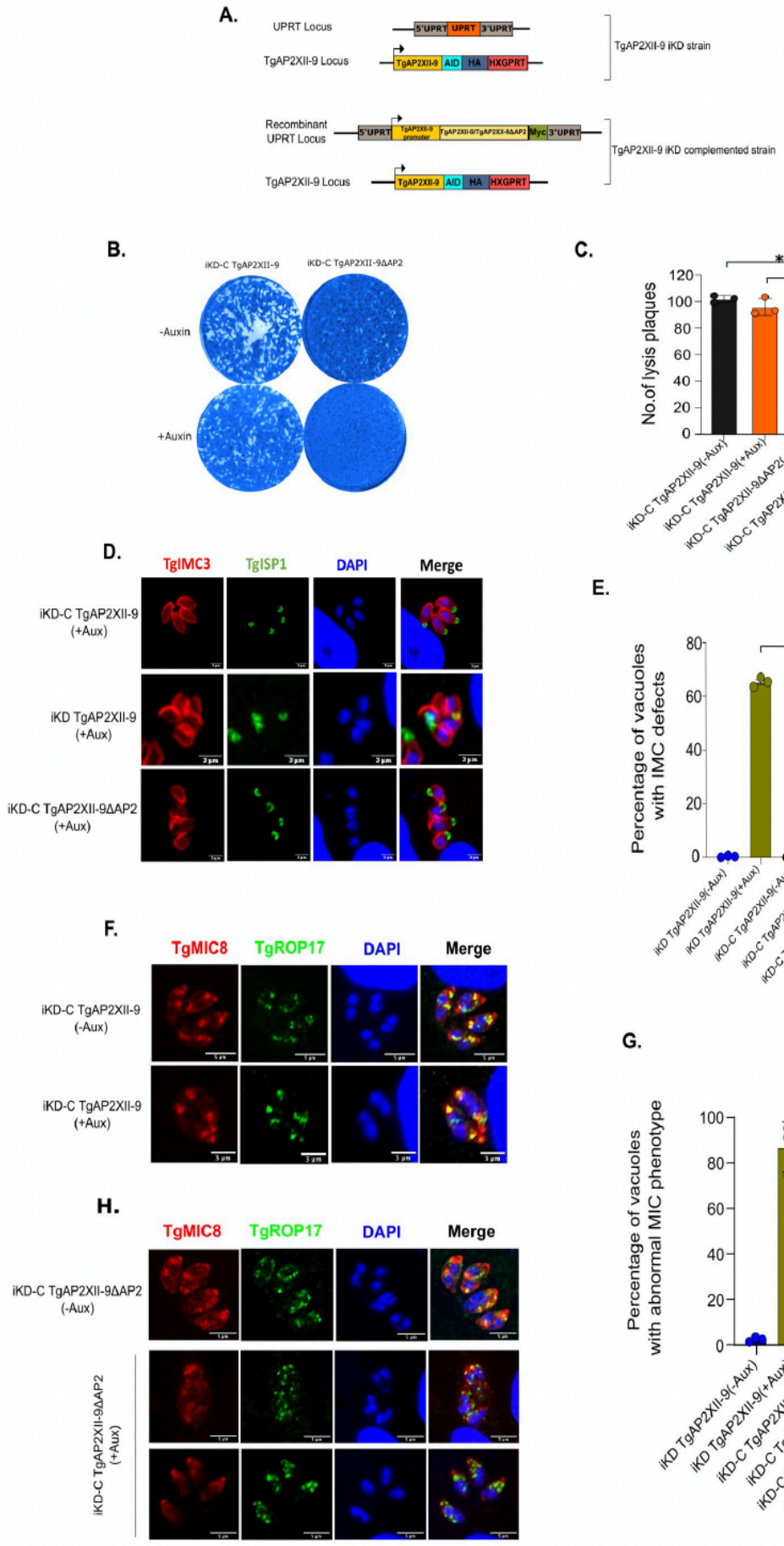
A.
UPRT Locus
5'UPRT
UPRT
3'UPRT
TgAP2XII-9 Locus
TgAP2XII-9
AID
HA
HXGPRT
TgAP2XII-9 iKD strain
Recombinant UPRT Locus
5'UPRT
TgAP2XII-9 promoter
TgAP2XII-9/TgAP2XII-9ΔAP2
Myc
3'UPRT
TgAP2XII-9 Locus
TgAP2XII-9 iKD complemented strain
B.
iKD-C TgAP2XII-9
iKD-C TgAP2XII-9ΔAP2
-Auxin
+Auxin
C.
No.of lysis plaques
120
100
80
60
40
20
0
****
***
*
iKD-C TgAP2XII-9(-Aux)
iKD-C TgAP2XII-9(+Aux)
iKD-C TgAP2XII-9ΔAP2(-Aux)
iKD-C TgAP2XII-9ΔAP2(+Aux)
D.
TgIMC3
TgISP1
DAPI
Merge
iKD-C TgAP2XII-9 (+Aux)
iKD TgAP2XII-9 (+Aux)
iKD-C TgAP2XII-9ΔAP2 (+Aux)
3 μm
E.
Percentage of vacuoles with IMC defects
80
60
40
20
0
***
**
*
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)
iKD-C TgAP2XII-9(-Aux)
iKD-C TgAP2XII-9(+Aux)
iKD-C TgAP2XII-9ΔAP2(-Aux)
iKD-C TgAP2XII-9ΔAP2(+Aux)
F.
TgMIC8
TgROP17
DAPI
Merge
iKD-C TgAP2XII-9 (-Aux)
iKD-C TgAP2XII-9 (+Aux)
3 μm
G.
Percentage of vacuoles with abnormal MIC phenotype
100
80
60
40
20
0
*
**
**
iKD TgAP2XII-9(-Aux)
iKD TgAP2XII-9(+Aux)
iKD-C TgAP2XII-9(-Aux)
iKD-C TgAP2XII-9(+Aux)
iKD-C TgAP2XII-9ΔAP2(-Aux)
iKD-C TgAP2XII-9ΔAP2(+Aux)
H.
TgMIC8
TgROP17
DAPI
Merge
iKD-C TgAP2XII-9ΔAP2 (-Aux)
iKD-C TgAP2XII-9ΔAP2 (+Aux)
3 μm

Fig 8. TgAP2XII-9 is responsible for the phenotypes observed and the AP2 domain is crucial for its function. (A) Schematic representation of the iKD TgAP2XII-9 complementation strategy. The UPRT locus was targeted for the insertion of exogenous myc-tagged TgAP2XII-9/TgAP2XII-9ΔAP2, driven by its native promoter, to generate the complemented TgAP2XII-9 iKD strain. (B) Plaque assay showing the proliferation of the iKD-C TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 strains in the presence and the absence of Auxin. (C) Quantification of the number of plaques in the iKD-C TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 strains. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{****}p<0.0001$, $^{***}p < 0.001$, $^{*}p<0.05$. Data are presented as mean ± s.d. (n = 3). (D) IFA and confocal imaging illustrate the IMC defect phenotype seen by using TgIMC3 and TgISP1 in the iKD TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 in presence of Auxin but not in the iKD-C TgAP2XII-9, scale bar = 3 μm. (E) Quantification of the IMC defect phenotype using TgIMC3 between iKD TgAP2XII-9, iKD-C TgAP2XII-9 and iKD-C TgAP2XII-9ΔAP2 strains. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p < 0.001$, Data are presented as mean ± s.d. (n = 3).

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TgAP2IX-5 controls genes that are essential for budding initiation, while TgAP2XII-9 seems important for the subsequent phases of daughter cell construction. When examining the genes that are directly controlled by TgAP2XII-9 (genes both present in the RNA-seq and CUT&Tag dataset), we noticed that genes encoding for TgISP3 and Apical Annuli proteins TgAAP5 and TgAAMT were downregulated. These proteins are expressed after the IMC apical cap deposition and are present at the central IMC sub compartment (TgISP3) or the apical annuli (TgAAP5 and TgAAMT). Interestingly, TgISP3 maternal staining dissipates as daughter parasites form, indicating that TgISP3 may be synthesized in daughters and degraded in mothers [38]. Our data confirm this hypothesis and suggest an active role of TgAP2XII-9 in this process. Apical annuli proteins may be inserted in the suture of the IMC plaques and therefore are needed once the apical and central IMC plaques are formed [39]. While TgAP2XII-9 seems to activate the expression of some IMC proteins, our analysis also detected IMC and basal complex genes that were directly repressed by TgAP2XII-9. Notably, the expression of transcripts encoding TgAC1 and TgIAP2, two apical cap proteins, and TgBCC3 and TgBCC7, two early and late markers of the basal complex [40–42] are directly repressed by TgAP2XII-9. These data indicate that TgAP2XII-9 seems to exert a dual activity of repressing the early budding markers (e.g apical cap and early basal complex component) and activating the expression of transcript encoding for proteins needed during IMC elongation. Overall, our data indicate that the production and assembly of each daughter cell IMC subcompartments correspond to a strictly controlled process that involves the timely expression of IMC encoded transcripts and proteins in different temporal waves that are controlled at least by TgAP2IX-5 and TgAP2XII-9. Indeed, it has been hypothesized that the apical cap of the IMC is assembled in the apical direction while the central and basal compartments are in the basal direction [43].

Defects in daughter cell formation have downstream effects on other phenotypes such as nuclear segregation. For example, TgILP1 overexpression, which predominantly localizes to budding daughters, leads to severely deformed cytoskeletons and abnormally large nuclei, suggesting a disruption in mitotic coordination similar to the phenotype that we see in TgAP2XII-9 depleted parasites [43]. Interestingly, *TgILP1* transcript is overexpressed in TgAP2XII-9 depleted parasites, recapitulating some of the phenotypes we observed. However, the IMC phenotype observed is probably due to the collective deregulation (up or downregulation upon TgAP2XII-9 depletion) of multiple genes targeted to the IMC and whose expression is controlled directly or indirectly by TgAP2XII-9.

Much like daughter cell IMC formation, the *de novo* production of rhoptries and micronemes are tightly regulated. Cell-cycle transcript expression profiles show that the temporal expression of rhoptries (early S phase) and micronemes (early M phase) are different [40]. Consistent with these gene expression patterns, we showed that transcripts encoding rhoptry proteins, which peak prior to TgAP2XII-9 expression are repressed by this TF, whereas expression of transcripts encoding microneme proteins is activated by TgAP2XII-9. We also showed

that TgAP2XII-9 ensures the proper biogenesis of micronemes probably through the activation of the expression of micronemes genes. TgAP2XI-5 and TgAP2X-5 were already shown to be important in regulating the expression of virulence factor genes, specifically as activators of rhoptry-encoded transcripts [20]. Our findings indicate that transcript expression profiles linked to the cell cycle, similar to those observed during differentiation, must be kept repressed until their expression becomes necessary. This regulatory pattern is particularly evident for transcripts encoding rhoptry and microneme proteins, which need to be expressed precisely when these organelles are formed *de novo*. Thus, TgAP2XII-9 is positioned as a repressor of a subset of rhoptry protein encoded transcripts and an activator of a subset of microneme protein encoded transcripts, contrasting with the roles of TgAP2XI-5 and TgAP2X-5, which primarily activate transcripts encoding rhoptry proteins.

Upon TgAP2XII-9 depletion, some kinase transcripts were upregulated. Cyclin-related kinases like TgCRK1 and TgCRK6 are significantly dysregulated after TgAP2XII-9 depletion as suggested by our RNAseq data. Notably, the TgCRK6 promoter is also directly bound by TgAP2XII-9 and its transcript expression is being repressed. It is interesting to note that TgCRK6 interacts with TgAP2IX-5 and has similar temporal expression and localization to that of TgAP2IX-5 [44] It was also speculated that the role of TgCRK6 might be to inactivate TgAP2IX-5 just after budding has been initiated [44]. If in fact that is the case, TgAP2XII-9 (activated by AP2IX-5) might be repressing the gene expression of TgCrk6 when it is no longer needed, for example, after budding initiation. Overall, our data show that TgAP2XII-9 is acting as a crucial transcription factor at a turning point during the cell cycle when daughter cell buds are formed and microneme biogenesis must occur.

We identified that a majority of the genes that are downregulated in response to *TgAP2XII-9* knockdown are preferentially expressed in bradyzoites or sexual stages compared to tachyzoites (Fig 6C). This is probably linked to the list of ApiAP2 TFs that are dysregulated after TgAP2XII-9 depletion. Nine AP2 transcription factors are significantly upregulated (TgAP2IV-4, TgAP2IX-8, TgAP2XI-2, TgAP2IX-9, TgAP2XII-9, TgAP2IV-2, TgAP2XI-3, TgAP2XI-4 and TgAP2III-2) and 4 are significantly downregulated (TgAP2IV-3, TgAP2Ib-1, TgAP2IX-3 and TgAP2IV-1). Of these TgAP2XII-9 binds to the promoters of TgAP2IV-4, TgAP2IX-8, TgAP2XI-2, TgAP2IX-9, TgAP2XII-9, TgAP2IV-3, and TgAP2Ib-1. TgAP2IV-4 and TgAP2IX-9 are known repressors of bradyzoite-specific genes [45,46], whereas TgAP2IV-3, TgAP2Ib-1 and TgAP2XI-4 are activators [47,48]. These data suggest that TgAP2XII-9 directly represses the transcripts of other AP2s, such as TgAP2IV-4 and TgAP2IX-9, which in turn may repress bradyzoite-specific gene expression, while it activates the expression of AP2IV-3 and AP2Ib-1, which stimulate the bradyzoite-specific gene expression. This is reminiscent of the data published on TgAP2IX-5 [27], which was shown to activate the expression of TgAP2IV-4 (a repressor of bradyzoite differentiation). These data reinforce the link between the cell cycle and differentiation that was previously shown [49,50] where AP2 TFs important for differentiation are expressed at a specific point of the cell cycle (early M phase). At this specific point, by controlling the expression of a subset of AP2 TFs, TgAP2XII-9 might create a more permissive environment for the bradyzoite expression program and offer a possible getaway toward bradyzoite differentiation. In contrast, TgAP2IX-5, which acts to initiate budding, promotes the repression of the bradyzoite-specific expression program.

The complementation using the AP2 deleted copy of the TgAP2XII-9 gene was instrumental in showing the importance of the AP2 domain in TgAP2XII-9's biological activity. However, the phenotypes could be partially complemented by this deleted copy of the genes indicating that TgAP2XII-9 may interact with other proteins to exert its action. We and other have previously shown that ApiAP2 protein can bind to DNA as hetero or homodimers, suggesting that the cooperative nature of their activity [20,51].

By the use of different complementation constructs, we examined the role of the AP2 domain in the function of TgAP2XII-9. As expected, the AP2 domain is critical for the essential function of TgAP2XII-9. However, when complementing the iKD strain using a construct deleted for the AP2 domain, some of the phenotypes, such as the disordered IMC phenotype, were partially complemented. This indicates that TgAP2XII-9 may exert some of this function through other parts of the proteins. ApiAP2 TFs are known to heterodimerize and cooperate to exert their function [20,51]. TgAP2XII-9 might therefore interact and cooperate with other proteins to regulate this phenotype independently from the presence of the AP2 domain.

Finally, we observed that TgAP2XII-9 binds to its own promoter and represses it which seems to be a typical characteristic of other AP2s. This feature was also shown for TgAP2IX-5 [27], TgAP2XI-5 [32], and TgAP2XII-2 [33]. This indicates that negative feedback loops are a common regulatory mechanism for these TFs and during the tachyzoite cell cycle, adding another layer of complexity to gene regulation in *T. gondii*.

In conclusion, we showed that TgAP2XII-9 plays a crucial role as a TF during daughter cell formation by activating genes that are required during the process of daughter cell IMC elongation and microneme *de novo* synthesis and repressing the expression of genes necessary during budding initiation.

Materials & methods

Parasite culture, transfection, and purification

The tachyzoites from the RH Δku80 Tir1 strain of *Toxoplasma gondii* were grown in human foreskin fibroblasts (HFF) under controlled laboratory conditions, using Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and 1% penicillin-streptomycin. This particular strain, RH Δku80 Tir1, is recognized for its rapid proliferation due to the deletion of the ku80 gene, which promotes successful homologous recombination and transfection. Moreover, this strain produces the Tir1 protein, facilitating the regulated breakdown of labelled proteins upon the introduction of auxin to the culture medium. The cultivation process occurred in ventilated tissue culture flasks within a HERA cell VLOS 160i CO2 incubator (Thermo Scientific) maintained at 37˚C and 5% CO2. Transgenes were delivered through electroporation utilizing a BTX Harvard apparatus electroporator (ECM 630), and stable transformants were identified by growing them in media containing specific concentrations of mycophenolic acid (MPA)- 25 μg/ml, xanthine (50 μg/ml), pyrimethamine (2 μM), or FUDR (5 μg/ml). Clonal lines were isolated through a process of limited dilution. Before extracting total RNA, genomic DNA, or protein, intracellular parasites underwent purification via sequential syringe passage, first through a 17 gauge and then 26-gauge needles (Terumo AGANI needles) and filtration of the parasite through 3-μm polycarbonate membrane filter (Whatman).

Generation of transgenic *T. gondii* strains

The iKD TgAP2XII-9 strain was developed by utilizing the RH Δku80 Tir1 strain, in conjunction with a Cas9 plasmid engineered to target the gene's 3' end post the stop codon, and a PCR product containing the HA-AID cassette flanked by homology regions. The primer sequences utilized in this experiment are detailed in the S7 Table. To produce the iKD and ΔAP2 complementation line, a plasmid containing 3-kb upstream of the predicted ATG of the TgAP2XII-9 gene and the full-length or AP2 domain-deleted c-myc-tagged TgAP2XII-9 gene flanked by 2 kb homology fragments for the uprt gene was co-transfected with the pSAG1::Cas9-U6::sgUPRT plasmid in the iKD TgAP2XII-9 strain to ensure insertion into the UPRT locus. The parasites were then selected using 5 μM 5-fluoro-2'-deoxyruridine (FUDR). To produce the

double mutant strain, specifically a clean knockout of the TgAP2III-2 gene in the inducible knockdown (iKD) TgAP2XII-9 background, two gRNAs were designed to target the 5' and 3' ends of the TgAPIII-2 gene. This strategy facilitated the insertion of a DHFR selection cassette flanked by 35 base pair (bp) homology regions at both the 5' and 3' ends. The procedure involved transfecting the iKD TgAP2XII-9 parasite with two Cas9 plasmids, each targeting one end of the TgAPIII-2 gene.

Growth assays

To assess growth, we introduced 8 x 10^4 parasites of both parental Tir1 and iKD AP2XII-9 mutant strains onto HFF cell monolayers cultivated on coverslips in a 24-well plate. This setup was maintained for 24 hours under conditions with and without 0.5mM auxin (AID/indole-acetic acid) in the medium. The purpose of incorporating auxin was to trigger the degradation of TgAP2XII-9 protein. Following 24-hour duration, infected coverslips were treated with 4% paraformaldehyde (PFA) for fixation. The fixed parasites were then subjected to staining using anti-TgEno2 to visualize parasite nuclei and anti-TgIMC1 antibodies to visualize the Inner Membrane Complex (IMC). The quantification involved counting the number of parasites per vacuole, with 100–200 vacuoles analyzed per biological replicate. Each growth assay experiment comprised three biological replicates.

Plaque assay

Plaque assays were conducted by inoculating either 500 parasites of the Parental Tir1 strain or the iKD TgAP2XII-9/ TgAP2III-2 or the Double Mutant strain onto a monolayer of HFF cells cultivated in a 6-well plate, with the choice of normal media or media supplemented with 0.5mM auxin. The parasites were allowed to proliferate for 7 days before fixation with 100% ethanol. Plaques were visualized by staining with Crystal Violet. To assess plaque size under each experimental condition, an Excel macro was utilized for quantification.

Organelle labelling

The Parental Tir1 and iKD TgAP2XII-9, TgAP2III-2 parasites were cultured on HFF cell monolayers on coverslips within 24-well plates. They were grown in both regular media and media supplemented with auxin for either overnight or 6 hours. Subsequently, the parasites were fixed using 4% PFA and stained with antibodies. The nucleus was marked using anti-TgEno2, and the count of nuclei per parasite was conducted. For the labeling of the Inner Membrane Complex, both the parental and iKD TgAP2XII-9 strains were allowed to grow on HFF cells for 18–20 hours, followed by a 6- or 18-hour treatment with auxin. Intracellular parasites were then labelled using anti-TgISP1 and anti-TgIMC1 antibodies. The components of the centrosome were labelled after overnight growth of both the parental and iKD TgAP2XII-9 strains, followed by a 6-hour auxin treatment, using anti-TgCentrin1 and anti-TgChromo1 antibodies. Golgi and plastid labelling were performed after overnight growth of both the parental and iKD TgAP2XII-9 strains in auxin-containing media, using anti-TgSortilin and anti-TgACP antibodies, respectively.

Immunofluorescence assays (IFA)

Immunofluorescence experiments were conducted following the fixation of intracellular parasites cultivated on coverslips using 4% PFA for 30 minutes. Subsequently, the coverslips were washed three times with 1X PBS buffer. Permeabilization was achieved by incubating the samples for 30 minutes in a buffer composed of 1X PBS, 0.1% Triton 100X, 0.1% glycine, and 5% FBS. Following permeabilization, primary antibody incubation was performed for 1 hour,

with the antibodies diluted in the same buffer used for permeabilization. Afterward, the coverslips containing the fixed intracellular parasites were washed three times with 1X PBS and incubated for 1 hour with DAPI and secondary antibodies conjugated to either Alexa-594 or Alexa-488. Following another three washes with 1X PBS buffer, the coverslips were mounted onto microscope slides using Moviol. Primary antibodies used included anti-TgIMC1 (a gift from Prof. Ward, University of Vermont), anti-TgEno2, anti-TgISP1, anti-TgCentrin1 (a gift from Prof. Gubbels, College of Boston), anti-TgACP (a gift from Pr. Striepen, U. Penn), anti-TgSortilin, and anti-HA (Sigma Aldrich), anti-myc (abcam), anti-TgIMC3(a gift from Prof. Gubbels, College of Boston) were used at the following dilutions: 1:500, 1:1000, 1:500, 1:500, 1:500, 1:500, 1:1000, 1:200 and 1:2000 respectively. Signal visualization involved manually counting 100–300 parasites for each replicate, with a total of three replicates carried out for each experiment. Immunofluorescence assay experiments were visualized using the ZEISS LSM880 confocal microscope at 63X magnification, and image processing was conducted using CARL Zeiss Zen software.

Ultrastructure Expansion Microscopy (ExM) Procedure

Coverslips with HFF monolayers were inoculated with the iKD TgAP2XII-9 strain. The iKD strain was cultured either in normal media or media treated with auxin for 6 hours. Subsequently, cells were fixed with 4% paraformaldehyde (PFA) and prepared for ultrastructure expansion microscopy (U-ExM) following previously described protocol [52]. Briefly, the coverslips were incubated for 5 hours in a 2× 1.4% acrylamide (AA)/2% formaldehyde (FA) mix at 37˚C. Gelation was performed by incubating in a solution containing ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and a monomer mixture (19% sodium acrylate, 10% AA, and 0.1% bis-acrylamide in 10× PBS) for 1 hour at 37˚C. The gels were then denatured at 95˚C for 1.5 hours. Following denaturation, gels were incubated in double-distilled H2O (ddH2O) overnight to allow for expansion. The next day, gels were washed three times in PBS (10 minutes each) before incubation with primary antibodies for 3 hours at 37˚C. After primary antibody incubation, gels were washed three times in PBS-Tween 0.1%, followed by incubation with secondary antibodies for 3 hours at 37˚C. The gels were washed again three times in PBS-Tween 0.1% and then incubated in ddH2O for a second round of expansion before imaging. Confocal imaging was conducted using a ZEISS LSM880 Confocal Microscope at 63x magnification. Primary antibodies used were anti-TgIMC3 (a gift from Prof. Gubbels, Boston College) at a dilution of 1:1000, and acetylated α-tubulin (Santa Cruz Biotechnology) at a dilution of 1:200.

RNA sample preparation and extraction

RNA samples were prepared by infecting HFF cell monolayers in T175 flasks with iKD TgAP2XII-9 parasites for 24 hours, followed by a 2 or 6-hour treatment with auxin before collecting the samples and adding Trizol (Invitrogen). Control samples were cultured in regular media. RNA extraction was conducted according to the manufacturer's instructions, followed by removal of genomic DNA and purification using the RNase-free DNase I Amplification Grade Kit (Sigma). The quality of all RNA samples was assessed using an Agilent 2100 Bioanalyzer, with only samples having an integrity score of 8 or higher included in the RNA library preparation. 5 biological replicates were generated for Auxin treated conditions and 3 biological replicates were generated for control conditions.

RNA library preparation and validation

RNA libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina), following the manufacturer's protocol. Validation of the libraries was performed using

DNA high-sensitivity chips on an Agilent 2100 Bioanalyzer. Quantification of the libraries was conducted using quantitative PCR (12K QuantStudio).

RNA-sequencing analysis

Bcl2fastq 2.17 (Illumina) was utilized for demultiplexing. The quality of the dataset was assessed using FastQC v0.11.8–0, while adapter treatment for sequencing was performed using Cutadapt v1.18. Trimmomatic v0.39 was employed to filter out reads shorter than 30 bp and those with low-quality bases. Following data cleaning, alignment against the *T. gondii* ME49 genome from ToxoDB was carried out using HiSAT2 v2.2.1. Gene expression quantification was performed on annotated genes using htseq-count from the HTseq suite v1.99.2. Differential gene expression analysis was conducted using DeSeq2 v1.22.1, with P-values adjusted using the Benjamin-Hochberg method. Gene expression exhibiting a fold change >2 or < -2 and an adjusted P value < 0.01 was deemed significantly differentially expressed.

Western blotting

Western blot analysis was conducted by cultivating 2x10^6 parasites of the iKD TgAP2XII-9 strain in regular media overnight, followed by the addition of auxin for durations of 30 minutes, 1, 2, and 6 hours. Control samples were left to grow in normal media. Parasite samples were harvested by filtration and subsequent centrifugation. The resulting pellet was re-suspended in a loading buffer composed of 240 mM Tris-HCl pH 6.8, 8% SDS, 40% sucrose, 0.04% bromophenol blue, and 400 mM DTT. This was followed by denaturation through incubation of the parasite samples at 95˚C for 10 minutes. Protein extracts were separated by electrophoresis on an 8% polyacrylamide gel and then transferred onto a nitrocellulose membrane (GE Healthcare) for 90 minutes at 100V. To block the membrane, a blocking buffer containing 5% milk in TBS buffer comprising 100 mM Tris pH 8, 150 mM NaCl, and 0.1% Tween was employed. The Western blot membranes were then subjected to incubation with primary antibodies for 1 hour, followed by four washes and an additional hour of incubation with secondary antibodies. Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was utilized to visualize protein bands, with ChemiDocTM XRS+ (Biorad) employed for band visualization. The antibodies used included anti-HA, anti-Ty, anti-Myc, and anti-TgMIC3, each at a dilution of 1:1000, 1:500, 1:500, and 1:400, respectively. The secondary antibody utilized was species-specific and conjugated to HRP.

Cleavage under targets and tagmentation (CUT & Tag)

CUT&Tag was employed to identify the genomic localization of TgAP2XII-9. For each sample, intracellular parasites cultured for 24 hours were harvested from a T-175 flask, lysed using a syringe, filtered through a 3 μm filter, and quantified. A total of 20 million (2 × 10^7) parasites were centrifuged at 2,000 × g for 10 minutes, and the resulting pellets were directly processed using the CUT&Tag-IT Assay Kit (Active Motif 53160).

Indexed libraries for each sample were evaluated using Agilent Bioanalyzer, pooled, and sequenced on a NovaSeq6000 to generate paired-end reads. The reads were demultiplexed using bcl2fastq version 2.20.0 and processed with cutadapt v3.4 to eliminate sequencing adapters from the 3' end of reads, discarding any reads with less than 30 base pairs. The remaining reads were aligned to version 64 of the *Toxoplasma gondi*i ME49 reference obtained from ToxoDB using HISAT2 v2.2.1. For each sample, peaks were called using the callpeak command within MACS2. Overlapped peaks across the three biological replicates were determined using the bedtools overlap function. Peak annotation was conducted with CHIPSeeker (an R

package) employing a 2-kb cutoff distance, and the peaks were finally annotated against version 64 of the *T. gondii* reference genome in ToxoDB.

Supporting information

S1 Fig. iKD TgAP2XII-9 and iKD TgAP2III-2 mutant construction and TgAP2III-2 is dispensable for parasite proliferation *in vitro*. **(A)** Illustration of strategy used to construct the iKD mutants of TgAP2XII-9 and TgAP2III-2. A CRISPR/Cas9-assisted homologous recombination was used to generate the iKD strains, in which the endogenous TgAP2XII-9 and tgAP2III-2 is tagged with an AID domain, HA tag and HXGPRT selection cassette. **(B)** PCR verification of the integration of the HXGPRT-HA-AID cassette at the correct genomic locus of the iKD TgAP2XII-9 **(i)** and TgAP2III-2 **(ii)** mutant. A band corresponding to 2776bp and 2045bp using iKD TgAP2XII-9 and iKD TgAP2III-2 genomic DNA respectively, confirms cassette integration, compared to the absence of this band using Tir1(WT)genomic DNA. A positive control was used to confirm the presence of the genomic DNA. **(C)(i)** Plaque assay depicting the proliferation and growth of the iKD AP2III-2 and Parental strains in presence and absence of Auxin. **(C)(ii)** Quantification of the number of lysis plaques in the iKD TgAP2III-2 and parental strain reveals the non-essentiality of TgAP2III-2. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns>0.05. Data are presented as mean ± s.d. (n = 3).
(TIF)

S2 Fig. Plastid and Golgi segregation in the iKD AP2XII-9 throughout the tachyzoite stage. **(A)** IFA and confocal imaging depicting iKD TgAP2XII-9 parasites labelled plastid (red) and Golgi (green) in the presence and absence of overnight Auxin treatment. The IFA revealed no segregation defects in Golgi and plastid. **(B)(i)** Bar graph representing the ratio of Golgi: nucleus using the parental and iKD TgAP2XII-9 strains in the absence and presence of overnight auxin treatment. A Student's *t*-test was performed, significance denoted by ns>0.05; mean ± s.d. (n = 3). **(B)(ii)** Bar graph representing the ratio of Plastid: nucleus using the parental and iKD TgAP2XII-9 strains in the absence and presence of overnight auxin treatment. A Student's *t*-test was performed, significance denoted by ns>0.05; mean ± s.d. (n = 3).
(TIF)

S3 Fig. TgAP2XII-9 and TgAP2III-2 have no combinatorial effect on the parasite biology *in vitro*. **(A)** **(i)** Plaque assay depicting the proliferation of the double mutant in presence and absence of auxin. **(A)(ii)** Quantification of the number of plaques in the iKD TgAP2XII-9 and Double mutant strains. A two tailed Student's *t*-test was performed, significance denoted by *p<0.05, **p<0.01, ****p<0.0001; mean ± s.d. (n = 3). (B) Growth assay for parental and iKD TgAP2XII-9, iKD TgAP2III-2 and the Double mutant strains with and without 24-hour auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by *p<0.05, ns>0.05. Data are presented as mean ± s.d. (n = 3). **(C)** IFA and confocal imaging illustrating the multiple nuclei and IMC defect phenotype labelled by TgIMC3(red) and TgENO2(green) in the Double mutant strain in presence and absence of auxin, scale bar = 5 μm. **(D)** Quantification of the multiple nuclei phenotype in the **parental and iKD TgAP2XII-9, iKD TgAP2III-2 and the Double mutant strains with and without overnight auxin treatment.** Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ns>0.05. Data are presented as mean ± s.d. (n = 3). **(E)** Quantification of the IMC defect phenotype in the parental and iKD TgAP2XII-9 and the Double mutant strains after 6 hrs. auxin treatment. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated by ****p<0.0001, ns>0.05. Data are

presented as mean ± s.d. (n = 3).
(TIF)

S4 Fig. TgAP2XII-9 regulates key genes important for daughter parasite formation. (A) Heatmap showing the cell cycle expression all the upregulated transcripts upon the depletion of TgAP2XII-9. Majority if the upregulated transcripts show peak expression across the cell cycle. (B) Heatmap showing the cell cycle expression all the downregulated transcripts upon the depletion of TgAP2XII-9. Majority if the downregulated transcripts show peak expression during the late S, M,C and G1 phases of the cell cycle.
(TIF)

S5 Fig. TgAP2XII-9 has a different biological role from TgAP2XII-2 and MORC. (A) Cell cycle expression of the 25 genes that are directly regulated and targeted by MORC and TgAP2-XII-9 and bound by TgAP2XII-2 at their promoters. Most of them show basal level of expression throughout the cell cycle, while some of them show expression peaks during the M, C and the G1 phase. **(B)** Heatmap of the 25 genes during the different life stages of the parasite show that these genes are preferentially expressed in the bradyzoite and sexual stages of the life cycle.
(TIF)

S6 Fig. Complementation of the iKD TgAP2XII-9 demonstrate that the TgAP2XII-9 protein is responsible for the phenotypes observed in the mutant and the AP2 domain is crucial for its function. (A) CUT & Tag track s of 3 replicates of TgAP2XII-9-HA and Tir1 strains showing the targeting of TgAP2XII-9 to its own promoter suggesting a negative feedback loop. **(B)** IFA and confocal imaging illustrating the localisation of the complemented TgAP2XII-9 to the nucleus in presence of auxin when the native TgAP2XII-9-HA is depleted. Endogenous TgAP2XII-9 tagged with HA is represented in red while exogenous TgAP2XII-9 tagged with myc is represented in green. Scale bar = 5 μm. **(C)** Bar graph representing the expression of TgAP2XII-9 using anti-HA and anti-myc antibodies in the complemented strain. mean ± s.d. (n = 3 independent experiments). **(D)** Quantification of the IMC defect phenotype in the iKD-C TgAP2XII-9 and iKD TgAP2XII-9 strains. Statistical analysis was performed using a two-tailed Student's t-test, with significance indicated ns>0.05. Data are presented as mean ± s.d. (n = 3). **(E)** IFA and confocal imaging illustrating the localisation of the complemented TgAP2XII-9 to the nucleus in presence of auxin when the native TgAP2XII-9-HA is depleted. Endogenous TgAP2XII-9 tagged with HA is represented in red while exogenous TgAP2XII-9 tagged with myc is represented in green. Scale bar = 5 μm. **(D)** IFA and confocal imaging illustrating the localisation of the complemented TgAP2XII-9ΔAP2 to the nucleus in presence of auxin when the native TgAP2XII-9-HA is depleted. Endogenous TgAP2XII-9 tagged with HA is represented in red while exogenous TgAP2XII-9ΔAP2 tagged with myc is represented in green. Scale bar = 3 μm.
(TIF)

S1 Table. List of genes significantly affected 2 hours after the depletion of TgAP2XII-9.
(XLSX)

S2 Table. List of genes significantly affected 6 hours after the depletion of TgAP2XII-9.
(XLSX)

S3 Table. List of genes that are upregulated in both 2-hour and 6-hour transcriptomic data following the depletion of TgAP2XII-9.
(XLSX)

S4 Table. List of genes that are down-regulated in both 2-hour and 6-hour transcriptomic data following the depletion of TgAP2XII-9.
(XLSX)

S5 Table. List of genes associated with peaks identified by CUT&Tag experiments conducted with three biological replicates.
(XLSX)

S6 Table. List of genes present in both transcriptomic and CUT&Tag datasets to identify direct targets of TgAP2XII-9.
(XLSX)

S7 Table. List of primers used in this study.
(XLSX)

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ANNEXE-2

PLOS BIOLOGY

RESEARCH ARTICLE

PP1 phosphatase controls both daughter cell formation and amylopectin levels in *Toxoplasma gondii*

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Abstract

Virulence of apicomplexan parasites is based on their ability to divide rapidly to produce significant biomass. The regulation of their cell cycle is therefore key to their pathogenesis. Phosphorylation is a crucial posttranslational modification that regulates many aspects of the eukaryotic cell cycle. The phosphatase PP1 is known to play a major role in the phosphorylation balance in eukaryotes. We explored the role of TgPP1 during the cell cycle of the tachyzoite form of the apicomplexan parasite *Toxoplasma gondii*. Using a conditional mutant strain, we show that TgPP1 regulates many aspects of the cell cycle including the proper assembly of the daughter cells' inner membrane complex (IMC), the segregation of organelles, and nuclear division. Unexpectedly, depletion of TgPP1 also results in the accumulation of amylopectin, a storage polysaccharide that is usually found in the latent bradyzoite form of the parasite. Using transcriptomics and phospho-proteomics, we show that TgPP1 mainly acts through posttranslational mechanisms by dephosphorylating target proteins including IMC proteins. TgPP1 also dephosphorylates a protein bearing a starch-binding domain. Mutagenesis analysis reveals that the targeted phospho-sites are linked to the ability of the parasite to regulate amylopectin steady-state levels. Therefore, we show that TgPP1 has pleiotropic roles during the tachyzoite cell cycle regulation, but also regulates amylopectin accumulation.

Introduction

Apicomplexa is a phylum that comprises single-celled, obligate, intracellular protozoan parasites. Within this phylum, there are several species of human pathogens, such as *Plasmodium* spp. (the causative agent of malaria), *Toxoplasma* (which causes toxoplasmosis), and

PXD043539. The RNA-seq data was deposited to the SRA database under the identifier PRJNA1002988.

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Abbreviations: AG, amylopectin granules; AID, auxin-inducible degron; APS, ammonium persulfate; DMSO, dimethyl sulfoxide; EM, electron microscopy; FDR, false discovery rate; HCD, higher-energy collisional dissociation; HFF, human foreskin fibroblast; IMC, inner membrane complex; MPA, mycophenolic acid; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; PM, plasma membrane; PFA, paraformaldehyde; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase.

Cryptosporidium spp. (the cause of cryptosporidiosis). Interestingly, *T. gondii* has been used as a model for other apicomplexan parasites due to its genetic tractability. Acute toxoplasmosis is caused in the intermediate host by rapid proliferation of the tachyzoite form. Chronic toxoplasmosis is due to the establishment of the encysted form of the parasite (bradyzoites) in specific tissues (mainly brain and muscles). The establishment of the bradyzoite cysts in neurons or muscle cells is of interest in terms of pathology in humans, since reactivation of the cysts can lead to the deadly form of the disease (cerebral toxoplasmosis). It is also crucial for transmission since cysts contained in muscles are one of the main infectious routes for humans through the consumption of undercooked meat. Therefore, the ability to rapidly proliferate as a tachyzoite and to switch to the latent bradyzoite form are key elements of pathogenesis in humans and other intermediate hosts.

Diseases triggered by *T. gondii* involve an uncontrolled increase in parasite numbers, leading to inflammation and destruction of host cells. Although this parasite undergoes a sexual cycle in the definitive host, the pathogenesis is primarily driven by asexual replication cycles occurring within the host's cells. The tachyzoite proliferation involves a tightly regulated control of the cell cycle, ultimately resulting in the production of new daughter cells containing 1 nucleus and a complete set of organelles [1]. Although regulation of the cell cycle involves transcriptional control of gene expression [2,3], posttranslational regulation of critical mechanisms has been shown to occur in these parasites [1]. Among these posttranslational modifications, phosphorylation and dephosphorylation regulate important molecular functions including parasite cell division [4,5] and both *T. gondii*'s and *P. falciparum*'s global phosphoproteome show extensive phosphorylation of a large proportion of proteins [6] suggesting an important contribution of these posttranslational marks in the life cycle of these parasites. Moreover, phosphatases and kinases have been shown to play a crucial role during the *Plasmodium* life cycle [7,8].

T. gondii kinases have been demonstrated to have crucial roles in the division of the tachyzoite [9]. Among the 40 kinases that were inspected, 15 were essential to tachyzoite growth, many of which showed cell cycle defects linked to daughter cell formation and nuclear division [9]. Tachyzoite division is controlled by a bipartite centrosome, for which the inner core controls nuclear division while the outer core controls the daughter cell production [10]. The proper division of the centrosome is regulated by TgCDPK7 [11], TgMAPK-L1 [10], and TgMAPK2 [12]. Moreover, cell division of the *T. gondii* tachyzoite is regulated by 5 cyclically expressed kinases denoted as TgCrks; these kinases control several cell cycle checkpoints and ensure the smooth progression of the cell cycle [13,14]. Only a handful of protein phosphatases have been characterized in depth in this parasite and many protein phosphatase functions remain to be unraveled [5]. Seventeen serine/threonine protein phosphatases were mutated using the CRISPR/Cas9 system and only TgPP7 demonstrated a prominent role in virulence [15]. TgPP2A has been shown to play a role in differentiation from the tachyzoite to the bradyzoite stage by regulating starch metabolism [16,17], a process that is also controlled by the kinase TgCDPK2 [18]. TgPPKL depletion leads to the uncoupling of the cell cycle, with normal DNA duplication but severe defects in forming daughter parasites [19]. Moreover, TgPP1, a serine/threonine phosphatase with homologs that have been extensively studied in higher eukaryotes, has been shown to play a critical role in promoting motility of the parasite in response to Ca^{2+} upon egress from the host cell [20]. Notably, upon the release of Ca2+ from intracellular stores, TgPP1 exhibited relocalization to the apex of the parasite [20]. Phosphoproteomics analysis after treating parasites with zaprinast (a cGMP activator that ultimately stimulates egress of the parasite), revealed that TgPP1 is likely involved in dephosphorylating crucial targets during egress and motility [20]. The affected pathways included transmembrane transport, cyclic nucleotide synthesis, ubiquitin transfer, and cytoskeletal motor activity [20],

revealing the crucial roles of TgPP1 in the control of the ultimate steps of the intracellular cycle of the parasite. The *P. falciparum* PP1 showed similar defects in egress of the host cell and also exhibited a deficiency in DNA replication [21]. *P. berghei* PP1 was shown to be crucial for the erythrocytic cycle [22] as well as for maturation of the male gametocyte and exflagellation, a process that requires multiple rounds of nuclear division [23]. The TgPP1 depleted parasites were also shown to exhibit growth defects independent from the egress phenotype but this was not explored further [20]. Therefore, the role of TgPP1 during the cell cycle and in controlling intracellular growth has not been investigated. Indeed, TgPP1, as well as *Plasmodium* PP1 [24–26], forms a complex with TgLRR1 [27] and TgI2 [28], 2 homologs of known regulators of cell cycle function in other eukaryotes [29], suggesting a role in controlling the cell cycle in this parasite.

In this study, we produced an inducible knockdown mutant of TgPP1 and characterized its phenotypes during the tachyzoite cell cycle. We demonstrate that TgPP1 has a crucial role in regulating the multiple pathways that are essential for the production of daughter cells such as inner membrane complex (IMC) assembly, organelle division, and chromosome segregation. TgPP1 also ensures normal assembly of the daughter cell's IMC. Phospho-proteomics demonstrated the differential phosphorylation of several IMC proteins and other targets that are linked to the regulation of the cell cycle. Moreover, we demonstrated the role of TgPP1 in starch production and identified an unknown protein that is crucial for the balance of starch production and whose activity is, at least partially, regulated through dephosphorylation by TgPP1.

Materials and methods

Parasite culture, transfection, and purification

Toxoplasma gondii tachyzoites belonging to the Type I RH Δ*ku80* Tir1 strain were grown in vitro in human foreskin fibroblast (HFF) cells using Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum (FCS), 2 mM glutamine, and 1% penicillin-streptomycin. The Type I Δ*ku80* Tir1strain has the *Ku80* gene deleted with the aim of promoting homologous recombination. In addition to expressing the Transport Inhibitor Response (Tir1) protein which has a role in the inducible degradation of targeted proteins using an auxin-inducible degradation (AID) system. Parasite strains were grown in T25 ventilated flasks containing HFF cell monolayers and maintained within a HERAcell VLOS 160i CO_2 incubator (Thermo Scientific) at 37˚C and 5% CO_2. Electroporation was used to introduce transgenes into the parasite's genome by using a BTX Electro Cell Manipulator 600 at 1.5 kV.cm^{-1}, 25 μF capacitance, and 24 Ω resistance. Transgenic parasites were selected by using 25 μg/ml mycophenolic acid (MPA) and 50 μg/ml xanthine. Parasite clones were produced by using limiting dilution. Experiments consisting of DNA, total RNA, and protein purification involved tachyzoite collection by mechanical lysis using a 17-guage needle followed by a 24-guage needle (Terumo AGANI) followed by the filtration of the lysate using a 3 μm polycarbonate membrane (Whatman).

Generation of transgenic *T. gondii* strains

The iKD TgPP1 line was generated by utilizing the Type I RH Δ*ku80* Tir1 strain as a parental strain. A CRISPR/Cas9 plasmid containing a gRNA targeting the 5′ end of the endogenous *TgPP1* gene (TGGT1_310700) and a PCR product consisting of the HXGPRT selection cassette (HXGPRT-T2A-AID-Ty) flanked by 30 bp of homology were used for transfection. The PCR product contained a Skip peptide (T2A) allowing for the dual expression of protein under a single promoter followed by cleavage of the TgPP1 protein from the HXGPRT protein

in order to promote the degradation of the TgPP1 protein by the proteasome once the auxin hormone is added. The PCR product was amplified by using the pHXGPRT- 2TA-AID-Ty plasmid as a template. Ten μg of PCR product was transfected with 30 μg of pSAG1:: Cas9-U6 targeting the 5′ end of the TgPP1 gene in the RH Δ*ku80* Tir1 strain. Primers used to generate transfection material are included in S1 Table.

Generation of trehalose phosphatase mutants

TGGT1_297720 gene was tagged at the 3′ end with 3xmyc tag in the RH Δ*ku80* strain using a CrispR/Cas9 expressing construct targeting the 3′ end of the gene. To generate point mutation in the TGGT1_297720 gene, a CrispR/Cas9 construct was designed in the 15th intron of the gene. A DNA fragment that included the 2 phosphosites to be mutated was synthesized with a point mutation in the PAM site of the gRNA (located in the 15th intron of the gene). The mutated DNA fragments were generated by mutagenesis using the Q5 mutagenesis kit to induce point mutations in the codons corresponding to Serine 1054 and Serine 1073. They were mutated to either Alanine or Aspartate amino acids. These DNA fragments were co-transfected together with the CrispR/Cas9 construct into the RH Δ*ku80* strain. FACS sorting based on the CrispR/Cas9 construct's GFP expression was used to isolate the mutant strains and directly clone them into 96-well plates. Each mutant clone was verified using sequencing.

Growth assays

Growth assays were carried out by the inoculation of 8×10^4 parasites of parental Tir1 and iKD TgPP1 mutant parasites on HFF cell monolayers grown on coverslips in a 24-well plate for 24 h and 48 h in the presence and absence of 0.5 mM of auxin (AID/indoleacetic acid). Auxin was introduced into the media with the aim of inducing TgPP1 protein degradation. After either 24 h or 48 h, infected coverslips were fixated with 4% of paraformaldehyde (PFA). The fixated parasites were stained using anti-TgEno2 in order to stain parasite nuclei as well as anti-TgIMC1 antibodies in order to stain the IMC. The number of parasites per vacuole was counted for a total of 100 vacuoles per biological replicate. Each growth assay experiment included a total of 3 to 5 biological replicates.

Plaque assays

In order to carry out the plaque assays, a total of 1,000 parasites of either the Parental Tir1 strain or iKD TgPP1 mutant strain were inoculated on a monolayer of HFF cells grown in a 6-well plate either in normal media or media treated with 0.5 mM of auxin. Parasites were left to grow for 7 days before fixation using 100% ethanol. Plaques were stained using Crystal Violet for the purpose of plaque visualization. An Excel macro was used to quantify the plaque size for each experimental condition.

Organelle labeling

Parental Tir1 and iKD TgPP1 parasites were left to grow on monolayers of HFF cells grown on coverslips of 24-well plates in normal media as well as media treated with auxin for 24 h and 48 h before fixating parasites with 4% PFA and staining with antibodies. The IMC was labeled using anti-TgIMC1, anti-TgISP1, and anti-TgGAP45. Other organelles consisting of the Golgi, plastid, and centrosome were labeled using anti-TgSORT, anti-TgACP, and anti-TgCentrin1 antibodies, respectively.

Immunofluorescence assays

Immunofluorescence experiments were carried out after fixation of intracellular parasites grown on coverslips using 4% of PFA for 30 min. This was followed by coverslip washing 3 times using 1× PBS buffer. Permeabilization was carried out for 30 min using the following buffer (1× PBS, 0.1% Triton 100×, 0.1% glycine, 5% FBS). This was followed by primary antibody incubation for 1 h. Antibodies were diluted using IFA buffer used for permeabilization. Coverslips containing fixated intracellular parasites were then washed 3 times using 1× PBS. Coverslips were then incubated for 1 h in DAPI and secondary antibodies coupled to either Alexa-594 or Alexa-488. Coverslips were then washed 3 times using 1× PBS buffer and mounted onto microscope slides using Moviol. Primary antibodies used included anti-TgIMC1 (a gift from Prof. Ward, University of Vermont), anti-TgEno2, anti-TgISP1 [30], anti-TgCentrin1 (a gift from Prof. Gubbels, College of Boston), anti-TgACP (a gift from Pr. Striepen, U. Penn), anti-TgSortilin, and anti-TgGAP45 (Prof. Soldati, Geneve University), anti-HA, and anti-Ty (a gift from Dr. Bastin, Institut Pasteur de Paris) were used at the following dilutions: 1:500, 1:1,000, 1:500, 1:500, 1:500, 1:500, 1:10,000, 1:500, and 1:10,000, respectively. Signal was visualized manually by counting 100 parasites for each replicate. A total of 3 replicates were carried out for each experiment.

Immunofluorescence assay experiments were visualized using the ZEISS LSM88O confocal microscope at a magnification of 63×. Image processing was carried out using the CARL Zeiss Zen software.

Electron microscopy and cytochemical localization of glycogen

iKD TgPP1 parasites were grown on monolayers of HFF cells in normal media or media treated with auxin for 48 h in T25 flasks. Intracellular parasites were fixated using the following solution: 1% glutaraldehyde in 0.1 M sodium cacodylate at a pH of 6.8 and at 4˚C. Parasite samples were then post-fixated using 1% osmium tetraoxide and 1.5% potassium ferricyanide. This was then followed by using 1% uranyl acetate. Post-fixation was carried out for 1 h using the following conditions: distilled water, in the dark, and at room temperature. Increasing ethanol concentration solutions were used to dehydrate fixated samples after washing. Epoxy resin was used with the aim of infiltrating parasite samples. This was followed by curation for 24 h at 60˚C. Deposition of 70 to 80 nm-thick sections was carried out in formvar-coated grids. Images were observed using 80 kV on a Hitachi H7500 TEM (Federal University of Rio de Janeiro University, Brazil). Acquisition of images was carried out by using an Mpixel digital camera (Federal University of Rio de Janeiro University, Brazil). For the cytochemical localization of glycogen, parasites were fixed and processed as described for TEM. Subsequently, 90-nm sections were collected on gold grids and incubated for 15 min in a solution containing 1% periodic acid, washed 3 times, and then incubated with 1% thiosemicarbazide in 10% acetic acid for 24 h. Successive washes were performed in 10%, 5%, and 2% acetic acid for 10 min each. Afterward, the sections were incubated with 1% silver proteinate for 30 min, protected from light. Finally, the sections were washed in distilled water for 10 min each and observed unstained on a FEI Tecnai SPIRIT transmission electron microscope.

Ultrastructure expansion microscopy

Coverslips with HFF monolayers were inoculated with the iKD TgPP1 or ikD TgPP1-IMC17-myc parasite strains. They were cultured either in normal media or media treated with auxin for 24 h. Following this, cells were fixed using 4% PFA for 20 to 30 min and prepared for ultrastructure expansion microscopy (U-ExM). In brief, coverslips were incubated for 5 h at 37˚C in a solution of 2× 1.4% acrylamide (AA) and 2% formaldehyde (FA). Gelation was achieved

by incubating the samples in a mixture containing ammonium persulfate (APS), tetramethylethylenediamine (TEMED), and a monomer solution (19% sodium acrylate, 10% AA, and 0.1% bis-acrylamide in 10× PBS) for 1 h at 37˚C. Post-gelation, the gels were denatured at 95˚C for 1.5 h. The denatured gels were then expanded by incubating overnight in double-distilled H2O (ddH2O). The following day, gels were washed 3 times with PBS (10 min each wash) before incubating with primary antibodies for 3 h at 37˚C. After primary antibody incubation, the gels were washed 3 times in PBS-Tween 0.1%, followed by an overnight incubation with secondary antibodies at 4˚C. Subsequent washes in PBS-Tween 0.1% were performed 3 times before a second round of expansion in ddH2O prior to imaging. Confocal imaging was performed using a ZEISS LSM880 Confocal Microscope with a 63× magnification. Primary antibodies used were anti-TgIMC3 (provided by Prof. Gubbels, Boston College) at a dilution of 1:1,000, acetylated α-tubulin (Santa Cruz Biotechnology) at a dilution of 1:200, anti-Myc (abcam) at a dilution of 1:200, and anti-TgNuf2 (provided by Prof. Gubbels, Boston College) used at a dilution of 1:200.

RNA sample preparation and extraction

RNA samples of iKD TgPP1 parasites were collected after inoculating parasite onto monolayers of HFF cells grown in T175 flasks. iKD TgPP1 parasites were grown in normal media (control) as well as media treated with auxin for 24 h. RNA extraction was carried out by re-suspending the parasite sample in Trizol (Invitrogen). This was followed by the addition of chloroform (4˚C) to the sample allowing for the separation of the RNA-containing aqueous phase and the protein-containing organic phase. This was followed by a centrifugation step at room temperature for 10 min. The aqueous phase was then transferred into a new tube consisting of cold isopropanol in order to precipitate the RNA followed by a centrifugation step. Washing of the precipitated RNA pellet was carried out using 70% ethanol followed by air-drying the pellet and re-suspension in RNase-free water (Gibco). RNA samples were purified using the RNase-free DNaseI Amplification Grade Kit (Sigma). Extracted RNA quality was verified using an RNA 6000 Nano (Agilent) chip and RNA samples with an integrity score of 8 or greater were used for RNA library preparation.

RNA-sequencing library preparation

Libraries for the RNA samples were prepared by using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina). Libraries were prepared as per the manufacturer's instructions. Validation of the libraries was carried out using DNA high sensitivity chips read by an Agilent 2100 Bioanalyzer. Libraries were then quantified by using the KAPA library quantification kit (Illumina) using a 12K QuantStudio qPCR thermocycler.

RNA-sequencing analysis

RNA libraries were sequenced using a HiSeq 2500 as 50 bp reads by using the sequence by synthesis technique. HiSeq control software and real-time analysis component were used for image analysis. Bcl2fastq 2.17 (Illumina) was used to demultiplex. Data set quality was verified using FastQC v0.11.8–0. Cutadapt v1.18. was used to treat the adapters for sequencing. Filtering of reads shorter than 50 bp and low-quality bases was carried out by using Trimmomatic v0.38.1. Alignment of data sets using HiSAT2 v2.2.0 was carried out after cleaning of data sets against the *T. gondii* ME49 genome from ToxoDB. Annotated gene expression was quantified using htseq-count from the HTseq suite v0.9.1. DeSeq2 v1.22.1 was used to carry out differential gene expression analysis. *P*-values were adjusted using the Benjamin–Hochberg method.

Adjusted *p*-values less than 0.05 and a log2 fold change greater than 2 corresponding to differentially expressed genes were kept.

Phosphoproteomics sample extraction

Mutant iKD TgPP1 parasites were left to grow in T175 flasks before the addition of auxin for 2 h and 24 h in T175 flasks. iKD TgPP1 parasites grown in normal media were considered as a control. After parasite purification by filtration and centrifugation, parasite pellet was re-suspended using 8 M Urea consisting of Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Parasite was re-suspended in urea solution to a final concentration of 35 million parasites/50 μl of re-suspension solution. Extraction steps were carried out on ice. A Bioruptер Sonicator was used at 4˚C for 10 cycles (30 s on/off per cycle). This was followed by a centrifugation step for 20 min at 4,000 rpm at 4˚C. The supernatant was separated from the pellet and put into a new tube. The Pierce BCA Protein Assay (Life Technologies) was used to quantify the protein concentration of the samples. Samples were then stored at −80˚C prior to analysis.

Tryptic digestion

S-Trap mini spin column (Protifi, Hutington, United States of America) digestion was performed on 100 μg of cell lysates, according to manufacturer's instructions. Briefly, SDS concentration was first adjusted to 5% and samples were reduced with 20 mM TCEP and alkylated with 50 mM chloracetamide for 15 min at room temperature. Aqueous phosphoric acid was then added to a final concentration of 1.2% following by the addition of S-Trap binding buffer (90% aqueous methanol, 100 mM TEAB, pH 7.1). Mixtures were loaded on S-Trap columns by 30 s centrifugation at 4,000 × g; 6 washes were performed before adding the trypsin (Promega) at 1/20 ratio for 2 h at 47˚C. After elution, peptides were vacuum dried.

Phosphopeptides enrichment by titanium dioxide (TiO_2) and phosphopeptides purification by graphite carbon (GC)

Phosphopeptide enrichment was carried out using a Titansphere TiO_2 Spin tip (3 mg/200 μl, Titansphere PHOS-TiO, GL Sciences, Japan) on 90 μg of digested proteins for each biological replicate. Briefly, the TiO_2 Spin tips were conditioned with 20 μl of solution A (80% acetonitrile, 0,1% TFA), centrifuged at 3,000 × g for 2 min and equilibrated with 20 μl of solution B (75% acetonitrile, 0,075% TFA, 25% lactic acid) followed by centrifugation at 3,000 × g for 2 min. Peptides were resuspended in 20 μl of 10% acetonitrile, 2% TFA in HPLC-grade water, mixed with 100 μl of solution B and centrifuged at 1,000 × g for 10 min. Sample was applied back to the TiO2 Spin tips 2 more times in order to increase the adsorption of the phosphopeptides to the TiO2. Spin tips were washed with, sequentially, 20 μl of solution B and 2 times with 20 μl of solution A. Phosphopeptides were eluted by the sequential addition of 50 μl of 5% NH_4OH and 50 μl of 5% pyrrolidine. Centrifugation was carried out at 1,000 × g for 5 min.

Phosphopeptides were further purified using GC Spin tips (GL-Tip, Titansphere, GL Sciences, Japan). Briefly, the GC Spin tips were conditioned with 20 μl of solution A, centrifuged at 3,000 × g for 2 min and equilibrated with 20 μl of solution C (0,1% TFA in HPLC-grade water) followed by centrifugation at 3,000 × g for 2 min. Eluted phosphopeptides from the TiO2 Spin tips were added to the GC Spin tips and centrifuged at 1,000 × g for 5 min. GC Spin tips were washed with 20 μl of solution C. Phosphopeptides were eluted with 70 μl of solution A (1,000 × g for 3 min) and vacuum dried.

nanoLC-MS/MS protein identification and quantification

Samples were resuspended in 42 μl of 0.1% TFA in HPLC-grade water. For each run, 5 μl was injected in a nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000, Thermo Scientific, Massachusetts, USA). Phosphopeptides were loaded onto a μ-precolumn (Acclaim PepMap 100 C18, cartridge, 300 μm i.d. × 5 mm, 5 μm, Thermo Scientific, Massachusetts, USA) and were separated on a 50 cm reversed-phase liquid chromatographic column (0.075 mm ID, Acclaim PepMap 100, C18, 2 μm, Thermo Scientific, Massachusetts, USA). Chromatography solvents were (A) 0.1% formic acid in water; and (B) 80% acetonitrile, 0.08% formic acid. Phosphopeptides were eluted from the column with the following gradient 1% to 40% B (120 min), 40% to 80% (1 min). At 121 min, the gradient stayed at 80% for 5 min and, at 126 min, it returned to 5% to re-equilibrate the column for 20 min before the next injection. Two blanks were run between each replicate to prevent sample carryover. Phosphopeptides eluting from the column were analyzed by data-dependent MS/MS, using top-10 acquisition method. Phosphopeptides were fragmented using higher-energy collisional dissociation (HCD). Briefly, the instrument settings were as follows: resolution was set to 70,000 for MS scans and 17,500 for the data-dependent MS/MS scans in order to increase speed. The MS AGC target was set to 3.10^6 counts with maximum injection time set to 200 ms, while MS/MS AGC target was set to 1.10^5 with maximum injection time set to 120 ms. The MS scan range was from 400 to 2,000 m/z. Dynamic exclusion was set to 30 s duration.

For total proteomic analysis, peptides were eluted from the column with the following gradient 5% to 40% B (120 min), 40% to 80% (6 min). At 127 min, the gradient returned to 5% to re-equilibrate the column for 20 min before the next injection. MS parameters were the same as those used for LC-MS/MS analysis described above.

Data processing following nanoLC-MS/MS acquisition

The MS files were processed with the MaxQuant software version 1.6.14.0 and searched with the Andromeda search engine against the UniProtKB/Swiss-Prot *Homo sapiens* database (release February 2021, 20,396 entries) and *Toxoplasma gondii* strain ATCC 50853/GT1 (release November 2020, 8,450 entries). To search parent mass and fragment ions, we set an initial mass deviation of 4.5 ppm and 0.5 Da, respectively. The minimum peptide length was set to 7 amino acids and strict specificity for trypsin cleavage was required, allowing up to 2 missed cleavage sites. Carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met), N-term acetylation, and phosphorylation (Ser, Thr, Tyr) were set as variable modifications (only for phosphoproteomics analysis). The match between runs option was enabled with a match time window of 0.7 min and an alignment time window of 20 min. The false discovery rates (FDRs) at the protein and peptide level were set to 1%. Scores were calculated in MaxQuant as described previously [31]. The reverse and common contaminants hits were removed from MaxQuant output.

The phosphopeptide output table and the corresponding logarithmic intensities were used for phosphopeptide analysis. The phosphopeptide table was expanded to separate individual phosphosites, and all sites identified in all 4 replicates were kept in at least 1 group for the 2 h condition and in all 3 replicates in at least 1 group for the 24 h experiment. Missing values were inputed using width = 0.3 and down-shift = 3. Significantly altered phosphosites were represented by volcano plots (*t* test S0 = 1, FDR = 0.05).

For total proteome analysis, only proteins identified in all 4 replicates were kept in at least 1 group. Missing values were inputed using width = 0.3 and down-shift = 2.5. The significantly altered proteins were represented on a volcano plot s (*t* test S0 = 1, FDR = 0.05).

Western blotting

Western blotting was carried out by inoculating 2×10^6 parasites of the iKD TgPP1 strain in T175 flasks grown in normal media for 24 h (control) and grown in media treated with auxin for 1 and 2 h. Parasite samples were collected by filtration, followed by centrifugation. The obtained pellet was re-suspended in loading buffer consisting of 240 mM Tris-HCl (pH 6.8), 8% SDS, 40% saccharose, 0.04% bromophenol blue, and 400 mM DTT. This was followed by a denaturing step by incubating the parasite samples at 95˚C for 10 min. Protein extracts were separated by electrophoresis on an 8% polyacrylamide gel and then transferred onto a nitrocellulose membrane (GE Healthcare) for 90 min at 100 V. Blocking buffer containing 5% milk in TNT buffer consisting of 100 mM Tris (pH 8), 150 mM NaCl, and 0.1% Tween was used to block the membrane. Western blot membranes were incubated in primary anti-body for 1 h, washed 4 times, incubated for another hour in the secondary antibody. Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used to reveal protein bands and ChemiDoc XRS$^+$ (Biorad) was used to visualize protein bands. Antibodies used were anti-Ty used at a dilution of 1/500. Secondary antibody used is species specific and conjugated to HRP.

PAS staining

For the PAS staining, Parental Tir 1 and iKD TgPP1 parasites were left to grow on HFF cell monolayers grown on coverslips for 48 h in the presence and absence of auxin. Periodic acid-Schiff stain was used in order to determine the presence of polysaccharides within the parasites. Visualization of polysaccharides was carried out by using a confocal microscope.

Amylopectin quantification

Parental Tir1 and iKD TgPP1 parasites were left to grow for 48 h in the presence and absence of auxin before parasite filtration and centrifugation. Around 200 million parasites were collected for each sample. After purification, parasite samples were stored at −80˚C and sent for analysis. Purified *T. gondii* cells were resuspended in ice-cold phosphate-buffered saline (PBS) at a concentration of 20 million parasites per ml. Cell suspensions were then disrupted 3 times by a French press (13,000 p.s.i.) and centrifuged at 10,000g for 30 min at 4˚C. The pellets containing amylopectin and cell debris were passed through a self-formed 90% Percoll gradient at 10,000g for 30 min at 4˚C. The purified amylopectin pellets were washed in ultrapure water, centrifuged twice at 10,000g, and kept dry at 4˚C. Polysaccharide amounts were measured by an amyloglucosidase assay using the Enzytec starch kit following the manufacturer's recommendations. In parallel, 30 μg of polysaccharide were resuspended in 20 μl of 100% dimethyl sulfoxide (DMSO), boiled 10 min, and diluted to 10% DMSO. Twenty μl of a freshly prepared iodine solution (0,2% I2; 2% KI) were added to 80 μl of the boiled sample and the absorbance of the complex was monitored from 700 to 400 nm allowing the determination of its λmax (wavelength at the maximal absorbance). Controls including commercial potato amylopectin, potato amylose, and rabbit liver glycogen (Sigma) were used and displayed λmax values of 542 nm, 631 nm, and 495 nm, respectively.

Statistics

Graph pad Prism software version 8/9 (San Diego, California, USA) was used to analyze all data concerning growth assays, proliferation assays, and ratio counts. Student *t* tests were used to determine significant differences between data sets where *p*-values <0.05 were considered

as significant. All experiments were carried out in biological triplicates. For each independent experiment, a total of 100 parasites/vacuoles was counted.

Results

TgPP1 is essential for the normal growth and proliferation of *T. gondii* tachyzoites

We generated an inducible knock down mutant of TgPP1(iKD TgPP1) with the aim of determining the role of the TgPP1 protein using the auxin-inducible degron (AID) system. The mutant parasite line was produced using CRISPR/Cas9 to insert an *hxgprt-t2a-AID-2ty* insert at the 5′ end of the TgPP1 gene (Fig 1A). Previous attempts to generate the transgenic parasite strain by inserting an *hxprt-AID-HA* cassette at the 3′ end were unsuccessful. Insertion of the *hxgprt-t2a-AID-2xty* cassette at the appropriate locus within the iKD TgPP1 genome was confirmed by insertion PCR (S1A Fig). The localization of Ty-tagged TgPP1 protein was investigated using IFA and TgPP1 was demonstrated to be localized mainly in the nucleus but also in the cytoplasm when compared with a cytoplasmic marker (TgAlba1 [32]) during the tachyzoite intracellular cell cycle (Fig 1B). The depletion of TgPP1 was obtained within 1 h of auxin treatment as verified by western blot of total protein extracts (Fig 1C). A growth assay was carried out for 24 h in the presence and absence of auxin. We noticed that the growth of the strain

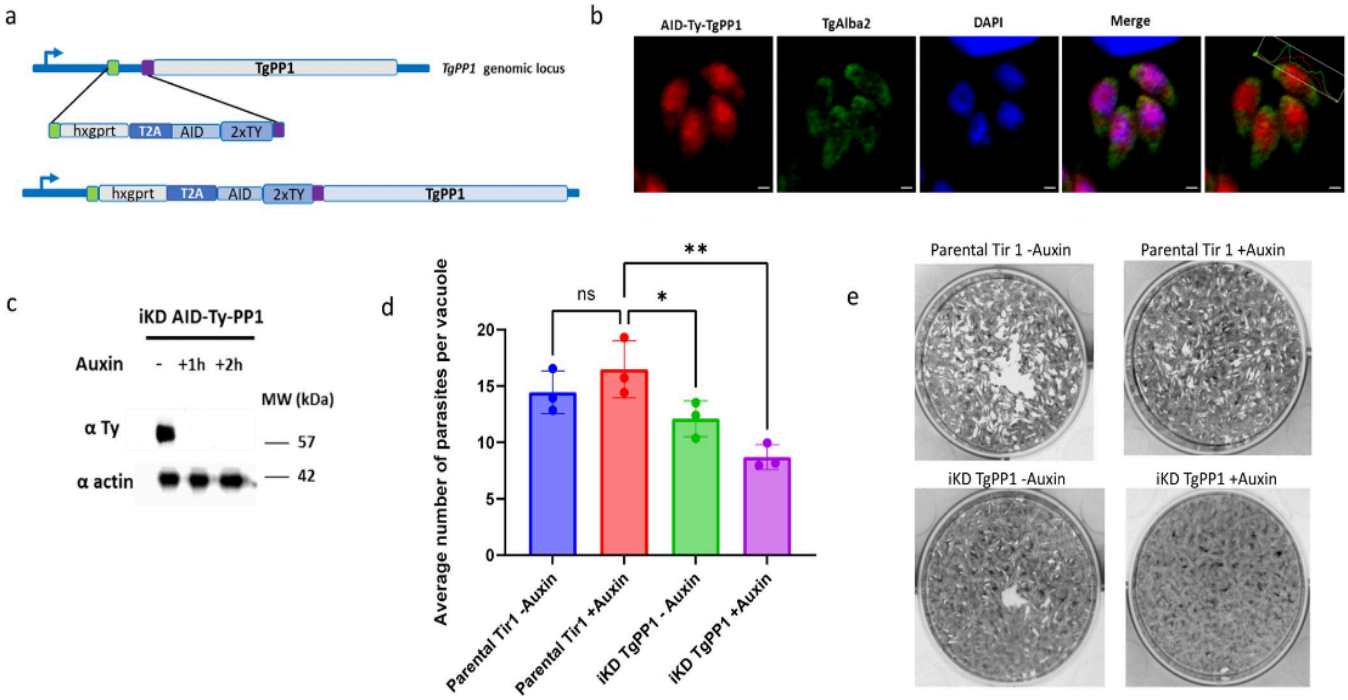

Fig 1. TgPP1 is required for parasite growth and proliferation. **(a)** Schematic representation of the construct used to generate iKD TgPP1 mutant parasites using the AID system that allows for the inducible degradation of the TgPP1 protein. The system involves introducing the AID domain into the gene of interest. In the presence of Auxin, the AID domain will be recognized by the Tir1 protein and the protein degraded by the proteasome. The HXGPRT-T2A-AID-2Ty cassette is inserted at the 5′ end of the gene using the CRISPR/Cas9 gene-editing strategy. **(b)** Confocal microscopy imaging of the AID-Ty iKD TgPP1 parasite in the absence of auxin labeled with anti-Ty (red, TgPP1) and TgAlba2 (green), a protein that localized to the cytoplasm. DAPI (blue) was used to stain the nucleus. The scale bar is indicated in the lower right corner of each image. Measurements of red and green fluorescence throughout length of the parasite is indicated in the upper right corner of the overlayed TgPP1 and TgAlba2 image. **(c)** Western blot of total protein extract from the iKD TgPP1strain in absence and presence of auxin (1 h and 2 h) displaying depletion of the TgPP1 protein after the addition of Auxin. Western blots were probed with anti-Ty antibodies to determine the presence of the TgPP1 protein (upper panel). Western blot was normalized using anti-TgActin antibodies (lower panel). **(d)** Growth assay of the Parental Tir1 and iKD TgPP1 strains in the absence and presence of Auxin for 48 h. The average number of parasites per vacuole was recorded. A Student's *t* test was performed. $^{*}p < 0.05$, $^{**}p < 0.01$; mean ± SD ($n = 3$). **(e)** Plaque assay demonstrating proliferation of the Parental Tir 1 and iKD TgPP1 strain in the presence and absence of auxin. The data underlying this figure can be found in S1 Data. AID, auxin-inducible degron.

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was affected in absence of auxin, a phenotype that was also observed for another published TgPP1 conditional mutant [20] (S1B Fig). However, the iKD TgPP1 mutant in presence of auxin demonstrated a significant growth defect after 48 h (Fig 1D). We then performed a plaque assay that measures the ability of the parasite to form plaques in the host cell monolayer over a period of 7 days. In this assay, the iKD TgPP1 was unable to form plaques in the presence of auxin thus demonstrating that TgPP1 is important for parasite proliferation as a tachyzoite (Fig 1E). This experiment also showed that the size of the plaque produced by the mutant in absence of auxin was smaller than the parental parasite strain, confirming that the insertion of the AID construct was deleterious to the parasite's growth (S1C Fig). Overall, these results suggest the essentiality of TgPP1 for the normal growth and proliferation of the tachyzoite.

TgPP1 is important for the formation of the inner membrane complex (IMC)

To better understand the biological role of TgPP1 during the intracellular tachyzoite cell cycle, we investigated its ability to form the IMC, a double membrane structure located below the tachyzoite's plasma membrane (PM). Upon depletion of TgPP1, the parasites exhibited an abnormal IMC structure as labeled by an anti-IMC3 antibody, featuring an unstructured network of intertwined IMC3 protein. Upon auxin treatment, the IMC was unable to form properly (Fig 2A, lowest panel) as opposed to the IMC structures of the iKD TgPP1 mutant in the absence of auxin treatment as well as the IMC structures of the Parental Tir1 strain (Fig 2A, upper and medium panels). We also performed expansion microscopy to better asses this phenotype (Fig 2B), showing parasites with similar defect of IMC formation. The quantification of the percentage of vacuoles with this IMC phenotype demonstrated that 40% of the vacuoles showed a similar phenotype after 48 h of auxin treatment (Fig 2C). This phenotype was accompanied by the presence of the parasite's IMC without nuclear material or nuclear material without formed IMC reminiscent of a missegregation of nuclear material as illustrated in Fig 2A (arrowed, lower panel, iKD TgPP1 + Auxin) or Fig 2B (arrowed, lower panel, iKD TgPP1 + Auxin). Quantification revealed that a similar percentage (40%) of vacuoles showed an important missegregation of nuclear material in the iKD TgPP1 mutant in the presence of auxin (Fig 2D) while this phenotype was almost lacking in the parental strain and in absence of auxin. The effect of TgPP1 depletion on additional components of the IMC was also investigated. Upon auxin treatment, TgGAP45, a protein targeted to the PM and connected with the IMC at the C-terminal end, revealed similar defects in IMC formation in the iKD TgPP1 mutant parasites treated with auxin (S2A Fig). Similar results were obtained with the IMC Sub-compartment protein 1 (TgISP1) which in normal instances localizes to the apical cap of the IMC and was instead dispersed along the periphery of the iKD TgPP1 parasite in the presence of auxin (S2B Fig). TgIMC1 was also used as an IMC marker and showed essentially similar phenotypes to TgIMC3 (S2C Fig, quantified in S2D Fig). To further confirm the effect of TgPP1 depletion on the IMC structure, electron microscopy (EM) was carried out. In absence of auxin, the iKD TgPP1 mutant parasites showed both the PM and the IMC normally formed and these 2 structures appeared intact (Fig 2D). In presence of auxin, the iKD TgPP1 mutant parasites exhibited an intact and continuous PM along the parasite. In contrast, the IMC was only partially present and discontinuous along the formed PM (black arrows, Fig 2E). Quantification of these EM observations (S2E Fig) further validate the role of TgPP1 in correctly forming the IMC.

The effect of TgPP1 depletion affects the division and segregation of the Golgi and plastid

During the tachyzoite's cell cycle, the subcellular organelles are replicated according to a well-determined timeline [2]. Indeed, the centrosome divides first and is then followed by the Golgi

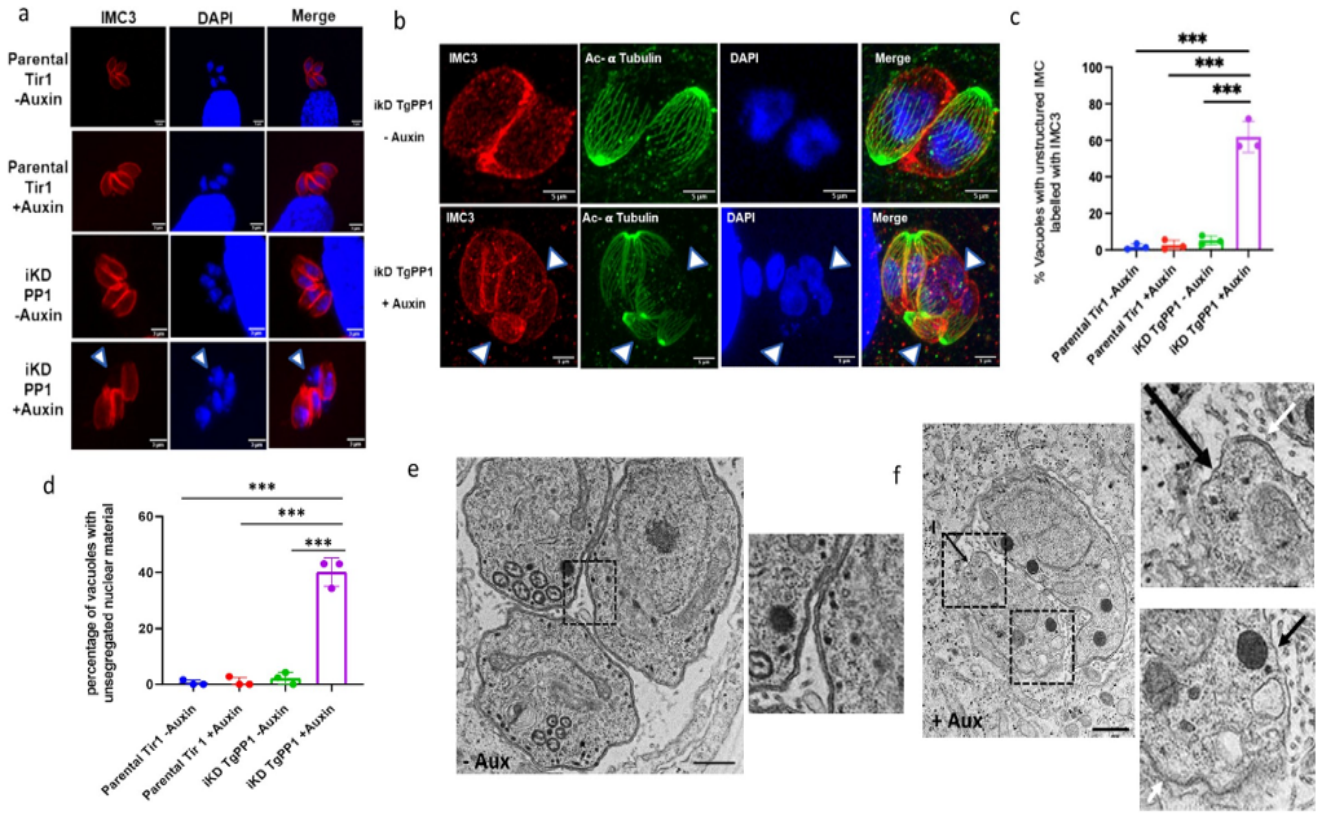

Fig 2. Depletion of TgPP1 results in a collapsed IMC and unsegregated nuclei. **(a)** Confocal imaging of the Parental Tir1 and iKD TgPP1 strains labeled TgIMC3 (red) in the presence and absence of auxin treatment. DAPI was used to stain the nucleus. Scale bar (1 μm) is indicated in the lower right corner of each individual image. A nucleus without formed parasite body is indicated by a white arrow. **(b)** Expansion microscopy images of the iKD TgPP1 strain in absence and presence of auxin. The parasite IMC (IMC3, red) and cytoskeleton (acetylated tubulin, green) were labeled as well as the nucleus by DAPI (blue). A parasite without nucleus and a parasite bearing 2 nuclei are indicated by a white arrow. **(c)** Bar graph representing the percentage of Parental Tir1 and iKD TgPP1 vacuoles possessing a collapsed IMC by using anti-TgIMC3 antibodies for labeling the IMC in the absence and presence of auxin treatment for 48 h. A Student's *t* test was carried out, $^{***}p < 0.001$; mean ± SD ($n = 3$). **(d)** Bar graph displaying the quantification of vacuole with nuclear segregation defects in the Parental Tir1 and iKD TgPP1 strain in the absence and presence of auxin treatment for 48 h. A Student's *t* test was carried out, $^{***}p < 0.001$; mean ± SD ($n = 3$). **(e)** EM image demonstrating the structural morphology characteristics of the IMC and PM of the iKD TgPP1 mutant parasite in the absence of auxin treatment. In this case, both IMC and PM remain intact Magnified region is boxed. **(f)** EM image demonstrating the structural morphology of the IMC and PM of the iKD TgPP1 parasite after auxin treatment for 48 h. In this case, PM membrane remains intact, but the IMC is absent. (I) stand for IMC. Scale bar (5 μm) is demonstrated in the lower right region of each EM. Magnified regions are boxed. Region missing the IMC are indicated by black arrows. A region with both the IMC and PM is indicated by a white arrow. The data underlying this figure can be found in S1 Data. EM, electron microscopy; IMC, inner membrane complex; PM, plasma membrane.

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and then plastid [33]. We investigated the effect of TgPP1 depletion on the division of the centrosome (using TgCentrin1 as a marker of the outer core centrosome) by IFA. However, when counting more than 100 vacuoles in each of the 3 biological replicates, no significant change of the TgCentrin1 to nucleus ratio was identified, suggesting that the centrosome division is mainly unaffected in absence of TgPP1. We then investigated the effect of TgPP1 on the division of organelles. We, therefore, studied the effect of TgPP1 on the division of the plastid and the Golgi. By IFA, we observed that the plastid (TgCpn60) and Golgi (TgSORT) were missegregated after TgPP1 depletion (Fig 3A). Representative pictures presenting the normal and abnormal phenotype observed are presented in Fig 3B. The depletion of TgPP1 after 48 h of auxin treatment resulted in a significant percentage of parasite vacuoles with this phenotype (Fig 3C). To better assess the role of TgPP1 during division and cell cycle, we performed expansion microscopy (S3C Fig) and assessed the number of parasites undergoing mitosis (S3D Fig). While parasites were undergoing mitosis at a similar rate in auxin treated and control samples, the number of parasites in metaphase was much higher in the auxin treated

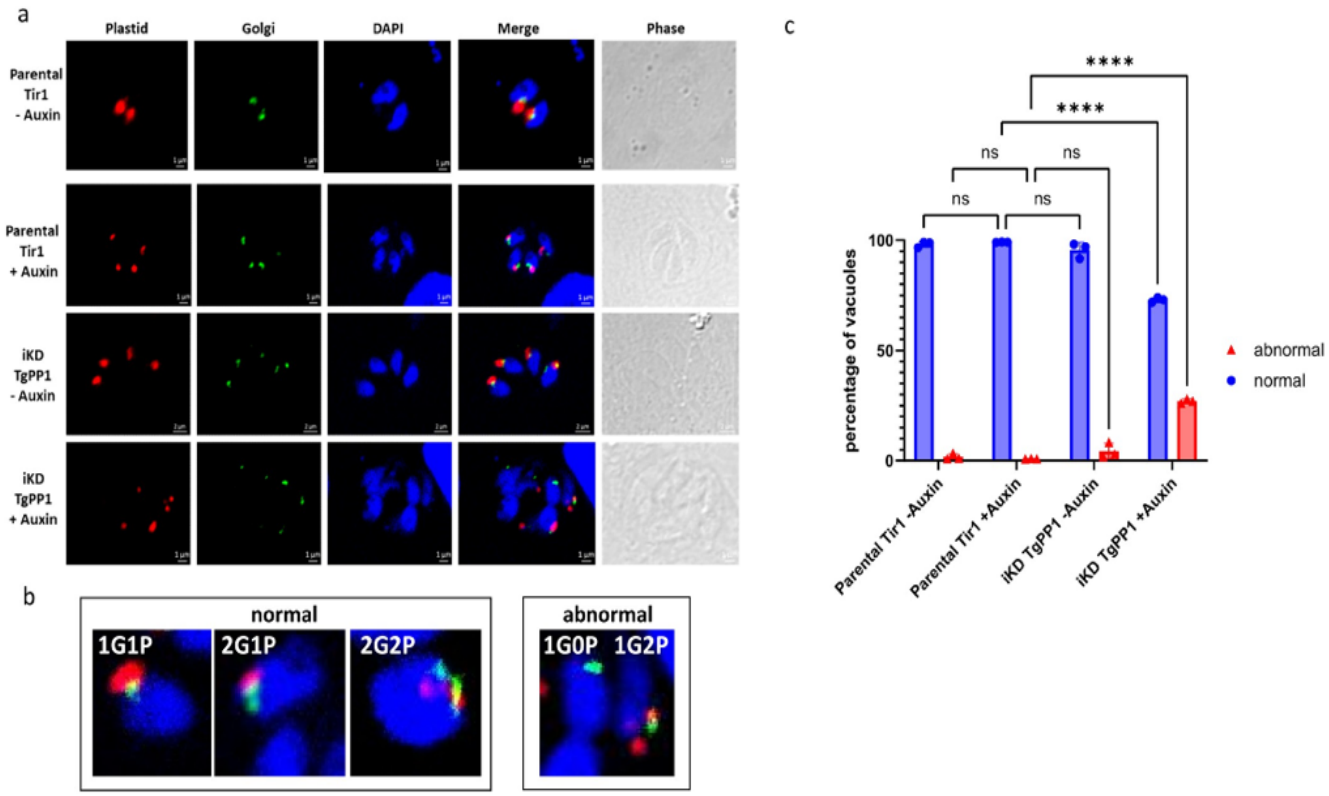

Fig 3. TgPP1 depletion induce plastid and Golgi missegregation. (a) Confocal imaging of the Parental Tir1 and iKD TgPP1 strains with labeled plastid (red) and Golgi (green) in the presence and absence of auxin treatment. DAPI was used to stain the nucleus. Scale bar (1 μm) is indicated in the lower right corner of each individual image. **(b)** Zoom images representing the normal segregation of Golgi (G, green) and plastid (P, red). A zoom image representing the abnormal segregation of Golgi (G, green) and plastid (P, red) is also shown on the right panel. DAPI was used to stain the nucleus. **(c)** Graph bar demonstrating the percentage of Parental Tir1 and iKD TgPP1 vacuoles possessing normal and abnormal plastid and Golgi segregation in the absence and presence of auxin treatment for 48 h. A Student's *t* test was carried out. $^{****}p < 0.0001$; mean ± SD ($n = 3$). Blue bars represent normal plastid and Golgi segregation. Red bars represent abnormal plastid and Golgi segregation. The data underlying this figure can be found in S1 Data.

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parasites, indicating that these parasites may be slowed down at this stage after depletion of TgPP1. Collectively, these results demonstrate that TgPP1 has an important role in coordinating the cell cycle steps after centrosome division.

TgPP1 depletion affects amylopectin steady-state levels

The control of amylopectin levels has been reported to be regulated by the phosphorylation [18] and dephosphorylation [16,17] of enzymes of amylopectin metabolism in *T. gondii*. We hypothesized that TgPP1 could also play a role in starch storage. To measure the effect of TgPP1 depletion on the amylopectin steady-state levels, we used the periodic acid–Schiff (PAS) method that labels polysaccharides. After 48 h of TgPP1 depletion, we clearly identified the appearance of structures positive for PAS staining accumulating in the parasites (Fig 4A, lower panel). This labeling is almost inexistent in the parental strain (in presence or absence of auxin) but can be detected in the iKD TgPP1 mutant in absence of auxin (Fig 4A). The presence of amylopectin granules was further confirmed through EM using a similar staining. Amylopectin granules (AG) were rare in the iKD TgPP1 parasites in the absence of auxin (Fig 4Bi) but observed in the iKD TgPP1 parasites in the presence of auxin at 24 h and 48 h (Fig 4Bii and 4Biii, respectively). AG accumulated in the cytoplasm of the parasite but not in the residual body, a phenotype that is different from what is observed in absence of TgCDPK2 or TgPP2A. The percentage of PAS positive vacuoles was quantified in the iKD TgPP1 mutant and parental strains in the absence and presence of

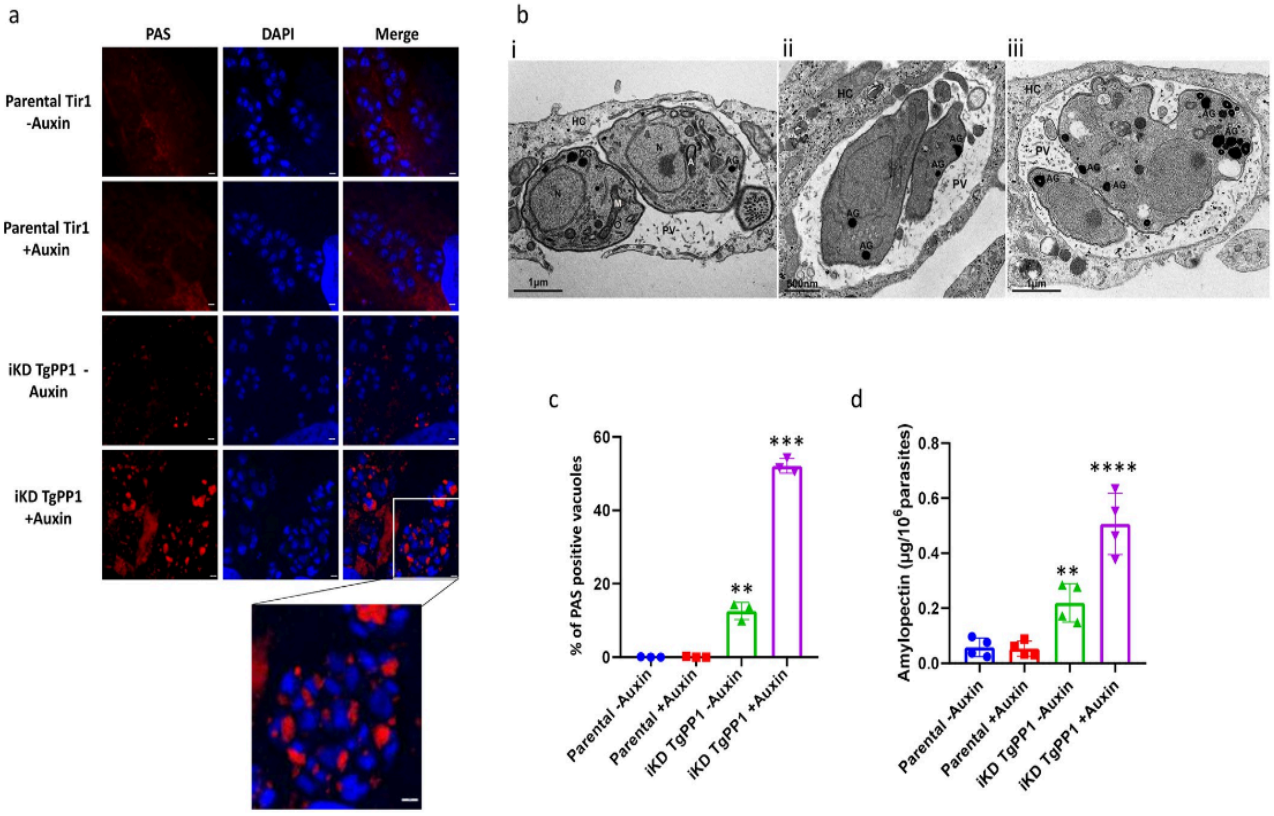

Fig 4. Absence of TgPP1 results in the accumulation of amylopectin granules. (a) Confocal imaging of the Parental Tir1 and iKD TgPP1 strain stained with PAS (red) in the absence and presence of auxin for 48 h. DAPI was used to stain the nucleus. Scale bar (1 μm) is located in the far-right corner of each image. **(b)** EM scan of the iKD TgPP1 parasites in the absence (i) or presence (ii, 24 h and iii, 48 h) of auxin treatment depicting the presence of AG in the iKD mutant. Starch was labeled using periodic acid. Note the accumulation of AG in the tachyzoites in (ii) and (iii). AG, amylopectin granules; DG, dense granules; N, nucleus; PV, parasitophorous vacuole; HC, host cell. Scale bar (1 μm or 0.5 μm) is demonstrated in the lower left region of each EM scan. **(c)** Bar graph representing the percentage of PAS positive vacuoles in the Parental Tir1 and iKD TgPP1 strains in the absence and presence of auxin for 48 h. A Student's *t* test was carried out. $^{**}p < 0.01$, $^{***}p < 0.001$; mean ± SD ($n = 3$). **(d)** Quantification of the amount of amylopectin, as measured by biochemical assay, present within Parental Tir1 and iKD TgPP1 parasites in the absence and presence of auxin for 48 h. A Student's *t* test was carried out. $^{***}p < 0.001$, $^{****}p < 0.0001$; mean ± SD ($n = 3$). The data underlying this figure can be found in S1 Data. EM, electron microscopy; PAS, periodic acid–Schiff.

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auxin for 48 h. There was a significantly increased percentage of PAS positive vacuoles (approximately 50%) in the iKD TgPP1 mutant in the presence of auxin when compared to the parental strain or the iKD TgPP1 mutant in absence of auxin (Fig 4D). However, as observed in Fig 4A, a significant number of vacuoles were PAS positive in the iKD TgPP1 mutant in absence of auxin (around 15%). We independently verified the accumulation of amylopectin in the iKD TgPP1 mutant in absence and presence of auxin by a biochemical dosage of amylopectin produced by this strain and the parental strain. In the absence and presence of auxin, the iKD TgPP1 mutant parasite possesses a significant amount of amylopectin (Fig 4E). The amount of amylopectin was significantly increased in presence of auxin in the iKD TgPP1 mutant (Fig 4E), indicating that the depletion of TgPP1 induces the accumulation of amylopectin in the parasite. Therefore, TgPP1 plays pleiotropic roles during the intracellular growth of the tachyzoite including regulation of the amylopectin steady-state levels.

TgPP1 regulates the biology of the tachyzoite through dephosphorylation of a large set of proteins

Since TgPP1 was localized to the nucleus of the parasite, we investigated its potential role in gene regulation. For that we carried out RNA-sequencing analysis by examining changes in

the transcriptome of the iKD TgPP1 strain after treatment with or without auxin for 24 h from 4 biological replicates. Deseq2 was used to determine significant differential expression of genes based on an adjusted *p*-value cutoff of 0.05 and a minimum fold-change of 2 (S4A Fig). To our surprise, only a few genes were considered differentially regulated following the depletion of TgPP1 and none of them pass the minimum fold-change of 2 cutoff (S4A Fig and S1 Table). This suggested that TgPP1 had a minimal role in transcriptional regulation despite its nuclear localization.

We then investigated the role of TgPP1 in differential phosphorylation of proteins during the intracellular growth of the parasite. For that, the phosphoproteome of the iKD TgPP1 mutant parasite was investigated at 2 time points. A first time point, after 24 h of normal growth and then 2 h of auxin treatment, was aimed at discovering the direct targets of TgPP1, a short time period after its complete depletion (as measured by western blots). A second time point, after 24 h of auxin treatment was aimed at measuring the global effect of TgPP1 on the phosphoproteome. The presence of significantly hyper or hypo-phosphorylated peptides was identified in a minimum of 3 biological replicates.

After 2 h of auxin treatment, a total of 7,822 phosphorylation sites were identified (S2 Table), among the 4 biological replicates that were analyzed. Among these, 104 phosphopeptides exhibited significant differential phosphorylation with an FDR less than 0.01 (Fig 5A and S2 Table). Among the 104 phosphopeptides (representing 93 proteins), 37 proteins were considered significantly hyper-phosphorylated (40 phospho-peptides associated) and 56 proteins (64 phospho-peptides associated) were considered significantly hypo-phosphorylated (Fig 5A). We focused on the hyper-phosphorylated proteins that are likely the effect of the phosphatase depletion. We noticed that IMC1 was identified with the highest hyper-phosphorylation ratio (S2 Table). Moreover, the Apical Cap protein 2 (AC2, TGME49_250820), the CPH1-interacting protein 2 (CIP2, TGME49_257300) and TGME49_285850 are predicted to localize to the apical compartment or IMC [34] and were found to be hyperphosphorylated after 2 h of auxin treatment. Concordant with the TgPP1 nuclear localization, a high number of hyper-phosphorylated proteins (19/37) are predicted to localize to the nucleus. Among them, the DNA replication licensing factor MCM7 is likely involved in DNA replication. We also identified hyper-phosphorylation of 2 fitness-conferring kinases: CDPK6 [35] and TKL1 [36]. Moreover, we identified the hyper-phosphorylation of a trehalose phosphatase (TGGT1_297720), an uncharacterized protein containing a CBM20 domain, known to bind to starch. In the list of hypo-phosphorylated proteins, we also found 5 other IMC proteins such as IMC17, ISC1, PMCAA1, and uncharacterized IMC proteins (TGGT1_217510 and TGGT1_306190). Moreover, 4 putative kinases (CDPK2a, the cell cycle associated GSK (TGME49_265330), TGME49_225960, and TGME49_320000) and 1 phosphatase (TGME49_269460) were also found to be hypo-phosphorylated. A motif analysis of the differentially phosphorylated peptides indicates that serine is the main target of phosphorylation. There was no obvious enrichment of kinase substrates among these motifs (S5 Fig).

We then wanted to investigate further by studying the effect of TgPP1 depletion on the differential phosphorylation of phospho-peptides after a longer period of auxin treatment. After 24 h of auxin treatment, a total of 7,509 phospho-sites were identified. Out of these phospho-sites, 376 were considered as significantly differentially phosphorylated and corresponded to a total of 284 proteins (Fig 5B and S2 Table). The total number of hyper-phosphorylated peptides was 152, whereas the total number of hypo-phosphorylated peptides was 224, associated with 114 and 178 proteins, respectively. A striking number of IMC proteins (10/114) were discovered to be significantly hyper-phosphorylated such as IMC4, IMC18, IMC20, AC2, and ISC7. On the other hand, 17 proteins associated with the IMC or the apical compartment were identified as hypo-phosphorylated, suggesting a global perturbation of the phosphorylation

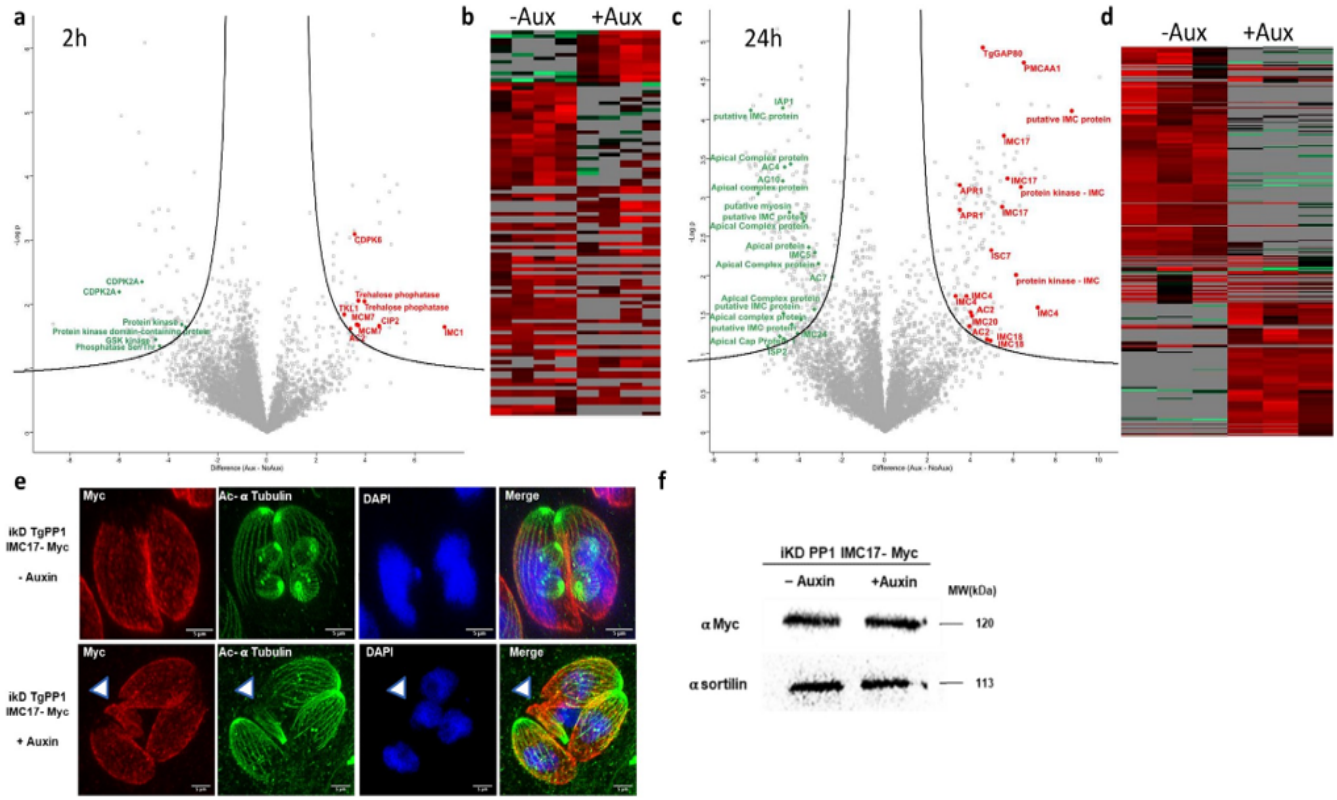

Fig 5. TgPP1 depletion results in differentially phosphorylated proteins including a high number of IMC proteins. **(a)** Volcano plot of the total phosphosites in the iKD TgPP1 mutant parasite after the treatment of auxin for 2 h resulting from phosphoproteomics analysis ($n = 7,822$). Selected proteins presenting hyperphosphorylated peptides in absence of TgPP1 are highlighted in red. Selected proteins presenting hypophosphorylated peptides are highlighted in green. See the full list in S2 Table. **(b)** Heat map of phosphosites which are differentially phosphorylated as a result of 2 h of auxin treatment in the iKD TgPP1 mutant. **(c)** Volcano plot of the total phosphosites in the iKD TgPP1 mutant parasite after the treatment of auxin for 24 h as demonstrated following phosphoproteomics analysis ($n = 7,509$). Selected proteins presenting hyperphosphorylated peptides in absence of TgPP1 are highlighted in red. Selected proteins presenting hypophosphorylated peptides are highlighted in green. See the full list in S2 Table. **(d)** Heat map displaying phosphosites which are differentially phosphorylated following the treatment of auxin for 24 h. **(e)** Expansion microscopy images of the iKD TgPP1 strain in absence and presence of auxin. The parasite IMC (IMC17, red) and cytoskeleton (acetylated tubulin, green) were labeled as well as the nucleus by DAPI (blue). A parasite with IMC formation defects is indicated by a white arrow. **(f)** Western blot image showing the unchanged level of expression of the TgIMC17 (IMC17-myc) protein in presence or absence of Auxin after 24 h. Sortilin was used as a loading control. IMC, inner membrane complex.

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status of this compartment. As an illustration, a single IMC protein, IMC17, was found both hyper-phosphorylated at particular phospho-sites and hypo-phosphorylated at other phospho-sites. Of note, the Trehalose phosphatase (TGME49_ 297720) was also found hyper-phosphorylated after 24 h of treatment. Similar to what has been shown for the 2 h auxin treatment data set, there was no obvious enrichment of kinase substrates among the differentially phosphorylated peptide motifs (S6 Fig) although the GO analysis revealed an enrichment of proteins in kinase, enzyme regulator, and DNA-binding transcription factor activities (S6D Fig).

To assess the effect of the differential phosphorylation on IMC proteins, we tagged IMC17 in the iKD TgPP1 mutant and performed expansion microscopy after 24 h auxin treatment (Fig 6E). This experiment confirmed the IMC phenotypes described before.

When comparing our phosphoproteome analysis to that of Herneisen and colleagues [20], we identified a small overlap: around 22% of the protein present in our data set (21/93 at 2 h and 64/284 at 24 h) were common. Interestingly, among the hyperphosphorylated proteins that are common with our data sets, we found the apical/IMC proteins IMC4, AC2, AC13, and CIP2 and the centrosomal Cep250 protein (S2 Table). Among the hypo-phosphorylated proteins, the IMC/apical proteins AC4, IMC24, and AAP2 and the centromeric protein Cenp-C were present in both data sets.

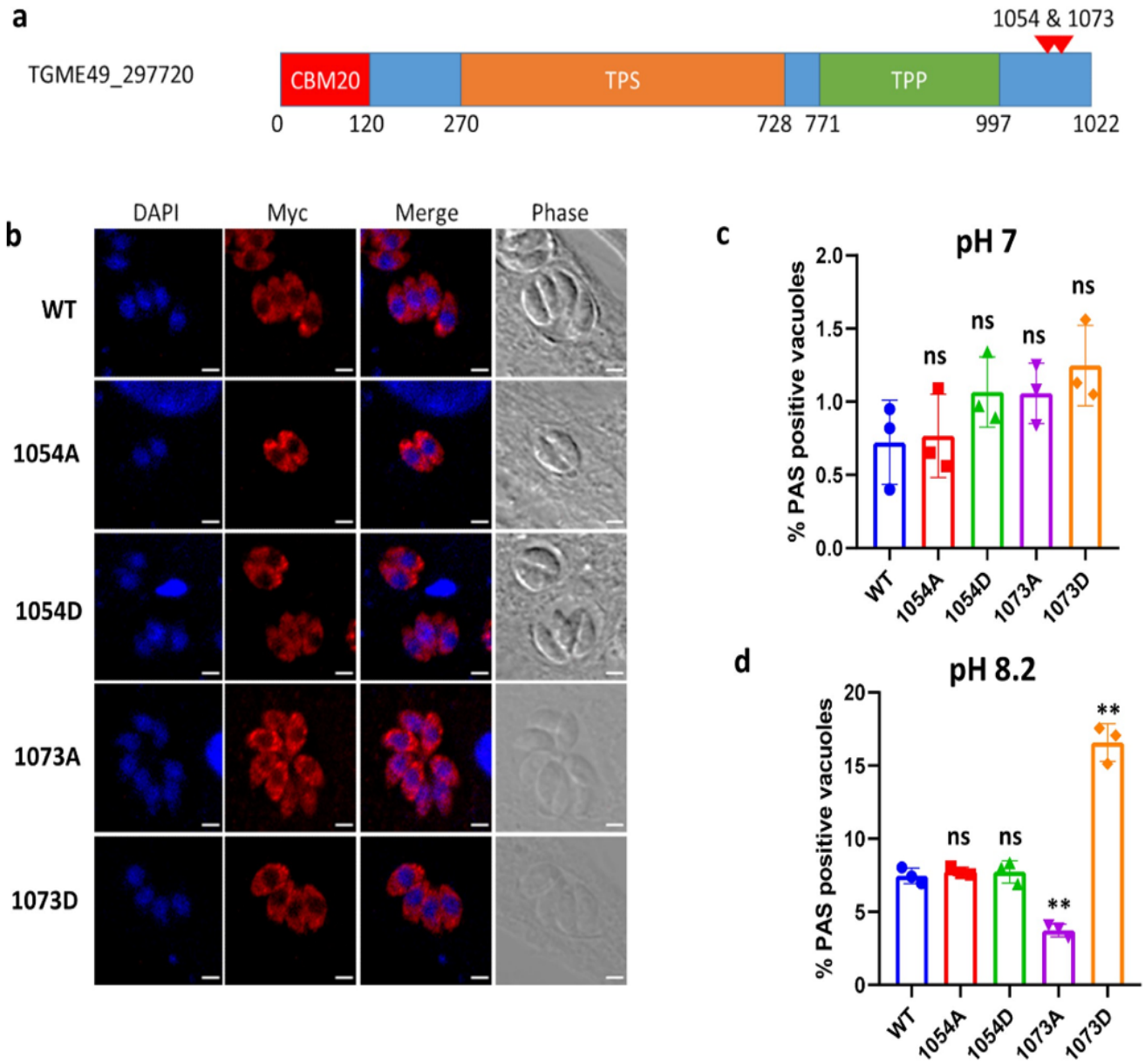

Fig 6. The phosphorylation status of the TGGT1_297720 protein affects amylopectin steady-state levels. (a) Schematic representation of the trehalose synthase-phosphatase protein (TGGT1_297720) that consisting of CBM20, trehalose synthase (TPS), and the trehalose phosphatase (TPP) domains. Differentially phosphorylated sites after TgPP1 depletion at Serine 1054 and Serine 1073 are indicated by red arrow heads. **(b)** Confocal imaging of the mutated TGGT1_297720 proteins. The myc tagged TGGT1_297720 protein is labeled with anti-myc antibodies (red). DAPI was used to stain the nucleus. Scale bar (2 μm) is indicated in the lower right corner of each individual image. The identity of the mutation is indicated on the left side. **(c)** Bar graph representing the percentage of PAS positive vacuoles at a pH of 7.0 in the WT and mutant 1054A, 1054D, 1073A, and 1073D parasites. A Student's *t* test was carried out. ns > 0.05; mean ± SD ($n = 3$). **(d)** Bar graph representing the percentage of PAS positive vacuoles at a pH of 8.2 in the WT and mutant 1054A, 1054D, 1073A, and 1073D parasites. A Student's *t* test was carried out. ns > 0.05, $^{**}p < 0.01$; mean ± SD ($n = 3$). The data underlying this figure can be found in S1 Data. PAS, periodic acid–Schiff; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase.

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As a control, we performed a global proteome analysis of the same samples that were treated for 24 h with or without auxin (S1 Table and S4B Fig) and did not identify significant differences in protein content in these samples, suggesting that TgPP1 mainly acts through modulating the phosphorylation status of proteins and does not affect global proteome content. These results were independently confirmed by a western blot of the iKD TgPP1 IMC17-myc strain showing that the IMC17 level of protein expression was unchanged in presence of auxin for 24 h (Fig 6F).

TgPP1 dephosphorylation of the TGME49_297720 protein may influence starch metabolism

TgPP1 depletion influences starch metabolism (Fig 4). We identified the 2 differentially phosphorylated sites on the TGME49_297720 protein. This protein encodes for a starch binding domain (CBM20), a trehalose-6-phosphate synthase (TPS), and a trehalose-6-phosphate phosphatase (TPP) domain (Fig 6A). Both identified hyper-phosphorylated sites (Serine 1054 or Serine 1073) are situated outside of the putative enzymatic domains (Fig 6A). We explored whether these phospho-sites were significantly linked to the starch accumulation phenotype observed previously. For that, we produced 4 transgenic parasite mutants bearing single point mutations corresponding to Serine 1054 or Serine 1073 changed to an Alanine or Aspartate amino acid. A parental strain (RH ΔKu80) with the Trehalose phosphatase gene bearing a C-terminal Myc tag was used to generate the mutants using the CRISPR/Cas9 system and direct FACS sorting into 96-well plates (S7 Fig). To identify whether the WT or mutated fragment had been inserted into the correct locus of the genome, PCR fragments representing the targeted locus were amplified and sent for sequencing (S7 Fig). First, we verified that the mutation had no effect on the enzyme localization (Fig 6B). We then proceed to verify the effect of the induced mutation on accumulation of starch in these mutant parasites. For that, we quantified the number of PAS positive vacuoles in normal culture conditions (pH 7) or in conditions known to induce bradyzoite differentiation (pH 8.2), the bradyzoite form accumulates amylopectin. In normal culture conditions, there was no significant difference between the WT and the mutant parasites, when measuring the percentage of PAS positive vacuoles. However, in bradyzoite inducing conditions (pH 8.2), the percentage of PAS positive vacuoles significantly decreased in the 1073A mutant, whereas it significantly increased in the 1073D mutant compared to their wild-type counterpart. These results suggest that the phosphorylation status of the Trehalose phosphatase is linked to the ability of the parasite to accumulate starch, mimicking the phenotype observed after TgPP1 depletion.

Discussion

Phosphorylation is a widespread posttranslational modification in apicomplexan parasites [6]. In *T. gondii*, it is involved in the regulation of the tachyzoite cell cycle [37] and of starch metabolism [18]. Here, we explored the pleiotropic phenotypes caused by the depletion of the TgPP1 protein. A previous study investigated TgPP1 roles by focusing on the late stage of the tachyzoite cycle and examining the critical roles of this protein in egress and motility of the parasite [20], a feature that appeared common with PfPP1 [21]. In our study, we focused on the division of the tachyzoite and showed that TgPP1 is involved in a wide range of molecular mechanisms including nuclear and organelle segregation. Therefore, we can conclude that TgPP1 is a crucial phosphatase that regulates both the intracellular cell cycle (this study) and the extracellular mobility [20]. Genetic manipulation of this locus has proven challenging and the resulting independent mutants [20] have shown a decreased fitness in conditions where TgPP1 was still expressed, suggesting that the activity of this protein is highly important for the parasite. The mutant produced in this study, although noticeably affected in absence of auxin, showed stronger phenotypes under auxin treatment.

Among the phenotypes observed, IMC formation was one of them and, as confirmed by IFA and EM. Differential phosphorylation in absence of TgPP1 of multiple IMC targeted proteins such as IMC1, IMC4, IMC5, IMC17, IMC18, IMC20, IMC24, ISP1, and ISC1 is a hallmark of the phospho-proteomics experiments we performed. This was only partially confirmed by the previous study where IMC4, AC2, AC13, and CIP2 were found differentially phosphorylated, although technical and analytical differences impede a direct comparison

between the 2 data sets. A short time after TgPP1 depletion, TgIMC1 was found to be hyperphosphorylated at the threonine 62, with the greatest amplitude when comparing the auxin-treated samples to the non-treated ones. IMC proteins are targeted sequentially to the IMC and form a rigid meshwork onto the flattened alveolar sacs [38]. Posttranslational modifications can influence the function and assembly of the IMC proteins. For example, TgIMC1 undergoes proteolytic cleavage and the cleaved form is associated with filament rigidity [39]. Numerous phosphorylation sites have been detected on IMC proteins although their biological significance remained to be investigated. Of note, assembly and rigidity of intermediate filament proteins (e.g., nuclear lamina network) in other organisms have been shown to be regulated through phosphorylation [40]. Phosphorylation on serine and threonine residues would generally promote disassembly and dephosphorylation increasing stability of the intermediate filament [41]. In a genome-wide screen for kinase function, among the 15 kinases that were essential for tachyzoite growth, at least 9 showed phenotypes linked to IMC formation [9], indicating the importance of phosphorylation in this process. After TgPP1 depletion, we have found a global collapse of the IMC and hyper-phosphorylation of multiple IMC proteins that suggest that the IMC network assembly and stability may be controlled by similar phosphorylation mechanisms as for the assembly of other intermediate filaments. Of note, the phosphorylation status of the IMC proteins does not seem to control their overall stability. Indeed, the global proteome was unaffected after 24 h auxin treatment although IMC defects were seen in a majority of vacuoles. In support of this hypothesis, the amount the TgIMC17 protein remained unchanged after depletion of TgPP1, although its phosphorylation status is drastically changed (hyperphosphorylated and hypophosphorylated) at several amino acid locations.

The specific role in the assembly of the IMC of the TgIMC1 Thr62 phosphorylation would be of interest to investigate in the future, although individual phosphorylation sites on TgIMC12 were not shown to impact the IMC assembly [42]. Rather than the limited phosphorylation status of individual IMC proteins, TgPP1 depletion seems to induce a global imbalance in the phosphorylation status of IMC proteins that may be the cause of the visual phenotype observed. This is reminiscent to the PfPP1 mutant phenotype for which the IMC failed to form when depletion of the protein was achieved mid-intraerythrocytic developmental cycle [21].

Phosphorylation of TgGAP45 has been shown to control its assembly to the MyoA-glidosome [43], a structure essential for the extracellular movement of the parasite. Interestingly, TgGAP80, a protein belonging to the TgGAP45 protein family, was found to be hyperphosphorylated after TgPP1 depletion. Moreover, IAP1, a protein associated with TgGAP80 is also differentially phosphorylated in presence of auxin. Both proteins are part of the MyoC-glideosome, a complex associated with IMC proteins that localizes at the basal pole of the parasite [44]. However, the mutation of individual phosphorylation sites on the TgGAP45 protein did not affect glideosome assembly and parasite growth, indicating the modest contribution of individual phosphorylation site to the glideosome or pellicle assembly [45].

Taken together, these data indicate that the phosphorylation status of IMC proteins might be an important determinant of their ability to assemble and rigidify of the IMC network. The structural morphology of the iKD TgPP1 mutant is impacted as was indicated through EM. In the presence of auxin, the usual bow-shaped tachyzoite is absent and instead an abnormally shaped tachyzoite takes its place. Despite that the plasma membrane remains intact, it appears to exhibit unusual curvatures and invaginations, this might possibly be due to the collapsed IMC structure which is absent underneath it. In normal conditions, the tachyzoite structure is maintained through IMC proteins resulting in tachyzoite pellicle strength. Our data indicates that TgPP1 influence on IMC may be independent from its potential role in modifying centrosome activity.

The presence of nuclear and organelle segregation phenotypes after depletion of TgPP1 suggests that this phosphatase may have targets that are organizing cell division in the parasite. Although the centrosome division remains mainly unaffected in absence of TgPP1, TgCep250 was identified as differentially phosphorylated in our data set and in Hernesien and colleagues [20]. TgCep250 mutants led to a high number of parasites presenting nuclear segregation defects [46], a phenotype that is also observed in the iKD TgPP1 parasite in presence of auxin. Overall, the mitosis defects after depletion of TgPP1 points toward a desynchronization of the budding and nuclear cycle leading to production of parasites without nucleus or nucleus without parasite body. Detailed examination of mitosis after depletion of TgPP1 indicates that metaphase may be slowed in this mutant but mitosis eventually processed. This in contrast of the function observed in *Plasmodium* PP1 for which DNA replication was strongly affected [21,23].

RNA-seq revealed that TgPP1 depletion did not change the transcriptome of the parasite after 24 h of auxin treatment. Despite TgPP1 nuclear localization and the presence of differential phosphorylation of ApiAP2 transcription factors in the phospho-proteome at 24 h auxin treatment, the phenotypes observed are not the consequence of differential transcript expression or proteome expression. ApiAP2 posttranslational modifications have been suggested to modify their activity [2] but the data presented here does not support this hypothesis.

The formation of amylopectin granules in the tachyzoite following the depletion of TgPP1 was a striking finding. This suggests that TgPP1 has a role in the regulation of amylopectin steady-state levels during tachyzoite proliferation. Phosphorylation has been shown to regulate amylopectin metabolism in *T. gondii*. TgCDPK2 phosphorylates a wide range of enzymes that are involved in this process [18] among which a glycogen phosphorylase, an alpha-glucan water dikinase, and a pyruvate phosphate dikinase that are known to bind to amylopectin through CBM20 domains [47]. Mutating the phosphorylation sites of glycogen phosphorylase was later shown to phenocopy the TgCDPK2 knock-out strain [48] suggesting a crucial role for phosphorylation in regulating the steady-state levels of amylopectin in this parasite. A phosphatase was also recently implicated in this pathway: TgPP2A contributes to the regulation of amylopectin metabolism via dephosphorylation of TgCDPK2 at a particular site (S679) [16] suggesting a pivotal role for TgCDPK2 in amylopectin accumulation. We found that a protein annotated as a Trehalose phosphatase was differentially phosphorylated in absence of TgPP1. Mutagenesis of these phosphosites recapitulates partially the TgPP1 depletion phenotype with amylopectin accumulation being detected after induction of bradyzoite differentiation. This may be due to the fact that single phosphosite mutants could not recapitulate the extend of the perturbation that are caused to the parasite environment when TgPP1 is depleted. Moreover, our phosphoproteomic data may not be exhaustive as other phosphosites on this protein have been discovered [6] and could play a role in the regulation of the activity of this protein. Interestingly, the TGME49_297720 protein was not listed as the potential target of TgCDPK2 or TgPP2A suggesting that it acts through an independent pathway that also controls the metabolism of amylopectin. In favor of this hypothesis, accumulation of amylopectin was only observed in the cytoplasm of the parasites lacking TgPP1 while it was observed both in the cytoplasm and residual body of parasites mutated for TgCDPK2 [47] or TgPP2A [17], suggesting a different regulation mechanism. TGME49_297720 encompasses a starch-binding domain (CBM20) that further supports the hypothesis of a function linked to starch metabolism regulation. This protein is annotated as a Trehalose phosphatase. However, its sequence encodes a TPS and a TPP domain. Dual activity enzymes are found in some bacteria and fungi [49]. The TGME49_297720 protein may therefore be misannotated as a trehalose phosphatase and should be annotated as a bifunctional TPS-TPP protein. The presence of this dual-activity enzyme indicates that *T. gondii* may have the ability to produce trehalose from UDP-Glucose

and Glucose-6-Phosphate as it is the case for numerous eukaryotes. However, these activities have to be confirmed experimentally. Trehalose is a nonreducing disaccharide consisting of 2 glucose subunits with an α,α′-1,1′-glycosidic bond. This carbohydrate occurs in a wide range of species and is synthesized by bacteria, fungi, plants, and various invertebrates. Trehalose has roles in development, stress tolerance, energy storage, and the regulation of carbon metabolism in plants and fungi [50]. In stress conditions, trehalose is also known to act as a stabilizing factor for protein structures [51]. Starch accumulation is linked to stress-induced differentiation in *T. gondii*; therefore, trehalose may be acting as a chaperone during this process. Alternatively, trehalose-6-phosphate, the product of the enzymatic reaction catalyzed by TPS, is known as a signal molecule in plants [50]. Trehalose-6-phosphate acts by inhibiting starch degradation by preventing the early steps in the starch degradation pathway within the chloroplast, although this effect may be indirect through the regulation of kinases and phosphatases that control the activity of the enzymes responsible for starch degradation [52]. Further investigations are warranted to discover if trehalose-6-phosphate plays a similar role in regulating starch degradation in the parasite. While trehalose or trehalose-6-phosphate have not been identified so far in the parasite [53], the data presented here suggests that it may have a role in regulating starch metabolism in the parasite.

Overall, we showed that TgPP1 acts through the phosphorylation of a wide range of targets to regulate a large set of molecular mechanisms that are essential for the completion of the tachyzoite cell cycle. Our study puts an emphasis on the role of phosphorylation in the IMC protein network assembly and stability. It also confirms the importance of phosphorylation in regulating the amylopectin steady-state levels in the parasite and suggests the involvement of a new regulatory pathway involving a new enzyme in this pathway.

Supporting information

S1 Table. RNA-seq analysis of the iKD TgPP1 strain in absence and presence of auxin for 24 h.
(XLSX)

S2 Table. Phosphoproteomics results for the iKD TgPP1 strain in absence and presence of auxin for 2 h or 24 h. Quantification of each differentially phosphorylated peptide is listed along with the targeted amino acid and the kinase family potentially phosphorylating the peptide.
(XLSX)

S3 Table. Results of the global proteome analysis of the iKD TgPP1 strain in absence and presence of auxin for 24 h.
(XLSX)

S1 Data. Raw data for figures: Figs 1D, 2C, 2D, 3C, 4C, 4D, 6C, 6D, S1B, S1C, S2D, S2E, S3B, S3D, S4A and S4B.
(XLSX)

S1 Raw Images. Uncropped version of the western blots and gel images.
(PDF)

S1 Fig. iKD TgPP1 mutant construction. (a) PCR verifying integration of the HXGPRT-2TA-AID-Ty cassette at the correct genome locus of the iKD TgPP1 mutant. A band corresponding to 1536 using iKD TgPP1 genomic DNA confirms cassette integration compared to using WT genomic DNA. **(b)** Growth assay of the Parental Tir1 and iKD TgPP1 mutant strains in the absence and presence of auxin treatment for 24 h. A Student's *t* test was

performed, ns > 0.05, $^{**}p < 0.01$; mean ± SD ($n = 5$). **(c)** Bar graph indicating plaque size produced by the Parental Tir1 and iKD TgPP1 strain in the presence and absence of auxin. Plaque size was determined by measuring the percentage of lysed surface of the plaque assay. Three independent experiments were carried out. A Student's *t* test was performed, $^{**}p < 0.01$; mean ± SD ($n = 3$). The data underlying this figure can be found in S1 Data.
(PDF)

S2 Fig. iKD TgPP1 demonstrates a collapsed IMC verified through TgGAP45 and TgISP1 labeling. **(a)** Confocal imaging of the Parental Tir1 and iKD TgPP1 strains labeled with anti-TgGAP45 (red) in the presence and absence of auxin treatment. DAPI was used to stain the nucleus. Scale bar (1 μm) is indicated in the lower right corner of each individual image. **(b)** Confocal imaging of the Parental Tir1 and iKD TgPP1 strains labeled with anti-TgEno2 (red) and anti-TgISP1 (green) in the presence and absence of auxin treatment. DAPI was used to stain the nucleus. Scale bar (1 μm) is indicated in the lower right corner of each individual image. **(c)** Confocal imaging of the Parental Tir1 and iKD TgPP1 strains labeled with TgEno2 (red) and TgIMC1 (green) in the presence and absence of auxin treatment. DAPI was used to stain the nucleus. Scale bar (1 μm) is indicated in the lower right corner of each individual image. **(d)** Bar graph representing the percentage of Parental Tir1 and iKD TgPP1 vacuoles possessing a collapsed IMC by using anti-TgIMC1 antibodies for labeling the IMC in the absence and presence of auxin treatment for 48 h. A Student's *t* test was carried out, $^{***}p < 0.001$; mean ± SD ($n = 3$). **(e)** Bar graph representing the number of parasite observed by EM with inner membrane defects in the iKD TgPP1 strain in absence of auxin ($n = 53$), after 24 h of auxin treatment ($n = 44$) or after 48 h of auxin treatment ($n = 46$). The data underlying this figure can be found in S1 Data.
(PDF)

S3 Fig. Conditional depletion of TgPP1 has a qualitative impact on the outer core centrosome. **(a)** Confocal imaging of Parental Tir1 and iKD TgPP1 parasites in the absence and presence of auxin treatment for 48 h labeled with anti-TgCentrin1 antibodies (green). DAPI was used to stain the nucleus. Scale bar (1 μm) is indicated in the lower right corner of each image. **(b)** Bar graph demonstrating TgCentrin1: nucleus ratio of Parental Tir1 and iKD TgPP1 in the absence and presence of 48-h auxin treatment. A Student's *t* test was performed, ns: $p > 0.05$; mean ± SD ($n = 3$). For each nucleus, the number of centrin dots is accounted independently from their size. Overall, more than 100 individual nuclei are counted for each biological replicate. **(c)** Representative expansion microscopy images of the iKD TgPP1 parasites in presence of auxin treatment for 24 h labeled with anti-Nuf2 (kinetochores, red) and anti-acetyl Tubulin (cytoskeleton, green) antibodies. DAPI was used to stain the nucleus. Scale bar (5 μm) is indicated in the lower right corner of each image. **(d)** Bar graph comparing the number of parasite undergoing Metaphase of Anaphase of the iKD TgPP1 in the absence ($n = 414$) and presence ($n = 426$) of 24-h auxin treatment. A Chi^2-test was performed, ****: $p < 0.001$; the number of parasites scored is indicated on top of the graph. The data underlying this figure can be found in S1 Data.
(PDF)

S4 Fig. RNA-sequencing and proteome analysis at 24 h of auxin treatment displays only a few differentially regulated genes. **(a)** Volcano plot demonstrating the differentially expressed genes analyzed from RNA-sequencing of the iKD TgPP1 mutant parasite treated with auxin for 24 h. Differential expression is based on the analysis of 3 biological replicates. Statistically significant differentially expressed genes are indicated in red. However, these do not pass the +/− 1 $\log_2$ expression ratio criteria. **(b)** MA (Bland–Altman) plot demonstrating