Establishing a reporter system to identify membrane contact site components between the apicoplast and mitochondrion of *Toxoplasma gondii*

By

Nicole Khamsa

(Under the Direction of Diego Huet)

ABSTRACT

In eukaryotes, membrane contact sites (MCS) are defined as specialized regions between two organelles in close apposition to each other. These regions enable inter-organellar communication by creating microdomains maintained via protein complexes. *Toxoplasma gondii* (*T. gondii*), an obligate intracellular protozoan parasite capable of infecting virtually any nucleated cell, is the causative agent of one of the most prevalent parasitic infections in the world, disseminated toxoplasmosis. Disseminated toxoplasmosis is clinically relevant for the mothers of unborn fetuses and immunocompromised individuals. *T. gondii* goes through a complex life cycle that involves intracellular and extracellular forms and numerous hosts. The parasite harbors two essential organelles: the apicoplast, a non-photosynthetic plastid conserved in most apicomplexans, and the mitochondrion. The two organelles have been observed in close proximity for many years and share essential metabolic pathways. Despite their close apposition and their metabolic exchanges, little is known about the nature of their interaction. We hypothesize that apicomplexan-specific MCS proteins could mediate the

interaction between the apicoplast and the mitochondrion of *T. gondii*. To identify those interactors, we are creating a reporter system using Split-GFP, which consists of two portions of a green fluorescent protein (GFP) that do not fluoresce on their own but can self-complement and fluoresce when they interact. This Split-GFP system has been used as a fluorescent tag to study protein-protein interactions in other organisms. First, as a proof of concept, we are generating *T. gondii* strains with each component of the Split-GFP system expressed in different subcellular compartments. Once established, we will then use the Split-GFP components as a reporter system in a genetic screen to identify MCS components between the apicoplast and mitochondrion *of T. gondii*. Understanding apicomplexan-specific MCS will shed light on inter-organelle communication in these organisms and on essential proteins that may be targeted in the on-going search to find new therapeutic drug targets against *T. gondii* infections.

INDEX WORDS: *Toxoplasma gondii*, Membrane contact sites, Split-GFP, GFP1-10 and GFP11, Reporter system, Apicoplast, Mitochondrion

ESTABLISHING A REPORTER SYSTEM TO IDENTIFY MEMBRANE CONTACT SITE COMPONENTS BETWEEN THE APICOPLAST AND MITOCHONDRION OF *TOXOPLASMA GONDII*

BY

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER 1

INTRODUCTION

1.1 *Toxoplasma gondii*, discovery and disease

Apicomplexans are obligate intracellular protozoan parasites that cause diseases such as malaria, toxoplasmosis, and cryptosporidiosis in humans. Together, these diseases contribute to significant global morbidity, mortality, and economic loss worldwide. The apicomplexan *Toxoplasma gondii (T. gondii)* is a parasite capable of infecting virtually any nucleated cell. *T. gondii* is the causative agent of one of the most prevalent parasitic infections in the world, disseminated toxoplasmosis, and is found to infect approximately one-third of the world's population (Dubey, 2004). *T. gondii* was first described by Nicolle and Manceaux in 1908 mistakenly as *Leishmania* (Nicolle and Manceaux, 2009). In the same year, Nicolle realized it was not *Leishmania* and that he had just discovered a new organism (Dubey, 2008). The parasite later obtained its name from *Ctenodactylus gundis*, a hamster-like rodent, where it was originally discovered (Dubey, 2004).

T. gondii is one of the leading causes of foodborne pathogens that may infect humans when consuming contaminated foods or water infected with oocysts (Saadatnia and Golkar, 2012; Esch and Petersen, 2013). Individuals may also get infected by eating raw or undercooked meat containing tissue cysts (Saadatnia and Golkar, 2012; Esch and

Petersen, 2013). Other ways of infection include receiving an organ transplant infected with tissue cysts or transplacentally from mother to fetus. If the mother is infected during pregnancy, this may lead to loss of the fetus (Saadatnia and Golkar, 2012; Esch and Petersen, 2013). Although *T. gondii*'s chronic infections are typically asymptomatic or present as mild flu-like symptoms in healthy individuals, it may cause morbidity and mortality in immunocompromised people. Morbidities are due to reactivations of a chronic infection that may lead to diseases such as cardiac toxoplasmosis, retinal chorioretinitis and encephalitis.

1.2 Current diagnoses, prevention, and treatment

A variety of diagnostic tests have been developed to detect past or current *Toxoplasma* infections. One test that is commonly used to determine infection is by the detection of IgM and IgG antibodies. These antibodies start presenting around 2 weeks after initial infection and may persist for years (Liu *et al.*, 2015). Another test commonly used to diagnose the acute infection of *Toxoplasma* is the isolation of the parasite from blood or other body fluids (McCabe and Remington, 1983; Liu *et al.*, 2015). PCR tests against specific *Toxoplasma* genes have also been used to clinically diagnose Toxoplasmosis (Liu *et al.*, 2015).

Although limited, treatment for toxoplasmosis currently consists of drugs that target the acute infection of the parasite. A combination of pyrimethamine and sulfadiazine, along with folic acid agonists, are used to inhibit the replication of *T. gondii* (McCabe and Remington, 1983; Dubey, 2004; Deng *et al.*, 2019). This treatment, however, loses its efficacy and causes toxicity resulting in the discontinued use of the drugs (Konstantinovic

et al., 2019). Another drug combination used to target the acute infection of *Toxoplasma* is clindamycin and azithromycin, which are also shown to have fewer toxic effects (Shiojiri *et al.*, 2019).

Preventative measures are currently the main method used to lessen the transmission rate of *T. gondii.* It is advised for pregnant women to avoid changing the litter boxes of cats since cat feces are a culprit for the transmission of *Toxoplasma (Bobić, Villena and Stillwaggon, 2019)*. It is also advised that people do not consume contaminated or undercooked foods (Djurković-Djaković *et al.*, 2019), making adequate education of sanitation practices and food consumption important preventative measures in lowering the transmission of *T. gondii* (Pinto-Ferreira *et al.*, 2019).

1.3 Life cycle

T. gondii can infect a variety of warm-blooded animals (Smith, 1995). The parasite undergoes a complex life cycle that is composed of asexual replication in intermediate hosts, and sexual replication inside cells of its definitive hosts (Figure 1.1). The life cycle begins when the definitive host, animals in the family *Felidae* which include domestic cats, ingests meat contaminated with oocytes (Frenkel and Dubey, 1972). These oocytes contain sporozoites, which will rupture inside the feline's intestine (Furtado *et al.*, 2011). After several rounds of replication, the parasites undergo merogony, a form of asexual reproduction where an organism replicates its own nucleus inside its host's cell and then induces cell segmentation, and develop into male or female gametes (Ferguson *et al.*, 1979a). These gametes will then fuse to form zygotes and form immature oocysts that are released into the environment in the cat's feces (Ferguson *et al.*, 1979b).

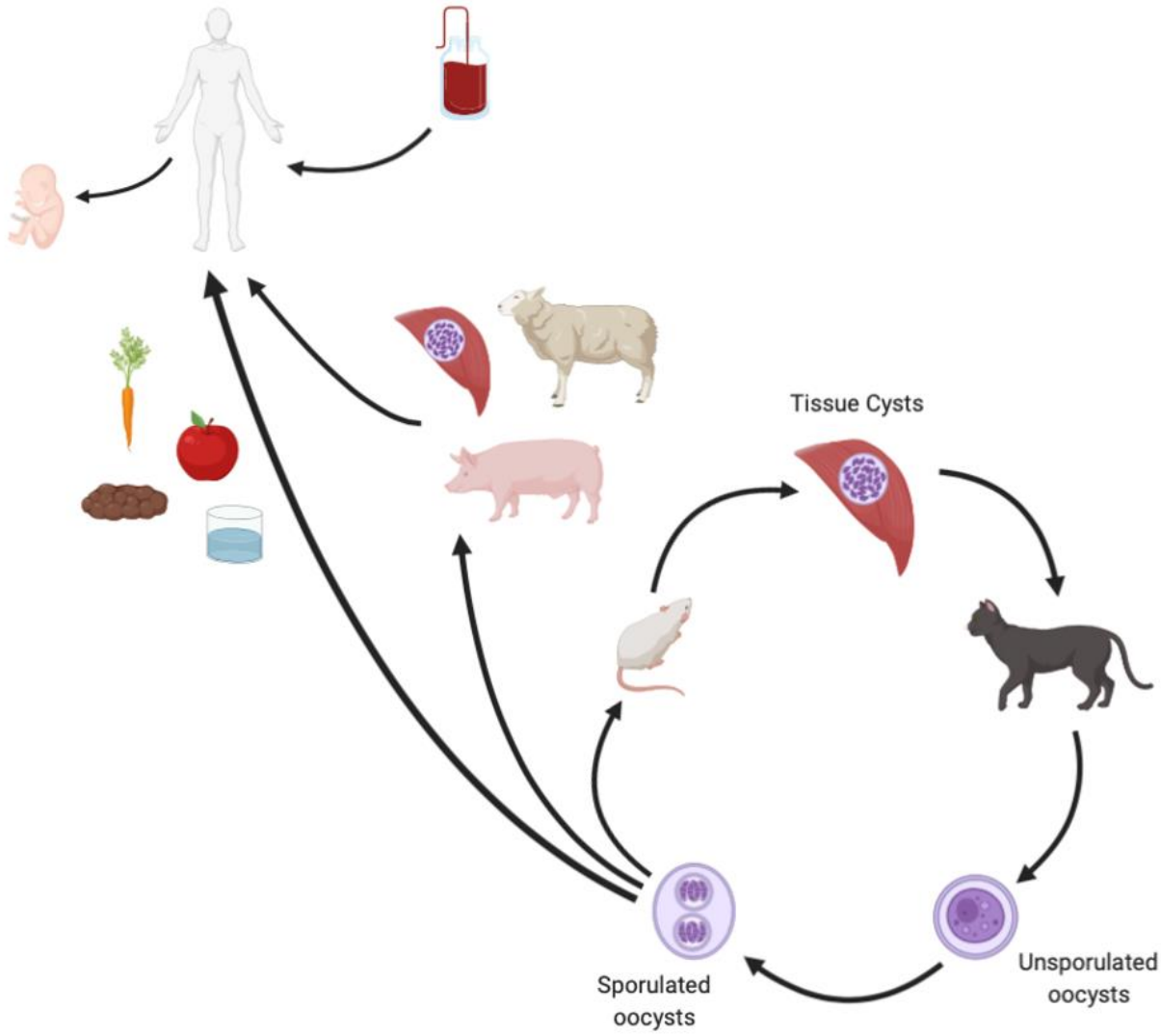

Figure 1.1 Life cycle of *Toxoplasma gondii.* The definitive hosts of *T. gondii* are animals in the family *Felidae*, this is where the parasite goes through sexual replication. Infected cats shed oocysts into the environment through their feces which can infect intermediate hosts, warm-blooded animals and humans. Humans can get infected through consumption of contaminated foods or infected and undercooked meats. In humans, *T. gondii* infection has the ability to cross the placental barrier to infect the fetus and may also spread through organ transplant. Created with Biorender.com

Asexual replication occurs when sporulated oocysts get ingested by intermediate hosts which consists of virtually any warm-blooded animals such as livestock and mice (Skariah, McIntyre and Mordue, 2010). In the intermediate host, the oocysts rupture and differentiate into tachyzoites, the fast-replicating form of the parasite (Skariah, McIntyre and Mordue, 2010). Tachyzoites then go to invade any nucleated cell and divide by a process called endodyogeny, where two cells are formed within a mother cell (Gubbels *et al.*, 2020). Cells replicate every 6 to 8 hours and will eventually fill the host cell, leading to egress and the invasion of other cells to continue replication. With time and the immune system pressure, parasites will eventually encyst in tissue cysts and differentiate into bradyzoites (Sullivan and Jeffers, 2012). Tissue cysts containing bradyzoites are characteristic of the asexual chronic stage of *Toxoplasma* infection. This form of the parasite can persist for the length of the host's life. Unfortunately, immunosuppression may cause the reactivation of the acute infection, leading to pathology and the disease toxoplasmosis (Montoya and Liesenfeld, 2004) .

1.4 Lytic cycle

Toxoplasma's lytic cycle consists of four steps: attachment, invasion, replication, and egress (Figure 1.2). The lytic cycle begins with attachment, which occurs when tachyzoites forms a tight bond to a potential host cell via a receptor-ligand interaction between the heparan-sulfate proteoglycans on the host cell surface and the surface antigens on the parasite (Black and Boothroyd, 2000; He *et al.*, 2002). Once attachment to the host cell occurs, invasion begins. Invasion is an active process that utilizes *Toxoplasma*'s secretory organelles as well as the parasite's actin and myosin motors

(Black and Boothroyd, 2000; Blader *et al.*, 2015). A tight interaction called a moving junction is formed between the parasite and the host cell, and is established by the secretion of specialized secretory organelles called micronemes and rhoptries from the apical end of the parasite (Alexander *et al.*, 2005). Once the moving junction is made, the parasite starts entering the host cell through invagination of the host cell's plasma membrane, which becomes the outer membrane of the parasitophorous vacuole (PV). The PV is a specialized vacuole that apicomplexans like *T. gondii* inhabit while they are inside a cell, and it serves as a sieve where proteins and ions can cross to be utilized by the parasite (Joiner *et al.*, 1994; Schwab, Beckers and Joiner, 1994). Once the PV is formed, a third set of secretory organelles called dense granules are secreted to make the environment inhabitable for the parasite. After invasion and the formation of the PV, the parasite begins replication, which occurs via endodyogeny. Endodyogeny is a form of asexual reproduction that forms two daughter cells inside a mother cell. After several rounds of replication, an unknown signal triggers parasite egress, which is the process where the parasite lyses out of the host cell. Once the parasites egress, they may use gliding motility, the term to describe parasites actively moving, to find new cells to invade. This lytic cycle is responsible for tissue damage since it ends with host cell lysis (Blader *et al.*, 2015).

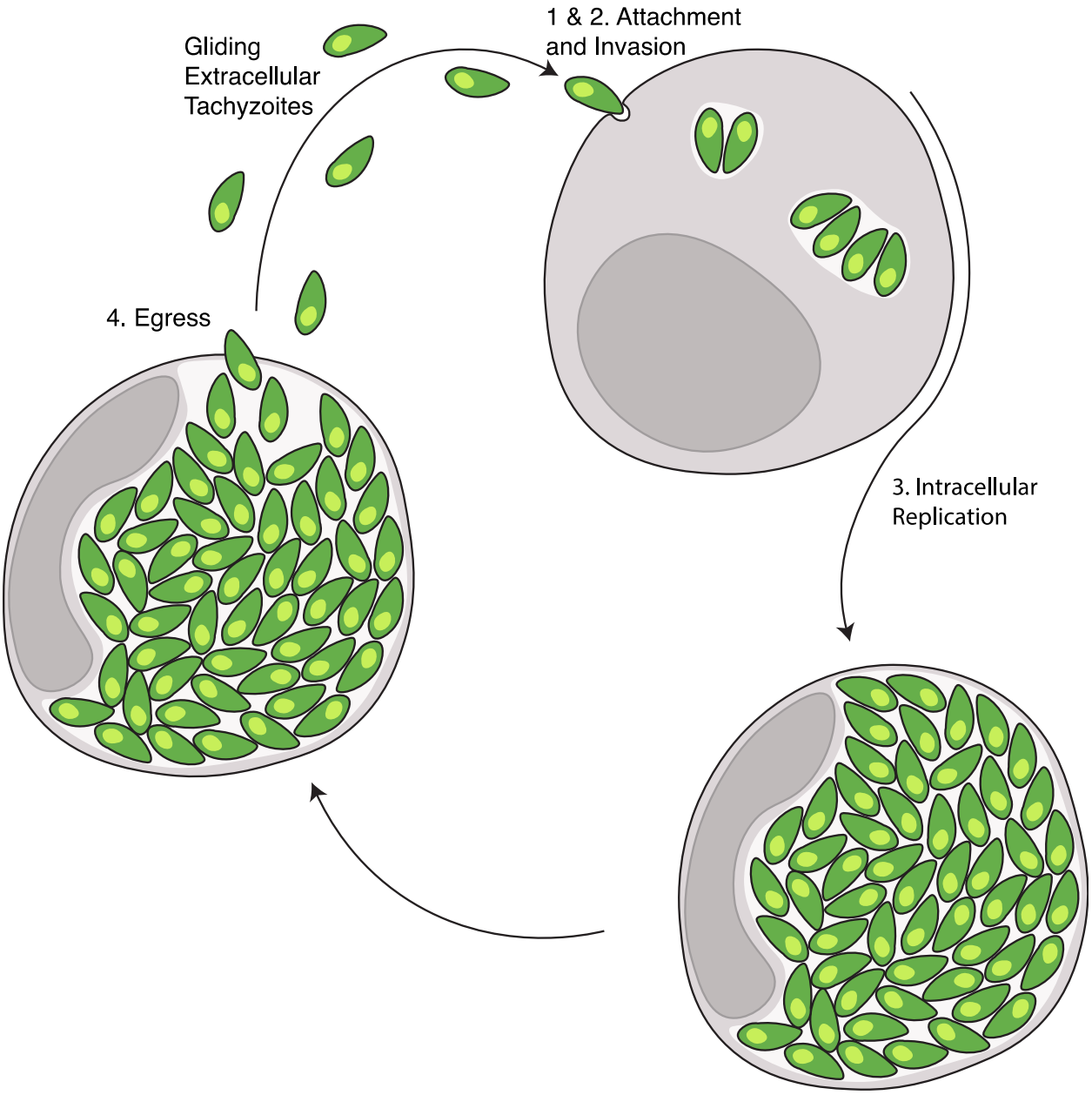

Figure 1.2 Lytic cycle of *Toxoplasma gondii*. *T. gondii*'s lytic cycle begins with attachment and invasion into a host cell. Parasites inside host cells will go through asexual replication in a parasitophorous vacuole (PV). Once the host cell is full, an unknown signal will trigger parasite egress. Extracellular tachyzoites will then glide to find another host cell to invade.

1.5 The mitochondrion and apicoplast

T. gondii, like many apicomplexans, has a plant-derived organelle called the apicoplast. This organelle contains 35 kilobase pairs of its own circular DNA (Wilson *et al.*, 1996) and has 4 membranes that resulted from two separate endosymbiotic events (van Dooren *et al.*, 2008; McFadden, 2011). The first endosymbiotic event occurred when a heterophobic eukaryote endocytosed a cyanobacterium creating an eukaryotic alga, and the second endosymbiotic event occurred when the descendant of the eukaryotic alga was then engulfed by a heterotrophic eukaryote (van Dooren *et al.*, 2008). Because of these endosymbiotic events, the apicoplast has a number of genes that have plant-like characteristics (van Dooren *et al.*, 2008; McFadden, 2011) that would be advantageous to target since mammals lack these plastid-derived proteins and biosynthetic pathways.

Another organelle essential to *T. gondii* is its single mitochondrion. Like in other eukaryotes, *T. gondii* has a double membrane-bound organelle that carries out a central role in the organism's energy production and cellular metabolism. Some key functions of *T. gondii's* mitochondrion include harboring the electron transport chain (ETC) and the enzymes of the tricarboxylic acid (TCA) cycle, along with the enzymes responsible for oxidative phosphorylation. The organelle also aids in pyrimidine, iron-sulfur cluster and heme biosynthesis (Vercesi *et al.*, 1998; Seeber, Limenitakis and Soldati-Favre, 2008; MacRae *et al.*, 2012; Hortua Triana *et al.*, 2016). Toxoplasma's mitochondrion is divergent, with approximately 400 proteins sharing no homologs to humans or yeast (Seidi *et al.*, 2018; Barylyuk *et al.*, 2020). Because of this, the mitochondrion and its proteins could serve as a good target for anti-parasitics. In fact, some drugs that are currently used to target the mitochondrion of *Toxoplasma* include Atovaquone (Dunay *et*

al., 2018) and endochin-like quinolones like ELQ316 and ELQ400 which target complex III in the ETC (Doggett *et al.*, 2012; McConnell *et al.*, 2018).

1.5.1 The mitochondrion and the apicoplast: shared metabolic pathways

T. gondii's two essential and divergent organelles, the apicoplast and mitochondrion, also share essential metabolic pathways such as the isoprenoid and heme biosynthesis pathways (Figure 1.3). Isoprenoids are lipid compounds that play many important roles in the parasite. Unlike mammals who use the mevalonate pathway for the synthesis of isoprenoid precursors, *T. gondii* has the prokaryotic-type 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway that is carried out in the apicoplast (Li *et al.*, 2013). This pathway generates important groups of molecules that include steroids, cholesterols and ubiquinone that are important for the membrane of the mitochondria as well as ETC composition (Ling *et al.*, 2007). In the shared heme biosynthesis pathway, Succinyl-CoA and glycine get converted into 5-aminolevulinic acid in the mitochondrion, is trafficked to the apicoplast where it gets converted into Coproporphyrinogen III, enters the cytoplasm and re-enters the mitochondrion to be converted into heme (Bergmann *et al.*, 2020) (Figure 1.3).

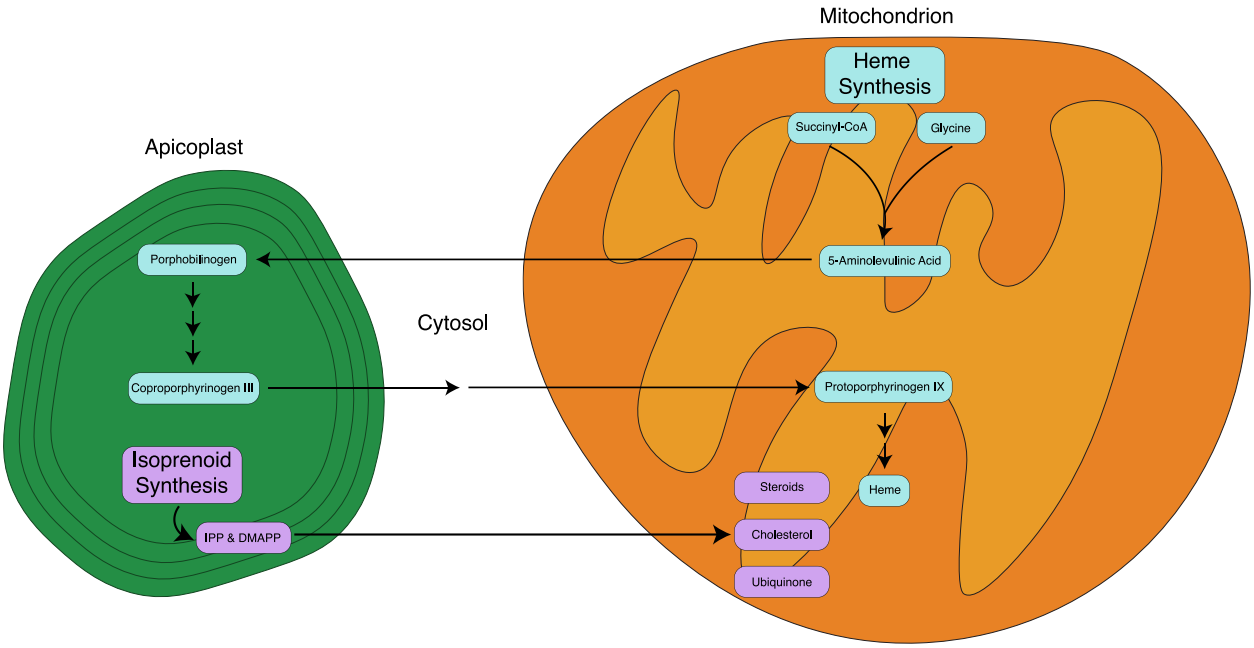

Figure 1.3 Shared metabolic pathways between the apicoplast and mitochondrion. The isoprenoid pathway precursors such as steroids, cholesterol and ubiquinone are used by the mitochondrion in its membrane and its ETC. Heme synthesis begins in the mitochondrion with succinyl-CoA and glycine, it is then trafficked to the apicoplast and gets converted to coproporphyrinogen III. This molecule is transported into the cytosol, then back to the mitochondrion where the final steps of the heme biosynthetic pathway take place.

1.5.2 The mitochondrion and the apicoplast: physical evidence of their close association

Along with sharing metabolites, *T. gondii's* apicoplast and mitochondrion have also been seen to localize alongside each other since the mid 1960s. In 1966, while using electron microscopy to study the ultrastructure's of the blood stage of *Plasmodium fallax*, a type of apicomplexan that infects birds, a team of scientist consistently found that the apicoplast and mitochondrion exist in a very close relationship to each other (Hepler, Huff and Sprinz, 1966). More recently, while scientists were using time-lapse microscopy of fluorescent markers targeted to various subcellular structures of *Toxoplasma* to study its organelle packaging and organization, they found consistently that the mitochondrion and apicoplast existed in a close proximity to each other (Nishi *et al.*, 2008). Additionally, scientists who used nitrogen cavitation to isolate the mitochondrion of *Plasmodium falciparum* found that their mitochondrial fractions were contaminated with apicoplasts, suggesting the existence of a physical binding between the two organelles (Kobayashi *et al.*, 2007).

Despite their close apposition and their metabolic exchanges, little is known about the nature of their interaction. In eukaryotes, close associations of organelles, otherwise known as membrane contact sites (MCS) are created and held together by tethering proteins. We hypothesize that a similar phenomenon is happening in *T. gondii,* and that apicomplexan-specific MCS proteins could mediate the interaction between the apicoplast and the mitochondrion.

1.6 Membrane contact sites and tethers

Membrane-bound organelles evolved to allow for segregation of biochemical reactions into specific compartments, which allow specialized microenvironments to perform specific tasks. Although membranes help with creating microenvironments, having membrane-bound organelles hinders diffusion of metabolites to all parts of the cell freely and may crowd the inside of cells if not organized properly. Because of this, cells need a way to organize their organelles depending on their cellular needs.

In eukaryotes, MCS are known to be specialized regions between two organelles in close apposition to each other (Helle *et al.*, 2013; Scorrano *et al.*, 2019). In other words, MCS are the interface of two membranes that are tethered, lacking fusion, having a specific function and having a functional protein and/or a membrane composition (Scorrano *et al.*, 2019). These regions are necessary for inter-organellar communication, like the exchange of metabolites between two different compartments. Molecularly, tethers are proteins that create MCS by holding two organelles in a close proximity to each other (Eisenberg-Bord *et al.*, 2016). They are defined as having a defined location, having structural and functional capacity, and their primary job is to bridge the membranes of organelles to mediate their physical attachment (Eisenberg-Bord *et al.*, 2016). The type of tether depends on what stage the cell is in the cell cycle or what the particular needs of that cell are at the moment. Because of this, scientists were interested in defining the types of tethers.

As defined by Eisenberg-Bord *et. al.*, 2016, tethers can be defined as principal or auxiliary tethers, static or dynamic tethers, and dedicated or multifunctional tethers. Principal tethers are defined as both necessary and sufficient in forming and maintaining MCS interdependently or other membrane components (Eisenberg-Bord *et al.*, 2016).

Loss of a principal tether typically also means loss of the membrane contact site. Auxiliary tethers, on the other hand, work with other tethers to create membrane contact sites, so loss of the tether does not affect membrane proximity (Eisenberg-Bord *et al.*, 2016). Static tethers result in a persistent tethering regardless of the environment while dynamic tethers may respond to environmental stimuli, allowing them to be regulated (Eisenberg-Bord *et al.*, 2016). Dedicated tethers are proteins that have no function other than holding two organelles in close apposition to each other, while multifunctional tethers may fulfill additional functions(s) like acting as transporters or channels for metabolites such as lipids or Ca^{2+} (Eisenberg-Bord *et al.*, 2016). Many MCS components have been characterized in *Saccharomyces cerevisiae* and metazoan cells (Eisenberg-Bord *et al.*, 2016). Some examples include proteins such as Scs2 and Scs22 between the endoplasmic reticulum and plasma membrane of *Saccharomyces cerevisiae* and VAPs and Nir2 between the endoplasmic reticulum and Golgi in metazoan cells (Eisenberg-Bord *et al.*, 2016). Although MCS components are extensively studied in metazoan and yeast cells, there are few identified and little knowledge about them in *T. gondii.* Because of their biological roles in organellar organization, identifying apicomplexan specific MCS components and discovering more about the biology in *T. gondii* would be advantageous in the ongoing search for therapies to combat apicomplexan diseases including toxoplasmosis.

1.7 Examples of membrane contact sites identified in *T. gondii*

Recently, MCS proteins and genes that regulate the maintenance of MCS in *T. gondii* have been discovered. The protein LMF1 has been seen to interact with Fis1 on

the outer mitochondrion membrane and another or other proteins in the parasites inner membrane complex to establish membrane contact sites and maintain the mitochondrion's shape (Jacobs, Charvat and Arrizabalaga, 2020). Recent work has further characterized this interaction and it has been found that protein IMC10 and LMF1 may be acting as tethers between *T. gondii's* mitochondrion and its inner membrane complex (Souza, Jacobs and Arrizabalaga, 2022). It has also been found that a plastid two-pore channel (TPC) may be responsible for regulating MCS formation between the ER and apicoplast of *T. gondii (Li et al., 2021)*. These findings are evidence that MCS proteins that regulate the maintenance of MCSs in *T. gondii* exist. Many MCS are still left uncovered, creating a need for a technique to discover components that maintain them.

1.8 Split-GFP system

The first recorded split protein was created in 1958 by Fred Richards, a scientist interested in studying the effects of nucleases on protein structures (Richards, 1958). He cut a well-studied ribonuclease A at a single site and found that the protein was able to spontaneously refold and maintain its enzymatic activity upon reconstitution (Richards, 1958). Fast forward to 2000, scientists used this concept on the green fluorescent protein (GFP). Scientists cut GFP between residue 157 and 158 (seventh and eighth β-strand) and found that it was able to spontaneously re-from a noncovalent bond (Ghosh, Hamilton and Regan, 2000). This spontaneous recombination allowed for the maturation of the chromophore, which could not mature while the two halves were apart (Ghosh, Hamilton and Regan, 2000). Since their discovery, split fluorescent proteins have been used widely to study scaffold protein assembly, cell-cell contacts, gene expression and protein-protein

interactions (Feng *et al.*, 2019; Kamiyama *et al.*, 2021). Recently, an improved Split-GFP system that is able to self-complement without the assistance of other protein-protein interactions has been developed, where a sfGFP is broken between its tenth and eleventh β-strand creating a GFP1-10 and a GFP11 fragment (Cabantous, Terwilliger and Waldo, 2005). Superfolder GFP, otherwise known as sfGFP, refers to a GFP with a series of mutations that allows for it to rapidly fold and mature even when it is fused to poorly folding peptides. The larger GFP1-10 contains three residues that constitute the GFP chromophore, while the smaller 16 amino acid GFP11 piece has the required E222 residue needed for chromophore maturation. When the two portions are near each other, they self-complement and the reconstituted GFP becomes fluorescent once the chromophore maturation is complete (Cabantous, Terwilliger and Waldo, 2005; Kent, Childs and Boxer, 2008). Additionally, GFP11x7, a repeating chain of GFP11, has been developed to amplify GFP fluorescence. GFP11x7 may be used in lowly expressed proteins since it allows for more GFP1-10 to bind, which in turn amplifies GFP fluorescence.

Fluorescent proteins have been utilized by cell biologists to study protein-protein interactions for over three decades. Self-complementing split fluorescent proteins have been successfully used in mammalian and yeast cells to study the interface of organelles (Kamiyama *et al.*, 2016), and they can be used as epitope tags in live cells to study live cell protein-protein interactions. Many applications of this split GFP1-10 and GFP11 proteins have used the smaller GFP11 fragment as an epitope tag. A few proteins that have been previously tagged with the smaller GFP11 fragment include MeCP2, FKBP, and H-Ras in N2A cells or HEK cells (Kaddoum *et al.*, 2010); PipB2, SteA, and SteC in

Salmonella (Van Engelenburg and Palmer, 2010); and disulfide isomerase (PDI) and the N- and C-termini of tapasin (Hyun, Maruri-Avidal and Moss, 2015). Split GFP1-10 and GFP11 have also been observed to be expressed and recombine on the interface of two organelles in yeast, with one portion of the split protein on the mitochondria and the other portion on the ER (Kakimoto *et al.*, 2018). Because Split GFP1-10 and GFP11 has been used in many organisms to study the interface of organelles, we believe that it will be an excellent tool to use as a reporter system between the apicoplast and mitochondrion of *T. gondii.*

1.9 Thesis outline

This thesis will focus on establishing a reporter system between the apicoplast and mitochondrion of *T. gondii* using Split GFP1-10 and GFP11 for the purpose of identifying MCS components between the two organelles (Figure 2.2A). Although there is strong physical evidence and knowledge of molecular exchanges between the apicoplast and mitochondrion which suggest that these two organelles are positioned next to each other, the components that contribute to their close association have not yet been identified. Chapter 2 focuses on creating a cytoplasmic Split GFP1-10 and GFP11 as a proof of concept that each half of the protein is capable of recombining in our system. Additionally, the chapter focuses on the progress of creating a reporter system between the mitochondrion and apicoplast of *T. gondii*. We also have evidence that GFP1-10 and GFP11 can recombine and result in GFP fluorescence and that this fluorescence is strong enough to be detected via Flow Cytometry.

CHAPTER 2

ESTABLISHING A REPORTER SYSTEM TO IDENTIFY MEMBRANE CONTACT SITE COMPONENTS BETWEEN THE APICOPLAST AND MITOCHONDRION OF *TOXOPLASMA GONDII*

2.1 Introduction

Membrane-bound organelles evolved to allow for the segregation of biochemical reactions in specific compartments. This segregation allows for the creation of microenvironments that may perform specific and specialized tasks. Although membranes allow for specific compartmentalized production of metabolites, organellar membranes hinder the diffusion of metabolites to all parts of the cell and create a need for organellar organization based on their cellular needs. A way cells have evolved to organize their organelles is by creating membrane contact sites (MCS), which are defined as specialized regions between two organelles in close apposition to each other (Helle *et al.*, 2013; Scorrano *et al.*, 2019). These regions are necessary for inter-organellar communication, like the exchange of metabolites or lipids (Helle *et al.*, 2013; Scorrano *et al.*, 2019) and are held together by proteins called tethers.

Toxoplasma gondii is an intracellular parasite from the Apicomplexa phylum that causes Toxoplasmosis in humans (Blader *et al.*, 2015). Infection with *T. gondii* may lead to severe complications in immunocompromised patients like encephalitis, myocarditis,

and retinal chorioretinitis. Pathogenicity is due to T. gondii's tachyzoite stage that is responsible for its lytic cycle which results in host cell lysis (Figure 1.2) (Sullivan and Jeffers, 2012). Due to their complex life cycle involving different hosts along with intracellular and extracellular environments, *T. gondii* must adapt its metabolic pathways to its ever-changing environment, which requires inter-cellular communication. *T. gondii* harbors two essential organelles: the apicoplast, a non-photosynthetic plastid conserved in many apicomplexans, and the mitochondrion. Many metabolic pathways converge in these two organelles, and they have been seen in close proximity for many years (Hepler, Huff and Sprinz, 1966; Kobayashi *et al.*, 2007; Nishi *et al.*, 2008) and share essential metabolic pathways including the isoprenoid and heme biosynthesis pathways (Ling *et al.*, 2007; Bergmann *et al.*, 2020). Despite their close apposition and their metabolic exchanges, little is known about the nature of their interaction. We hypothesize that apicomplexan-specific MCS proteins mediate the interaction between the apicoplast and mitochondrion of *T. gondii*.

As previously mentioned in section 1.7, scientists have begun to uncover MCS genes that regulate the maintenance of MCS in *T. gondii*. Proteins like LMF1 has been seen to interact with Fis1 on the outer mitochondrion membrane and other proteins in the parasites IMC to establish MCSs. This interaction contributes to the maintenance of the mitochondrion's lasso shape in intracellular parasites (Jacobs, Charvat and Arrizabalaga, 2020). Additionally, a plastid two-pore channel (TPC) was found to potentially play a role in regulating membrane contact site formation between the ER and apicoplast of *T. gondii* (Li *et al*., 2021). These findings are evidence that MCS proteins or genes that regulate the maintenance of MCSs in *T. gondii* exist.

To identify MCS between the apicoplast and the mitochondrion of *T. gondii,* we are creating a reporter system between the two organelles using Split-GFP. As a proof of concept, we are generating strains that express GFP1-10 in the cytoplasm and GFP11 on a protein with its extremities facing the cytoplasm to confirm that both halves of the Split-GFP can be expressed and recombine within the parasite. Once established, we will use the split system as a reporter system in a genetic screen to identify MCS components between the apicoplast and mitochondrion of *T. gondii*.

In our study, we were able to successfully generate strains expressing GFP11 fused to the IMC and outer mitochondrion membrane of *T. gondii*. We were also able to recombine GFP1-10 protein with GFP11 in fixed cells and found that recombined Split-GFP fluorescence can be recognized via Flow Cytometry in live permeabilized cells. Unfortunately, we also found that GFP1-10 seems to not be well expressed freely in the cytosol. Understanding apicomplexan-specific MCS will shed light on inter-organelle communication in these organisms. This knowledge may then be used in studies to uncover essential proteins that may be targeted in the on-going search to find new therapeutic drug targets against *T. gondii*.

2.2 Results

2.2.1 Expressing Split-GFP components in different compartments.

To test if Split-GFP could self-assemble in *T. gondii*, we set out to create a cytoplasmic Split-GFP system in RH-ΔKu80 as part of a proof of concept (Figure 2.1A). We generated a plasmid that would allow for expression of GFP1-10 fused to an HA tag in the parasite's cytoplasm (Figure 2.1C). Despite several attempts at transfections, we

were unable to obtain a clonal population expressing cytoplasmic GFP1-10. However, immunofluorescence directly after transfection of the plasmid (Table 2.2) showed transient cytoplasmic expression of HA-GFP1-10 co-localizing with CDPK1, a known cytoplasmic marker (Figure 2.1C). It is noteworthy that parasites after transfection were able to survive and recover from drug selection but did not express the HA tag or recombine with GFP11 in GFP11x7 expressing stains.

It has been shown previously that GFP11x7 is capable of giving off a stronger GFP fluorescence that was more detectable through IFA in comparison to just a single GFP11 when recombined with GFP1-10 (Kamiyama *et al.*, 2016) (Figure 2.1B). We selected a structural IMC protein named GAPM1a and generated a plasmid that would express GAPM1a exogenously with a Ty tag and a GFP11x7 fused to its C-terminus (Figure 2.1D; Table 2.2). The plasmid has the endogenous GAPM1a promoter, the GAPM1a gene, a Ty tag, GFP11x7, a skip peptide and the hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT), which confers resistance to mycophenolic acid in the presence of xanthine (Xa/MPA). Localization of our exogenous GAPM1a-Ty-GFP11x7 was determined by immunofluorescence showing the protein colocalized with the IMC marker IMC6.

In summary, we see transiently expressed GFP1-10 in the cytoplasm and found that GFP11x7 can be fused to the IMC protein GAPM1a. Because GFP1-10 seems to be toxic when expressed in *T. gondii's* cytoplasm, future directions may involve using an inducible system, like the inducible cre-loxP system (Kim *et al.*, 2018), to express a cytoplasmic HA-GFP1-10 upon addition of a drug, in the case of the inducible cre-loxP this drug would be rapamycin. Another alternative approach could be utilizing the

inducible gene expression system that was established based on a transactivator composed of the TetRep fused to a nonendogenous activating domain (TATi1) (Meissner, Schlüter and Soldati, 2002). In this system, the presence of anhydrotetracycline (ATc) reduces the affinity of the TetRep for the TetO and transcription is turned off (Bujard, 1999; Meissner *et al.*, 2001).

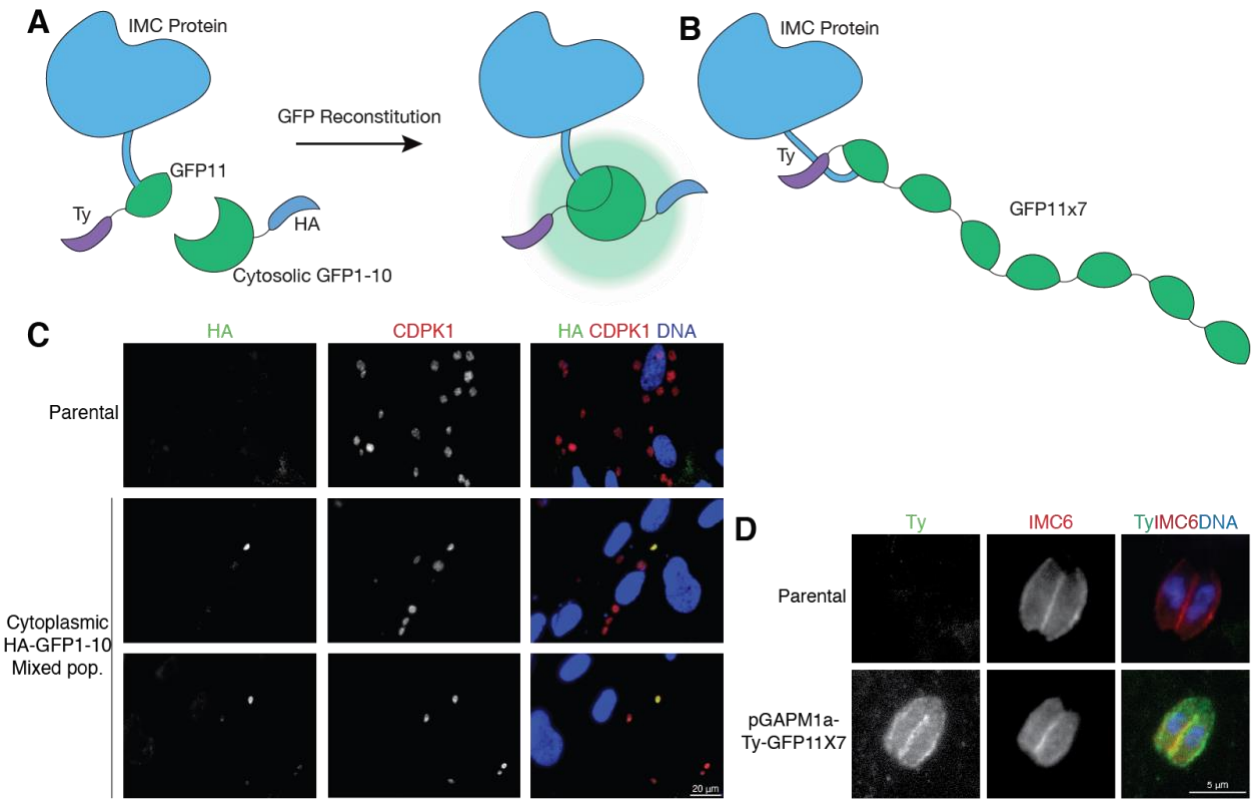

Figure 2.1 Expressing Split-GFP components in different compartments. A. Representative schematic for the cytoplasmic Split GFP1-10 and GFP11 system. GFP1-10 is shown expressing freely in the cytoplasm and GFP11 is fused to an IMC protein with its extremities facing the cytoplasm. **B.** Representative schematic of GFP11x7 on an IMC protein. **C.** IFA of intracellular tachyzoites showing mixed population of cytoplasmic GFP1-10 fused to HA in comparison to its parental. Cytoplasmic HA-GFP1-10 colocalizes with the cytoplasmic protein CDPK1 **D.** Comparative IFA of intracellular tachyzoites showing exogenous expression of GAPM1a fused to a Ty tag and GFP11x7. pGAPM1a-Ty-GFP11x7 colocalizes with IMC6.

2.2.2 Expression of GFP11x7 at the mitochondrial surface

In addition to the cytoplasmic proof of concept expression of the Split-GFP components, we set out to create a reporter system between the apicoplast and mitochondrion of *T. gondii*. We attempted to use APT1, an apicoplast triosephosphate translocator that has been described to have both its C and N terminus in the cytoplasm (Karnataki *et al.*, 2007) and has been used in other apicomplexans such as *Plasmodium falciparum* to isolate apicoplasts (Botté *et al.*, 2013), as our protein handle to express GFP1-10 on the apicoplast. Unfortunately, we were unable to obtain a clone of 3xHA-GFP1-10 fused to APT1 on its C-terminus.

We were able to use OMP25, a human targeting signal to the outer mitochondrial membrane that has been previously in our lab to fuse GFP and TurboID to the outer mitochondrial membrane, to fuse GFP11 to the outer mitochondrial membrane of *T. gondii*. We created a plasmid expressing a Ty tag fused to GFP11x7 and the human outer mitochondrial targeting signal OMP25 (Table 2.2) that we transfected into RH-ΔKu80. After transfection, parasites were selected using XA/MPA and a clone was isolated by limiting dilution subcloning. Localization of our Ty-GFP11x7-OMP25 was determined by immunofluorescence where the protein colocalized with the mitochondrion marker TOM40 (Figure 2.2B). Additionally, a band of approximately 20.7 kDa was observed in lysates of Ty-GFP11x7-OMP25 tachyzoites which validated the correct incorporation of the tag in a western blot analysis using anti-Ty antibodies (Figure 2.2B).

In conclusion, we were able to express and localize GFP11x7 to the outer mitochondrial membrane of *T. gondii* tachyzoites. We were unable, however, to isolate a clone of APT1 with 3xHA-GFP1-10 fused to its C-terminus. Using inducible systems like

the ones described in the last section could be used to aid in conditional and stable expression of APT1 fused to 3xHA-GFP1-10. Other approaches that should be considered is introducing a drug resistance to the repair template to create the APT1-3xHA-GFP1-10 strain. Although the repair template would be larger in size, the pressure of drug selection could increase the possibility of obtaining a stably expressing clone of APT1 fused to 3xHA-GFP1-10.

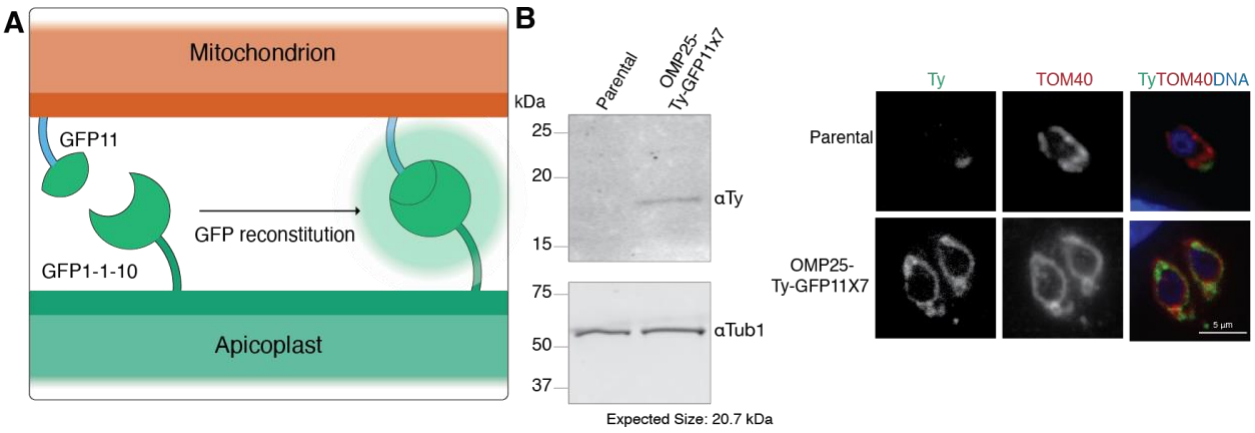

Figure 2.2 Expression of GFP11x7 at the mitochondrial surface. A. Representative schematic of Split-GFP reporter system between the apicoplast and mitochondrion. **B.** Left, Western blot confirming expression of exogenous Ty and GFP11x7 with an outer mitochondrial targeting signal. αTub1 was used as the loading control. Right, IFA of intracellular tachyzoites using αTy co-localized with αTOM40.

2.2.3 GFP1-10 protein can bind to permeabilized GFP11 expressing cells

Since we were unable to co-express GFP1-10 and GFP11 in *T. gondii*, we set out to investigate if GFP1-10 and GFP11 could recombine in our parasite model. We were able to generate a plasmid that enables the expression of GFP1-10 in *E. coli* (Table 2.2). Since GFP1-10 is only around 50% soluble, we isolated the GFP1-10 protein by forcing the protein to aggregate into inclusion bodies by inducing the bacteria for 3 hours and then performing several rounds of sonication and centrifugation to break up the inclusion bodies and pellet down GFP1-10. This pellet containing GFP1-10 was then be dissolved in 9M urea and 5mM DTT, filtered through a 0.22 µm syringe filter and kept for up to two weeks in -20 °C (Figure 2.3A). Once we obtained GFP1-10 protein, we set out to use it in an *in vivo* GFP1-10 assay.

Our GFP11 expressing parasites were fixed, permeabilized, blocked and incubated with the extracted GFP1-10 protein for 3 hours. We were able to observe GFP fluorescence by IFA after the 3-hour incubation (Figure 2.3B) indicating that GFP1-10 protein could successfully recombine with GFP11 fused to either the IMC and facing the cytoplasm or the outer mitochondrial membrane also facing the cytoplasm expressed in our parasites. Additionally, the GFP signal co-localized with the Ty tags fused to their target protein and GFP11x7, indicating that recombination was happening at the site of GFP11 protein expression (Figure 2.3B).

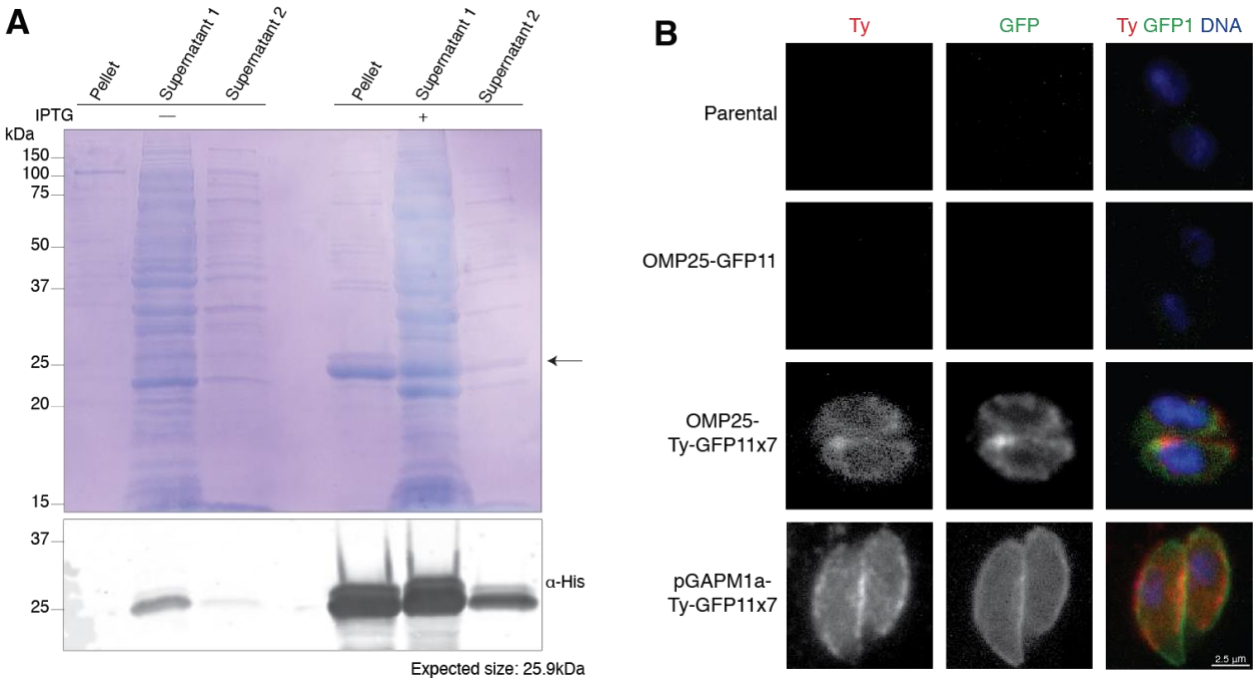

Figure 2.3 GFP1-10 protein can bind to permeabilized GFP11-expressing cells. A Above, Coomassie showing samples form GFP1-10 expressing E. coli that are either induced or non-induced with IPTG for 3h. Below, Western Blot showing samples described above. GFP1-10 is partially soluble and fused to an 8xHis tag. **B.** GFP1-10 assay with parental, OMP25-GFP11, OMP25-Ty-GFP11x7 and pGAPM1a-Ty-GFP11x7. GFP1-10 can recombine with fixed cells tagged with GFP11. GFP11x7 has an amplified GFP signal in comparison to GFP11.

2.2.4 Reconstituted Split-GFP can be recognized via Flow Cytometry

After we confirmed that GFP1-10 could recombine with GFP11 in fixed cells, we set out to answer the question of whether or not the recombined Split-GFP fluorescence would be bright enough to be detected by Flow Cytometry. This was an essential step in assessing if our split system could be used as a reporter system. For this experiment, we permeabilized cells expressing pGAPM1a-Ty-GFP11x7 and its parental, RH-ΔKu80, with 12.5µM digitonin and then incubated with GFP1-10 protein for 3h (Figure 2.4A). We then looked at the fluorescence of the cells with Flow Cytometry and were able to confirm that the reconstituted Split-GFP was bright enough to be recognized and differentiated from non-fluorescent cells.

In summary, GFP1-10 can bind to GFP11x7 expressed on either the IMC or mitochondrion and facing the cytoplasm in live *Toxoplasma* cells, allowing for GFP chromophore maturation. Additionally, the GFP fluorescence resulting from GFP1-10 and GFP11 recombination in live cells can be identified using Flow Cytometry. These findings combined are evidence that, if co-expressed, GFP1-10 and GFP11 can be used as a reporter system between two organelles in *T. gondii.*

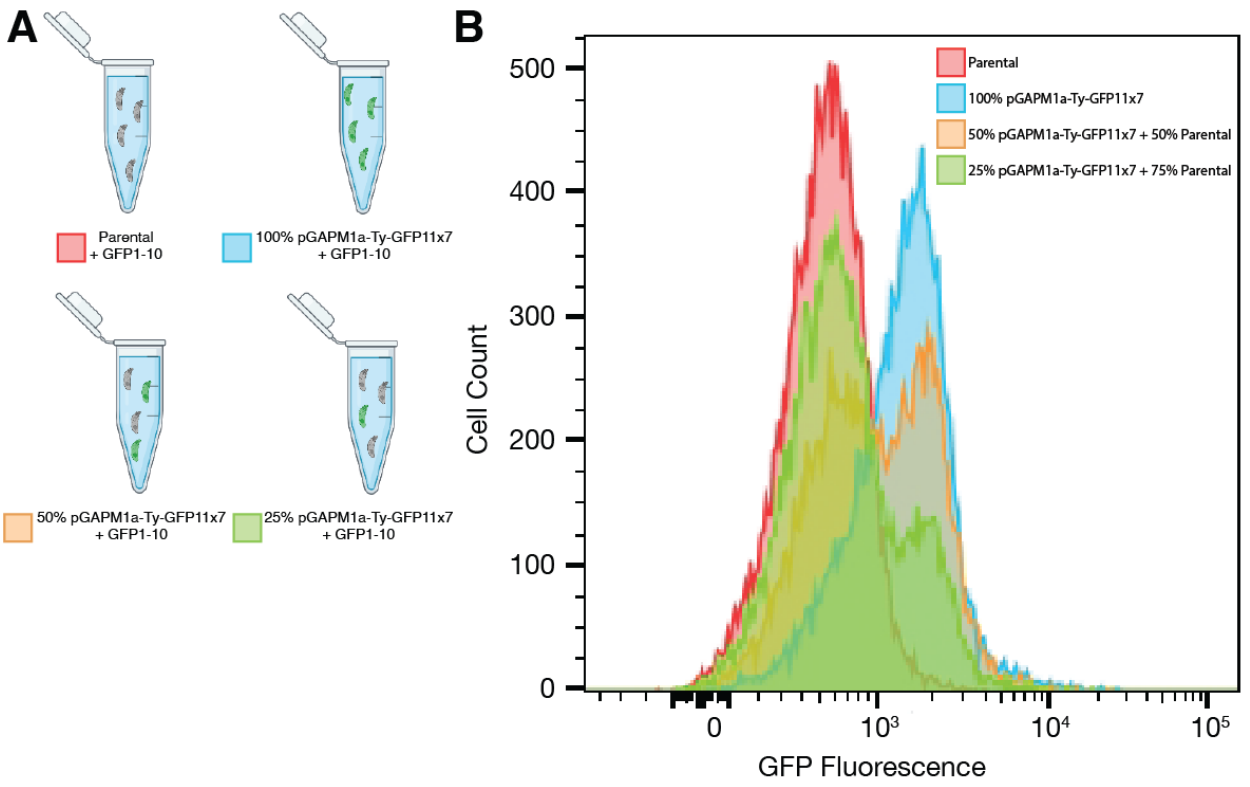

Figure 2.4 Reconstituted Split-GFP can be recognized via Flow Cytometry. A. Schematic of experiment using BioRender.com. Each tube contained 4 x 106 parasites of parental, pGAPM1a-Ty-GFP11x7 or a mixture of the two. Parasites were permeabilized with 12.5µM digitonin and incubated with GFP1-10 protein for 3h. **B.** Graph depicting cells analyzed for GFP fluorescence using flow cytometry and cells described above.

2.3 Discussion

In this study, we worked to establish a fluorescence-based reporter system between the apicoplast and mitochondrion in the apicomplexan parasite *Toxoplasma gondii* using Split GFP1-10 and GFP11. Establishing this reporter system would be the first step in carrying out a CRISPR genetic screen to identify MCS components between the apicoplast and mitochondrion of *T. gondii*. In this screen, we will first create a fluorescence-based reporter system between the apicoplast and mitochondrion using Split GFP1-10 and GFP 11. Next, we will perform a CRISPR screen to knock out MCS candidate genes. Following that step, we will FACS sort cells to find the ones that lose fluorescence since this will indicate that the gene may be involved in creating MCS. After identifying potential MCS component genes, those genes may be further characterized to understand their involvement in creating MCS. What all of these candidate genes have in common are that they are localized to the outer membrane of the mitochondrion or apicoplast, according to a recently published subcellular localization atlas of the *T. gondii* proteome (Sidik, Huet and Lourido, 2018), and they have at least one transmembrane domain. Based on these criteria, we generated a list of 122 genes. FACS sorting would follow to assess if the loss of a candidate gene results in a decrease of fluorescence from our reporter system. This loss in fluorescence may be due to one of two possibilities, either a loss of parasite viability or a loss in the association of the apicoplast and mitochondrion. To further investigate which reason is responsible for the loss of the association, the proteins that cause a loss in fluorescence will then be further characterized to assess if they are MCS components that aid in the interaction of the mitochondrion and apicoplast. First, we could tag any potential candidate to learn more

about its subcellular localization using IFA. Additionally, immunoprecipitations may be performed to see if other proteins may be pulled down with the tagged protein. These proteins may then be accesses in a similar way to see if they are involved in protein tethering. Then, we could design a knockout or knockdown approach depending on if the gene is essential or not and study how the gene knockout or knockdown affects the parasite. Affects include a loss of mitochondrial and apicoplast association, a loss of parasite viability, or limitations in the parasite's lytic cycle. A few techniques that may be used to study these effects may include observing organelle localization and morphology via IFA and electron cryo-tomography (cryo-ET), performing plaque assays to assess if there is a lytic cycle effect, or using assays such as a heme assay to see if a shared pathway is still being carried out after a gene knockout or knockdown. Similarly, to the heme assay, lipidomics analysis may also be used to analyze the knockout or knockdowns to access if the mutants have an effect on isoprenoid synthesis, since a disruption in isoprenoid synthesis would disrupt lipid levels. Similarly, since isoprenoids are involved with ETC function and the synthesis of isoprenoids may be disrupted in the knockout and knockdown mutants, ETC function can also be accessed using ouroboros, a membrane potential assay, or in-gel activity assays of each ETC complex. As mentioned, ToxoDB uses LOPIT data which is very limiting and only accounts for proteins that show one subcellular localization. To account for and screen dual localization proteins that may be added to this CRISPR screen, future studies could look for targeting signals to either the outer membrane of the mitochondrion or apicoplast. An example of this kind of targeting sequence is that a bipartite N-terminal targeting sequence has been found to be responsible for protein trafficking to the apicoplast (Pino *et al.*, 2007) while

proteins targeted to the outer mitochondrial membrane contain a N-terminal pre-sequences (van Dooren *et al.*, 2016). Along these lines, there has been a discovery of bimodal targeting to the mitochondrion and the apicoplast found to occur in *T. gondii* (Pino *et al.,* 2007). Another way dual localization proteins could be identified is by utilizing Split-TurboID on both the outer membrane or the mitochondrion and apicoplast as a tool to label nearby proteins in order to identify them. This approach has been used recently in mammalian cells to map the proteome between the ER and mitochondria (Cho *et al.*, 2020).

As a proof of concept, we wanted to express Split GFP1-10 and GFP11 in the cytoplasm to see if they could assemble in *T. gondii*. Our goal was to show that GFP1-10 and GFP11 could be co-expressed and recombine in *T. gondii* when both split components are expressed in the cytoplasm of the parasite. We chose to express GFP1-10 by transfecting in a plasmid that would allow for the cytoplasmic expression of the protein. We chose this approach because it has been used previously in mammalian cells (Kamiyama *et al.*, 2016). Unfortunately, we were unable to isolate a clonal population of cytoplasmic GFP1-10-expressing parasites even after attempting to with several cytoplasmic expression plasmids. However, we were able to see expression of GFP1-10 transiently after transfection (Figure 2.1C). Because we were unable to isolate a clone of GFP1-10 expressing in the cytoplasm of *T. gondii,* but were able to see it transiently expressed, these results suggest that GFP1-10 expression in the *T. gondii's* cytoplasm is toxic and that it may be getting degraded. A few ways that may overcome this issue could involve using an inducible system, such as the conditional U1 gene silencing system (Pieperhoff *et al.*, 2015). The conditional U1 gene silencing system would allow for

expression of a cytoplasmic HA-GFP1-10 only upon addition of the drug rapamycin. Another alternative approach could be to use the inducible gene expression system called the tet-transactivator system that was established based on a transactivator composed of the TetRep fused to a nonendogenous activating domain (TATi1) (Meissner, Schlüter and Soldati, 2002). The tet-transactivator system, similar to the conditional U1 gene silencing system, would allow for expression of cytoplasmic HA-GFP1-10 upon addition of the drug ATc. Being able to control gene expression would allow for expression of the potentially toxic GFP1-10 to only be expressed when necessary. Another option is using a weaker promoter to express GFP1-10. Previously, we have tried the Tub1 and the Sag1 promoter (strong and moderate promoters). Accordingly, a weak promoter such as the DHFR promoter may be used to express the protein at a low level. Along with trying to express HA-GFP1-10 in the cytoplasm, we also tried to fuse it to the C-terminus of the cytoplasmic protein CDPK1. Perhaps due to the fact CDPK1 is an essential protein, we were unable to isolate a clone that expressed GFP1-10 on the protein. Alternatively, we could try to tag a non-essential protein, such as the Fbx014 protein (TGGT1_259880). This is a non-essential protein that localizes to the cytoplasm of *T. gondii* that has been successfully tagged by a personal communication. For the rest of our proof of concept, we exogenously fused Ty-GFP11x7 to the cytoplasmic facing C-terminus of the structural IMC protein GAPM1a (Figure 2.1D). The GAMP1-Ty-GFP11x7 strain was created to be used as a proof of concept along with a cytoplasmic GFP1-10. Since we were unable to generate a cytoplasmic GFP1-10 strain, future studies may use GAPM1a-Ty-GFP11x7 as the parental while trying the techniques mentioned above to attempt to co-express both portions of Split GFP1-10 and GFP11.

While trying to express the Split-GFP components in the cytoplasm, we were simultaneously creating our reporter system between the mitochondrion and apicoplast using GFP1-10 and GFP11. We were able to fuse GFP11x7 to the outer mitochondrial membrane using a human targeting signal to the outer membrane of the mitochondrion from the human protein OMP25 (Figure 2.2B). Unfortunately, we were unable to obtain a clone that expressed GFP1-10 on the apicoplast using APT1. A potential way to combat this issue would be to introduce a drug resistance cassette to the repair template. A drug resistance cassette would allow for selection of APT1-GFP1-10 expressing cells. Another potential approach could be to fuse the bulkier GFP1-10 to the outer mitochondrial membrane using the OMP25 targeting signal and fusing the smaller GFP11 on the C-terminus of APT1 to avoid fusing a large protein to an essential protein, which may be hindering its function, thus making the parasites less fit.

An additional reason we suggest that the co-expression of GFP1-10 and GFP11 may be toxic for *T. gondii* is because transient expression showed that in dividing vacuoles, only individual parasites were able to express Ty-GFP11x7 when co expressed with GFP1-10. Because of the lack of success we had with expressing GFP1-10, we tested if GFP1-10 and GFP11 could bind in our parasite model in an alternative way. We isolated GFP1-10 protein to use in a GFP1-10 assay with our parasites that expressed GFP11x7 fused to GAPM1a's C-terminus or the outer mitochondrial membrane. In our assay, we were able to show GFP fluorescence colocalized to a Ty tag that was fused to GFP11x7 on either the IMC or the outer mitochondrial membrane (Figure 2.3B). These results supported that GFP1-10 and GFP11 could recombine in fixed *Toxoplasma* cells

which supports that we may use Split GFP1-10 and GFP11 as a reporter system between the apicoplast and mitochondrion of *T. gondii.*

As a final step, we investigated whether or not Split GFP1-10 and GFP11 could recombine in live cells and if this recombination would result in a fluorescence that was bright enough to be recognized by Flow Cytometry. In our assay, we permeabilized *T. gondii* cells that had GFP11x7 fused to GAPM1a's C-terminus in the parasite's IMC, its parental (RH-ΔKu80) and a combination of these cells at a certain percentage in a test tube (Figure 2.4A). After analyzation via Flow Cytometry, the results of this experiment showed that GFP1-10 can bind to GFP11 in live cells and that this binding is bright enough to be recognized by Flow Cytometry (Figure 2.4B) This result suggests that Split GFP1-10 and GFP11 could be used as a reporter system between the apicoplast and mitochondrion in a genetic screen to discovering tethering proteins in *T. gondii.*

Altogether, our results provide insight on creating a reporter system between the apicoplast and mitochondrion of *T. gondii* to use in a CRISPR screen to find potential MCS components. Our work could help shed light on unraveling the organellar biology of *Toxoplasma gondii* and in turn uncover potential drug targets against apicomplexan infections.

2.4 Materials and Methods

Parasite culture and strains

T. gondii tachyzoites from the strain RH and its derivatives were maintained at 37°C with 5% CO_2 growing in human foreskin fibroblasts (HFFs) cultured in Dulbecco's modified minimal essential media (DMEM) supplemented with 3% heat-inactivated fetal bovine

serum, 10 µg/ml gentamicin and 2mM glutamine. Parasites were continuously maintained *in vitro* by serial passages into new HFFs (ATCC).

Parasite transfection

Parasites were transfected as described previously by (Sidik *et al.*, 2014). For selection, MPA (mycophenolic acid) (Mycophenolic acid, cat. No. M3536-50MG) and XA (xanthine) (Sigma-Aldrich, cat. no. X4002-5G) were used at 50 µg/ml and 25 µg/ml, and chloramphenicol (Sigma Aldrich, cat. no. C3175-100MG) was used at either 40µM or 20µM concentration.

Cytoplasmic GFP1-10 strain construction

To generate a strain expressing cytoplasmic GFP1-10, a plasmid called cytoplasmic-mcherry (Table 2) for short was digested by NHe1 and Mfe1 to create a 7788 bp backbone. The GFP1-10 was amplified by PCR using NEB Q5® High-Fidelity DNA Polymerase (NEB, cat. no. M0492L) with a forward primer containing 20 bp homology to the backbone, an HA tag and a reverse primer containing 20 bp homology to the backbone. Both reverse and forward primers contained 15bp homology to a plasmid (P1 and P2) with GFP1-10 and were used to amplify a 750 bp GFP1-10 DNA fragment. The backbone and GFP1-10 DNA fragment were assembled into a plasmid using the NEB Hifi DNA Assembly kit (NEB cat. no. E2621L). For simplicity, this plasmid will be referred to as Cytoplasmic-GFP1-10. Plasmid was sequenced through Sanger sequencing and transformed into DH5α, grown with 100 mg/ml Ampicillin (Millipore Sigma, cat. no. A0166-5G) and collected with a Maxi prep kit (VWR cat. no. 77001-470). 100 µg of Cytoplasmic-GFP1-10 plasmid were transfected into RH-ΔKu80-ΔHXGPRT tachyzoites. Transfection was followed by chloramphenicol (Sigma Aldrich, cat. no. C3175-100MG) selection at

20μM concentration. Parasites were subcloned by limiting dilution and screening for the clones was done by PCR using Taq DNA polymerases (NEB cat. no. B9014S) and P3 and P4.

Ty-GFP11x7 plasmid construction

To generate a plasmid expressing a Ty tag fused to GFP11x7, pACUH-GFP11x7 (Addgene) was digested with EcoRI and P23 and P24 was duplexed together to be inserted into the vector.The insert contains a Ty tag along with 15 bp homology to pAUCH-GFP11x7 (Table 2). The backbone and duplexed DNA fragment were assembled into a plasmid using the NEB Hifi DNA Assembly kit (NEB cat. no. E2621L). pAUCH-Ty-GFP11x7 (Table 2) plasmid was sequenced with P25 and P26 with Sanger Sequencing and transformed into DH5α, grown with Amp (Millipore Sigma, cat. no. A0166-5G) and the plasmid was isolated with a Maxi prep kit (VWR cat. no. 77001-470).

IMC GFP11x7 strain construction (exogenous expression)

To generate a strain expressing GFP11x7 exogenously on the IMC and facing the cytoplasm, we chose to express GAPM1a, an IMC protein with its C-terminus in the cytoplasm, in a plasmid. For this, we needed a backbone and 4 DNA fragments containing DNA encoding the GAPM1a endogenous promoter region, the GAPM1a gene, a Ty tag, GFP11x7, T2A (a skip peptide) and HXGPRT (which will allow for selection using Xa/MPA). The 3393 bp backbone was obtained by digesting a parental plasmid called 3xHA-TurboID-OMP25 (Table 2) with HindIII and AvrII. Next, a 1040 bp insert with the GAPM1a endogenous promoter was amplified by PCR using primer P4 and P5 and genomic DNA from the RH strain. A 945 bp insert with the GAPM1a gene was amplified by PCR from cDNA using P7 and P8. A 529 bp insert with Ty and GFP11x7 was amplified

by PCR from a mitochondrial GFP11x7 template (OMP25-TY-GFP11x7-HXGPRT, Table 2) using primers P9 and P10. Finally, a 750 pb insert with HXGPRT was amplified by PCR using P11 and P12. PCRs of the inserts were performed using NEB Q5® High-Fidelity DNA Polymerase (NEB, cat. no. M0492L) with forward primers containing 20 bp homology to the backbone or adjacent inserts and reverse primers containing 20 bp homology to the backbone or adjacent inserts. All primers can be found in Table 1. The backbone and four inserts described above were assembled into a plasmid using the NEB Hifi DNA Assembly kit (NEB cat. no. E2621L). The resulting plasmid, pGAPM1a-Ty-GFP11x7-HXGPRT, was sequenced with Sanger Sequencing and transformed into DH5α grown with Ampicillin (Millipore Sigma, cat. no. A0166-5G). The plasmid was isolated with a Maxi prep kit (VWR cat. no. 77001-470). 100 µg of plasmid was transfected into RH-ΔKu80-ΔHXGPRT tachyzoites. Transfection was followed by 50µg/ml XA and 25 µg/ml MPA selection. Parasites were subcloned by limiting dilution and screening for the clones were done by PCR using Taq DNA polymerases (NEB cat. no. B9014S) and P13 and P14. Further validation was performed by IFA.

IMC GFP11x7 strain construction

For this approach, we choose to tag the C-terminus of the GAMP1a locus to generate a strain fused to GFP11x7 on the IMC and facing the cytoplasm. To do so, a CRISPR Cas9 guide targeting the C-terminus of GAPM1a. To create the repair template with DNA that could fuse a Ty tag and GFP11x7 to the C-terminus of GAPM1a, we performed a PCRs using P2, P22 and NEB Q5® High-Fidelity DNA Polymerase (NEB, cat. no. M0492L). This resulted in a 560 bp repair template with a Ty tag and GFP11x7 with 40 bp of homology on each side to the C-terminus of GAPM1a. 100 µg of guide and 30 µg of repair

template was used to transfect RH-ΔKu80-ΔHXGPRT tachyzoites. PCR using Taq DNA polymerases (NEB cat. no. B9014S) was performed with P27 and P28 on the DNA of the mixed population to confirm repair template integration.

Mitochondrial GFP11x7 strain construction

To generate the strain expressing GFP11x7 on the mitochondrial membrane facing the cytoplasm, a plasmid was created by digesting the 3xHA-TurboID-OMP25 plasmid (Table 2) with NheI and AvrII creating a 6368 bp backbone. An insert containing a small linker and the OMP25 human outer mitochondrial targeting signal was duplexed using oligos P15 and P16 and a second 482 bp insert was amplified from pACUH-Ty-GFP11x7 using P17 and P18. PCRs were performed using NEB Q5® High-Fidelity DNA Polymerase (NEB, cat. no. M0492L) with forward primers containing 20 bp homology to the backbone or adjacent inserts and reverse primers containing 20 bp homology to the backbone or adjacent inserts. All primers can be found in Table 1. The backbone and inserts were assembled into a plasmid using the NEB Hifi DNA Assembly kit (NEB cat. no. E2621L). Plasmid was sequenced with Sanger Sequencing and transformed into DH5α, grown with Ampicillin at 100 mg/ml (Millipore Sigma, cat. no. A0166-5G) and the plasmid was isolated with a Maxi prep kit (VWR cat. no. 77001-470). 100 µg of plasmid for Mitochondrial GFP11x7 strains was transfected into RH-ΔKu80-ΔHXGPRT tachyzoites. Transfection was followed by XA/MPA selection. Parasites were subcloned by limiting dilution and screening for the clones was done by PCR using Taq DNA polymerases (NEB cat. no. B9014S) and P19 and P20.

Immunofluorescence microscopy

Intracellular parasites that have invaded HFF-covered coverslips were fixed with 4% formaldehyde (VWR, cat. no. 100503-917) at 4°C for 10 min, then permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, cat. no. T8787-100ML) in PBS for 8 min and blocked for 10 min with a PBS solution containing 5% normal goat serum (NGS) (Thermo Fisher Scientific, cat. no. 16210072) and 5% heat-inactivated fetal bovine serum (IFS) (Sigma-Aldrich, cat. no. A9418-5G). Staining was performed with mouse-anti-Ty, rabbit-anti-HA, guinea pig-anti-CDPK1, rabbit-anti-IMC6, or rabbit-anti-TOM40. Alexa-488-conjugated goat-anti-mouse (Invitrogen, cat. no. A11029), Alexa-647-conjugated goat-anti-rabbit (Invitrogen, cat. no. A11037) Alexa-488-conjugated goat-anti-rabbit (Fisher Scientific, cat. no. A32731), and Alexa-647-conjugated goat-anti-guinea pig (Thermo Scientific, cat. no. A-21450) were used as secondary antibodies. Nuclei were stained with Hoechst (Santa Cruz, cat. no. sc-394039), and coverslips were mounted in Prolong Diamond (Thermo Fisher, cat. no. P36965). Images were acquired using an Echo Revolve epifluorescence microscope and the d373 imaging software. FIJI was used for image analysis, and Adobe Photoshop and Illustrator CC 2022 were used for image processing.

Western Blot Analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed established protocols (Laemmli, 1970). Lysates were prepared by resuspending a pellet of 1 x 10^7 tachyzoites in 50 µL of cell lysis buffer containing 10% Triton X-100 (Sigma-Aldrich, cat. no. T8787-100ML), HALT protease inhibitor cocktail (VWR, cat. no. PI78440), DNAse I (NEB, cat. no. M0303L) and Xenopus buffer containing 50 mM KCl, 20 mM HEPES (Sigma-Aldrich, cat. no. H4034-100G), 2 mM $MgCl_2$ and 0.1 mM EDTA (Sigma-Aldrich, cat. no. 59417C-100MLall) at pH 7.5. Total lysates were boiled in Laemmli sample buffer (BioRad).

Immunoblotting followed established protocols using rabbit anti-HA monoclonal antibody (1:10,000), mouse anti-Ty antibody (1:10,000), guinea pig anti-CDPK1 (1:1,000). Detection was done using the Odyssey Clx LICOR system using goat anti-mouse IRDye 800CW (1:10,000), goat anti-rabbit IRDye 800CW (1:10,000) and goat anti-guinea pig IRDDye 800CW as secondary antibodies.

Bacterial expression of GFP1-10 and the collection of GFP1-10

To isolate GFP1-10, a plasmid was created to enable GFP1-10 production *E. coli.* To create this, a plasmid named DH013 (Table 2.2) was digested with NsiI and XmaI. A PCR was performed to amplify GFP1-10 with homology to DH013 using P35 and P36 (Table 2.1) on the DNA template pcDNA-GFP1-10 (Table 2.2) and NEB Q5® High-Fidelity DNA Polymerase (NEB, cat. no. M0492L). The backbone and insert were assembled into a plasmid using the NEB Hifi DNA Assembly kit (NEB cat. no. E2621L). The plasmid was sequenced with Sanger Sequencing and transformed into DH5α, grown with Ampicillin at 100 mg/ml (Millipore Sigma, cat. no. A0166-5G) and collected with a Maxi prep kit (VWR cat. no. 77001-470). DNA was then transformed into BL21 *E. coli*. On day 0, GFP1-10 BL21 *E. coli was* grown in 3 ml of LB overnight as a starter culture. On day 1, 3 ml of the overnight culture were inoculated into 500 ml of LB containing 35 µg/ml kanamycin for 2 hours or until it was in its exponential stage with an OD between 0.5-0.8. 5mL of this sample was placed in a falcon tube and kept as the non-induced sample (this sample went through all the same steps besides induction). To force GFP 1–10 to aggregate into inclusion bodies, bacteria were induced with 1 mM IPTG for 5 h at 37 °C. After the induction, cells were collected by centrifugation for 15 min at 3,500g. All centrifugations were done at 4°C. The medium was discarded, and cell pellets were resuspended in 15

ml of TNG buffer which consists of 100 mM Tris-HCl (pH 7.4), 150 mM NaCl and 10% glycerol vol/vol. Next, the solution was sonicated three times for 2 min on ice at 50% duty cycle. Cell debris containing crude inclusion bodies was pelleted by centrifugation for 30 min at 30,000g. The supernatant containing the GFP1-10 was kept and 5 ml of Cell LyticB, Lysozyme, Benzonase and Halt Protease inhibitor was added to the solution. This solution was then sonicated to resuspend the crude inclusion bodies. 30ml CellLytic, 300µl lysozyme at 10 mg/ml, 5µl benzonase, and 300µl HALT protease inhibitor were mixed together for 30ml of cell Lytic buffer. Solution was then centrifuged for 10 min at 30,000g and the supernatant was kept. This wash process was repeated three times with Cell LyticB/lysozyme solution, then twice with 5 ml of TNG buffer (this step removes residual detergent from the pellet mass). The pellet was weighed and resuspended in TNG buffer to obtain a concentration of ~75 mg/ml of washed and packed pellet. The inclusion bodies were resuspended by briefly sonicating. 1mL aliquots were transferred to 1.7 ml microcentrifuge tubes. The contents were then centrifuged for 10 min at 16,000g. The pellet can be stored in the Eppendorf tubes at −80 °C. These pellets may be stored at −80 °C for up to 6 months. Pellets were weighed and their weights were recorded. One GFP1-10 inclusion body pellet was then dissolved in 1 mL of 9 M urea containing 5 mM DTT. The pellet was broken up with a pipette tip. This solution was incubated in a 37 °C water bath to help dissolution of the inclusion bodies. After complete dissolution, the microcentrifuge tube was centrifuged for 2 min at 16,000g to remove any aggregated material. To a 15 ml Falcon tube, X ml of TNG buffer was added and the 1 ml of the soluble urea suspension. The tube was mixed gently by inversion. The soluble solution

was then filtered through a 0.2 µm syringe filter. This solution was then stored at -20°C for up to 2 weeks.

In vivo protein solubility assays using Split-GFP

GFP1-10 protein was collected and prepared as described above. For the Split-GFP assay, intracellular parasites expressing GFP11x7 on the outer mitochondrial membrane or the C-terminus of GAPM1a that have invaded HFF-covered coverslips were fixed with 4% formaldehyde (VWR, cat. no. 100503-917) at 4°C for 10 min, then permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, cat. no. T8787-100ML) in PBS for 8 min and blocked for 10 min with a PBS solution containing 5% normal goat serum (NGS) (Thermo Fisher Scientific, cat. no. 16210072) and 5% heat-inactivated fetal bovine serum (IFS) (Sigma-Aldrich, cat. no. A9418-5G). Each coverslip was then incubated in a solution of 0.15 mg/mL of GFP1-10 protein at RT for 3h. After incubation with GFP1-10 protein, coverslips were washed with PBS 7.0 pH then processed for an IFA as previously described above. Staining was performed with mouse-anti-Ty. Alexa-488-conjugated goat-anti-mouse (Invitrogen, cat. no. A11029) was used as a secondary antibody. Nuclei were stained with Hoechst (Santa Cruz, cat. no. sc-394039), and coverslips were mounted in Prolong Diamond (Thermo Fisher, cat. no. P36965). Images were acquired using an Echo Revolve epifluorescence microscope and the d373 imaging software. FIJI was used for image analysis, and Adobe Photoshop and Illustrator CC 2022 were used for image processing.

Mitochondrial GFP11 strain construction

To generate a strain expressing GFP11 on the mitochondrial membrane facing the cytoplasm, a plasmid was created first by digesting the 3xHA-TurboID-OMP25 plasmid

(Table 2) with Nhe1 and AvrII creating a 6408 bp backbone. A 219 bp gene block insert was ordered from IDT containing GFP11 and 20 bp of homology to the backbone on each side. The backbone and insert were assembled into a plasmid using the NEB Hifi DNA Assembly kit (NEB cat. no. E2621L). Plasmid transformed into DH5α bacteria, grown with Amp (Millipore Sigma, cat. no. A0166-5G) sequenced with P29 and P30 Sanger Sequencing and collected with a Maxi prep kit (VWR cat. no. 77001-470). 100 µg of plasmid for mitochondrial expression of GFP11 strains was transfected into RH-ΔKu80-ΔHXGPRT tachyzoites (Sidik *et al.*, 2014). Transfection was followed by 2x chloramphenicol selection. Parasites were subcloned by limiting dilution and screening for the clones was done by PCR PCR using Taq DNA polymerases (NEB cat. no. B9014S) and P29 and P30.

Cytoplasmic GFP11x7 on CDPK1

To generate a strain expressing GFP11 endogenously on the C-terminus of CDPK1, we generated a CRISPR Cas9 guide to cut the C-terminus of CDPK1. PCRs using NEB Q5® High-Fidelity DNA Polymerase (NEB, cat. no. M0492L) were used to amplify 560 bp repair template using P31 and P32 that 20 bp of homology to a plasmid expressing a Ty tag and GFP11x7 and 40 bp of homology on each side to the C-terminus of GAPM1a. 100 µg of guide and 30 µg of repair temp was used to transfect RH-ΔKu80-ΔHXGPRT tachyzoites (Sidik *et al.*, 2014). PCR using Taq DNA polymerases (NEB cat. no. B9014S) was performed with P33 and P34 on the DNA of the mixed population to confirm repair template integration.

Parasite preparation with GFP1-10 for flow cytometry

Parasites were kept on ice for the length of their preparation. The parasites were filtered. The filtered parasites were then resuspended in 5 ml of ice-cold PBS pH 7.0. They were then counted, and that number was recorded. The parasites were then resuspended in 1000uL of ice-cold PBS. The desired number of parasites per tube is 4×10^6. We added contents to each tube as follows: 100% parental (negative control), 100% GFP expressing cells (positive control), 100% GFP11 expressing strain, 50% GFP11 expressing strain + 50% parental, 75% GFP11 expressing strain + 25% parental. Each tube containing parasites were then centrifuged at 800 rcf for 10 minutes at 4°C. The continents were then resuspended in ice cold 1 ml fluorobrite + 1% IFS + 2mM glutamate. 12.5uM digitonin was added to each tube. GFP1-10 was then added to a concentration of 0.15 mg/ml. The tubes were incubated while rotating at 4°C for 3h. Data was then quantified on the Agilent Quanteon.

Tables

Table 2.1: List of primers and oligos used in this study

Cytoplasmic GFP1-10 construction		
P1	Fwd-Cyto-GFP1-10-insert	CTTGAATTCCCTTTTgctagcGCCACCATGTACCCTTATGATGTGCCTGATTATGCTGGAGGGAGCGGCATGTCCAAAGGAGAAGAACT
P2	Rev-Cyto-GFP1-10-insert	cgggcagcttctgtcaattgCTAACTTCCGCCGCCACCTG
P3	Sequencing-Fwd-Cyto-GFP1-10-insert	CCAAACGGTGTTACACAATCAC
P4	Sequencing-Rev-Cyto-GFP1-10-	aaggacacaggggtctctgg

	insert	
IMC GFP11x7 strain construction (exogenous)		
P5	Fwd-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 1	CGAGGTCGACggtatcgataagcttctcgtcaggtctactccagtagtct
P6	Rev-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 1	GTAGGTGAAGTACATtttgcggaaaaagggattttc
P7	Fwd-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 2	ccctttttccgcaaaATGTACTTCACCTACGTTGTG
P8	Rev-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 2	gtggacttccccgggGGATCTGCAGGACAGGCAAG
P9	Fwd-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 3	CTGCAGATCCcccggggaagtccacacgaaccagg
P10	Rev-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 3	TGGATTTTCTTCTACATCTCCACATGTTAATAAACTTCCTCTTCCTTCGGTGATACCGGCAGCATTG
P11	Fwd-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 4	GGAGATGTAGAAGAAAATCCAGGACCAGCCGCGTCCAAACCCATTGAAGACTAC
P12	Rev-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT Insert 4	aaacgagagacgggcagcttctgtcctaggTTACTTCTCGAACTTTTTGCGAGCGG
P13	Sequencing-Fwd-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT	tggaaaaacgccagcaacgc
P14	Sequencing-Rev-pGAPM1a-Ty-GFP11x7-T2A-HXGPRT	cttcgataaaggacacaggggtct
Mitochondrial GFP11x7 strain construction		

P15	Top-oligo-OMP25-Ty-GFP11x7 Insert 1	ACCGGCGGCAAATTCCCGCGGCATCGAGGCGACGGAGAGCCTAGTGGAGTTCCTGTAGCTGTGGTGCTGCTGCCAGTGTTTGCCCTTACCCTGGTAGCAGTTTGGGCCTTCGTGAGATACCGAAAGCAGCTCTGAcctaggacagaagctgcccgt
P16	Bottom-oligo-OMP25-Ty-GFP11x7 Insert 1	acgggcagcttctgtcctaggTCAGAGCTGCTTTCGGTATCTCACGAAGGCCCAAACTGCTACCAGGGTAAGGGCAAACACTGGCAGCAGCACCACAGCTACAGGAACTCCACTAGGCTCTCCGTCGCCTCGATGCCGCGGGAATTTGCCGCCGGT
P17	Fwd-OMP25-Ty-GFP11x7 Insert 2	CAAACCTTTGCGAAAGATATCATGgaagtccacacgaaccaggac
P18	Rev-OMP25-Ty-GFP11x7 Insert 2	CGCCTCGATGCCGCGGGAATTTGCCGCCGGTGATACC
P19	Sequencing-Fwd-OMP25-Ty-GFP11x7	TTCCCTGTGCTGTCTCACCC
P20	Sequencing-Rev-OMP25-Ty-GFP11x7	aaggacacaggggtctctgg
IMC Ty-GFP11x7 strain construction (endogeneous)		
P21	Fwd-GAPM1a-Ty-GFP11x7-RT insert	TGCTGCGGAGCAGGCTCAGGCTTGCCTGTCCTGCAGATCCATGgaagtccacacgaaccaggac
P22	Rev-GAPM1a-Ty-GFP11x7-RT insert	ctgttcaccacatttcccagtgcttgctaattagcctTTAGAATTTGCCGCCGGTGATACC
P27	Sequencing-Fwd-GAPM1a-Ty-GFP11x7	CCTGCAGATCCATGgaagtccaca
P28	Sequencing-Rev-GAPM1a-Ty-GFP11x7	attagcctTTAGAATTTGCCGCCGG
Plasmid with Ty-GFP11x7		

P23	Top oligo with Ty with 15 bp homology to pACUH-GFP11x7 insert	AACTCTGAATAGGGAATTGGGAATTCATGgaagtccacac gaaccaggacccgctcgatGGCGGATCCCGTGACCACATGGT CCTTCA
P24	Bottom oligo with Ty with 15 bp homology to pACUH-GFP11x7 insert	TGAAGGACCATGTGGTCACGGGATCCGCCatcgagcgggt cctggttcgtgtggacttcCATGAATTCCCAATTCCCTATTCAGA GTT
P25	Sequencing Fwd-pAUCH-Ty-GFP11x7	GCAAATAAACAAGCGCAGCTGAAC
P26	Sequencing Rev-pAUCH-Ty-GFP11x7	GTGCACCTTGAAGCGCATGAA
Mitochondrial GFP11 strain construction		
P29	Sequencing Fwd-OMP25-GFP11 insert	ctctccgacccgaggcactc
P30	Sequencing Rev-OMP25-GFP11 insert	ACGCGCACATTTCCCTGTGC
Cytoplasmic GFP11 and GFP11x7 on CDPK1		
P31	Fwd-CDPK1-Ty-GFP11x7-RT insert	TGACGAGTTTCAACAGATGCTCTTGAAGCTCTGCGGA AACGGCGGATCCCGTGACCACATGGTCCTTCA
P32	Rev-CDPK1-Ty-GFP11x7-RT insert	ggagaatagacgtcacgcacagggagagtgcaagcttTTAGAATTTG CCGCCGGTGATAC
P33	Sequencing-Fwd-CDPK1-Ty-GFP11x7	TTCGAGGGGAAGTGCCAAGA
P34	Sequencing-Rev-CDPK1-Ty-GFP11x7	CATGTGGTCACGGTTTCCGC

GFP1-10 Protein		
P35	Fwd-GFP1-10-bacterial-expression-insert	catcaccatcaccatgcatccATGTCCAAAGGAGAAGAACTGTTTACCG
P36	Rev-GFP1-10-bacterial-expression-insert	cgactctagaggatccccgggCTAACTTCCGCCGCCACCTG

Table 2.2: List of plasmids used in this study

Plasmids	
Cytoplasmic-mcherry	DH5α e-coli expression for ampicillin resistance. For expression in *T. gondii*, CmR and Tub1 promoter for HA and sfmcherry1-10 expression.
Mitochondrial TurboID 3xHA-TurboID-OMP25	DH5α e-coli expression for ampicillin resistance. For expression in T. gondii, CmR and a Tub1 promoter for 3xHA tag, TurboID and OMP25 (a human targeting signal to the outer membrane of the mitochondrion) expression.
GFP11x7 pAUCH-GFP11x7	From Addgene. DH5α e-coli expression for ampicillin resistance. Used as template for GFP11x7.
Ty and GFP11x7 pAUCH-Ty-GFP11x7	Modified from pAUCH-GFP11x7 to be used as a template with a Ty tag fused to GFP11x7.
Exogenous IMC Ty and GFP11x7 pGAPM1a-Ty-GFP11x7	DH5α e-coli expression for ampicillin resistance. For expression in *T. gondii*, endogenous GAPM1a promoter for GAPM1a, a Ty tag, GFP11x7, T2A and HXGPRT for resistance selection using Xa/MPA.
Mitochondrial Ty and GFP11x7 OMP25-Ty-GFP11x7	DH5α e-coli expression for ampicillin resistance. For expression in T. gondii, for HXGPRT and a Tub1 promoter for a Ty tag, GFP11x7 and OMP25 (a human targeting signal to the outer membrane of the mitochondrion) expression.
Mitochondrial GFP11 OMP25-GFP11	DH5α e-coli expression for ampicillin resistance. For expression in T. gondii, for CmR and a Tub1 promoter for GFP11 and OMP25 (a human targeting signal to the outer membrane of the mitochondrion) expression.
GFP1-10 protein	BL21 e-coli expression for kanamycin resistance. For protein expression of GFP1-10.
Cytoplasmic-Ha-GFP1-10	DH5α e-coli expression for ampicillin resistance. For expression in T. gondii, for CmR and a Sag1 promoter for free GFP1-10 expression in the cytoplasm.
GFP1-10 pcDNA3.1-GFP1-10	From Addgene. DH5α e-coli expression for ampicillin resistance. Used as template for GFP1-10

3xHA-GFP1-10	DH5α e-coli expression for ampicillin resistance. Used to amplify repair templates with 3xHA fused to GFP1-10

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