UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

VALIDATION OF *TOXOPLASMA GONDII* GENES THAT DETERMINE FITNESS IN IFNγ-ACTIVATED MACROPHAGES

BY

PRISCILLA OFOSUA GYAN (11009034)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL IN MOLECULAR CELLULAR BIOLOGY OF INFECTIOUS DISEASES DEGREE

NOVEMBER 2024

DECLARATION

I, Priscilla Ofosua Gyan, hereby declare that, except for references to work conducted in other studies, which have been properly acknowledged through citations, this research was carried out by me in the Department of Microbiology and Immunology at the University of Michigan, Ann Arbor. The project was conducted under the supervision of Dr. Yifan Wang and Professor Osbourne Quaye.

---------- Date: 11/26/2024

Priscilla O Gyan (Student)

Yifan Wang

---------- Date: 11/26/2024

Dr. Yifan Wang (Supervisor)

---------- Date: 11/26/2024

Prof. Osbourne Quaye (Supervisor)

DEDICATION

I dedicate this work to my family for their unfailing support and to my Lord for His love, which keeps me going even amid the storm.

ACKNOWLEDGEMENTS

I want to acknowledge Dr. Yifan Wang and Prof. Osbourne Quaye, and Dr. Alice Telesnitsky for their guidance, corrections, support, and training during my master's program. I would also like to thank all members of the Wang lab for their invaluable contributions to my work during presentations and experiments. I would finally like to thank my family for their moral support during my program.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AMA1	Apical membrane antigen 1
BMDM	Bone marrow-derived macrophages
CD	Cluster of differentiation
CDC	Center for Disease Control
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DBS	Double-strand break
DCs	Dendritic cells
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GAC	Glideosome associated connector
GBP	Guanylate-binding protein
GOI	Gene of Interest

GRA	Dense granule protein
HA	Hemagglutinin
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HDR	Homology-directed repair
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HFF	Human foreskin fibroblasts
HIV	Human Immunodeficiency Virus
IFNγ	Interferon-gamma
IL	Interleukin
INDELs	Insertions and deletions
iNOS	inducible Nitric Oxide Synthase
IRG	Immune-related GTPase
ISGs	Interferon-stimulated genes
LD	Lethal dose
MAPK	Mitogen-activated protein kinases
MIC	Microneme protein
MJ	Moving junction
MYD88	Myeloid differentiation primary response 88

MyoA	Myosin heavy chain A
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NK cell	Natural killer cell
NuRD	Nucleosome Remodeling and Deacetylase
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
Pyr-sulf	Pyrimethamine-sulfadiazine
QTL	Quantitative trait locus
RON	Rhoptry neck protein
ROP	Rhoptry protein
sgRNA	single guide RNA
SOCS	Suppressor of cytokine signaling
STAT1	Signal Transducer And Activator Of Transcription 1
*Tg*IST	*T. gondii* inhibitor of STAT transcription

TgMLC1	*Toxoplasma gondii* Myosin light chain 1
TgWIP	*Toxoplasma gondii* WAVE-complex-interacting protein
Th	T-helper
TLRs	Toll-like receptors
TMP-SMX	Trimethoprim-sulfamethoxazole
TNFα	Tumor necrosis factor-alpha

ABSTRACT

Toxoplasma gondii is an important pathogen to human and veterinary health. This study validated two *T. gondii* genes that were determined to confer fitness in IFNγ-stimulated macrophages from a loss-of-function CRISPR screen. Using CRISPR/Cas9, individual knockout of candidate genes was generated. Plaque assay was used to determine if knockouts had general fitness defects. IFNγ stimulation of murine and rat BMDMs in luciferase assays was used to determine the knockout phenotype *in vitro*. The phenotype of candidate genes was determined *in vivo* during acute murine infection. Finally, the genes were endogenously tagged and their localization in the parasite was determined by immunofluorescent assays.

Positive clones of single-gene knockout parasites were successfully obtained and determined not to have general growth defects in HFF cells. Clone Δ*TGGT1_205350 #1-6F* was determined to have reduced growth with increased concentration of IFNγ in rat BMDMs, while all clones of Δ*TGGT1_232670* grew unrestricted like the wild-type parasites. Murine BMDMs were consistently unresponsive to IFNγ stimulation hence growth phenotype of candidate genes could not be assessed. Murine infection revealed that TGGT1_205350 was dispensable for parasite fitness during acute infection, while the likely subcellular localization of the TGGT1_205350 was observed to be in the Golgi apparatus. In conclusion, candidate gene TGGT1_205350 was demonstrated to confer fitness in IFNγ-stimulated rat BMDMs and it is possibly localized in the Golgi compartment of *T. gondii*.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The Apicomplexan protozoan *Toxoplasma gondii* (*T. gondii*) is one of the most successful obligate intracellular parasites, given its ability to invade all nucleated cells and infect a wide range of warm-blooded vertebrates (Sibley et al., 2007). Members of the same phylum, like *Plasmodium spp*. and *Cryptosporidium*, are significant disease-causing organisms for both humans and animals. *T. gondii* is known for its persistent infection, as thirty percent of the global human population is chronically infected with the parasite (Kim & Weiss, 2004). Humans usually get infection via the ingestion of tissue cysts (chronic stage) from infected animal tissues or oocysts excreted by feline, the definitive hosts of *T. gondii* (Ybañez et al., 2020). Following ingestion, a primary infection site is established in the tissue surrounding the intestines, resulting in inflammation (Jensen et al., 2011). Oocysts or tissue cysts can develop into tachyzoites (acute stage), which infect leukocytes and exploit these cells to further disseminate within infected hosts via parasite-induced migratory capabilities (Harker et al., 2015). After invasion into the host cells, *T. gondii* creates a parasitophorous vacuole (PV) that does not fuse with the lysosomes and endosomes of the host and keeps a neutral pH. Disruption of the PV leads to the loss of the parasite's intracellular replication niche and, ultimately, the death of *T. gondii* (Frickel & Hunter, 2021). As the cell type that is predominantly infected by *T. gondii* at the site of infection *in vivo* (Jensen et al., 2011), macrophages play an essential role in early host defense against the parasite during infection (Sasai & Yamamoto, 2019). In addition, the host cytokine IFNγ, generated by NK and T cells, contributes significantly to host resistance against *T. gondii* infection, mostly by activating macrophage anti-

microbial responses(Sasai & Yamamoto, 2019) (Frickel & Hunter, 2021). IFNγ signaling induces a group of interferon-stimulated genes (ISGs) via STAT1 activation and nuclear translocation (Schneider et al., 2014). While host immunological status is recognized to be crucial in the clinical outcome of toxoplasmosis, the severity of the disease that results from *T. gondii* infection is highly dependent on the host species and parasite strain and remains variable among individuals (Mukhopadhyay et al., 2020). Despite various host immune mechanisms to control *T. gondii* infection, the parasite has an arsenal of effectors to oppose these mechanisms, ensuring its survival (Sanchez & Besteiro, 2021). For instance, proteins from secretory organelles like the rhoptries and dense granules have been shown to directly or indirectly antagonize important host mechanisms responsible for restricting *T. gondii* (Seizova et al., 2024; Hakimi et al., 2017; Hunter & Sibley, 2012).

1.2 Rationale

A study conducted by (Wang et al., 2020) employed a genome-wide loss-of-function CRISPR (clustered regularly interspaced short palindromic repeats) screen in the *T. gondii* RH strain to identify over 160 genes that determined fitness in IFNγ-activated macrophages. They generated single-gene knockouts of their top candidate genes to verify the screen results, and one of these genes was *GRA45*, encoding a dense granule protein. It was demonstrated that Δ*gra45* parasites had a strong fitness defect in IFNγ-activated murine, rat, and human macrophages as well as reduced virulence in mice. As IFNγ is a potent cytokine released by the host to control *T. gondii* infections, studying the genes that contribute to parasite fitness in this condition will provide insights into the natural mechanisms of parasite resistance. Hence, validation of fitness-conferring genes will provide candidates for drug targets, leading to translational discoveries for new

therapeutics. This study sought to follow up on two genes that had significant fitness defects from the screen (Wang et al., 2020) by generating single-gene knockouts to validate their importance in parasite survival in IFNγ-activated macrophages.

1.3 Problem statement

One of the challenges associated with toxoplasmosis is the adverse effects of treatment. Current first-line drugs used to treat *T. gondii* infection mainly target inhibition of the folate pathway of the parasite. To develop novel therapeutics that can effectively eliminate *T. gondii* infection while minimizing toxic side effects, a comprehensive understanding of parasite biology and pathogenesis is crucial. Therefore, identifying parasite genes that determine fitness in different hosts will provide potential drug targets specific to the parasite.

1.4 Research questions

i. Do *T. gondii* candidate genes, *TGGT1_232670* and *TGGT1_205350*, contribute to survival in IFNγ-stimulated mouse and rat macrophages?

ii. Do *T. gondii* candidate genes, *TGGT1_232670* and *TGGT1_205350*, contribute to survival during acute infection in mice?

iii. Where do these genes localize in *T. gondii?*

1.5 Aim

To determine the *T. gondii* effectors contributing to parasite fitness in IFNγ-stimulated murine and rat macrophages.

1.6 Specific objectives

1. To generate CRISPR/Cas9-mediated knockout of *T. gondii* candidate genes
2. To verify the phenotype of individual gene knockout parasites *in vivo* and *in vitro* in murine models
3. To determine the subcellular localization of candidate genes in intracellular *T. gondii* by endogenous tagging.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Toxoplasma gondii* structure

T. gondii is a unicellular eukaryotic organism. The primary structure of this parasite is the same for all its infective forms, but the tachyzoite is the most studied form. They are elongated with a rounded posterior end and pointed apical end with an approximate size of 5µm in length and 2µm in width (Attias et al., 2020). As a eukaryotic organism, it has a nucleus, endoplasmic reticulum (ER), Golgi apparatus, mitochondria, and ribosomes. Similar to other members of the phylum Apicomplexa, it possesses an apicoplast, a cytoskeletal structure known as the conoid at its apical end. Also, in the apical region, secretory organelles like micronemes, and rhoptries are found while dense granules are distributed in the cytoplasm (Paredes-Santos et al., 2012).

Of the three secretory organelles, micronemes are the most abundant. They are cylindrical structures, and the first to secrete their proteins. Proteins secreted from this organelle are known as MICs (Carruthers & Tomley, 2008) and are important for host cell membrane attachment, parasite motion, and formation of the moving junction during cell invasion (Soldati et al., 2001; Carruthers & Sibley, 1997). Rhoptries are club-shaped, much larger than micronemes, and consist of the neck (anterior) and bulb (posterior) regions (Paredes-Santos et al., 2012). Proteins secreted from the rhoptry neck (RONs) work with MICs to aid parasite invasion. Rhoptry bulb proteins (ROPs) are involved in subverting the host defense mechanisms (Bradley & Sibley, 2007). They also decorate and modify the parasitophorous vacuole membrane (PVM) that is formed during invasion (El Hajj et al., 2007). Dense granules are the third secretory organelle that secrete proteins

known as GRAs. They are spherical structures distributed in the parasite (Paredes-Santos et al., 2012) and are secreted once the parasite has invaded the cell. These proteins may be secreted into the parasitophorous vacuole (PV) lumen, unto the PVM, or beyond the PVM into the host cytosol and nucleus (Griffith et al., 2022; Panas & Boothroyd, 2021). The nucleus is large, sphere-like, and centrally positioned. Lateral and anterior to the nucleus is the apicoplast which has four membranes (Sabine Köhler, 2005) and is a product of a secondary endosymbiotic event of a photosynthetic unicellular alga (Roos et al., 1999; Köhler et al., 1997). It is an important site for metabolic activity such as isoprenoid, fatty acids, and heme biosynthesis (Mazumdar et al., 2006). Close to the apicoplast and the apical side of the nucleus is the Golgi. It is made of about four to six stacks of membrane-bound lamellae and functions in the sorting and maturation of secretory proteins (Pelletier et al., 2002). The ER is associated with the posterior side of the nucleus and a single mitochondrion is present (Melo et al., 2000).

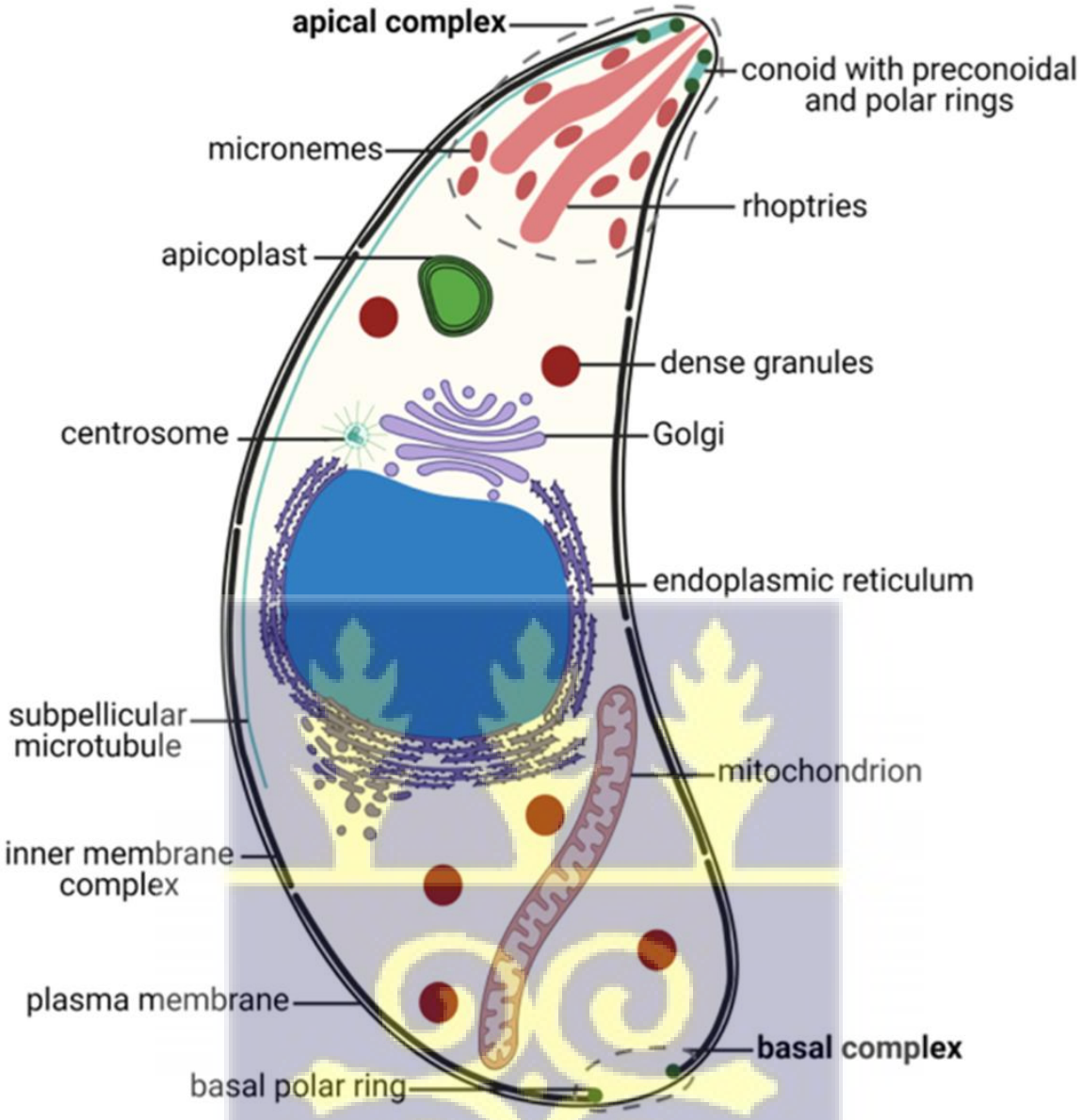


Figure 2.1: Labelled diagram of *T. gondii* tachyzoite stage (Delgado et al., 2022)

2.2 History and life cycle

T. gondii was discovered by Nicolle and Manceaux in 1908 in the tissue of a rodent, gundi of the family *Ctenodactylidae* but the protozoan was mistaken for *Leishmania* initially (Weiss & Dubey, 2009). Simultaneously, Splendore also discovered it in a laboratory rabbit in Brazil (Splendor,

2009). *T. gondii* has different morphological forms based on its life cycle. The crescent-shaped tachyzoite (Frenkel, 1973) ultrastructure was first studied in 1954 by Gustafson et. al (Gustafson et al., 1954). It actively proliferates and divides by a process known as endodyogeny to form daughter cells (Sheffield & Melton, 1968; Goldman et al., 1958). The slow-growing stage, bradyzoites are found encysted in tissues of the host. The term "bradyzoites" was first proposed by Frenkel (Frenkel, 1973) and the first description of the ultrastructure of the tissue cysts was by Wanko et. al (Wanko et al., 1962). The earliest rigorous study on the development of tissue cysts showed that they formed in mouse tissue 3 days post-infection with tachyzoites (Dubey & Frenkel, 1976).

T. gondii undergoes three infectious phases during its life cycle: tachyzoites, bradyzoites located in tissue cysts, and sporozoites present within sporulated oocysts (Dubey et al., 1998). All three stages are infectious to intermediate hosts, such as humans, sheep, pigs, rabbits, mice, and rats. Definitive hosts, which include domestic and wild cats, usually are infected by ingestion of tissue cysts or sporulated oocysts (Attias et al., 2020). Sexual reproduction takes place only in the definitive hosts. When definitive hosts ingest tissue cysts from an intermediate host, the bradyzoites are released from these cysts and invade the epithelium of the small intestine. The cyst wall of bradyzoites was found to be digestible by gastric enzymes such as trypsin and pepsin, releasing bradyzoites, which were resistant when subjected to treatment (Jacobs et al., 1960). After undergoing several rounds of asexual replication, schizonts containing merozoites are formed in the intestinal epithelium. At this stage, some of the organisms undergo sexual differentiation, leading to the formation of gametes (a process known as gametogony) (Di Genova & Knoll, 2020). Oocysts are formed within enterocytes after fertilization and are shed in feces unsporulated. Infected felines can shed about 100 million oocysts in the feces, and oocyst shedding can last three

to twenty days after ingesting tissue cysts. In the environment, they become sporulated (sporogony), forming two sporocysts, each containing four sporozoites after meiosis. These are highly infectious to intermediate hosts by contaminating food and water (Shapiro et al., 2019). Once sporulated oocysts are ingested by intermediate hosts, sporozoites convert to rapidly dividing tachyzoites. Tachyzoites continue to proliferate by endodyogeny in the intestinal epithelium and further disseminate to invade virtually any cell type. After rounds of replication, the host mounts a strong immunological response, leading to tachyzoites differentiation to bradyzoites, and tissue cysts form primarily in the brain and muscles (Hunter & Sibley, 2012). Tissue cysts form within a week of infection and can persist throughout the lifetime of the host.

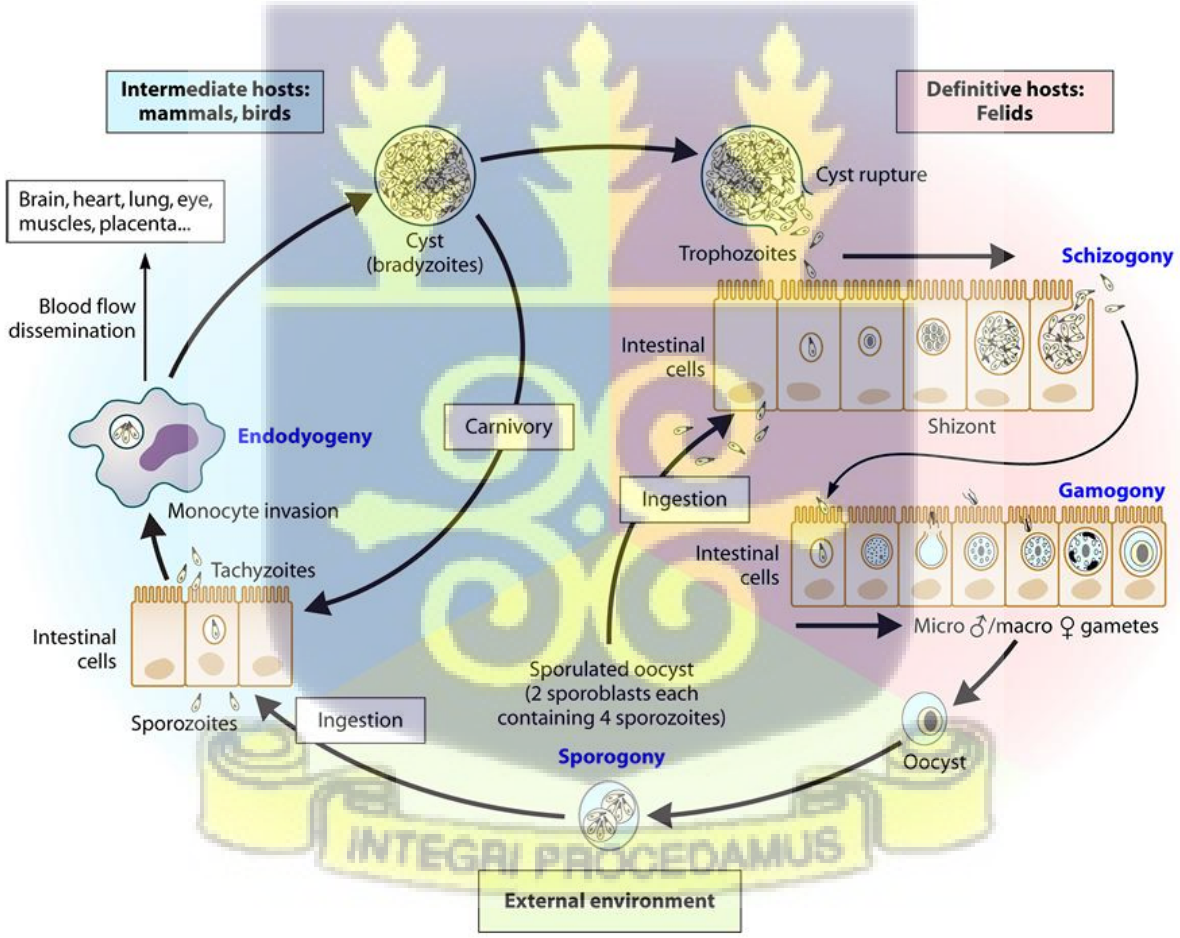


Figure 2.2: The life cycle of *T. gondii* (Robert-Gangneux & Dardé, 2012)

2.3 Transmission

Transmission of *T. gondii* can be horizontal between intermediate hosts or between an intermediate host and the definitive host. One of the main sources of horizontal transmission in humans is by ingesting raw or undercooked meat containing *T. gondii* tissue cysts (Opsteegh et al., 2016; Dubey et al., 2005; Cook et al., 2000). In Europe, 60% of acquired toxoplasmosis was attributed to transmission by consumption of contaminated meat with the highest implicated sources from pigs and sheep (Koutsoumanis et al., 2018). This makes pork and mutton more common sources for *T. gondii* infection than beef and chicken (Lafrance-Girard et al., 2018; Belluco et al., 2018, 2016).

Sporulated oocysts are also an important source of transmission able to contaminate the soil, food, and water (Almeria & Dubey, 2021). Unwashed fruits and raw vegetables pose a risk of oocyst contamination and *T. gondii* infection when consumed (Ekman et al., 2012; Alvarado-Esquivel et al., 2011). Also, several outbreaks of *T. gondii* infection have been reported to be a result of water sources contaminated with oocysts (Shapiro et al., 2019; de Moura et al., 2006; Benenson et al., 1982). In water bodies, filter feeders such as oysters, clams, and mussels have been shown to harbor *T. gondii* oocysts and because they can be consumed raw, they present significant danger for infection (Tedde et al., 2019; Coupe et al., 2018; Cong et al., 2017; Putignani et al., 2011).

Vertical transmission by tachyzoites across the placental barrier is another important route by which *T. gondii* is transmitted in humans and other intermediate hosts (Montoya & Remington, 2008). Interestingly, tachyzoites have also been found in the milk of intermediate hosts (Boughattas, 2017), and consumption of unpasteurized goat milk has been associated with past outbreaks of toxoplasmosis (Chiari & Neves, 1984; Sacks et al., 1982). Reports of *T. gondii* tachyzoite transmission in blood and blood products for transfusion have been published over the years (Gouda et al., 2024; Wesołowski, Pawłowska, & Mila-Kierzenkowska, 2023; Alvarado-

Esquivel et al., 2018; Mansouri et al., 2017), though the frequency of acquiring the infection through this route is minimal. Organ transplants from a *T. gondii*-infected donor to a *T. gondii* seronegative recipient have been implicated as a route of transmission (Robert-Gangneux & Dardé, 2012; Hill & Dubey, 2002). Solid organs such as kidneys, liver, heart, or others like bone marrow transplants could house tissue cysts or tachyzoites of *T. gondii* and hence serve as a source of infection (Wesołowski, Pawłowska, & Mila-Kierzenkowska, 2023; Fabiani et al., 2018; Khurana & Batra, 2016; Robert-Gangneux & Dardé, 2012).

Table 2.1: Summary of the routes of transmission of *T. gondii* in humans

Transmission routes	Infectious stage
Consumption of raw or undercooked meat (pork, lamb, chicken)	Bradyzoites (tissue cysts)
Consumption of raw oysters, clams, mussels	Sporulated oocysts and/or bradyzoites
Contamination of fruits, vegetables, water, soil	Sporulated oocysts
Organ transplant (heart, liver, bone marrow)	Tachyzoites/Bradyzoites
Vertical transmission (transplacental), transfusion of blood and blood products, needle stick injury in the laboratory	Tachyzoites
Unpasteurized milk	Tachyzoites

2.4 Toxoplasmosis in humans

Toxoplasma gondii is widespread globally and is significant in veterinary and medical fields, as it can lead to abortion or congenital diseases in its intermediate hosts (Tenter et al., 2000). Approximately 25 to 30% of the world's human population is generally assumed to be infected with *Toxoplasma* (Robert-Gangneux & Dardé, 2012). *Toxoplasma gondii* has a broad host range, including most warm-blooded animals, and the parasite can invade all nucleated cells (Sibley et al., 2007). Most horizontal transmissions in humans occur either through ingesting tissue cysts in infected meat (Tenter et al., 2000) or through contaminated soil contact, consuming water, or food contaminated with sporulated oocysts from the environment (Shapiro et al., 2019).

The majority of *T. gondii* infections in healthy individuals do not show symptoms. Sometimes, mild signs like fever, headaches, fatigue, or malaise may present, with lymphadenopathy being the most notable clinical feature (Weiss & Dubey, 2009). *T. gondii* can be passed on to the fetus in women with a competent immune system by crossing the placental barrier and entering the fetal circulation and tissues (Moghaddami et al., 2024). This transmission, depending on the trimester of pregnancy, can lead to complications such as abortion or fetal abnormalities, and it may result in vision impairment, mental retardation, or hearing loss for children who survive the infection in utero (Bollani et al., 2022). The organism may cause opportunistic infections in individuals with weakened immune systems, like those diagnosed with AIDS. This typically occurs due to the reactivation of latent infection, with severe encephalitis being the most common clinical presentation in these patients (Wesołowski, Pawłowska, Smoguła, et al., 2023).

2.5 Toxoplasmosis in Ghana

Like the rest of the world, toxoplasmosis is a health concern in Ghana, particularly in at-risk groups. As a sub-Saharan country, however, there are several infectious diseases of significant concern, such as Malaria, that often overshadow *T. gondii.* Given the overlap in symptoms between toxoplasmosis and diseases such as malaria and influenza, there is a heightened risk of misdiagnosis, particularly in light of the continued endemicity of malaria in Ghana (Reynolds, 2020).

The earliest documented case of human toxoplasmosis in Ghana was recorded in 1962. The study presented clinical evidence of congenital toxoplasmosis in four pediatric patients from Kumasi (Anteson et al., 1980). Since then, epidemiological studies have been conducted in mostly high-risk groups in Ghana. Current reports suggest a high seroprevalence among pregnant women in Ghana, with estimates ranging from 51.2% to 92.5%. (Ayi, et al., 2016; Sefah-Boakye et al., 2016). The average prevalence of anti-*Toxoplasma gondii* antibodies in children is 58% with evidence of congenital transmission in sampled placental tissues (Blay et al., 2015; Kwofie et al., 2016) and among women of reproductive age, a prevalence of 61% has been reported (Ayeh-Kumi et al., 2010). Studies show that 25.0% to 74.4% of HIV-infected patients have toxoplasmosis (Ayi, et al., 2016; Pappoe et al., 2017). Less than half of these infected individuals, only 44.1%, were receiving highly active antiretroviral therapy (HAART), posing a significant risk of acquiring opportunistic infections such as *Toxoplasma gondii* (Reynolds, 2020). These surveys indicate a high prevalence of *Toxoplasma gondii* infections among a cross-section of the Ghanaian population. Most of the findings focus on Southern Ghana, and there is a lack of published data on the Northern regions of the country. It is important to note that data may not be transferable from one location to another due to differences in epidemiological and cultural settings (Reynolds, 2020).

In Ghana, while there is documented evidence of congenital transmission of toxoplasmosis, screening during pregnancy mainly focuses on *Plasmodium*, Hepatitis B virus (HBV), and HIV (Helegbe et al., 2018). Although policies exist for screening several maternal infections to improve pregnancy and neonatal health, there is no specific policy for screening for *T. gondii*. Recently, attention has increased regarding toxoplasmosis, but a lack of evidence-based information on T. gondii infections among high-risk groups complicates the development of a national policy for its management (Ayi et al., 2009). Reports on risk factors for acquiring infection include education, place of settlement, and sanitation. Higher education is associated with a lower risk of *T. gondii* infections. Additionally, living in peri-urban and urban areas also reduces the risk compared to rural areas. (Assoah et al., 2025). The retrieval of parasites from various farm animals, soils, and irrigation water, along with fresh produce and salads available in Ghanaian markets, indicates the potential for environmental spread of *T. gondii* in Ghana (Amissah-Reynolds et al., 2020; Duedu et al., 2014). Overall, developing countries, like Ghana, struggle with sanitation, access to quality health services, and the implementation of public health policies. Addressing these issues can improve health outcomes and overall quality of life.

2.6 Treatment, control, and prevention of infection

The standard treatment for toxoplasmosis in humans involves combination therapy with drugs that target the enzymes involved in the folate synthesis pathway including dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). (Dunay et al., 2018). Regimens include pyrimethamine-sulfadiazine (pyr-sulf) and trimethoprim-sulfamethoxazole (TMP-SMX), as well as combinations of pyrimethamine with antimicrobials such as atovaquone, clarithromycin, azithromycin or clindamycin (Alday & Doggett, 2017). The synergistic activity of pyr-sulf was

experimentally demonstrated in mice infection in the 1950s (Eyles & Coleman, 1953), establishing the foundation for utilizing pyr-sulf as a primary treatment for toxoplasmosis. However, the efficacy of sulfonamides was reported in the early 1940s (Sabin & Warren, 1942). Despite the efficacy of pyrimethamine-based combination therapies, high rates of toxic side effects have been reported, requiring discontinuation or change of drug for treatment (Shammaa et al., 2021; Borkowski et al., 2016;Balaskas et al., 2012; Chirgwin et al., 2002;). Adverse events reported from treatment include hepatocellular injury, bone marrow suppression, nausea, rash, fever, and vomiting (Shammaa et al., 2021). The anti-toxoplasmic activity of spiramycin has also been demonstrated (Garin & Eyles, 1958). Since it is not toxic, it is administered as a prophylactic drug to prevent *T. gondii* from crossing the placental barrier during pregnancy (Goldstein et al., 2008). However, it is less effective when used to treat an established infection (Nguyen & Stadtsbaeder, 1983; Coradello & Kretschmer, 1978). The present challenge in treating toxoplasmosis is the availability of effective yet non-toxic alternatives. An ideal medication for toxoplasmosis should effectively target the bloodstream, brain, and eyes, tackling both the rapid tachyzoite phase and the dormant bradyzoite phase of the organism. Additionally, new drugs should aim for fewer and milder side effects, improving upon the current first-line treatments (Alday & Doggett, 2017).

Control and preventive measures primarily involve educating the public on the risk of toxoplasmosis. The effectiveness of public education is, however, questionable given the issue of people's understanding of the risks and compliance with preventive measures (Andiappan et al., 2014; Chandrasena et al., 2016; Elsafi et al., 2015; Gollub et al., 2008; Millar et al., 2014; Pereboom et al., 2013; Smereka et al., 2018; Velázquez-Hernández et al., 2019). Regardless, measures such as thoroughly washing fruits and vegetables before eating and avoiding raw or undercooked meats, are important for controlling transmission (Shapiro et al., 2019). Daily

disposal of cat feces is recommended by the Center for Disease Control (CDC) since oocysts in the environment take about two days to sporulate. To avoid contaminating oneself after handling and disposing of feces, wearing gloves and handwashing is encouraged. Pregnant women who have not been previously exposed to *T. gondii* infection and other immunocompromised individuals should avoid changing the cat litter (Smith et al., 2021). Reinforcing prophylactic administration for transplant patients who are seronegative for *T. gondii* (Derouin et al., 2008) and in HIV-positive *T. gondii*-positive patients (Miro et al., 2006) can minimize the risk of opportunistic and reactivation of latent infection respectively.

Systemic efforts to control the infection during pregnancy involve implementing serological screening for anti-*T. gondii* antibodies during monthly antenatal visits (Goldstein et al., 2008). Countries such as France have had great success in reducing the burden of congenital toxoplasmosis by this approach (Peyron et al., 2017). To minimize food contamination by oocysts, systematic approaches like reducing cats and rodent access on farmlands and inactivating tissue cysts in meat products by irradiation or other processing methods have been suggested (Kijlstra & Jongert, 2008; Opsteegh et al., 2015). Also, routine methods used in water treatment like chlorination are not enough to kill *T. gondii* oocyst since it is resistant to most strong chemicals, and detergents (Krueger et al., 2014; Wainwright et al., 2007). Implementing filtration systems in the treatment of water is strongly encouraged (Opsteegh et al., 2015; Krueger et al., 2014; Villena et al., 2004). The only commercially available vaccine in use against *T. gondii* infection is for the prevention of abortions in sheep and is not safe for use in humans (Innes et al., 2019). Despite the efforts made so far in developing a vaccine for human toxoplasmosis, none of the candidates have been approved for clinical trials in humans (Smith et al., 2021).

2.5 Host cell invasion

The ability of *T. gondii* to invade virtually all nucleated cells (Sibley et al., 2007) is remarkable. The process of cellular invasion is active and is facilitated by the successive release of proteins from the secretory organelles. MICs are the first to be released into the parasite's plasma membrane followed by the secretion of RONs that integrate into the host cell plasma membrane (Rastogi et al., 2019). Adhesin molecules secreted from micronemes such as MIC2-M2AP, MIC4-1-6, and MIC3-8 complexes enhance parasite adherence to the host cell which is an important first step (Gras et al., 2017; Wang & Yin, 2015 Meissner, Reiss, et al., 2002; Rabenau et al., 2001; Soldati et al., 2001). A strong association and attachment formed between the host cell plasma membrane and the apical region of *T. gondii* referred to as the moving junction (MJ). This propels the parasite forward, moving from the apex to the bottom until it is fully internalized into the cell, forming a PV (Alexander et al., 2005). The complex forming the MJ involves apical membrane antigen 1 (AMA1) which secretes to the parasite surface from the micronemes, and rhoptry neck protein, RON2 (as well as RON4, RON5, and RON8) secreted into the host cell membrane (Guérin et al., 2017; Lamarque et al., 2011; Tyler & Boothroyd, 2011; Besteiro et al., 2009). A semblance of a receptor-ligand interaction is formed between AMA1 and the extracellular segment of RON2 resulting in a robust interaction between the parasite surface and host membrane (Lamarque et al., 2011; Tonkin et al., 2011; Tyler & Boothroyd, 2011).

The invasion mechanism also involves a motor complex known as the glideosome. Myosin heavy chain A (MyoA), myosin light chain (TgMLC1), and three gliding-associated proteins (TgGAP45, TgGAP50, and TgGAP40) make up this complex of actin-myosin motors (Frénal et al., 2010; Herm-Götz et al., 2002; Meissner, Schlüter, et al., 2002). Aldolase was initially thought to mediate the interaction between the glideosome and the MJ (Jewett & Sibley, 2003). However, the

glideosome-associated connector (GAC) was discovered to be the linker between the adhesin molecules on the surface of the cell and the parasite's actin (Jacot et al., 2016). Enough power is generated from the activities of the glideosome and MJ that thrust the parasite into the host cell, dissociating it from the host cell plasma membrane (Zhu et al., 2019). A PV is formed from the modification of the host membrane cytoskeleton and the release of ROPs and GRAs (Clough & Frickel, 2017). These changes to the membrane make the PV non-fusogenic with host lysosomal and endosomal components establishing a replicative niche and ensuring parasite survival (Pavlou et al., 2018; Mordue & Sibley, 1997).

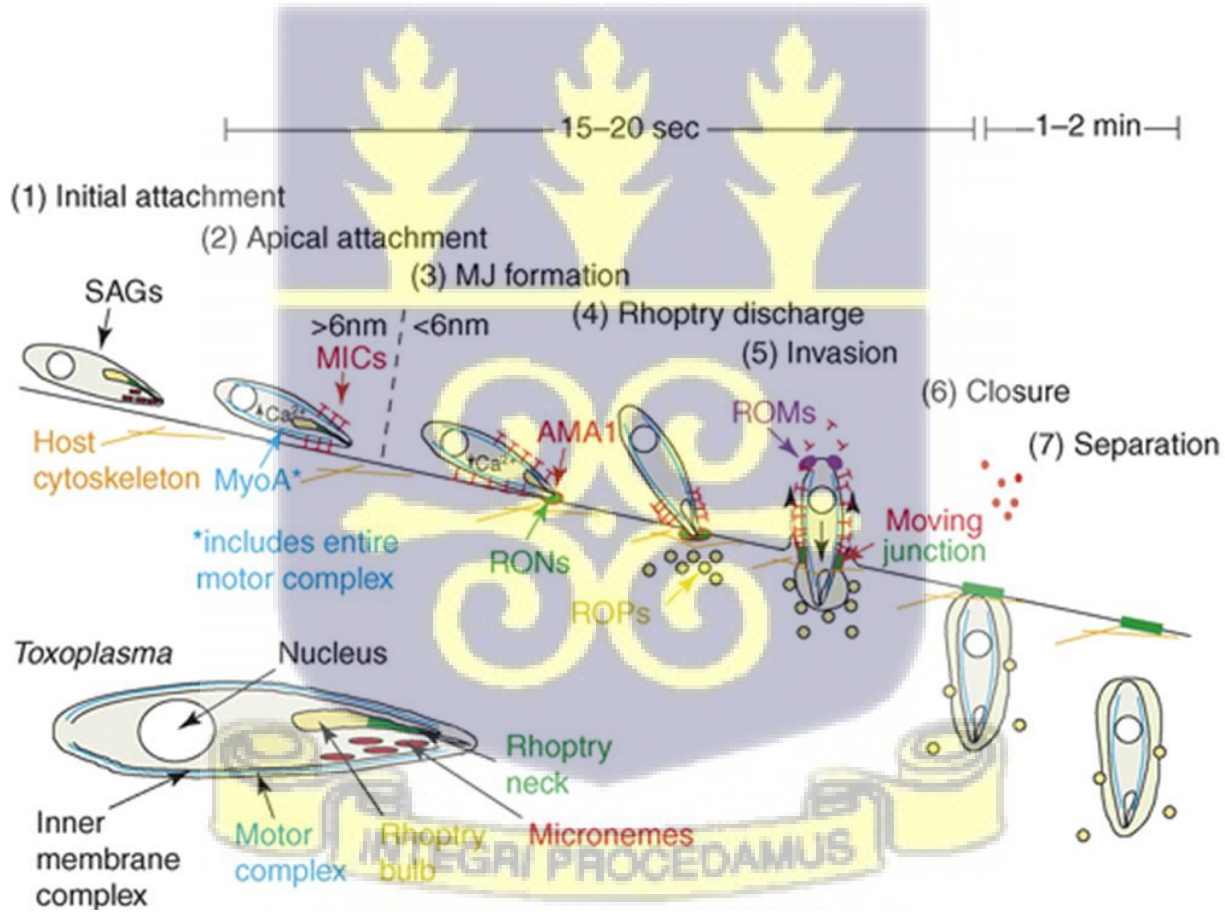


Figure 2.3: An illustration of the mechanism of host cell invasion by *T. gondii* (Carruthers & Boothroyd, 2007)

2.6 Genotype and virulence

Toxoplasma gondii strains in Europe and North America consist of three distinct clonal lineages, that is type I, II, and III (Howe and Sibley, 1995) with ~1% or less genetic diversity (Su et al., 2003). Strains in South America, however, diverge from this clonal population structure and are more genetically diverse, making them mostly atypical (Sibley & Ajioka, 2008). Genotyping studies conducted in Ghana also identified clonal types I, II, and a combination of both (types I and II) in human subjects. Notably, 93.8% of the positive samples corresponded to clonal type II (Ayi, Kwofie, et al., 2016), which correlates with other findings in Europe and America. Animal studies also led to the identification of unique genotypes of *T. gondii* in Ghana. An earlier study identified TgCkGh1 and TgCkGh2 in chickens (Dubey et al., 2008). In contrast, another independent study conducted in Kumasi years later identified a different genotype, TgCtGh1, in cats, which had not been previously reported (Pappoe et al., 2017). These suggest the possibility of recombination of strains in the felid population in Ghana. However, more data is needed to understand the prevailing genotypic landscape of *T. gondii* in Ghana.

Parasite strain virulence has been experimentally defined using a mouse model after intraperitoneal injection with tachyzoites (Dubremetz & Lebrun, 2012). Type I strains are highly virulent due to their ability to cause the death of mice with a lethal dose $(LD)_{100} = 1$ after a week of infection. Type II and type III strains, however, have an LD_{50} of 10^3 and 10^5 tachyzoites hence, are generally considered less virulent (Sanchez & Besteiro, 2021; Sibley & Boothroyd, 1992). In

the US, type II strains are responsible for most human toxoplasmosis in immunocompromised individuals, regardless of severity (Dardé, 2004). However, reports of virulent type I and atypical strains have been documented and implicated in severe ocular disease in otherwise healthy individuals (Grigg et al., 2001). Severe congenital toxoplasmosis caused by type I was reported in Europe (Fuentes et al., 2001). These findings present a correlation between *T. gondii* genotype and virulence.

2.7 Host Immunity to *T. gondii*

The innate immune system is the initial responder to *T. gondii* infections, producing interleukin (IL)-12 from dendritic cells (DCs), neutrophils, and monocytes (Tosh et al., 2016; Aldebert et al., 2007; Bliss et al., 1999). In mice, profilin secreted by *T. gondii* is sensed by toll-like receptors (TLRs)-11 and 12, leading to activation of myeloid differentiation primary-response protein 88 (MYD88) and IL-12 production downstream (Koblansky et al., 2013; Yarovinsky et al., 2005). This pathway plays a crucial role in controlling infections, as demonstrated by the vulnerability of TLR adaptor protein MYD88 knockout mice to *T. gondii* and decreased IL-12 and IFNγ production (Scanga et al., 2002). Human cells lack these TLRs and immune cells such as monocytes sense infection when alarmin S100A11 is released by nearby infected cells leading to chemokine production (Safronova et al., 2019). *T. gondii* recognition in the cytosol can also be a result of inflammasome activation, leading to the production of IL-1β and eventually cell death, eliminating the parasite replicative niche (Gov et al., 2017; Witola et al., 2011).

The production of cytokines in inflamed tissue stimulates natural killer (NK) cells and T-cells to produce interferon-gamma (IFNγ) (Wilson et al., 2008; Gazzinelli et al., 1993, 1994; C. A. Hunter et al., 1994). This response results in a strong adaptive Th1 immune response that effectively controls *T. gondii* infection (Däubener et al., 1995; Suzuki et al., 1988). IFNγ signaling is facilitated by the transcription factor known as signal transducer and activator of transcription 1 (STAT1), which enhances the expression of numerous genes collectively referred to as IFN-stimulated genes (ISGs). STAT1 has subsequently been described as a vital regulator of antimicrobial effectors in toxoplasmosis control (Gavrilescu et al., 2004; Lieberman et al., 2004). The stimulation of IFNγ leads to limited growth of parasites through the activation of inducible nitric oxide synthase (iNOS) (Bando et al. 2018; Li et al. 2012; Scharton-Kersten et al. 1997), the generation of reactive oxygen species (Murray et al. 1983), and the limitation of nutrients like tryptophan (Pfefferkorn 1984) and iron (Dimier and Bout 1997). Upregulation of specific pathways targeting the parasitophorous vacuole membrane (PVM), such as the recruitment of immunity-related GTPases (IRGs) (Zhao et al., 2009), guanylate-binding proteins (GBPs) (Kravets et al., 2016; Selleck et al., 2013; Yamamoto et al., 2012), ubiquitination, and autophagy molecules (Choi et al., 2014; Hernandez et al., 2022; Matta et al., 2023; Ohshima et al., 2014; Wang et al., 2024), leads to the vacuole disruption and death of the parasite.

Additional cytokines like tumor necrosis factor α (TNFα) and IL-6 have been associated with controlling chronic *T. gondii* infections in the brain (Chao et al., 1994). A partial compensatory role of TNFα in individuals with IFNγ receptor 1 deficiency has been also described to control *T. gondii* infection in these patients (Janssen et al., 2002). The autophagic disruption of the parasitophorous vacuole (PV) through Cluster of Differentiation 40 (CD40) is recognized as a

pathway that operates independently of IFNγ (Andrade et al., 2006). Altogether, numerous immune mechanisms that control *T. gondii* have been described in different cell types and host species.

2.8 *Toxoplasma gondii* effectors involved in virulence and host modulation

Organelles such as the ROPs and GRAs release effector molecules that are usually agonistic to host immune mechanisms (Panas & Boothroyd, 2021; Rommereim et al., 2019). These enable their survival in different cells and hosts despite the present immune mechanisms. For instance, in murine macrophages, ROP5, ROP18 & ROP17 (Behnke et al., 2012; Etheridge et al., 2014; Reese et al., 2011) complexes have been shown to phosphorylate IRGs, leading to their failure to accumulate at PVM. A PVM-associating dense granule protein, GRA7, can further interact with these ROP complexes and facilitate the turnover of IRGs (Alaganan et al., 2014). STAT1 activation and nuclear translocation are vital for the transcription of ISGs. However, if infection occurs prior to IFNγ stimulation, the parasite deploys a GRA protein, known as *T. gondii* inhibitor of STAT transcription (*Tg*IST) that can inhibit this signaling pathway (Gay et al., 2016). *TgIST* is released beyond the PVM into the host nucleus, where it strongly interacts with activated STAT1 and proteins that modify the chromatin, such as those present in the nucleosome-remodeling and deacetylase (NuRD) complex (Olias et al., 2016). When parasite-infected cells are stimulated by IFNγ, STAT1 moves to the nucleus as expected, but remains inactive because it is secluded with the NuRD complex by *Tg*IST (Gay et al., 2016; Olias et al., 2016), hence transcription of ISGs is impeded. Also, different *T. gondii* strains have polymorphic effectors used to modulate the host cell environment. For example, the type I and III alleles of rhoptry kinase, ROP16, efficiently phosphorylate STAT3 and STAT6. This leads to the production of anti-inflammatory cytokines

such as IL-4 and the downregulation of IL-12 while the type II allele of ROP16 is unable to elicit the same outcome (Saeij et al., 2007; Yamamoto et al., 2009).

Other effectors seem to be beneficial to host survival. Though this seems counterintuitive, *T. gondii,* being an obligatory intracellular parasite, requires its host to survive and has evolved mechanisms to ensure it. GRA15 (Gov et al., 2013), GRA6 (Ma et al., 2014), and GRA24 (Braun et al., 2013) modulate independent pathways that lead to the production of chemokines and cytokines and promote a proinflammatory host environment. GRA15 and GRA6 are restricted to the vacuole and are not exported beyond the PVM yet can modulate host transcription pathways (Hakimi et al., 2017). Type II GRA15 has been demonstrated to facilitate the activation and movement of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) into the nucleus, which results in the secretion of proinflammatory cytokines (Rosowski et al., 2011) and promotes M1 polarization of macrophages (Jensen et al., 2011). GRA6 also activates another host transcription factor, the nuclear factor of activated T cells 4 (NFAT4), which leads to chemokine production and recruitment of inflammatory cells to the infection site (Ma et al., 2014).

GRA16 and GRA24 are exported beyond the PVM to the host nucleus where they interact with nuclear proteins to modulate host transcription pathways (Hakimi et al., 2017). GRA16 can translocate to the host nucleus and beneficially modulate pathways involved in host cell survival (Bougdour et al., 2013). It is trafficked to the host nucleus with a complex of host phosphatase PP2A-B55 and the herpesvirus-associated ubiquitin-specific protease (HAUSP). In concert with HAUSP, GRA16 regulates and maintains steady levels of tumor suppressor p53 thereby promoting cell survival (Bougdour et al., 2013). GRA24, like other dense granules mentioned above, provokes a strong inflammatory response with the production of chemokines and cytokines and enhanced activity of phagocytic cells, clearing the infection. It induces persistent phosphorylation

of p38α in a non-canonical pathway, independent of mitogen-activated protein kinase (MAPK) (Braun et al., 2013). Overall, *T. gondii* is unique in its ability to infect a variety of hosts and the ability to counteract host mechanisms could be why it is such a successful pathogen (Zhu et al., 2019).

2.9 Genetic manipulation of *T. gondii*

Unlike other Apicomplexan parasites, *T. gondii* is experimentally tractable and easy to culture in nucleated cells, which makes it agreeable to genetically manipulate (Pfefferkorn & Wyler, 1990) and explore gene functions that may be involved in parasite virulence, survival, or host immune modulation. Forward and reverse genetic approaches have been employed to study the gene function in *T. gondii* (Weilhammer & Rasley, 2011). Forward genetic approaches, including genetic crosses, chemical mutagenesis, and random insertional mutagenesis, have been useful in associating specific genes with a phenotype (Wang et al., 2016). For instance, genetic crosses between combinations of type I, II, and III and quantitative trait locus (QTL) analysis led to the discovery of ROP18 and ROP5 responsible for differences in strain virulence (Behnke et al., 2011; Reese et al., 2011; Saeij et al., 2006; Taylor et al., 2006). And insertional mutagenesis enabled identification of the localization of ROP4 to the rhoptries (Bradley et al., 2004). Despite the significant contributions these techniques made, the limitations of intractable, tedious, and laborious methods, as well as the lack of specificity, led to the exploration of alternatives (Wang et al., 2016).

Reverse genetics explores the phenotypic outcome of genetic manipulation and provides a way to study essential genes in an organism. Currently, one of the most widely used genome-editing

techniques in organisms, including *T. gondii,* is the CRISPR/Cas9 system due to its specificity and ease of use. The system uses a single guide RNA (sgRNA) molecule to direct an endonuclease (Cas9) to induce a double-strand break (DBS) at a specific genomic locus. After DBS is introduced, it can be repaired by inserting a foreign DNA sequence of choice by homology-directed repair (HDR) or nonhomologous end-joining (NHEJ) (Haber, 2000). In *T. gondii,* the latter is favored (Fox et al., 2009; Donald & Roos, 1994) and HDR only occurs effectively when Ku80, a subunit of the DNA repair protein, is knocked out (Fox et al., 2009). With the advantages of being scalable (on a genome-wide scale), efficient, specific, and less expensive, CRISPR has proved to be very useful in studying parasite biology (Bryant et al., 2019).

The versatility of CRISPR/Cas9 enables its use on a whole-genome scale. The first genome-wide loss-of-function CRISPR screen in *T. gondii* employed a pooled approach where sgRNA libraries targeting most of the coding sequences in the genome were transfected into Cas9-expressing parasites. This creates a diverse mixture of genetic modifications. Hit gene candidates are then identified by applying selection pressure and comparing the results to control pools (Sidik et al., 2016). The screen identified many fitness-conferring genes, particularly claudin-like Apicomplexan microneme (clamp) protein, validated to be important for parasite invasion. Since then, other research groups have used CRISPR screens *in vitro* to identify genes that contribute to parasite survival, such as GRA45 (Wang et al., 2020), GRA57, GRA70, and GRA71 (Krishnamurthy et al., 2023) in IFNγ-primed host cells. Other genes linked with drug resistance and susceptibility were identified by this method (Harding et al., 2020). *In vivo* screens in mice using a pool of mutant *T. gondii* also facilitated the identification of a protein, *Toxoplasma gondii* WAVE-complex-interacting protein (TgWIP) that induces a hypermotility phenotype of infected cells, allowing dissemination of the parasites to distant organs (Sangaré et al., 2019). Another

study targeted *T. gondii*-secreted proteins that promote immune evasion, elucidated the novel role of a previously characterized rhoptry protein ROP1 (Butterworth et al., 2022). The results highlight the significance of CRISPR screens in exploring the biology of parasites and their interactions with hosts. However, one drawback of screening methods is that numerous genes are critical for growth, which may lead to them not being directly included in the screen or having a specific function assigned (Hesping & Boddey, 2024). To address these limitations, a conditional CRISPR/Cas knockout screening method was recently introduced (Li et al., 2022).

In summary, the interplay between host immune mechanisms to suppress or eliminate the parasite and the various pathways of evasion by *T. gondii* is complex and fascinating. The research on host-parasite interactions provides a basis for understanding the natural mechanisms of host resistance and for identifying the pathogen effectors that interact with them. Molecular tools such as CRISPR/Cas9 editing provide scientists with effective machinery to manipulate parasite and host genes and decipher their functions and relevance. As new host-pathogen pathways are discovered, more host-directed therapeutic approaches and novel pathogen targets can be tested and validated for drug and vaccine development. That makes these exploratory studies necessary for the future of basic and translational sciences of successful pathogens such as *T. gondii* and for infectious diseases as a whole.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study site and design

This study employed an exploratory design as part of a broader investigation into the factors influencing *Toxoplasma gondii* parasite fitness in bone marrow-derived macrophages (BMDMs) from mice and rats activated by IFNγ. Following previously established protocols, plasmids were constructed to target and disrupt selected candidate genes. The phenotypes of the resulting genetic mutations were evaluated *in vitro* and *in vivo* to validate the genes' importance to *T. gondii.* Finally, the localizations of candidate genes were determined by endogenously tagging to help further elucidate their functions. The site of the study was the Wang lab at the Department of Microbiology and Immunology at the University of Michigan for a duration of 10 months (January 2024 to October 2024).

3.2 Laboratory animals

Female C57BL/6J mice, aged six to eight weeks (Stock No: 000664), were acquired from The Jackson Laboratory (ME, USA). Six to eight-week-old female Brown Norway rats (Strain Code: 091) were obtained from Charles River Laboratories (MA, USA). Both mice and rats were kept in ventilated cages with corn bedding and had unrestricted access to water and chow. All cages were kept on a single rack with a housing density of five mice per cage and three rats per cage. The mice and rats were allowed to acclimate in our vivarium for at least a week without disturbance. The animal room operated on a 12-hour light/12-hour dark cycle, maintained a temperature of 22–25 °C, and kept humidity levels between 30–70%. Veterinarians monitored the mice twice daily, they

were weighed each day, and their cage bedding was changed biweekly. Mice and rats were housed in pathogen-specific free (SPF) conditions within the animal facility at the University of Michigan. All animal studies were conducted in full compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals provided by the National Institutes of Health, as well as the Animal Welfare Act, with approval obtained from the Institutional Animal Care and Use Committee at the University of Michigan (assurance number PRO00011886).

3.3 Plasmid construction

The pU6-Universal plasmid (Addgene plasmid #52694, MA, USA) was utilized to create the plasmid that contains sgRNAs aimed at the target genes. In summary, the constructs were produced by annealing oligos with the sgRNA sequences, then inserted into the BsaI (New England Biolabs, MA, USA)-digested pU6-Universal plasmid. To create the plasmid for the generation of the HA-tagged strain of TGGT1_232670 and TGGT1_205350 endogenously, approximately 1300 bp upstream of the TGGT1_232670 and TGGT1_205350 stop codons were amplified through PCR and inserted into pLIC-3xHA DHFR via ligation-independent cloning (Huynh & Carruthers, 2009). All PCR primers and sgRNA oligos are listed in Appendix 1.

3.4 Cell and parasite culture

Human foreskin fibroblasts (HFFs) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 10 mg/mL gentamicin. Murine bone marrow-derived macrophages (BMDMs) were derived by culturing bone marrow cells from the tibia and femur of C57BL/6J

mice in DMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, 10 mg/mL gentamicin, and 20% L929 conditioned medium for 5–7 days. Rat BMDMs were produced by culturing bone marrow cells isolated from the tibia and femur of Brown Norway rats in DMEM, 20% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1x non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, 10 mg/mL gentamicin, and 30% L929 conditioned medium for 5–7 days. The *T. gondii* strains *RH-Luc+/Δhxgprt* (wild-type) and *RHΔku80Δhxgprt* were regularly cultured in vitro on HFF monolayers at 37 °C in a 5% CO_2.

3.5 Isolation of *Toxoplasma* genomic DNA

Freshly lysed-out parasites were scraped from T25 flasks or 24-well plates and harvested after centrifugation at 1700 rpm for 7 minutes or 12,000 rpm for 2 minutes, respectively (Eppendorf centrifuge 5810R, 15 Amp version, Hamburg, Germany). The supernatant was aspirated, followed by the addition of 500 µl or 250 µl of DNAzol (Invitrogen, Cat#10503027, CA, USA) to lyse cells. Samples were transferred to 1.5 mL Eppendorf tubes and 500 µl or 250 µl of 100% ethanol added, respectively, mixed by inverting several times and spun at 13,000 rpm for 10 minutes (Eppendorf centrifuge 5425R, Hamburg, Germany). Supernatant aspirated and 1 mL or 500 µl of 70% ethanol was added and spun at 13,000 rpm for 1 minute. Supernatant aspirated, and this step was repeated. A final short spin to remove all ethanol and aspirated liquid before adding 100 µl or 50 µl of buffer EB (Qiagen, Cat#19086, Hilden, Germany). Samples were placed on a heating block at 60 ºC for 20 minutes to dissolve DNA.

3.6 Generation of parasite strains

3.6.1 Knockout generation of candidate genes

The candidates' single-gene knockout was achieved using CRISPR/Cas9 technology. To create the knockout strains, plasmids containing sgRNA were co-transfected into RH-Luc+/Δhxgprt parasites alongside NotI (New England Biolabs, MA, USA)-linearized pTKO (Rosowski et al., 2011), using a ratio of 5:1 for sgRNA to linearized pTKO plasmid. Two T25 flasks with confluent HFF cells were infected with 200 µl of the transfected parasites each, obtaining two knockout populations for each gene. A control group was set up by infecting HFF cells with untransfected parasites. 24 hours post-transfection, the populations were selected using 50 µg/mL of mycophenolic acid (Sigma–Aldrich, Cat#89287, MO, USA) and 50 µg/mL of xanthine (Sigma–Aldrich, Cat#X3627, MO, USA).

Following the successful detection of knockouts in transfected populations, single independent clones had to be obtained by limiting dilutions in a 96-well plate. Single GFP-positive plaques were transferred for each gene population to 24-well plates. DNA was isolated for genotyping of clones for both genes.

3.6.2 Diagnostic PCR to detect gene knockouts

To verify the disruption of a candidate gene locus, four sets of PCR were conducted to identify gene knockouts in the parasites: (1) a PCR was performed to check for an undisrupted gene locus (referred to as locus disruption check; P1+P2); a PCR to identify the insertion of the repair template in either (2) the forward direction or (3) the reverse direction (indicated by P1+P3/P2+P3 respectively); and (4) a control PCR was carried out to amplify the locus of a non-relevant gene in

the *T. gondii* genome, ensuring the presence of genomic DNA in the samples (known as DNA control PCR). Briefly, the total reaction volume of 20 µl comprised 10 µl of OneTaq Quick-load 2X master mix (New England BioLabs, Cat#M0486L, MA, USA), 1 µl of each 10 µM forward and reverse primers (refer to Appendix 1), 7 µl of nuclease-free water, and 1 µl of genomic DNA extracted from the parasite. The thermocycling process was conducted using a Bio-Rad Touch™ Thermal Cycler (C1000, CA, USA), starting with an initial denaturation at 94 °C for 30 seconds, followed by 30 cycles that included denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 68 °C for 1 minute. This was concluded with a final extension at 68 °C for 5 minutes and then held at 12 ºC indefinitely. The resulting PCR products were analyzed using a 0.8% agarose gel stained with GelRed (Biotium, Cat#41003, CA, USA) and were visualized under UV light on a Bio-Rad GelDoc Go Imaging System (CA, USA).

3.6.3 Generation of 3xHA epitope endogenously tagged parasites

TGGT1_232670 and TGGT1_205350 endogenously tagged parasites were generated from the parental RH*Δku80Δhxgprt* strain by transfection with XcmI enzyme-linearized pLIC-TGGT1_232670-3xHA-DHFR plasmid and XcmI enzyme-linearized pLIC-TGGT1_205350-3xHA-DHFR plasmid, respectively. Two T25 flasks with confluent HFF cells were infected with 200 µl of the transfected parasites each, obtaining two populations for each gene. A control group was set up by infecting HFF cells with untransfected parasites. Transfected parasite populations were selected with 3 µM pyrimethamine. The presence of TGGT1_232670-3×HA and TGGT1_205350-3×HA in the transfected population was determined by immunofluorescence assays.

3.7 Luciferase assays

Luciferase assays were conducted to evaluate the fitness of knockout strains within IFNγ-activated macrophages. For both murine and rat macrophages, bone marrow-derived macrophages (BMDMs) were plated in 96-well formats at a density of 1×10^5 cells per well and treated with 50 U/mL or 100 U/mL of murine IFNγ (Gibco, Cat#31505100UG, MA, USA) and 25 U/mL or 50 U/mL of rat IFNγ (Gibco, Cat#40020100UG, MA, USA) for 24 hours, followed by infection with luciferase-expressing wild-type or knockout parasites at two different Multiplicity of Infection (MOIs) values of 0.5 and 1. A plaque assay was set up in HFF monolayers to evaluate the viability of different parasite strains at the time of infection. After a 24-hour infection period, the cells in the 96-well plates were lysed using a homemade lysis buffer (Mukhopadhyay and Saeij 2020), followed by the addition of luciferin solution to assess luciferase activity with a Synergy H1 microplate reader (BioTek, Vermont, USA). The raw luciferase readings (RLU) from unstimulated infected cells were regarded as 100 percent, and the relative growth of parasites in IFNγ-activated macrophages was calculated. To compare wild-type and knockout parasites, the "real" MOI was calculated with the plaque assay results.

3.8 Plaque assay to determine knockout growth phenotype in HFF

Freshly confluent 24-well plates containing HFF cells were infected with 100 parasites per well, and the plates were allowed to incubate undisturbed at 37 °C for 5 days. The total number of plaques was examined using a Nikon Eclipse Ti2 microscope (Tokyo, Japan). To assess the growth of the parasites in HFFs, the area of each individual plaque was photographed and analyzed using the aforementioned microscope, which was equipped with a Hamamatsu ORCA-Fusion digital

camera (Shizuoka, Japan) and NIS Elements Imaging Software (Tokyo, Japan). For each separate experiment, a minimum of 40 plaques from each parasite strain were captured and evaluated.

3.9 *In vivo* infection

At the beginning of the experiment, four mice were randomly placed into different experimental groups. *T. gondii* tachyzoites were collected from cell cultures and released using two 27-gauge needles, followed by a 30-gauge needle. C57BL/6J mice were infected intraperitoneally (i.p.) with 100 tachyzoites from each strain, and the viability of the parasites in the inoculum was assessed through a plaque assay post-infection. The mice were observed for a duration of 9 days after infection, and the daily count of deceased mice in each group was recorded.

3.10 Immunofluorescence assays

24-well plates with confluent HFF cells on coverslips were infected with parasites at MOI = 1 for 24 hours. Briefly, media was aspirated, and the cells were washed with 1 ml 1×PBS twice, followed by the addition of 300 µl of 3% formaldehyde to fix the cells for 20 minutes at room temperature. Formaldehyde was aspirated, and the cells were washed with 1 mL 1×PBS twice. The cells were incubated with 300 µl of blocking buffer (PBS/3%BSA/5% Goat Serum/ 0.2% Triton/0.01% Sodium Azide) at room temperature for 1 h. Blocking buffer was aspirated, and 200 µl of primary antibodies diluted in blocking buffer (Rat anti-HA (Sigma Cat#11867431001, MO, USA)) - 1:500 dilution, Goat anti-Toxo Biotin (Thermo Scientific Cat#PA1-73002, MA, USA) - 1:2000 dilution) were added to each well and incubated at 4 ºC overnight. Primary antibodies were retrieved, and the cells were washed with 1ml 1×PBS three times. The cells were then incubated with 200 µl of

secondary antibodies diluted in blocking buffer (Goat anti-Rat IgG Alexa Fluor 594 (Thermo Scientific Cat#A48264, MA, USA) - 1:2000 dilution, Streptavidin Alexa Fluor 488 (Thermo Scientific Cat#S32354, MA, USA) - 1:2000 dilution, DAPI - 1:2000 dilution) for 1 h at room temperature, avoiding light. Secondary antibodies were aspirated, and the cells were washed with 1 ml 1×PBS five times before mounting each coverslip on slides with 5 µl Mowiol mounting medium. Microscopic images were taken using a Nikon Eclipse T*i*2 microscope equipped with Hamamatsu ORCA-Fusion digital camera (Hamamatsu Photonics, Shizuoka, Japan) and NIS Elements Imaging Software (Tokyo, Japan).

3.11 Statistics and Data Analysis

Statistical analysis and comparisons of data sets were performed utilizing GraphPad Prism version 10.3.1(San Diego, CA, USA). All data are expressed as mean ± standard error of the mean (SEM), with the precise n values provided in the figure legends. A significance level of $p < 0.05$ was applied for all calculations. For data involving more than two groups for a single variable, a One-way ANOVA accompanied by Tukey's multiple comparisons test was employed. Survival analyses were carried out using the Log-rank (Mantel-Cox) test. Inhibitory Concentrations (IC) were calculated by subtracting the luminescence readings of IFNγ-activated wells from "No IFNγ" wells (baseline- little to no restriction expected) for each MOI and IFNγ concentration, respectively. The concentrations at which 30-50% growth restriction was observed (compared to baseline at 100%) were used for downstream experiments.

CHAPTER FOUR

4.0 RESULTS

4.1 A loss-of-function genome-wide CRISPR screen identifies fitness-conferring *Toxoplasma* genes in IFNγ-activated macrophages

Wang et al. (2020) used a genome-wide loss-of-function CRISPR/Cas9 screen in the type I RH strain to identify T. gondii genes that impact parasite fitness in IFNγ-activated BMDMs. Transfecting Cas9-expressing RH parasites with a library of linearized sgRNAs that targeted 8,156 *T. gondii* genes allowed them to produce a *T. gondii* mutant pool. They cultured the remaining mutants in naïve and IFNγ-activated murine and rat BMDMs for three passages after passing the mutants in HFFs to eliminate those with fitness problems (Figure 4.1). Illumina sequencing was used to determine the relative abundance of sgRNAs, and the input library was compared to the parasite DNA from naïve and IFNγ-activated BMDMs to obtain the "fitness" score for each gene. They determined parasite genes that selectively improve fitness in IFNγ-activated murine and/or rat BMDMs by deducting the naïve fitness score from the IFNγ score for each gene.

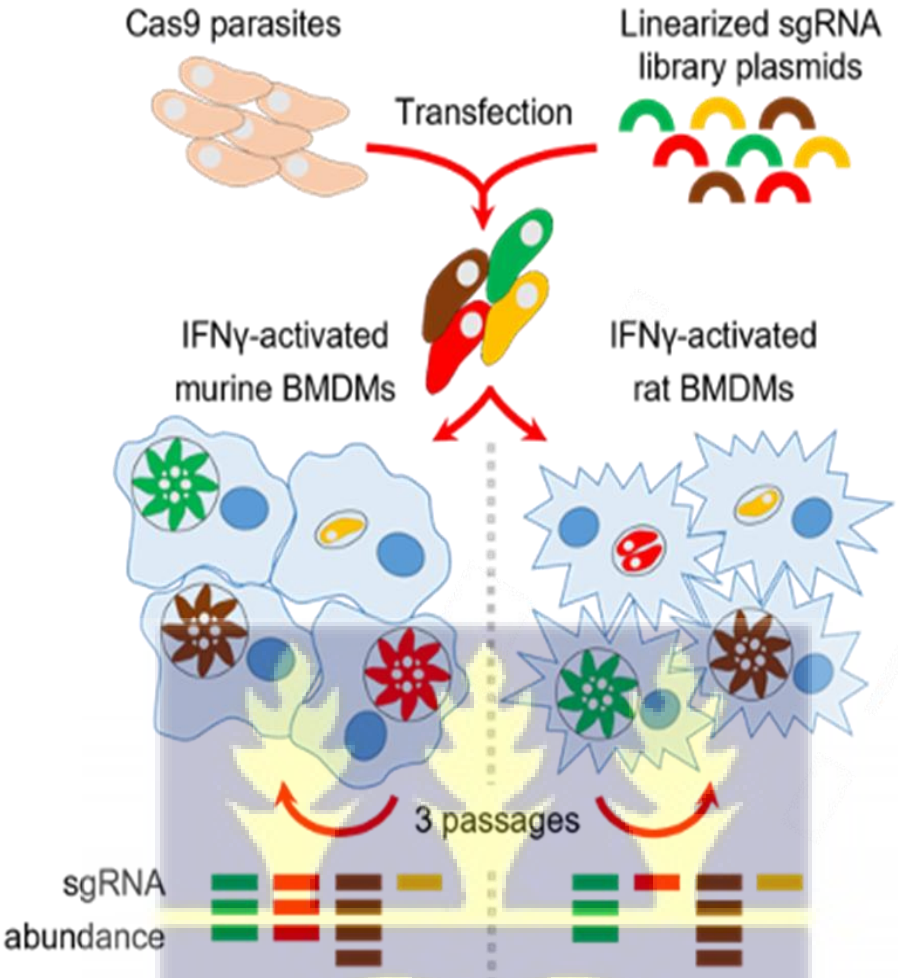


Figure 4.1: Schematic illustration of loss-of-function CRISPR screen in IFNγ-activated BMDMs

4.2 Candidate genes from CRISPR/Cas9 screen in IFNγ-activated macrophages

To further investigate the genes from the screen, I chose two *T. gondii* genes for validation, specifically *TGGT1_232670* and *TGGT1_205350*. The genes were selected because they had significantly decreased fitness scores in IFNγ-activated BMDMs isolated from mice and rats, indicating that they may be important for the parasite survival in different host species. Since a

loss-of-function approach was used here, if a gene may be important in the condition tested, a depletion of sgRNA abundance is expected after comparing the input sgRNA library to the final DNA isolated from the parasite after rounds of passaging in BMDMs. The mean log2 fold change in sgRNA abundance is calculated for a specific gene to obtain fitness scores. If the gene is fitness-conferring, a significant negative score is obtained. A summary of their description, localization, and fitness scores is shown in (Table 1) below.

Table 4.1: *Toxoplasma gondii* genes that determine fitness in IFNγ-activated murine and rat BMDMs

Gene ID	Description	Localization	IFNγ murine BMDM fitness	IFNγ rat BMDM fitness
TGGT1_232670	Hypothetical protein	Unknown	-3.3	-2.14
TGGT1_205350	Putative integral membrane protein GNS1/SUR4 family	Unknown	-3.25	-2.74

4.3 Detection of gene knockouts by PCR

4.3.1 Schematic of CRISPR knockout strategy

The schematic diagram (Figure 4.2) illustrates the genomic loci of the genes of interest (GOI) at the top, alongside the CRISPR/Cas9 targeting site indicated by a red box. In the middle, the linearized pTKO plasmid, which contains a GFP-coding sequence and an HXGPRT (HPT) selection cassette, is depicted. This plasmid serves as the repair template for disrupting GOI, as illustrated at the bottom, following selection with Mycophenolic acid and Xanthine. Primers P1 + P2 are employed to confirm the disruption of the locus, while combinations of P1 + P3 and P2 + P3 are used to verify the insertion of the repair template into the GOI locus in the forward or reverse orientation, respectively (Yifan Wang et al., 2020).

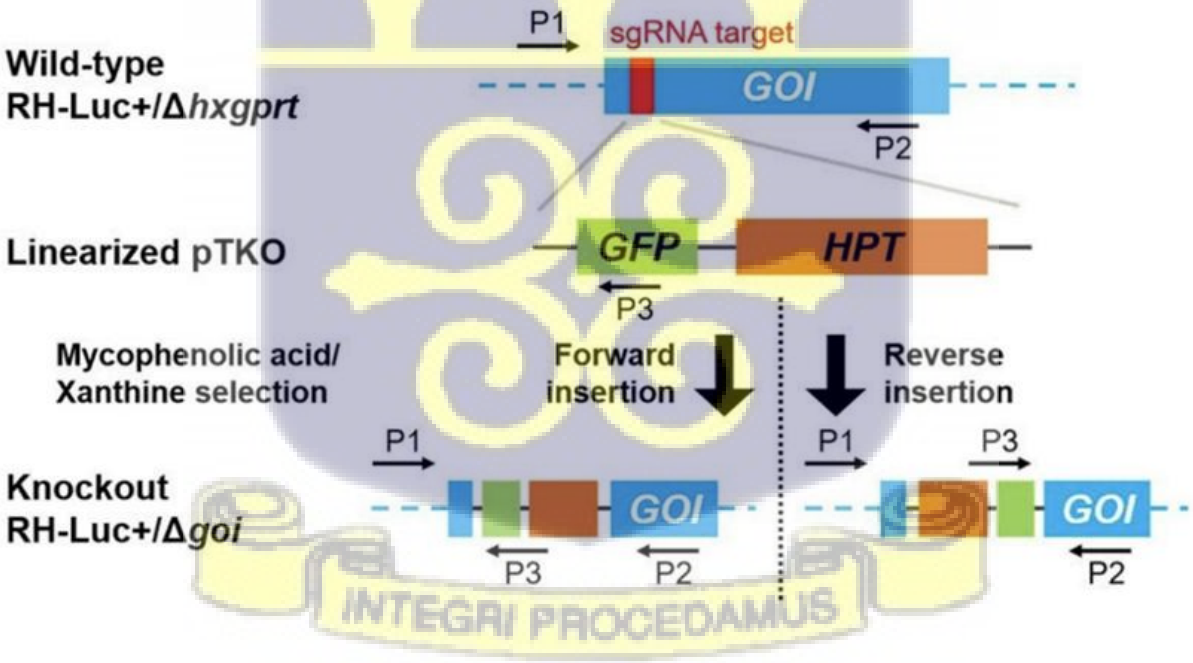
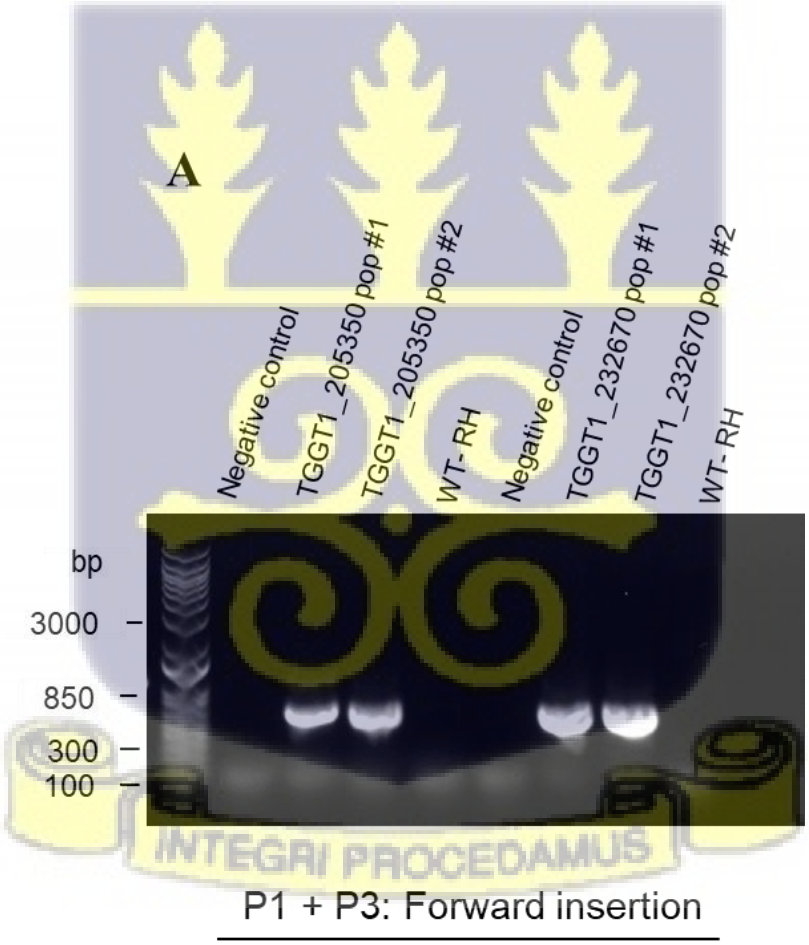


Figure 4.2: Diagram illustrating knockout strategy and primer sets used for genotyping PCR

4.3.2 Detection of positive knockout parasites in transfected populations

Each gene was transfected with the repair template and two sgRNAs designed to target ~100 bp from the start codon to ensure gene disruption. After transfected populations emerged from Mycophenolic acid /xanthine selection, genomic DNA was isolated to detect knockout parasites in the population. Primers to detect the insertion of the repair template in the forward and reverse directions were used to screen the positive knockout parasites (Appendix 1). The populations 1 and 2 of both *TGGT1_205350* and *TGGT1_232670* knockouts were positive for forward insertion (Figure 4.3A). Both populations of *TGGT1_205350* knockout were positive for reverse insertion, while only population 2 of *TGGT1_232670* knockout was positive for reverse insertion (Figure 4.3B).



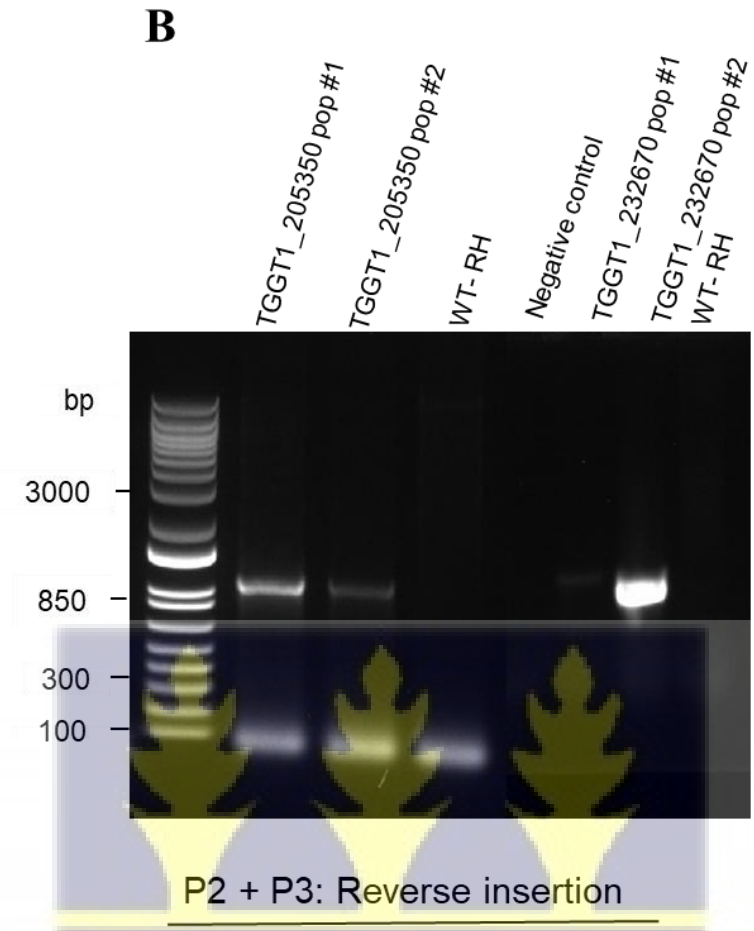


Figure 4.3: Detection of TGGT1_205350 and TGGT1_232670 knockouts in the transfection populations. (A) The PCR detected forward insertion and (B) reverse insertion of linearized pTKO plasmid using the indicated primers. Negative control: nuclease-free water was added instead of genomic DNA

4.4 Genotyping PCR of independent clones for gene knockouts

Following the successful detection of knockout parasites in transfected populations, single clones were isolated by limiting dilutions in a 96-well plate and confirmed by genotyping PCR. For *TGGT1_232670* knockout, clones 7, 8 & 9 from population 1 and clone 4 from population 2 were

confirmed by final genotyping to be true knockouts (Figure 4.4). To test the phenotype of *ΔTGGT1_232670* parasites, two independent clones were selected, they are clone 8 from population 1 (Figure 4.4 A), which had a forward insertion of the repair template, and clone 4 from population 2 (Figure 4.4 B), which had a reverse insertion.

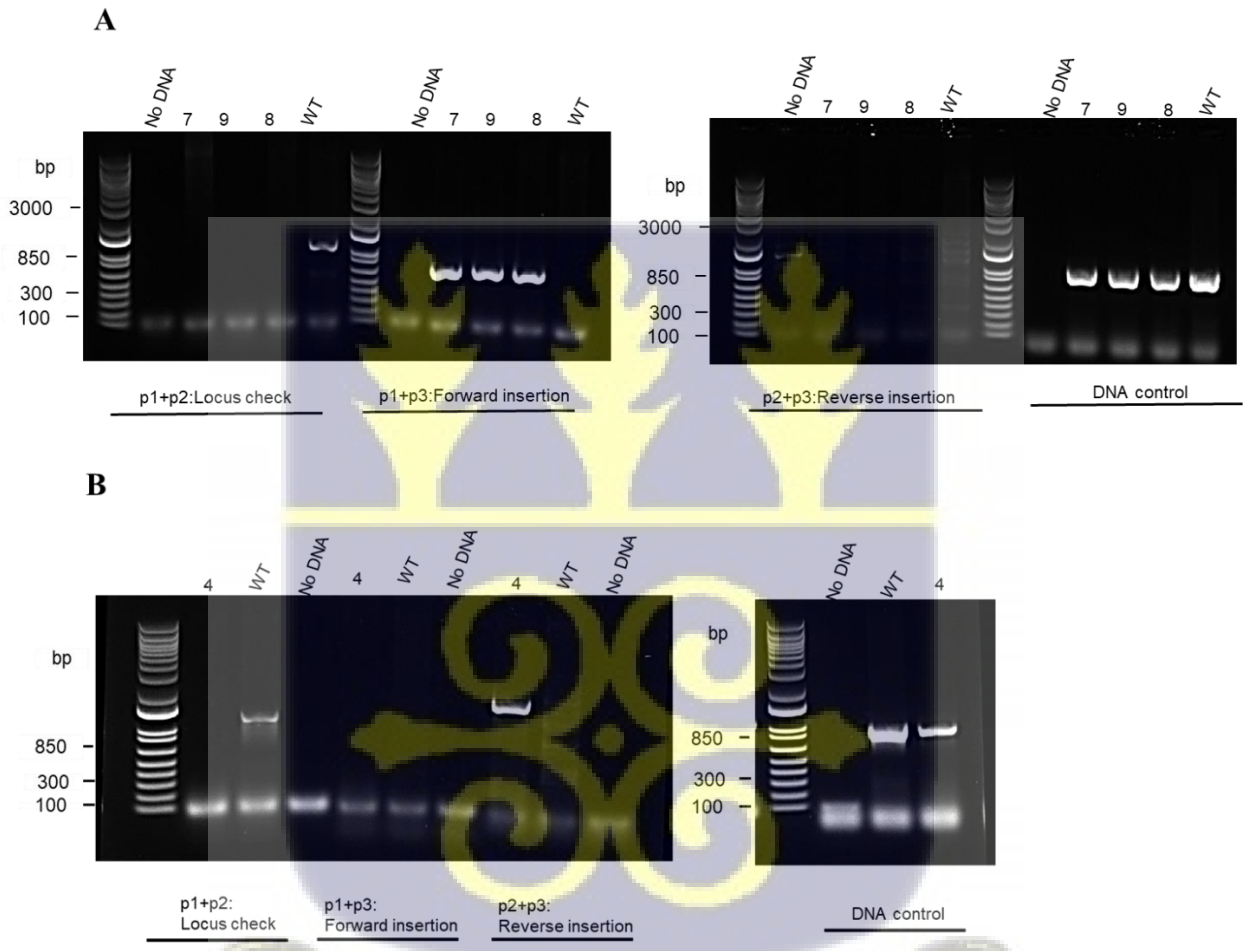


Figure 4.4: Final selection of TGGT1_232670 independent clones with (**A**) forward insertion (**B**) and reverse insertion of the repair template. DNA control PCR amplified *TGGT1_246178* gene using primers TGGTI_246178_Mut-F & TGGTI_246178_Mut-R (Appendix 1) to verify that each sample contains genomic DNA from *T. gondii*.

For *TGGT1_2205350* knockout, **c**lone 6 from population 1 and clones 4 & 11 from population 2 were confirmed by final genotyping PCR to be the true knockouts (Figure 4.5). Three clones were selected to be the independent clone to test their phenotype. They are clone 6 from population 1 and clone 4 from population 2, which both contain forward insertions of the repair template, and clone 11 from population 2, which had a reverse insertion.

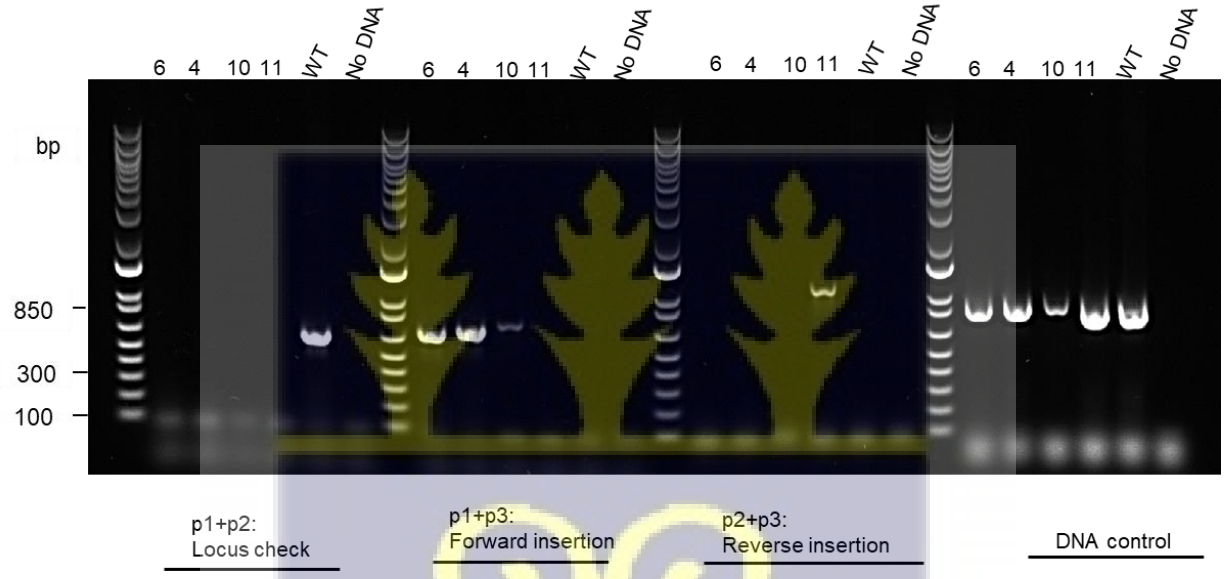


Figure 4.5: Final genotyping of TGGT1_205350 independent clones. DNA control PCR amplified *TGGT1_246178* gene using primers TGGTI_246178_Mut-F & TGGTI_246178_Mut-R (Appendix 1) to verify that each sample contains genomic DNA from *T. gondii*.

4.5 Plaque area quantification to determine fitness in HFF cells

To ensure that individual gene knockouts did not have general fitness defects in parasite intracellular proliferation, plaque assays were set up in HFF cells since this is the cell line used for

the routine culture of *T. gondii*. After measuring the plaque sizes compared to wild-type (WT) parasites, all clones were seen to have no fitness defect (Figure 4.6A and B).

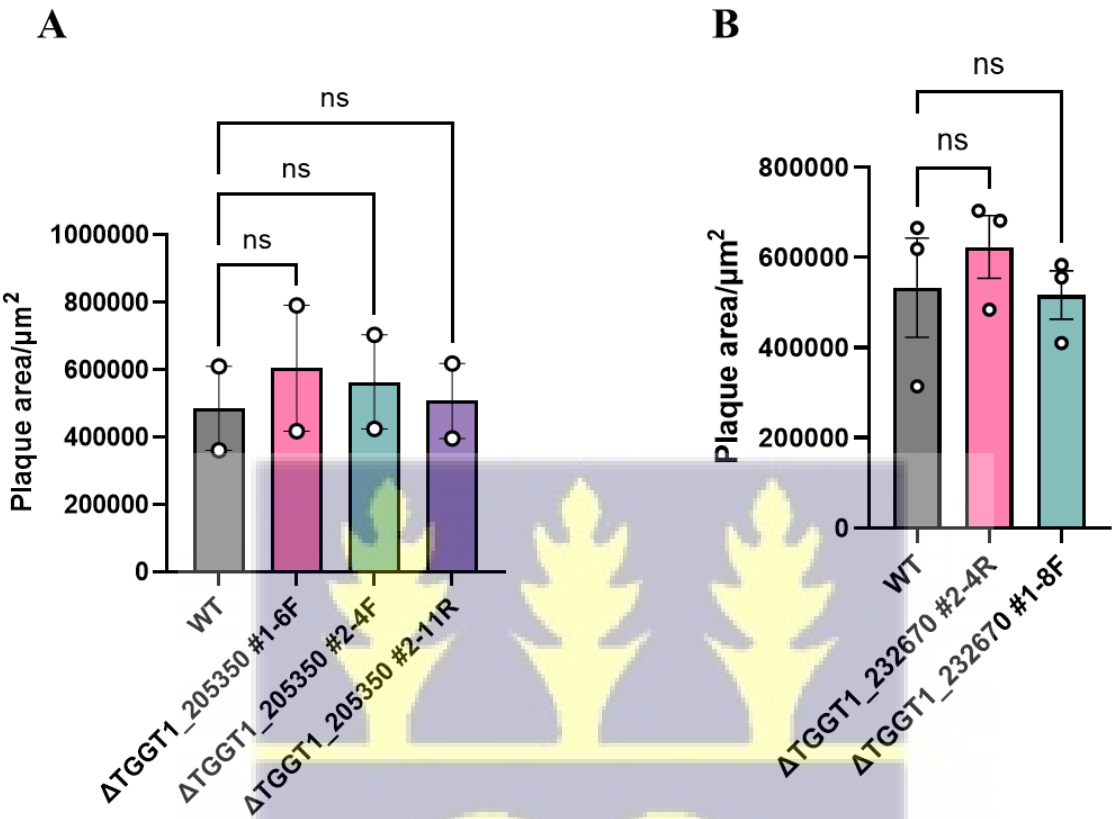
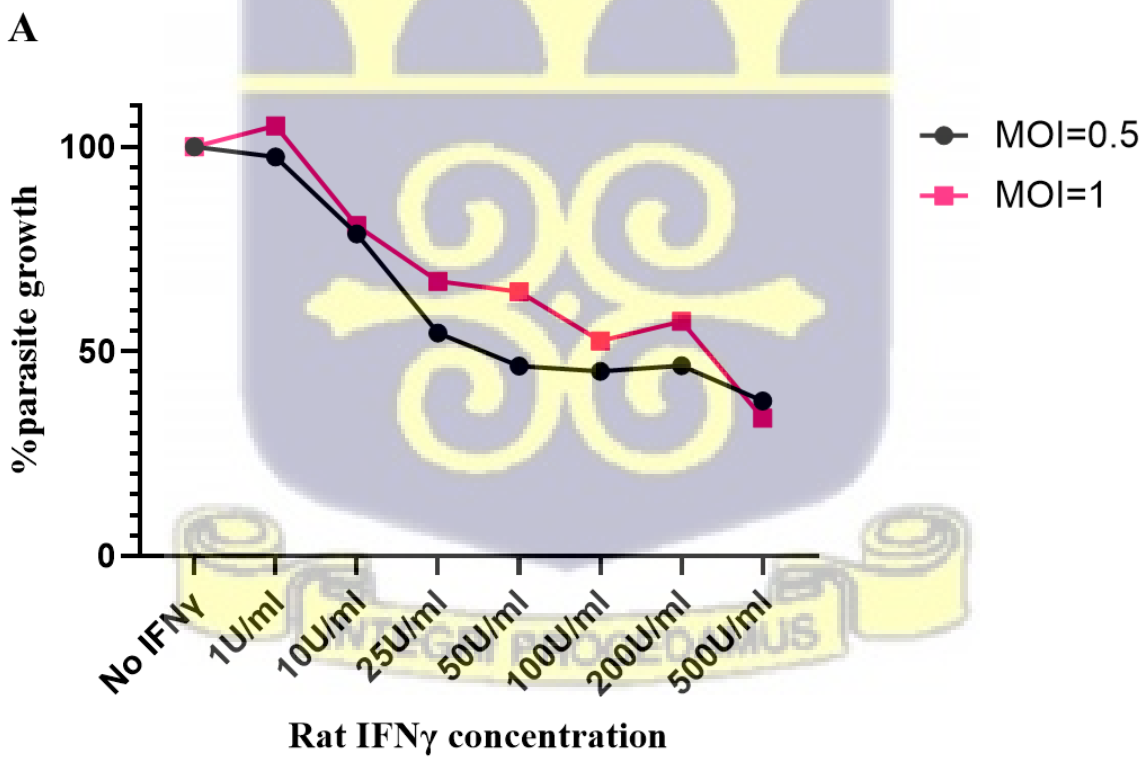


Figure 4.6: Gene knockouts have no growth defect in HFF cells. Plaque assay was set up by infecting confluent monolayers of HFF cells in 24-well plates with 100 tachyzoites and incubated at 37 ºC for 5 days without moving the plates. Plaque area was quantified for (**A**) Δ*TGGT1_205350* clones or **(B)** Δ*TGGT1_232670* clones under the microscope. The imaging of at least 40 plaques from technical duplicate wells was done for each independent experiment. The data are shown as mean±SEM, and the number of dots represents the number of independent experiments. One-way ANOVA along with Tukey's multiple comparisons test was used for statistical analysis.

4.6 IFNγ concentration curves to determine IC_{30} to IC_{50}

To test the phenotype of newly generated knockout parasites in IFNγ-stimulated macrophages, the concentrations of IFNγ that inhibited about 30% to 50% of WT-RH parasite growth had to be determined. Rat or murine BMDMs were stimulated with rat or murine IFNγ, respectively. Seven concentrations were tested and a "No IFNγ" control was set up along with these concentrations. Using a luciferase assay, WT parasite growth was determined after 24 hours of infection, and two concentrations that inhibited parasite growth by 30-50% were chosen to test the knockout phenotype. The inhibitory concentrations (IC) of 25 U/ml and 50 U/ml were selected when rat BMDMs were stimulated with rat IFNγ since these concentrations inhibited 30-50% of parasite growth (Figure 4.7A). For murine BMDMs stimulated with murine IFNγ, concentrations of 50 U/ml and 100 U/ml gave the required range of inhibition (Figure 4.7B)



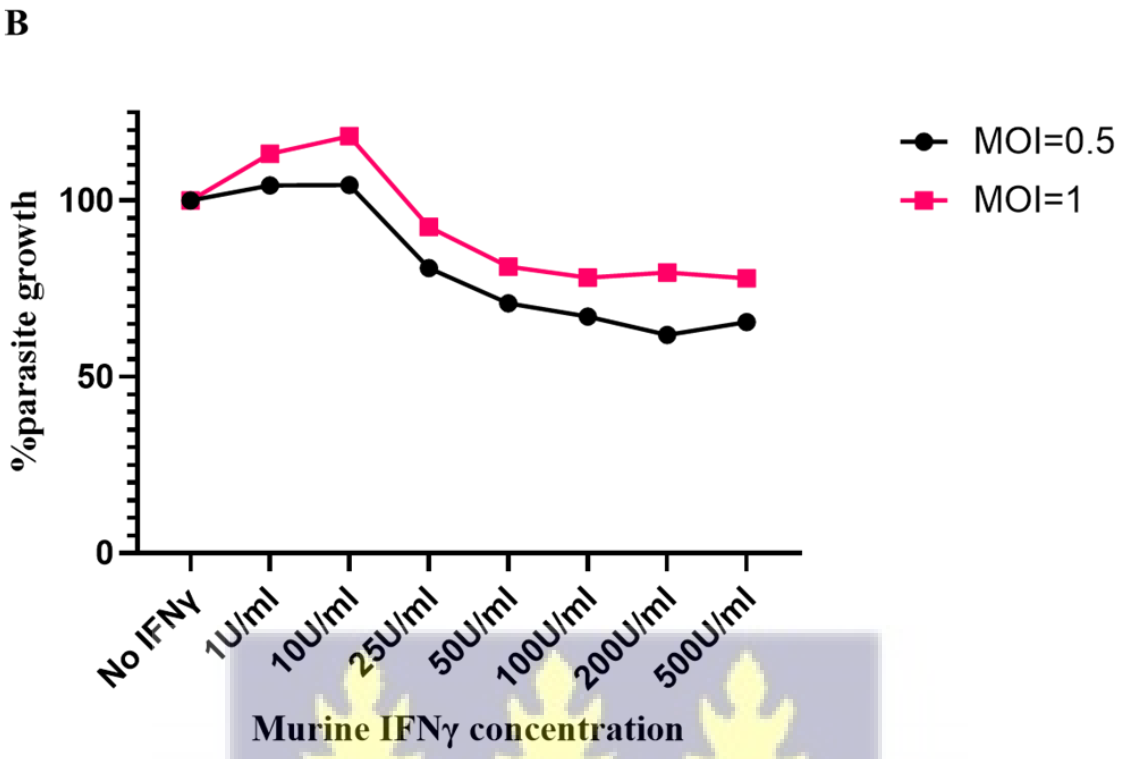
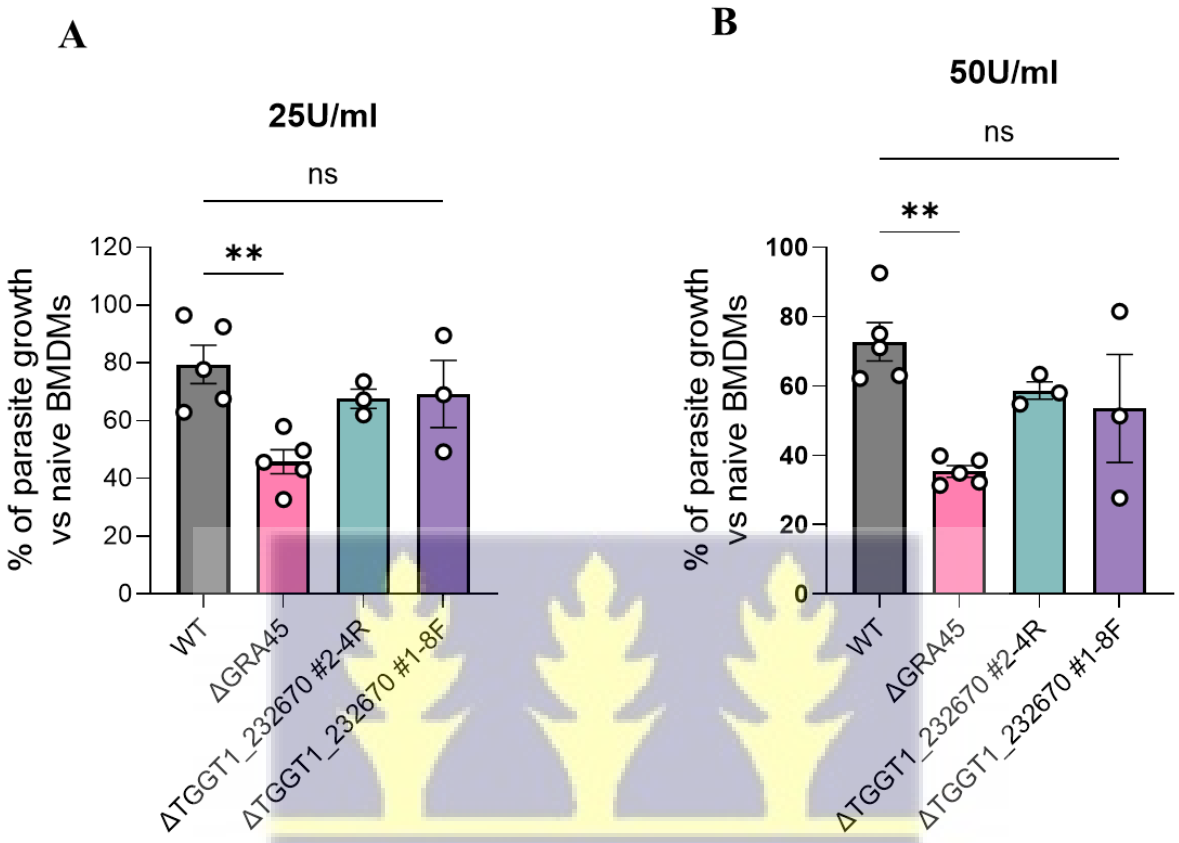


Figure 4.7: IFNγ concentration curves to determine IC30 & IC50 using (A) rat IFNγ to stimulate rat BMDMs and (B) murine IFNγ to stimulate murine BMDMs. Cells were left unstimulated or pre-stimulated with indicated concentration for 24 hours before infection with WT parasites at MOI = 0.5 or 1. The luciferase assay was used to quantify the parasite growth for each concentration 24 hours post-infection. Three technical replicates for each concentration, murine and rat, respectively.

4.7 Phenotype of knockout parasites in IFNγ-stimulated BMDMs

Since individual knockouts were generated in luciferase-expressing RH parasites, a luciferase assay was employed to determine the growth phenotype of newly generated knockout parasites. Δ*gra45*, previously validated as the parasite strain having reduced growth in this condition, was

used as positive control. The knockout parasites were first tested in the BMDMs isolated from the Brown Norway rats. Even with increasing concentrations of IFNγ (Figure 4.8A, B), the two clones of Δ*TGGT1_232670* tested in rat BMDMs stimulated with IFNγ had comparable growth phenotypes to WT parasites (Figure 4.8A, B). Out of the three clones of Δ*TGGT1_205350* tested, Δ*TGGT1_205350* #1-6F demonstrated a consistent growth defect at 50 U/mL concentration (Figure 4.8 D). The other two clones had comparable growth phenotypes to WT parasites (Figure 4.8C, D).



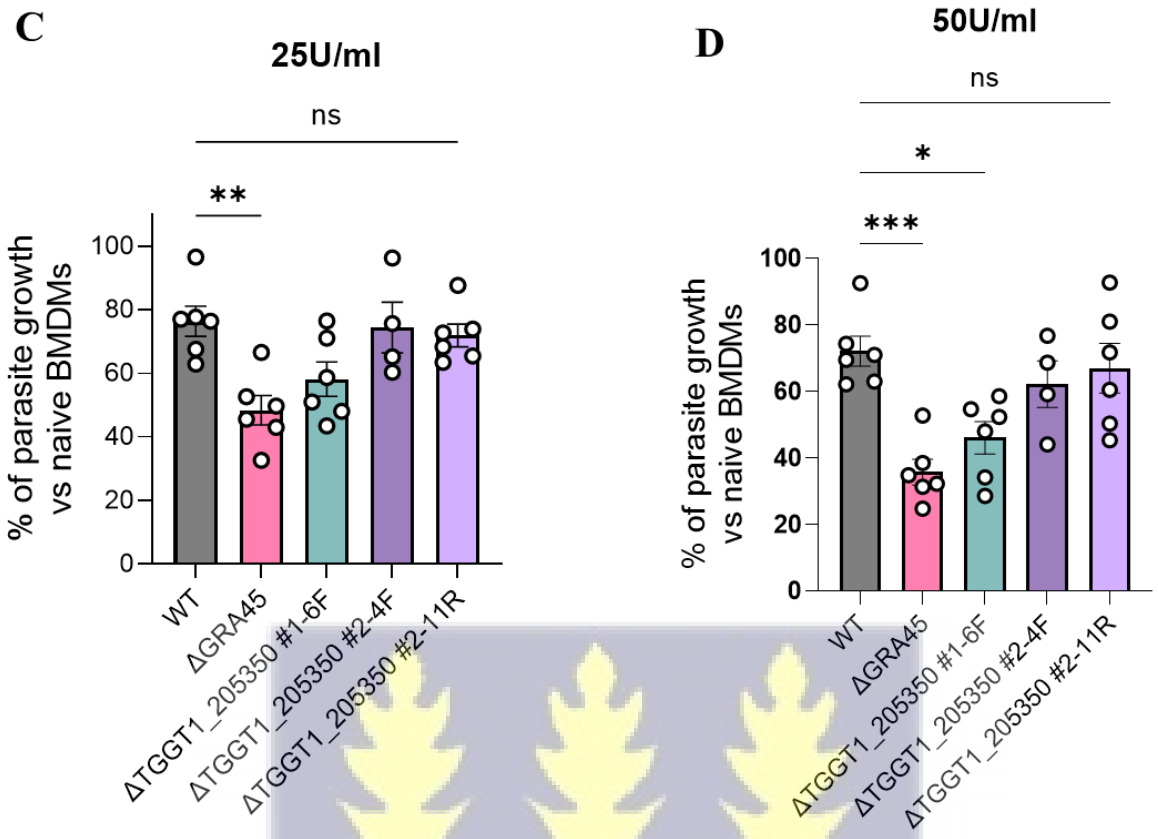
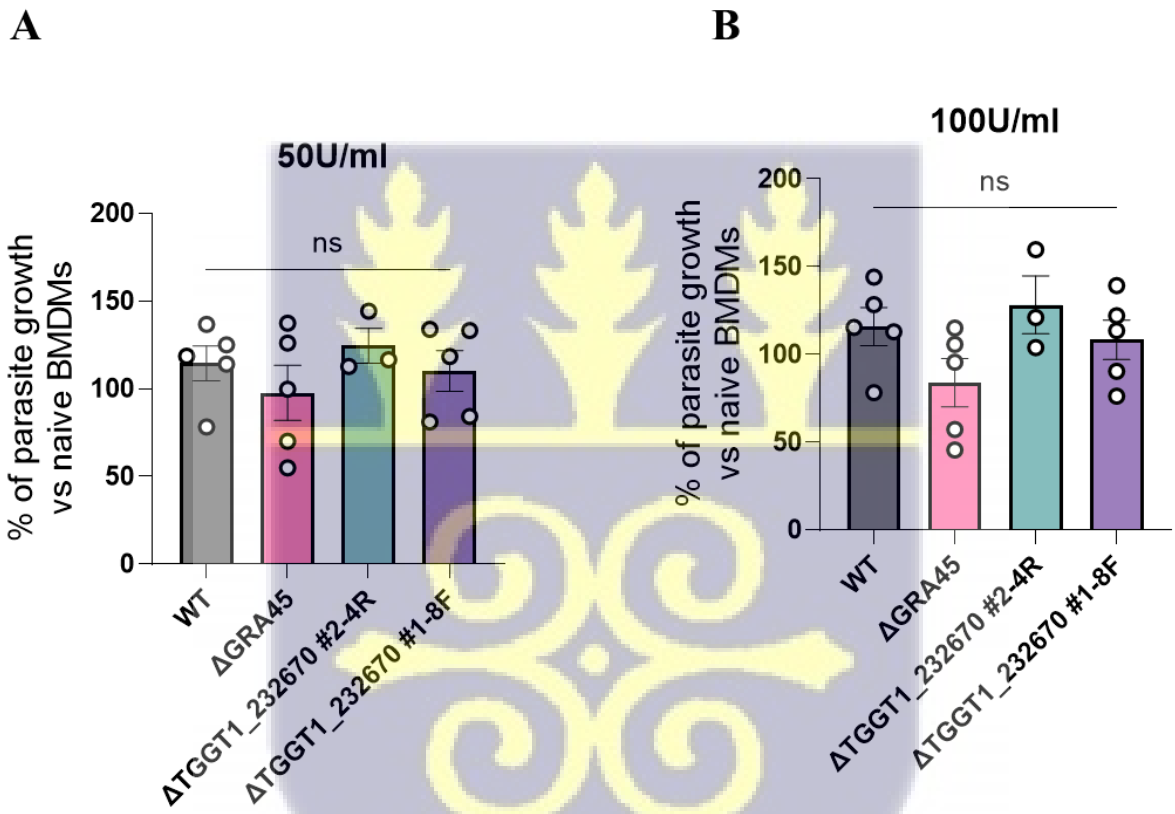


Figure 4.8: Evaluation of candidate genes influencing parasite fitness in rat BMDMs stimulated by IFNγ. A-D Rat BMDMs were infected with luciferase-expressing wild-type (WT) parasites after being either unstimulated for 24 hours or pre-stimulated with IFNγ (25 or 50 U/mL) or (A-B) Δ*gra45* (n=5), Δ*TGGT1_232670* #1-8F (n=3), Δ*TGGT1_2323670* #2-4R (n=3), and (C-D) Δ*gra45* (n=6), Δ*TGGT1_205350* #1-6F (n=6), Δ*TGGT1_205350* #2-4F (n=4), or Δ*TGGT1_205350* #2-11R (n=6) parasites (MOI of 0.3). The luciferase assay was used to quantify the parasite growth for each strain 24 hours post-infection. In IFNγ-activated BMDMs, parasite growth is expressed relative to naïve BMDM growth. The data are shown as mean ± SEM, and the number of dots represents the number of independent experiments. One-way ANOVA using Tukey's multiple comparisons test was used for statistical analysis.

The *TGGT1_232670* knockout clones were then tested in murine BMDMs stimulated with IFNɣ, which had comparable growth phenotypes to WT parasites (Figure 4.9A, B). Even though Δ*gra45* had previously been validated to have a growth defect in this condition, that was not demonstrated here (Figure 4.9). Knockout clones Δ*TGGT1_205350* also demonstrated no growth defect with increasing concentrations of IFNɣ (Figure 4.9C and D) when compared to WT parasites.



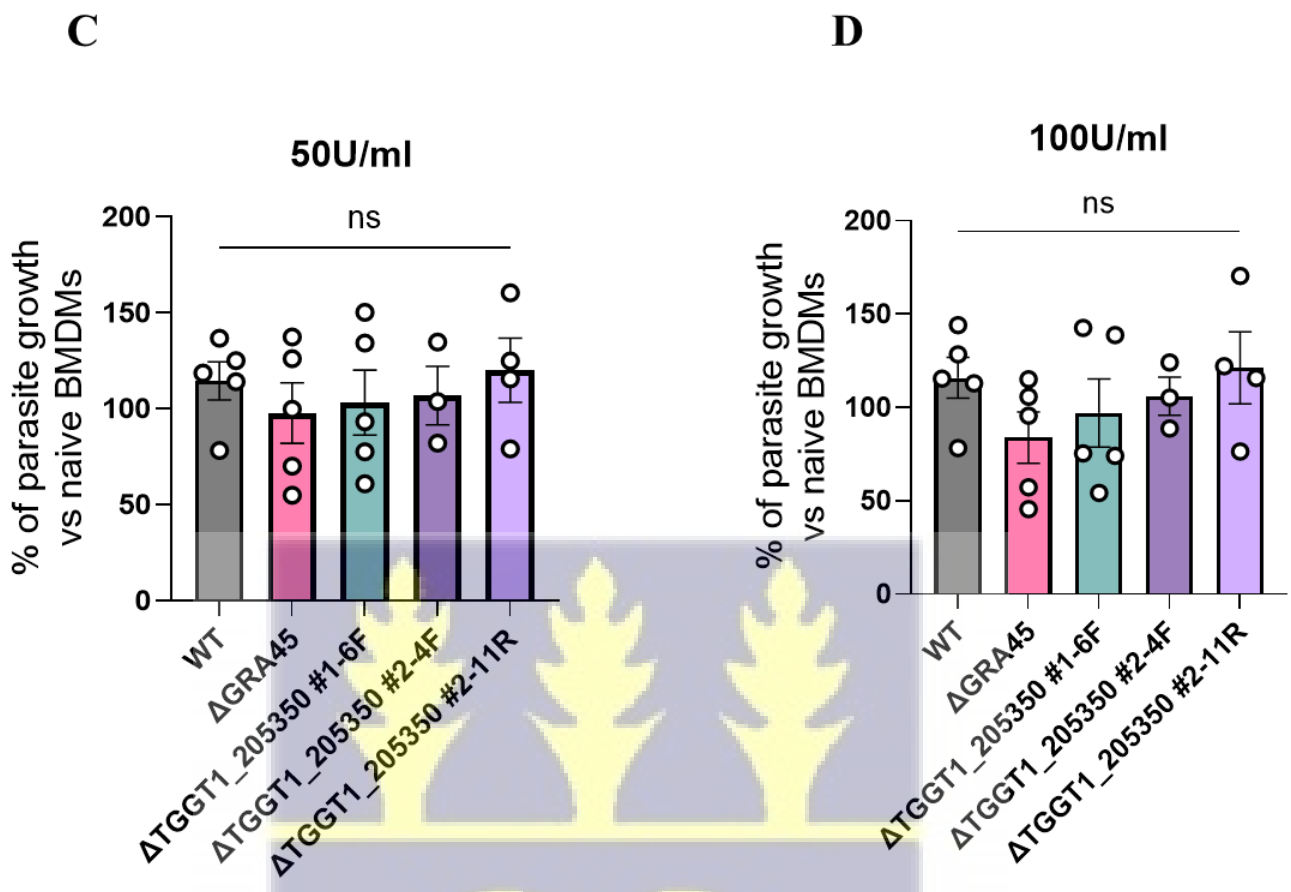


Figure 4.9: Evaluation of candidate genes influencing parasite fitness in murine BMDMs stimulated by IFNγ. A-D Murine BMDMs were infected with luciferase-expressing wild-type (WT) parasites after being either unstimulated for 24 hours or pre-stimulated with IFNγ (50 or 100 U/mL). or (A-B) Δ*gra45* (n=5), Δ*TGGT1_232670* #1-8F (n=5), Δ*TGGT1_2323670* #2-4R (n=3), and (C-D) Δ*gra45* (n=5), Δ*TGGT1_205350* #1-6F (n=5), Δ*TGGT1_205350* #2-4F (n=3), or Δ*TGGT1_205350* #2-11R (n=4) parasites (MOI of 0.3). The luciferase assay was used to quantify the parasite growth for each strain 24 hours post-infection. In IFNγ-activated BMDMs, parasite growth is expressed relative to naïve BMDM growth. The data are shown as mean ± SEM, and the

number of dots represents the number of independent experiments. One-way ANOVA using Tukey's multiple comparisons test was used for statistical analysis.

4.8 Phenotype determination of gene knockouts *in vivo* in mice

C57BL/6J mice were infected with knockouts to determine if genes contribute to parasite fitness *in vivo.* Symptoms of acute infection started at 5 dpi. After monitoring infected mice for 9 days, all mice died or were euthanized with no significant difference was observed between WT and knockout infection (Figure 4.10).

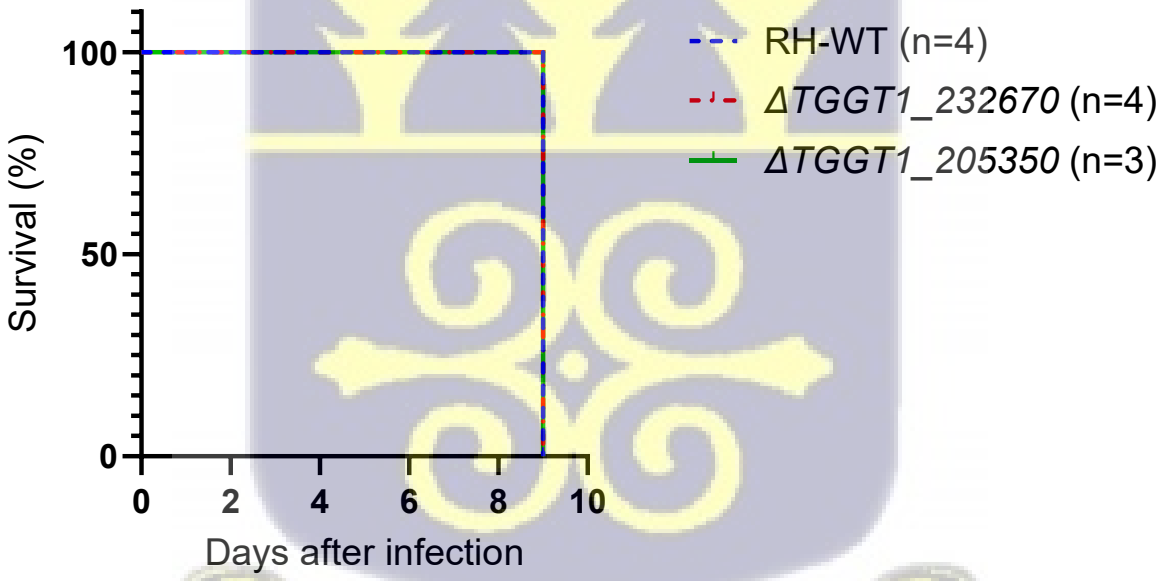


Figure 4.10: Evaluation of candidate genes influencing parasite fitness in mice during acute infections. The survival of C57BL6/J mice was observed for 9 days after they were intraperitoneally (i.p.) infected with 100 tachyzoites of WT, *ΔTGGT1_232670*, or

ΔTGGT1_205350 parasites. (n=4 mice infected with WT or *ΔTGGT1_232670* parasites, n=3 mice infected with *ΔTGGT1_205350* parasites) The data are presented as Kaplan–Meier survival curves.

4.9 Determine the localization of TGGT1_205350 and TGGT1_232670

The subcellular localizations of TGGT1_205350 and TGGT1_232670 in *T. gondii* were previously unknown. Therefore, a plasmid construct was used to generate HA-epitope endogenously tagged TGGT1_205350 and TGGT1_232670, respectively. After the transfection of the plasmid, an immunofluorescence assay was performed to detect the HA epitope. TGGT1_205350 was observed to possibly be localized to the Golgi apparatus (Figure 4.11). For TGGT1_232670 endogenously tagged parasites, about 400 vacuoles were observed and no HA-positive parasites were found, which the localization of TGGT1_232670 remains unknown (data in Appendix 5).

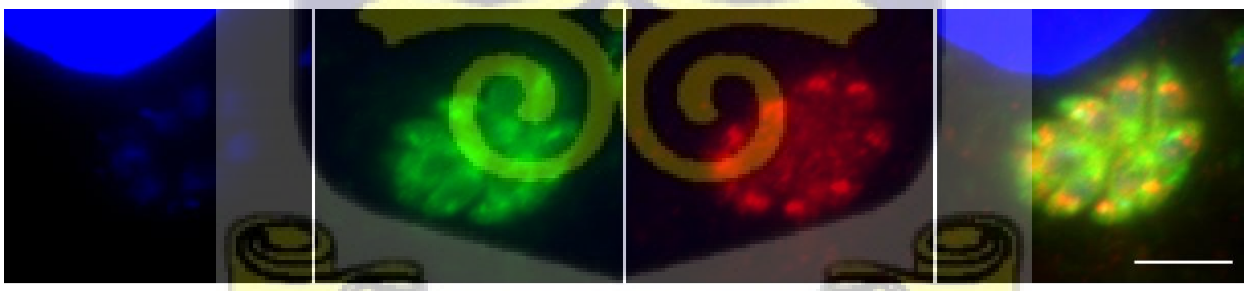


Figure 4.11: Representative immunofluorescent image of TGGT1_205350 localization 20 µL of parasites that had emerged from pyrimethamine selection were added to freshly confluent HFF

cells seeded on coverslips. Cells were fixed at 24 hours post-infection prior to being subjected to the immunofluorescent test using antibodies that target the HA epitope. In both populations, the tagged protein appears to be located in the Golgi apparatus. The image (scale bar =5 μm) is representative of HA-positive parasites seen in the population.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION, LIMITATION & RECOMMENDATION, FUTURE DIRECTIONS

5.1 DISCUSSION

This study aimed to validate two *T. gondii* genes that were hits from a genome-wide CRISPR screen and determined fitness in IFNγ-activated macrophages. Generating individual mutations at a specific genomic locus allows us to decipher its relevance or function. Here, knockouts of *TGGT1_232670* and *TGGT1_205350* were successfully generated in *T. gondii*. By designing plasmids containing sgRNAs (results shown in Appendix 2 and 3) targeting the beginning of their coding sequences, the gene disruption method of CRISPR/Cas9 technology (Shen et al., 2017) was employed. Independent knockout clones were confirmed by PCR before proceeding to assess their phenotypes *in vitro* and *in vivo*. This was necessary to ensure that only true knockout clones were used in phenotypic assays and the reliability of results obtained (clone selection results shown in Appendix 4).

After successfully obtaining knockout clones of *TGGT1_232670* and *TGGT1_205350*, their growth phenotype in HFF cells was tested by plaque assay. HFF is the cell type routinely used in the laboratory to culture *T. gondii* tachyzoites *in vitro*. From the screen results, the candidate genes were seen to be fitness-conferring in IFNγ-stimulated macrophages only (hence the negative fitness score) and not HFF cells. To be certain that any growth difference of the knockouts observed in IFNγ-stimulated macrophages is truly reflective of this condition, the possibility of a growth defect in HFF cells had to be determined. Indeed, compared to the WT parasites, no growth

differences in terms of plaque size were observed for any of the clones. This confirmed that knockout clones did not have a general growth defect, allowing accurate interpretation of data that was obtained when the clones were tested in macrophages stimulated with IFNγ.

To determine the IFNγ concentrations that would be suitable to test the phenotype of the knockout parasites, growth inhibition curves were obtained in both rat and murine BMDMs using infection with WT parasites. We reasoned those concentrations of IFNγ that inhibited 30-50% of WT parasite growth (hence allowed 50-70% growth) would be suitable to observe significant growth differences, if any, in the knockout parasites when compared. Therefore, the IC_{30} and IC_{50} were determined in BMDMs from both species.

Several *in vitro* assays are available for testing the growth and replication phenotype of mutant strains of *T. gondii* such as growth competition assay, luciferase assay, parasite per vacuole, and plaque assays. Luciferase assay was employed because the parental strain used to generate the knockouts was luciferase-expressing, allowing estimation of parasite growth or restriction in macrophages by quantifying the expression of luciferase enzyme by substrate conversion. In this study, TGGT1_232670 was observed to have a comparable growth phenotype to the WT strain in IFNγ-stimulated rat BMDMs after multiple independent replicates, suggesting that the gene may be dispensable for parasite fitness in this condition. Genome-wide CRISPR screens are effective high-throughput methods for investigating the relationship between a genotype and its resulting phenotype. As powerful as this method is, it does have certain limitations. For instance, off-target cleavage events may occur when the potential target sequence is similar to other sequences in the genome, resulting in considering dispensable genes as fitness-conferring (Tsai et al., 2015). This is why hits from such screens are validated with individual gene knockouts to confirm if the genes are truly fitness-conferring in a particular condition and rule out false positives. Also, events where

there is inadequate interaction between Cas9 and sgRNA due to inaccessibility of the target site or inefficient transcription of sgRNA (T. Wang et al., 2014) have been noted as a limitation. Even though TGGT1_232670 was not observed to confer fitness in the conditions tested, it still provides valuable information for parasite biology, as the function of this gene is currently unknown. In the drug discovery pipeline, it adds to existing knowledge that the gene is non-fitness-conferring in the conditions tested. This, therefore, helps to prioritize other target genes for validation and streamline time and resources in the pursuit of translational discovery.

A clone of Δ*TGGT1_205350* parasites demonstrated consistently reduced growth phenotype at 50 U/ml IFNγ in rat BMDMs compared to WT, suggesting it is fitness conferring in rat macrophages. *T. gondii* undergoes nonhomologous end joining (NHEJ) to repair DNA after a break (David S. Roos et al., 1995). This results in random high-frequency insertions and deletions (INDELS) leading to loss-of-function mutation after Cas9 nuclease creates a double-stranded break. These INDELS may account for the differences observed in the phenotype of the independent clones of Δ*TGGT1_205350* in this condition.

The growth phenotypes of Δ*TGGT1_232670* and Δ*TGGT1_205350* parasites in IFNγ-stimulated murine BMDMs were inconclusive. A candidate gene *GRA45*, from the genome-wide CRISPR screen, was previously validated in both rat and murine IFNγ-stimulated BMDMs and observed to have reduced growth in both conditions (Yifan Wang et al., 2020). Hence, it was used as a positive control for all *in vitro* assays. For all independent replicates of luciferase assay in IFNγ-stimulated murine BMDMs, Δ*gra45* parasites did not demonstrate growth defect. This suggests that murine BMDMs were unresponsive to IFNγ stimulation between different batches of macrophages isolated. Macrophage activation involves multiple mediators, such as cytokines, and recognition of PAMPs, leading to a myriad of signal transduction pathways that result in

their antimicrobial activity. IFNγ is a known potent activator of macrophages, typically via a STAT1-dependent pathway. Conversely, it is also able to induce the expression of inhibitory protein, suppressor of cytokine signaling (SOCS) (Hu et al., 2008). It has been shown that a delicate balance must be maintained between STAT1 and SOCS1 expression during IFNγ stimulation to achieve macrophage activation. The model suggests that higher doses of IFNγ induce sustained expression of SOCS1, while low priming doses maintain expression of STAT1 (Hu et al., 2002). This may be a probable reason for the unresponsive macrophages, but it is presently difficult to conclude without further experiments.

Also, the murine model has been demonstrated to have varied clinical outcomes for *T. gondii* depending on the strain of infection. For instance, a single tachyzoite of the type I RH strain can kill all infected mice after about a week post-infection ($LD_{100} = 1$). While infection with either type II or III requires a thousand to a hundred thousand fold more tachyzoites to kill half of the infected mice (LD_{50}) (Sanchez & Besteiro, 2021; L. D. Sibley & Boothroyd, 1992). The molecular mechanism underlying the high virulence of the RH strain in mice has been attributed to polymorphic effectors from the rhoptry organelle such as ROP5, ROP17, and ROP18. The kinase and pseudokinase activity of these ROPs facilitates the phosphorylation of IRGs and GBPs on the PVM ROP17 (Behnke et al., 2012; Etheridge et al., 2014; Reese & Boothroyd, 2011). In murine macrophages, IRGs (Zhao et al., 2009) and GBPs (Kravets et al., 2016; Selleck et al., 2013; Yamamoto et al., 2012) are important ISGs upregulated to control *T. gondii* infection. These host proteins, once phosphorylated, are unable to oligomerize and accumulate at the PVM hence, there is no vacuole disruption and death of the parasite, ultimately promoting parasite survival (Hakimi et al., 2017). Given that the knockouts were generated in the RH strain, antagonistic effectors present such as the ones discussed above, were likely efficient in subverting the host IFNγ-induced

mechanisms in the murine BMDMs. Therefore, masking the phenotype of *TGGT1_232670* and *TGGT1_205350* in these cells if any.

While completing in vitro validation of candidate genes, a CRISPR genome-wide screen during acute murine infection was published (Giuliano et al., 2024), and a database was created based on that study (https://toxo-mouse-screen.wi.mit.edu/). From this database, *TGGT1_232670* was predicted to be fitness-conferring during acute mouse infection, specifically in the peritoneum, liver, spleen, and lung, while *TGGT1_205350* was fitness-conferring in the liver, spleen, and lung. Since knockouts had already been generated for these genes, a validation of their phenotype during acute mouse infection was performed. It was hypothesized that if candidate genes were fitness-conferring during acute infection in mice, then mice infected with the knockout strains should survive longer or overcome infection compared with wild-type infected mice. By 9 d p.i, all Δ*TGGT1_205350* parasites-infected mice had died along with two WT-infected mice. This suggests that *TGGT1_205350* may not be fitness-conferring during *in vivo* infection in mice. The remaining WT-infected mice and Δ*TGGT1_232670* parasites-infected mice were euthanized on the same day. Therefore, the endpoint of death was not naturally observed, making it challenging to draw conclusions on the fitness of *TGGT1_232670* during murine infection.

Determining gene localization helps to further explore its function. The localization of TGGT1_232670 and TGGT1_205350 was previously unknown, and this study set out to tag their gene products endogenously to observe their localization. Tagging genes directly on the chromosome better mimics natural expression and permits the use of conventional antibodies or protein tags to view localization. Using the method described (Huynh & Carruthers, 2009) with minimal modification, TGGT1_205350 seemed to localize to the Golgi apparatus. The Hyperplexed Localization of Organelle Proteins by Isotope Tagging (hyperLOPIT) on

ToxoDB.org predicts an endoplasmic reticulum (ER) localization of this protein, however. This highlights the importance of validating the localization of individual genes to enhance knowledge to study their functions. The localization of the TGGT1_232670 gene product could not be obtained after transfection and drug selection. The HA tag may have been inaccessible to antibodies during the staining process due to the conformation of the folded protein, hence reducing the probability of acquiring HA-positive parasites in the transfected population. Given that knocking out TGGT1_205350 produced a growth defect in IFNγ-stimulated rat macrophages, it has been validated as a candidate gene. The localization of this gene in the Golgi apparatus could suggest a role in sorting, modification, and trafficking of parasite proteins. As most parasite effectors are proteins that need to be trafficked to the appropriate organelles for proper function, it would be illuminating to further investigate the mechanistic role of TGGT1_205350 in this process.

5.2 CONCLUSIONS

This study validated two *T. gondii* genes that were hits from a genome-wide CRISPR screen in both IFNγ-activated mice and rat BMDMs. A clone of *TGGT1_205350* was observed to confer fitness in IFNγ-activated rat BMDMs but not during acute phase infection in mice. Endogenously tagging the gene revealed a possible localization to the Golgi. *TGGT1_205350* could be explored as a drug target if further experiments validate its importance to parasite fitness. *TGGT1_232670* was found not to confer fitness in IFNγ-activated rat BMDMs. However, its fitness during the acute phase infection in mice and localization remains unknown. Overall, this study successfully validated that *TGGT1_205350* confered fitness in IFNγ-activated rat BMDMs and showed that its gene product localized to the Golgi, which was previously unknown.

5.3 LIMITATIONS AND RECOMMENDATIONS

This study showed evidence that *TGGT1_205350* may be fitness-conferring in IFNγ-activated rat BMDMs, however, it could not confirm this. Generating a complementation strain of the gene to observe if the growth phenotype can be restored is recommended. Also, it could not be validated whether the candidate genes were fitness conferring in IFNγ-activated murine BMDMs. Future experiments could employ synergistic stimulation of IFNγ, TNFα, and bacterial LPS to promote macrophage activation and overcome the unresponsive state. TGGT1_232670's fitness in mice during acute infection was not verified in this study. It is recommended that infected mice be observed till the endpoint of death to confidently ascertain the gene's role in acute murine infection.

5.4 FUTURE DIRECTIONS

As part of the effort to understand *T. gondii* biology, genome-wide CRISPR screens have become an important technique. *T. gondii* is such a dynamic parasite in its ability to adapt to different host species and variable cellular environments. CRISPR screens enable us to explore and discover the myriad of genes *T. gondii* uses to thrive in remarkably different conditions. The collective goal is to find suitable drug targets or vaccine candidates that will provide effective and non-toxic alternatives to currently available drugs.

Moving forward from these two validated candidate genes, I am interested in validating other genes that were hits from the screen (both in naïve and IFNγ primed conditions). From the IFNγ screen data, I am following up on two genes that were previously validated. GRA22 was found to be fitness-conferring in both murine and rat BMDMs stimulated with IFNγ by having reduced growth compared to WT parasites when the gene was knocked out. I am generating complementation of GRA22 in the knockout strains to determine if the phenotype that was previously observed is solely attributable to the loss of GRA22. TGGT1_263560 is another gene that was validated to have reduced growth in only murine BMDMs stimulated with IFNγ. Similarly, a complementation strain will be generated for this gene and tested to ascertain if the growth phenotype will be restored. To understand the molecular mechanisms underlying the observed phenotypes, determining the localization of these gene products is important. This first step can help us determine if the genes are directly or indirectly interacting with host proteins and what these proteins are.

In the naïve murine BMDM screen data, TGGT1_249580 (also known as TgApiAT6-3) was a top hit. TgApiAT6-3 is a predicted apicomplexan amino acid transporter and hence may play an important role in nutrient acquisition. As a parasite, *T. gondii* is unable to synthesize several

proteins and molecules it requires for survival and therefore scavenges them from its host. A knockout strain of this gene has been generated in the lab and I am currently determining its growth phenotype in murine cells and HFF cells. I am also testing how the presence or absence of amino acids in the cell culture media affects the phenotype of the knockout strains.

At the Wang lab, we want to understand how important these genes are for parasite survival. Studying these genes in the context of a physiologically relevant cell type like the macrophage is essential given the crucial role these cells play in controlling *T. gondii* infection within the host.

REFERENCES

Alaganan, A., Fentress, S. J., Tang, K., Wang, Q., & Sibley, L. D. (2014). Toxoplasma GRA7 effector increases turnover of immunity-related GTPases and contributes to acute virulence in the mouse. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(3), 1126–1131.

Alday, P. H., & Doggett, J. S. (2017). Drugs in development for toxoplasmosis: advances, challenges, and current status. *Drug Design, Development and Therapy*, *11*, 273–293.

Aldebert, D., Durand, F., Mercier, C., Brenier-Pinchart, M.-P., Cesbron-Delauw, M.-F., & Pelloux, H. (2007). Toxoplasma gondii triggers secretion of interleukin-12 but low level of interleukin-10 from the THP-1 human monocytic cell line. *Cytokine*, *37*(3), 206–211.

Alexander, D. L., Mital, J., Ward, G. E., Bradley, P., & Boothroyd, J. C. (2005). Identification of the moving junction complex of Toxoplasma gondii: a collaboration between distinct secretory organelles. *PLoS Pathogens*, *1*(2), e17.

Almeria, S., & Dubey, J. P. (2021). Foodborne transmission of Toxoplasma gondii infection in the last decade. An overview. *Research in Veterinary Science*, *135*, 371–385.

Alvarado-Esquivel, C., Estrada-Martínez, S., & Liesenfeld, O. (2011). Toxoplasma gondii infection in workers occupationally exposed to unwashed raw fruits and vegetables: a case control seroprevalence study. *Parasites & Vectors*, *4*(1), 235.

Alvarado-Esquivel, C., Sánchez-Anguiano, L. F., Hernández-Tinoco, J., Ramos-Nevarez, A., Estrada-Martínez, S., Cerrillo-Soto, S. M., Medina-Heredia, G. E., Guido-Arreola, C. A., Soto-Quintero, A. A., & Beristain-Garcia, I. (2018). Association between Toxoplasma gondii infection and history of blood transfusion: a case-control seroprevalence study. *The Journal of International Medical Research*, *46*(4), 1626–1633.

Amissah-Reynolds, P. K., Yar, D. D., Gyamerah, I., Apenteng, O. Y., & Sakyi, S. (2020). Fresh vegetables and ready-to-eat salads: Sources of parasitic zoonoses in mampong-Ashanti, Ghana. *European Journal of Nutrition & Food Safety*, 47–55.

Andiappan, H., Nissapatorn, V., Sawangjaroen, N., Khaing, S.-L., Salibay, C. C., Cheung, M. M. M., Dungca, J. Z., Chemoh, W., Xiao Teng, C., Lau, Y.-L., & Mat Adenan, N. A. (2014). Knowledge and practice on Toxoplasma infection in pregnant women from Malaysia, Philippines, and Thailand. *Frontiers in Microbiology*, *5*, 291.

Andrade, R. M., Wessendarp, M., Gubbels, M.-J., Striepen, B., & Subauste, C. S. (2006). CD40 induces macrophage anti-Toxoplasma gondii activity by triggering autophagy-dependent fusion of pathogen-containing vacuoles and lysosomes. *The Journal of Clinical Investigation*, *116*(9), 2366–2377.

Assoah, E., Yar, D. D., Amissah-Reynolds, P. K., Balali, G. I., Addy, R., & Zineyele, J. K. (2025). Co-infections and risk factors of Toxoplasma gondii infection among pregnant women in Ghana: A facility-based cross-sectional study. *PloS One*, *20*(5), e0324950.

Attias, M., Teixeira, D. E., Benchimol, M., Vommaro, R. C., Crepaldi, P. H., & De Souza, W. (2020). The life-cycle of Toxoplasma gondii reviewed using animations. *Parasites & Vectors*, *13*(1), 588.

Ayi, I., Edu, S. A., Apea-Kubi, K. A., Boamah, D., Bosompem, K. M., & Edoh, D. (2009). Sero-epidemiology of toxoplasmosis amongst pregnant women in the greater accra region of ghana. *Ghana Medical Journal*, *43*(3), 107–114.

Ayi, Irene, Kwofie, K. D., Blay, E. A., Osei, J. H. N., Frempong, K. K., Koku, R., Ghansah, A., Lartey, M., Suzuki, T., Boakye, D. A., Koram, K. A., & Ohta, N. (2016). Clonal types of

Toxoplasma gondii among immune compromised and immune competent individuals in Accra, Ghana. *Parasitology International*, *65*(3), 238–244.

Ayi, Irene, Sowah, A. O.-K., Blay, E. A., Suzuki, T., Ohta, N., & Ayeh-Kumi, P. F. (2016). Toxoplasma gondii infections among pregnant women, children and HIV-seropositive persons in Accra, Ghana. *Tropical Medicine and Health*, *44*(1), 17.

Balaskas, K., Vaudaux, J., Boillat-Blanco, N., & Guex-Crosier, Y. (2012). Azithromycin versus Sulfadiazine and Pyrimethamine for non-vision-threatening toxoplasmic retinochoroiditis: a pilot study. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *18*(5), CR296-302.

Behnke, M. S., Fentress, S. J., Mashayekhi, M., Li, L. X., Taylor, G. A., & Sibley, L. D. (2012). The polymorphic pseudokinase ROP5 controls virulence in Toxoplasma gondii by regulating the active kinase ROP18. *PLoS Pathogens*, *8*(11), e1002992.

Behnke, M. S., Khan, A., Wootton, J. C., Dubey, J. P., Tang, K., & Sibley, L. D. (2011). Virulence differences in Toxoplasma mediated by amplification of a family of polymorphic pseudokinases. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(23), 9631–9636.

Belluco, S., Mancin, M., Conficoni, D., Simonato, G., Pietrobelli, M., & Ricci, A. (2016). Investigating the determinants of Toxoplasma gondii prevalence in meat: A systematic review and meta-regression. *PloS One*, *11*(4), e0153856.

Belluco, S., Simonato, G., Mancin, M., Pietrobelli, M., & Ricci, A. (2018). Toxoplasma gondii infection and food consumption: A systematic review and meta-analysis of case-controlled studies. *Critical Reviews in Food Science and Nutrition*, *58*(18), 3085–3096.

Benenson, M. W., Takafuji, E. T., Lemon, S. M., Greenup, R. L., & Sulzer, A. J. (1982). Oocyst-transmitted toxoplasmosis associated with ingestion of contaminated water. *The New England Journal of Medicine*, *307*(11), 666–669.

Besteiro, S., Michelin, A., Poncet, J., Dubremetz, J.-F., & Lebrun, M. (2009). Export of a Toxoplasma gondii rhoptry neck protein complex at the host cell membrane to form the moving junction during invasion. *PLoS Pathogens*, *5*(2), e1000309.

Blay, E. A., Ghansah, A., Otchere, J., Koku, R., Kwofie, K. D., Bimi, L., Takashi, S., Ohta, N., & Ayi, I. (2015). Congenital toxoplasmosis and pregnancy malaria detection post-partum: Effective diagnosis and its implication for efficient management of congenital infection. *Parasitology International*, *64*(6), 603–608.

Bliss, S. K., Marshall, A. J., Zhang, Y., & Denkers, E. Y. (1999). Human polymorphonuclear leukocytes produce IL-12, TNF-alpha, and the chemokines macrophage-inflammatory protein-1 alpha and -1 beta in response to Toxoplasma gondii antigens. *The Journal of Immunology*, *162*(12), 7369–7375.

Bollani, L., Auriti, C., Achille, C., Garofoli, F., De Rose, D. U., Meroni, V., Salvatori, G., & Tzialla, C. (2022). Congenital toxoplasmosis: The state of the art. *Frontiers in Pediatrics*, *10*, 894573.

Borkowski, P. K., Brydak-Godowska, J., Basiak, W., Świtaj, K., Żarnowska-Prymek, H., Olszyńska-Krowicka, M., Kajfasz, P., & Rabczenko, D. (2016). The impact of short-term, intensive antifolate treatment (with pyrimethamine and sulfadoxine) and antibiotics followed by long-term, secondary antifolate prophylaxis on the rate of toxoplasmic retinochoroiditis recurrence. *PLoS Neglected Tropical Diseases*, *10*(8), e0004892.

Bougdour, A., Durandau, E., Brenier-Pinchart, M.-P., Ortet, P., Barakat, M., Kieffer, S., Curt-Varesano, A., Curt-Bertini, R.-L., Bastien, O., Coute, Y., Pelloux, H., & Hakimi, M.-A. (2013). Host cell subversion by Toxoplasma GRA16, an exported dense granule protein that targets the host cell nucleus and alters gene expression. *Cell Host & Microbe*, *13*(4), 489–500.

Boughattas, S. (2017). Toxoplasma infection and milk consumption: Meta-analysis of assumptions and evidences. *Critical Reviews in Food Science and Nutrition*, *57*(13), 2924–2933.

Bradley, P. J., Li, N., & Boothroyd, J. C. (2004). A GFP-based motif-trap reveals a novel mechanism of targeting for the Toxoplasma ROP4 protein. *Molecular and Biochemical Parasitology*, *137*(1), 111–120.

Bradley, P. J., & Sibley, L. D. (2007). Rhoptries: an arsenal of secreted virulence factors. *Current Opinion in Microbiology*, *10*(6), 582–587.

Braun, L., Brenier-Pinchart, M.-P., Yogavel, M., Curt-Varesano, A., Curt-Bertini, R.-L., Hussain, T., Kieffer-Jaquinod, S., Coute, Y., Pelloux, H., Tardieux, I., Sharma, A., Belrhali, H., Bougdour, A., & Hakimi, M.-A. (2013). A Toxoplasma dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation. *The Journal of Experimental Medicine*, *210*(10), 2071–2086.

Bryant, J. M., Baumgarten, S., Glover, L., Hutchinson, S., & Rachidi, N. (2019). CRISPR in parasitology: Not exactly cut and dried! *Trends in Parasitology*, *35*(6), 409–422.

Butterworth, S., Torelli, F., Lockyer, E. J., Wagener, J., Song, O.-R., Broncel, M., Russell, M. R. G., Moreira-Souza, A. C. A., Young, J. C., & Treeck, M. (2022). Toxoplasma gondii virulence factor ROP1 reduces parasite susceptibility to murine and human innate immune restriction. *PLoS Pathogens*, *18*(12), e1011021.

Carruthers, V. B., & Sibley, L. D. (1997). Sequential protein secretion from three distinct organelles of Toxoplasma gondii accompanies invasion of human fibroblasts. *European Journal of Cell Biology*, *73*(2), 114–123.

Carruthers, V., & Boothroyd, J. C. (2007). Pulling together: an integrated model of Toxoplasma cell invasion. *Current Opinion in Microbiology*, *10*(1), 83–89.

Carruthers, Vern B., & Tomley, F. M. (2008). Microneme proteins in apicomplexans. *Sub-Cellular Biochemistry*, *47*, 33–45.

Chandrasena, N., Herath, R., Rupasinghe, N., Samarasinghe, B., Samaranayake, H., Kastuririratne, A., & de Silva, N. R. (2016). Toxoplasmosis awareness, seroprevalence and risk behavior among pregnant women in the Gampaha district, Sri Lanka. *Pathogens and Global Health*, *110*(2), 62–67.

Chao, C. C., Gekker, G., Hu, S., & Peterson, P. K. (1994). Human microglial cell defense against Toxoplasma gondii. The role of cytokines. *The Journal of Immunology*, *152*(3), 1246–1252.

Chiari, C. de A., & Neves, D. P. (1984). Human toxoplasmosis acquired by ingestion of goat's milk. *Memorias do Instituto Oswaldo Cruz*, *79*(3), 337–340.

Chirgwin, K., Hafner, R., Leport, C., Remington, J., Andersen, J., Bosler, E. M., Roque, C., Rajicic, N., Mc Auliffe, V., Morlat, P., Jayaweera, 10 D. T., Vilde, J.-L., Luft, B. J., & the AIDS Clinical Trials Group 237/Agence Nationale de Recherche sur le SIDA. (2002). *Randomized Phase II Trial of Atovaquone with Pyrimethamine or Sulfadiazine for Treatment of Toxoplasmic Encephalitis in Patients with Acquired Immunodeficiency Syndrome: ACTG 237/ANRS 039 Study*. https://academic.oup.com/cid/article/34/9/1243/464079

Choi, J., Park, S., Biering, S. B., Selleck, E., Liu, C. Y., Zhang, X., Fujita, N., Saitoh, T., Akira, S., Yoshimori, T., Sibley, L. D., Hwang, S., & Virgin, H. W. (2014). The parasitophorous vacuole membrane of Toxoplasma gondii is targeted for disruption by ubiquitin-like conjugation systems of autophagy. *Immunity*, *40*(6), 924–935.

Clough, B., & Frickel, E.-M. (2017). The Toxoplasma parasitophorous vacuole: An evolving host-parasite frontier. *Trends in Parasitology*, *33*(6), 473–488.

Cong, W., Zhang, N.-Z., Hou, J.-L., Wang, X.-C., Ma, J.-G., Zhu, X.-Q., & Chen, G.-J. (2017). First detection and genetic characterization of Toxoplasma gondii in market-sold oysters in China. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *54*, 276–278.

Cook, A. J., Gilbert, R. E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P. A., Foulon, W., Semprini, A. E., & Dunn, D. T. (2000). Sources of toxoplasma infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis. *BMJ (Clinical Research Ed.)*, *321*(7254), 142–147.

Coradello, H., & Kretschmer, S. (1978). A comparison of Ultrax, Diazil, Bactrim and Spiramycin in experimental toxoplasmosis in mice (author's transl). *Wiener klinische Wochenschrift*, *90*(1), 25–29.

Coupe, A., Howe, L., Burrows, E., Sine, A., Pita, A., Velathanthiri, N., Vallée, E., Hayman, D., Shapiro, K., & Roe, W. D. (2018). First report of Toxoplasma gondii sporulated oocysts and Giardia duodenalis in commercial green-lipped mussels (Perna canaliculus) in New Zealand. *Parasitology Research*, *117*(5), 1453–1463.

Daniel K. Howe and L. David Sibley. (1995). Toxoplasma gondii comprises three clonal lineages: Correlation of parasite genotype with human disease. *The Journal of Infectious Diseases*, *172:1561-6*.

Dardé, M.-L. (2004). Genetic analysis of the diversity in Toxoplasma gondii. *Annali Dell'Istituto Superiore Di Sanita*, *40*(1), 57–63.

Däubener, W., Mackenzie, C., & Hadding, U. (1995). Establishment of T-helper type 1- and T-helper type 2-like human Toxoplasma antigen-specific T-cell clones. *Immunology*, *86*(1), 79–84.

de Moura, L., Bahia-Oliveira, L. M. G., Wada, M. Y., Jones, J. L., Tuboi, S. H., Carmo, E. H., Ramalho, W. M., Camargo, N. J., Trevisan, R., Graça, R. M. T., da Silva, A. J., Moura, I., Dubey, J. P., & Garrett, D. O. (2006). Waterborne toxoplasmosis, Brazil, from field to gene. *Emerging Infectious Diseases*, *12*(2), 326–329.

Delgado, I. L. S., Zúquete, S., Santos, D., Basto, A. P., Leitão, A., & Nolasco, S. (2022). The Apicomplexan parasite Toxoplasma gondii. *Encyclopedia*, *2*(1), 189–211.

Derouin, F., Pelloux, H., & ESCMID Study Group on Clinical Parasitology. (2008). Prevention of toxoplasmosis in transplant patients. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *14*(12), 1089–1101.

Donald, R. G., & Roos, D. S. (1994). Homologous recombination and gene replacement at the dihydrofolate reductase-thymidylate synthase locus in Toxoplasma gondii. *Molecular and Biochemical Parasitology*, *63*(2), 243–253.

Dubey, J. P., & Frenkel, J. K. (1976). Feline toxoplasmosis from acutely infected mice and the development of Toxoplasma cysts. *The Journal of Protozoology*, *23*(4), 537–546.

Dubey, J. P., Hill, D. E., Jones, J. L., Hightower, A. W., Kirkland, E., Roberts, J. M., Marcet, P. L., Lehmann, T., Vianna, M. C. B., Miska, K., Sreekumar, C., Kwok, O. C. H., Shen, S. K., & Gamble, H. R. (2005). Prevalence of viable Toxoplasma gondii in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. *The Journal of Parasitology*, *91*(5), 1082–1093.

Dubey, J. P., Huong, L. T. T., Lawson, B. W. L., Subekti, D. T., Tassi, P., Cabaj, W., Sundar, N., Velmurugan, G. V., Kwok, O. C. H., & Su, C. (2008). Seroprevalence and isolation of Toxoplasma gondii from free-range chickens in Ghana, Indonesia, Italy, Poland, and Vietnam. *The Journal of Parasitology*, *94*(1), 68–71.

Dubey, J. P., Lindsay, D. S., & Speer, C. A. (1998). Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clinical Microbiology Reviews*, *11*(2), 267–299.

Dubremetz, J. F., & Lebrun, M. (2012). Virulence factors of Toxoplasma gondii. *Microbes and Infection*, *14*(15), 1403–1410.

Duedu, K. O., Yarnie, E. A., Tetteh-Quarcoo, P. B., Attah, S. K., Donkor, E. S., & Ayeh-Kumi, P. F. (2014). A comparative survey of the prevalence of human parasites found in fresh vegetables sold in supermarkets and open-aired markets in Accra, Ghana. *BMC Research Notes*, *7*(1), 836.

Dunay, I. R., Gajurel, K., Dhakal, R., Liesenfeld, O., & Montoya, J. G. (2018). Treatment of toxoplasmosis: Historical perspective, animal models, and current clinical practice. *Clinical Microbiology Reviews*, *31*(4). https://doi.org/10.1128/CMR.00057-17

Ekman, C. C. J., Chiossi, M. F. do V., Meireles, L. R., Andrade Júnior, H. F. de, Figueiredo, W. M., Marciano, M. A. M., & Luna, E. J. de A. (2012). Case-control study of an outbreak of

acute toxoplasmosis in an industrial plant in the state of São Paulo, Brazil. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *54*(5), 239–244.

El Hajj, H., Lebrun, M., Arold, S. T., Vial, H., Labesse, G., & Dubremetz, J. F. (2007). ROP18 is a rhoptry kinase controlling the intracellular proliferation of Toxoplasma gondii. *PLoS Pathogens*, *3*(2), e14.

Elsafi, S. H., Al-Mutairi, W. F., Al-Jubran, K. M., Abu Hassan, M. M., & Al Zahrani, E. M. (2015). Toxoplasmosis seroprevalence in relation to knowledge and practice among pregnant women in Dhahran, Saudi Arabia. *Pathogens and Global Health*, *109*(8), 377–382.

Etheridge, R. D., Alaganan, A., Tang, K., Lou, H. J., Turk, B. E., & Sibley, L. D. (2014). The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host & Microbe*, *15*(5), 537–550.

Eyles, D. E., & Coleman, N. (1953). Synergistic effect of sulfadiazine and daraprim against experimental toxoplasmosis in the mouse. *Antibiotics & Chemotherapy (Northfield, Ill.)*, *3*(5), 483–490.

Fabiani, S., Fortunato, S., & Bruschi, F. (2018). Solid organ transplant and parasitic diseases: A review of the clinical cases in the last two decades. *Pathogens*, *7*(3), 65.

Fox, B. A., Ristuccia, J. G., Gigley, J. P., & Bzik, D. J. (2009). Efficient gene replacements in Toxoplasma gondii strains deficient for nonhomologous end joining. *Eukaryotic Cell*, *8*(4), 520–529.

Frénal, K., Polonais, V., Marq, J.-B., Stratmann, R., Limenitakis, J., & Soldati-Favre, D. (2010). Functional dissection of the apicomplexan glideosome molecular architecture. *Cell Host & Microbe*, *8*(4), 343–357.

Frenkel, J. K. (1973). Toxoplasma in and around Us. *Bioscience*, *23*(6), 343–352.

Frickel, E.-M., & Hunter, C. A. (2021). Lessons from Toxoplasma: Host responses that mediate parasite control and the microbial effectors that subvert them. *The Journal of Experimental Medicine*, *218*(11). https://doi.org/10.1084/jem.20201314

Fuentes, I., Rubio, J. M., Ramírez, C., & Alvar, J. (2001). Genotypic characterization of Toxoplasma gondii strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. *Journal of Clinical Microbiology*, *39*(4), 1566–1570.

Garin, J. P., & Eyles, D. E. (1958). Spiramycin therapy of experimental toxoplasmosis in mice. *Presse Medicale*. https://www.semanticscholar.org/paper/09d9d69e81ec5710ed11361ac4dde02356b6c420

Gavrilescu, L. C., Butcher, B. A., Del Rio, L., Taylor, G. A., & Denkers, E. Y. (2004). STAT1 is essential for antimicrobial effector function but dispensable for gamma interferon production during Toxoplasma gondii infection. *Infection and Immunity*, *72*(3), 1257–1264.

Gay, G., Braun, L., Brenier-Pinchart, M.-P., Vollaire, J., Josserand, V., Bertini, R.-L., Varesano, A., Touquet, B., De Bock, P.-J., Coute, Y., Tardieux, I., Bougdour, A., & Hakimi, M.-A. (2016). Toxoplasma gondii TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-γ-mediated host defenses. *The Journal of Experimental Medicine*, *213*(9), 1779–1798.

Gazzinelli, R. T., Hieny, S., Wynn, T. A., Wolf, S., & Sher, A. (1993). Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(13), 6115–6119.

Gazzinelli, R. T., Wysocka, M., Hayashi, S., Denkers, E. Y., Hieny, S., Caspar, P., Trinchieri, G., & Sher, A. (1994). Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii. *The Journal of Immunology*, *153*(6), 2533–2543.

Giuliano, C. J., Wei, K. J., Harling, F. M., Waldman, B. S., Farringer, M. A., Boydston, E. A., Lan, T. C. T., Thomas, R. W., Herneisen, A. L., Sanderlin, A. G., Coppens, I., Dvorin, J. D., & Lourido, S. (2024). CRISPR-based functional profiling of the Toxoplasma gondii genome during acute murine infection. *Nature Microbiology*, *9*(9), 2323–2343.

Goldman, M., Carver, R. K., & Sulzer, A. J. (1958). Reproduction of Toxoplasma gondii by internal budding. *The Journal of Parasitology*, *44*(2), 161–171.

Goldstein, E. J. C., Montoya, J. G., & Remington, J. S. (2008). Management of Toxoplasma gondii infection during pregnancy. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *47*(4), 554–566.

Gollub, E. L., Leroy, V., Gilbert, R., Chêne, G., Wallon, M., & European Toxoprevention Study Group (EUROTOXO). (2008). Effectiveness of health education on Toxoplasma-related knowledge, behaviour, and risk of seroconversion in pregnancy. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *136*(2), 137–145.

Gouda, M. A., Saied, S. A., Ashry, W. M. O., Abd-Eltwab, R. A.-R., Aldesoky, M. M., El-Dydamoni, O. A., Yousef, M., & El-Derbawy, M. M. (2024). Trends of Toxoplasma gondii and common transfusable venereal infections among blood donors in Menoufia Province, Egypt. *Scientific Reports*, *14*(1), 20920.

Gov, L., Karimzadeh, A., Ueno, N., & Lodoen, M. B. (2013). Human innate immunity to Toxoplasma gondii is mediated by host caspase-1 and ASC and parasite GRA15. *MBio*, *4*(4). https://doi.org/10.1128/mBio.00255-13

Gov, L., Schneider, C. A., Lima, T. S., Pandori, W., & Lodoen, M. B. (2017). NLRP3 and potassium efflux drive rapid IL-1β release from primary human monocytes during Toxoplasma gondii infection. *The Journal of Immunology*, *199*(8), 2855–2864.

Gras, S., Jackson, A., Woods, S., Pall, G., Whitelaw, J., Leung, J. M., Ward, G. E., Roberts, C. W., & Meissner, M. (2017). Parasites lacking the micronemal protein MIC2 are deficient in surface attachment and host cell egress, but remain virulent in vivo. *Wellcome Open Research*, *2*, 32.

Griffith, M. B., Pearce, C. S., & Heaslip, A. T. (2022). Dense granule biogenesis, secretion, and function in Toxoplasma gondii. *The Journal of Eukaryotic Microbiology*, *69*(6), e12904.

Grigg, M. E., Ganatra, J., Boothroyd, J. C., & Margolis, T. P. (2001). Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *The Journal of Infectious Diseases*, *184*(5), 633–639.

Guérin, A., El Hajj, H., Penarete-Vargas, D., Besteiro, S., & Lebrun, M. (2017). RON4L1 is a new member of the moving junction complex in Toxoplasma gondii. *Scientific Reports*, *7*(1), 17907.

Gustafson, P. V., Agar, H. D., & Cramer, D. I. (1954). An electron microscope study of Toxoplasma. *The American Journal of Tropical Medicine and Hygiene*, *3*(6), 1008–1022.

Haber, J. E. (2000). Partners and pathwaysrepairing a double-strand break. *Trends in Genetics: TIG*, *16*(6), 259–264.

Hakimi, M.-A., Olias, P., & Sibley, L. D. (2017). Toxoplasma effectors targeting host signaling and transcription. *Clinical Microbiology Reviews*, *30*(3), 615–645.

Harding, C. R., Sidik, S. M., Petrova, B., Gnädig, N. F., Okombo, J., Herneisen, A. L., Ward, K. E., Markus, B. M., Boydston, E. A., Fidock, D. A., & Lourido, S. (2020). Genetic screens reveal a central role for heme metabolism in artemisinin susceptibility. *Nature Communications*, *11*(1), 4813.

Harker, K. S., Ueno, N., & Lodoen, M. B. (2015). Toxoplasma gondii dissemination: a parasite's journey through the infected host. *Parasite Immunology*, *37*(3), 141–149.

Helegbe, G. K., Aryee, P. A., Mohammed, B. S., Wemakor, A., Kolbila, D., Abubakari, A.-W., Askanda, S., Alhassan, R., Barnie, C., Donkoh, A. A., & Ofosu, E. (2018). Seroprevalence of malaria and hepatitis B coinfection among pregnant women in Tamale Metropolis of Ghana: A cross-sectional study. *Journal Canadien Des Maladies Infectieuses et de La Microbiologie Medicale [The Canadian Journal of Infectious Diseases & Medical Microbiology]*, *2018*, 5610981.

Herm-Götz, A., Weiss, S., Stratmann, R., Fujita-Becker, S., Ruff, C., Meyhöfer, E., Soldati, T., Manstein, D. J., Geeves, M. A., & Soldati, D. (2002). Toxoplasma gondii myosin A and its light chain: a fast, single-headed, plus-end-directed motor. *The EMBO Journal*, *21*(9), 2149–2158.

Hernandez, D., Walsh, S., Saavedra Sanchez, L., Dickinson, M. S., & Coers, J. (2022). Interferon-inducible E3 ligase RNF213 facilitates host-protective linear and K63-linked ubiquitylation of Toxoplasma gondii parasitophorous vacuoles. *MBio*, *13*(5), e0188822.

Hesping, E., & Boddey, J. A. (2024). Whole-genome CRISPR screens to understand Apicomplexan-host interactions. *Molecular Microbiology*, *121*(4), 717–726.

Hill, D., & Dubey, J. P. (2002). Toxoplasma gondii: transmission, diagnosis and prevention. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *8*(10), 634–640.

Hu, X., Chakravarty, S. D., & Ivashkiv, L. B. (2008). Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunological Reviews*, *226*(1), 41–56.

Hu, X., Herrero, C., Li, W.-P., Antoniv, T. T., Falck-Pedersen, E., Koch, A. E., Woods, J. M., Haines, G. K., & Ivashkiv, L. B. (2002). Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation. *Nature Immunology*, *3*(9), 859–866.

Hunter, C. A., Subauste, C. S., Van Cleave, V. H., & Remington, J. S. (1994). Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infection and Immunity*, *62*(7), 2818–2824.

Hunter, Christopher A., & Sibley, L. D. (2012). Modulation of innate immunity by Toxoplasma gondii virulence effectors. *Nature Reviews. Microbiology*, *10*(11), 766–778.

Huynh, M.-H., & Carruthers, V. B. (2009). Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. *Eukaryotic Cell*, *8*(4), 530–539.

Innes, E. A., Hamilton, C., Garcia, J. L., Chryssafidis, A., & Smith, D. (2019). A one health approach to vaccines against Toxoplasma gondii. *Food and Waterborne Parasitology*, *15*(e00053), e00053.

Jacobs, L., Remington, J. S., & Melton, M. L. (1960). The resistance of the encysted form of Toxoplasma gondii. *The Journal of Parasitology*, *46*(1), 11–21.

Jacot, D., Tosetti, N., Pires, I., Stock, J., Graindorge, A., Hung, Y.-F., Han, H., Tewari, R., Kursula, I., & Soldati-Favre, D. (2016). An Apicomplexan actin-binding protein serves as a connector and lipid sensor to coordinate motility and invasion. *Cell Host & Microbe*, *20*(6), 731–743.

Janssen, R., Van Wengen, A., Verhard, E., De Boer, T., Zomerdijk, T., Ottenhoff, T. H. M., & Van Dissel, J. T. (2002). Divergent role for TNF-alpha in IFN-gamma-induced killing of Toxoplasma gondii and Salmonella typhimurium contributes to selective susceptibility of patients with partial IFN-gamma receptor 1 deficiency. *The Journal of Immunology*, *169*(7), 3900–3907.

Jensen, K. D. C., Wang, Y., Wojno, E. D. T., Shastri, A. J., Hu, K., Cornel, L., Boedec, E., Ong, Y.-C., Chien, Y.-H., Hunter, C. A., Boothroyd, J. C., & Saeij, J. P. J. (2011). Toxoplasma polymorphic effectors determine macrophage polarization and intestinal inflammation. *Cell Host & Microbe*, *9*(6), 472–483.

Jewett, T. J., & Sibley, L. D. (2003). Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. *Molecular Cell*, *11*(4), 885–894.

Khurana, S., & Batra, N. (2016). Toxoplasmosis in organ transplant recipients: Evaluation, implication, and prevention. *Tropical Parasitology*, *6*(2), 123–128.

Kijlstra, A., & Jongert, E. (2008). Control of the risk of human toxoplasmosis transmitted by meat. *International Journal for Parasitology*, *38*(12), 1359–1370.

Kim, K., & Weiss, L. M. (2004). Toxoplasma gondii: the model apicomplexan. *International Journal for Parasitology*, *34*(3), 423–432.

Koblansky, A. A., Jankovic, D., Oh, H., Hieny, S., Sungnak, W., Mathur, R., Hayden, M. S., Akira, S., Sher, A., & Ghosh, S. (2013). Recognition of profilin by Toll-like receptor 12 is critical for host resistance to Toxoplasma gondii. *Immunity*, *38*(1), 119–130.

Köhler, S., Delwiche, C. F., Denny, P. W., Tilney, L. G., Webster, P., Wilson, R. J., Palmer, J. D., & Roos, D. S. (1997). A plastid of probable green algal origin in Apicomplexan parasites. *Science (New York, N.Y.)*, *275*(5305), 1485–1489.

Köhler, Sabine. (2005). Multi-membrane-bound structures of Apicomplexa: I. the architecture of the Toxoplasma gondii apicoplast. *Parasitology Research*, *96*(4), 258–272.

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cacciò, S., Chalmers, R., … Robertson, L. (2018). Public health risks associated with food-borne parasites. *EFSA Journal*, *16*(12), e05495.

Kravets, E., Degrandi, D., Ma, Q., Peulen, T.-O., Klümpers, V., Felekyan, S., Kühnemuth, R., Weidtkamp-Peters, S., Seidel, C. A., & Pfeffer, K. (2016). Guanylate binding proteins directly attack Toxoplasma gondii via supramolecular complexes. *ELife*, *5*. https://doi.org/10.7554/eLife.11479

Krishnamurthy, S., Maru, P., Wang, Y., Bitew, M. A., Mukhopadhyay, D., Yamaryo-Botté, Y., Paredes-Santos, T. C., Sangaré, L. O., Swale, C., Botté, C. Y., & Saeij, J. P. J. (2023). CRISPR screens identify Toxoplasma genes that determine parasite fitness in interferon gamma-stimulated human cells. *MBio*, *14*(2), e0006023.

Krueger, W. S., Hilborn, E. D., Converse, R. R., & Wade, T. J. (2014). Drinking water source and human Toxoplasma gondii infection in the United States: a cross-sectional analysis of NHANES data. *BMC Public Health*, *14*(1), 711.

Kwofie, K. D., Ghansah, A., Osei, J. H. N., Frempong, K. K., Obed, S., Frimpong, E. H., Boakye, D. A., Suzuki, T., Ohta, N., & Ayi, I. (2016). Indication of risk of mother-to-child Toxoplasma gondii transmission in the greater Accra region of Ghana. *Maternal and Child Health Journal*, *20*(12), 2581–2588.

Lafrance-Girard, C., Arsenault, J., Thibodeau, A., Opsteegh, M., Avery, B., & Quessy, S. (2018). Toxoplasma gondiiin retail beef, lamb, and pork in Canada: Prevalence, quantification, and risk factors from a public health perspective. *Foodborne Pathogens and Disease*, *15*(12), 798–808.

Lamarque, M., Besteiro, S., Papoin, J., Roques, M., Vulliez-Le Normand, B., Morlon-Guyot, J., Dubremetz, J.-F., Fauquenoy, S., Tomavo, S., Faber, B. W., Kocken, C. H., Thomas, A. W., Boulanger, M. J., Bentley, G. A., & Lebrun, M. (2011). The RON2-AMA1 interaction is a critical step in moving junction-dependent invasion by apicomplexan parasites. *PLoS Pathogens*, *7*(2), e1001276.

Li, W., Grech, J., Stortz, J. F., Gow, M., Periz, J., Meissner, M., & Jimenez-Ruiz, E. (2022). A splitCas9 phenotypic screen in Toxoplasma gondii identifies proteins involved in host cell egress and invasion. *Nature Microbiology*, *7*(6), 882–895.

Lieberman, L. A., Banica, M., Reiner, S. L., & Hunter, C. A. (2004). STAT1 plays a critical role in the regulation of antimicrobial effector mechanisms, but not in the development of Th1-type responses during toxoplasmosis. *The Journal of Immunology*, *172*(1), 457–463.

Ma, J. S., Sasai, M., Ohshima, J., Lee, Y., Bando, H., Takeda, K., & Yamamoto, M. (2014). Selective and strain-specific NFAT4 activation by the Toxoplasma gondii polymorphic dense granule protein GRA6. *The Journal of Experimental Medicine*, *211*(10), 2013–2032.

Mansouri, A., Adhami Mojarad, M. R., Badfar, G., Abasian, L., Rahmati, S., Kooti, W., YektaKooshali, M. H., Soleymani, A., & Azami, M. (2017). Epidemiology of Toxoplasma gondii among blood donors in Iran: A systematic review and meta-analysis. *Transfusion and Apheresis Science: Official Journal of the World Apheresis Association: Official Journal of the European Society for Haemapheresis*, *56*(3), 404–409.

Martorelli Di Genova, B., & Knoll, L. J. (2020). Comparisons of the sexual cycles for the Coccidian parasites Eimeria and Toxoplasma. *Frontiers in Cellular and Infection Microbiology*, *10*, 604897.

Matta, S. K., Kohio, H. P., Chandra, P., Brown, A., Doench, J. G., Philips, J. A., Ding, S., & David Sibley, L. (2023). *Genome- wide and targeted CRISPR screens identify RNF213 as a mediator of interferon gamma–dependent pathogen restriction in human cells*. https://doi.org/10.1073/pnas.2315865120#supplementary-materials

Mazumdar, J., H Wilson, E., Masek, K., A Hunter, C., & Striepen, B. (2006). Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in Toxoplasma gondii. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(35), 13192–13197.

Meissner, M., Reiss, M., Viebig, N., Carruthers, V. B., Toursel, C., Tomavo, S., Ajioka, J. W., & Soldati, D. (2002). A family of transmembrane microneme proteins of Toxoplasma gondii contain EGF-like domains and function as escorters. *Journal of Cell Science*, *115*(Pt 3), 563–574.

Meissner, M., Schlüter, D., & Soldati, D. (2002). Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. *Science (New York, N.Y.)*, *298*(5594), 837–840.

Melo, E. J., Attias, M., & De Souza, W. (2000). The single mitochondrion of tachyzoites of Toxoplasma gondii. *Journal of Structural Biology*, *130*(1), 27–33.

Millar, P. R., Moura, F. L. de, Bastos, O. M. P., Mattos, D. P. B. G. de, Fonseca, A. B. M., Sudré, A. P., Leles, D., & Amendoeira, M. R. R. (2014). Toxoplasmosis-related knowledge among pregnant and postpartum women attended in public health units in Niterói, Rio De Janeiro, Brazil. *Revista Do Instituto de Medicina Tropical de Sao Paulo*, *56*(5), 433–438.

Miro, J. M., Lopez, J. C., Podzamczer, D., Peña, J. M., Alberdi, J. C., Martínez, E., Domingo, P., Cosin, J., Claramonte, X., Arribas, J. R., Santín, M., Ribera, E., & GESIDA 04/98 Study Group. (2006). Discontinuation of primary and secondary Toxoplasma gondii prophylaxis is safe in HIV-infected patients after immunological restoration with highly active antiretroviral therapy: results of an open, randomized, multicenter clinical trial. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *43*(1), 79–89.

Moghaddami, R., Mahdipour, M., & Ahmadpour, E. (2024). Inflammatory pathways of Toxoplasmagondii infection in pregnancy. *Travel Medicine and Infectious Disease*, *62*(102760), 102760.

Montoya, J. G., & Remington, J. S. (2008). Management of Toxoplasma gondii infection during pregnancy. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *47*(4), 554–566.

Mordue, D. G., & Sibley, L. D. (1997). Intracellular fate of vacuoles containing Toxoplasma gondii is determined at the time of formation and depends on the mechanism of entry. *The Journal of Immunology*, *159*(9), 4452–4459.

Mukhopadhyay, D., Arranz-Solís, D., & Saeij, J. P. J. (2020). Influence of the host and parasite strain on the immune response during Toxoplasma infection. *Frontiers in Cellular and Infection Microbiology*, *10*, 580425.

Nguyen, B. T., & Stadtsbaeder, S. (1983). Comparative effects of cotrimoxazole (trimethoprim-sulphamethoxazole), pyrimethamine-sulphadiazine and spiramycin during avirulent infection with Toxoplasma gondii (Beverley strain) in mice. *British Journal of Pharmacology*, *79*(4), 923–928.

Ohshima, J., Lee, Y., Sasai, M., Saitoh, T., Su Ma, J., Kamiyama, N., Matsuura, Y., Pann-Ghill, S., Hayashi, M., Ebisu, S., Takeda, K., Akira, S., & Yamamoto, M. (2014). Role of mouse and human autophagy proteins in IFN-γ-induced cell-autonomous responses against Toxoplasma gondii. *The Journal of Immunology*, *192*(7), 3328–3335.

Olias, P., Etheridge, R. D., Zhang, Y., Holtzman, M. J., & Sibley, L. D. (2016). Toxoplasma effector recruits the Mi-2/NuRD complex to repress STAT1 transcription and block IFN-γ-dependent gene expression. *Cell Host & Microbe*, *20*(1), 72–82.

Opsteegh, M., Kortbeek, T. M., Havelaar, A. H., & van der Giessen, J. W. B. (2015). Intervention strategies to reduce human Toxoplasma gondii disease burden. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *60*(1), 101–107.

Opsteegh, M., Schares, G., Blaga, R., & van der Giessen, J. (2016). Experimental studies on Toxoplasma gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report. *EFSA Supporting Publications*, *13*(2). https://doi.org/10.2903/sp.efsa.2016.en-995

Panas, M. W., & Boothroyd, J. C. (2021). Seizing control: How dense granule effector proteins enable Toxoplasma to take charge. *Molecular Microbiology*, *115*(3), 466–477.

Pappoe, F., Cheng, W., Wang, L., Li, Y., Obiri-Yeboah, D., Nuvor, S. V., Ambachew, H., Hu, X., Luo, Q., Chu, D., Xu, Y., & Shen, J. (2017). Prevalence of Toxoplasma gondii infection in HIV-infected patients and food animals and direct genotyping of T. gondii isolates, Southern Ghana. *Parasitology Research*, *116*(6), 1675–1685.

Paredes-Santos, T. C., de Souza, W., & Attias, M. (2012). Dynamics and 3D organization of secretory organelles of Toxoplasma gondii. *Journal of Structural Biology*, *177*(2), 420–430.

Pavlou, G., Biesaga, M., Touquet, B., Lagal, V., Balland, M., Dufour, A., Hakimi, M.-A., & Tardieux, I. (2018). Toxoplasma parasite twisting motion mechanically induces host cell membrane fission to complete invasion within a protective vacuole. *Cell Host & Microbe*, *24*(1), 81-96.e5.

Pelletier, L., Stern, C. A., Pypaert, M., Sheff, D., Ngô, H. M., Roper, N., He, C. Y., Hu, K., Toomre, D., Coppens, I., Roos, D. S., Joiner, K. A., & Warren, G. (2002). Golgi biogenesis in Toxoplasma gondii. *Nature*, *418*(6897), 548–552.

Pereboom, M. T. R., Manniën, J., Spelten, E. R., Schellevis, F. G., & Hutton, E. K. (2013). Observational study to assess pregnant women's knowledge and behaviour to prevent toxoplasmosis, listeriosis and cytomegalovirus. *BMC Pregnancy and Childbirth*, *13*(1), 98.

Peyron, F., Mc Leod, R., Ajzenberg, D., Contopoulos-Ioannidis, D., Kieffer, F., Mandelbrot, L., Sibley, L. D., Pelloux, H., Villena, I., Wallon, M., & Montoya, J. G. (2017). Congenital toxoplasmosis in France and the United States: One parasite, two diverging approaches. *PLoS Neglected Tropical Diseases*, *11*(2), e0005222.

Pfefferkorn, E. R., & Wyler, D. J. (1990). Modern parasite biology. Cellular, immunological & molecular aspects. *WH Freeman and Co.*

Putignani, L., Mancinelli, L., Del Chierico, F., Menichella, D., Adlerstein, D., Angelici, M. C., Marangi, M., Berrilli, F., Caffara, M., di Regalbono, D. A. F., & Giangaspero, A. (2011). Investigation of Toxoplasma gondii presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG). *Experimental Parasitology*, *127*(2), 409–417.

Rabenau, K. E., Sohrabi, A., Tripathy, A., Reitter, C., Ajioka, J. W., Tomley, F. M., & Carruthers, V. B. (2001). TgM2AP participates in Toxoplasma gondii invasion of host cells and is tightly associated with the adhesive protein TgMIC2: TgM2AP and Toxoplasma invasion. *Molecular Microbiology*, *41*(3), 537–547.

Rastogi, S., Cygan, A. M., & Boothroyd, J. C. (2019). Translocation of effector proteins into host cells by Toxoplasma gondii. *Current Opinion in Microbiology*, *52*, 130–138.

Reese, M. L., & Boothroyd, J. C. (2011). A conserved non-canonical motif in the pseudoactive site of the ROP5 pseudokinase domain mediates its effect on Toxoplasma virulence. *The Journal of Biological Chemistry*, *286*(33), 29366–29375.

Reese, M. L., Zeiner, G. M., Saeij, J. P. J., Boothroyd, J. C., & Boyle, J. P. (2011). Polymorphic family of injected pseudokinases is paramount in Toxoplasma virulence. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(23), 9625–9630.

Reynolds, P. K. A.-. (2020). Toxoplasmosis in humans and animals in Ghana (1962 – 2020): A review. *International Journal of Tropical Disease & Health*, *41*(14), 20–31.

Robert-Gangneux, F., & Dardé, M.-L. (2012). Epidemiology of and diagnostic strategies for toxoplasmosis. *Clinical Microbiology Reviews*, *25*(2), 264–296.

Rommereim, L. M., Fox, B. A., Butler, K. L., Cantillana, V., Taylor, G. A., & Bzik, D. J. (2019). Rhoptry and dense granule secreted effectors regulate CD8+ T cell recognition of Toxoplasma gondii infected host cells. *Frontiers in Immunology*, *10*, 2104.

Roos, D. S., Crawford, M. J., Donald, R. G., Kissinger, J. C., Klimczak, L. J., & Striepen, B. (1999). Origin, targeting, and function of the apicomplexan plastid. *Current Opinion in Microbiology*, *2*(4), 426–432.

Roos, David S., Donald, R. G. K., Morrissette, N. S., & Moulton, A. L. C. (1995). Chapter 3: Molecular tools for genetic dissection of the protozoan parasite Toxoplasma gondii. In D. G. Russell (Ed.), *Methods in Cell Biology* (Vol. 45, pp. 27–63). Elsevier.

Rosowski, E. E., Lu, D., Julien, L., Rodda, L., Gaiser, R. A., Jensen, K. D. C., & Saeij, J. P. J. (2011). Strain-specific activation of the NF-kappaB pathway by GRA15, a novel Toxoplasma gondii dense granule protein. *The Journal of Experimental Medicine*, *208*(1), 195–212.

Sabin, A. B., & Warren., J. (1942). *Therapeutic Effectiveness of Certain Sulfonamides on Infection by an Intracellular Protozoon (Toxoplasma)*. Soc Exp Biol Med 51:19–23. https://doi.org/10.3181/00379727-51-13809

Sacks, J. J., Roberto, R. R., & Brooks, N. F. (1982). Toxoplasmosis infection associated with raw goat's milk. *JAMA: The Journal of the American Medical Association*, *248*(14), 1728–1732.

Saeij, J. P. J., Boyle, J. P., Coller, S., Taylor, S., Sibley, L. D., Brooke-Powell, E. T., Ajioka, J. W., & Boothroyd, J. C. (2006). Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science (New York, N.Y.)*, *314*(5806), 1780–1783.

Saeij, J. P. J., Coller, S., Boyle, J. P., Jerome, M. E., White, M. W., & Boothroyd, J. C. (2007). Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature*, *445*(7125), 324–327.

Safronova, A., Araujo, A., Camanzo, E. T., Moon, T. J., Elliott, M. R., Beiting, D. P., & Yarovinsky, F. (2019). Alarmin S100A11 initiates a chemokine response to the human pathogen Toxoplasma gondii. *Nature Immunology*, *20*(1), 64–72.

Sanchez, S. G., & Besteiro, S. (2021). The pathogenicity and virulence of Toxoplasma gondii. *Virulence*, *12*(1), 3095–3114.

Sangaré, L. O., Ólafsson, E. B., Wang, Y., Yang, N., Julien, L., Camejo, A., Pesavento, P., Sidik, S. M., Lourido, S., Barragan, A., & Saeij, J. P. J. (2019). In vivo CRISPR screen identifies TgWIP as a Toxoplasma modulator of dendritic cell migration. *Cell Host & Microbe*, *26*(4), 478-492.e8.

Sasai, M., & Yamamoto, M. (2019). Innate, adaptive, and cell-autonomous immunity against Toxoplasma gondii infection. *Experimental & Molecular Medicine*, *51*(12), 1–10.

Scanga, C. A., Aliberti, J., Jankovic, D., Tilloy, F., Bennouna, S., Denkers, E. Y., Medzhitov, R., & Sher, A. (2002). Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *The Journal of Immunology*, *168*(12), 5997–6001.

Schneider, W. M., Chevillotte, M. D., & Rice, C. M. (2014). Interferon-stimulated genes: a complex web of host defenses. *Annual Review of Immunology*, *32*(1), 513–545.

Sefah-Boakye, J., Frimpong, E. H., Dompreh, A., & Turpin, A. C. (2016). Seroprevalence of toxoplasma gondii infection among pregnant women in the ashanti region of ghana. *International Journal of Microbiology & Advanced Immunology*, *4*(1), 70–74.

Seizova, S., Ferrel, A., Boothroyd, J., & Tonkin, C. J. (2024). Toxoplasma protein export and effector function. *Nature Microbiology*, *9*(1), 17–28.

Selleck, E. M., Fentress, S. J., Beatty, W. L., Degrandi, D., Pfeffer, K., Virgin, H. W., 4th, Macmicking, J. D., & Sibley, L. D. (2013). Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against Toxoplasma gondii. *PLoS Pathogens*, *9*(4), e1003320.

Shammaa, A. M., Powell, T. G., & Benmerzouga, I. (2021). Adverse outcomes associated with the treatment of Toxoplasma infections. *Scientific Reports*, *11*(1), 1035.

Shapiro, K., Bahia-Oliveira, L., Dixon, B., Dumètre, A., de Wit, L. A., VanWormer, E., & Villena, I. (2019). Environmental transmission of Toxoplasma gondii: Oocysts in water, soil and food. *Food and Waterborne Parasitology*, *15*(e00049), e00049.

Sheffield, H. G., & Melton, M. L. (1968). The fine structure and reproduction of Toxoplasma gondii. *The Journal of Parasitology*, *54*(2), 209–226.

Shen, B., Brown, K., Long, S., & Sibley, L. D. (2017). Development of CRISPR/Cas9 for Efficient Genome Editing in Toxoplasma gondii. *Methods in Molecular Biology (Clifton, N.J.)*, *1498*, 79–103.

Sibley, L. D., & Boothroyd, J. C. (1992). Virulent strains of Toxoplasma gondii comprise a single clonal lineage. *Nature*, *359*(6390), 82–85.

Sibley, L. David, & Ajioka, J. W. (2008). Population structure of Toxoplasma gondii: clonal expansion driven by infrequent recombination and selective sweeps. *Annual Review of Microbiology*, *62*(1), 329–351.

Sibley, L. David, Charron, A. J., Hakansson, S., & Mordue, D. G. (2007). Invasion and intracellular survival by Toxoplasma. *Protozoans in Macrophages*, 16–24.

Sidik, S. M., Huet, D., Ganesan, S. M., Huynh, M.-H., Wang, T., Nasamu, A. S., Thiru, P., Saeij, J. P. J., Carruthers, V. B., Niles, J. C., & Lourido, S. (2016). A genome-wide CRISPR screen in Toxoplasma identifies essential Apicomplexan genes. *Cell*, *166*(6), 1423-1435.e12.

Smereka, J., Szarpak, L., Ruetzler, K., Schacham, Y., Smereka, A., Dabrowski, M., Terpilowska, M., Terpilowski, L., & Adam, I. (2018). A multicenter survey on toxoplasmosis knowledge among pregnant women in Poland (the TOWER study). *BMC Pregnancy and Childbirth*, *18*(1), 389.

Smith, N. C., Goulart, C., Hayward, J. A., Kupz, A., Miller, C. M., & van Dooren, G. G. (2021). Control of human toxoplasmosis. *International Journal for Parasitology*, *51*(2–3), 95–121.

Soldati, D., Dubremetz, J. F., & Lebrun, M. (2001). Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite Toxoplasma gondii. *International Journal for Parasitology*, *31*(12), 1293–1302.

Splendor, A. (2009). A new protozoan parasite in rabbits. *International Journal for Parasitology*, *39*(8), 861–862.

Su, C., Evans, D., Cole, R. H., Kissinger, J. C., Ajioka, J. W., & Sibley, L. D. (2003). Recent expansion of Toxoplasma through enhanced oral transmission. *Science (New York, N.Y.)*, *299*(5605), 414–416.

Suzuki, Y., Orellana, M. A., Schreiber, R. D., & Remington, J. S. (1988). Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. *Science (New York, N.Y.)*, *240*(4851), 516–518.

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S. J., Tang, K., Beatty, W. L., Hajj, H. E., Jerome, M., Behnke, M. S., White, M., Wootton, J. C., & Sibley, L. D. (2006). A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. *Science (New York, N.Y.)*, *314*(5806), 1776–1780.

Tedde, T., Marangi, M., Papini, R., Salza, S., Normanno, G., Virgilio, S., & Giangaspero, A. (2019). Toxoplasma gondii and Other Zoonotic Protozoans in Mediterranean Mussel (Mytilus galloprovincialis) and Blue Mussel (Mytilus edulis): A Food Safety Concern? *Journal of Food Protection*, *82*(3), 535–542.

Tenter, A. M., Heckeroth, A. R., & Weiss, L. M. (2000). Toxoplasma gondii: from animals to humans. *International Journal for Parasitology*, *30*(12–13), 1217–1258.

Tonkin, M. L., Roques, M., Lamarque, M. H., Pugnière, M., Douguet, D., Crawford, J., Lebrun, M., & Boulanger, M. J. (2011). Host cell invasion by apicomplexan parasites: insights from the co-structure of AMA1 with a RON2 peptide. *Science (New York, N.Y.)*, *333*(6041), 463–467.

Tosh, K. W., Mittereder, L., Bonne-Annee, S., Hieny, S., Nutman, T. B., Singer, S. M., Sher, A., & Jankovic, D. (2016). The IL-12 response of primary human dendritic cells and monocytes to Toxoplasma gondii is stimulated by phagocytosis of live parasites rather than host cell invasion. *The Journal of Immunology*, *196*(1), 345–356.

Tsai, S. Q., Zheng, Z., Nguyen, N. T., Liebers, M., Topkar, V. V., Thapar, V., Wyvekens, N., Khayter, C., Iafrate, A. J., Le, L. P., Aryee, M. J., & Joung, J. K. (2015). GUIDE-seq enables

genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology*, *33*(2), 187–197.

Tyler, J. S., & Boothroyd, J. C. (2011). The C-terminus of Toxoplasma RON2 provides the crucial link between AMA1 and the host-associated invasion complex. *PLoS Pathogens*, *7*(2), e1001282.

Velázquez-Hernández, N., Avilés Ávila, A. Y., Rivas-González, M. A., Delgado-González, S. P., Alvarado-Félix, G. A., Alvarado-Félix, Á. O., Beristain-Garcia, I., & Alvarado-Esquivel, C. (2019). Knowledge and practices regarding toxoplasmosis in housewives: A cross sectional study in a northern Mexican city. *PloS One*, *14*(9), e0222094.

Villena, I., Aubert, D., Gomis, P., Ferté, H., Inglard, J.-C., Denis-Bisiaux, H., Dondon, J.-M., Pisano, E., Ortis, N., & Pinon, J.-M. (2004). Evaluation of a strategy for Toxoplasma gondii oocyst detection in water. *Applied and Environmental Microbiology*, *70*(7), 4035–4039.

Wainwright, K. E., Miller, M. A., Barr, B. C., Gardner, I. A., Melli, A. C., Essert, T., Packham, A. E., Truong, T., Lagunas-Solar, M., & Conrad, P. A. (2007). Chemical inactivation of Toxoplasma gondii oocysts in water. *The Journal of Parasitology*, *93*(4), 925–931.

Wang, J.-L., Huang, S.-Y., Behnke, M. S., Chen, K., Shen, B., & Zhu, X.-Q. (2016). The past, present, and future of genetic manipulation in Toxoplasma gondii. *Trends in Parasitology*, *32*(7), 542–553.

Wang, T., Wei, J. J., Sabatini, D. M., & Lander, E. S. (2014). Genetic screens in human cells using the CRISPR-Cas9 system. *Science (New York, N.Y.)*, *343*(6166), 80–84.

Wang, Yanhua, & Yin, H. (2015). Research advances in microneme protein 3 of Toxoplasma gondii. *Parasites & Vectors*, *8*(1), 384.

Wang, Yifan, Hollingsworth, L. R., Sangaré, L. O., Paredes-Santos, T. C., Krishnamurthy, S., Penn, B. H., Wu, H., & Saeij, J. P. J. (2024). Host E3 ubiquitin ligase ITCH mediates Toxoplasma gondii effector GRA35-triggered NLRP1 inflammasome activation and cell-autonomous immunity. *MBio*, *15*(3), e0330223.

Wang, Yifan, Sangaré, L. O., Paredes-Santos, T. C., Hassan, M. A., Krishnamurthy, S., Furuta, A. M., Markus, B. M., Lourido, S., & Saeij, J. P. J. (2020). Genome-wide screens identify Toxoplasma gondii determinants of parasite fitness in IFNγ-activated murine macrophages. *Nature Communications*, *11*(1), 5258.

Wanko, T., Jacobs, L., & Gavin, M. A. (1962). Electron microscope study of Toxoplasma cysts in mouse brain. *The Journal of Protozoology*, *9*(2), 235–242.

Weilhammer, D. R., & Rasley, A. (2011). Genetic approaches for understanding virulence in Toxoplasma gondii. *Briefings in Functional Genomics*, *10*(6), 365–373.

Weiss, L. M., & Dubey, J. P. (2009). Toxoplasmosis: A history of clinical observations. *International Journal for Parasitology*, *39*(8), 895–901.

Wesołowski, R., Pawłowska, M., & Mila-Kierzenkowska, C. (2023). The medical relevance of Toxoplasma infections in terms of the safety of blood recipients under immunosuppression-A meta-analysis. *Microorganisms*, *11*(8). https://doi.org/10.3390/microorganisms11081980

Wesołowski, R., Pawłowska, M., Smoguła, M., & Szewczyk-Golec, K. (2023). Advances and challenges in diagnostics of toxoplasmosis in HIV-infected patients. *Pathogens*, *12*(1), 110.

Wilson, D. C., Matthews, S., & Yap, G. S. (2008). IL-12 signaling drives CD8+ T cell IFN-gamma production and differentiation of KLRG1+ effector subpopulations during Toxoplasma gondii Infection. *The Journal of Immunology*, *180*(9), 5935–5945.

Witola, W. H., Mui, E., Hargrave, A., Liu, S., Hypolite, M., Montpetit, A., Cavailles, P., Bisanz, C., Cesbron-Delauw, M.-F., Fournié, G. J., & McLeod, R. (2011). NALP1 influences susceptibility to human congenital toxoplasmosis, proinflammatory cytokine response, and fate of Toxoplasma gondii-infected monocytic cells. *Infection and Immunity*, *79*(2), 756–766.

Yamamoto, M., Okuyama, M., Ma, J. S., Kimura, T., Kamiyama, N., Saiga, H., Ohshima, J., Sasai, M., Kayama, H., Okamoto, T., Huang, D. C. S., Soldati-Favre, D., Horie, K., Takeda, J., & Takeda, K. (2012). A cluster of interferon-γ-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. *Immunity*, *37*(2), 302–313.

Yamamoto, M., Standley, D. M., Takashima, S., Saiga, H., Okuyama, M., Kayama, H., Kubo, E., Ito, H., Takaura, M., Matsuda, T., Soldati-Favre, D., & Takeda, K. (2009). A single polymorphic amino acid on Toxoplasma gondii kinase ROP16 determines the direct and strain-specific activation of Stat3. *The Journal of Experimental Medicine*, *206*(12), 2747–2760.

Yarovinsky, F., Zhang, D., Andersen, J. F., Bannenberg, G. L., Serhan, C. N., Hayden, M. S., Hieny, S., Sutterwala, F. S., Flavell, R. A., Ghosh, S., & Sher, A. (2005). TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science (New York, N.Y.)*, *308*(5728), 1626–1629.

Ybañez, R. H. D., Ybañez, A. P., & Nishikawa, Y. (2020). Review on the current trends of toxoplasmosis serodiagnosis in humans. *Frontiers in Cellular and Infection Microbiology*, *10*, 204.

Zhao, Y. O., Khaminets, A., Hunn, J. P., & Howard, J. C. (2009). Disruption of the Toxoplasma gondii parasitophorous vacuole by IFNgamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. *PLoS Pathogens*, *5*(2), e1000288.

Zhu, W., Li, J., Pappoe, F., Shen, J., & Yu, L. (2019). Strategies Developed by Toxoplasma gondii to Survive in the Host. *Frontiers in Microbiology*, *10*, 899.

APPENDICES

Appendix 1

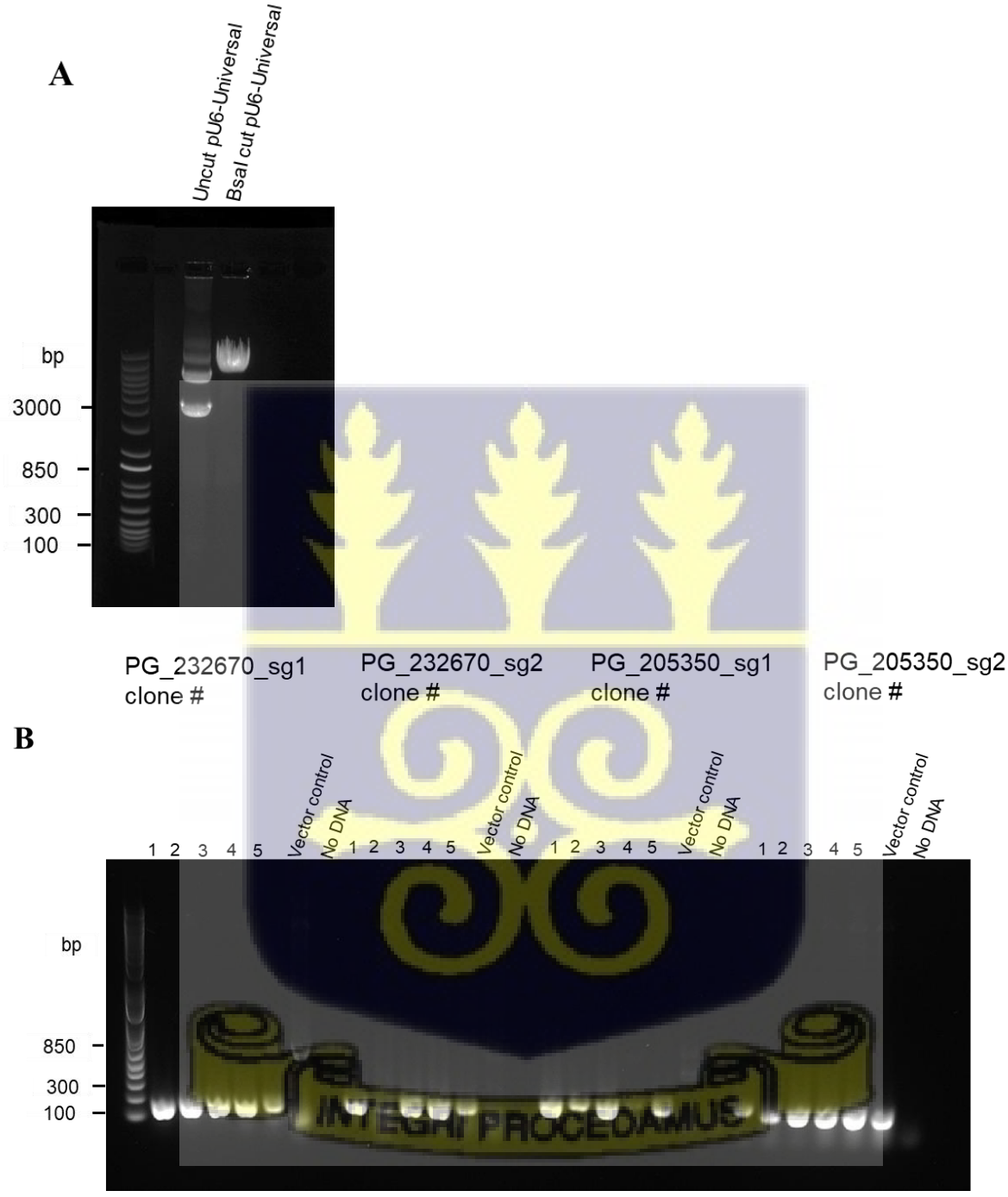
Table A1: Primers used in the study

Primer name	Primer sequence
232670_Mut-F	AGAACGGATGGAACTTCTGGACGG
232670_Mut-R	AGTTGCCGTCTTCCACCGTCAG
232670_sg1-F	AAGTTGACAGCAGCAACTGTTCGCAGG*
232670_sg1-R	AAAACCTGCGAACAGTTGCTGCTGTCA*
232670_sg2-F	AAGTTGAAGCAGCGACGAATTGTGCCG*
232670_sg2-R	AAAACGGCACAATTCGTCGCTGCTTCA*
205350_Mut-F	CGAGTTTCTCGCGTACTACCGAC
TGGT1_205350_R	GGTCTGTTCTTCATCCATCTCGTG
205350_sg1-F	AAGTTGTATCCGAGAAACGAGTAGCAG*
205350_sg1-R	AAAACTGCTACTCGTTTCTCGGATACA*
205350_sg2-F	AAGTTGGGCATGCCTAGCACCTATCG*
205350_sg2-R	AAAACGATAGGTGCTAGGCATGCCCA*
TGGT1_246178_Mut-F	GTACACAAAGCTGTTCCCCTGTC
TGGT1_246178_Mut-R	AGTGATGACAGCAATCCGGCAAC
pLIC-232670-F	#TACTTCCAATCCAATTTAATGCGCCAGCGTTCGACTGGAAAAGG
pLIC-232670-R	#TCCTCCACTTCCAATTTTAGCAATTACACCGAAACGTTCAGCCATC
pLIC-205350-F	#TACTTCCAATCCAATTTAATGCAGGCGCCCACTGAAATACTGGAAC
pLIC-205350-R	#TCCTCCACTTCCAATTTTAGCCTGAGCCTTTTCCGCCTGGATTTTC
pU6_Seq-F	CAAATGGCGACCTGCAGAGG
GFPinpTKO_R	ATCCCAACGAAAAGAGAGACCAC

*BsaI adaptor sequence & #pLIC plasmid adaptor sequence for cloning (in red)

Appendix 2

Constructing pU6-sgRNA plasmid



Constructing pLIC plasmid

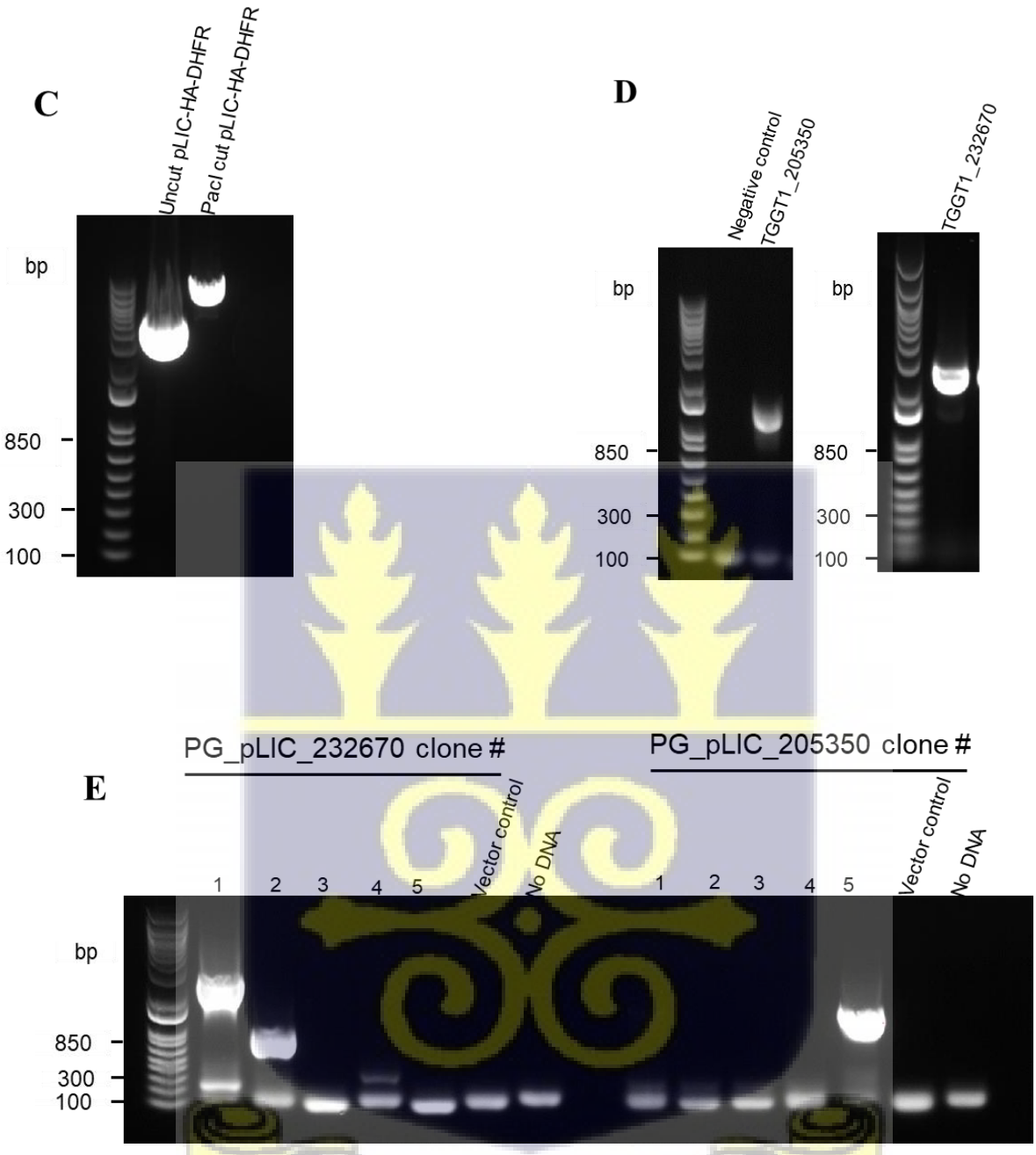
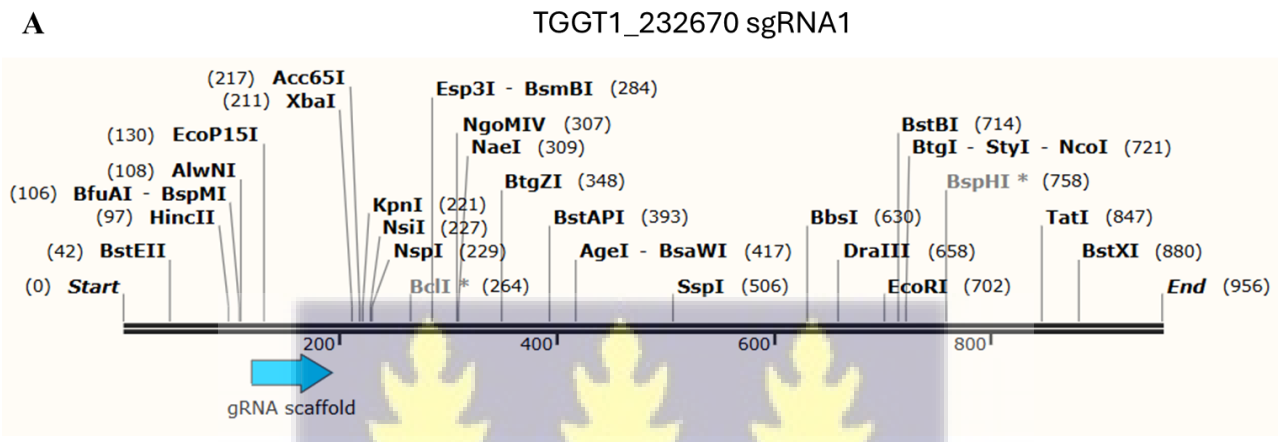


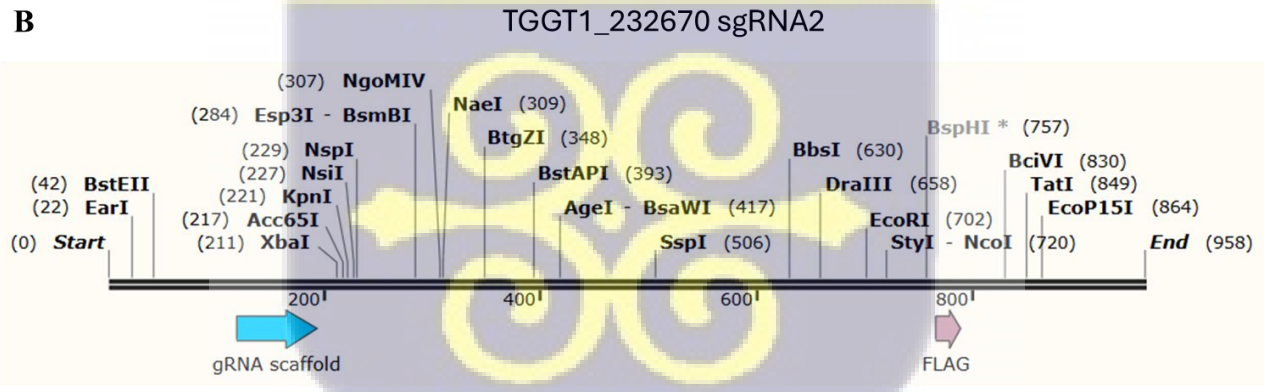
Figure A1: Plasmid construction and colony PCR. (A) BsaI linearized pU6-Universal plasmid (B) sgRNA Colony PCR (C) PacI linearized pLIC-HA-DHFR plasmid (D) Homologous arm

amplification of TGGT1_205350 and TGGT1_232670 for tagging (E) pLIC colony PCR to detect positive clones for plasmid sequencing

Appendix 3

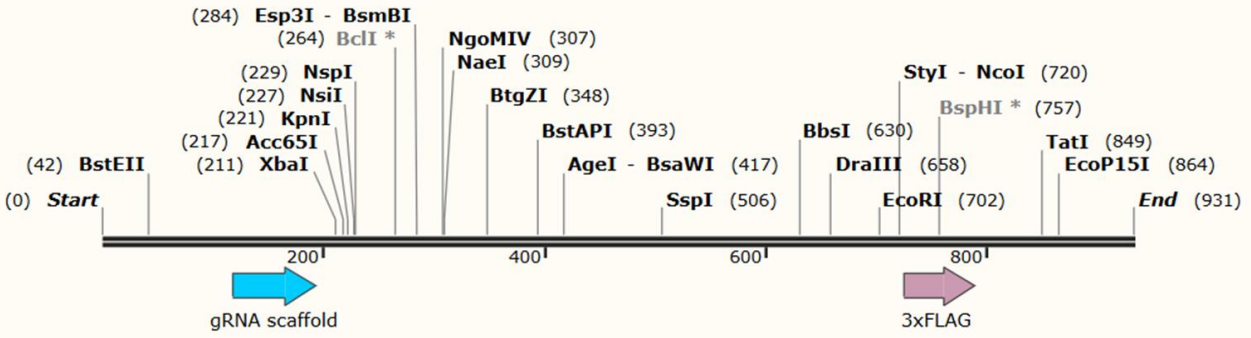
Sanger sequencing results: TGGT1_232670 sgRNA



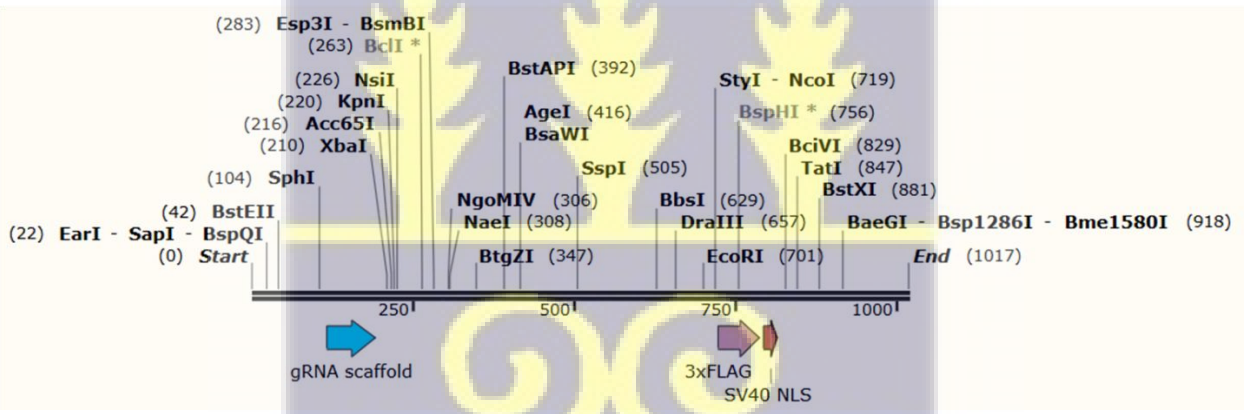


Sanger sequencing results: TGGT1_205350 sgRNA

C TGGT1_205350 sgRNA1

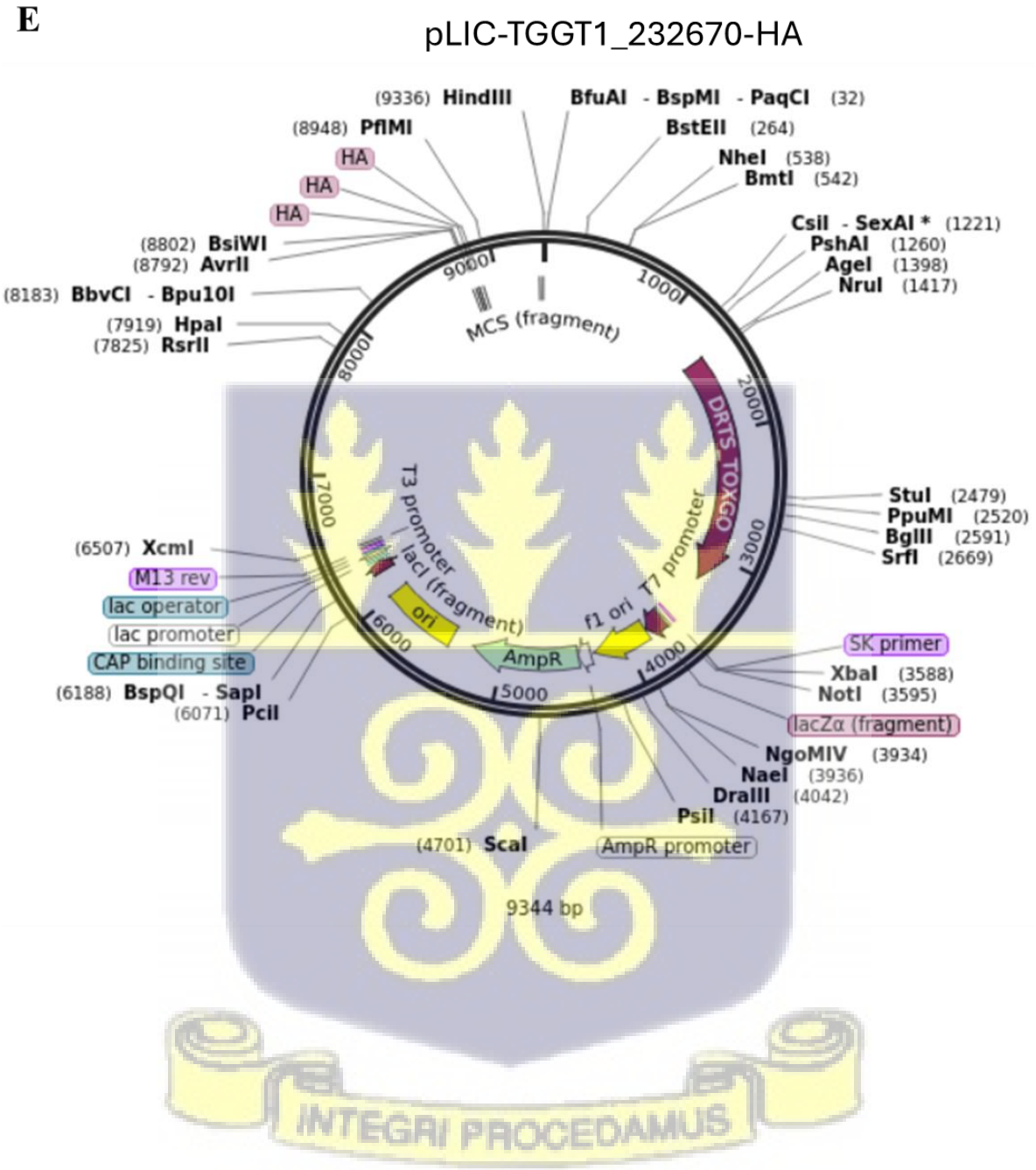


D TGGT1_205350 sgRNA2



Nanopore whole plasmid sequencing results: pLIC_TGGT1_232670-HA

E



Nanopore whole plasmid sequencing results: pLIC_TGGT1_205350-HA

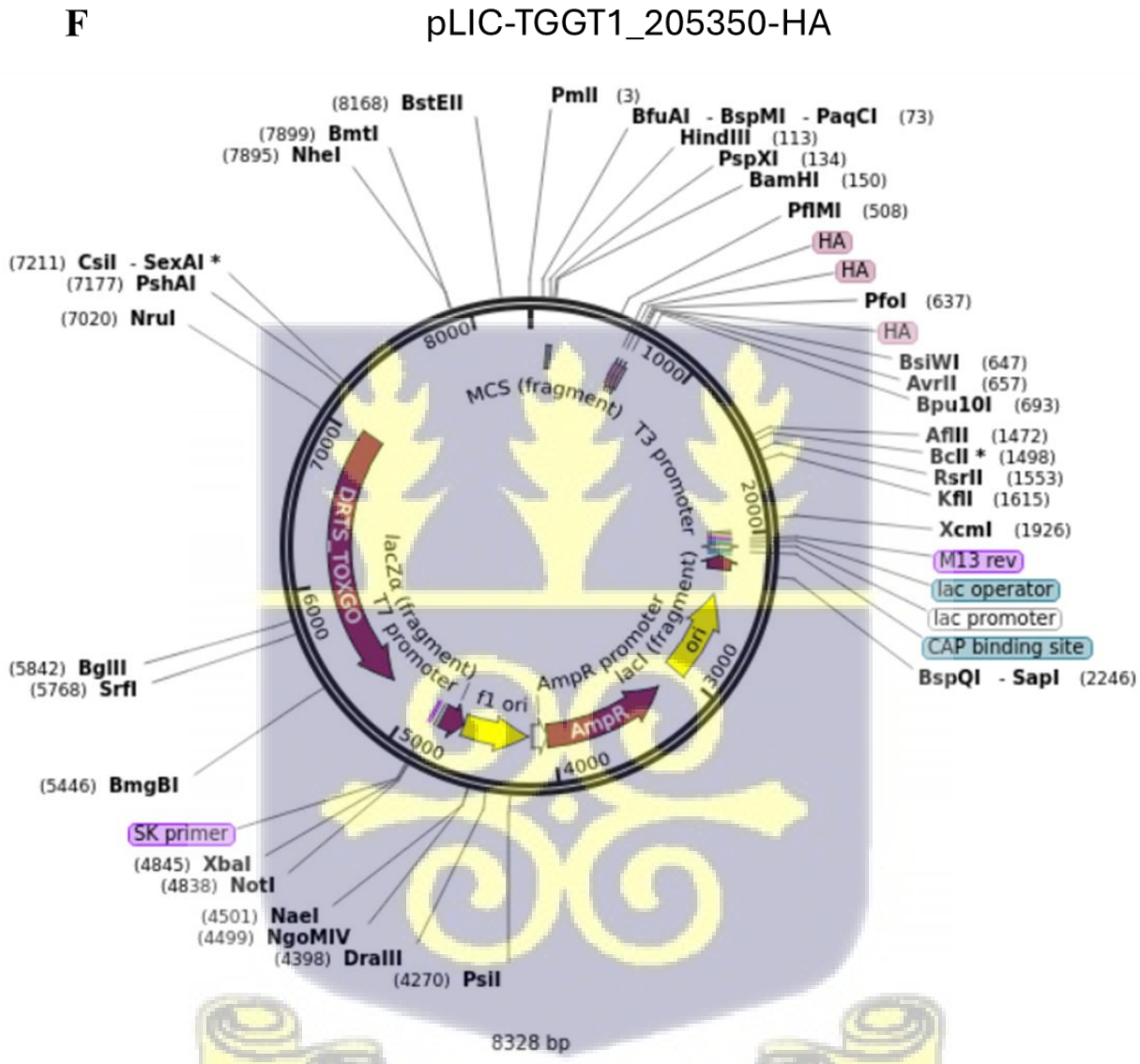
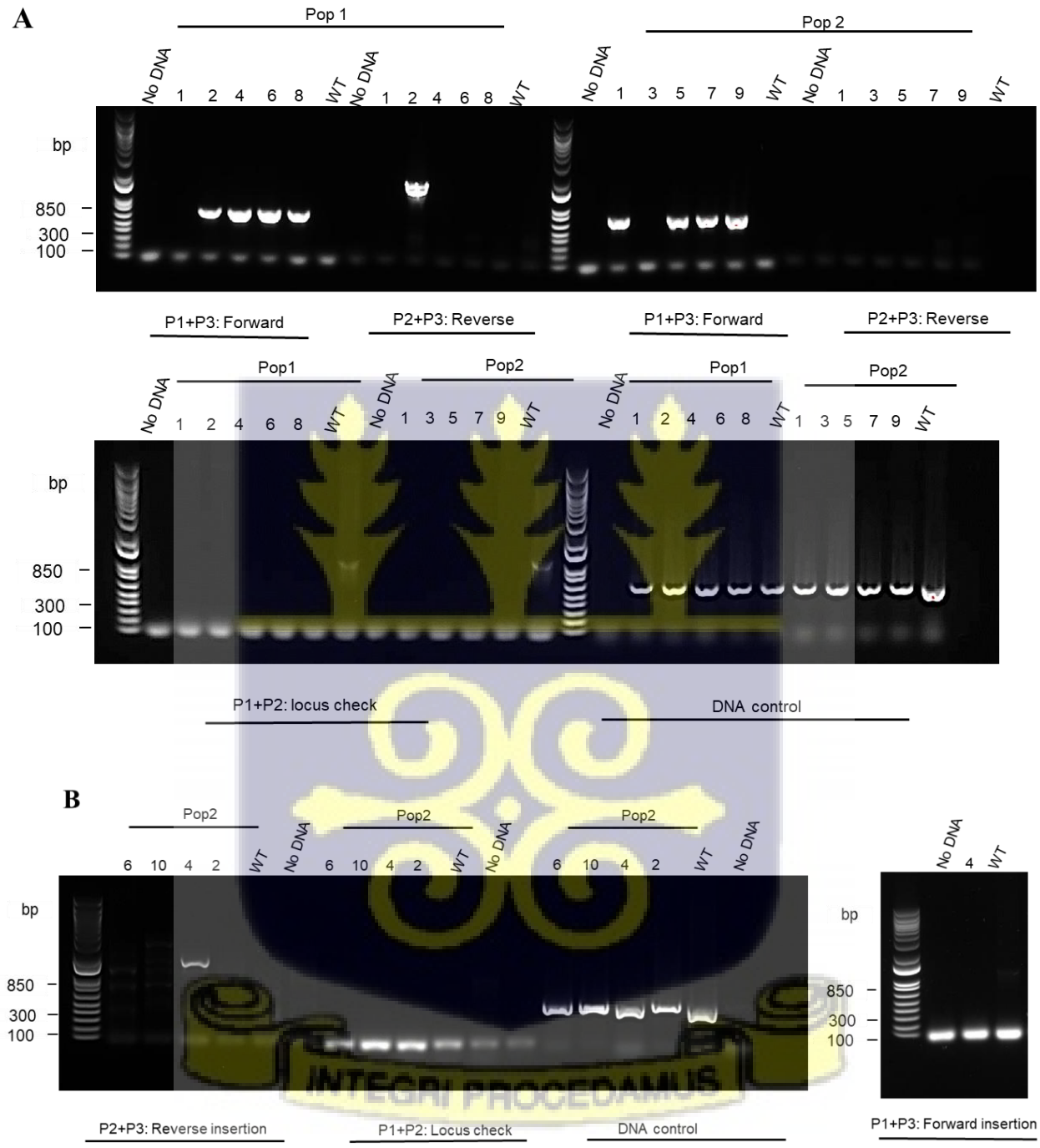


Figure A2: Sanger sequencing to confirm sgRNA fragment and Nanopore sequencing results confirming accurate pLIC plasmid (A) TGGT1_232670 sgRNA 1 (B) TGGT1_232670 sgRNA 2 (C) TGGT1_205350 sgRNA 1 (D) TGGT1_205350 sgRNA 2 targeting the ~100 – 200bp from

the start codon of the coding sequence of each gene (E) pLIC-TGGT1_232670-HA (F) pLIC-TGGT1_205350-HA plasmid sequenced to confirm accurate insertion of TGGT1_232670 or TGGT1_205350 homologous arm in pLIC-3×HA-DHFR backbone.

Appendix 4

Knockout clone identification PCR: Δ*TGGT1_232670*



Knockout clone identification PCR: Δ*TGGT1_205350*

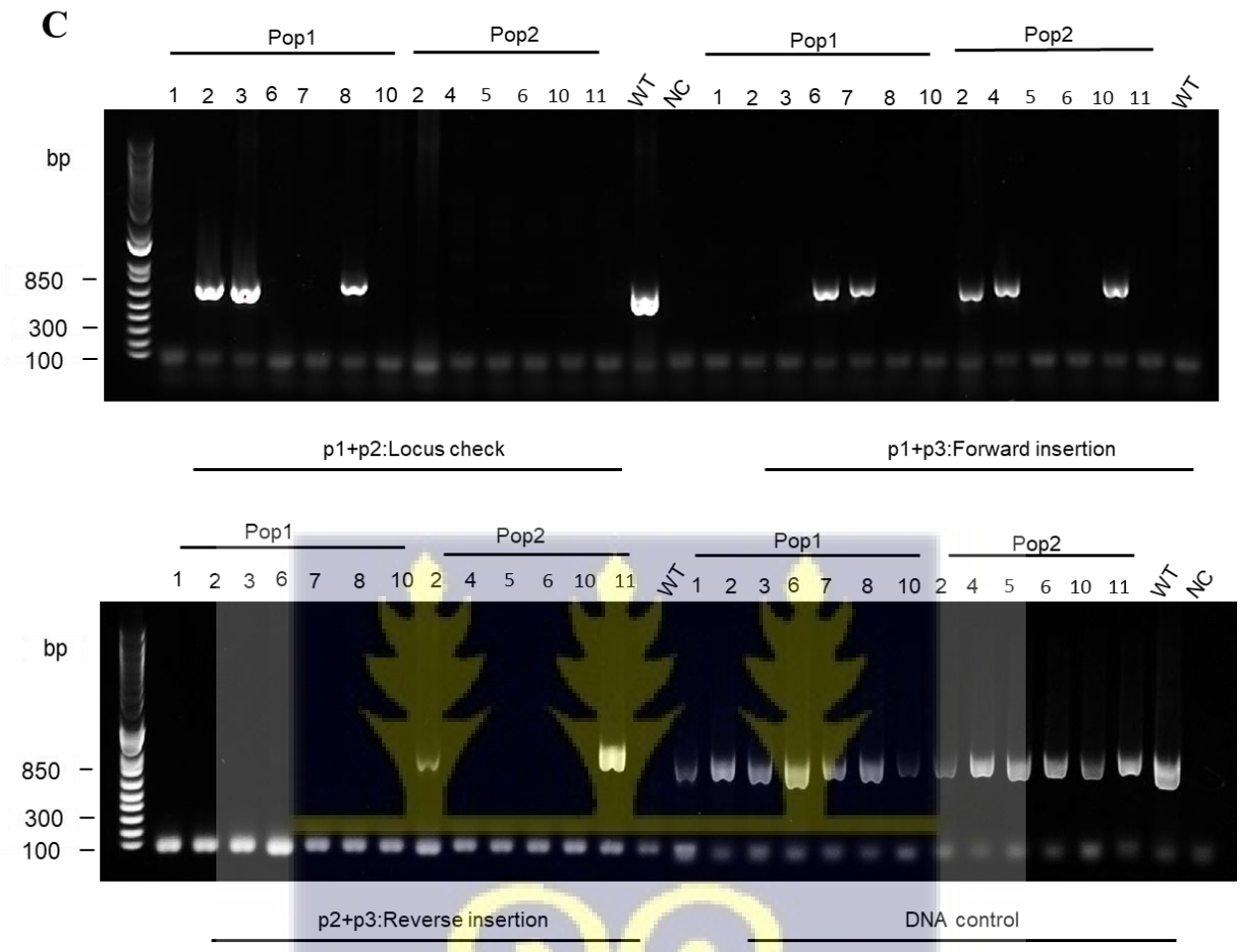


Figure A3: Detection of positive single clones for TGGT1_232670 populations. (A) forward and reverse insertion, locus check, and DNA control and (B) additional screening of population 2 clones (C) Detecting positive single clones for TGGT1_205350 populations by forward insertion, reverse insertion, locus check, and DNA control. DNA control PCR amplified TGGT1_246178 gene using primers AKL_TGGTI_246178_Mut-F & AKL_TGGTI_246178_Mut-R (Appendix 1) to verify that each sample contains genomic DNA from *T. gondii*.

Appendix 5

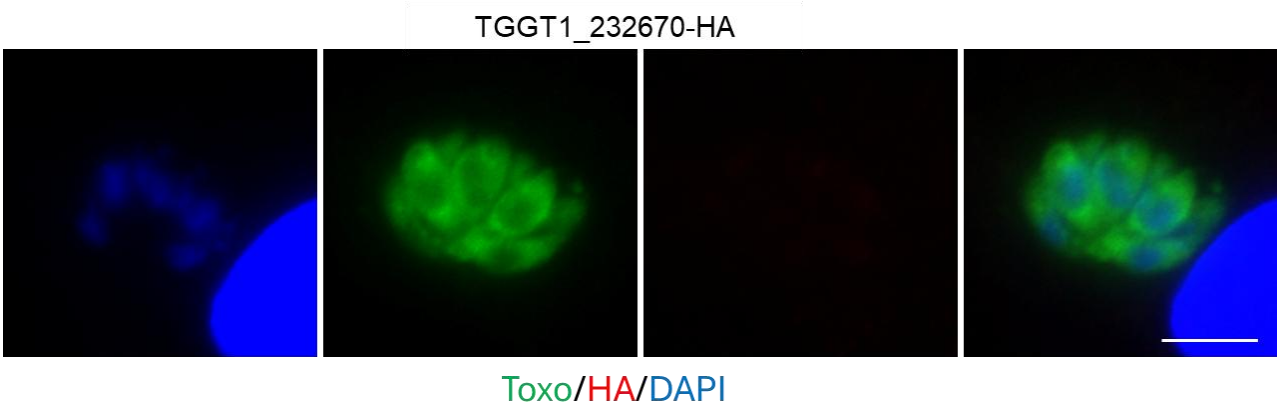


Figure A4: Representative immunofluorescent image of TGGT1_232670 localization 20 µL of parasites that had emerged from pyrimethamine selection were added to freshly confluent HFF cells seeded on coverslips. Cells were fixed at 24 hours post-infection prior to being subjected to the immunofluorescent test using antibodies that target the HA epitope. In both populations, the tagged protein appears to be located in the Golgi apparatus. The image (scale bar =5 µm) is representative of HA-positive parasites seen in the population.