A TOXOPLASMA GONDII MODEL TO DISSECT BRADYZOITE RECRUDESCENCE IN VITRO

by

OMAR SALAS-ORTEGA

(Under the Direction of Ronald Drew Etheridge)

ABSTRACT

Toxoplasma gondii's chronic infection is critical for the parasite's persistence and unfortunately, this aspect of the parasite is vastly understudied. This is due to the challenges of culturing the cyst forming bradyzoites *in vitro* as they are often outcompeted by the rapidly growing tachyzoite form. To overcome this challenge, we generated an Me49 Type II strain containing a plasmid allowing the suppression of tachyzoite growth, selection for bradyzoites, and verification of conversion by stage specific fluorescent protein expression. Optimization of conversion using the cyclic-GMP protein kinase-inhibiting Compound 1 provided mature cysts *in vitro* to study bradyzoite biology. Addition of the calcium ionophore Ionomycin showed an inability by bradyzoites to egress from mature cysts and mechanical lysis showed an additional inability to re-invade new host cells. Treatment with conditions that mimic the gastrointestinal tract, acidic pH and pepsin protease (Acid/Pepsin) or the pancreatic protease Trypsin, activated bradyzoite invasion with the latter being the most effective activator. Chemical mutagenesis with ENU combined with an enrichment protocol involving mechanical lysis and outgrowth of mutants that no longer required protease treatment allowed the isolation of a parasite population, along with clones, that via whole genome sequencing would allow the identification of genes

involved in this process. The ability to study bradyzoite biology in vitro provides a unique opportunity to observe and interrogate unexplored aspects of this crucial form of the parasite.

INDEX WORDS: *Toxoplasma gondii*, bradyzoites, Cpd1, reinvasion, protease, mutagenesis

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CHAPTER 1

INTRODUCTION

1. *Toxoplasma gondii* disease prevalence and life cycle

The intracellular protozoan *Toxoplasma gondii* is an Apicomplexan parasite currently infecting a third of all humans as well as more than half of warm-blooded animals (1). It can infect the central nervous system (CNS) which during pregnancy results in congenital infection leading to developmental diseases in the fetus (2) and encephalitis in immunocompromised individuals (3). It follows a heteroxenous life cycle in which the sexual stage is confined to felids, the definitive host, while other stages affect a broad range of intermediate hosts (4,5). Three different parasite forms comprise the life cycle; the rapidly replicating tachyzoite that causes the acute form of the disease, the slow growing bradyzoite which forms tissue cysts responsible for the chronic infection, and sporozoites which are shed from the definitive host in highly resistant oocysts as a result of sexual recombination (Fig. 1). *T. gondii* is transmitted in one of two ways, either by ingestion of sporozoites in oocysts shed by infected felids or the consumption of improperly cooked meat harboring bradyzoite tissue cysts (6). The success of this parasite is, in part, due to its unique ability to be transmitted through intermediate hosts and thus circumventing the often obligatory need to transit through the sexual stage to be transmitted as is seen in other tissue coccidians.

2. Bradyzoites and the cyst wall

Bradyzoites in cysts and sporozoites in oocysts can survive long periods of time to continue transmission, but once inside a host, they convert to tachyzoites and commence the

acute infection. One aspect that separates *T. gondii* from other Apicomplexans is the capability tachyzoites possess to convert back into bradyzoites which creates a continuous cycle of infection responsible for the untreatable chronic state of the disease (6-8). The continuous cycle between bradyzoites and tachyzoites is our point of interest. As tachyzoites invade cells, they convert to bradyzoites, either stochastically or as a result of environmental stress, to form cysts in intermediate hosts. While the immune system is well equipped for handling tachyzoites, the cysts are sequestered within host cells and are generally considered to be immunologically hidden. This makes cysts an important environmental reservoir that leads to transmission, or in the case of immunocompromised hosts, results in reactivation of toxoplasmosis (9).

The conversion of tachyzoites into bradyzoites has been studied for decades with results shedding light on the effects that stresses like high pH, immune pressure, and chemical agonists have in driving this stage switch; however, the mechanism by which the parasite achieves the transformation is still not fully understood (10, 11). What is known is that bradyzoites express a drastically different transcriptional program compared to tachyzoites resulting in distinct metabolisms, surface protein expression, and most prominently the production of a cyst wall beneath the membrane of the parasitophorous vacuole (PV) (12-14). The cyst wall is a network of complex proteins and carbohydrates which forms a matrix that provides the cyst with structural rigidity but interestingly also with the flexibility to expand and grow as parasites replicate (15-17). The canonical cyst walls formed by other protozoans, such as *Giardia lamblia* and *Cryptosporidium parvum*, are often impervious shells that allow survival in harsh environments outside the host, unlike *T. gondii*'s cyst wall which does not provide environmental protection and is permeable to ions and molecules under 10 kDa (18, 19). As a result, it isn't entirely clear what function the cyst wall plays in long term survival of the

bradyzoite within cells. Eventually, a signal, believed to be external, triggers the release of bradyzoites and the tissue cyst form reverts to tachyzoites restarting the infection cycle. With this unique developmental capacity, *T. gondii* bypasses the requirement for sexual replication in a definitive host and alternatively propagates via the consumption of bradyzoite tissue cysts (20).

Unfortunately, we still lack a basic understanding of how the reactivation of bradyzoites into active tachyzoites actually occurs. This is due in part to our inability to culture bradyzoites *in vitro* resulting in limited research into this medically relevant life cycle stage. Being able to easily culture tachyzoites *in vitro* has led to the discovery of drugs like pyrimethamine that can target the replicating stage. But even though bradyzoites are responsible for transmission and the life-long lasting infection, we have no treatment for it. With the penetrance of *Toxoplasma*, aging global populations, and an increase in organ transplants, it is imperative that we find a way to combat the cystogenic form of this parasite in to reduce the potential for recrudescence (21). This process begins with the long-term culturing of bradyzoites.

2. N-ethyl-N-nitrosourea (ENU) mutagenesis

Elmer Pfefferkorn pioneered the forward genetic system of *T. gondii* in the 1970s by taking advantage of the ability to culture the parasite *in vitro* along with its short replication time (22). Pfefferkorn began dissecting the nucleotide salvage and synthesis pathway using this system to create mutagenized parasites (23, 24). Since Pfefferkorn's work, chemical mutagenesis has been combined with forward genetic analyses to study various aspects of parasite biology like invasion (25). The selection of chemical mutagen is often driven by the profile of the organism's DNA composition. The mechanism by which ENU mutates the genome, alkylating DNA by transferring its ethyl group, makes it biased towards A/T base pair mutations (26) but also allows it to mutate the other bases (27). Due to the fact that ENU creates point mutations in

the genome, we can cause missense mutations in coding regions, regulatory mutations in promoters, as well as null mutations. Each of which provide us with varying effects on genes of interest. The advantage is having mutants with varying degrees of the same phenotype which can be more informative and useful, especially in the case of essential genes (28). Pfefferkorn extended his innovation of forward genetics by combining the mutagenesis with whole genome sequencing (WSG) to identify genetic mutations related to biological phenotypes (29). While this type of study has been known for decades, the mapping of mutations leading to phenotypes was not routine until recently (30). This is in large part thanks to advances in technology and sequencing techniques.

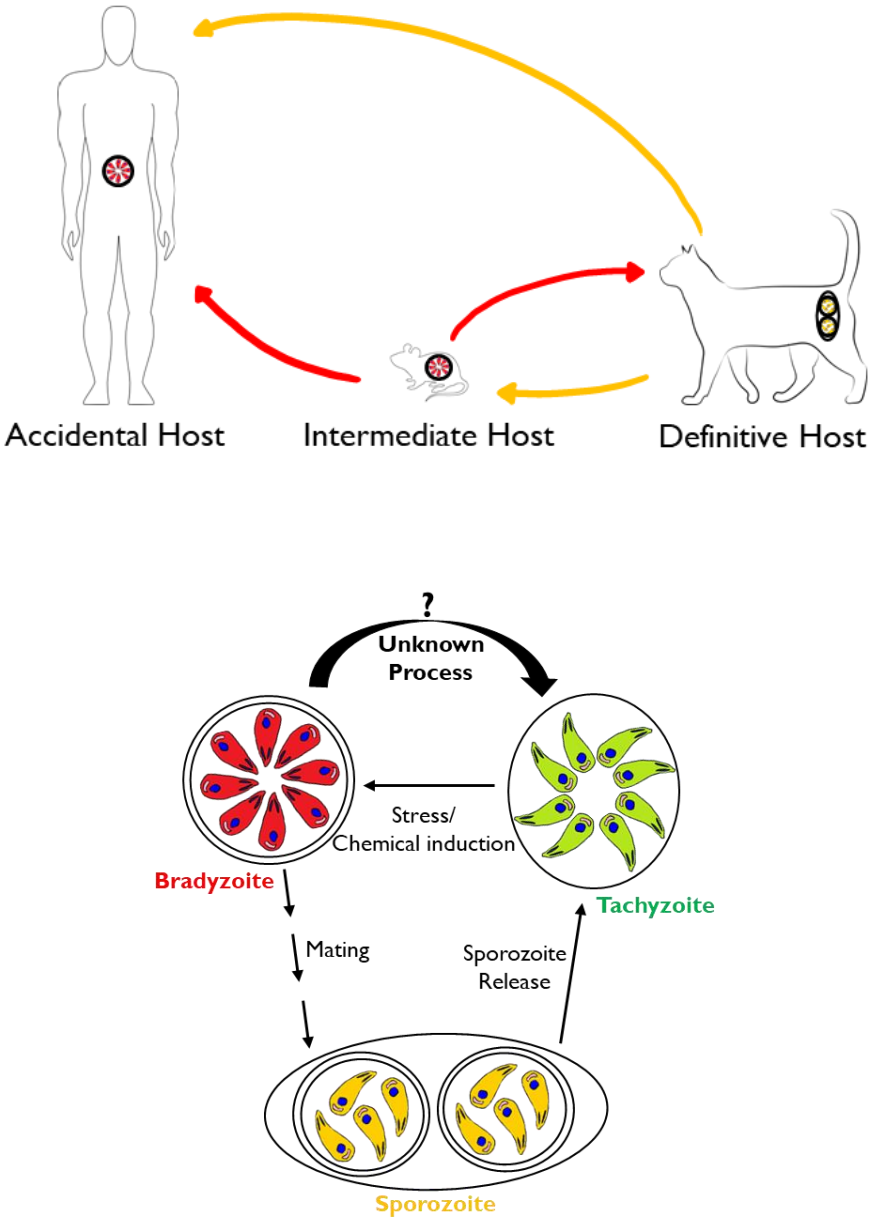


Figure 1. *T. gondii* life cycle stages. Schematic of *Toxoplasma* life cycle and stage transitions. Tachyzoites convert to bradyzoites in response to changes in the host cell which happens naturally in hosts but can also be induced chemically. After ingestion, intracellular tissue cysts in raw meat are digested and the parasite transforms into sporozoites in the definitive host (felids) through sexual reproduction or revert to tachyzoites in intermediate hosts through an unknown process.

CHAPTER 2

ME49^{Cyst} AND COMPOUND 1 CONVERSION

1. Function of the ME49^{Cyst} plasmid

Bradyzoite tissue cysts are vastly understudied and as a result poorly understood, yet it is the most clinically relevant stage of *T. gondii* (31). The reason for the lack of research on this form has been the inability to efficiently culture bradyzoites in a laboratory setting. We overcame this problem by using a cystogenic type II strain, ME49, that remains competent to form cysts both *in vitro* and *in vivo* unlike the common RH (type I) strain which has lost this ability due to prolonged *in vitro* cultivation. The ME49^{Cyst} strain provides two major advantages for this project: 1) a fluorescent reporter line allowing for visual monitoring of stage conversion, and 2) a means for the positive selection of bradyzoites while simultaneously selecting against tachyzoites. Our strain contains a vector that uses a tachyzoite-specific promoter (SAG1) (32) to drive the expression of the HXGPRT (HXG) (33, 34) drug resistance marker fused to a green fluorescence protein (GFP). The tachyzoite-specific promoter leads to both proteins being expressed only by the tachyzoite form and allows for the negative selection of tachyzoites by suppressing growth with 340 µg/ml 6-thioxanthine (6-Thio). The HXGPRT gene stalls the DNA replication of the parasite by impeding purine synthesis while the GFP fusion provides visual verification that parasites are in the tachyzoite stage. As part of the same vector, the drug marker for chloramphenicol resistance (CAT) (35) is fused to a red fluorescence protein (mCherry) and their expression is driven by the bradyzoite-specific promoter (BAG1) (36). This combination makes it possible to positively select for bradyzoites with 10.2 µg/ml chloramphenicol and verify

the life stage visually (Fig. 2). This greatly improves upon the current methods for creating mature cysts. The production of bradyzoites typically requires the generation of either banks of infected mice for tissue harvesting followed by cyst isolation (37), or alternatively, the use of stress-inducing conditions *in vitro* to promote conversion and suppress tachyzoite growth. The first method is cost and labor intensive while the second suffers from tachyzoite overgrowth and stress-induced impacts on the health and viability of the host cells (38).

2. Bradyzoite conversion using Compound 1

To grow mature bradyzoites cysts (> 2 weeks old), we first needed to find a way to induce conversion of the parasite without harming the host cells. Our goal was to identify a molecule that, while harmless to host cells, could induce bradyzoite differentiation efficiently *in vitro*. Compound 1 (Cpd1), the trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl] pyridine cGMP protein kinase inhibitor, was identified as a candidate as it dramatically lowers the replication rate of *T. gondii* (39) and induces expression of bradyzoite-specific antigen and other known cyst wall proteins in type II strains (40). While the exact mechanism for induction of differentiation is unknown, we know that 3 µM Cpd1 is capable of inducing conversion, host cells must be exposed prior to infection or while infected, and differentiation continues after a short exposure to Cpd1 and removal of the molecule (40). This provided a great starting point because we could induce differentiation of the parasites, limit the length of host cell exposure, and grow the culture under normal growth conditions (37 °C, 5% CO_2) allowing host cells to remain healthy.

With this information we tested multiple methods of exposure to Cpd1 and estimated the efficiency of the conversion to bradyzoites as well as impact on the health of the cysts and the host cell monolayer. Additionally, these various methods included the advantages provided by

the ME49^{Cyst} vector for positive and negative selection of the two life stages. A total of 5 methods were tested and compared (Fig 3). Conversion was monitored by observing fluorescence with BioTek's Lionheart FX Automated Live Cell Imager (Lionheart). Green fluorescence representing tachyzoite vacuoles and abundance of red fluorescence representing conversion to bradyzoites and potential cyst formation.

While methods 3, 4, and 5 showed significant conversion, method 5 demonstrated the most efficient conversion of ME49^{Cyst} tachyzoites into bradyzoites by providing the highest number of bradyzoite cysts with the fewest remaining tachyzoite vacuoles. As a result we employed the following method in the subsequent assays to study bradyzoites which consists of infecting a confluent monolayer of Human Foreskin Fibroblasts (HFFs) cultured in a VWR® 25cm^2 Tissue Culture Flask (T25) with 200 µl of freshly lysed parasites (~ 4x10^5)and allowing for 2 hrs of infection in 5 ml 1% FBS media (DMEM). After the 2 hrs, the media is removed, the monolayer is gently washed with 3ml Hanks' Balanced Salt Solution (HBSS) once, and 5 ml of 1% FBS/6-Thioxanthine/Chloramphenicol/Cpd1 are added and the parasites under normal growing conditions for 24 hrs. After the 24 hrs, the media is removed, the culture is gently washed with 3ml HBSS again in order to remove any residual Cpd1, and the media is replaced with 5 ml 1% FBS/6-Thioxathine/Chloramphenicol and allowed to grow for 12-14 days.

To verify the formation of mature cysts, cultures were grown for 12 days, to allow the maturation of the cyst and replication of the bradyzoites, followed by analysis via immunofluorescence assay. The cyst wall formed within the PV by bradyzoites is glycan-rich, and the *Dolichos biflorus* agglutinin (DBA) lectin serves as a marker for cyst wall formation (reviewed in 41). We used a version of this lectin coupled to fluorescein isothiocyanate (FITC) (Vector Laboratories Inc.) to check for the generations of the cyst wall and were able to show

that our ME49^{Cyst} strain produced bradyzoite cysts containing the classical signs of mature cysts (Fig. 4). Our data supports the idea that the ME49^{Cyst} strain is competent to undergo stage transition and produce bradyzoite cysts amenable for further studies. Having an efficient system for growing bradyzoites *in vitro* in place, we were able to proceed with investigations to further understand how bradyzoites initiate infection of new host cells.

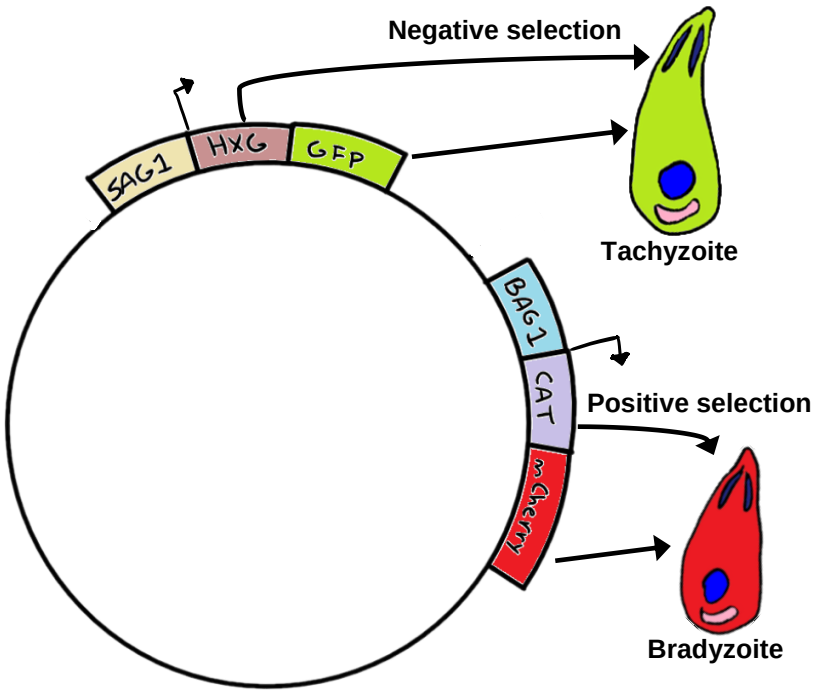


Figure 2. Generating the *in vitro* cyst forming *T. gondii* strain ME49^{Cyst}. Schematic of ME49^{Cyst} plasmid detailing fluorescence reporters (GFP and mCherry), selectable markers (HXG and CAT), and life stage-specific promoters (SAG1 for tachyzoites and BAG1 for bradyzoites).

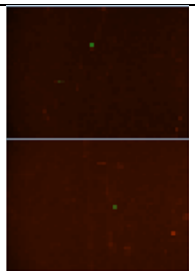
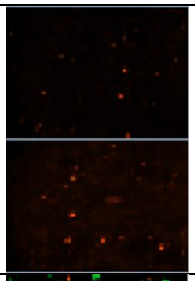
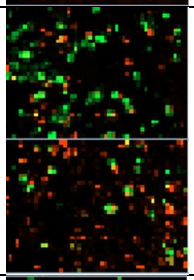
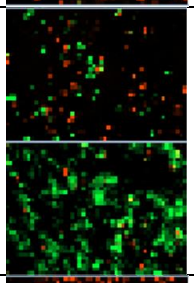
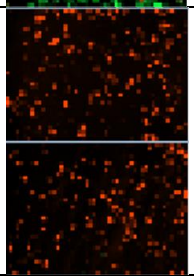
Conversion Method	Observed Fluorescence
1. 3 hrs pretreatment of HFF with 3 µM Cpd1, remove media and wash with HBSS, infect with fresh parasites in 1% FBS/6-Thioxathine/Chloramphenicol media, wash after 2 hrs, add 1% FBS/6-Thioxanthine/Chloramphenicol.	
2. 3 hrs pretreatment of HFF with 3 µM Cpd1, remove media and wash with HBSS, infect with fresh parasites in 1% FBS/Cpd1 media, wash after 2 hrs, add 1% FBS/6-Thioxanthine/Chloramphenicol/Cpd1.	
3. 3 hrs pretreatment of HFF with 3 M Cpd1, remove media and wash with HBSS, infect with fresh parasites in 1% FBS/Cpd1 media.	
4. Infect HFF with fresh parasites for 2 hrs in 1% FBS, remove media and wash with HBSS, add 1% FBS/Cpd1 media.	
5. Infect HFF with fresh parasites for 2 hrs in 1% FBS, remove media and wash with HBSS, add 1% FBS/6-Thioxanthine/Chloramphenicol/Cpd1 media.	

Figure 3. Identification of long-term method for ME49^{Cyst} conversion to bradyzoites. List of methods tested for bradyzoite conversion with observed results. Conversion was done in HFF monolayers cultured in a Greiner Bio-One glass bottom 96-well cell culture microplate for better imaging. Images were taken with Lionheart's "GFP" and "Texas Red" imaging settings.

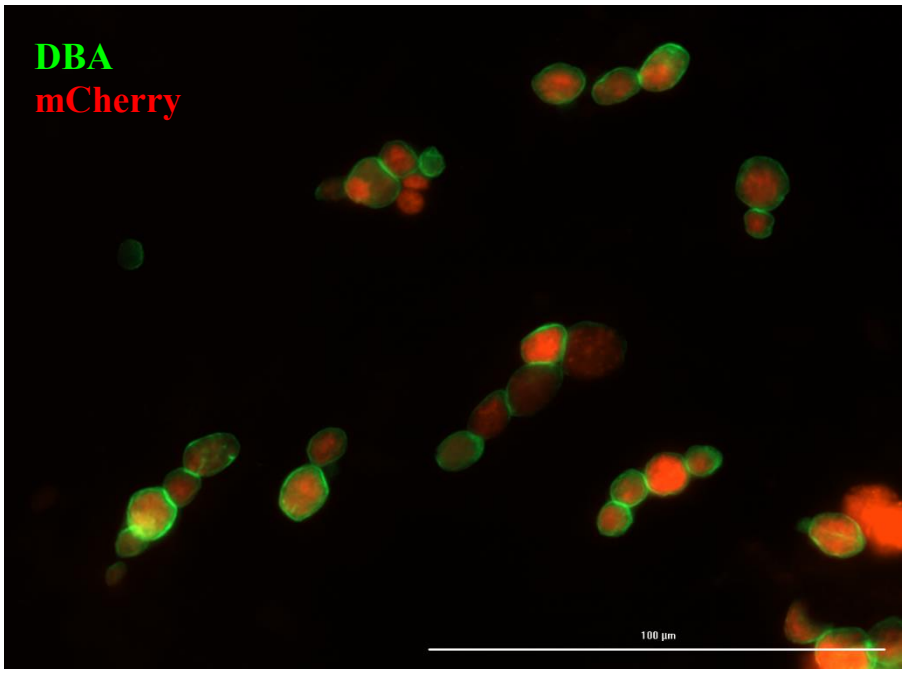


Figure 4. ME49^{Cyst} forms mature bradyzoite cysts. Verification of bradyzoite conversion and mature cyst formation of ME49^{Cyst} by immunofluorescence assay (IFA) using DBA lectin as a marker for the N-acetylgalactosamine commonly found in the cyst wall matrix. 5 µg/ml of DBA diluted in 5% FBS in 1x PBS was used for staining cysts while mCherry belonged to fluorescence reporter driven by BAG1 expression. Scale = 100 µm.

CHAPTER 3

DISCOVERIES ABOUT BRADYZOITE BIOLOGY

1. Differences in egress

In order for *Toxoplasma* to infect new host cells, it first needs to leave its cyst. Because of this, our initial step towards studying recrudescence was to examine egress in the presence of ionomycin, an egress-inducing calcium ionophore (42). A culture consisting of mature bradyzoite cysts (shown by red fluorescence) and tachyzoites (green fluorescence) in a monolayer of HFFs were relieved of drug pressure overnight to allow tachyzoites to behave normally. This lets the tachyzoites act as an internal control as their egress in the presence of ionomycin is well characterized. As expected, tachyzoites egressed from their PV shortly after addition of 1 µM ionomycin and began invasion of new host cells. However, bradyzoites failed to egress after the addition of ionomycin. Instead they were observed moving within the cyst 15 minutes after the ionomycin addition (Fig. 5) which suggested two key things; a) the bradyzoites were able to sense the ionomycin and engage their gliding motility machinery within the cyst verifying their viability, but b) regardless of their movement, they were unable to escape the cyst wall to begin the invasion process. This piqued our interest and led us to wonder if the cyst wall may serve as a self-generated cage rather than a protective barrier. It is possible that it functions to prevent accidental egress and promote long term viability of the cyst thereby extending the propagation of the parasite during the chronic stage of infection.

2. Mechanical lysis and reinvasion

To test if escaping the cyst wall is the only requirement for bradyzoites prior to reinfection, we mechanically disrupted the cyst with a dounce homogenizer. This allowed us to physically release the bradyzoites from their cysts onto a monolayer of fresh HFFs in the absence of selecting drugs and check if they could invade new cells by measuring tachyzoite outgrowth. While it is unknown whether bradyzoites convert to tachyzoites prior to the invasion or after penetration of the new cell, we have observed that replication takes place exclusively as tachyzoites, and so as a result tachyzoite growth is a readout for productive bradyzoite invasion in our assay. As a control for background infection caused by the contaminating tachyzoites that remain in the culture, a bradyzoite culture with a similar number of cysts was left untreated to assess the amount of growth due to unconverted tachyzoites in the bradyzoite culture and was compared against the growth observed from a culture after mechanical lysis (Fig. 6A). Growth is measured with a counting algorithm from the Lionheart's software that allows us to adjust for both size and fluorescence intensity. Despite releasing bradyzoites from 80% of the cysts, there was no significant change in the amount of tachyzoite growth between a culture where cysts remained untouched and the culture where the bradyzoites were released. These results furthered our curiosity as to what requirements bradyzoites have in order to invade. We hypothesized that some component of the gastrointestinal (GI) tract may be mandatory for bradyzoites to become invasion competent.

3. Acid/Pepsin-based activation

For decades, low pH and pepsin protease have been used to release bradyzoite cysts from infected tissues (43). This information led us to investigate the effects of treating the bradyzoite cysts with 0.0125 mg/ml pepsin protease dissolved in a low pH solution (pH 1.3) composed of

170 mM Sodium Chloride (NaCl) and 60 mM Hydrochloric Acid (HCl) (Acid/Pepsin). For this experiment we compared treatments between ME49^{Cyst} tachyzoites and the *in vitro* generated bradyzoites. Both cultures were identically treated with Acid/Pepsin for increasing amounts of time followed by neutralization of the reaction with Sodium Bicarbonate, and the resulting growth was measured after 3 days (Fig. 6B). A portion of each culture was left untreated to serve as a control. Two results emerged from this experiment; a) The treatment of bradyzoite cysts with Acid/Pepsin dramatically increased their ability to infect new host cells, and b) the treatment eliminated any contaminating tachyzoites in the bradyzoite culture. This showed an ability by bradyzoites to resist the low pH environment lacking by the tachyzoite form, something which has been reported (44, 45). After further testing, we discovered that it was the acidic conditions which killed the tachyzoites and the pepsin protease played no role in this effect. What was still unclear to us was whether the invasion competence shown by the bradyzoites was stimulated by the low pH or by the protease.

4. Neutral pH and Trypsin activation

To separate the differential effects of either pH or protease in bradyzoite activation, we chose to perform the previous experiment but with a protease active at neutral pH. Both tachyzoite and bradyzoite cultures were treated with 0.04 mg/ml Trypsin while a portion was left untreated as control. We observed that although the tachyzoite population demonstrated a minor increase in infection efficiency with this treatment, the bradyzoite invasion and growth had a 9-fold increase compared to the untreated control (Fig. 6C). This invasion was greater than what was observed with the Acid/Pepsin treatment. Based on these results, we believe that *Toxoplasma* has adapted a requirement for bradyzoites to undergo a proteolytic cleavage event to become invasion competent. The advantage of this adaptation could be as that of a molecular

compass that prevents the parasite from beginning invasion until it has entered the proper region of the GI tract. This might explain the "preference" that *T. gondii* has for the duodenum in the small intestine (46). While our development of an *in vitro* culture system allows us to reproducibly observe these unique differences, it also provides us with the opportunity and means to identify the molecules responsible for these stage-specific phenotypes.

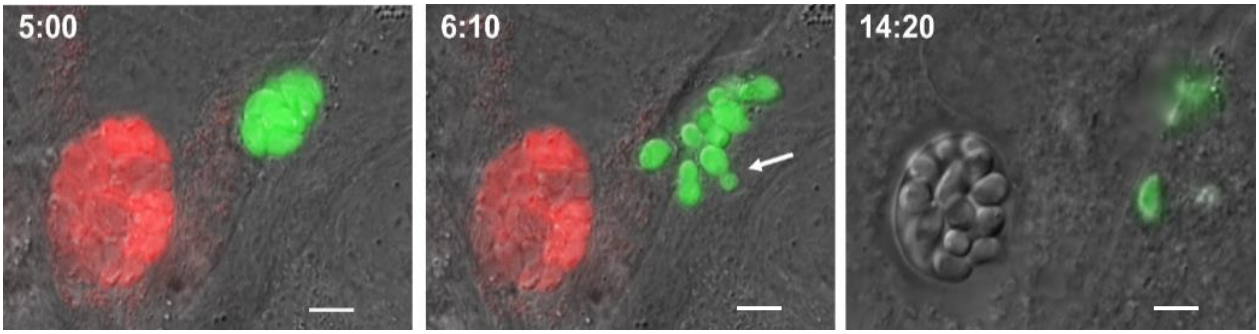


Figure 5. Bradyzoites do not egress from *in vitro* cysts in the presence of ionomycin.

Individual frames from time-lapse video showing behavior of tachyzoites in PV (green) and bradyzoites in cyst (red) after addition of calcium ionophore. Time = minutes after ionophore addition. Arrow = tachyzoite's invasion tight junction. Scale = 2 µm.

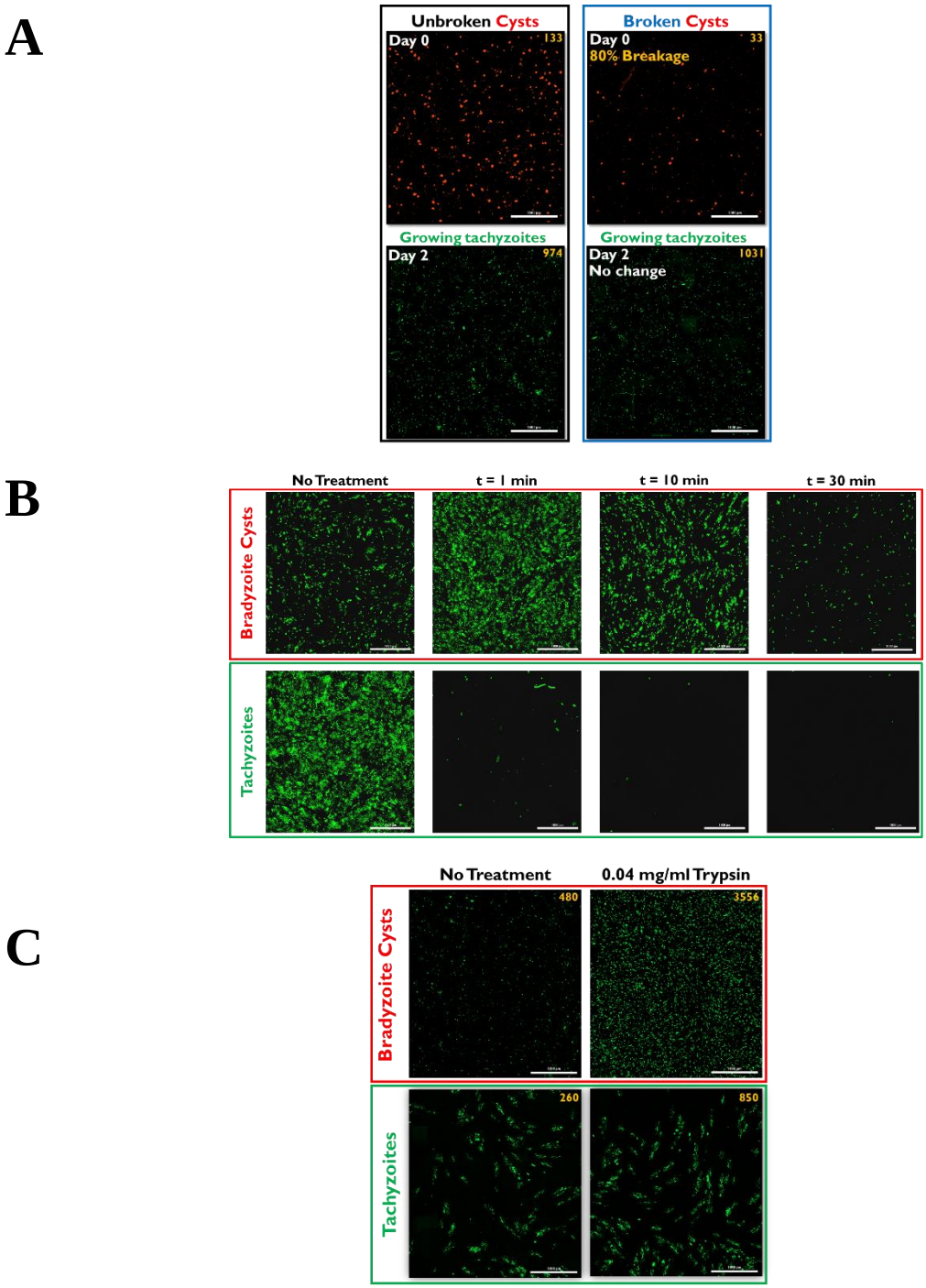


Figure 6. Protease treatment of bradyzoites is required for invasion competence. Bradyzoites were released from their cysts through various means to test their ability to invade new cells as compared to tachyzoites. A) Mechanically lysed bradyzoites are not invasion competent. Growth seen is due to remaining tachyzoites in the culture. B) Acid/Pepsin treatment activates bradyzoite invasion. C) Trypsin at neutral pH treatment of bradyzoites and subsequent outgrowth. Experiments performed 3 times.

CHAPTER 4

MAKING INVASION-COMPETENT BRADYZOITES

1. ENU mutagenesis and population enrichment

Our approach to identify potential targets responsible for the protease requirement of bradyzoites consists of an unbiased, positive selection screen. The screen was carried out by subjecting our ME49^{Cyst} strain to N-ethyl-N-nitrosourea (ENU) to randomly induce mutations throughout the genome. Parasites were treated with 1.25, 2.5, 5, and 10 mM of the mutagen along with a control containing no mutagen to identify a concentration which led to 30% survival of exposed parasites. This concentration has been shown previously to generate approximately 100 mutations per genome which is the standard for this type of screen (47). Based on our experiments, 5 mM ENU was chosen to generate our mutant population. After determining the correct concentration, we subcloned our ME49^{Cyst} strain in order to begin our experiments with a clonal population rather than a population of genetically different parasites due to prolonged passage in culture. By doing this we were able to begin from a genetically identical population which will improve the analysis of any potential results after whole genome sequencing.

The selected single clone was mutagenized, and the surviving parasite population was induced to convert into bradyzoite cysts and allowed to mature for two weeks. After maturing, the cysts were mechanically lysed and allowed to grow on a new monolayer of HFFs, the goal of which was to enrich for those parasites no longer requiring protease treatment to become invasion competent. The resulting tachyzoites were then expanded and reconverted again to bradyzoites followed by mechanical rupture and outgrowth. This process of enriching a

population of mutant bradyzoites that no longer need proteolysis was repeated a total of 3 times (Fig. 7A).

2. Mechanical lysis comparison

After three rounds of enrichment, we assessed the phenotype of the mutant population. We employed the mechanical lysis and outgrowth protocol to compare the ENU-treated/enriched strain to our input ME49^{Cyst} parasites. For this, both strains were grown and converted at the same time. Once mature cysts were ready, both cultures were treated with 170 mM NaCl and 60 mM HCl (pH 1.3) to kill unconverted tachyzoites so that only bradyzoites remained. Bradyzoites were mechanically lysed with the dounce homogenizer and the released parasites were placed on HFFs monolayers and allowed to grow. After 3 days, we observed no growth from the input strain but observable growth solely from the ENU-treated population (Fig. 7B). This verified that that the enriched mutants were both resistant to low pH, as is typical of bradyzoites, but lacked the protease requirement for reinvasion. Additionally, it suggested that the protease target is inhibitory because if the protease produced an activating signal, we would not have been able to enrich a mutant in the population.

With the population verified, we subcloned and recovered 10 single clones, labeled ENU Clones 1-10, out of which ENU Clones 3, 4, 5, 6, 9, and 10 demonstrated the strongest phenotype (Fig. 8). While the pooled population prior to subcloning can be used for further identification of genes, a single clone lowers the complexity of the data, and when combined with our analysis of the input strain, will increase the accuracy of our results. It is also possible that different mutations cause the phenotype and analysis of multiple clones will shed light on multiple mechanisms that have been altered. We can use these generated strains to identify single

nucleotide polymorphisms (SNPs) created by the ENU treatment which might identify gene(s) originally responsible for the requirement.

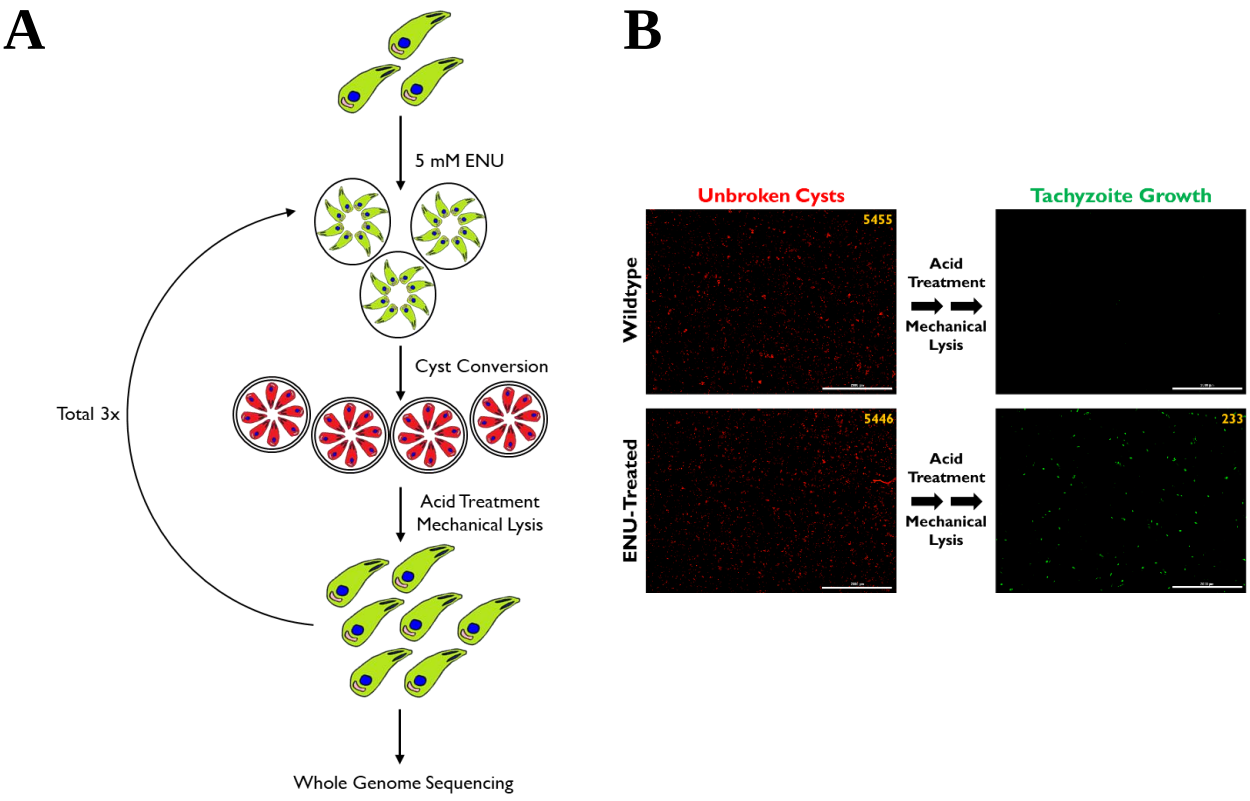


Figure 7. Generating ME49^{Cyst} strain capable of protease independent invasion. 5 mM ENU was used to perform an unbiased positive screen in which ME49^{Cyst} was mutated and enriched for a population able to convert to bradyzoites and reinvade after mechanical lysis from mature cysts. A) Step-by-step schematic of mutagenesis, growth of surviving parasites, conversion, acid treatment to remove tachyzoites, and enrichment after mechanical lysis. B) Comparison of growth after mechanical lysis between final population after 3 rounds of enrichment versus initial ME49^{Cyst} wildtype. Count of initial cysts or PVs after growth shown in upper right corner. Scale = 2000 µm.

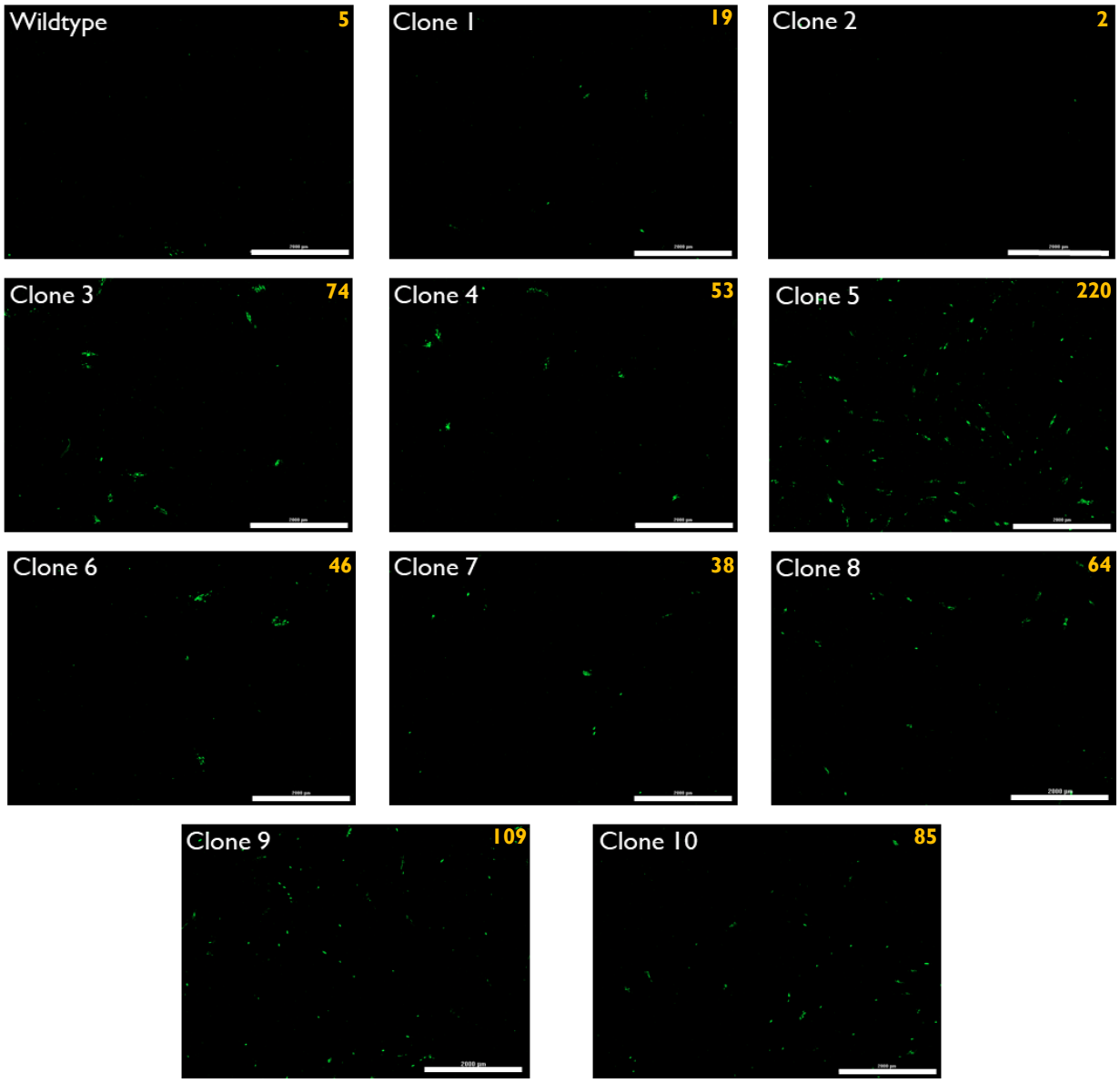


Figure 8. Isolation of parasite clones lacking proteolytic requirement for host cell invasion. Individual clones recovered after verification of ENU-treated pool compared for growth after mechanical lysis as compared to the wildtype parental clone. Clones able to grow after only mechanical lysis have been mutated to no longer need protease activation for reinfection. Count of PVs after growth shown in upper right corner. Scale = 2000 µm.

CHAPTER 5

FUTURE DIRECTIONS

The new culturing system we have created for growing bradyzoites *in vitro* has given us the means to make some novel observations in *Toxoplasma gondii*. It will now be possible to study aspects of bradyzoite invasion and growth as well as other important biology. Initially, we have observed differences in egress compared to tachyzoites as ionomycin leads to motility of bradyzoites, but not the release from cysts. This was followed by a phenomenon where bradyzoites that were released by physical means were still not capable of propagation. We managed to create release from the cysts and induce invasion with Acid/Pepsin and noticed that bradyzoites survive harsh acidic environments that kill tachyzoites within minutes. We then separated the two conditions using Trypsin and discovered that protease is required for bradyzoites to be invasion competent. These observations and discoveries comprise the early stages of a series of in-depth studies that can now be performed on bradyzoites.

With a focus on identifying the target of the protease, we unbiasedly mutagenized a single clone from our ME49^{Cyst} strain with ENU and enriched for parasites able to convert to bradyzoites and reinvade after only mechanical lysis. The resulting ENU-treated pool and single sub-clones will be used for Whole Genome Sequencing (WGS) to search for mutations that explain the loss of the protease requirement. It is possible that a consensus mutation in a genomic locus or a specific gene is responsible in which case this should be seen in all the single clones. It is also possible that multiple points of the same mechanism were affected by the mutagen in which case we might see different patterns of mutations in different sets of clones. Either result

would be interesting and beneficial towards identifying the source of the requirement. With the help of the Georgia Genomics and Bioinformatics Core (GGBC) and Rodrigo Baptista from the Kissinger Lab at the University of Georgia, we will create a list of candidate genes.

The genes on the candidate list produced will then be filtered for bradyzoite-specific expression. Because the tachyzoites do not demonstrate a need for protease activation to invade, we believe the target of the protease will be highly expressed by bradyzoites but not by tachyzoites. Next, because the protease would need to interact with the protein without entering the parasite, we believe the cleavage most likely takes place on the surface of the parasite and as such we will prioritize proteins containing signal sequences and/or transmembrane domains/GPI anchor sites as these are expected to be able to be presented on the parasite membrane. It is worth noting that as a result of the genome-wide CRISPR/Cas9 screen of *T. gondii*, a distinct population showed a positive fitness score after the mutation (48). Several of these positive fitness genes have been further characterized with no observable increase in replication rate, however many are surface expressed proteins that once removed may slightly increase the invasion efficiency of the parasite as we observe with protease treatment. Once the target list is narrowed down to a few candidates, these genes will be used for further validation.

The selected genes will be tagged using gene-specific guide ribonucleic acids (gRNAs) and CRISPR-Cas9 in conjunction with a TY protein tag fused onto the gene of interest. This will allow us to first verify the localization of the protein on bradyzoites. Once the localization is known, the gene will be knocked out using the same CRISPR-Cas9 approach but instead of a tagged form of the gene, a drug resistance marker will be added to select for the mutants. After generating a mutant for the genes, we will use our mechanical lysis assay to assess the phenotype. Any mutants able to invade without a protease requirement will have the gene

complemented to restore the need for protease treatment. As a secondary approach, genes that force a protease requirement phenotype during the validation process could also be expressed in tachyzoites with an overexpression vector (Fig. 9) that would drive expression using the SAG1 promoter to check if expression of any of these proteins impedes invasion in tachyzoites.

Understanding recrudescence is a crucial part of alleviating the problems caused by *T. gondii*. The finding that bradyzoites require a protease for release as well as activation prior to becoming invasion competent is the first key to understanding the chronic aspect of the infection. The protease requirement aligns with the events that take place upon the initial infection as both Pepsin and Trypsin are encountered throughout the gastrointestinal tract. This creates the question of how reinfection occurs later in the infection period since neither Pepsin nor Trypsin are encountered throughout muscles or in the brain where cysts are commonly found.

One idea worth exploring is the role that host cells may play in this reactivation. The immune system is present throughout the body and their proteases could trigger the release and activation of bradyzoites. This would mean that recrudescence is a constantly occurring act which would normally be hidden by the immune system's ability to kill tachyzoites. In the event of immunocompromised individuals, however, the innate immune system might trigger the activation of bradyzoites but not have the means to clear the ensuing tachyzoite infection. This is a possible explanation that ties the previous knowledge that tachyzoite infection cannot be controlled in an immunocompromised or immunosuppressed state with our current observation that protease activation is mandatory for chronic infection. It would be interesting to further explore this idea by inducing chronic infection in mice with different members of the immune system lacking and searching for differences in recrudescence.

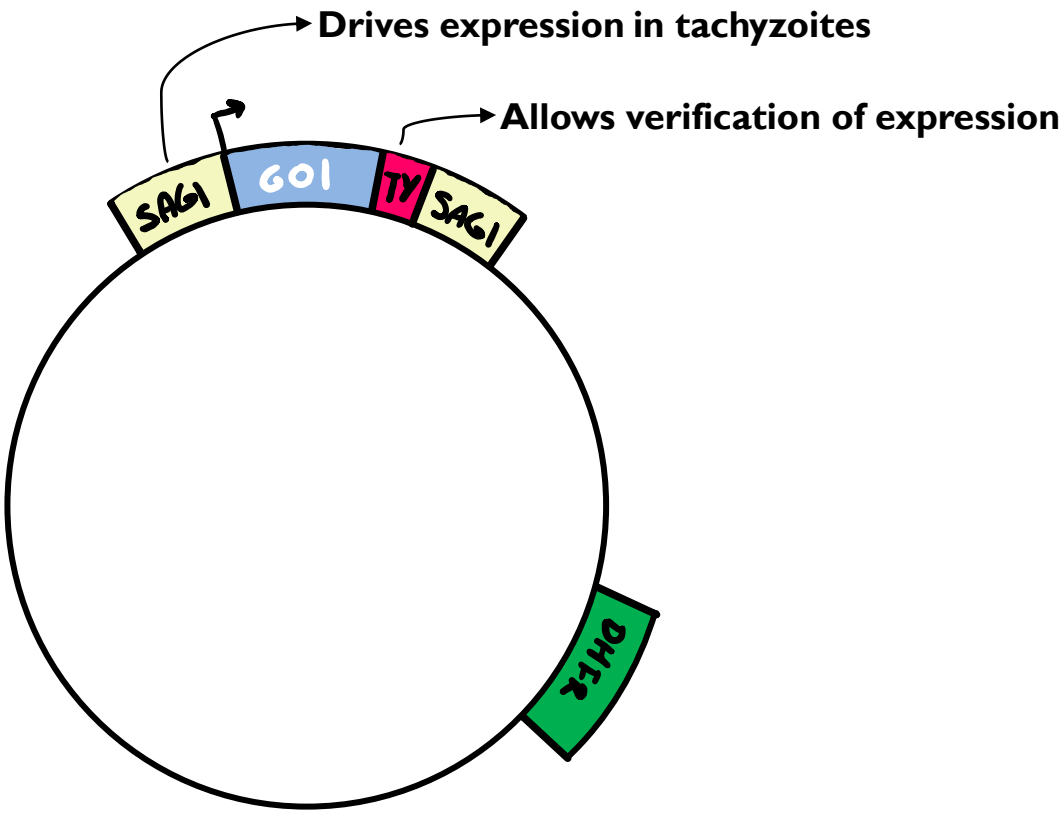


Figure 9. Expression of bradyzoite-specific genes in tachyzoites. Expression of genes of interest will be driven by the SAG1 promoter. TY tag followed by a stop codon will allow verification of expression by IFA and Western Blot. DHFR cassette provides parasites that have integrated the plasmid after transfection with Pyrimethamine resistance for positive selection.

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