

Nutrient Acquisition via the Ingestion Pathway in *Toxoplasma gondii*

by

Patrick Asher Rimple

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in the University of Michigan
2025

Doctoral Committee:

Professor Vern B. Carruthers, Chair
Assistant Professor Teresa Rodgers O'Meara
Professor Pat D. Schloss
Professor Yatrik M. Shah
Associate Professor Andrew W. Tai
Professor Lois S. Weisman

Patrick Asher Rimple

primple@umich.edu

ORCID iD: [0009-0000-6834-8343](https://orcid.org/0009-0000-6834-8343)

© Patrick Asher Rimple 2025

Dedication

I want to dedicate this work to everyone who has been a part of my academic journey.

Thank you, Mr. Nale, for kindling my love for biology and prepping me for college.

Thank you to the SEA PHAGE program at the University of Pittsburgh for giving me the opportunity to try research and teaching as an undergraduate and to Dr. Graham Hatfull for allowing me to continue researching in his lab.

Thank you to my family, who has supported all of my academic endeavors, even if that meant deciding to be in school for 25 years.

Acknowledgements

My PhD journey was a long, difficult process. Many people have helped support me through it. I cannot express how greatly I appreciate my environment and lab mates in the Carruthers lab. Whether it was the personnel when I first joined the lab – Alfredo, Yolanda, Geetha, or Mae – from whom I learned innumerable skills and guidance to help me develop from a fresh graduate student into a more seasoned researcher. Or the current group of TeamToxo – Pha, Anu, Christophe, Fengrong, Einar, and Tracey – who have kept me sane when my results were disappointing or provided support when confidence in my abilities waned in my seemingly endless PhD journey. Without a doubt, spearheading the lab, offering support, and mentorship, I have to thank Vern. Thank you for putting together a wonderful team.

I also have to thank my cohort of PhD students—Yuan, Saddle, Madison, Jenn, Austin, and Jaime. We have been through a lot together in this program. The bond we made studying for our checkpoint 2 exam together says everything it needs to about our cohort.

I am also grateful to my friends—Alex, Gwop, Johnathon, Sam, Scott, and Shane. Whether we met through running, games, or classes, I am glad we have continued to stick together. Hopefully, I will be able to see everyone in person more often now!

Finally, I want to thank my family. I have always had the unwavering love and support of my Mom, Dad, Brother, Nana, Nanny, and Pop. Thank you for always picking up the phone when I called.

Table of Contents

Dedication	ii
Acknowledgements	iii
List of Tables	vi
List of Figures	vii
List of Appendices	viii
Abstract	ix
Chapter 1 Introduction	1
1.1 Apicomplexans	1
1.2 Basic Biology of <i>Toxoplasma gondii</i>	1
1.2.1 Life cycle and basic epidemiology	1
1.2.2 The replicative niche of <i>Toxoplasma gondii</i>	2
1.3 Nutrients Metabolism and Salvage in <i>Toxoplasma gondii</i>	2
1.3.1 Soluble Nutrients	2
1.3.2 Macromolecular nutrients	7
1.3.3 The Ingestion Pathway.....	8
1.4 In this Study	8
Chapter 2 Metabolic Adaptability and Nutrient Scavenging in <i>Toxoplasma gondii</i> : Insights from Ingestion Pathway-Deficient Mutants.....	11
2.1 Introduction.....	11
2.2 Results.....	16
2.2.1 Generation of CRISPR Screen Mutants.....	16
2.2.2 CRISPR Screen.....	17
2.2.3 Attempt to validate synthetic lethality	22

2.2.4 Pathway enrichment analysis	24
2.2.5 Bulk metabolomics of ingestion mutants.....	27
2.2.6 Ingestion mutant growth under amino acid limitation.....	30
2.3 Discussion	33
2.4 Materials and methods	36
2.5 Notable contributions.....	43
Chapter 3 Characterizing <i>T. gondii</i> S1/P1 Nuclease 1, TgSPN1	44
3.1 Introduction.....	44
3.2 Results.....	46
3.2.1 Identifying Candidate S1/P1 nuclease in <i>Toxoplasma</i>	46
3.2.2 TGGT1_285180 resembles <i>P. citrinum</i> P1 nuclease P24289.1.....	47
3.2.3 Tagging TgSPN1 indicates it occupies the <i>T. gondii</i> endolysosomal system	48
3.2.4 Generation of recombinant TgSPN1.....	51
3.2.5 Mature TgSPN1 Localizes to an Unknown Compartment	53
3.3 Discussion	55
3.4 Materials and Methods.....	58
3.5 Notable Contributions.....	62
Chapter 4 Discussion	63
4.1 Overview	63
4.2 The Ingestion Pathway as a Conduit for Acquisition of Amino Acids and other Metabolites.....	64
4.3 Metabolic Plasticity and Biosynthetic Compensation	65
4.4 S1/P1 Nuclease (TgSPN1) and Nucleoside Salvage	66
4.5 Conclusions.....	68
Appendices.....	70
Bibliography	88

List of Tables

Table 2-1 Shared genes from Δ <i>gral4</i> , Δ <i>cpl</i> , and Δ <i>crt</i> fitness conferring hits.....	21
Table 3-1 Summary of S1/P1 nucleotide candidates in <i>Toxoplasma gondii</i>	46

List of Figures

Figure 1-1 The <i>Toxoplasma</i> ingestion pathway.	10
Figure 2-1 Validation of RHCas9 knockouts.	16
Figure 2-2 Quality control analysis of genome-wide CRISPR screens.	18
Figure 2-3 Genome-wide CRISPR screen identifies fitness-conferring genes related to the ingestion pathway.	20
Figure 2-4 . Analysis of plaque efficiency and size for screen hits.	24
Figure 2-5 . Ingestion mutants rely on several metabolic pathways..	25
Figure 2-6 Consistent clustering of groups in metabolic data.	28
Figure 2-7 Metabolic analysis of ingestion mutants shows a decrease in amino acids and glycolic end products.	30
Figure 2-8 Nutrient-limited conditions inhibit ingestion mutant replication.	32
Figure 2-9 Growth of <i>Toxoplasma</i> in phenylalanine-, arginine-, or tyrosine- limiting conditions.	33
Figure 3-1 TgSPN1 has substantial homology with S1/P1 nuclease from <i>P. citrinum</i>.	48
Figure 3-2 TgSPN1-AID3HA localizes to the endosome-like compartment.	50
Figure 3-3 Generation of recombinant TgSPN1 to create antibodies.	52
Figure 3-4 Mature TgSPN1 is in an unknown intracellular compartment.	54
Figure 3-5 Antibody made from refolded rTgSPN1-core works similarly to that of denatured rTgSPN1-core.	55

List of Appendices

Appendix A: Pathways Enrichment Analysis on Hits Shared Between $\Delta gra14$, Δcpl , and Δcrt screens	70
Appendix B: Patway Enrichment Analysis on Significant Hits in $\Delta gra14$ screens	72
Appendix C: Log2FC of Metabolites in Ingestion Mutants	74
Appendix D: Strains Used in This Study	79
Appendix E: Primers Used in This Study	80
Appendix F: Plasmids Used in This Study	84
Appendix G: Antibodies Used in This Study	85
Appendix H: SpaCr Settings for Training Model	86
Appendix I: Media Recipes for Amino Acid Limited Media	87

Abstract

The obligate intracellular parasite *Toxoplasma gondii* replicates within a specialized compartment called the parasitophorous vacuole (PV). Recent work showed that despite living within a PV, *Toxoplasma* endocytoses proteins from the cytosol of infected host cells via a so-called ingestion pathway. The ingestion pathway is initiated by dense granule protein GRA14, which binds host ESCRT machinery to bud vesicles into the lumen of the PV. The protein-containing vesicles are internalized by the parasite and trafficked to the Plant Vacuole-like compartment (PLVAC), where cathepsin protease L (CPL) degrades the cargo and the chloroquine resistance transporter (CRT) exports the resulting peptides and amino acids to the parasite cytosol. However, although the ingestion pathway was proposed to be a conduit for nutrients, there is limited evidence for this hypothesis. We reasoned that if *Toxoplasma* uses the ingestion pathway to acquire nutrients, then parasites lacking GRA14, CPL, or CRT should rely more on biosynthetic pathways or alternative scavenging pathways. To explore this, we conducted a genome-wide CRISPR screen in wild-type (WT) parasites and GRA14, CPL, or CRT knockout mutants to identify genes that are synthetically lethal in the ingestion-deficient parasites. Our screen revealed a significant overlap of genes that potentially become more fitness-conferring in the ingestion mutants. Pathway analysis indicated that CPL and CRT knockout mutants relied more on pyrimidine biosynthesis, fatty acid biosynthesis, TCA cycle, and lysine degradation. Bulk metabolomic analysis showed reduced levels of glycolytic intermediates, amino acids, and nucleosides in the ingestion mutants compared to WT,

highlighting the pathway's potential role in host resource scavenging. Interestingly, CPL and CRT mutants showed an exacerbated growth defect when cultured in amino acid-depleted media, suggesting that disrupting proteolysis or the export of proteolytic products from the PLVAC affects parasite survival during nutrient scarcity. The reduced level of nucleosides in the metabolomics data challenged our understanding that the ingestion pathway only acquired host cytosolic proteins. For nucleotide salvage to occur in the PLVAC, a nuclease must be present. We identified a putative S1/P1 nuclease, TGGT1_285180 (TgSPN1), that is of a protein family capable of targeting RNA at low pH. Surprisingly, TgSPN1 is potentially proteolytically processed in the endosome-like compartment before accumulating in an unknown multi-punctate structure in the parasite cytoplasm.

Chapter 1 Introduction

1.1 Apicomplexans

Apicomplexans are unicellular eukaryotic protozoans, most of which are obligate intracellular parasites. Many members of this phylum are clinically or agriculturally relevant pathogens, such as *Cryptosporidium spp.* (cryptosporidiosis), *Babesia spp.* (babesiosis), *Plasmodium falciparum* (malaria), and *Toxoplasma gondii* (toxoplasmosis). These early branching eukaryotes have undergone significant divergent evolution that has resulted in unique organelles such as the apicoplast, a plastid like organelle wherein fatty acids are synthesized, and an apical complex that houses secretory rhoptries, micronemes and dense granules required for cell invasion and the intracellular lifestyle of these organisms.

1.2 Basic Biology of *Toxoplasma gondii*

1.2.1 Life cycle and basic epidemiology

Toxoplasma gondii has a complex life cycle that includes both sexual and asexual stages. *Toxoplasma* replicates sexually within the intestinal epithelial cells of its definitive hosts, which includes any member of the felid family. Sexual replication culminates in the shedding of a spore-like cyst, termed oocyst, in the feces of infected felids. Oocysts promote the spread of *Toxoplasma* through ingestion of contaminated foodstuffs or inhalation of the spores, including during litter changes in domesticated cats. Upon infection of a new intermediate host, the parasite undergoes a shift into the fast replicate asexual stage of the parasite, the tachyzoite.

While most humans experience asymptomatic acute infections, the host immune system actively targets the infection and drives the parasite into a slow-growing, persistent stage that avoids immune clearance. The chronic form of the asexual life stage, the bradyzoite, can persist within tissue cysts in infected hosts for life, including in livestock animals. This can allow for the ingestion of meat contaminated with such tissue cysts, which will spread the parasite to other intermediate hosts, an ability unique to *Toxoplasma*. In addition to the multiple ways it can infect intermediate hosts, *Toxoplasma* is one of the world's most prolific pathogens since the parasite can asexually reproduce in nearly all warm-blooded animals. Given the multiple life stages and large host range, it is not surprising that *Toxoplasma* has developed robust mechanisms to facilitate survival in host cells.

1.2.2 The replicative niche of Toxoplasma gondii

Critical to the survival of all of the life stages of *Toxoplasma* is its isolation from the host cell in a replicative niche known as the parasitophorous vacuole (PV), which helps protect the parasite from the host defenses¹. The PV membrane (PVM) is formed during cell invasion when secreted rhoptry and microneme proteins facilitate active entry into a host cell, with the parasite using the host plasma membrane to create its intracellular compartment^{2,3}. Once the PVM is established, it is remodeled by dense granule (GRA) proteins to facilitate interaction with the host cell. These modifications are critical to the parasite's ability to access to nutrients in the host cytoplasm.

1.3 Nutrient Metabolism and Salvage in *Toxoplasma gondii*

1.3.1 Soluble Nutrients

1.3.1.1 Nutrient pores on the PVM

To acquire soluble nutrients from the infected cell, the parasite must overcome the permeability barrier of the PVM. *Toxoplasma* does this by secreting nutrient pore proteins GRA17, 23, 72, 47, which have been shown to be localized to the PVM and allow for the diffusion of small, <1.7 kDa, soluble molecules to pass into the parasitophorous vacuole (PV) lumen⁴⁻⁷. GRA72 and 47 were recently discovered as part of a synthetic lethal CRISPR screen in GRA17-deficient parasites. Loss of GRA17 substantially reduces the permeability of the PVM, drastically lowering the ability of the parasites to replicate due to reduced nutrient availability. It also introduces a “bubble vacuole” phenotype likely due to decreased transfer of material across the PVM, thus changing the osmotic balance. Similar phenotypes are seen in strains deficient in GRA23, 72, and 47^{5,6}. Generation of strains deficient in more than one nutrient pore protein has not been accomplished yet, suggesting that this is an essential pathway for nutrient acquisition of small soluble molecules such as glucose, nucleosides, and amino acids. However, it should be noted that not all small molecules cross the PVM due to the nutrient pore proteins. ABCG₁₀₇ is an ATP-binding cassette (ABC) G family transporter that has been shown to localize to both the PV and parasite plasma membrane and facilitate the transfer of cholesterol, a nutrient for which *Toxoplasma* is an auxotroph^{8,9}.

1.3.1.2 Sugars and Central Carbon Metabolism

Toxoplasma can engage in glycolysis, gluconeogenesis, the TCA cycle, and the pentose phosphate pathway (PPP) without any metabolic gaps. Commonly, central carbon metabolism is fed by glucose. *Toxoplasma* accesses host glucose in the PV lumen through a high-affinity transporter in the parasite plasma membrane TgGT1^{10,11}. TgST2 is another putative sugar transporter on the parasite plasma membrane, but its substrate is not known¹¹. Once inside the parasite, glucose is phosphorylated by hexokinase (HK) to prevent it from diffusing back to the

PV lumen. Parasites deficient in TgGT1 or HK can still cause a lethal infection in mice, showing that glycolysis is not critical to acute infection by *Toxoplasma*^{11,12}. *Toxoplasma* compensates for the loss of glucose by increasing its utilization of glutamine¹¹. This relies on the TCA cycle to process the glutamine into oxalacetate to feed back into gluconeogenesis. Conversely, impairment of the TCA cycle completely blocks parasite growth¹³.

Intermediates of glycolysis feed into other pathways besides the TCA cycle. Phosphoenolpyruvate (PEP) and dihydroxyacetone phosphate (DHAP) feed into the fatty-acid biosynthesis pathway, found in the apicoplast, via the apicoplast phosphate transporter (APT)¹⁴. Fatty acid biosynthesis produces C16:0 and C14:0 fatty acids for the parasite. The FAS II pathway found in the apicoplast is one of two FAS pathways in the parasite, though the other, FAS I, is dispensable. This is not the case with FAS II, as targeting of the pathway through the FAS II inhibitor triclosan, or depletion of APT, acyl carrier protein, and FabZ, is detrimental to the parasite¹⁴⁻¹⁷. Glucose-6-P, fructose-6-P, and glyceraldehyde-3-P all feed into the PPP. While less work has been done on the PPP in *Toxoplasma*, the end product of the pathway, phosphoribosyl pyrophosphate (PRPP), is critical to the parasite's ability to synthesize pyrimidines¹⁸.

1.3.1.3 Nucleotide Synthesis and Salvage in Toxoplasma

Toxoplasma can produce pyrimidines *de novo*, but it is auxotrophic for purines⁹. Pyrimidine biosynthesis in *Toxoplasma* is driven through the conversion of glutamine and bicarbonate into uracil monophosphate (UMP) by six enzymes¹⁹. UMP is then phosphorylated to uracil triphosphate (UTP) and can be converted to cytosine triphosphate (CTP)²⁰. While parasites lacking pyrimidine biosynthesis due to loss of the first enzyme in this pathway, carbamoyl phosphate synthetase II (CPS II), can replicate in rescue medium supplemented with 0.2 mM

uracil, they fail to replicate in mice and are highly attenuated in virulence²¹. This shows that while *Toxoplasma* can salvage enough pyrimidines for its replication when pyrimidines are abundantly available, it does not have sufficient access to pyrimidines during *in vivo* infection.

The inability of the parasite to produce purines underscores the importance of pilfering nutrients from the host for a parasite. *Toxoplasma* has multiple equilibrative nucleoside transporters (ENTs) on the plasma membrane to acquire nucleosides from the host. *T. gondii* Adenosine Transporter 1 (TgAT1) is the only ENT identified and characterized to date, and it has a low affinity for adenosine and inosine^{22,23}. These studies have described two other ENTs, TgAT2, which has high affinity for adenosine, inosine, guanosine, uridine, and thymidine, as well as low affinity for cytidine, and TgNBT1, which transports xanthine²³. However, TgAT2 and TgNBT1 were only identified by the characteristics of radiolabeled nucleoside incorporation assays, so their genetic identity remains unknown. Of the other recognizable ENTs, TgAT1 and TgENT3 localize to the plasma membrane. Although substrate studies have not been completed, TgENT3 is likely either TgAT2 or TgNBT1, as TgENT3 is localized to the parasite plasma membrane, where these observed transporters operate²⁴. Surprisingly, $\Delta ent3$, $\Delta at1$, and $\Delta ent3\Delta at1$ tachyzoites are viable *in vitro*, suggesting they can still salvage purines, likely via TgAT2 or TgNBT1. Two other ENTs are known, TgENT1 and TgENT2. TgENT2 is not expressed in the tachyzoite or bradyzoite stage. TgENT1 is expressed in tachyzoite and bradyzoite stages and is associated with the Plant-like Vacuole compartment (PLVAC), implying a potential role for the PLVAC in nucleoside salvage²⁴.

There are two purine salvage pathways and one pyrimidine salvage pathway that make use of the nucleosides the parasite scavenges. *Toxoplasma* can convert host adenosine into adenosine monophosphate (AMP) via adenosine kinase, which is an efficient purine salvage

mechanism²⁵. However, using PRPP, hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT), and purine nucleobases like hypoxanthine, xanthine, and guanine, it can produce the monophosphorylated version of those purines²⁶. Pyrimidine salvage in *Toxoplasma* is controlled by uracil conversion into UMP by uracil phosphoribosyltransferase (UPRT)^{27,28}. With these redundancies in nucleoside metabolism, two salvage pathways for purines, and a biosynthesis and salvage pathway for pyrimidines, *Toxoplasma* has robust mechanisms to supply the resources it needs.

1.3.1.4 Amino Acid Metabolism

Toxoplasma is auxotrophic for many amino acids, necessitating transporters at the parasite plasma membrane to scavenge these nutrients from the host. In *Toxoplasma*, sixteen Apicomplexan Amino Acid Transporters (ApiATs) have been identified²⁹. Eight of these transporters (TgApiAT1, TgApiAT2, TgApiAT3-1, TgApiAT3-2, TgApiAT3-3, TgApiAT5-3, TgApiAT6-1, and TgApiAT6-3) localize to the parasite plasma membrane in tachyzoites²⁹. Other ApiATs are not detectable in tachyzoites and may be specific to different life stages²⁹

TgApiAT1 specifically mediates high-affinity uptake of arginine and is critical for virulence, although it becomes dispensable in arginine-rich media³⁰. Subsequently, TgApiAT6-1 was revealed to be a high affinity transporter for lysine and to have lower affinity for arginine^{31,32}. The lower affinity transport of arginine by TgApiAT6-1 explains how parasites deficient in TgApiAT1 are viable in high arginine. However, TgApiAT6-1 is likely the only lysine transporter, as it cannot be knocked out²⁹. TgApiAT5-3 has been characterized as a high-affinity tyrosine transporter²⁹, although it can also transport other aromatic and large neutral amino acids like phenylalanine, tryptophan, and leucine²⁹.

Additionally, at least two amino acid transporters are associated with the Plant Vacuole-like compartment (PLVAC), the lysosome-like organelle of *Toxoplasma*. Parasites deficient in amino acid transporter 1 (TgAAT1) were more susceptible to toxicity from growth in esterified-arginine, suggesting that arginine accumulated in the PLVAC due to loss of TgAAT1³³. While no study has identified the substrates that chloroquine resistance transporter (TgCRT) transports out of the PLVAC, multiple studies have analyzed the PfCRT, due to its critical role in chloroquine resistance in *Plasmodium*. These studies have found that PfCRT transports small peptides 4-11 amino acids in length and neutral or basic amino acids like arginine, lysine, and histidine, likely a similar function to TgCRT^{34,35}. These results emphasize the tendency to have redundant pathways for acquiring or producing nutrients likely in response to the diverse environments *Toxoplasma* encounters in different host cells.

1.3.2 Macromolecular nutrients

1.3.2.1 The Micropore

A potential site of import of vesicles and other material into the parasite cytoplasm is the micropore, a specialized structure that houses an invagination of the parasite plasma membrane^{36,37}. This structure is similar to the cytostome in *Plasmodium* species, which is critical for acquiring hemoglobin from the red blood cell³⁸. However, it has been shown that *Toxoplasma* does not rely on external sources of heme³⁹. Multiple proteins associated with the *Toxoplasma* nutrient pore have been identified, but the most studied is Kelch13^{36,37}. Parasites deficient in Kelch13 do not produce plaques due to stalled lytic growth³⁶. Knockdown of Kelch13 results in decreased turnover of the parasite plasma membrane-derived vesicles³⁶. One study also reported reduced salvage of host cytosolic proteins and ceramide from the host Golgi, suggesting the micropore is a site for uptake of at least certain nutrients³⁷.

1.3.2.2 Lipid uptake

The PVM acts as a recruitment site for host organelles or other structures⁴⁰. Lipids are scavenged by sequestering host-derived vesicles derived from the host lysosome, Golgi, and lipid droplets within folds of the PVM before they are released into the lumen by GRA7 or through a GRA14- and GRA64- mediated interaction with host Endosomal Sorting Complex Required for Transport (ESCRT) components^{41–44}. However, this material has only been observed in the PV lumen.

1.3.3 The Ingestion Pathway

However, lipids are not the only macromolecular molecule scavenged at the PVM. Another potential nutrient acquisition route for *Toxoplasma* is the ingestion pathway, which entails parasite uptake of host cytosolic material, including polypeptides⁴⁵. At the PVM, GRA14 co-opts the host's ESCRT machinery via its interaction with TSG101 and ALIX to generate vesicles that the parasite internalizes^{46,47}. Whether these vesicles are internalized via the micropore is debated, with one study³⁶ providing evidence of a key role for the micropore for ingesting host protein, while another study³⁷, failed to see such an effect. Regardless, other work showed the ingested material traffics through the endosome-like compartment toward the PLVAC⁴⁸. Once inside the PLVAC, the ingested proteins are degraded by TgCPL⁴⁵, the major protease of the PLVAC^{49,50}, and the digested products are likely liberated by a small peptide and amino acid transporter, TgCRT^{51,52} (Figure. 1-1).

1.4 In this Study

Toxoplasma gondii has a myriad of ways to scavenge nutrients from the host cell to support its own biosynthetic processes. While we have proposed that the ingestion pathway acts as a salvage pathway for the parasite, direct evidence of the ingested material is limited to host

cytosolic proteins from our fluorescent protein-based assays. In chapter 2, we use unbiased survey methods, a genome-wide CRISPR screen and bulk metabolomics, to uncover the effects that disruption of the ingestion pathway has on $\Delta gra14$, Δcpl , and Δcrt mutant parasites. In our CRISPR screen, we observed that PLVAC mutant parasites had an increased reliance on biosynthetic pathways, including pyrimidine biosynthesis, the citric acid cycle, and fatty acid biosynthesis, compared to WT. Our bulk metabolomics showed decreased levels of nearly all the amino acids, many of the glycolytic intermediates, and nucleic acids. These surprising observations made us consider the hypothesis that the ingestion pathway was involved in the salvage of host RNA. In chapter 3, we characterize a putative S1/P1 nuclease in *Toxoplasma*, while the pro-form may exist in the endolysosomal system, the mature enzyme is not localized to the PLVAC. In chapter 4, we summarize the work herein and discuss future directions for these projects.

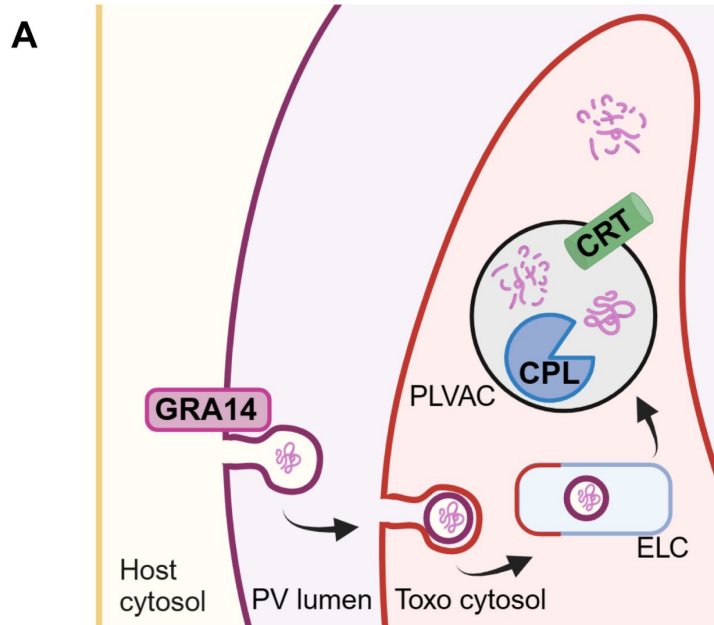


Figure 1-1 The *Toxoplasma* ingestion pathway **(A)** Schematic of the ingestion pathway wherein GRA14 is at the PVM to facilitate uptake of host cytosolic protein, the ingested material is trafficked through the parasite's endosome like-compartment (ELC), delivered to the PLVAC for degradation by CPL and liberated by CRT.

Chapter 2 Metabolic Adaptability and Nutrient Scavenging in *Toxoplasma gondii*: Insights from Ingestion Pathway-Deficient Mutants ¹

2.1 Introduction

The obligate intracellular parasite *Toxoplasma gondii* infects up to 30% of the world's human population⁵³. This success expands beyond humans as *Toxoplasma* can infect any nucleated cell in most warm-blooded animals. The extremely large host range suggests that *Toxoplasma* has evolved versatile mechanisms to support its growth.

The recent advent of CRISPR-based genetic manipulation in *Toxoplasma* has made genetic manipulation in the most genetically tractable apicomplexan even easier. This technology facilitated rapid advances in our understanding of the determinants of parasite fitness, especially through the use of large-scale CRISPR screens. The first genome-wide CRISPR screen assayed parasite growth in normal culture conditions, and a measure of how important each gene was to parasite fitness was calculated and termed the fitness score⁵⁴. For many known essential genes, the fitness score reflected that categorization⁵⁴. The authors were also able to identify novel essential genes from their study, like claudin-like apicomplexan microneme protein (CLAMP)⁵⁴.

This initial screen was just the first step, as new screens were designed that all innovated in different areas. One drawback of this initial screen was that the fitness pressure only compared

¹ The data in this chapter has been published in *mSphere*

Rimple PA, Olafsson EB, Markus BM, Wang F, Augusto L, Lourido S, Carruthers VB. Metabolic adaptability and nutrient scavenging in *Toxoplasma gondii*: insights from ingestion pathway-deficient mutants. *mSphere*. 2025. 0:e01011-24. <https://doi.org/10.1128/msphere.01011-24>

mutants to each other in nutrient-rich environments without immune pressure. A few more recent studies have address these shortcomings. For example, *in vitro* screens in IFN γ stimulated Human Foreskin Fibroblasts (HFFs) and murine macrophages have been done to assay the genes required to survive in these more hostile environments⁵⁵⁻⁵⁷. The two studies in IFN γ stimulated host cells led to the discovery of a complex of proteins consisting of GRA57, 70, 71 that contributed to parasite survival^{56,57}. Additionally, members of the MYR protein secretion complex, responsible for the secretion of GRA proteins into the host cytoplasm to modulate host transcription, were also more fitness conferring in this screen, likely due to preventing these parasites from secreting an effector⁵⁷. In IFN γ -stimulated macrophages, a chaperone-like protein GRA45 was found to convey resistance to IFN γ -based clearance⁵⁵. Notably, these studies did not find great overlap between their hits, highlighting that the diverse host range of *Toxoplasma* necessitates robust mechanisms for interacting with the host.

While these screens brought more relevant cell types and additional selection pressure to the experiments, they were ultimately done *in vitro*. Doing CRISPR screens *in vivo* would be an even greater increase in biological relevance for the data. However, some technical drawbacks make this difficult. Notably, the number of animals needed to test the entire genome of *Toxoplasma in vivo* is a potentially limiting factor. To get around these, most *in vivo* screens have used smaller subset libraries to test genes that are secreted by the parasite, reasoning that genes that are modulating the host response or the interaction of the parasite with the host will be most likely involved in fitness determinants. In an *in vivo* CRISPR screen, GRA12 and GRA38 were shown to be important for parasite survival in a mouse⁵⁸. Surprisingly, this study showed that MYR1, a component of the protein export apparatus at the PVM, was not required for parasite survival in pooled screens, but is critical when the mutant is injected into the mouse

alone. Further research on this phenotype showed that MYR1 exported proteins modulate the host environment to make a replicative niche permissible to parasite survival⁵⁹. Beyond looking for survival at the site of infection *in vivo*, CRISPR screens have looked at what parasite genes promote dissemination in the host⁶⁰. By looking for the loss of mutant parasites in distant organs compared to the site of infection, genes involved in dissemination may be identified. This study identified GRA25, MYR1, and TgWIP (WAVE complex interacting protein) as genes in dissemination *in vivo*⁶⁰. GRA25 likely had this phenotype due to its CCL2 and CXCL1 secretion modulation⁶¹. The reduced dissemination of MYR1-deficient parasites is likely due to MYR1-deficient parasites trafficking to other organs in isolation, unable to benefit from host manipulation facilitated by MYR1-competent parasites. No defects were detected in MYR1-deficient parasites at the site of infection, which likely encountered host cells primed by MYR1-competent parasites due to the high inoculum. This study showed that TgWIP was an uncharacterized protein that induced hypermotility and transmigration of dendritic cells⁶⁰. While these screens were all done using subset libraries, one screen has done a full genome-wide *in vivo* screen of parasite survival and dissemination in mice by using multiple subset libraries⁶². This screen replicated the identification of many known parasite virulence and dissemination contributors, like GRA12 and GRA25. It also identified new contributors to virulence, like TgMYST-B, which was dispensable in cell culture⁶². However, this study did not replicate the rescue of MYR1 deficient parasites in a pooled screen or the decreased dissemination of TgWIP deficient parasites. These data highlight that while CRISPR screens are powerful tools, there can be limitations to these methods and challenges to reproducing complicated phenotypes.

While these CRISPR screens are very powerful tools that look at parasite determinants of fitness in different environments, CRISPR screens in *Toxoplasma* can do a lot more. Screens

have been conducted using fluorescent readouts to identify parasites deficient in converting to the chronic stage of the parasite, leading to the identification of BFD1 (Bradyzoite Formation Deficient 1)⁶³. Fluorescent readouts have also been used to study mutants for abnormal phenotypes in invasion, replication, and egress⁶⁴. Using a splitCas9 system, which would only knock out target genes after the addition of rapamycin, the authors were able to induce gene disruption after parasite invasion. These screens allowed the authors to look for directly observable phenotypes, instead of just shifts in relative ratios of gRNAs.

Recently, a genome-wide synthetic lethal CRISPR screen involving GRA17-deficient parasites was performed. GRA17 deficient parasites will have decreased permeability of the PVM to soluble nutrients, disrupting their access to many nutrients. This is reflected in the frail nature of these parasites. The authors looked at the change in phenotype score for genes in the *Δgra17* mutant compared to the WT and identified another nutrient pore protein, GRA72⁷, that is required in *Δgra17* parasites but not in WT. Additionally, the authors also analyzed their more fitness-conferring hits and an enrichment of genes that were involved in glycerophospholipid metabolism and glycogen degradation, suggesting these are pathways the parasite uses to compensate for the loss of GRA17⁷. This screen mirrors what we attempt to do with our work on the ingestion pathway.

Beyond large-scale genetic screens, metabolic profiling has emerged as a powerful tool for analyzing how parasites scavenge and synthesize nutrients. One notable study examined the metabolic profile of infected host cells over the course of infection by *Toxoplasma*⁶⁵. During infection, changes to intermediates in glycolysis, the TCA cycle, the PPP, amino acid, and nucleotide synthesis were all observed. Notably, increases in tryptophan and the precursor to arginine were observed⁶⁵. This is particularly important as *Toxoplasma* cannot produce its own

tryptophan or arginine and must scavenge them from the host, likely indicating this increase in key host metabolites aids parasite scavenging of such resources^{66,67}. Focusing on another key auxotrophy for *Toxoplasma*, the study noted increased levels of guanosine, inosine, uridine, deoxynucleoside thymidine, xanthine, and phosphoribosyl pyrophosphate (PRPP)⁶⁵. The increased levels of the purines guanosine, inosine, and xanthine likely supports parasite scavenging of these nutrients for conversion by one of the parasite's purine salvage pathways controlled by hypoxanthine-xanthine-guanine-phosphoribosyltransferase. While this study shows the power of tracking metabolites over the course of infection, single-time-point metabolomics on parasites with disrupted salvage pathways can identify metabolites acquired by that pathway. When studying the micropore, researchers used knockdown lines to disrupt this structure, then observed changes in the metabolites of the parasites compared to the parental³⁷. When the micropore was disrupted, ceramide levels were reduced in the mutants, suggesting that ceramide is acquired via the micropore³⁷. These studies highlight how metabolomics can be a powerful tool in dissecting nutrient salvage in *Toxoplasma*.

Despite previous observations demonstrating that the ingestion pathway facilitates the acquisition of host cytosolic proteins, the exact reason for its employment by *Toxoplasma* remains unknown, particularly because disrupting this pathway has little impact on parasite fitness *in vitro*⁵¹. We hypothesize that in the absence of the ingestion pathway, *Toxoplasma* compensates by tapping into alternative nutrient and metabolite sources, such as through upregulation of biosynthesis pathways or increased utilization of other nutrient uptake systems. To test this, we employed a genome-wide CRISPR-mediated gene-disruption screen to identify genes that become more fitness-conferring in ingestion-deficient mutants than WT parasites. By analyzing mutants deficient in three different steps of the pathway ($\Delta gra14$, Δcpl , and Δcrt), we

aimed to uncover genes and pathways that compensate for the loss of ingestion. Additionally, we attempted to identify potential nutrient targets of the ingestion pathway by using bulk metabolomics on these same mutants to determine which metabolites were differentially present compared to the WT. Our results indicate that multiple changes due to the loss of the ingestion pathway are detectable in parasite metabolism. Still, broader metabolic adaptability and nutrient acquisition strategies of *Toxoplasma* reduce the physiological impact of these changes.

2.2 Results

2.2.1 Generation of CRISPR Screen Mutants

Before performing a genome-wide CRISPR screen to identify genes that are differentially fitness-conferring in ingestion mutants, we first generated ingestion mutant-strains in an RHCas9 strain background. We transfected RHCas9 parasites⁶⁸ with sgRNAs targeting the first exon of *GRA14*, *CPL*, or *CRT* to generate loss-of-function INDEL mutants. We verified the disruption of *GRA14*, *CPL*, and *CRT* by Sanger sequencing across the sgRNA-targeted sites (data not shown) and western blotting (Figure 2-1A–C). We also confirmed with an ingestion assay that RHCas9 Δ *gra14* parasites have reduced uptake of host cytosolic protein (Figure 2-1D).

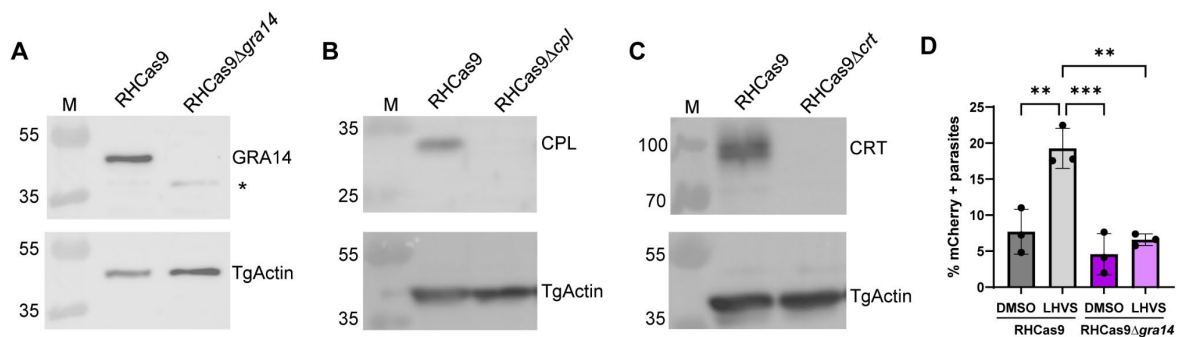


Figure 2-1 Validation of RHCas9 knockouts. (A) Parasite lysates of RHCas9 and RHCas9 Δ *gra14* were probed with Rb α GRA14 or Ms α TgActin (as a loading control). The * denotes a non-specific band. (B) Parasite lysates of RHCas9WT and RHCas9 Δ *cpl* were probed with Ms α CPL or Rb α TgActin. (C) Parasite lysates of RHCas9WT and RHCas9 Δ *crt* were probed with Rb α CRT or Ms α TgActin. Molecular weight markers (M) are shown in kDa. (D) Quantification of host mCherry ingestion 24 h post-infection by RHCas9 or RHCas9 Δ *gra14* parasites treated with DMSO (vehicle) or 1 μ M LHSV to inhibit degradation of host mCherry in the PLVAC ** $p < 0.01$, *** $p < 0.001$. One-way ANOVA with Tukey's multiple comparison. $n = 3$ biological replicates.

2.2.2 CRISPR Screen

A schematic of the screen is presented in Figure 2-3A. Briefly, we screened the WT, *Δgra14*, *Δcpl*, and *Δcrt* strains using an established protocol⁶⁹ with minor modifications, including passaging the library of mutant parasites four times instead of three times to give more time for fitness mutants to drop out of the population. After the final passage, genomic DNA was extracted and used to PCR amplify sgRNAs for sequencing and comparison to the sgRNAs in the initial library. We calculated the phenotype score for each gene in WT or mutant parasites by determining the Log2 fold change in relative abundance from the initial sgRNA pool to the final population. To minimize the effects of a bottleneck, we performed this analysis only for the top 5 most abundant sgRNAs per gene.

A modest bottleneck occurred in our screens, with our final libraries (P4) missing about 30-60% of the sgRNAs (Figure 2-2A). Part of this bottleneck was probably due to random chance since the dispensable genes, those with neutral or positive selection, on average retained 8/10 sgRNAs per gene, which was significantly smaller than the input of 10 sgRNAs at P0 ($p < 0.05$ for WT, $p < 0.01$ for mutants). The essential genes, those that undergo negative selection, on average retained only 3/10 sgRNAs, which was significantly different from the input and the dispensable genes ($p < 0.0001$ for all samples), suggesting that the loss of sgRNAs for the essential genes were due to both random loss and phenotypic selection (Figure 2-2B-D). Despite the modest bottleneck, phenotype scores for the WT screen had an R^2 value of 0.85 compared to the original CRISPR screen in this strain⁵⁴, which is comparable to or higher than other published screens^{55,56} (Figure 2-2E). We also observed a high correlation among WT replicates, suggesting reproducibility (Figure 2-2F). Additionally, previously known essential and dispensable genes stratified along a phenotype score of -2.5, with essential genes having lower

scores and dispensable genes having higher scores, consistent with previous screens⁵⁴ (Figure 2-2G).

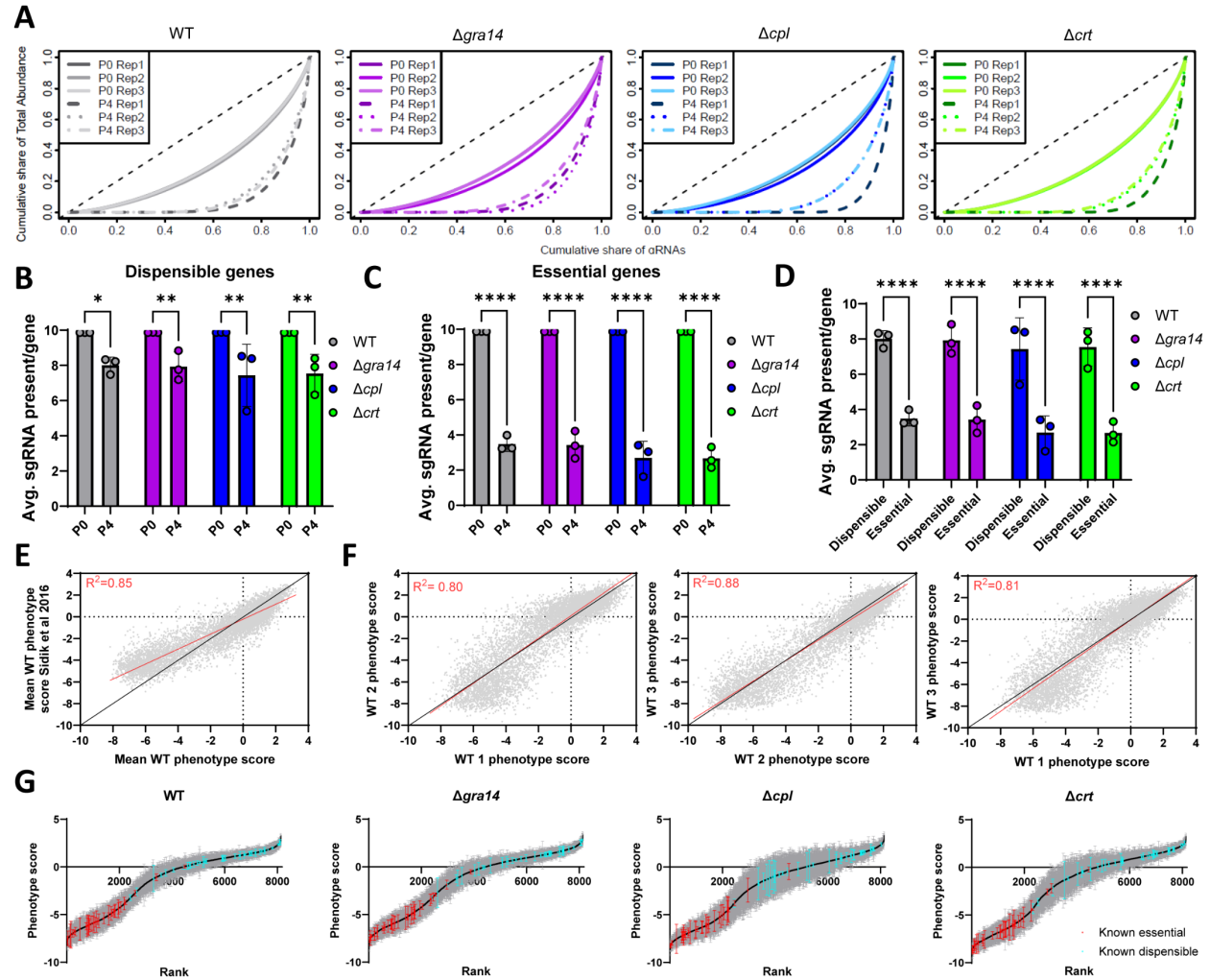


Figure 2-2 Quality control analysis of genome-wide CRISPR screens. (A) Lorenz curves of each CRISPR screen. P0 indicates the initial library for transfection and P4 is the final passage population. (B, C) The average number of unique sgRNA that were present in the initial and final populations for the dispensable (B) or essential (C) genes. (D) The average number of unique sgRNAs that were present per gene for the essential and dispensable genes in the final population of the of each screen. (E) Correlation of our RHCas9 phenotype scores mapped to the previously published scores from Sidiki et al., 2016. (F) Internal correlation of the phenotype score for each WT-screen replicate against the others. (G) Ranked phenotype score graphs with SEM for each screen with known essential (red) and dispensable (cyan) genes marked. (C) * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Two-way ANOVA with Tukey's multiple comparison. $n = 3$ biological replicates.

After calculating the phenotype scores, we assessed for each gene whether the shift in phenotype score between WT and mutants was statistically significant. We used a set of 497 genes expressed only in the sexual stages of the parasite as a reference cohort of dispensable genes to account for random variation in phenotype scores in WT and mutant parasites⁶⁰. We applied two criteria for significance: first, identifying genes with a shift greater than 2 standard deviations from the mean shift among the sexual stage genes, and second, performing a Wilcoxon-signed rank test using the shift in sexual stage genes as the control group to identify significantly shifted genes (FDR <0.01). Genes meeting both criteria were labeled as significantly shifted.

This analysis identified 149, 307, and 845 genes with significantly lower phenotype scores in *Δgral4*, *Δcpl*, and *Δcrt* mutants, respectively, and 210, 96, and 34 genes with significantly higher phenotype scores, respectively (Figure 2-3B, C). We looked for hits shared among all three mutants and found 30 genes that become significantly more fitness-conferring in all three mutants compared to WT (Table 1).

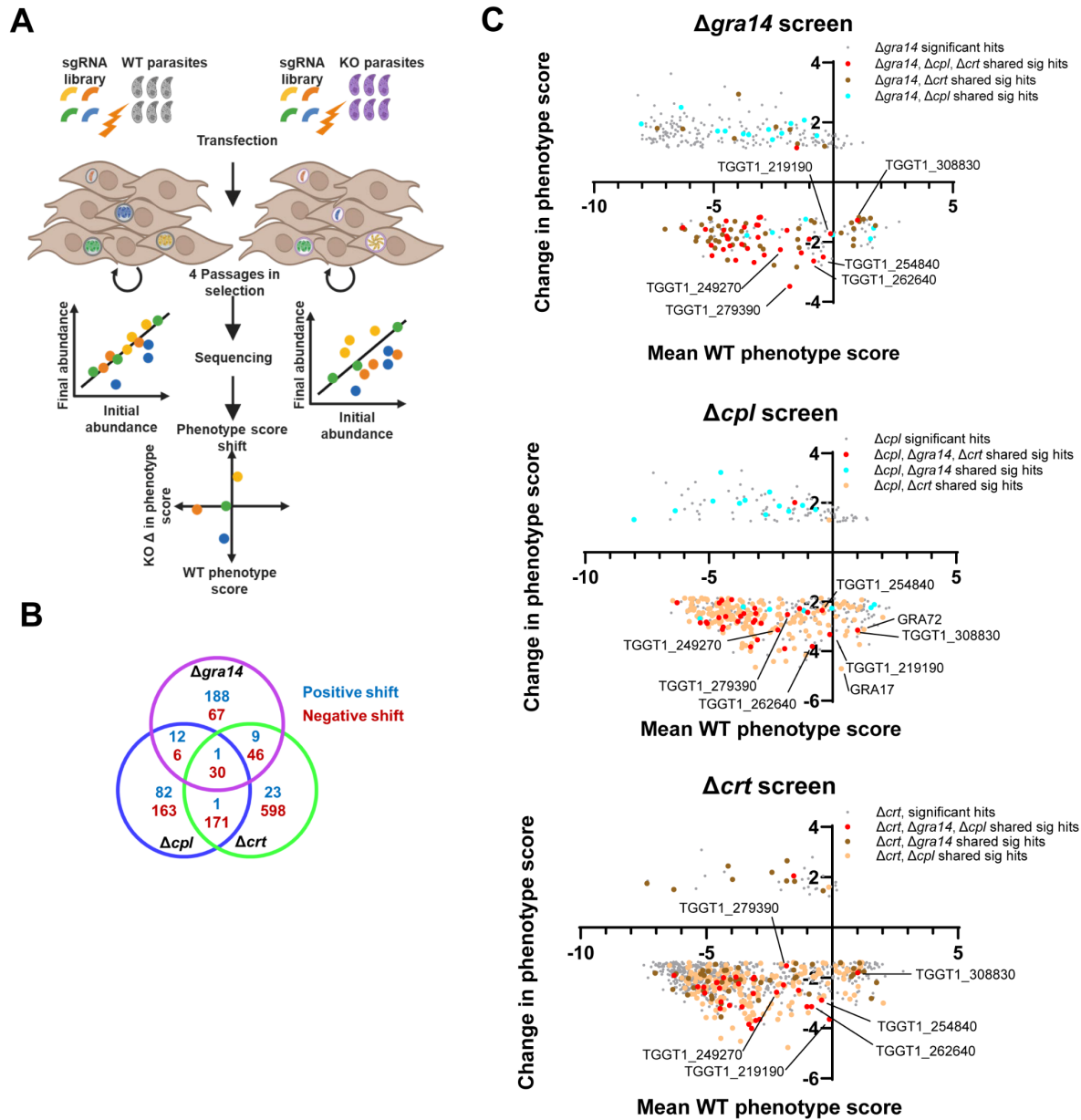


Figure 2-3 Genome-wide CRISPR screen identifies fitness-conferring genes related to the ingestion pathway. (A) Schematic of the CRISPR screens. 6×10^8 parasites were transfected with a linearized plasmid library containing 10 sgRNA per gene of the *Toxoplasma* genome. Parasites were passed 4 times in pyrimethamine to select for integration of the sgRNA-expression cassettes, subsequent disruption of the gene. sgRNA abundance was determined from the initial library and final population through Illumina sequencing, and the phenotype score was calculated based on the top 5 sgRNAs for each gene. (B) Venn diagram indicating significant hits shared between samples. Cyan indicates genes shifted positively; red indicates genes shifted negatively. (C) Graphs depicting the significantly shifted hits for each screen, with the change in phenotype score on the y axis and the phenotype score for the WT screen on the x axis. Grey indicates hits pertaining to just the mutant on the graph, red indicates shared between all mutants, cyan indicates shared between *Δgra14* and *Δcpl*, tan indicates shared between *Δcpl* and *Δcrt*, and gold indicates shared between *Δgra14* and *Δcrt*. $n=3$ biological replicate screens for each strain.

Table 2-1 Shared genes from *Δgral4*, *Δcpl*, and *Δcrt* fitness conferring hits

Gene ID	Product description	LoPIT	WT mean phen score	<i>Δgral4</i> mean phen score	<i>Δcpl</i> mean phen score	<i>Δcrt</i> mean phen score	Sidik et al 2016 phen score
TGGT1_308830	dual specificity protein phosphatase	N/A	1.01	-0.26	-2.14	-0.78	-0.17
TGGT1_219190	nuclear movement protein domain containing protein	cytosol	-0.11	-1.83	-3.43	-3.75	-1.68
TGGT1_254840	tetratricopeptide repeat-containing protein	N/A	-0.42	-2.92	-2.77	-3.31	-1.4
TGGT1_262640	ApiCox23	mitochondrion - membranes	-0.82	-3.46	-4.62	-3.97	-3.49
TGGT1_221320	acetyl-CoA carboxylase ACC1	apicoplast	-1.01	-2.91	-3.44	-4.15	-3.6
TGGT1_286530	ApiCox24	mitochondrion - membranes	-1.34	-3.70	-3.62	-3.84	-2.82
TGGT1_279390	proliferation-associated protein 2G4, putative	nucleus - non-chromatin	-1.82	-5.31	-4.33	-3.35	-0.88
TGGT1_288880	hypothetical protein	N/A	-1.94	-3.55	-5.84	-4.22	-4.6
TGGT1_249270	protein disulfide isomerase-related protein (provisional), putative	apicoplast	-2.21	-4.47	-5.35	-4.78	-4.56
TGGT1_235398	hypothetical protein	mitochondrion - soluble	-2.89	-5.32	-5.76	-6.54	-4.79
TGGT1_267560	folate-binding protein YgfZ protein	mitochondrion - soluble	-3.03	-4.21	-6.57	-6.73	-3.34
TGGT1_254135	hypothetical protein	N/A	-3.11	-4.29	-5.58	-5.09	-2.94
TGGT1_220940	ribosomal RNA methyltransferase (FtsJ) family protein	N/A	-3.13	-4.57	-5.88	-5.76	-2.38
TGGT1_313140	isocitrate dehydrogenase	mitochondrion - soluble	-3.15	-4.99	-5.41	-5.18	-3.04
TGGT1_254530	hypothetical protein	N/A	-3.20	-4.63	-6.04	-7.21	-4.69
TGGT1_273840	brix domain-containing protein	nucleus - non-chromatin	-3.31	-6.00	-7.13	-7.16	-3.18
TGGT1_319930	hypothetical protein	nucleus - chromatin	-3.58	-5.80	-6.07	-6.74	-3.76
TGGT1_227970	histone family DNA-binding protein	apicoplast	-3.72	-5.22	-6.30	-5.95	-2.15
TGGT1_254800	hypothetical protein	mitochondrion - soluble	-3.83	-5.91	-6.63	-5.80	-3.51
TGGT1_218770	carrier superfamily protein	mitochondrion - membranes	-4.08	-6.68	-5.99	-7.17	-4.06
TGGT1_271400	hypothetical protein	N/A	-4.32	-6.45	-6.92	-6.32	-4.91
TGGT1_233930	CAF1 family ribonuclease	mitochondrion - soluble	-4.42	-6.31	-7.44	-7.38	-4.53
TGGT1_267570	hypothetical protein	N/A	-4.45	-6.25	-7.56	-7.67	-4.14
TGGT1_243780	hypothetical protein	nucleus - chromatin	-4.48	-5.77	-6.41	-6.89	-4.68
TGGT1_229620	hypothetical protein	mitochondrion - soluble	-4.58	-6.18	-6.62	-6.76	-4.47
TGGT1_243440	histone lysine acetyltransferase GCN5-B	nucleus - chromatin	-4.60	-6.85	-7.23	-6.78	-2.96
TGGT1_261070	apicoplast triosephosphate translocator APT1	apicoplast	-5.07	-7.53	-7.94	-7.68	-4.44
TGGT1_236210	peptidase M16 family protein, putative	mitochondrion - membranes	-5.10	-7.21	-7.92	-7.47	-4.74

TGGT1_256970	vacuolar ATP synthase subunit A, putative	PM - peripheral 2	-5.34	-6.92	-8.19	-7.70	-3.61
TGGT1_205470	translation elongation factor 2 family protein, putative	cytosol	-6.29	-7.81	-8.33	-8.20	-4.31

2.2.3 Attempt to validate synthetic lethality

To validate whether the genes identified as more fitness conferring in the mutants were actual hits, we tested six genes (TGGT1_308830, TGGT1_219190, TGGT1_254840, TGGT1_262640, TGGT1_279390, and TGGT1_249270) from among those identified in all three mutants, particularly those with phenotype scores of >-2.0 in WT parasites, indicating knock-out feasibility. WT, $\Delta gra14$, or Δcpl parasites were transfected with sgRNAs targeting the six genes. sgRNAs targeting *sag1* and *cdpk1* were included as negative and positive controls, respectively⁷⁰. Nascent transfected populations were directly plaqued in the presence of solvent (ethanol, no drug selection) or 3 μ M pyrimethamine (drug selection) for 7 or 10 days, respectively. The wells were fixed, stained with crystal violet, and imaged. Using a modified Cellpose pipeline, we automated measuring the number and size of plaques (Figure 2-4A). The no-drug selection wells were a correction factor determining the number of viable parasites within drug-selected wells.

As expected, the $\Delta cdpk1$ wells produced few to no plaques since *CDPK1* is an essential gene⁷⁰. This result confirms that disrupting fitness-conferring genes reduced the WT and mutants' fitness. There were trends toward $\Delta gra14\Delta254840$ and $\Delta cpl\Delta254840$ having lower plaque efficiencies than WT $\Delta254840$ (Figure 2-4B), but the differences were not statistically significant. Compared to $\Delta sag1$, WT $\Delta262640$ plaques were statistically smaller, while there was a similar trend in the $\Delta gra14\Delta262640$ plaque size. Additionally, $\Delta247290$ parasites trended towards smaller plaque sizes in WT and $\Delta gra14$ parasites. The smaller plaque size in the WT strain for these mutants suggests these genes are false positives (Figure 2-4C). In previous

screens, the phenotype scores of $\Delta 262640$ and $\Delta 247290$ were notably lower (-4.0 and -4.8, respectively) than we observed (-0.8 and -2.2, respectively). None of the other genes had significantly smaller plaque sizes, indicating an inability to identify synthetic lethal genes conclusively. However, we verified that disrupting fitness-conferring genes reduced the WT and mutants' fitness.

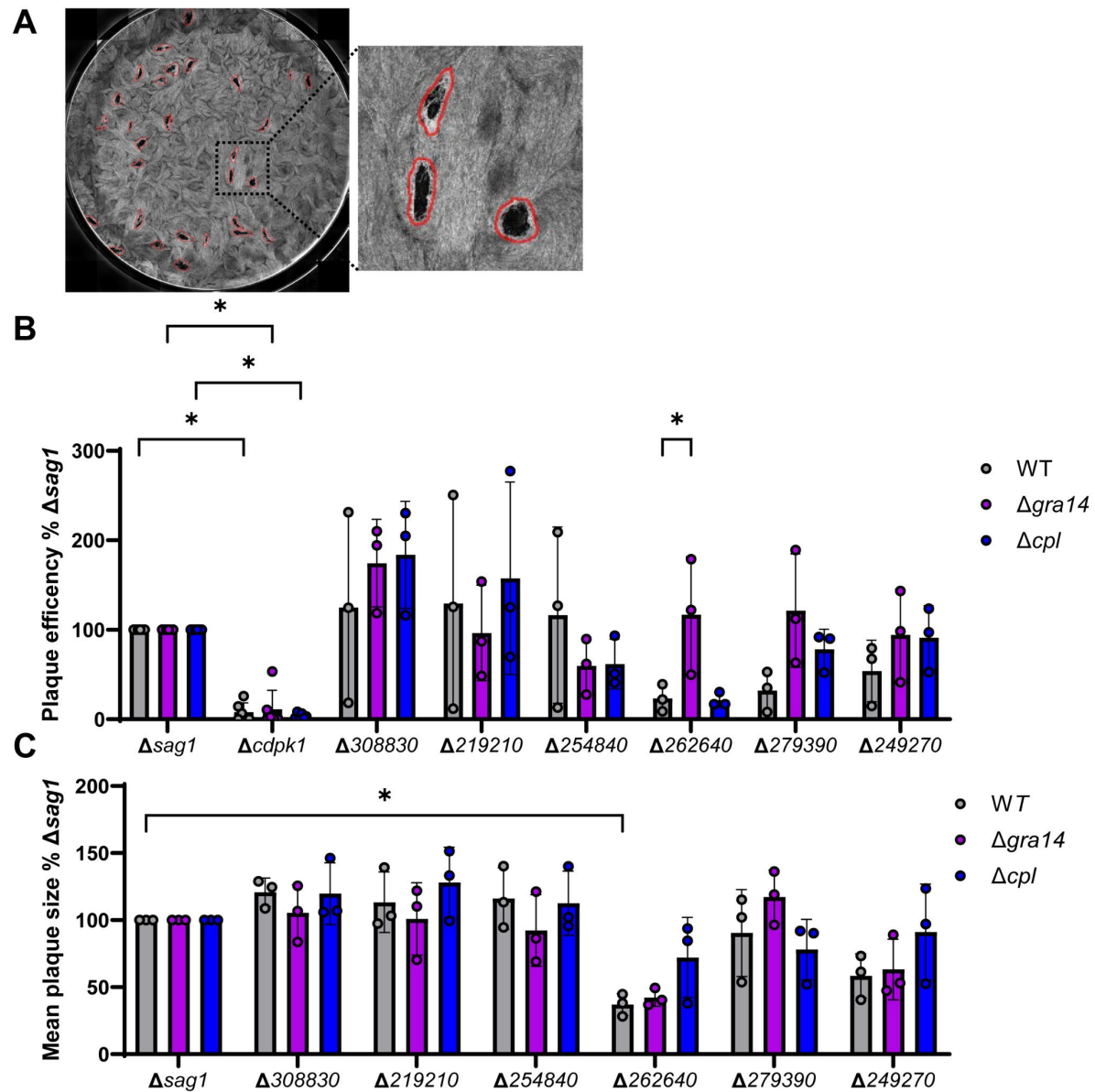


Figure 2-4 . **Analysis of plaque efficiency and size for screen hits.** (A) Representative image of crystal violet stained well after 4x magnification images were stitched together using Nikon Elements. Masks were created after automated plaque identification was performed using the plaque assay module in SpaCr, a modified cellpose cyto model trained to detect plaques in monolayers of cells, on two wells per condition. Masks were counted for plaque count, and size was recorded for plaque size analysis. Red outlines trace the mask area onto the input image. (B) RHCas9WT, RHCas9 Δ *gral4*, and RHCas9 Δ *cpl* parasites were transfected with an sgRNA targeting the indicated gene, and 1×10^3 or 1×10^4 parasites were inoculated into a 6-well plate under 3 μ M pyrimethamine selection for 10 days. Wells were fixed in 4% formaldehyde and stained with 2% crystal violet before being imaged. Plaque efficiency was calculated as the number of plaques counted versus the number of parasites added to the well, as determined from the plaque counts of non-drug selected wells, then normalized to the Δ *sag1* value for the given strain. (C) Plaque size was calculated and normalized to the Δ *sag1* value for the given strain. **B, C** The dots are means with SD as the error bars. * $p < 0.5$, two-way ANOVA with Tukey's multiple comparison, only comparisons between the same strain Δ *sag1*: Δ GOI and WT:ingestion mutant Δ GOI are being shown. $n=3$, except for Δ *sag1* and Δ *cdbl* in **B** which was $n=6$.

2.2.4 Pathway enrichment analysis

We reexamined our data to assess patterns in the ingestion mutant pairs. The largest overlap in significant genes was between Δ *cpl* and Δ *crt* with 201 total genes, likely due to both proteins being in the PLVAC. Thus, some hits could be involved in compensating for phenotypes associated only with the PLVAC. The Δ *gral4* and Δ *cpl* mutants and the Δ *gral4* and Δ *crt* mutants shared 76 and 36 genes, respectively. This suggests these genes probably do not function together in other pathways outside the ingestion pathway (Figure 2-3B).

To determine if this overlap was significant or due to random chance, we tested hypergeometric overlap and found significant overlap in all three gene pairs. This led us to conclude that our CRISPR screens successfully identified genes that were more fitness conferring due to the disruption of the ingestion pathway or the PLVAC.

To identify potential compensatory mechanisms among the more fitness-conferring genes, we conducted a metabolic pathway analysis using tools available on ToxoDB. No clear compensatory pathway emerged from the 30 genes more fitness-conferring shared by all three mutants (Appendix A). When we did pathway enrichment analysis on each screen's hits individually, we saw that Δ *gral4* did not show significant impacts on metabolic pathways, with

the pathway analysis returning only 1-2 genes per pathway (Appendix B). However, the Δcpl and Δcrt hits contained many genes involved in metabolic pathways, and had multiple similar pathways become enriched. Unique to the Δcpl screens were enrichment in genes required for lipoate biosynthesis, lysine degradation, and glycine, serine, and threonine metabolism (Figure 2-5A). Potentially suggesting some direct effects of disrupted protein turnover. Unique to the Δcrt screen was enrichment in NAD metabolism and ubiquinol biosynthesis (Figure 2-5B).

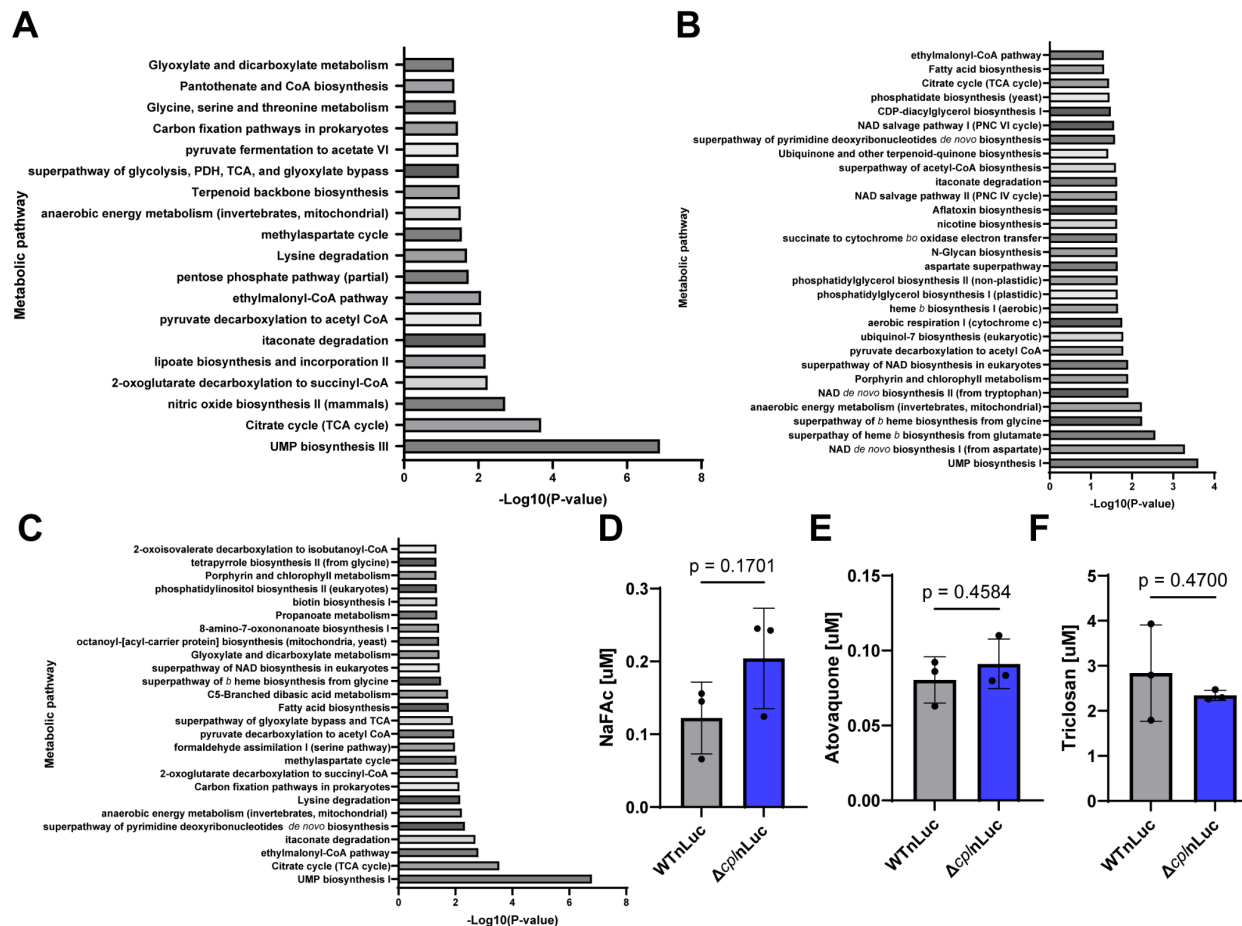


Figure 2-5 . Ingestion mutants rely on several metabolic pathways. (A, B, C) Pathway enrichment analysis of the significantly more fitness conferring hits in the RHCas9 Δcpl screen (A), RHCas9 Δcrt screen (B), and shared between the RHCas9 Δcpl and RHCas9 Δcrt screens (C) showing Kegg and Metacyc hits from ToxDB using default settings, with duplicate pathways removed and single gene pathways excluded. (D, E, F) Confluent HFF monolayers in 96-well plates were infected in triplicate with 2,000 WTnLuc or $\Delta cplnLuc$ parasites in different

concentrations of the TCA-cycle inhibitor sodium fluoroacetate (NaFAc) (D), the electron transport chain inhibitor atovaquone (E), or the FA biosynthesis inhibitor triclosan (F). After 4 days at 37 °C in 5% CO₂, parasite growth was measured by luciferase activity. Luminescence values were normalized to growth in vehicle (DMSO), and the IC₅₀ was calculated for parasite growth in each drug. The dots are mean IC₅₀ values with SD as the error bars from 3 biological replicates. Mann-Whitney test.

The Δcpl and Δcrt samples had a large overlap of shared hits. Interestingly, all six genes required for pyrimidine biosynthesis (TGGT1_210790, TGGT1_259660, TGGT1_259690, TGGT1_291640, TGGT1_293610, TGGT1_308580) were among the overlapping genes. Also, four overlapping genes are involved in fatty acid (FA) biosynthesis (TGGT1_217740, TGGT1_221320, TGGT1_225990, TGGT1_251930), eight in the TCA cycle (TGGT1_215280, TGGT1_219550, TGGT1_244200, TGGT1_290600, TGGT1_305980, TGGT1_309752, TGGT1_313140, TGGT1_314400), and five in lysine degradation (TGGT1_219550, TGGT1_236570, TGGT1_244200, TGGT1_301120, TGGT1_305980) (Figure 2-5C).

This data suggests that the Δcpl and Δcrt mutants might be more reliant on their own biosynthetic pathways due to decreased turnover of material in the PLVAC. In contrast, reduced uptake through the ingestion pathway in $\Delta gra14$ parasites does not appear to increase the parasites' reliance on biosynthetic pathways.

To determine whether PLVAC disruption increased reliance on metabolic processes, we tested the ability of RH $\Delta ku80\Delta hxgnLuc:hxg$ (WTnLuc) and RH $\Delta ku80\Delta hxgnLuc:hxg\Delta cpl:dhfr$ ($\Delta cplnLuc$) parasites⁷¹ to grow in the presence of drugs targeting these pathways. Nano luciferase expression allowed us to measure parasite growth via luminescence. We hypothesized that increased reliance on biosynthetic pathways would heighten sensitivity to their disruption. However, WTnLuc and $\Delta cplnLuc$ parasites did not have significantly different IC₅₀ values when grown in the presence of sodium acetate, atovaquone, or triclosan, which target the TCA cycle, the electron transport chain (ETC), and FA biosynthesis, respectively (Figure 2-5B-D). While

Δcpl was not more susceptible to these drugs, it remains unclear if there was an effect on the metabolomic profile of these parasites.

2.2.5 Bulk metabolomics of ingestion mutants

Our metabolic pathway analysis motivated us to conduct bulk metabolomics on the ingestion mutants to determine the direct impact of the ingestion pathway loss on the resources available to the parasite. We harvested WT, $\Delta gra14$, Δcpl , and Δcrt mutants after 44 hours of growth in confluent HFF monolayers. Additionally, we included two WT samples treated with LHVS, a chemical inhibitor of CPL, and DMSO, as a vehicle control, providing comparative points for our WT and Δcpl samples.

Principal component analysis (PCA) of detected metabolites showed that WT and WT-DMSO clustered together, while $\Delta gra14$, Δcpl , and Δcrt , and 4 out of 6 WT-LHVS samples clustered together and away from the WT samples, indicating conserved changes in these parasites (Figure 2-6A). We calculated the Log2 fold change of metabolites in the ingestion mutants vs WT and LHVS vs DMSO condition (Appendix C, Figure 2-6B). However, this left us with many metabolites that did not slot into pathways detected in our pathway analysis, so we restricted further analysis to the central carbon metabolism, pentose phosphate pathway (PPP), the citric acid cycle (TCA), energy molecules, nucleosides, and amino acids. We noticed that most metabolites in the Δcpl and LHVS samples followed similar trends (Figure 2-6C), but were not identical, possibly due to LHVS inhibiting both parasite CPL and host cathepsin L, thus impacting the host resources available for parasitic scavenging.

Continuing the analysis with only the WT, $\Delta gra14$, Δcpl , and Δcrt samples, we observed several trends from our bulk metabolomics data. Intermediates and end products of glycolysis were reduced in the ingestion mutants (Figure 2.7A–B). Specifically, pyruvate was significantly

mutants. Within the PPP, 6-O-phosphono-D-gluconic acid was increased in all the ingestion mutants. Since ribose is a main product of the PPP, these findings suggest the need for increased pyrimidine biosynthesis due to disrupted nucleoside salvage. This was further supported by significantly lower levels of guanosine, adenosine, and cytidine in $\Delta gra14$ parasites (Figure 2-7A). Precisely how the ingestion pathway is connected to nucleoside salvage remains to be determined.

The ingestion mutants exhibited markedly reduced levels of most amino acids, with $\Delta gra14$ mutants showing significantly reduced levels of all amino acids except alanine and valine, which still trended lower (Figure 2-7A). The Δcpl mutant had higher levels of glycine, while leucine and isoleucine trended upward. While the Δcrt mutant showed higher levels of glutamic acid, serine, and three other amino acids trended upward. This data supports a role for the ingestion pathway in scavenging host cytosolic proteins. Despite fewer amino acids in the mutants, there is little to no growth defect compared to WT parasites, suggesting the parasite has an excess pool of amino acids and can acquire sufficient amino acids through other means, such as amino acid transporters.

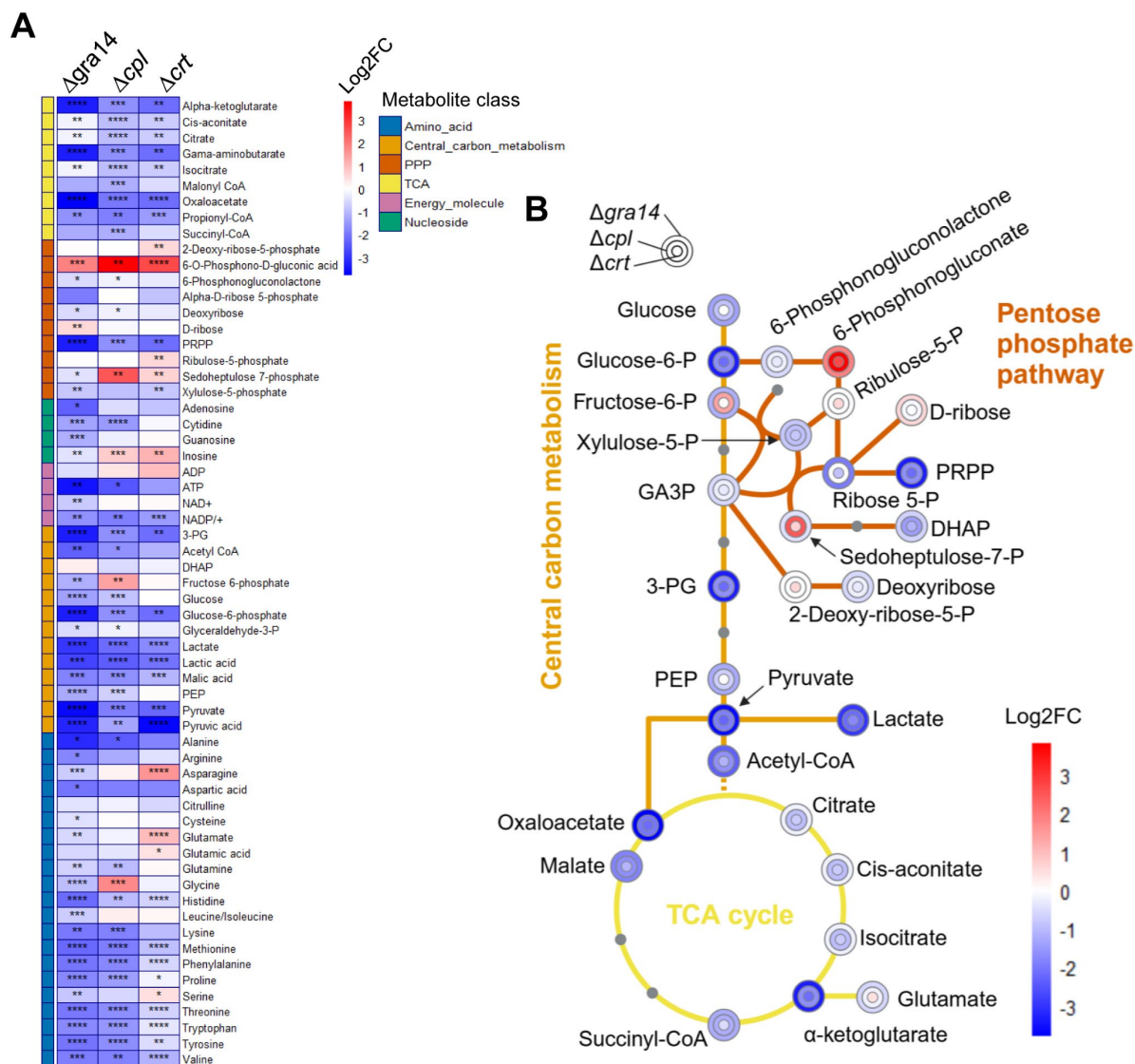


Figure 2-7 Metabolic analysis of ingestion mutants shows a decrease in amino acids and glycolic end products. (A) Heat map of select metabolites from bulk metabolomics data for the ingestion mutants, RHCas9 Δ *gra14*, RHCas9 Δ *cpl*, RHCas9 Δ *crt*. Parasites infected HFFs for 44 h before the monolayer was harvested. Parasites were freed from host cells and washed to remove residual media then subjected to LC-MS. Each metabolite was normalized to the RHCas9WT condition then the Log2FC was calculated. * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, t-test. $n = 6$ biological replicates. (B) Metabolic pathway schematic for RHCas9 Δ *gra14*, RHCas9 Δ *cpl*, and RHCas9 Δ *crt* depicting the central carbon metabolism (glycolysis and fermentation) in orange, feeding into the citric acid cycle in yellow, or the pentose phosphate pathway in dark orange. The color of the dot is the Log2FC that the metabolite experienced in the mutant parasite. Grey dots are metabolites that were not looked at in our analysis

2.2.6 Ingestion mutant growth under amino acid limitation

To assess if the ingestion pathway significantly contributes to amino acid acquisition under nutrient-limited conditions, we grew the mutants in single amino acid-depleted media.

Using the nano-luciferase strain, we generated $\Delta gra14nLuc$ and $\Delta crt nLuc$ strains by disrupting the gene locus and replacing the coding sequence with a *dhfr* resistance cassette, like what was done for the $\Delta cpnLuc$ strain⁷¹ (Figure 2-8A-D). Starving HFFs for 24 hours before parasite infection ensured reduced internal amino acid stores. We focused on tryptophan and phenylalanine, which were significantly reduced in all three mutants, and included tyrosine and arginine due to tyrosine being the other aromatic amino acid, host arginine levels being controlled by host cells, and *Toxoplasma* being auxotrophic for both amino acids^{9,66,30,72}.

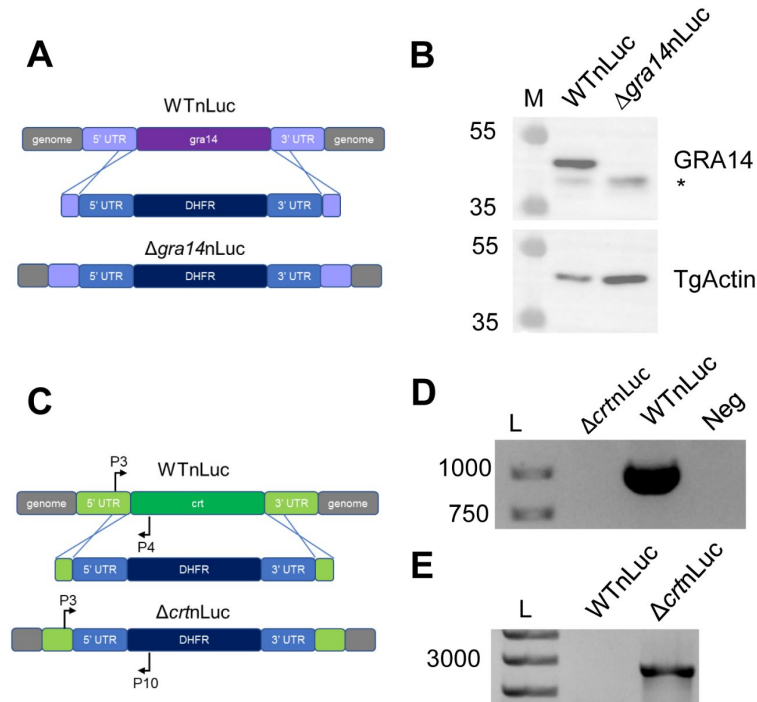


Figure 2-8 Generation of RH $\Delta ku80\Delta gra14nLuc$. (A) Schematic of replacing the *gra14* locus with DHFR (B) Parasite lysates of RH $\Delta ku80nLuc$, RH $\Delta ku80\Delta gra14nLuc$ with Rb α GRA14 or Ms α TgActin as a loading control. Molecular weight markers (M) are shown in kDa. The * denotes a nonspecific band. (C) Schematic of replacing the *CRT* locus with DHFR (D) Genomic DNA of WTnLuc and $\Delta crt nLuc$ PCR'd with P3 and P4 to look for the native *CRT* locus. (E) Genomic DNA of WTnLuc and $\Delta crt nLuc$ PCR'd with P3 and P10 to assay 5' integration. D, E DNA Ladder (L) is shown in bp.

We assayed parasite growth in complete (D1) and tryptophan-free (D1 -W) media to determine doubling time over 3 days. There was no difference in doubling time for WTnLuc, $\Delta gra14nLuc$, $\Delta cpnLuc$, and $\Delta crtLuc$ parasites in complete media. However, in tryptophan-free media, $\Delta cpnLuc$ and $\Delta crtLuc$ mutants grew significantly slower than WTnLuc parasites (Figure 2-9A). Parasites did not grow in phenylalanine, arginine, or tyrosine-free media, so we tested diminishing concentrations of these amino acids on WTnLuc parasites to identify minimal growth conditions (Figure 2-10A-C). Testing parasites in 6.5 μM F, 8.6 μM R, or 3.4 μM Y showed smaller shifts in overall doubling time, yet $\Delta cpnLuc$ still grew significantly slower in arginine- and tyrosine-limiting conditions (Figure 2-94B). Additionally, all nutrient-limited growth conditions grew slower than the complete media (Figure 2-9A, B). Overall, this data suggests that *Toxoplasma* may rely on material turnover in the PLVAC during nutrient-limited situations, facing difficulties in resource scavenging.

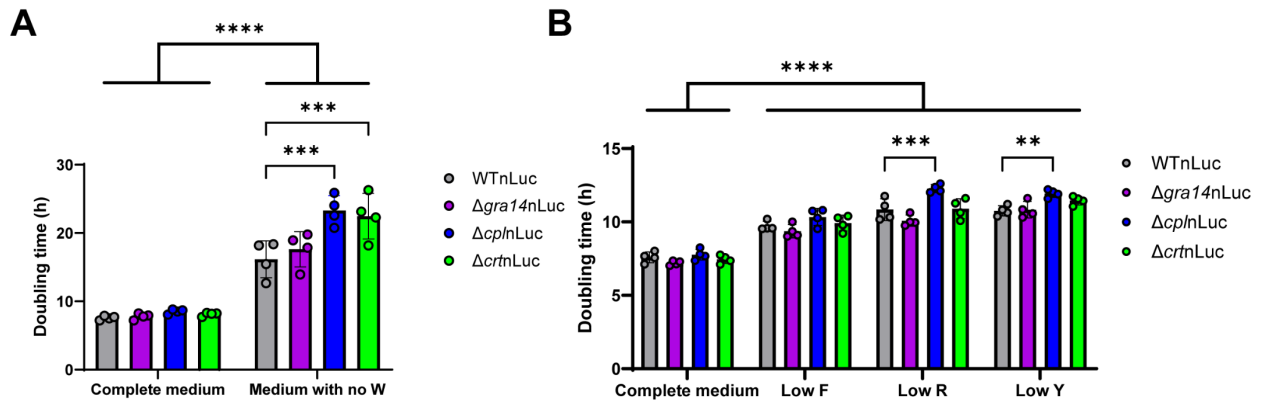


Figure 2-8 Nutrient-limited conditions inhibit ingestion mutant replication. (A, B) HFF monolayers grew to confluency over 1 week in 96-well plates. 24 h before infection, D10 was replaced with complete D1 or D1 without tryptophan (A) or with 6.5 μM phenylalanine, 8.6 μM arginine, or 3.4 μM tyrosine (B). 2,000 WTnLuc, $\Delta gra14nLuc$, $\Delta cpnLuc$, or $\Delta crtLuc$ parasites infected monolayers in triplicate per condition. Luciferin was added at 4, 24, 48, and 72 hours post-infection to measure parasite growth. Samples were normalized to the 4 hpi reading and then Log2-transformed. The slope of the linear regression is the doubling time of the parasites. The dots represent mean doubling times with SD as the error bars. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ two-way ANOVA with Tukey's multiple comparison. $n = 4$ biological replicates.

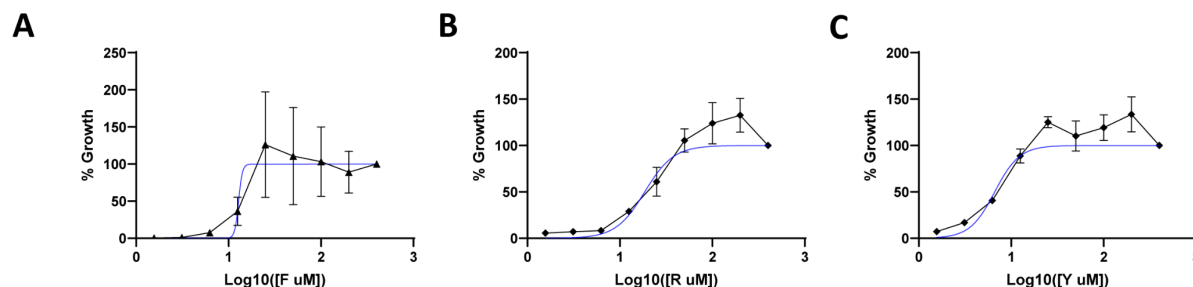


Figure 2-9 Growth of *Toxoplasma* in phenylalanine-, arginine-, or tyrosine- limiting conditions. (A, B, C) Confluent HFF monolayers in 96-well plates were prestarved for 24 hours in D1 complete media serially diluted in D1 missing phenylalanine (A), arginine (B), or tyrosine (C) to create D1 with concentration gradients for the experimental amino acid before infecting with 2,000 WTnLuc parasites per well with 3 technical replicates per dilution. Four hours post-invasion, the media was changed, keeping the dilution series, and the first reading was taken. The rest of the samples grew for 4 days at 37 °C in 5% CO₂. Readings were normalized with the 4 hours post-infection readings and plotted as a percentage of growth in the complete media. A non-linear fit was used to determine the EC₅₀. The dots are the mean growth with SD represented by the error bars. *n*=3 biological replicates.

2.3 Discussion

Our results provide insights into the adaptive mechanisms employed by *Toxoplasma* in the absence of a functional ingestion pathway. Despite modest bottlenecks in our CRISPR screens, comparison of the WT screen done herein to the original screen by the Lourido Lab⁵⁴ showed a strong correlation. Further, we noted statistically significant overlaps in hits shared by pairwise analysis of $\Delta gra14$, Δcpl , and Δcrt . The overlap between Δcpl and Δcrt was particularly strong and involved pathways for pyrimidine biosynthesis, FA biosynthesis, the TCA cycle, and lysine degradation. This suggests a pivotal compensatory role for these biosynthetic pathways when the PLVAC is disrupted. However, we were unable to confirm increased reliance on the pathways identified by metabolic pathway analysis, via our drug sensitivity assay. Our bulk metabolomic profiles of the ingestion mutants complemented our pathway analysis findings, revealing a pronounced deficiency in glycolytic intermediates and certain amino acids, suggesting a marked alteration of these biosynthetic pathways when the ingestion pathway is compromised.

Our detailed metabolomic analysis supports the hypothesis that loss of the ingestion pathway significantly impacts amino acid acquisition. Mutant strains exhibited a global decrease in most amino acids, indicative of disrupted amino acid salvage from the host cytosol. Notably, the $\Delta gra14$ mutant displayed markedly reduced levels of all amino acids except alanine and valine, while the Δcpl and Δcrt mutants showed a slightly more complex but similar amino acid profile disruption. This supports the notion that the ingestion pathway functions in scavenging key nutrients, particularly amino acids, from the host.

We explored this hypothesis by growing the mutants in single amino acid-depleted media. The lack of a differential growth defect in $\Delta gra14$ vs WT parasites during amino acid deprivation, despite amino acid levels being most disrupted in this mutant, suggests sufficient access to the resources for growth. This may be due to redundant GRA proteins still acquiring sufficient nutrients from the host in the form of other macromolecule uptake systems or diffusion of soluble nutrients across the nutrient pore. Conversely, if the PLVAC is a hub of exogenous and endogenous amino acid turnover, even with reduced amino acid uptake in $\Delta gra14$ parasites, sufficient resources may be recycled for parasite growth. This is consistent with our findings that, under nutrient limitation, PLVAC mutants displayed growth impairments, especially in conditions deficient in tryptophan, arginine, and tyrosine. Our findings suggest that *Toxoplasma* uses the PLVAC as a site of protein turnover for amino acids during host nutrient deprivation, such as during host infection-induced amino acid starvation.

Interestingly, in our Δcpl CRISPR screens, two of the four known nutrient pore genes, GRA17 and GRA72, were detected as more fitness conferring (Figure 2-3C). Supportive of this interaction, the GRA17 synthetic lethal screen also observed a negative shift in the phenotype score of CPL, though it was not interrogated for a synthetic lethal phenotype⁷. This raises an

interesting hypothesis that when protein turnover is impaired by loss of CPL, the parasite might rely more heavily on soluble nutrients as ready-to-use material. Conversely, when soluble resources are scarce in GRA17-deficient parasites, protein turnover recycles useful material. In partial support of this hypothesis, the glucose transporter TgGT1 and hexokinase are more fitness-conferring in the Δcpl screen. This could indicate that *Toxoplasma* needs to generate more glycolytic intermediates or energy when protein turnover is impaired. Speculatively, indications of what material is being turned over in the PLVAC might also be inferred from our Δcpl CRISPR screens. Interestingly, we also noticed that LipA, the lipoic acid synthase, was more fitness-conferring in the Δcpl mutant. Lipoic acid is needed for enzymes in both the apicoplast and mitochondria. However, *Toxoplasma* can only produce lipoic acid via its synthase in the apicoplast, while the lipoic acid in the mitochondria is scavenged from the host⁷³. This suggests that some lipoic acid may be turned over in the PLVAC for reuse.

Our study provides valuable insights into the adaptive resilience and metabolic flexibility of *Toxoplasma*. This is highlighted in the metabolomic data and pathway analysis that showed a decrease in nucleosides and an increased reliance on pyrimidine biosynthesis in these mutants. This data suggests that the ingestion pathway may also scavenge host cytosolic nucleosides, possibly in the form of host rRNA, mRNA, and tRNA. Recently, a preprint study localized TgENT1, an essential equilibrative nucleoside transporter, to the PLVAC²⁴. This transporter could play a role in liberating host nucleosides acquired by the parasite via the ingestion pathway.

Among the limitations of this study, we were unable to directly validate any overlapping hits as being synthetically lethal. Of the 30 genes that were consistently more fitness conferring in all three ingestion-deficient mutants, only 9 of the hits had WT phenotype scores that were

indicative that they might be dispensable in WT parasites. However, upon looking at data from previous screens, we can see that 5 of these genes, TGGT1_262640, TGGT1_221320, TGGT1_286530, TGGT1_288880, and TGGT1_249270 have phenotype scores < -2 , which indicates another screen found them fitness conferring in WT parasites⁵⁴. This highlights the importance of recognizing a degree of uncertainty with phenotype scores. Also, we attempted to validate the hits by measuring viability (plaqueing efficiency) and overall progression through the lytic cycle (plaque size). These assays do not measure fitness in the same way as the CRISPR screen, which is essentially a competition assay. Alternative ways of validating hits for synthetic lethal screens, including by pairwise competition assays, should be considered.

Our findings underscore the reliance of *Toxoplasma* on a multiplicity of adaptive pathways to compensate for the loss of nutrients. This flexibility ensures the parasite can thrive under various constraints, highlighting its evolutionary success as an intracellular pathogen.

2.4 Materials and methods

Host cells and cell culture: Human foreskin fibroblasts, HFFs, (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose supplemented with 2mM L-glutamine, 10% Cosmic calf serum, 20 mM HEPES, and 5 μ g/mL pen/strep (D10) at 37°C and 5% CO₂ unless otherwise stated. *Toxoplasma gondii* strains were passaged in the above HFFs.

Parasite strain generation: A list of all parasite strains, primers, and plasmids can be found in Appendix D-F, respectively. To generate RHCas9*Agra14*, RHCas9 parasites were transfected with 50 μ g of circular pU6-DHFR-GRA14gRNA1, which targets the first exon of the gene. To prevent integration of the resistance maker, the plasmid was not linearized. After 24 h, the media was changed. Once the HFF monolayer was lysed, parasites were syringed, filtered, and diluted into 96-well plates to isolate single clones. Clones were grown for one week and

expanded to 24-well plates for DNA extraction. DNA was extracted using a Qiagen DNeasy Blood and Tissue kit and subjected to PCR by the Q5 polymerase with primers, P1 and P2, which flank the gRNA cut site. Purified fragments were sent for Sanger sequencing to identify INDEL mutations. The same procedure was used to generate RHCas9 Δcrt using pU6-DHFR-CRTgRNA 1 and primers 3 and 4. RHCas9 Δcpl was generated similarly, but pCas9-GFP-Ble-CPLgRNA1 was used to allow for sorting of GFP⁺ parasites. Twenty-four hours after transfection, the monolayer was scraped, syringed, and filtered. Parasites were resuspended in PBS + 5% FBS and sorted by flow cytometry. GFP⁺ parasites were used to infect HFFs, and once this monolayer was lysed, the procedure above was followed, and primers 5 and 6 verified the INDEL. Protein disruption was validated by western blotting using antibodies specific to these proteins.

To generate RH $\Delta ku80nLuc\Delta gra14$, RH $\Delta ku80nLuc$ parasites were transfected with 50 μ g of pCas9-GFP-Ble-GRA14gRNA1 and 10 μ g of repair template that had 40 bp of homology to the 5' and 3' ends of the genomic DNA, flanking a DHFR resistance cassette. 24 h post-transfection, the media was changed, and 3 μ M pyrimethamine was added for selection. Once the population was lysing, the monolayer was scraped, syringed, filtered, and cloned into 96-well plates. After one week, the Phire Tissue Direct PCR kit was used to test for integration of the DHFR resistance cassette using primers P9 and P10 for 5' integration. This was also validated at the protein level by western blotting. A similar approach was used to generate RH $\Delta ku80nLuc\Delta crt$. However, two sgRNAs, pCas9-GFP-Ble-CRTgRNA1 and pCas9-GFP-Ble-CRTgRNA2, were used as *CRT* is a multi-exon gene. Primers P3 and P10 were used to test for 5' integration.

Ingestion assay: Inducible mCherry HeLa cells and harvesting steps were used from a previous study⁴⁷. After harvest, parasites were fixed with 4% methanol-free formaldehyde and imaged on a Zeiss Axiovert Observer fluorescence microscope. Samples were blinded, and mCherry+ parasites were counted. Data were graphed and analyzed in Prism.

Immunoblot: A list of all antibodies is in Appendix G. Parasite lysates were harvested using RIPA buffer solution (Thermo Scientific, 89900) with EDTA-free miniComplete protease inhibitor (Roche, 11836153001). PVDF membranes were activated with methanol for protein transfer, then blocked with 5% milk in PBS-Tween. Primary antibody was incubated O/N at 4°C, or at 1 h at RT for loading controls, followed by horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at RT. Signal was developed with 2 min incubation in SuperSignal West Pico PLUS Chemiluminescent or SuperSignal West Femto Maximum sensitivity substrate. The chemiluminescent signal was visualized using the Syngene PXi6 imaging system. Membranes were stripped using 4% trichloroacetic acid, then blocked and re-probed as needed.

CRISPR screens: Genome-wide screens used an sgRNA library (CrLib) containing 10 sgRNAs per gene for 8,156 genes in *Toxoplasma*, following an established protocol⁶⁹ with few changes. The CrLib was linearized using AseI, split into 12 cuvettes for transfection (50 µg of linearized plasmid with 5×10^7 parasites each), and used to infect twelve D150 dishes of HFFs. 24 h post-infection, the media was changed to D10 with 40 µM chloramphenicol, 3 µM pyrimethamine, and 10 µg/mL DNase I. To maintain parasite diversity, 1.5×10^8 total parasites were passaged every 48-72 h, based on monolayer integrity, for 4 passages. After the final passage, 1×10^8 parasites were pelleted and genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue kit. The sgRNAs were amplified from genomic DNA using primers

listed in Appendix E, and gel purified for Illumina NovaSeq sequencing. Three sequential screens were performed for each mutant and the WT parasites.

Data analysis: A custom KNIME workflow trimmed the sequencing reads to begin with the sgRNA sequence, making them compatible with the Countess R script (https://github.com/LouridoLab/CRISPR_Analysis), detailed in the protocol paper⁶⁹. The raw counts can be found in Table S1. The phenotype score was the log2 fold change for the 5 most abundant gRNAs for each gene. To determine which genes changed phenotype score significantly, we compared the average mutant score to the average WT score. A shift was significant if it was greater than 2 standard deviations above the average shift of the 497 sexual genes⁶⁰ experienced in the mutant compared to the WT. Additionally, a one-sided Wilcoxon signed rank test was used to determine the probability of each gene having a significant shift compared to the shift in the 497 sexual genes, FDR<0.01. Genes significant by both methods were classified as hits. Significant overlap between screens was assessed by hypergeometric overlap testing (http://nemates.org/MA/progs/overlap_stats.html).

Gene enrichment analysis: Genes with significant shifts were subject to gene enrichment analysis using the metabolic pathway enrichment analysis from ToxoDB, using default settings.

Validation of screens: Thirty genes were fitness-conferring genes in all three mutants. Of these, 6 genes had fitness scores > -2.5 in the WT, suggesting they were dispensable. To validate synthetic lethality, these genes were disrupted in the RHCas9WT, RHCas9 Δ *gral4*, and RHCas9 Δ *cpl* parasites, and the fitness of the resulting single or double mutants was determined. pU6-DHFR-SAG1 and pU6-DHFR-CDPK1 sgRNA served as a negative and positive lethality control, respectively. For knockouts, 5×10^7 parasites were transfected with 50 μ g of linearized

plasmid and plated in 6-well plates, in duplicate. Ethanol-treated wells were seeded with 100 and 1,000 tachyzoites. 1.5 μ M pyrimethamine-treated wells were seeded with 1,000 or 10,000 tachyzoites. Ethanol-treated plates grew for 8 days, and pyrimethamine-treated plates grew for 10 days. On the final day of growth, supernatant from the pyrimethamine-selected RHCas9WT plates was collected for validation of gene disruption (detailed below). For the plaque assay, monolayers were washed with PBS, fixed with 4% methanol-free formaldehyde, and stained with 2% crystal violet in 20% ethanol. Wells were imaged on a Nikon Ti-2E microscope at 4x magnification and stitched together using NIS Elements. Two wells were analyzed per condition. Plaques were detected and quantified using the plaque assay module in SpaCR (<https://github.com/EinarOlafsson/spacr>, <https://pypi.org/project/spacr/>, v. 0.3.62). First, plaques were manually annotated using the SpaCR module `spacr.make_masks` on 100 whole well stitched images. The cellpose cyto model⁷⁴ was fine-tuned⁷⁵ on 90 images with the `spacr.train` module (Appendix H). 10 images were reserved for testing. The plaque counts and sizes were computed by SpaCR. The data was imported to R where outlier analysis was conducted, average plaque size and count for each condition were calculated, and normalized to the EtOH and *Δ sgl* controls. The data was exported to PRISM for graphing and statistical analysis.

To confirm successful gene knockout using this strategy, the supernatant from plaques was collected and plated for single clones in 96-well plates. After 7 days, the Phire Tissue Direct PCR kit was used to probe for disruption of the gene through the integration of the sgRNA fragment at the targeted gene locus with genomic and sgRNA primer pairs (P57–P70, and P55 and P56). Clones negative for a genomic-sgRNA PCR fragment were subject to PCR with genomic primer pairs that flanked the cut site. That PCR fragment was cleaned up and sent for Sanger sequencing to identify INDELS.

Luciferase growth assay: To determine if $\Delta cp\ln$ Luc parasites were more susceptible to drugs that targeted the metabolic pathways that were more fitness-conferring in the $\Delta cp\ln$ and Δcrt mutants, we employed a growth assay. Briefly, 96-well plates were seeded with HFFs 1 week prior to infection. On the day of infection, WTnLuc and $\Delta cp\ln$ Luc parasites were harvested by scraping, syringing, filtering, and centrifugation at 1,200 g for 10 min, then washed and resuspended in HBSS. Triplicate wells for each strain were infected with 2,000 tachyzoites per well. Parasites invaded for 4 h before the wells were washed with PBS, and fresh media was added containing either DMSO or 3-step serial dilutions of sodium fluoroacetate from 5.4 μ M, atovaquone from 5.4 μ M, or triclosan from 17 μ M. After 4 days, media was aspirated from the wells and monolayers were lysed for 10 min in 50% 1 $\times$ PBS 50% 2 $\times$ Lysis buffer (100 mM MES, 1 mM CDTA, 0.5% Tergitol, 0.05% Antifoam 204, 150 mM KCl, 1 mM DTT, 35 mM Thiourea) with 12.5 μ M h-coelenterazine (NanoLight Technology, 301-10), luciferase substrate. Luminescence was measured on a Syngene plate reader using the following settings: Integration time: 1s, Optics: top, Gain: 135, Delay: 100 ms, read height: 4.5 mm. Data were normalized to the DMSO controls, growth curves were plotted, and the IC₅₀ was calculated in Prism.

To assess growth under nutrient-limited conditions, we adapted a luciferase growth assay similar to⁷¹. Briefly, 96-well plates were seeded 1 week before infection in D10. Twenty-four h before infection the D10 was replaced with amino acid free DMEM (US Biological, D9800-13) supplemented with 1% dialyzed fetal bovine serum, 20 mM HEPES, 5 μ g/mL pen/strep and further supplemented as described in Appendix I to make D1 complete, D1 -tryptophan, D1 -phenylalanine, D1 -arginine and D1 -tyrosine. The media was brought to pH 7.4–7.6 before filter sterilization. D1 complete was mixed with an amino acid-free media to make the necessary 2-step concentrations. To determine inhibitory concentrations for phenylamine, arginine, and

tyrosine, triplicate wells were infected as above. After invasion and washing, the 2-step serial dilution of D1 was reapplied to the wells. The first reading for normalization was gathered at this time. The reading of parasite growth was accomplished as above. For inhibitory concentrations of F, R, and Y, samples were assayed after 4 days of growth and normalized to the 4 h post-infection reading and plotted as a percentage of the D1 complete growth. A nonlinear curve was fit to the graphs in PRISM to estimate the EC₉₀.

For growth assays in nutrient-limited media 96 96-well plates were seeded with HFFs and grown for 1 week in D10. Twenty-four hours prior to infection, D10 was swapped for amino acid limited D1. *WTnLuc*, *Δgral4nLuc*, *ΔcplnLuc*, and *ΔcrtnLuc* parasites were harvested as above. Triplicate wells for each strain and time point captured were infected as above. Readings were taken at 4, 24, 48, and 72 h post-infection and were normalized to the 4 h post-infection readings for each sample before fold change was calculated. Prism was used to calculate the doubling time of the parasites.

Metabolomics: HFF monolayers were infected with an equal number of RHCas9, RHCas9 Δ *gral4*, or RHCas9 Δ *cpl*, and RHCas9 Δ *crt* parasites. After 2 h of invasion, the monolayer was washed with DMEM, and infection proceeded for 18 h in D1 complete. Samples were either treated with 1 μ m LHVS for 24 h or DMSO as the vehicle control before parasites were syringe lysed and harvested for metabolomic analysis using LC-MS/MS. Untargeted metabolomics was conducted as previously published⁷⁶. Data was analyzed in R to identify outliers, calculate the log2 fold change, and the significance of the metabolites between the WT, ingestion mutants, and the DMSO and LHVS conditions.

2.5 Notable contributions

We thank Dr. Zhicheng Dou from Clemson University for gifting us the *RH Δ ku80nLuc:HXG* and *RH Δ ku80nLuc:HXG Δ cpl:DHFR* strains. We thank Dr. Benedikt Marcus for sharing the RHCas92aCat strain with us prior to publishing with it. We also thank Dr. Fengrong Wang for completing the FACs sorting on our difficult to obtain RHCas9 Δ cpl strain. We appreciate our colleagues Mary O’Riordan, Joel Swanson, Yoshifumi Nishikawa, David Sibley, and Manlio di Cristina for supplying key reagents. We thank the Advanced Genomics Core at the University of Michigan for sequencing our libraries. We especially thank Dr. Einar Olafson author level contribution in developing SpaCR so the plaque analysis could be automated. We are grateful for Dr. Leonardo Augusto’s author-level help in preparing the metabolic samples for the Mass Spectrometry and Proteomics Core Facility at the University of Nebraska Medical Center, allowing the core to perform the metabolomics analysis.

Chapter 3 Characterizing *T. gondii* S1/P1 Nuclease 1, TgSPN1

3.1 Introduction

RNA turnover is important for maintaining cellular homeostasis⁷⁷. Recently, the lysosome in animals, the lysosome in *C. elegans*, the vacuole in yeast, and the vacuole in plants have been shown to be a site of RNA turnover during RNaphagy, which mostly targets rRNA^{78–83}. Turnover of the RNA in the lysosome is driven by the T2 family of RNases, which are optimally active at low pH^{81,83–85}. T2 exonuclease activity on RNA generates 5' nucleoside monophosphates (5' NMPs), which are further processed into nucleosides by 5' nucleoside phosphatases, and transported out of the vacuole by ENTs^{81,86,80,84}. However, T2 nucleases are not the only nucleases known to function at low pH.

The optimal pH range of activity of S1/P1 nucleases ranges from 4.0 to 7.0^{87,88}. Members of the S1/P1 nuclease family are sugar-nonspecific phosphoesterases first identified in fungi⁸⁹. Early work described hallmark representatives such as S1 nuclease from *Aspergillus oryzae* and P1 nuclease from *Penicillium citrinum*^{90,91}. However, this family of enzymes has also been found in plants and, more recently, protists like *Leishmania donovani* and *Trypanosoma cruzi*^{92–95}.

S1/P1 nucleases process single-stranded and double-stranded nucleic acids to generate 5'-NMPs, having little discrimination between RNA and DNA^{96,97}. S1/P1 nucleases typically require metal ions, often Zn²⁺, for catalysis^{97,98}. However, some also exhibit activity with Ca²⁺, Mg²⁺, or Mn²⁺^{99,100}. The binding pocket begins with a conserved tryptophan¹⁰¹. Nine residues involved in the stabilization of the three Zn²⁺ ions are conserved in the whole S1/P1 nuclease family⁹⁹. Finally, an arginine or lysine residue is typically found near the active site¹⁰¹.

In protists, S1/P1 nucleases have clear links to purine salvage and virulence^{102–104}. *Leishmania donovani* expresses a surface S1/P1 nuclease that appears critical for scavenging nucleotides under purine-limiting conditions⁹⁴. Vaccine studies have demonstrated that immunization with S1/P1 enzymes, or homologs like P4 nuclease, elicits partial protection against leishmaniasis in animal models, suggesting these are good immunization targets^{105,106}.

Given that *Toxoplasma* is an obligate intracellular pathogen with multiple auxotrophies, including for purines, one possibility is that an unidentified T2 or S1/P1 nuclease might aid in nucleotide salvage, paralleling the roles of these nucleases in other parasites^{26,103,104}. The recent discovery of TgENT1 being localized to the PLVAC, the presumed site of RNA turnover given these enzyme families, suggests that, like other organisms, *Toxoplasma* may turn over RNA in the PLVAC²⁴. Due to the macromolecular nature of RNA, scavenging it from the host would require a bulk acquisition pathway, like the ingestion pathway. Interestingly, we observed a large decrease in nucleoside levels in our ingestion deficient mutants, suggesting that the ingestion pathway may acquire host RNA (Figure 2-7A). Alternatively, lysosomal RNases might function in processing parasite RNA under stress conditions, similar to S1/P1 homologs in *Leishmania*¹⁰⁷. Notably, no nucleases or 5' phosphatases have been identified in the PLVAC, which would be necessary for processing both host and parasite RNA for transport via TgENT1.

Despite the broad distribution of T2 and S1/P1 nucleases, their presence in *Toxoplasma* has not been documented. Here, we present initial identification and characterization of a putative S1/P1 nuclease TGGT1_285180. TGGT1_285180 has an S1/P1 nuclease core domain but many unstructured regions in the predicted structure as well. The protein is proteolytically processed, potentially at these unstructured regions. We used tagged strains and antibodies to attempt to localize the protein to the PLVAC. However, we found that the mature form of the

protein is not exclusively associated with the PLVAC. Interestingly, the localization of the core protein, as determined by antibodies made to the S1/P1 core of the protein, does not localize to any known structure.

3.2 Results

3.2.1 Identifying Candidate S1/P1 nuclease in *Toxoplasma*

The *Toxoplasma* genome (ToxoDB) has no genes annotated as members of the T2 family of RNases. Also, when the protein sequence of the T2 RNase, RNY1, from *S. cerevisiae* was BLASTed against the *Toxoplasma* genome no genes were identified with significant homology. However, TGGT1_240280 is annotated as an S1/P1 nuclease. When the protein sequence of *P. citrinum* P1 nuclease, P24289.1, was BLASTed against the *Toxoplasma* genome three genes with S1/P1 nuclease domains were identified, TGGT1_228370, TGGT1_240280, and TGGT1_285180 (Table 3-1). TGGT1_228370 only has proteomic peptides detected in the oocyst, the progeny of the sexual stage of the parasite, and lacks a signal peptide, indicating it is likely not expressed in the endolysosomal system of tachyzoites. TGGT1_240280 has a signal peptide and is expressed in the tachyzoite stage. However, it has previously been localized to the Golgi based on LoPIT data and gene tagging¹⁰⁸. TGGT1_285180 is expressed in tachyzoites and contains a signal sequence. While the LoPIT data has predicted the localization of TGGT1_285180 to be at the plasma membrane, this is an estimate and needs to be verified experimentally, as it was for TGGT1_240280. Based on these data, we pursued TGGT1_285180 to determine if it was an S1/P1 nuclease in the PLVAC.

Table 3-1 Summary of S1/P1 nucleotide candidates in *Toxoplasma gondii*

ID	Product description	WT mean phenotype score	LoPIT	Signal sequence	Proteomic detection
TGGT1_228370	hypothetical protein	-0.11	N/A	No	Oocyst

TGGT1_240280	S1/P1nuclease	-5.12	Golgi	Yes	Tachyzoite, Bradyzoite
TGGT1_285180	hypothetical protein	0.18	PM - peripheral 2	Yes	Tachyzoite, Bradyzoite

3.2.2 TGGT1_285180 resembles *P. citrinum* P1 nuclease P24289.1

TGGT1_285180 is predicted to be 632 amino acids in length, which is longer than the 270 amino acids of P24289.1. This is due to TGGT1_285180 having predicted large unstructured regions in the S1/P1 nuclease core domain and at the C-terminus of the core. This C-terminal tail contains a transmembrane (TM) domain flanked by large unstructured regions. (Figure 3-1A). We used a predicted structure from AlphaFold 2 to compare TGGT1_285180 with the solved crystal structure of P24289.1. The predicted S1/P1 nuclease core of TGGT1_285180 aligned closely with P24289.1, with the unstructured regions creating large loops in the predicted structure (Figure 3-1-B). These loops are predicted to be stabilized by disulfide bonds near their site of emanation. To further assess whether TGGT1_285180 might possess S1/P1 nuclease activity, we looked for the residues that are associated with the binding to the 3 Zn^{2+} ions that are required for S1/P1 nuclease activity^{99,101}. Using AlphaFold 3, we generated a predicted structure of TGGT1_285180 with 3 Zn^{2+} ions as ligand; we had to remove the signal sequence for this since it otherwise occludes the binding pocket. The 3 Zn^{2+} ions are predicted to bind to the active site of TGGT1_285180, similar to how the 3 Zn^{2+} ions are found in the crystal structure of P24289.1 (Figure 3-1C). Additionally, 10 residues are predicted to stabilize the Zn^{2+} ions; TGGT1_285180 has 9 of these residues, and they are oriented in a similar manner to the corresponding residues in P24289.1. As such, we will refer to TGGT1_285180 as TgSPN1.

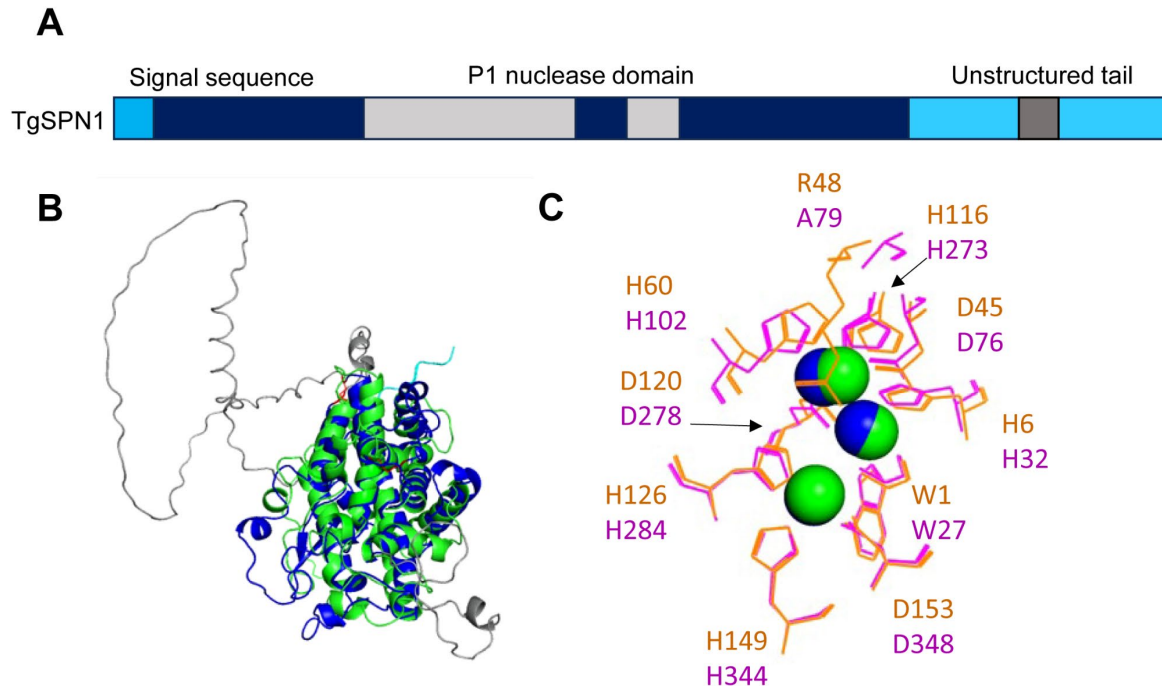


Figure 3-1 **TgSPN1 has substantial homology with S1/P1 nuclease from *P. citrinum*.** (A) Graphical representation of TgSPN1 (TGGT1_285180) with a signal sequence in blue, P1 nuclease domain in navy, large unstructured loops in grey, an unstructured tail in cyan, and a putative transmembrane domain in dark grey. (B) An alignment of a predicted alpha fold structure for TgSPN1 in green to the crystal structure of *P. citrinum* P1 nuclease (P24289) in navy. TgSPN1 unstructured loops are shown in grey. The unstructured tail is omitted for clarity, but the emanation point is shown in cyan. Predicted disulfide bridges are shown in red. (C) AlphaFold 3 predicted binding of three Zn^{2+} ions in TgSPN1 in green, aligned with the binding of three Zn^{2+} ions to P24289 in navy. Residues predicted to stabilize Zn^{2+} are shown in magenta for TgSPN1 and orange for P24289.

3.2.3 Tagging TgSPN1 indicates it occupies the *T. gondii* endolysosomal system

Recent work has shown that the equilibrative nucleoside transporter, TgENT1, which resides on the PLVAC, is essential for parasite survival²⁴. While the phenotype score of TgSPN1 in our WT CRISPR screen is neutral (Table 3.1), we nonetheless chose to generate a strain that was capable of conditional knockdown of TgSPN1 to determine localization and phenotype. In a genetically tractable RHΔku80 strain expressing the heterologous F-box protein TIR1 (hereafter TIR1WT), we tagged the C-terminus of TgSPN1 with an auxin-inducible degron (AID) fused with 3HA tags, TIR1*spn1*AID-3HA¹⁰⁹ (Figure 3-2A, B). We tested for tag expression via western blot. Full-length TgSPN1 is predicted to be ~70 kDa, while the AID tag should add about 30

kDa. We observed bands at roughly 110, 90, 40, and 35 kDa in our TIRI*spn1*/AID-3HA lysate (Figure 3-2C). This suggests that while we can detect full-length TgSPN1AID-3HA, this protein is multiply processed and the C-terminal tail is likely not associated with the mature protein.

Since we hypothesized that TgSPN1 is a ribonuclease in the PLVAC, we tested the localization of TgSPN1AID-3HA by IFA, looking for colocalization of the HA tag with a known marker of the PLVAC. Using newly invaded parasites, we did not see TgSPN1AID-3HA localize to the PLVAC (Figure 3-2D). Instead, the single punctate signal localized to the endosome-like compartment (ELC) (Figure 3-2E). The ELC is upstream of the PLVAC and involved in the maturation of proteins destined for the apical secretory organelles^{50,110}. This localization suggests that immature TgSPN1 initially occupies the endolysosomal system and is processed within the ELCs.

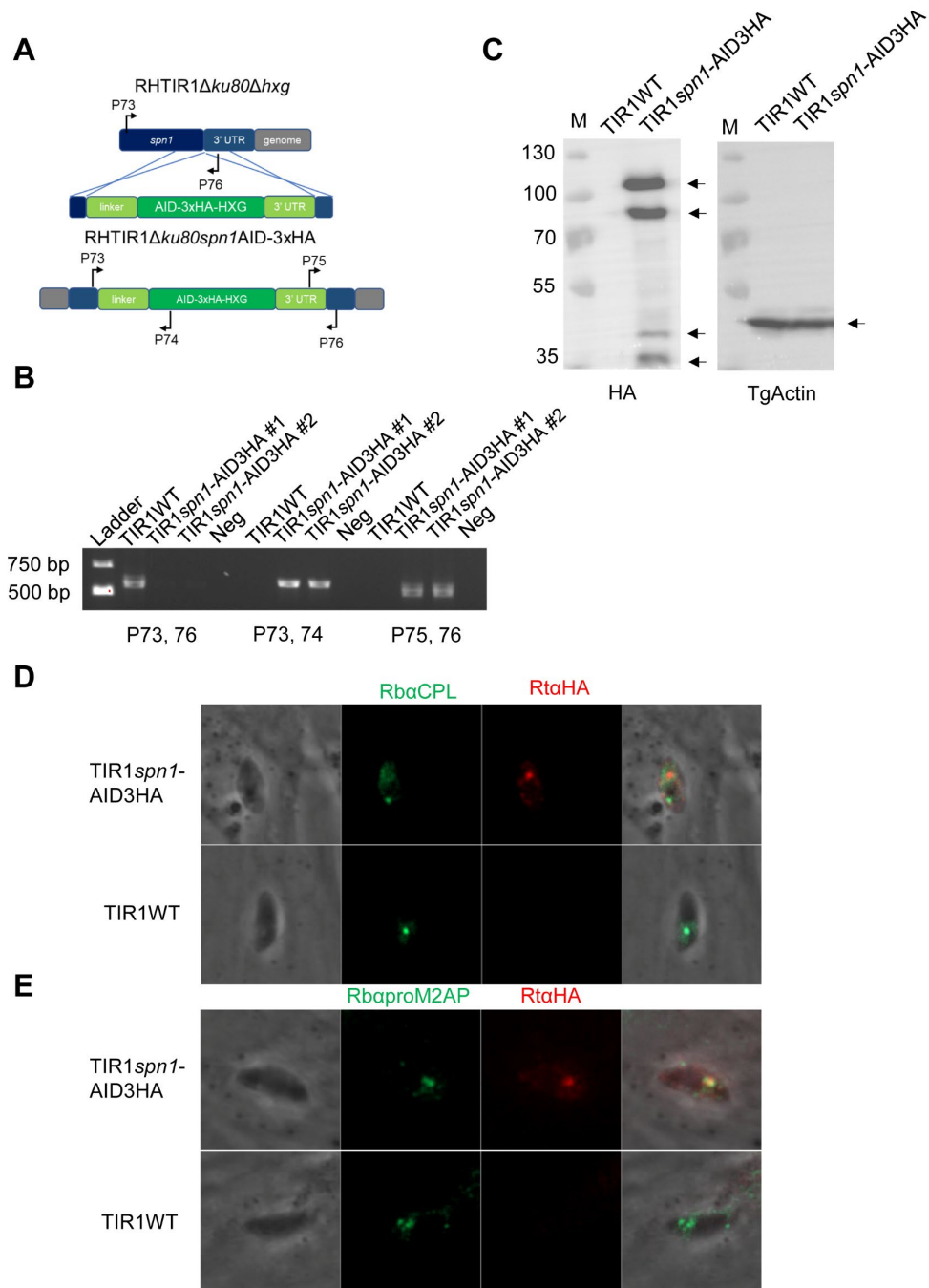


Figure 3-2 TgSPN1-AID3HA localizes to the endosome-like compartment. (A) Schematic of adding the AID-3HA tag to the C-terminus of TgSPN1, with primers denoted for verification of the inserted tag. (B) Genomic DNA of TIR1WT, TIR1*spn1*-AID3HA #1, and #2 were PCR amplified with primers P73 and P76 to look for the native sequence of TgSPN1, P73 and P74 to test for the 5' integration of the tag, and P75 and P76 to test for the 3' integration of the tag. (C) Lysates of TIR1WT or TIR1*SPN1*-AID3HA were probed with R α HA or R α TgActin. Molecular weight markers (M) are shown in kDa. Arrows indicate target bands. (D, E) Immunofluorescence assays of 30-min post-invasion TIR1WT or TIR1*spn1*-AID3HA parasites stained with antibodies R α HA (1:500), R α CPL (1:500) (D), or R α proM2AP (1:500) (E).

3.2.4 Generation of recombinant TgSPN1.

Since the proteolytic processing of TgSPN1 did not allow us to visualize the mature protein using antibodies to the C-terminal tag, we produced antibodies to the recombinant core S1/P1 nuclease domain to localize the mature enzyme. A schematic of our workflow can be found in Figure 3-3A. Briefly, we cloned an expression vector containing a codon-optimized version of the S1/P1 nuclease core domain (hereafter TgSPN1-core), with an *E. coli*-specific periplasmic signal sequence, MalE, swapped for the *Toxoplasma* signal sequence into the pETite-C-term-His-Kan plasmid. Periplasmic localization would potentially help stabilize rTgSPN1-core since disulfide bridges can only form in the periplasm of *E. coli*¹¹¹. We grew BL21 cells overnight at 20°C in IPTG to induce protein expression. Upon testing of the lysate and supernatant of our culture, we were only able to detect expression of the rTgSPN1-core in the whole culture lysate in the presence of BME, suggesting it was expressed in insoluble aggregates (Figure 3-3B). We extracted the protein from the insoluble fraction and tested it by western blotting to confirm that the band at 55 kDa was rTgSPN1-core (Figure 3-3C). We proceeded to column purify the rTgSPN1-core using two methods. First, we affinity purified the protein under denaturing conditions (8 M urea) to produce antibodies that recognize linear epitopes. Second, we performed an on-column refolding procedure to obtain rTgSPN1-core that potentially resembles its native fold with disulfide bonds. However, refolded rTgSPN1-core migrated the same in the presence and absence of BME, suggesting the disulfide bridges did not form correctly (Figure 3-3D). Nevertheless, after dialysis and concentration of the purified proteins, we immunized 5 mice with denatured rSPN1-core and 5 mice with refolded rSPN1-core. We injected the first round of rTgSPN1-core using Freud's complete adjuvant and the

challenge two weeks later with Freud's incomplete adjuvant as we had done previously⁵⁰. Serum was collected and the antibodies were tested below.

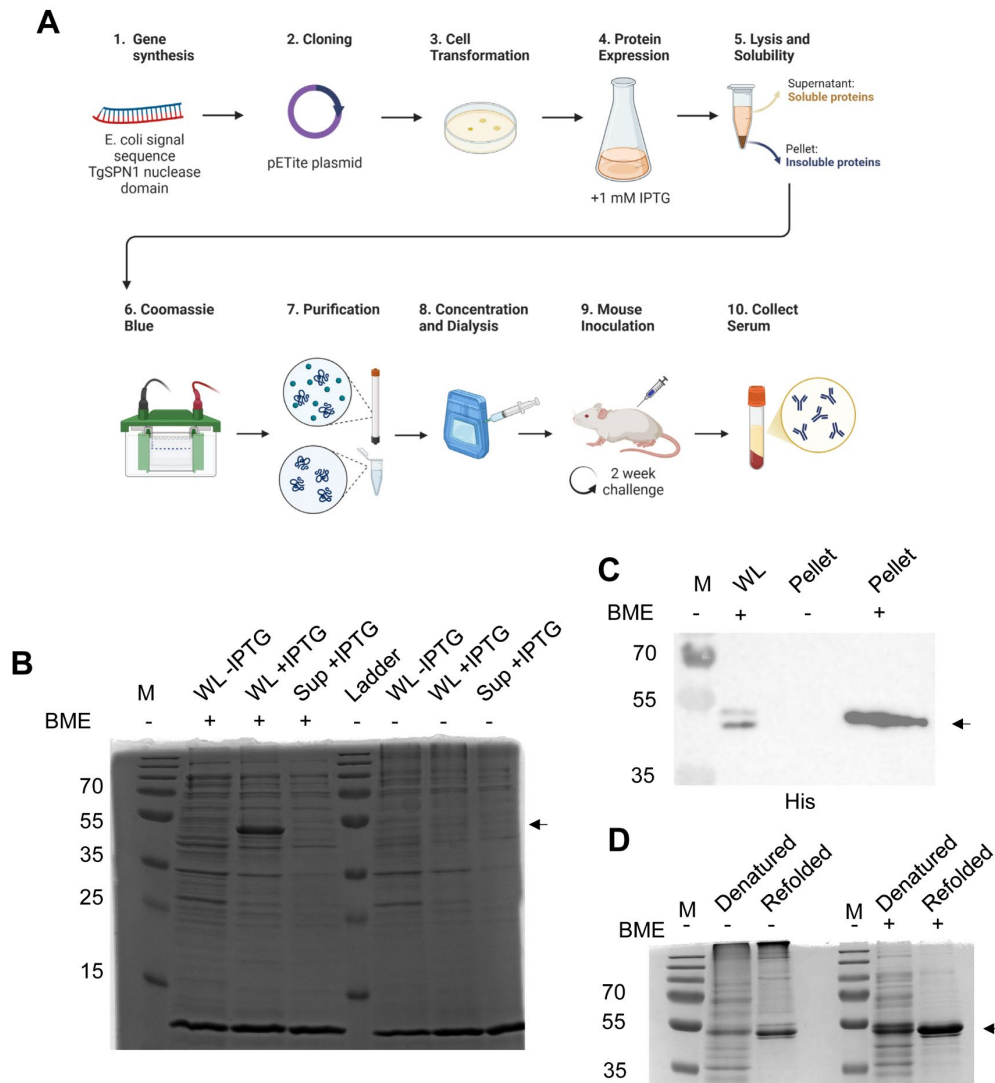


Figure 3-3 Generation of recombinant TgSPN1 to create antibodies. (A) Schematic of generation of antibodies from recombinant TgSPN1. 1. A linear DNA fragment was ordered that contained a MalE, periplasmic signal sequence attached to the codon optimized S1/P1 core domain of TgSPN1. 2. Gibson assembly of TgSPN1 insert into a pETite-C-term-His-Kan empty vector. 3. Transform pETite-MalE-spn1-core-C-term-His-Kan into BL21 cells. 4. Grow an O/N culture of protein-expressing cells and dilute it in LB. Once the large culture is at OD600, bring to a concentration of 1 mM IPTG for 20 h at 20°C. 5. Centrifuge at 4°C and then lyse bacteria before centrifugation again at 4°C, separating the supernatant and pellet. 6. Test for the presence of TgSPN1 in the supernatant. 7. Use column purification to acquire denatured and refolded rTgSPN1. 8. Dialyze the sample recombinant protein and concentrate it for mouse injections. 9. Inject mice with 50 ug of rTgSPN1-core and challenge again at 2 weeks. 10. Collect serum from mice. (B) Induced and uninduced fractions of the bacterial lysate were run on a polyacrylamide gel in the presence or absence of BME in the sample buffer and stained with Coomassie blue. (C) Whole cell (WL) lysate and protein extracted from the bacterial pellet in the presence or absence of BME were probed with MsaHis antibody. (D) Protein extracted from the bacterial pellet using denaturing buffer or after on-column refolding run on a polyacrylamide gel in the presence or absence of BME and stained with Coomassie blue.

3.2.5 Mature TgSPN1 Localizes to an Unknown Compartment

To verify if the TgSPN1 antibodies we created were specific to the protein, we created a parasite line deficient in TgSPN1. In the RH $\Delta ku80\Delta hxcg$ (hereafter RHWT) strain, we targeted the *SPN1* locus and replaced it using homologous recombination, with an HXG-resistance cassette (hereafter RH $\Delta spn1$) (Figure 3-4A). We verified the integration of the HXG marker via PCR (Figure 3-4B). We tested our antibodies from the mice injected with the denatured TgSPN1-core and found that three out of five mice produced an antibody that recognized TgSPN1. Data from mouse 2 (Ms2 α SPN1) is shown as an example. Western blot analysis showed similar evidence of proteolytic processing of TgSPN1 as seen previously, and minimal background staining of RH $\Delta spn1$ parasites, supporting the specificity of this antibody (Figure 3-4C).

Using the TgSPN1 antibody that was made against the enzymatic core to do IFA we probed for the localization of the of TgSPN1 in newly invaded parasites. The signal for TgSPN1 did not colocalize with TgCPL as a marker for the PLVAC (Figure 3-4D). The signal also did not colocalize with the ELC marker, possibly because the precursor protein is much less abundant than the mature protein. This has been seen in other proteins that are matured in the parasite endolysosomal system¹¹². Since the morphology for the TgSPN1 antibody is small punctate structures in the cytoplasm, we examined whether TgSPN1 localized to the dense granules. TgSPN1 signal did not colocalize with GRA14 (Figure 3-4E). There is no known cytoplasmic structure within *Toxoplasma* that resembles the localization pattern seen for TgSPN1.

All five of the mice immunized with the refolded protein produced antibodies that were specific for TgSPN1 when tested by IFA (Figure 3-5A). We will show the data pertaining to

Ms6 α SPN1. However, many of the western blots had at least one band appear in the RH Δ *spn1* that was similar in size to a band we would correspond to TgSPN1 (Figure 3-5B). Thus, these antibodies need more validation.

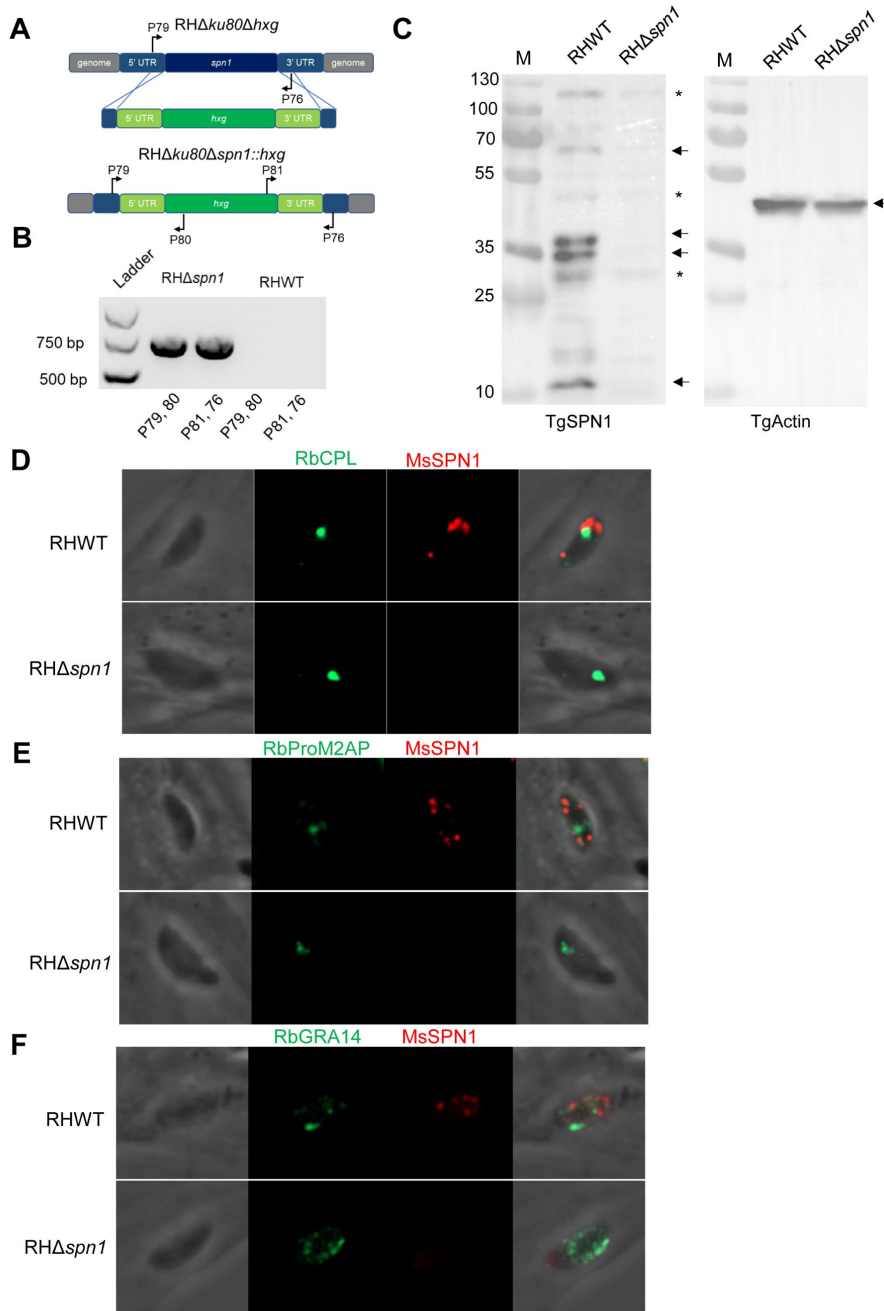


Figure 3-4 **Mature TgSPN1 is in an unknown intracellular compartment.** (A) Schematic of knocking out *SPN1* by replacing it with an *HXG* selectable marker, with primers denoted to verify the inserted tag. (B) Genomic DNA

of RHWT, RH Δ *spn1* PCR amplified with primers P79 and P80 to test for the 5' integration of the resistance marker, and P81 and P76 to test for the 3' integration of the resistance marker. (C) Lysates of RHWT or RH Δ *spn1* were probed with Ms2 α TgSPN1 or Rb α TgActin. Molecular weight markers (M) are shown in kDa. Arrows indicate target bands. The * denotes a nonspecific band. (D, E, F) Immunofluorescence assays of 30-min post-invasion RHWT or RH Δ *spn1* parasites stained with antibodies Ms2 α TgSPN1 (1:500), Rb α CPL (1:500) (C), Rb α proM2AP (1:500) (E), or Rb α GRA14 (1:500) (F).

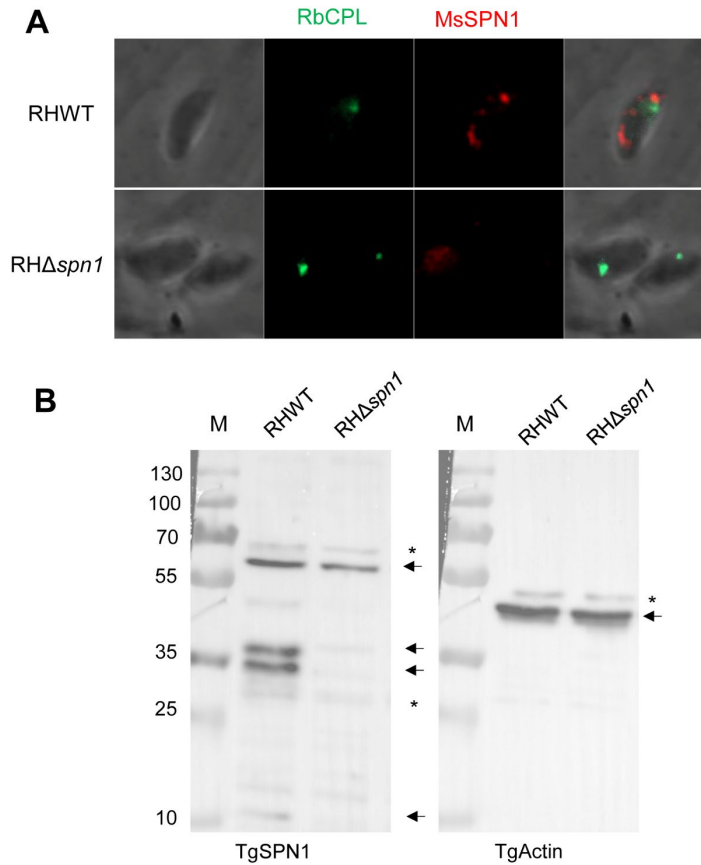


Figure 3-5 Antibody made from refolded rTgSPN1-core works similarly to that of denatured rTgSPN1-core. (A) Immunofluorescence assays of 30-min post-invasion RHWT or RH Δ *spn1* parasites stained with antibodies Ms6 α TgSPN1 (1:500), Rb α CPL (1:500). (B) Lysates of RHWT or RH Δ *spn1* were probed with Ms6 α TgSPN1 or Rb α TgActin. Molecular weight markers (M) are shown in kDa. Arrows indicate target bands. The * denotes a nonspecific band.

3.3 Discussion

In this work, we sought to identify and characterize a putative S1/P1 nuclease in *Toxoplasma gondii*, hypothesizing that such an enzyme might operate similarly to the S1/P1 nucleases implicated in purine salvage and virulence in other protozoan parasites¹⁰³. Our work

begins the characterization of TgSPN1 (TG GT1_285180), a predicted 632 amino-acid protein with an apparent S1/P1 nuclease core domain and multiple unstructured regions.

Our initial bioinformatic analyses suggested that TgSPN1 carries a signal peptide and a conserved active site containing nine of the ten canonical Zn^{2+} coordinating residues^{99,101}. Such conservation hinted at a functional nuclease, like the P1 nuclease of *Penicillium citrinum*⁹¹. However, subsequent localization experiments in tachyzoites provided no definitive evidence that TgSPN1 occupies the PLVAC. Instead, antibody-based IFAs showed that the core of this protein accumulates in punctate structures that are distinct from known compartments such as the ELC, dense granules, or the PLVAC. This is likely the terminal organelle for mature TgSPN1 enzyme as the signal is stronger in this compartment, masking the signal of the immature protein in the ELCs. Given the ambiguous subcellular localization, high-resolution immunogold EM could definitively place TgSPN1 within specific organelles or membranous structures¹¹³.

Since we were unable to replicate the ELC localization observed in the C-terminally tagged TIR1~~spn1~~AID-3HA strain with our antibodies, it is also possible that C-terminal tagging may have distorted TgSPN1 folding or hindered sorting signals, causing partial mislocalization. This is interesting in the context of TG GT1_240280, a putative S1/P1 nuclease, which we decided not to pursue, as its localization was determined by C-terminally tagging it with HA; potentially the tagged version is in the Golgi for maturation and the mature protein is elsewhere¹⁰⁸.

Our western blot analyses confirmed that TgSPN1 undergoes multiple proteolytic processing events, yielding fragments of varying molecular weights. This is consistent with the presence of numerous predicted unstructured loops predicted by AlphaFold 2. Based on the western blots it is likely the large unstructured loop that is stabilized by a disulfide bond is subject

to processing as there is a full length band and band underneath it that is smaller, but not missing the whole enzyme core size (Figure 3-2C).

However, while the antibodies produced by immunization with denatured protein showed a clean signal in our negative control RH Δ *spn1* lysates, there was potentially some reactivity to other proteins using antibodies produced by immunization of the refolded protein. Attempts to express rTgSPN1-core in *E. coli* yielded largely insoluble and partially aggregated material. While we successfully purified denatured and “refolded” protein for immunization, critical disulfide bonds likely did not form properly, due to the protein not reaching the periplasm, possibly altering antigenic epitopes. Given this, further optimization of producing refolded rTgSPN1-core is required before testing for functional nuclease activity, as has been seen in¹¹¹.

Our initial characterization of TgSPN1 provides a clarifies the hypothesis that *Toxoplasma gondii* may harness an S1/P1 nuclease in metabolism but it is likely not this gene. Still definitively determining TgSPN1’s precise localization and enzymatic capabilities is a critical next step in exploring how *T. gondii* recycles or salvages nucleic acids during infection. Given what we now know about the extensive proteolytic processing of TgSPN1, TGGT1_240280 should also be characterized with antibodies made to the catalytic domain to localize the mature protein, as the C-terminal tag previously used may indicate the localization of the immature protein¹⁰⁸. Ultimately, a deeper mechanistic grasp of S1/P1 nuclease functions, especially in relation to RNA turnover in the parasite’s endolysosomal system, could open doors to novel anti-parasitic strategies, whether by directly blocking nucleotide salvage or interfering with parasite RNA homeostasis.

3.4 Materials and Methods

Protein alignment and AlphaFold: We downloaded the predicted folded structure of TGGT1_285180/TgSPN1 from AlphaFold 2. Then, using PyMol, we aligned the TgSPN1 predicted structure to that of a known S1-P1 nuclease, P24289, from *Penicillium citrinum*, which we also downloaded from PDBe-KB.

We used AlphaFold 3 to predict how three Zn^{2+} ions would bind in the active site of TgSPN1. Then we aligned the predicted protein-ligand model of TgSPN1 with the known P24289 Zn^{2+} structure in PyMol to compare them.

Host cells and cell culture: Human foreskin fibroblasts, HFFs, (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and supplemented with 2 mM L-glutamine, 10% Cosmic calf serum, 20 mM HEPES, 5 ug/mL pen/strep (D10) at 37°C and 5% CO_2 unless otherwise stated.

Toxoplasma gondii strains were passaged in the above HFFs. The RH Δ ku80TIR1 (TIR1WT) and RH Δ ku80 Δ hxg (RHWT) parasites have been described previously^{109,114}.

Parasite strain generation: Lists of all parasite strains, primers, and plasmids can be found in Appendix D-F, respectively. To generate RHTIR1-285180AID, RHTIR1 parasites were transfected with 50 μg of pCas9-GFP-Ble-285180gRNA1, to target the C-terminus of the gene, and 10 μg of repair template that had 40 bp of homology to the genomic DNA directly up and downstream of the stop codon, flanking an X10 linker-AID-3xHA-HXG cassette. Twenty-four hours post-transfection, the media was changed, and 1 μM of MPA and xanthine were added for selection. Once the parasites were lysing out, the monolayer was scraped, syringed, filtered, and cloned into 96-well plates. After one week, the Phire Tissue Direct PCR kit was used to test for AID integration at the C terminus of the *SPN1* sequence using P73 and P74 and P75 and P76 for 5' and 3' integration, respectively. Protein expression was tested via IFA and Western blot.

To generate RH Δ ku80 Δ hxg Δ spn1, RH Δ ku80 Δ hxg parasites were transfected with 50 μ g of pCas9-GFP-Ble-285180gRNA1, pCas9-GFP-Ble-285180gRNA2, and 10 μ g of repair template that had 40 bp of homology to the 5' and 3' of the genomic DNA, flanking an HXG resistance cassette. The population was selected for and screened as above using the Phire Tissue Direct PCR kit, and integration of the HXG resistance cassette was assessed using primers P79 and P80 for 5' integration and P81 and P76 for 3' integration.

Immunofluorescence and immunoblotting: Appendix G lists all antibodies used in this study. Samples were fixed with 4% methanol-free formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 10% FBS for 30 min. Primary and secondary antibodies were diluted in 1% FBS and 1% Normal goat serum (NGS) prior to 1 h incubations at RT. Images were taken using a Zeiss Axiovert Observer fluorescence microscope.

Parasite lysates were harvested using radioimmunoprecipitation assay (RIPA) buffer solution (Thermo Scientific, 89900) with EDTA-free miniComplete protease inhibitor (Roche, 11836153001). PVDF membranes were activated with methanol for protein transfer, then blocked with 5% milk in PBS-Tween. Primary antibody was incubated O/N at 4°C, or at 1 h at RT for loading controls, followed by horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at RT. Signal was developed with 2 min incubation in SuperSignal West Pico PLUS Chemiluminescent or SuperSignal West Femto Maximum sensitivity substrate. The chemiluminescent signal was visualized using the Syngene PXi6 imaging system. Membranes were stripped using 4% trichloroacetic acid then blocked and re-probed as needed.

TgSPN1-core expression: We ordered a codon optimized DNA fragment that contained the *E. coli* MalE periplasmic signal sequence, attached to codon optimized TgSpn1-core (base

pairs 67-1359), and flanked by 60 bp homology arms to the pETite-C-His-Kan plasmid. The empty pETite-6-His-Kan vector was amplified using P82 and P83 to add 40 bp homology arms to the vector, and the MalE-TgSPN1-core was amplified using P83 and P84 to generate enough for an insert. Then the Gibson assembly was done following the manufacturer's protocol, and the resultant plasmid was transformed into 10G competent cells using the heat shock method. Colonies were recovered from LB-Kan (50 µg/mL) plates after O/N incubation at 37°C. We sequenced the junction where the insert and vector met to confirm it was intact. After plasmid prepping the new pETite-MalE-spn1-core-C-His-Kan plasmid using the Qiagen miniprep kit. The plasmid was transformed into BL21 (DE3) cells using standard protocol and plated on LB-Kan plates O/N at 37°C. Colonies were grown O/N in LB with 50 µg/mL Kanamycin at 30°C. The cultures were then diluted 1:100 in 600 mL LB with 50 µg/mL Kanamycin at 37°C until OD₆₀₀ of between 0.5 and 0.7. Cultures were cooled on ice, spiked with IPTG to a concentration of 1 mM, and grown O/N at 20°C. Bacterial pellets were collected by centrifugation for 30 min at 3000 x g at 4°C and either immediately subjected to lysis or frozen at -80°C until needed.

TgSPN1 purification: Resuspend pellets (from 100 ml induced cultures) in 30 ml of 50 mM TrisHCl pH 8.0; 150 mM NaCl; 2 mM EDTA; 1 cOmplete protease inhibitor tablet/50 mL. Lyse cells by sonication on ice (five pulses 30 sec each, with 30 sec rest in between). The lysate was spun at 13,000 rpm for 30 min at 4°C.

For denatured protein, the pellet was resuspended in denaturing buffer 20 mM Tris HCl pH 8.0; 300 mM NaCl; 8 M urea; 10 mM imidazole. A HisTrap™ High Performance column from Cytiva was equilibrated with denaturing buffer before our sample ran through the column. It was washed with 20 volumes of 10 mM Tris HCl pH 8.0, 100 mM NaH₂PO₄, and 8 M urea. Elute sample in 10 volumes of 10 mM Tris HCl pH 4.5, 100 mM NaH₂PO₄, and 8 M Urea.

Fractions containing TgSPN1 were dialyzed in 10000 MW cutoff Slide-a-lyzer dialysis cassettes O/N at 4°C in 20 mM Tris HCl pH 8.0; 200 mM NaCl. The lysates were concentrated by centrifugation at 3000 x g at 4°C to above 1 µg/µL.

For column refolded protein, the pellet was resuspended in denaturing buffer 10 mM Tris HCl pH 8.0; 100 mM NaH₂PO₄; 8 M Urea; 25 mM imidazole. A HisTrap™ High Performance column from Cytiva was equilibrated with denaturing buffer before our sample ran through the column. It was washed with 20 volumes of 10 mM Tris HCl pH 8.0; 100 mM NaH₂PO₄; 8 M Urea; 50 mM imidazole. The column was washed with 10 mM Tris HCl pH 8.0; 100 mM NaH₂PO₄; 25 mM imidazole for on-column refolding. Elute sample in 10 volumes of 10 mM Tris HCl pH 8.0; 100 mM NaH₂PO₄; 500 mM imidazole.

Fractions containing denatured or refolded rTgSPN1 were dialyzed in 10000 MW cutoff Slide-a-lyzer dialysis cassettes O/N at 4°C in 20 mM Tris HCl pH 8.0; 200 mM NaCl. The lysates were concentrated by centrifugation at 3000 x g at 4°C to above 1 µg/µL.

Generation of TgSPN1 antibody in mice. We injected 5 mice with 50-200 µl of 12.5-50 µg of denatured TgSPN1 in Complete Freund's Adjuvant. After 2 weeks, we challenge the mice with 200 µl of 50 µg of denatured rTgSPN1 in Incomplete Freund's Adjuvant. After 2 more weeks, the mice were sacrificed and serum was collected. We injected 5 mice with 150-200 µl of 37.5-50 µg of refolded rTgSPN1 in Complete Freund's Adjuvant. After 2 weeks, we challenge the mice with 200 µl of 50 µg of denatured TgSPN1 in Incomplete Freund's Adjuvant. After 2 more weeks, the mice were sacrificed and serum was collected. These antibodies were tested via IFA and WB.

3.5 Notable Contributions

This work would not have been accomplished without the mentorship of Dr. Hargobinder Kaur, who taught me everything I know about making recombinant proteins. Additionally, Tracey Schultz was invaluable for handling the mouse injections and husbandry. I am also thankful to the Raghavan lab here at the University of Michigan for letting me use their equipment to complete these experiments.

Chapter 4 Discussion

4.1 Overview

Toxoplasma gondii has an extensive host range and multiple life stages that necessitate extensive metabolic flexibility from the parasite. While research on every parasite stage is critical to fully understand the biology of *Toxoplasma*, most research has focused on the fast-growing tachyzoite stage for its ease of culture and genetic accessibility compared to other forms. Thus, the methods by which *Toxoplasma* exploits host cells to acquire nutrients are best understood at this stage. Notably, the presence of the nutrient pore proteins, GRA17, 23, 72, and 47, is instrumental in making the PVM porous to small molecules⁴⁻⁷. Once soluble nutrients diffuse into the PV lumen through such pores, high-affinity transporters can facilitate the acquisition of these molecules for use by the parasite^{8,11,22-24,29-32}. However, due to the metabolic plasticity of *Toxoplasma*, single loss of many of these transporters is tolerated by the parasite with little fitness cost^{11,12,24,29-32}. Acquisition of macromolecular molecules, like lipid-rich vesicles scavenged from the host, is facilitated through budding at the PVM, at sites of tubular invaginations in a GRA7-dependant manner or due to interaction with host ESCRT proteins, in a GRA14- and GRA64-dependent manner⁴¹⁻⁴⁴. This is distinct from the ingestion pathway, though it may use similar initiation machinery, as the vesicles accumulate in the PV lumen instead of entering the parasite for delivery to the PLVAC^{42,44}. Finally, host cytosolic proteins are trafficked across the PVM by host ESCRT-dependent vesicular uptake by the parasite and degradation in the PLVAC through the ingestion pathway^{45,47,48}. Because the role of the

ingestion pathway in *Toxoplasma* biology has not been determined, we interrogated parasites deficient in the ingestion pathway to examine the pathway's potential role in nutrient acquisition.

4.2 The Ingestion Pathway as a Conduit for Acquisition of Amino Acids and other Metabolites

The ingestion pathway involves GRA14-mediated recruitment of host ESCRT machinery to the PVM, CPL-driven proteolysis of ingested material in the PLVAC, and CRT-mediated export of the resulting peptides and amino acids into the parasite cytosol^{45–52}. Our metabolomics data indicate that all three mutants ($\Delta gra14$, Δcpl , Δcrt) have reduced levels of amino acids and glycolytic intermediates compared to the wild type (WT).

Though these mutants replicate comparably to WT parasites in complete medium, we uncovered that growth defects for Δcpl and Δcrt parasites become apparent when cultured in amino acid-depleted conditions. Notably, Δcpl and Δcrt mutants showed an exacerbated growth defect in tryptophan-free media, and Δcpl parasites also displayed delayed growth under arginine- and tyrosine-limiting media. These observations highlight the potential importance of the PLVAC in generating a salvageable pool of amino acids during metabolic stress. This idea is supported by previous work in which bradyzoites, the slow-growing chronic stage of the parasite, which rely more heavily on autophagy for viability than tachyzoites, have reduced survival of Δcpl and Δcrt strains^{52,113,115,116}.

Interestingly, despite evidence that $\Delta gra14$ parasites also have significantly reduced amino acids, they did not show phenotypes in the amino acid-limiting assays. One possibility is that while the absence of GRA14 impairs vesicle budding at the PVM, even in nutrient-limited conditions, enough material from the host cell can be salvaged through the nutrient pore and high-affinity plasma membrane transporters to mask severe growth defects. Furthermore,

ongoing work in the lab is studying how additional proteins, like GRA8, have ESCRT-interacting domains and recruit ESCRT components in a GRA14-independent manner. It is unknown if GRA8 contributes to ingestion, but redundancy in GRA proteins is a common occurrence in *Toxoplasma*, as evidenced by the nutrient pores. Our data shows that the ingestion pathway contributes to *Toxoplasma*'s versatility in acquiring nutrients, yet its disruption alone is insufficient to completely abrogate parasite survival in standard conditions.

Another outstanding question about the ingestion pathway is where the ingested material enters the parasites. One study³⁶ that disrupted the micropore through Kelch13 knock down saw no reduction in host cytosolic protein uptake, while another did³⁷ potentially due to the differing amount of time the Tet and AID systems require to sufficiently knockdown the protein. In potential support of the ingestion pathway-derived vesicles entering the parasite at the micropore, the authors of the later study did metabolomics in their micropore knock down strains and saw decreases in three amino acids, proline, lysine, and glutamate³⁷. However, their metabolomics showed more variability due to only have 2 replicates and did not identify many metabolites, limiting comparison to our data.

4.3 Metabolic Plasticity and Biosynthetic Compensation

A striking outcome of our genome-wide CRISPR screens in Δcpl and Δcrt mutants is the increased reliance on biosynthetic pathways, such as pyrimidine biosynthesis, fatty acid synthesis, the TCA cycle, and lysine degradation. All six genes required for pyrimidine biosynthesis were identified as significantly more fitness-conferring in the PLVAC-deficient lines, suggesting that parasites compensate for decreased salvage of host-derived nucleotides by intensifying their *de novo* synthetic routes¹⁹. Further, we observed that some TCA cycle components and fatty acid biosynthesis enzymes became more fitness-conferring in these

mutants, consistent with earlier studies showing that *Toxoplasma* upregulates endogenous synthesis pathways when exogenous sources are restricted¹¹.

Despite these compensatory strategies, Δcpl and Δcrt parasites did not exhibit markedly increased susceptibility, compared to WT, to inhibitors of the TCA cycle or fatty acid synthesis, through drug challenge with sodium fluoroacetate, triclosan, or atovaquone, suggesting that reliance on a given pathway does not necessarily translate into heightened drug sensitivity. This illustrates the difficulties of confirming competition-based phenotypes in isolated parasite growth-based assays. Future validation experiments should be replicated in ways that most resemble the screens, such as direct competition of WT vs. single mutant during drug challenge to help exaggerate the subtle phenotype.

Further, our plaque-based validation of a subset of genes showing altered fitness in the ingestion mutants did not yield strong evidence for synthetic lethality. This is partially due to some of the genes, TGGT1_262640 and TGGT1_249270, having notably lower fitness scores in other screens, so the single knockout in the WT line was likely already compromised⁵⁴. However, plaque assays, while good for assessing parasite viability and lytic cycle growth, do not directly compete the mutants against each other, as was done in the screen.

4.4 S1/P1 Nuclease (TgSPN1) and Nucleoside Salvage

Another focus of our project was exploring whether *Toxoplasma* ingests host RNA and how it might be processed. Our metabolomic data revealed reduced nucleosides, guanosine, adenosine, and cytidine, in the ingestion mutants. Additionally, the metabolomics showed decreased glycolytic intermediates but increased pentose phosphate pathway intermediates, 6-O-phosphono-D-gluconic acid, suggesting that the parasites may be increasing their generation of phosphoribosyl phosphate to feed into the pyrimidine biosynthesis pathway. Taken together, this

hints that parasites may acquire host RNA through the ingestion pathway. Processing of these nucleotides to nucleosides for release from the PLVAC by TgENT1 would necessitate the presence of a nuclease and a 5'-phosphatase.

To explore this hypothesis, we identified TGGT1_285180 (TgSPN1) as a putative S1/P1 nuclease, an enzyme class known to degrade RNA and DNA into 5'-NMPs^{90-92,96,97}. Bioinformatic analysis suggested TgSPN1 retains most of the known Zn²⁺ binding residues necessary for S1/P1 nuclease activity⁹⁹⁻¹⁰¹. The ability of TgSPN1 to function as a nuclease needs to be confirmed through *in vitro* nuclease activity assays using properly refolded recombinant protein.

However, localization of TgSPN1 proved challenging. The tagged TgSPN1-AID-3HA initially appeared to localize to the ELC. At the same time, the mature protein, detected by custom anti-TgSPN1 antibodies, did not clearly overlap with any canonical organelle markers, including PLVAC, ELC, or dense granules. Additionally, TgSPN1 appears to undergo extensive proteolysis from a precursor form to a mature enzyme, complicating the task of localizing TgSPN1. Taken together, one potential interpretation of these results is that the pro-form of TgSPN1 is processed in the ELC before it traffics to the yet unknown, non-secretory organelles, where the antibody made against the TgSPN1-core preferentially recognizes it. This is a known phenomenon seen with other proteins processed in the parasite endolysosomal system and trafficked to other organelles such as the micronemes and rhoptries¹¹². Potential workarounds to conclusively localize TgSPN1 may involve immunogold electron microscopy to map TgSPN1 precisely and allow for the identification of the vesicle structure based on ultrastructural characteristics. Additionally, biotin proximity labeling could label other proteins, potentially elucidating the structure via known localizations or functions of the labeled proteins.

Although the paralog of TgSPN1, TGGT1_250280, was localized to the parasite Golgi by C-terminal tagging, the localization of this second S1/P1 nuclease should be revisited given our findings with TgSPN1. The negative fitness score associated with the loss of TGGT1_250280 makes it a reasonable candidate for a PLVAC nuclease, as it more closely resembles the phenotype of putatively disrupting nucleoside release from the PLVAC in the absence of TgENT1.

While *Toxoplasma* might indeed degrade RNAs to salvage nucleotides in the PLVAC, as evidenced by the presence of TgENT1, neither host nor parasite nucleotides have been shown in the PLVAC. To address this, fluorescently labeled nucleotides should be incorporated in host RNA prior to infection by *Toxoplasma*, and accumulation of the host nucleotides should be examined in TgENT1 knockdown parasites.

4.5 Conclusions

This work illuminates the nuanced role of the ingestion pathway in *Toxoplasma*'s metabolic adaptability. While $\Delta gra14$, Δcpl , and Δcrt parasites can proliferate under standard culture conditions, their altered amino acid profiles, compensatory biosynthetic pathway reliance, and growth defects in nutrient-poor environments underscore niches in which this pathway may be important. The marked shifts in pyrimidine biosynthesis, fatty acid synthesis, and TCA cycle reliance, particularly in Δcpl and Δcrt mutants, highlights how proteolytic turnover in the PLVAC helps buffer the parasite against variable nutrient availability.

Further, our metabolomics data raises the possibility that nucleic acid salvage may also occur via the ingestion pathway. We explored TgSPN1 as a potential nuclease in the PLVAC, but the precise role and localization of TgSPN1 remains to be firmly established.

Altogether, the results herein stress that *Toxoplasma gondii*, despite a highly robust and multifaceted nutrient acquisition network, experiences measurable metabolic shifts when ingestion functions are disrupted. This knowledge expands our fundamental understanding of parasite biology and opens potential avenues for targeting *Toxoplasma*'s scavenging mechanisms for the clinical management of toxoplasmosis.

Appendices

Appendix A: Pathways Enrichment Analysis on Hits Shared Between $\Delta gra14$, Δcpl , and Δcrt screens

Appendix Table A-1 Pathway enrichment analysis data from ToxoDB for significantly fitness-conferring hits from the CRISPR screen shared between $\Delta gra14$, Δcpl , and Δcrt mutants. Labels on the Bgd count – total genes in pathway, Result count – number of genes enriched.

Pathway ID	Pathway name	Bgd count	Result count	Result gene list	P-value
PWY0-1264 PK MetaCyc	biotin-carboxyl carrier protein assembly	2	1	TGGT1_221320	0.0119224
ec00254 PK KEGG	Aflatoxin biosynthesis	2	1	TGGT1_221320	0.0119224
PWY-6679 PK MetaCyc	jadomycin biosynthesis	2	1	TGGT1_221320	0.0119224
PWY-6117 PK MetaCyc	spermine and spermidine degradation I	2	1	TGGT1_243440	0.0119224
PWY-6728 PK MetaCyc	methyiaspartate cycle	30	2	TGGT1_221320, TGGT1_313140	0.01202235
PWY-5130 PK MetaCyc	2-oxobutanoate degradation I	3	1	TGGT1_221320	0.01783903
PROPIONMET-PWY PK MetaCyc	propanoyl CoA degradation I	3	1	TGGT1_221320	0.01783903
ec00720 PK KEGG	Carbon fixation pathways in prokaryotes	40	2	TGGT1_221320, TGGT1_313140	0.02098293
PWY-7980 PK MetaCyc	ATP biosynthesis	4	1	TGGT1_256970	0.02372611
PWY-5966-1 PK MetaCyc	fatty acid biosynthesis initiation (type I)	4	1	TGGT1_221320	0.02372611
PWY-0 PK MetaCyc	putrescine degradation III	5	1	TGGT1_243440	0.02958375
PWY-6549 PK MetaCyc	nitrogen remobilization from senescing leaves	6	1	TGGT1_313140	0.03541208
PWY-5744 PK MetaCyc	glyoxylate assimilation	6	1	TGGT1_221320	0.03541208
PWY-7185 PK MetaCyc	UTP and CTP dephosphorylation I	56	2	TGGT1_205470, TGGT1_256970	0.03966697
PWY-5743 PK MetaCyc	3-hydroxypropanoate cycle	7	1	TGGT1_221320	0.04121121
PWY-7024 PK MetaCyc	superpathway of the 3-hydroxypropanoate cycle	7	1	TGGT1_221320	0.04121121
PWY-7799 PK MetaCyc	Arg/N-end rule pathway (eukaryotic)	7	1	TGGT1_279390	0.04121121
PWY-7198 PK MetaCyc	pyrimidine deoxyribonucleotides <i>de novo</i> biosynthesis IV	60	2	TGGT1_205470, TGGT1_256970	0.04509694
PWY-6368 PK MetaCyc	3-phosphoinositide degradation	8	1	TGGT1_205470	0.04698125
PWY-7800 PK MetaCyc	Ac/N-end rule pathway	8	1	TGGT1_279390	0.04698125
PWY-7210 PK MetaCyc	pyrimidine deoxyribonucleotides biosynthesis from CTP	63	2	TGGT1_205470, TGGT1_256970	0.04935377

Appendix B: Patway Enrichment Analysis on Significant Hits in *Agra14* screens

Appendix Table B-1 Pathway enrichment analysis data from ToxoDB for significantly fitness-conferring hits from the CRISPR screen of *Δgra14* mutants. Labels on the Bgd count – total genes in pathway, Result count – number of genes enriched.

Pathway ID	Pathway name	Bgd count	Result count	Result gene list	P-value
PWY-6368 PK MetaCyc	3-phosphoinositide degradation	8	2	TGGT1_205470, TGGT1_238400,	0.00888301
PWY-7242 PK MetaCyc	D-fructuronate degradation	1	1	TGGT1_242300,	0.0189243
PWY-5907 PK MetaCyc	homospermidine biosynthesis I	1	1	TGGT1_235620,	0.0189243
PWY-7581 PK MetaCyc	<i>N</i>-acetylneuraminate and <i>N</i>-acetylmannosamine degradation II	1	1	TGGT1_242300,	0.0189243
PWY-7562 PK MetaCyc	3,6-anhydro-α-L- galactopyranose degradation	1	1	TGGT1_242300,	0.0189243
GLUCUROCAT- PWY PK MetaCyc	superpathway of β-D- glucuronosides degradation	1	1	TGGT1_242300,	0.0189243
GALACT-GLUCUROCAT- PWY PK MetaCyc	superpathway of hexuronide and hexuronate degradation	1	1	TGGT1_242300,	0.0189243
PWY-7310 PK MetaCyc	D-glucosamine degradation	1	1	TGGT1_242300,	0.0189243
PWY0-1324 PK MetaCyc	<i>N</i>-acetylneuraminate and <i>N</i>-acetylmannosamine degradation I	1	1	TGGT1_242300,	0.0189243
PWY-7130 PK MetaCyc	L-glucose degradation	1	1	TGGT1_242300,	0.0189243
GALACTUROCAT- PWY PK MetaCyc	D-galacturonate degradation I	1	1	TGGT1_242300,	0.0189243
HYDROXYPRODEG- PWY PK MetaCyc	<i>trans</i>-4-hydroxy-L-proline degradation I	1	1	TGGT1_242300,	0.0189243
GALACTCAT- PWY PK MetaCyc	D-galactonate degradation	1	1	TGGT1_242300,	0.0189243
PWY-6507 PK MetaCyc	4-deoxy-L-<i>threo</i>-hex-4- enopyranuronate degradation	1	1	TGGT1_242300,	0.0189243
PWY-6679 PK MetaCyc	jadomycin biosynthesis	2	1	TGGT1_221320,	0.03750899
ENTBACSYN- PWY PK MetaCyc	enterobactin biosynthesis	2	1	TGGT1_203420,	0.03750899
PWY-7761 PK MetaCyc	NAD salvage pathway II (PNC IV cycle)	2	1	TGGT1_305840,	0.03750899
ec00254 PK KEGG	Aflatoxin biosynthesis	2	1	TGGT1_221320,	0.03750899
PWY-6012-1 PK MetaCyc	acyl carrier protein activation	2	1	TGGT1_203420,	0.03750899
PWY-6012 PK MetaCyc	acyl carrier protein metabolism	2	1	TGGT1_203420,	0.03750899

PWY-6289 PK MetaCyc	petrobactin biosynthesis	2	1	TGGT1_203420,	0.03750899
ENTNER-DOUDOROFF-PWY PK MetaCyc	Entner-Doudoroff shunt	2	1	TGGT1_242300,	0.03750899
PWY-6117 PK MetaCyc	spermine and spermidine degradation I	2	1	TGGT1_243440,	0.03750899
PWY0-1264 PK MetaCyc	biotin-carboxyl carrier protein assembly	2	1	TGGT1_221320,	0.03750899
PWY-8004 PK MetaCyc	Entner-Doudoroff pathway I	18	2	TGGT1_222020, TGGT1_242300,	0.04335795
ec00562 PK KEGG	Inositol phosphate metabolism	45	3	TGGT1_205470, TGGT1_222020, TGGT1_238400,	0.04930439

Appendix C: Log2FC of Metabolites in Ingestion Mutants

Appendix Table C-1 Log2FC and p-values of each metabolite with outliers removed compared to WT for Δ grl4, Δ cpl, Δ crt and DMSO for LHVS samples.

Meta	Δ grl4 Log2FC	Δ cpl Log2FC	Δ crt Log2FC	LHVS Log2FC	Δ grl4 pval	Δ cpl pval	Δ crt pval	LHVS pval
(E)-p-coumaric acid	-2.24	-1.75	-0.63	-1.69	1.26E-03	8.63E-05	3.07E-01	3.20E-03
(Z,E)-9,12-Tetradecadienyl acetate	-1.02	-0.97	0.03	-0.22	1.58E-02	6.68E-03	5.17E-01	5.72E-01
1-Aminocyclopropane-1-carboxylic acid	-0.55	-0.31	-0.03	0.00	5.95E-02	1.23E-01	6.87E-01	9.97E-01
1-Octadecylamine	-1.27	-0.23	0.75	0.22	1.97E-02	6.82E-01	2.62E-01	5.69E-01
1-Oleoyl-2-stearoyl-sn-glycero-3-phosphocholine	0.20	0.62	1.36	0.85	7.03E-01	9.55E-02	2.29E-03	2.13E-02
1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine	-0.90	-1.90	0.07	0.79	1.58E-01	5.74E-03	7.96E-01	2.44E-02
1-Pentafuranosyl-2,4(1H,3H)-pyrimidinedione	-2.44	-2.18	-0.74	-0.28	2.54E-03	1.37E-03	2.32E-02	6.94E-01
11-Aminoundecanoic acid	-0.55	-0.09	-0.71	0.16	2.69E-02	5.67E-01	2.73E-04	3.29E-01
12-Oxo phytodienoic acid	-1.44	-0.74	-0.86	-0.20	3.96E-03	9.32E-03	9.93E-03	5.48E-01
18:1 Dimethyl PE	0.23	-1.67	1.22	-0.87	6.19E-01	1.71E-02	2.00E-01	1.96E-01
1palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine	-0.33	-1.73	0.13	-2.17	6.48E-02	4.94E-03	6.25E-01	6.81E-03
2-Deoxy-ribose-5-phosphate	0.05	-0.02	0.59	-0.02	1.77E-01	7.54E-01	9.68E-03	7.54E-01
2-Methylbutyrylcarnitine	-0.09	-0.02	0.16	1.08	8.28E-01	9.36E-01	4.55E-02	5.13E-02
2,2'-Iminodipropan-1-ol	-0.44	-0.25	-0.73	-0.74	1.58E-01	6.68E-01	1.19E-01	9.53E-02
3-Buten-1-amine	0.13	0.05	1.08	3.11	5.24E-01	6.19E-01	4.39E-01	1.67E-02
3-Oxopalmitic acid	-0.11	0.23	-0.28	0.46	5.26E-01	4.87E-01	4.13E-01	6.92E-02
3-PG	-3.36	-1.65	-2.16	-1.71	4.55E-05	3.63E-04	1.00E-03	1.30E-07
4-Amino-5-phosphomethyl-2-methylpyrimidine	-1.06	-2.08	-0.97	-0.85	4.62E-03	2.18E-04	5.63E-03	1.06E-01
4-Dodecyl-phenol	-0.92	-2.61	0.55	-1.57	4.03E-02	2.31E-04	1.87E-02	8.25E-02
4-Hydroxypropylleucine	-1.16	-1.25	0.18	0.58	5.07E-02	1.29E-02	7.73E-01	3.55E-01
4-Nitroaniline	-0.03	-0.07	-0.07	0.03	9.67E-01	9.00E-01	8.95E-01	9.09E-01
4-Undecylbenzenesulfonic acid	0.15	0.22	-1.07	0.73	7.57E-01	6.06E-01	1.81E-02	1.17E-01
6-O-Phosphono-D-gluconic acid	1.85	3.81	2.70	2.48	3.79E-04	4.38E-03	4.16E-05	7.16E-03
6-Phosphonogluconolactone	-0.55	-0.24	-0.30	-0.48	2.49E-02	1.89E-02	4.76E-01	1.45E-05
Acetylglutamide	-1.30	-1.25	-1.12	0.22	1.11E-02	1.48E-03	5.19E-03	5.29E-01

Acetyl-carnitine	-0.45	-0.06	0.04	0.96	5.26E-01	7.37E-01	9.44E-01	8.23E-02
Acetyl CoA	-2.37	-1.63	-1.12	-1.72	7.54E-03	3.65E-02	1.04E-01	1.90E-03
Adenosine	-2.32	-0.51	-0.89	0.98	4.17E-02	5.01E-01	3.41E-01	6.22E-04
Adenylthiomethylpentose	1.02	1.46	0.90	1.65	6.27E-02	1.45E-03	5.84E-02	4.89E-03
ADP	-0.50	0.38	0.97	1.72	5.28E-01	6.64E-01	2.49E-01	8.31E-03
Alanine	-3.18	-2.42	-1.84	-1.86	1.24E-02	2.00E-02	7.94E-02	1.55E-03
Alpha-D-ribose 5-phosphate	-1.95	-0.01	-0.90	1.40	6.15E-02	9.91E-01	2.93E-01	6.81E-03
Alpha-ketoglutarate	-3.36	-1.65	-2.16	-1.46	4.55E-05	3.63E-04	1.00E-03	2.93E-04
Arginine	-1.80	-1.27	-0.48	-0.28	4.61E-02	2.06E-01	4.95E-01	4.70E-01
Asparagine	-0.75	0.16	1.55	0.45	5.23E-04	5.63E-01	2.11E-06	1.30E-02
Aspartic acid	-2.07	-1.90	-1.79	-0.58	4.01E-02	7.63E-02	5.75E-02	6.44E-03
ATP	-3.50	-2.51	-1.46	-0.08	5.80E-03	2.00E-02	9.62E-02	9.04E-01
Benzylpenicillin	2.00	2.11	3.60	1.71	1.02E-03	7.75E-04	1.14E-03	3.09E-02
Butoctamide	0.10	-0.13	-0.54	-0.05	7.68E-01	5.95E-01	2.53E-03	8.70E-01
Carnitine	-0.14	-0.42	-0.22	0.54	6.86E-01	2.70E-02	3.56E-01	1.32E-01
Ceramide (d18:1/16:0)	0.24	-0.77	2.39	-0.57	5.55E-01	1.24E-02	6.36E-03	6.25E-02
Ceramide (d18:1/24:1(15Z))	-0.43	-3.44	0.81	-1.67	1.19E-01	1.41E-04	7.34E-02	5.59E-02
Cis-3-hexenyl phenylacetate	-0.23	0.40	0.46	0.38	7.08E-01	7.34E-01	6.43E-01	5.38E-02
Cis-aconitate	-0.19	-0.96	-0.76	-0.96	9.15E-03	3.80E-05	5.02E-03	3.80E-05
Citrate	-0.19	-0.96	-0.76	-0.96	9.15E-03	3.80E-05	5.02E-03	3.80E-05
Citrulline	-0.40	-0.23	-0.61	0.49	2.26E-01	3.14E-01	1.34E-01	3.37E-01
CMP	-0.45	0.38	1.45	1.25	4.50E-02	2.78E-01	5.16E-04	9.18E-02
Coprine	-2.12	-0.39	-0.41	0.57	3.71E-05	1.34E-01	5.78E-01	1.43E-02
CPAD(Ribofuranosylpicolinamide Adenine-din)	-0.09	0.24	1.15	0.90	7.73E-01	5.77E-01	9.33E-02	1.03E-02
Creatine	-2.03	-0.80	-0.75	0.01	1.82E-03	2.50E-02	2.46E-01	9.54E-01
CTP	-0.10	1.61	2.31	2.12	6.11E-01	7.59E-04	3.36E-03	9.91E-05
Cyclic ADP-ribose	-0.45	0.61	0.02	0.88	9.93E-02	9.44E-02	9.44E-01	5.86E-03
Cyprodenate	1.73	1.90	1.42	0.77	6.25E-02	9.78E-03	1.22E-01	1.83E-02
Cyromazine	-0.14	0.07	-0.60	-0.47	3.43E-01	7.99E-01	5.41E-04	8.13E-02
Cysteine	-0.48	0.05	-0.09	0.31	1.94E-02	7.32E-01	5.86E-01	3.12E-02
Cytidine	-1.37	-1.50	-0.12	-2.33	2.08E-04	8.37E-05	2.82E-01	2.06E-03
D-benzylpenicillioic acid	-1.22	-1.38	-0.02	0.31	5.59E-03	9.14E-03	9.72E-01	2.65E-01
D-ribose	0.59	-0.11	-0.02	-0.11	9.68E-03	4.50E-01	7.54E-01	4.50E-01
Deanol	-0.87	-0.23	-1.18	0.54	4.51E-02	7.10E-01	1.92E-02	6.10E-01

Deoxyribose	-0.55	-0.24	-0.30	-0.48	2.49E-02	1.89E-02	4.76E-01	1.45E-05
dGMP/AMP	0.56	2.22	2.10	2.55	1.21E-02	1.47E-04	1.63E-03	4.74E-04
dGTP	-0.71	0.80	0.79	1.87	3.24E-01	3.93E-01	2.19E-01	1.40E-03
DHAP	0.26	-0.54	-0.21	-0.65	3.15E-01	5.36E-02	2.74E-01	8.17E-02
Dihydrothymine	-1.25	0.03	-1.09	0.19	1.99E-02	7.71E-01	5.61E-04	2.09E-01
Dimorpholamine	-1.55	-0.96	-0.97	0.08	2.73E-02	6.14E-03	6.57E-03	7.21E-01
Diphenyl sulfoxide	-2.60	-2.69	-2.54	-0.80	3.56E-04	1.51E-04	1.48E-04	4.98E-01
Fructose 6-phosphate	-1.16	1.36	0.11	1.74	1.55E-03	8.67E-03	4.85E-01	2.10E-03
G-Butyrobetaine	0.86	0.51	0.56	0.25	2.88E-02	4.24E-02	8.64E-03	2.84E-01
Gama-aminobutarate	-3.36	-1.65	-2.16	-1.46	4.55E-05	3.63E-04	1.00E-03	2.93E-04
Glucose	-1.39	-0.88	-0.02	-0.57	4.08E-05	4.37E-04	5.56E-01	1.10E-01
Glucose-6-phosphate	-3.36	-1.65	-2.16	-1.71	4.55E-05	3.63E-04	1.00E-03	1.30E-07
Glutaconate	-0.87	-0.87	0.04	-0.73	2.75E-04	6.29E-04	8.83E-01	4.81E-03
Glutamate	-0.57	-0.16	1.00	0.70	6.71E-03	2.91E-01	9.29E-05	2.47E-02
Glutamic acid	-0.57	-0.36	0.44	1.19	2.52E-01	1.23E-01	1.63E-02	1.55E-01
Glutamine	-0.67	-1.00	0.11	-1.32	1.30E-03	3.30E-03	5.81E-01	9.46E-03
Glyceraldehyde-3-P	-0.55	-0.24	-0.30	-0.48	2.49E-02	1.89E-02	4.76E-01	1.45E-05
Glycerophosphoethanolamine	0.14	0.03	0.20	1.31	4.59E-01	9.04E-01	1.94E-01	3.70E-02
Glycidyl oleate	-0.06	-0.89	3.29	0.89	9.33E-01	1.63E-01	2.66E-03	3.88E-01
Glycine	-0.96	1.73	-0.24	2.10	3.01E-05	1.42E-04	7.76E-02	6.52E-04
GMP	-0.41	1.67	1.62	1.78	8.33E-02	2.54E-03	3.22E-04	4.76E-03
Guanine	-2.06	-1.23	-2.52	-0.13	1.25E-03	2.00E-03	1.31E-05	8.07E-01
Guanosine	-1.20	-0.24	0.07	0.79	1.19E-04	5.08E-02	6.82E-01	6.33E-02
Hex-2-ulose	-2.27	-1.88	-1.36	-0.57	2.13E-04	1.36E-04	1.06E-06	1.56E-01
Hexadecasphinganine	0.30	-0.05	-0.42	0.27	3.67E-01	9.29E-01	2.84E-01	3.81E-01
Hexitol	-2.26	-1.86	-0.16	-0.70	1.48E-04	3.19E-05	7.21E-01	1.87E-01
Hexylamine	-0.47	1.03	-1.26	0.47	3.93E-02	7.45E-04	1.06E-03	3.78E-02
Histidine	-2.19	-0.99	-0.63	-0.89	1.21E-06	6.78E-03	6.28E-05	8.78E-05
IMP	0.97	1.58	2.15	1.31	4.04E-04	1.90E-04	3.78E-06	6.07E-03
Indoleacrylic acid	-1.32	-1.22	-0.53	0.25	1.49E-02	8.24E-03	3.01E-02	4.40E-01
Inosine	-0.40	0.77	1.13	1.00	4.76E-03	1.39E-04	1.55E-03	6.45E-03
Isocitrate	-0.19	-0.96	-0.76	-0.96	9.15E-03	3.80E-05	5.02E-03	3.80E-05
Isopropaline	-1.42	-2.20	-1.52	-0.40	7.92E-04	1.82E-03	1.13E-03	5.34E-01
L-alpha-glycerylphosphorylcholine	-0.77	-1.07	0.77	1.24	9.83E-02	1.05E-02	2.32E-01	5.79E-02

L-gamma-glutamyl-L-leucine	-1.22	-0.85	-0.69	0.46	1.80E-02	7.09E-03	2.11E-03	2.44E-02
Lactate	-2.95	-2.18	-1.75	-2.31	8.03E-07	5.61E-08	3.48E-07	6.60E-06
Lactic acid	-2.77	-2.40	-2.08	-1.12	4.90E-04	2.78E-05	6.85E-05	1.14E-02
Leucine/Isoleucine	-0.76	0.25	0.14	0.29	4.14E-04	7.92E-02	1.26E-01	2.02E-02
Lipoic acid	-2.62	-2.30	-1.68	1.36	1.50E-03	2.09E-05	1.76E-02	5.72E-02
Lysine	-1.98	-1.87	-1.00	-1.23	1.49E-03	6.13E-04	7.28E-02	1.55E-02
Malic acid	-1.83	-1.66	-1.10	-1.45	2.03E-04	2.65E-04	6.79E-04	8.88E-05
Malonyl CoA	-1.24	-1.20	-0.53	1.18	6.29E-02	1.34E-04	3.63E-01	2.33E-02
Methionine	-2.22	-1.91	-0.93	-0.95	6.85E-06	1.39E-06	1.39E-05	1.78E-01
Methyl diisopropyl propionamide	1.22	1.29	-0.36	1.71	2.92E-02	4.22E-03	1.24E-01	2.33E-03
Methyl palmoxirate	-0.14	-1.60	0.25	-0.09	6.87E-01	3.40E-02	2.33E-01	6.73E-01
Myristyl sulfate	0.28	0.80	-0.36	0.20	5.20E-01	4.03E-02	2.54E-01	4.82E-01
N-(tert-butoxycarbonyl)-L-leucine	-1.08	-0.22	0.11	1.57	8.82E-02	6.62E-01	8.38E-01	1.29E-02
N-Acetyl-DL-histidine	-2.04	-2.16	-2.28	0.71	2.62E-03	5.97E-05	9.07E-05	2.61E-01
N-Acetyl-LL-2,6-diaminopimelic acid	-1.74	-1.58	-1.14	-0.48	4.07E-02	7.28E-04	7.24E-03	2.56E-01
N-Methylisopelletierine	-1.04	0.97	-0.23	1.16	2.74E-03	5.53E-03	3.96E-01	3.06E-02
N,N-Diethylethanolamine	-0.22	0.13	-1.21	0.59	4.75E-01	8.19E-01	5.97E-03	4.42E-01
NAD+	-0.79	0.08	0.08	0.19	2.77E-03	7.48E-01	6.60E-01	6.80E-01
NADP/+	-1.61	-1.88	-1.46	-0.76	1.34E-03	2.89E-03	3.77E-04	5.51E-02
Nicotinamide	-0.91	0.48	0.66	1.90	2.09E-02	9.01E-02	2.57E-01	8.84E-03
Nicotinamide adenine dinucleotide	-1.01	0.89	0.35	1.55	2.51E-02	1.10E-01	1.55E-01	4.77E-03
Octylamine	0.24	0.25	-0.87	0.06	4.19E-01	7.84E-02	7.76E-03	8.20E-01
Oxaloacetate	-3.75	-2.02	-2.16	-1.61	2.39E-08	5.34E-05	1.71E-07	3.07E-02
Palmitic acid	0.78	-0.23	0.11	-0.13	1.79E-01	3.46E-01	8.24E-01	7.55E-01
Pantoic acid	-1.79	-1.48	-1.39	-1.44	5.10E-03	5.42E-04	2.00E-03	2.56E-03
PEP	-1.25	-0.69	0.04	-0.77	4.29E-05	2.72E-04	2.56E-01	9.52E-02
Phenacetin	-0.59	-0.07	-0.63	0.75	7.14E-02	7.56E-01	3.08E-02	2.18E-01
Phenethylamine	-1.24	-0.53	-0.47	-0.69	2.03E-02	2.80E-02	1.69E-01	1.69E-01
Phenylacetyl glycine	0.32	-0.05	1.35	1.30	4.22E-01	7.60E-01	4.09E-02	3.46E-02
Phenylalanine	-2.03	-1.77	-0.61	-0.22	2.09E-08	3.68E-07	8.17E-05	6.72E-01
Pinidine	0.16	-0.05	-1.47	0.21	3.69E-01	9.23E-01	3.10E-03	3.76E-01
Proline	-1.76	-1.44	-0.17	-1.28	1.47E-05	4.86E-06	4.30E-02	5.43E-04
Propionyl-CoA	-1.61	-1.88	-1.46	-0.76	1.34E-03	2.89E-03	3.77E-04	5.51E-02
Propyl 1-(propylsulfinyl)propyl disulfide	1.27	1.67	0.35	0.60	3.60E-02	9.73E-04	1.55E-01	3.61E-01

Prosopinine	-1.03	-2.30	0.16	-0.14	2.80E-02	3.85E-05	6.50E-01	8.51E-01
PRPP	-3.36	-1.65	-2.16	-1.46	4.55E-05	3.63E-04	1.00E-03	2.93E-04
Pyruvate	-3.60	-1.89	-2.26	-1.72	2.07E-05	3.14E-04	3.47E-04	2.32E-04
Pyruvic acid	-3.23	-1.32	-3.69	-1.49	5.22E-05	9.53E-03	1.97E-05	3.20E-03
Reduced glutathione	-0.69	0.75	0.17	0.51	2.75E-01	3.45E-02	1.21E-01	8.03E-02
Riboflavin	-0.16	-0.51	-1.04	0.90	2.99E-01	1.72E-02	2.07E-02	7.04E-02
Ribulose-5-phosphate	0.05	-0.02	0.59	-0.02	1.77E-01	7.54E-01	9.68E-03	7.54E-01
Sedoheptulose 7-phosphate	-0.42	2.47	0.64	2.89	1.69E-02	4.12E-03	4.01E-03	1.03E-05
Serine	-0.86	-0.61	0.45	0.35	2.44E-03	7.86E-02	2.32E-02	5.21E-01
Succinyl-CoA	-1.24	-1.20	-0.53	1.18	6.29E-02	1.34E-04	3.63E-01	2.33E-02
Threonine	-1.99	-1.69	-0.64	-1.56	5.27E-07	4.73E-07	7.59E-06	7.40E-03
Tryptophan	-1.74	-1.56	-0.38	-1.17	4.55E-07	1.45E-06	5.91E-05	3.81E-02
Tyrosine	-2.03	-1.69	-0.51	-1.01	7.94E-08	1.94E-05	2.76E-03	2.39E-03
UDP	-0.12	0.97	1.12	1.46	3.20E-01	3.18E-02	7.04E-04	5.43E-03
UDP-D-glucuronate	0.08	1.68	2.20	1.62	3.33E-01	4.12E-04	7.32E-07	7.76E-04
UDP-GlcNAc	-0.92	1.69	0.42	1.61	2.42E-05	1.91E-03	1.10E-02	6.74E-03
UMP	-0.12	0.71	0.50	1.74	2.65E-01	1.02E-03	1.42E-03	3.55E-04
Undecanoyl carnitine	-1.81	-1.51	-0.09	0.40	2.54E-03	2.41E-03	8.51E-01	6.29E-01
UTP	-0.58	2.89	1.00	2.39	6.28E-02	1.73E-04	2.12E-02	6.72E-04
Valine	-1.90	-1.83	-1.10	-0.93	4.16E-04	1.76E-03	2.29E-05	1.29E-01
Xylulose-5-phosphate	-0.82	-0.93	-0.83	-0.87	2.08E-03	7.12E-02	1.72E-03	2.67E-02

Appendix D: Strains Used in This Study

Appendix Table D-1 Strains used in this study.

Genotype	Short name	Parental	Selectable marker	Provided by
RHCas92aChlor Δ hgx	Cas9WT		Chlor	Lourido lab
RHCas92aChlor Δ hgx Δ grl4	Cas9 Δ grl4	RHCas92aChlor Δ hgx	Chlor	
RHCas92aChlor Δ hgx Δ crt	Cas9 Δ crt	RHCas92aChlor Δ hgx	Chlor	
RHCas92aChlor Δ hgx Δ cpl	Cas9 Δ cpl	RHCas92aChlor Δ hgx	Chlor	
RH Δ ku80 Δ hxnLuc:hgx	WTnLuc		HXG	Dou lab
RH Δ ku80 Δ hxnLuc:hgx Δ cpl:dhfr	Δ cplnLuc	RH Δ ku80 Δ hxnLuc:hgx	HXG,DHFR	Dou lab
RH Δ ku80 Δ hxnLuc:hgx Δ grl4:dhfr	Δ grl4nLuc	RH Δ ku80n Δ hxnLuc:hgx	HXG,DHFR	
RH Δ ku80 Δ hxnLuc:hgx Δ crt:dhfr	Δ crtnLuc	RH Δ ku80n Δ hxnLuc:hgx	HXG,DHFR	
RH Δ ku80 Δ hgxTIR1:chlor	TIR1WT		Chlor	
RH Δ ku80 Δ hgxTIR1:chlorspn1-AID-3HA-HXG	TIR1spn1AID-3HA	RH Δ ku80 Δ hgxTIR1:chlor	Chlor, HXG	
RH Δ ku80 Δ hgx	RHWT			
RH Δ ku80 Δ hgx Δ spn1::hgx	RH Δ spn1	RH Δ ku80 Δ hgx	HXG	

Appendix E: Primers Used in This Study

Appendix Table E-1 Primers used in this study.

Primer	Description	Sequence
P1	TgGRA14.-490	GTCAGAAGAACTGAGCGTAATGC
P2	TgGRA14.520	CAGACGCAACAGAACCAAGGAAAC
P3	TgCRT.-463	GTCAAAGGTCTCTCCTGCG
P4	TgCRT.514	AAGTATGTTTCAGTTCGGGTGCAG
P5	TgCPL.-574.	CTAGGACGACGAGTGGAAGGAAAG
P6	TgCPL.452.	TGTGTGTGAATGTAGACGAGGTTG
P7	gra14-dhfr 5' utr KO repair FWD	TAAC TTCAGTCCACTTTCTAGCCCTGGTTTCCGGAGTTATCC TCAGGGAGACTGCCTGAC
P8	gra14-dhfr 3' utr KO repair REV	CTTGACTGAACAAATGTCAAGGAGTCATACACCAGAATAT ACCCCGGGCTGCAGGAATTC
P9	TGGT1_gra14.-656	CTTGCTTGGGCGAGTCC
P10	DHFR.REV	GAATCCTTGTA CTCTTCCTCCAGA
P11	CRT-dhfr 5' utr KO repair FWD	CCCTCATTCTCCCTCTCTGGATTTCCTGCACAGAGAGCCCCC TCAGGGAGACTGCCTGAC
P12	CRT-dhfr 3' utr KO repair REV	GGGGGGCAGATGGGAAAAGAGCGCGATGCTGTGCGACTGT CCCCGGGCTGCAGGAATTCA
P13	FWD end of DHFR	TGCGGGAAAGTCACGCATATGG
P14	2k downstream of CRT end REV	ACA CAG GAG GAC GAG GCA AAT
P15	Nova seq primer set 1 FWD	AATGATACGGCGACCACCGAGATCTACACAACCGCGGACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P16	Nova seq primer set 1 REV	CAAGCAGAAGACGGCATAACGAGATAGCGCTAGGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P17	Nova seq primer set 2 FWD	AATGATACGGCGACCACCGAGATCTACACGGTTATAAACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P18	Nova seq primer set 2 REV	CAAGCAGAAGACGGCATAACGAGATGATATCGAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P19	Nova seq primer set 3 FWD	AATGATACGGCGACCACCGAGATCTACACCAAGTCCACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P20	Nova seq primer set 3 REV	CAAGCAGAAGACGGCATAACGAGATCGCAGACGGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG

P21	Nova seq primer set 4 FWD	AATGATACGGCGACCACCGAGATCTACACTTGGACTTACAC TCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCGC TCTTCGA
P22	Nova seq primer set 4 REV	CAAGCAGAAGACGGCATACGAGATTATGAGTAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P23	Nova seq primer set 5 FWD	AATGATACGGCGACCACCGAGATCTACACCAGTGGATACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P24	Nova seq primer set 5 REV	CAAGCAGAAGACGGCATACGAGATAGGTGCGTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P25	Nova seq primer set 6 FWD	AATGATACGGCGACCACCGAGATCTACACTGACAAGCACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P26	Nova seq primer set 6 REV	CAAGCAGAAGACGGCATACGAGATGAACATACGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P27	Nova seq primer set 7 FWD	AATGATACGGCGACCACCGAGATCTACACCTAGCTTGACAC TCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCGC TCTTCGA
P28	Nova seq primer set 7 REV	CAAGCAGAAGACGGCATACGAGATACATGCGGTGACTGGA GTTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATCG AATGACATGCTAG
P29	Nova seq primer set 8 FWD	AATGATACGGCGACCACCGAGATCTACACTCGATCCAACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P30	Nova seq primer set 8 REV	CAAGCAGAAGACGGCATACGAGATGTGCGATAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P31	Nova seq primer set 9 FWD	AATGATACGGCGACCACCGAGATCTACACCCTGAACTACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P32	Nova seq primer set 9 REV	CAAGCAGAAGACGGCATACGAGATCCAACAGAGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P33	Nova seq primer set 10 FWD	AATGATACGGCGACCACCGAGATCTACACCCAACAGAACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P34	Nova seq primer set 10 REV	CAAGCAGAAGACGGCATACGAGATTTGGTGAGGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P35	Nova seq primer set 11 FWD	AATGATACGGCGACCACCGAGATCTACACAGTAGAGAACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P36	Nova seq primer set 11 REV	CAAGCAGAAGACGGCATACGAGATCGCGGTTCTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P37	Nova seq primer set 12 FWD	AATGATACGGCGACCACCGAGATCTACACGACGAGAGACA CTCTTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA

P38	Nova seq primer set 12 REV	CAAGCAGAAGACGGCATAACGAGATTATAACCTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCTCGGGGGGTGAAAATC GAATGACATGCTAG
P39	Nova seq primer set 13 FWD	AATGATACGGCGACCACCGAGATCTACACCGAGATATACA CTCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P40	Nova seq primer set 13 REV	CAAGCAGAAGACGGCATAACGAGATCAATTAACGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P41	Nova seq primer set 14 FWD	AATGATACGGCGACCACCGAGATCTACACTAGAGCGCACA CTCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P42	Nova seq primer set 14 REV	CAAGCAGAAGACGGCATAACGAGATTGGCCGGTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P43	Nova seq primer set 15 FWD	AATGATACGGCGACCACCGAGATCTACACAACCTGTTACAC TCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCGC TCTTCGA
P44	Nova seq primer set 15 REV	CAAGCAGAAGACGGCATAACGAGATAGTACTCCGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P45	Nova seq primer set 16 FWD	AATGATACGGCGACCACCGAGATCTACACGGTTCACCACA CTCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P46	Nova seq primer set 16 REV	CAAGCAGAAGACGGCATAACGAGATGACGTCTTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P47	Nova seq primer set 17 FWD	AATGATACGGCGACCACCGAGATCTACACCATTGTTGACAC TCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCGC TCTTCGA
P48	Nova seq primer set 17 REV	CAAGCAGAAGACGGCATAACGAGATTGCGAGACGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P49	Nova seq primer set 18 FWD	AATGATACGGCGACCACCGAGATCTACACTGCCACCAACA CTCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCG CTCTTCGA
P50	Nova seq primer set 18 REV	CAAGCAGAAGACGGCATAACGAGATCATAGAGTGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P51	Nova seq primer set 19 FWD	AATGATACGGCGACCACCGAGATCTACACCTCTGCCTACAC TCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCGC TCTTCGA
P52	Nova seq primer set 19 REV	CAAGCAGAAGACGGCATAACGAGATACAGGCGCGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGGTGAAAATCG AATGACATGCTAG
P53	Nova seq primer set 20 FWD	AATGATACGGCGACCACCGAGATCTACACTCTCATTACAC TCTTCCCTACACGACGCTCTTCCGATCTCCTCCCTGTGCGC TCTTCGA

P54	Nova seq primer set 20 REV	CAAGCAGAAGACGGCATACGAGATGTGAATATGTGACTGG AGTTCAGACGTGTGCTCTTCCGATCCGGGGGGTGAAAATCG AATGACATGCTAG
P55	pU6 DHFR REV insert	TTGGTACTCACTATTGTGACGCCG
P56	pU6 DHFR FWD insert	TTCTACGGGGTCTGACGCTCA
P57	TGGT1_219190.-274	GCAATCTTCTGTAAAGGCTACCGTTGT
P58	TGGT1_219190.1663	GGGTGTTCAACTAAGAGGCGCC
P59	TGGT1_227970.-448	GTCACAAAACGCCACGACAGC
P60	TGGT1_227970.1608	GGAACCCGACAAAGCGTTTCTCTAC
P61	TGGT1_249270.-425	GCGACAGAATGCGAGAAAACTGCTG
P62	TGGT1_249270.1270	ACAGTGGGGAAGCCTTGGAC
P63	TGGT1_254840.-442	TCTCTTGGCAGACAACGGCA
P64	TGGT1_254840.1576	TCCCAGGCGATTTCTCTCTCTC
P65	TGGT1_262640.-371	GATTTCGCGTCTTCGGGTAAAG
P66	TGGT1_262640.1648	CGCTCGGGGATAACTGGTCAG
P67	TGGT1_279390.-670	CCCACAAGAACCCTCCGTATGC
P68	TGGT1_279390.1108	CCTGGACGACTTCTGTCTCAACTTCTG
P69	TGGT1_308830.-504	TATCTCCGTATCTCTGTCTGCACAT
P70	TGGT1_308830.1514	GGAATGGACGCACACACAGCA
P71	285180 AID repair FWD	TACCCGCCTTTTGGAAAATCGAGAGACGATGCAGCGAGGC GCTAGCAAGGGCTCGGGCTC
P72	285180 AID repair REV	TCTTGGTCCGTTCCCAAGACAAAATGAAGTCCAAAGTTCCC ATCCTGCAAGTGCATAGAA
P73	285180 5' integration FWD	GCCGCTGTGTCGCTAACCAC
P74	AID 5' integration REV	GTGCTCCGTCCATTGATACCTTCAC
P75	DHFR 3' integration FWD	GGCATTTCACACAGTCTCA
P76	285180 3' integration REV	ATGTCCTACTCCTCCACTCT
P77	285180_KO_HXG HR repair FWD	TTCAATCTTGCTTCGTTTTCCGGTGAAATTCAAGAGTGCA ACCCGCGGAAGATCCGATCTTG
P78	285180_KO_HXG HR repair REV	CTCTCTTGGTCCGTTCCCAAGACAAAATGAAGTCCAAAGTT CCCATCCTGCAAGTGCATAGAA
P79	285180_-243_5'UTR	CTAAACTTCTCCGCTTGTCGC
P80	HXG coding Seq 7	CCGTAGTCTTCAATGGGTTTGG
P81	HXG coding Seq 614	CGACTTCAACGAGATGTTCCG
P82	Gibson_pElite_primer_Mal E REV	CGGATAATGCGAGGATGCGTGCACCTGTTTTTATTTTCATA TGTATATCTCCTTCTTATA
P83	Gibson_pElite_primer_285180 FWD	GGCGAGACTCGCATTGCTACTGGAAAGCTTGAGTGCTTCGC ATCATCACCACCATCACTA
P84	285180core_geneblock amplify FWD	ACAATTTACGTGGAACAGC
P85	285180core_geneblock amplify REV	GGTTATGCTAGTTATTGCTCAGC

Appendix F: Plasmids Used in This Study

Appendix Table F-1 Plasmids used in this study

Name	Expression	Notes
pU6-DHFR-SAG1gRNA1	gRNA that targets sag1 first exon	GGCAGTGAGACGCGCCGTCA
pU6-DHFR-GRA14gRNA1	gRNA that targets gra14 first exon	AACTCGACCACCCCGACGAG
pU6-DHFR-CRTgRNA1	gRNA that targets crt first exon	TTCCGCGTCCAGGCCGTCTG
pCas9-GFP-Ble-CPLgRNA1	gRNA that targets cpl first exon	CGGGTCGTGCAACGAACGG
pCas9-GFP-Ble-GRA14gRNA1	gRNA that targets gra14 first exon	AACTCGACCACCCCGACGAG
pDHFR-TSc3-M2M3	expresses DHFR resistance cassette with dhfr 5' and 3' UTRs	Only used to generate repair template for Δ gra14nluc and Δ crtmluc
pCas9-GFP-Ble-CRTgRNA1	gRNA that targets crt first exon	GTTCGCGTCCAGGCCGTCTG
pCas9-GFP-Ble-CRTgRNA2	gRNA that targets crt last exon	ACAGGAGACTTGCTTGGTTT
pU6-DHFR-CDPK1gRNA1	gRNA that targets cdpk1 first exon	GTACAGACTCACCGGAAAGA
pU6-DHFR-PLP1gRNA1	gRNA that targets plp1 first exon	GGCGTTGTCCGCCCTTCGTCT
pU6-DHFR-219190gRNA1	gRNA that targets 219190 first exon	GTCAGTAGAAGCTGCGTTCCGG
pU6-DHFR-220940gRNA1	gRNA that targets 220940 first exon	GCTTCTTCGACGCCTCGTCGA
pU6-DHFR-221320gRNA1	gRNA that targets 221320 first exon	GCTCTCGGCCGATAGATCAC
pU6-DHFR-227970gRNA1	gRNA that targets 227970 first exon	GCTGTCTCTCGTTCTGCTTG
pU6-DHFR-249270gRNA1	gRNA that targets 249270 first exon	GGTGGACTGTGGACGTTTGT
pU6-DHFR-254840gRNA1	gRNA that targets 254840 first exon	AGACACCCATTCCATACCAG
pU6-DHFR-262640gRNA1	gRNA that targets 262640 first exon	GGATTGTACTCTCTTGGTGG
pU6-DHFR-279390gRNA1	gRNA that targets 279390 first exon	GGATGCGCAGTTGTGTATAT
pU6-DHFR-286530gRNA1	gRNA that targets 286530 first exon	GTGAACGGGCTAATCCATTTG
pU6-DHFR-288880gRNA1	gRNA that targets 288880 first exon	GTTTCTCTTTCAACCGCCTCA
pU6-DHFR-308830gRNA1	gRNA that targets 308830 first exon	GTCTGGGAGGCCTCAAAGACG
pCas9-GFP-Ble- <i>spn1</i> gRNA1	gRNA that targets immediately up stream of 285180 stop codon	GAGACGATGCAGCGAGGCTG
pTub1YFP-AID-3HA-DHFR-TSHXGPR	expresses auxin inducible degron, with a leading linker sequence, 3copies of HA on the AID, and contains a resistance cassette with HXG under dhfr 5' and 3' UTRs	Used to generate AID-3HA-HXG repair template for TIR1285180-AID-3HA, and HXG repair template for RH Δ ku80 Δ 285180
pCas9-GFP-Ble- <i>spn1</i> gRNA2	gRNA that targets first exon of 285180	AGTGCAACATGGTGTGCCTC
pETite-MalE-spnl-core-C-term-His-Kan	expresses codon optimized SPN1 nuclease core with a periplasmic MalE signal sequence and C terminal His tag	

Appendix G: Antibodies Used in This Study

Appendix Table G-1 Antibodies used in this study.

Antibody	Dilution for Immunofluorescence	Dilution for Immunoblot
RbGRA14	1:500	1:10000
MsMic2		1:10000
RbCPL	1:500	1:8000
RbCRT	1:250	1:1000
MsActin		1:2000
RtHA	1:500	1:2500
RbTgActin		1:20000
RbproM2AP	1:500	
Ms2TgSPN1	1:500	1:5000
Ms6TgSPN1	1:500	1:5000

Appendix H: SpaCr Settings for Training Model

Appendix Table H-1 SpaCr settings used for training the model.

Key	Value
img_src	/path
model_name	toxoplasma
model_type	cyto
Signal to noise	10
background	200
remove_background	FALSE
learning_rate	0.2
weight_decay	1.00E-05
batch_size	8
n_epochs	25000
from_scratch	FALSE
diameter	30
resize	TRUE
width_height	[1120, 1120]
verbose	TRUE
channels	[0, 0]
normalize	TRUE
percentiles	
circular	FALSE
invert	FALSE
grayscale	TRUE
test	FALSE

Appendix I: Media Recipes for Amino Acid Limited Media

Appendix Table I-1 Recipes for amino acid limited media.

Nutrient	Formula DMEM (10-013-CV) [mg/L]	Formula AA free DMEM (D9800-13) [mg/L]	Supplementation for complete AA free DMEM [mg/L]	Supplementation for -tryptophan DMEM [mg/L]	Supplementation for -arginine DMEM [mg/L]	Supplementation for -phenylalanine DMEM [mg/L]	Supplementation for -Tyrosine DMEM [mg/L]
D-Glucose (Dextrose)	4500	1000	3500	3500	3500	3500	3500
Glycine	30	0	30	30	30	30	30
L-Arginine hydrochloride	84	0	84	84	0	84	84
L-Cystine HCl	31.6	0	31.6	31.6	31.6	31.6	31.6
L-Glutamine	584	0	584	584	584	584	584
L-Histidine hydrochloride-H ₂ O	42	0	42	42	42	42	42
L-Isoleucine	105	0	105	105	105	105	105
L-Leucine	105	0	105	105	105	105	105
L-Lysine	116.85	0	116.85	116.85	116.85	116.85	116.85
L-Methionine	30	0	30	30	30	30	30
L-Phenylalanine	66	0	66	66	66	0	66
L-Serine	42	0	42	42	42	42	42
L-Threonine	95	0	95	95	95	95	95
L-Tryptophan	16	0	16	0	16	16	16
L-Tyrosine	72.15	0	72.15	72.15	72.15	72.15	0
L-Valine	94	0	94	94	94	94	94
Sodium Bicarbonate (NaHCO ₃)	3700	0	3700	3700	3700	3700	3700
Sodium Phosphate monobasic	125	109	16	16	16	16	16
Sodium pyruvate	110	0	110	110	110	110	110
AA free DMEM powder			8320	8320	8320	8320	8320

Bibliography

1. Sibley, L. D. Invasion and Intracellular Survival by Protozoan Parasites. *Immunological reviews* **240**, 72 (2011).
2. Morisaki, J. H., Heuser, J. E. & Sibley, L. D. Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. *J Cell Sci* **108**, 2457–2464 (1995).
3. Bargieri, D. *et al.* Host Cell Invasion by Apicomplexan Parasites: The Junction Conundrum. *PLoS Pathog* **10(9)**, :e1004273. doi: 10.1371/journal.ppat.1004273. eCollection 2014 Sep. (2014).
4. Schwab, J. C., Beckers, C. J. & Joiner, K. A. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 509–513 (1994).
5. Gold, D. A. *et al.* The *Toxoplasma* Dense Granule Proteins GRA17 and GRA23 Mediate the Movement of Small Molecules between the Host and the Parasitophorous Vacuole. *Cell Host Microbe* **17**, 642–652 (2015).
6. Bitew, M. A. *et al.* Two *Toxoplasma gondii* putative pore-forming proteins, GRA47 and GRA72, influence small molecule permeability of the parasitophorous vacuole. *mBio* **15**, e03081-23 (2024).
7. Paredes-Santos, T. C. *et al.* Genome-wide CRISPR screen identifies genes synthetically lethal with GRA17, a nutrient channel encoding gene in *Toxoplasma*. *PLOS Pathogens* **19**, e1011543 (2023).

8. Ehrenman, K. *et al.* Novel roles for ATP-binding cassette G transporters in lipid redistribution in *Toxoplasma*. *Molecular Microbiology* **76**, 1232–1249 (2010).
9. Coppens, I. Exploitation of auxotrophies and metabolic defects in *Toxoplasma* as therapeutic approaches. *International Journal for Parasitology* **44**, 109–120 (2014).
10. Pomel, S., Luk, F. C. Y. & Beckers, C. J. M. Host Cell Egress and Invasion Induce Marked Relocations of Glycolytic Enzymes in *Toxoplasma gondii* Tachyzoites. *PLoS Pathog* **4**, e1000188 (2008).
11. Blume, M. *et al.* Host-derived glucose and its transporter in the obligate intracellular pathogen *Toxoplasma gondii* are dispensable by glutaminolysis. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 12998–13003 (2009).
12. Shukla, A. *et al.* Glycolysis is important for optimal asexual growth and formation of mature tissue cysts by *Toxoplasma gondii*. *Int J Parasitol* **48**, 955–968 (2018).
13. MacRae, J. I. *et al.* Mitochondrial Metabolism of Glucose and Glutamine Is Required for Intracellular Growth of *Toxoplasma gondii*. *Cell Host & Microbe* **12**, 682–692 (2012).
14. Brooks, C. F. *et al.* The *Toxoplasma* Apicoplast Phosphate Translocator Links Cytosolic and Apicoplast Metabolism and Is Essential for Parasite Survival. *Cell Host & Microbe* **7**, 62–73 (2010).
15. Mazumdar, J., Wilson, E. H., Masek, K., Hunter, C. A. & Striepen, B. 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**, 13192–13197 (2006).

16. Martins-Duarte, É. S., Carias, M., Vommoro, R., Surolia, N. & de Souza, W. Apicoplast fatty acid synthesis is essential for pellicle formation at the end of cytokinesis in *Toxoplasma gondii*. *Journal of Cell Science* **129**, 3320–3331 (2016).
17. Xu, X.-P. *et al.* The Role of Type II Fatty Acid Synthesis Enzymes FabZ, ODSCI, and ODSCII in the Pathogenesis of *Toxoplasma gondii* Infection. *Front. Microbiol.* **12**, (2021).
18. Schumacher, M. A. *et al.* Crystal structures of *Toxoplasma gondii* uracil phosphoribosyltransferase reveal the atomic basis of pyrimidine discrimination and prodrug binding. *The EMBO Journal* **17**, 3219–3232 (1998).
19. Asai, T. *et al.* Enzymes of the de novo pyrimidine biosynthetic pathway in *Toxoplasma gondii*. *Mol Biochem Parasitol* **7**, 89–100 (1983).
20. Narvaez-Ortiz, H. Y., Lopez, A. J., Gupta, N. & Zimmermann, B. H. A CTP Synthase Undergoing Stage-Specific Spatial Expression Is Essential for the Survival of the Intracellular Parasite *Toxoplasma gondii*. *Front. Cell. Infect. Microbiol.* **8**, (2018).
21. Fox, B. A. & Bzik, D. J. De novo pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*. *Nature* **415**, 926–9 (2002).
22. Chiang, C.-W. *et al.* The Adenosine Transporter of *Toxoplasma gondii*. *Journal of Biological Chemistry* **274**, 35255–35261 (1999).
23. Koning, H. P. D., Al-Salabi, M. I., Cohen, A. M., Coombs, G. H. & Wastling, J. M. Identification and characterisation of high affinity nucleoside and nucleobase transporters in *Toxoplasma gondii*. *International journal for parasitology* **33**, 821–831 (2003).
24. Goerner, A., Messina, G., Beckham, L. D. & Genova, B. M. D. Impact of Equilibrative Nucleoside Transporters TgAT1, TgENT1, and TgENT3 on *Toxoplasma gondii* Infection and

Differentiation. 2024.07.10.601519 Preprint at <https://doi.org/10.1101/2024.07.10.601519> (2024).

25. Chaudhary, K. *et al.* Purine salvage pathways in the apicomplexan parasite *Toxoplasma gondii*. *The Journal of biological chemistry* **279**, 31221–31227 (2004).
26. Krug, E. C., Marr, J. J. & Berens, R. L. Purine Metabolism in *Toxoplasma gondii*. *Journal of Biological Chemistry* **264**, 10601–10607 (1989).
27. Pfefferkorn, E. R. & Pfefferkorn, L. C. Specific labeling of intracellular *Toxoplasma gondii* with uracil. *J Protozool* **24**, 449–453 (1977).
28. Pfefferkorn, E. R. & Pfefferkorn, L. C. *Toxoplasma gondii*: characterization of a mutant resistant to 5-fluorodeoxyuridine. *Experimental parasitology* **42**, 44–55 (1977).
29. Parker, K. E. R. *et al.* The tyrosine transporter of *Toxoplasma gondii* is a member of the newly defined apicomplexan amino acid transporter (ApiAT) family. *PLOS Pathogens* **15**, e1007577 (2019).
30. Rajendran, E. *et al.* Cationic amino acid transporters play key roles in the survival and transmission of apicomplexan parasites. *Nature communications* **8**, 14455 (2017).
31. Kloehn, J., Lunghi, M., Varesio, E., Dubois, D. & Soldati-Favre, D. Untargeted Metabolomics Uncovers the Essential Lysine Transporter in *Toxoplasma gondii*. *Metabolites* **11**, 476 (2021).
32. Fairweather, S. J. *et al.* Coordinated action of multiple transporters in the acquisition of essential cationic amino acids by the intracellular parasite *Toxoplasma gondii*. *PLOS Pathogens* **17**, e1009835 (2021).

33. Piro, F. *et al.* A *Toxoplasma gondii* putative amino acid transporter localizes to the plant-like vacuolar compartment and controls parasite extracellular survival and stage differentiation. *mSphere* **9**, e00597-23.
34. Shafik, S. H. *et al.* The natural function of the malaria parasite's chloroquine resistance transporter. *Nat Commun* **11**, 3922 (2020).
35. Juge, N. *et al.* Plasmodium falciparum chloroquine resistance transporter is a H⁺-coupled polyspecific nutrient and drug exporter. *Proc Natl Acad Sci U S A* **112**, 3356–3361 (2015).
36. Koreny, L. *et al.* Stable endocytic structures navigate the complex pellicle of apicomplexan parasites. *Nat Commun* **14**, 2167 (2023).
37. Wan, W. *et al.* The *Toxoplasma* micropore mediates endocytosis for selective nutrient salvage from host cell compartments. *Nat Commun* **14**, 977 (2023).
38. Spielmann, T., Gras, S., Sabitzki, R. & Meissner, M. Endocytosis in Plasmodium and Toxoplasma Parasites. *Trends Parasitol* **36**, 520–532 (2020).
39. Bergmann, A. *et al.* *Toxoplasma gondii* requires its plant-like heme biosynthesis pathway for infection. *PLOS Pathogens* **16**, e1008499 (2020).
40. Wang, Y., Sangaré, L. O., Paredes-Santos, T. C. & Saeij, J. P. J. Toxoplasma Mechanisms for Delivery of Proteins and Uptake of Nutrients Across the Host-Pathogen Interface. *Annu Rev Microbiol* **74**, 567–586 (2020).
41. Coppens, I. *et al.* *Toxoplasma gondii* sequesters lysosomes from mammalian hosts in the vacuolar space. *Cell* **125**, 261–74 (2006).
42. Romano, J. D., Sonda, S., Bergbower, E., Smith, M. E. & Coppens, I. *Toxoplasma gondii* salvages sphingolipids from the host Golgi through the rerouting of selected Rab vesicles to the parasitophorous vacuole. *Molecular biology of the cell* **24**, 1974–1995 (2013).

43. Nolan, S. J., Romano, J. D. & Coppens, I. Host lipid droplets: An important source of lipids salvaged by the intracellular parasite *Toxoplasma gondii*. *PLoS Pathog* **13**, e1006362 (2017).
44. Romano, J. D. *et al.* *Toxoplasma gondii* scavenges mammalian host organelles through the usurpation of host ESCRT-III and Vps4A. *J Cell Sci* **136**, (2023).
45. Dou, Z., McGovern, O. L., Cristina, M. D. & Carruthers, V. B. *Toxoplasma gondii* ingests and digests host cytosolic proteins. *mBio* **5**(4), e01188-14 (2014).
46. Rome, M. E., Beck, J. R., Turetzky, J. M., Webster, P. & Bradley, P. J. Intervacuolar transport and unique topology of GRA14, a novel dense granule protein in *Toxoplasma gondii*. *Infection and immunity* **76**, 4865–4875 (2008).
47. Rivera-Cuevas, Y. *et al.* *Toxoplasma gondii* exploits the host ESCRT machinery for parasite uptake of host cytosolic proteins. *PLoS Pathog* **17**, e1010138 (2021).
48. McGovern, O. L., Rivera-Cuevas, Y., Kannan, G., Jr, A. N. & Carruthers, V. B. Intersection of Endocytic and Exocytic Systems in *Toxoplasma gondii*. *Traffic (Copenhagen, Denmark)* (2018) doi:10.1111/tra.12556 [doi].
49. Parussini, F., Coppens, I., Shah, P. P., Diamond, S. L. & Carruthers, V. B. Cathepsin L occupies a vacuolar compartment and is a protein maturase within the endo/exocytic system of *Toxoplasma gondii*. *Molecular microbiology* **76**, 1340–1357 (2010).
50. Dou, Z., Coppens, I. & Carruthers, V. B. Non-canonical Maturation of Two Papain-family Proteases in *Toxoplasma gondii*. *The Journal of biological chemistry* **288**, 3523–3534 (2013).
51. Warring, S. D., Dou, Z., Carruthers, V. B., McFadden, G. I. & Dooren, G. G. van. Characterisation of the Chloroquine Resistance Transporter homologue in *Toxoplasma gondii*. *Eukaryotic cell* (in press) (2014) doi:EC.00027-14 [pii].

52. Kannan, G. *et al.* Role of *Toxoplasma gondii* Chloroquine Resistance Transporter in Bradyzoite Viability and Digestive Vacuole Maintenance. *mBio* **10**, (2019).
53. Pappas, G., Roussos, N. & Falagas, M. E. Toxoplasmosis snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *International journal for parasitology* **39**, 1385–1394 (2009).
54. Sidik, S. M. *et al.* A Genome-wide CRISPR Screen in *Toxoplasma* Identifies Essential Apicomplexan Genes. *Cell* **166**, 1423-1435.e12 (2016).
55. Wang, Y. *et al.* Genome-wide screens identify *Toxoplasma gondii* determinants of parasite fitness in IFN γ -activated murine macrophages. *Nat Commun* **11**, 5258 (2020).
56. Krishnamurthy, S. *et al.* CRISPR Screens Identify *Toxoplasma* Genes That Determine Parasite Fitness in Interferon Gamma-Stimulated Human Cells. *mBio* **14**, e00060-23 (2023).
57. Lockyer, E. J. *et al.* A heterotrimeric complex of *Toxoplasma* proteins promotes parasite survival in interferon gamma-stimulated human cells. *PLOS Biology* **21**, e3002202 (2023).
58. Young, J. *et al.* A CRISPR platform for targeted in vivo screens identifies *Toxoplasma gondii* virulence factors in mice. *Nat Commun* **10**, 3963 (2019).
59. Torelli, F., da Fonseca, D. M., Butterworth, S. W., Young, J. C. & Treeck, M. Paracrine rescue of MYR1-deficient *Toxoplasma gondii* mutants reveals limitations of pooled in vivo CRISPR screens. *eLife* **13**, RP102592 (2024).
60. Sangaré, L. O. *et al.* In Vivo CRISPR Screen Identifies TgWIP as a *Toxoplasma* Modulator of Dendritic Cell Migration. *Cell Host Microbe* **26**, 478-492.e8 (2019).
61. Shastri, A. J., Marino, N. D., Franco, M., Lodoen, M. B. & Boothroyd, J. C. GRA25 Is a Novel Virulence Factor of *Toxoplasma gondii* and Influences the Host Immune Response. *Infection and Immunity* **82**, 2595–2605 (2014).

62. Giuliano, C. J. *et al.* CRISPR-based functional profiling of the *Toxoplasma gondii* genome during acute murine infection. *Nat Microbiol* **9**, 2323–2343 (2024).
63. Waldman, B. S. *et al.* Identification of a Master Regulator of Differentiation in *Toxoplasma*. *Cell* **180**, 359–372.e16 (2020).
64. Li, W. *et al.* A splitCas9 phenotypic screen in *Toxoplasma gondii* identifies proteins involved in host cell egress and invasion. *Nat Microbiol* **7**, 882–895 (2022).
65. Olson, W. J. *et al.* Dual metabolomic profiling uncovers *Toxoplasma* manipulation of the host metabolome and the discovery of a novel parasite metabolic capability. *PLOS Pathogens* **16**, e1008432 (2020).
66. Fox, B. A., Gigley, J. P. & Bzik, D. J. *Toxoplasma gondii* lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. *International journal for parasitology* **34**, 323–331 (2004).
67. Sibley, L. D., Messina, M. & Niesman, I. R. Stable DNA transformation in the obligate intracellular parasite *Toxoplasma gondii* by complementation of tryptophan auxotrophy. *Proc Natl Acad Sci U S A* **91**, 5508–12 (1994).
68. Markus, B. M., Bell, G. W., Lorenzi, H. A. & Lourido, S. Optimizing Systems for Cas9 Expression in *Toxoplasma gondii*. *mSphere* **4**, (2019).
69. Sidik, S. M., Huet, D. & Lourido, S. CRISPR-Cas9-based genome-wide screening of *Toxoplasma gondii*. *Nat Protoc* **13**, 307–323 (2018).
70. Lourido, S. *et al.* Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. *Nature* **465**, 359–362 (2010).

71. Key, M. *et al.* Determination of Chemical Inhibitor Efficiency against Intracellular Toxoplasma Gondii Growth Using a Luciferase-Based Growth Assay. *Journal of visualized experiments : JoVE* 10.3791/60985 (2020) doi:10.3791/60985.
72. Marino, N. D. & Boothroyd, J. C. Toxoplasma growth in vitro is dependent on exogenous tyrosine and is independent of AAH2 even in tyrosine-limiting conditions. *Experimental parasitology* **176**, 52–58 (2017).
73. Crawford, M. J. *et al.* Toxoplasma gondii scavenges host-derived lipoic acid despite its de novo synthesis in the apicoplast. *The EMBO journal* **25**, 3214–3222 (2006).
74. Stringer, C., Wang, T., Michaelos, M. & Pachitariu, M. Cellpose: a generalist algorithm for cellular segmentation. *Nat Methods* **18**, 100–106 (2021).
75. Pachitariu, M. & Stringer, C. Cellpose 2.0: how to train your own model. *Nat Methods* **19**, 1634–1641 (2022).
76. White, M. D. *et al.* Selective host autophagy is induced during the intracellular parasite Toxoplasma gondii infection controlling amino acid levels. *mSphere* **9**, e00369-24.
77. Kazibwe, Z., Liu, A.-Y., MacIntosh, G. C. & Bassham, D. C. The Ins and Outs of Autophagic Ribosome Turnover. *Cells* **8**, 1603 (2019).
78. Fujiwara, Y. *et al.* Discovery of a novel type of autophagy targeting RNA. *Autophagy* **9**, 403–409 (2013).
79. Aizawa, S. *et al.* Lysosomal putative RNA transporter SIDT2 mediates direct uptake of RNA by lysosomes. *Autophagy* **12**, 565–578 (2016).
80. Huang, H. *et al.* Bulk RNA degradation by nitrogen starvation-induced autophagy in yeast. *The EMBO Journal* **34**, 154–168 (2015).

81. MacIntosh, G. C., Bariola, P. A., Newbigin, E. & Green, P. J. Characterization of Rny1, the *Saccharomyces cerevisiae* member of the T2 RNase family of RNases: Unexpected functions for ancient enzymes? *Proceedings of the National Academy of Sciences* **98**, 1018–1023 (2001).
82. Floyd, B. E., Morriss, Stephanie C, MacIntosh, Gustavo C & Bassham, D. C. Evidence for autophagy-dependent pathways of rRNA turnover in Arabidopsis. *Autophagy* **11**, 2199–2212 (2015).
83. Hillwig, M. S. *et al.* RNS2, a conserved member of the RNase T2 family, is necessary for ribosomal RNA decay in plants. *Proceedings of the National Academy of Sciences* **108**, 1093–1098 (2011).
84. Liu, Y. *et al.* Autophagy-dependent ribosomal RNA degradation is essential for maintaining nucleotide homeostasis during *C. elegans* development. *eLife* **7**, e36588 (2018).
85. Haud, N. *et al.* rns2 mutant zebrafish model familial cystic leukoencephalopathy and reveal a role for RNase T2 in degrading ribosomal RNA. *Proceedings of the National Academy of Sciences* **108**, 1099–1103 (2011).
86. Bernard, C. *et al.* Equilibrative nucleoside transporter 1 (ENT1) is critical for pollen germination and vegetative growth in Arabidopsis. *Journal of Experimental Botany* **62**, 4627–4637 (2011).
87. Ko, C.-Y. *et al.* Arabidopsis ENDO2: Its Catalytic Role and Requirement of N-Glycosylation for Function. *J. Agric. Food Chem.* **60**, 5169–5179 (2012).
88. Vogt, V. M. Purification and Further Properties of Single-Strand-Specific Nuclease from *Aspergillus oryzae*. *European Journal of Biochemistry* **33**, 192–200 (1973).

89. Endo, K., Umeyama, Yōko, Nakajima, Junko & Kawai, H. Purification and Some Properties of Nucleases from Shii-take, *Lentinus edodes*. *Agricultural and Biological Chemistry* **44**, 1545–1551 (1980).
90. Ando, T. A nuclease specific for heat-denatured DNA isolated from a product of *Aspergillus oryzae*. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis* **114**, 158–168 (1966).
91. Kuninaka, A., Otsuka, Shin-ichiro, Kobayashi, Yasuo & Sakaguchi, K. Studies on 5'-Phosphodiesterases in Microorganisms: Part I. Formation of Nucleoside-5'-monophosphates from Yeast Ribonucleic Acid by *Penicillium citrinum*. *Bulletin of the Agricultural Chemical Society of Japan* **23**, 239–243 (1959).
92. Kowalski, D., Kroeker, W. D. & Laskowski, M. Mung bean nuclease I. 6. Physical, chemical, and catalytic properties. *Biochemistry* **15**, 4457–4463 (1976).
93. Oleykowski, C. A., Bronson Mullins, C. R., Godwin, A. K. & Yeung, A. T. Mutation detection using a novel plant endonuclease. *Nucleic Acids Research* **26**, 4597–4602 (1998).
94. Debrabant, A., Gottlieb, M. & Dwyer, D. M. Isolation and characterization of the gene encoding the surface membrane 3'-nucleotidase/nuclease of *Leishmania donovani*. *Molecular and Biochemical Parasitology* **71**, 51–63 (1995).
95. Debrabant, A., Bastien, P. & Dwyer, D. M. A unique surface membrane anchored purine-salvage enzyme is conserved among a group of primitive eukaryotic human pathogens. *Mol Cell Biochem* **220**, 109–116 (2001).
96. Matoušek, J. *et al.* Accumulation of viroid-specific small RNAs and increase in nucleolytic activities linked to viroid-caused pathogenesis. **388**, 1–13 (2007).

97. Gite, S., Reddy, G. & Shankar, V. Active-site characterization of S1 nuclease I. Affinity purification and influence of amino-group modification. *Biochemical Journal* **285**, 489–494 (1992).
98. Koval', T. *et al.* Crystallization of recombinant bifunctional nuclease TBN1 from tomato. *Acta Cryst F* **67**, 124–128 (2011).
99. Lesniewicz, K., Karlowski, W. M., Pienkowska, J. R., Krzywkowski, P. & Poreba, E. The Plant S1-Like Nuclease Family Has Evolved a Highly Diverse Range of Catalytic Capabilities. *Plant and Cell Physiology* **54**, 1064–1078 (2013).
100. Yang, B. *et al.* Purification, Cloning, and Characterization of the CEL I Nuclease. *Biochemistry* **39**, 3533–3541 (2000).
101. Koval, T. & Dohnálek, J. Characteristics and application of S1–P1 nucleases in biotechnology and medicine. *Biotechnology Advances* **36**, 603–612 (2018).
102. Paletta-Silva, R. *et al.* *Leishmania amazonensis*: Characterization of an ecto-3'-nucleotidase activity and its possible role in virulence. *Experimental Parasitology* **129**, 277–283 (2011).
103. Enzyme Regulation in a Trypanosomatid: Effect of Purine Starvation on Levels of 3'-Nucleotidase Activity. <https://www.science.org/doi/10.1126/science.2981117>
doi:10.1126/science.2981117.
104. Joshi, M. B. & Dwyer, D. M. Molecular and Functional Analyses of a Novel Class I Secretory Nuclease from the Human Pathogen, *Leishmania donovani**. *Journal of Biological Chemistry* **282**, 10079–10095 (2007).

105. Soong, L., Duboise, S. M., Kima, P. & McMahon-Pratt, D. Leishmania pifanoi amastigote antigens protect mice against cutaneous leishmaniasis. *Infection and Immunity* **63**, 3559–3566 (1995).
106. Campbell, K., Diao, H., Ji, J. & Soong, L. DNA Immunization with the Gene Encoding P4 Nuclease of Leishmania amazonensis Protects Mice against Cutaneous Leishmaniasis. *Infection and Immunity* **71**, 6270–6278 (2003).
107. Guimarães-Costa, A. B. *et al.* 3'-Nucleotidase/Nuclease Activity Allows Leishmania Parasites To Escape Killing by Neutrophil Extracellular Traps. *Infection and Immunity* **82**, 1732–1740 (2014).
108. Barylyuk, K. *et al.* A Comprehensive Subcellular Atlas of the Toxoplasma Proteome via hyperLOPIT Provides Spatial Context for Protein Functions. *Cell Host Microbe* **28**, 752-766.e9 (2020).
109. Brown, K. M., Long, S. & Sibley, L. D. Conditional Knockdown of Proteins Using Auxin-inducible Degron (AID) Fusions in Toxoplasma gondii. *Bio Protoc* **8**, (2018).
110. McDonald, C. *et al.* Toxoplasma Cathepsin Protease B and Aspartyl Protease 1 Are Dispensable for Endolysosomal Protein Digestion. *mSphere* **5**, (2020).
111. Hušťáková, B. *et al.* A highly active S1-P1 nuclease from the opportunistic pathogen Stenotrophomonas maltophilia cleaves c-di-GMP. *FEBS Letters* **597**, 2103–2118 (2023).
112. Smith, D. *et al.* Toxoplasma TgATG9 is critical for autophagy and long-term persistence in tissue cysts. *Elife* **10**, (2021).
113. Huynh, M. H. & Carruthers, V. B. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. *Eukaryotic cell* **8**, 530–539 (2009).

114. Thaprawat, P. *et al.* TgATG9 is required for autophagosome biogenesis and maintenance of chronic infection in *Toxoplasma gondii*. *Autophagy Reports* **3**, 2418256 (2024).
115. Di Cristina, M. *et al.* *Toxoplasma* depends on lysosomal consumption of autophagosomes for persistent infection. *Nature microbiology* **2**, 17096 (2017).
116. Harper, J. M. *et al.* A cleavable propeptide influences *Toxoplasma* infection by facilitating the trafficking and secretion of the TgMIC2-M2AP invasion complex. *Molecular biology of the cell* **17**, 4551–4563 (2006).