Defining the Apicomplexan Amino Acid Transporter (ApiAT) Family

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• The silhouette vector images used in the Figure 1.2 were created by Freepik.

• Multiple sequence alignments were generated using Clustal Omega.

• The multiple sequence alignment was edited using Jalview.

• The phylogenetic tree was generated using PHYLYP.

• Tree files were viewed and manipulated using Fig Tree v1.4.0.

• Statistical analysis of many data sets and plotting of some graphs was done in R using the RStudio integrated development environment.

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The Apicomplexan phylum is a large group of unicellular eukaryotic parasites. These parasites cause some notorious diseases of humankind, including malaria and toxoplasmosis. Apicomplexan parasites can also infect animal species, causing significant animal suffering and economic loss in the livestock industry. The advent of drug resistance and the poor side-effect profiles of some medications provides impetus for the development of new anti-parasitic drugs. Uncovering key aspects of parasite biology may aid in identifying and characterising novel inhibitors of parasite growth.

Disruption of nutrient acquisition is one avenue to halt parasite replication and thereby cure infections. Parasites, by definition, rely on their hosts for a source of nutrients. Most nutrients do not diffuse freely across cellular membranes and their acquisition relies on cell-derived processes. One means by which parasites can acquire nutrients is via transporter proteins. Transporters are integral membrane proteins that mediate the movement of solutes across biological membranes. Apart from nutrient acquisition, transporters are also a means by which cells remove waste products and toxins, and control their ionic composition. Despite their importance, the transporters that allow apicomplexan parasites to perform these cell functions are currently not well characterised.

Here, I define a family of amino acid transporters that are ubiquitous in apicomplexan parasites, which I name Apicomplexan Amino acid Transporters (ApiATs). I show that 10 of the 16 ApiATs in Toxoplasma gondii are expressed in the disease-causing tachyzoite stage, and 8 of them localise to the parasite plasma membrane. I demonstrate that four ApiAT proteins are important for growth of the tachyzoite stage of the parasite.

In T. gondii, the amino acid glutamine is of particular interest because, apart from its role in protein synthesis, it is also used as an energy source. Prior to this work the uptake mechanism for glutamine in T. gondii was unknown. I investigated the substrate specificities of one ApiAT protein, TgApiAT2, demonstrating that it functions as a neutral amino acid transporter and is the major glutamine transporter of the parasite.
Together, these findings identify a family of amino acid transporters in apicomplexans, and highlight the importance of amino acid scavenging for the biology of this important phylum of intracellular parasites.
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Chapter 1

Introduction

The Apicomplexa are a large phylum of unicellular eukaryotic parasites. They have complex lifecycles involving both sexual and asexual reproduction, multiple life stages and sometimes multiple hosts. It is thought that every species of animal hosts at least one species of apicomplexan parasite [Morrison, 2009]. Apicomplexans cause important human and animal diseases which continue to plague humanity despite progress in prevention and treatment.

1.1 The apicomplexan phylum

Apicomplexans evolved from a free-living photosynthetic ancestor. Their closest extant free-living relatives are the chromerids and colpodellids, which form a single monophyletic sister group to apicomplexans [Janouškovec et al., 2015]. The colpodellids are free living single-celled predators [Kuvardina et al., 2002] and the chromerids are free living photosynthetic protists. Taxonomically, the apicomplexans and chromerids/colpodellids group with the dinoflagellates, and these three lineages are collectively referred to as the Myzozoa. In turn, myzozoans group with the ciliates, together forming the superphylum Alveolata. [Morrison, 2009] (Figure 1.1). The uniting feature of the Alveolata are cellular structures termed alveoli, which are adjoining membranous sacks located beneath the plasma membrane. In dinoflagellates and ciliates, these are filled with cellulosic material [Gagat et al., 2014]. In apicomplexans, these serve as anchoring points for the actin/myosin-based motility system [Morrissette & Gubbels, 2014].

The defining features of apicomplexan parasites are as follows. In apicomplexans the alveoli are flattened to form parallel membranes that are stabilized by a membrane-associated protein meshwork [Striepen et al., 2007]. Together the alveoli and the pro-
§1.1 The apicomplexan phylum

The apicomplexan phylum

Babesia bovis
Theileria annulata
Plasmodium berghei
Plasmodium falciparum
Toxoplasma gondii
Neospora caninum
Eimeria tenella
Cryptosporidium parvum
Vitrella brassicaformis
Chromera velia
Colpodellids
Dinoflagellates
Ciliates
Chromerids
Apicomplexa
Myzozoa
Alveolata

Figure 1.1: Schematic of the relationships between apicomplexan species. A representative phylogenetic tree of the apicomplexan species used in this study, and the relationships to their closest free living relatives. The branch lengths on the tree are not to scale.

tein meshwork are referred to as the inner membrane complex [Striepen et al., 2007]. All apicomplexans have a structure at the anterior end of the parasites referred to as an apical complex. The apical complex is usually present in the infective stages and gives the phylum its name. It is made of cytoskeletal elements and secretory organelles which are critical for the active host cell invasion process employed by these parasites [Levine, 1988]. Most apicomplexans harbour a non-photosynthetic plastid of red algal origin called the apicoplast [McFadden et al., 1996; Janouškovec et al., 2010]. The apicoplast participates in important metabolic pathways including fatty acid, isoprenoid, iron-sulphur cluster and haem synthesis, rendering the apicoplast essential to survival [Lim & McFadden, 2010].

One could argue that, since every free-living organism usually harbours many species of parasites, parasitism is actually a more common way of life, yet it is the least studied of the two. Therefore the study of parasites is biologically interesting and important. The apicomplexan phylum is interesting from an evolutionary perspective, as almost
all members are obligate intracellular parasites [Levine, 1988]. The apicomplexans are a large phylum; approximately 6000 species have been described, though it has been estimated that there may be 1.2-10 million species [Adl et al., 2007], in which case as little as 0.1% of apicomplexan species have been named to date [Morrison, 2009]. Since the phylum is almost entirely parasitic, but has close free-living photosynthetic and predatory relatives, apicomplexans and their relatives provide important insights into the evolution of parasitism from free-living organisms [Woo et al., 2015; Kuvardina et al., 2002; Janouškovec & Keeling, 2016].

1.1.1 Diseases caused by apicomplexans

In addition to being biologically interesting, apicomplexan parasites cause a range of diseases in humans and economically important animal species. In humans, Plasmodium species cause malaria, Cryptosporidium infection causes diarrheal disease and Toxoplasma gondii can cause central nervous system damage [Antinori et al., 2012; Clark, 1999; Montoya & Liesenfeld, 2004]. In agriculture, cattle are affected by Babesia and Theileria species, which cause haemolytic or lymphoproliferative illnesses [Bock et al., 2004; Schnittger et al., 2012; Onuma et al., 1998], and also by Neospora caninum, which causes abortions [Dubey, 1999]. T. gondii causes abortions in sheep [Innes, 2010] and Eimeria species cause coccidiosis in poultry [Blake & Tomley, 2014]. Together, the human and animal diseases caused by apicomplexans constitute a large burden on humanity.

1.1.1.1 Animal diseases

Apicomplexan parasites are a scourge of livestock industries worldwide, causing considerable economic cost to humans and suffering to animals. Eimeria species cause the intestinal disease coccidiosis in poultry. Seven species of Eimeria specifically infect chickens; three of these cause haemorrhagic diseases and four of these cause malabsorptive diseases, leading to reduced egg production and failure to thrive [Blake & Tomley, 2014]. Coccidiosis can be controlled using in-feed anticoccidial drugs or by vaccination. However, drug resistance develops readily [Chapman, 1997], and there is increasing demand for organic poultry free from antimicrobial drugs [Sharman et al.,

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1 One exception to this rule has emerged in the form of the marine endosymbiont Nephromyces, an apicomplexan which has an obligate mutualistic, rather than parasitic, symbiotic relationship with marine invertebrates [Saffo et al., 2010].
Furthermore, live-attenuated vaccines are the only ones available and since *Eimeria* cannot yet be propagated *in vitro*, vaccine production is costly and thus vaccination is not always used [Blake & Tomley, 2014]. These factors, combined with the strong environmental persistence of the parasites, mean that most chicken flocks in the world are at risk of *Eimeria* infection [Blake & Tomley, 2014]. The economic cost of production losses combined with costs of prevention and treatment is estimated at USD $3 billion per annum globally [Blake & Tomley, 2014].

Cattle are another important livestock animal afflicted with diseases caused by apicomplexan parasites. Parasitism by apicomplexans adversely impacts the cattle industry through mortality, abortions, decreased meat and milk production, costly control measures, and cattle trade restrictions [Schnittger et al., 2012]. The majority of bovine babesiosis (red water fever) is caused by three species of *Babesia*, which invade erythrocytes. The symptoms associated with babesiosis depend on which species is causing the infection. The most virulent species, *B. bovis*, causes hypotensive shock syndrome with respiratory and neurological symptoms due to accumulation of infected erythrocytes in lung and brain capillaries. The other two species, *B. bigemina* and *B. divergens*, mainly cause severe anaemia due to massive erythrocyte destruction [Schnittger et al., 2012].

Parasites of the genus *Theileria* are closely related to *Babesia* (both are in the order Piroplasmida) and also infect the erythrocytes of cattle [Brayton et al., 2007]. *Theileria parva* causes an acute high-mortality disease in cattle called East Coast fever, prevalent in east Africa. Infected cattle have high fever, swollen lymph nodes, and difficulty breathing. Unlike *Babesia* spp., *T. parva* parasites also infect white blood cells, accounting for the lymph node swelling [Nene et al., 2016]. On entering its host, *T. parva* first infects lymphocytes, where it proliferates before developing into the stage that infects erythrocytes [Nene et al., 2016]. Intriguingly, *T. parva* (along with two other *Theileria* species) has the unique ability to induce cell division of its lymphocyte host cell [Nene et al., 2016]. The parasite divides in synchrony with the host cell so that daughter host cells remain infected [Striepen et al., 2007]. *T. parva* infected lymphocytes undergo extensive proliferation, giving them a cancer-like phenotype. The resulting lymphoproliferative disease is what causes symptoms, and can ultimately lead to death of the infected animal.
Neospora caninum and Toxoplasma gondii are major causes of abortion in cattle and sheep, respectively [Dubey et al., 2007; Dubey, 2009]. Both N. caninum and T. gondii can cause epidemic abortions (abortion storms), most likely due to a point source exposure to oocyst-contaminated food or water, resulting in many animals becoming infected at once [Dubey et al., 2007]. N. caninum may be responsible for over 30 per cent of all abortions in dairy and beef cattle in NSW, which costs the Australian dairy and beef industries AUD$85 million and $25 million per year, respectively [Walker, 2004]. Globally the N. caninum related losses in cattle are estimated to exceed US $1.298 billion per annum [Reichel et al., 2013]. Amongst all microbes of cattle, N. caninum is one of the most efficient transplacentally transmitted pathogens, with up to 95% transmission rate [Dubey et al., 2007]. This high transmission rate may be due to reactivation of latent N. caninum infection during gestation by an unknown mechanism, an event which seems to occur in multiple pregnancies of the same cow [Dubey et al., 2007], with repeated abortions reported in some cows.

In studies over the past 20 years, evidence of T. gondii infection was detected in up to 23% of aborted lambs [Dubey, 2009]. T. gondii also causes abortion and stillbirth in humans, goats and sometimes pigs. T. gondii-induced abortion in sheep is usually due to primary infection of a pregnant ewe through contaminated feed or water, rather than from reactivation in a chronically infected ewe. In contrast to N. caninum, congenital transmission of T. gondii from ewes persistently infected with the parasite may occur, but is infrequent, with potentially less than 4% of chronically infected ewes transmitting to the next generation [Dubey, 2009].

1.1.1.2 Human diseases

In 2017, Plasmodium species caused between 203-262 million new cases of malaria worldwide, and an estimated 435 000 deaths [World Health Organisation, 2018b]. This figure represents significant progress in containing malaria since the turn of the century: the World Health Organisation estimates that the malaria mortality rate decreased by 60% globally between 2000 and 2015 [World Health Organisation, 2016]. Malaria is a direct cause of poverty [Gallup & Sachs, 2001], meaning that the resultant gains in life-expectancy cumulatively over those 15 years have been valued at US$ 1 810 billion in sub-Saharan Africa and US$ 2 040 billion globally [World Health Organisation, 2016]. However, the World Health Organisation reports that progress in global malaria
control is stalling, with “no significant progress” made in the last two years [World Health Organisation, 2018b]. Malaria interventions therefore continue to be part of the global agenda and constitute a large area of research. There is an ongoing effort to develop new anti-malarial drugs, because the parasite is particularly adept at evolving resistance to drug treatment [Cowell et al., 2018]. The most recent threat to malaria-control efforts and life-saving treatments is emerging resistance to artemisinin-based combination therapies, the current front-line treatment [Ashley et al., 2014].

Cryptosporidium, another apicomplexan, has been recognised as a major cause of diarrheal disease in children in the developing world [Kotloff et al., 2013]. In this study, Cryptosporidium was the second most frequent cause of diarrhoea in children less than one year of age (behind Rotavirus) and the third most frequent cause in children aged between one and two years, behind Rotavirus and Shigella [Kotloff et al., 2013]. Every year, there are almost 1.7 billion cases of childhood diarrhoeal disease around the globe [World Health Organisation, 2017]. Diarrheal disease is the second leading cause of death of children under 5 worldwide. It caused 9% of all deaths in children under five in 2015, almost twice the number caused by malaria (5%) and nine times the number caused by HIV/AIDS (1%). Note that forty-five percent of all deaths of children under 5 occur in the neonatal period (0-27 days) and are mostly due to preterm birth complications or intrapartum-related events [United Nations Inter-agency Group for Child Mortality Estimation (UN-IGME), 2017]. Accordingly, if these neonatal deaths are excluded, the importance of diarrheal disease becomes even more evident, causing over 16% of the deaths of children.

Even when diarrhoea does not cause death directly, it contributes to other causes of morbidity and mortality, as diarrhoea is a top cause of undernutrition in children under five years old [World Health Organisation, 2017]. Undernutrition in turn is linked to approximately 45% of all child deaths [World Health Organisation, 2018a]. Cryptosporidium is a particular threat to infants and those with compromised immune systems, such as the significant number of people in sub-Saharan Africa living with AIDS. There is a significant gap in research effort when it comes to Cryptosporidium, as the parasite is hard to culture in vitro and manipulate genetically [Striepen, 2013]. The one drug available for Cryptosporidium infection works worst in the people who need it.

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2New estimates reveal that four pathogens cause the majority of cases of diarrhoea: Rotavirus, enterotoxigenic E. coli, Shigella and, somewhat surprisingly, Cryptosporidium [Kotloff et al., 2013].
most, the malnourished and immunosuppressed [Striepen, 2013].

Globally, *T. gondii* is a very common infection, with an average prevalence in humans of 40% [Tenter *et al.*, 2000]. *T. gondii* infection in humans is usually asymptomatic [Innes, 2010]. However, infected persons can develop toxoplasmosis if the parasite is allowed to replicate unchecked by the immune system [Black & Boothroyd, 2000]. Therefore, toxoplasmosis usually occurs in patients where the immune system is under-developed or compromised. There are, however, some more virulent strains of *T. gondii* which can cause disease, particularly ocular toxoplasmosis, even in healthy patients [Alday & Doggett, 2017]. The parasite displays tropism for the central nervous system [Black & Boothroyd, 2000], so unchecked replication destroys cells of the CNS, inducing mental status changes, seizures and focal neurological deficits (amongst other symptoms) [Alday & Doggett, 2017]. It also can cause life-threatening encephalitis [Luft & Remington, 1992]. Toxoplasmic encephalitis is life-threatening for immunocompromised patients and was a major killer during the AIDS epidemic of the 1980’s. Congenital toxoplasmosis may arise if the mother is infected for the first time whilst pregnant. This disease results in severe birth defects or death of the developing foetus [Montoya & Liesenfeld, 2004].

### 1.1.2 Study Species

#### 1.1.2.1 Life cycle

*T. gondii* is one of the most successful parasites in the world [Tenter *et al.*, 2000]. It is capable of infecting virtually all warm-blooded animals (birds and mammals) and within those animals, any nucleated cell [Su *et al.*, 2003]. This is a rare feat amongst protozoan parasites, which tend to be specialised in the cell types they infect. This incredible diversity has been suggested to contribute to *T. gondii*’s success [Tenter *et al.*, 2000].

A basic understanding of the life cycle of *T. gondii* is necessary to appreciate parts of this thesis. *T. gondii* has ‘predator-prey’ transmission (Figure 1.2). Felids, most commonly domestic cats, are the definitive host (in which sexual replication takes place) and birds and mammals are the intermediate hosts (in which asexual replication takes place) [Tenter *et al.*, 2000]. Sexual stage parasites are present in the intestine of infected cats, leading to shedding of oocysts in faeces. Oocysts sporulate in the envi-
§1.1 *The apicomplexan phylum*

Rononment, becoming infective to intermediate hosts. Ingestion of sporulated oocysts by intermediate hosts sparks the asexual stage of the life cycle, which can take two forms - the rapidly replicating tachyzoites and the slow-growing bradyzoites. Bradyzoites form microscopic cysts which persist in the central nervous systems and muscles of the intermediate hosts for the lifetime of the host organism. Cats become infected by consuming bradyzoite cysts in the tissues of their prey, perpetuating the cycle [Black & Boothroyd, 2000]. Therefore, the animals that cats eat (rodents, small birds etc.) are intermediate hosts which can perpetuate the cycle of transmission, whereas humans and livestock species are usually ‘dead-end’ intermediate hosts, as cats do not usually prey on these animals. Human infection can occur by three routes: (1) ingestion of sporulated oocysts, (2) ingestion of bradyzoite tissue cysts in food animals, and (3) vertical transmission from mother to foetus (Figure 1.2).

All three infectious stages of *T. gondii* (tachyzoites, bradyzoites in tissue cysts, and sporozoites in sporulated oocysts) are infectious to both intermediate and definitive hosts. Therefore, *T. gondii* may be transmitted between definitive and intermediate hosts, but also between two definitive or between two intermediate hosts (Figure 1.2) [Tenter *et al.*, 2000]. This is unlike most other apicomplexan parasites, which must cycle between definitive and intermediate hosts [Blader & Saeij, 2009] (for example, *Plasmodium* species must undergo sexual replication in the mosquito before becoming infectious to humans). Transmission of *T. gondii* between intermediate hosts is either vertical (mother-foetus) or horizontal (carnivorism) (Figure 1.2). The extraordinary flexibility in the life-cycle *T. gondii* employs probably plays a part in its massive prevalence worldwide [Blader & Saeij, 2009]. Theoretically, the life cycle could continue indefinitely in the absence of either host type by transmission of tissue cysts between intermediate hosts or transmission of oocysts between definitive hosts [Tenter *et al.*, 2000].

Tachyzoites are the disease-causing stage of the *T. gondii* life cycle. The research presented in this thesis focuses exclusively on this stage. Replication in the tachyzoite stage is referred to as the lytic cycle, because host cells are destroyed, i.e. lysed, in the process of parasite replication [Black & Boothroyd, 2000]. Extracellular parasites actively invade host cells, attaching to and penetrating their target cell using a specialised group of cytoskeletal structures and organelles termed the apical complex [Hu *et al.*, 2006] (Figure 1.3). The parasites invade using an actin-myosin based system, enclosing
Figure 1.2: The life cycle of *T. gondii*. *T. gondii* has predator-prey transmission, cycling between the definitive felid host and small vertebrate intermediate hosts. The arrows are colour coded to represent the mode of transmission. Blue arrows are transmission via oocysts, red via tissue cysts and green via tachyzoites. These three infective stages represent the three routes of infection for humans, numbered (1), (2) and (3). Cats becomes infected through ingesting bradyzoites as cysts in the tissues of a rodent (red arrow). In the cat intestine, parasites first replicate asexually then initiate the sexual phase of the lifecycle. Oocysts are shed into the environment in the faeces. Sporulation occurs once the oocysts are the environment, resulting in an infectious oocyst that can go on to infect many different intermediate hosts (blue arrows). Cats can also become infected via ingesting oocysts (blue arrows between cats). In this case, sporozoites initiate rounds of asexual multiplication as tachyzoites in extra-intestinal tissues before the parasites migrate to the intestinal tissues and initiate sexual reproduction. If an oocyst is ingested by an intermediate host, the sporozoites are released and invade the gut mucosa, and differentiate into tachyzoites. Tachyzoites replicate rapidly, constituting the acute phase of the infection. Bradyzoites constitute the chronic stage of the infection, and form tissue cysts that are ‘invisible’ to the immune system (grey dashed arrow, stage conversion). Carnivorism (red arrows) perpetuates *T. gondii* transmission, via ingestion of bradyzoites, completing the cycle. Rather uniquely, carnivorism allows transmission between intermediate hosts. The final mode of transmission is trans-placental, from mother to developing foetus (green arrows).
Figure 1.3: The Lytic cycle of *T. gondii* tachyzoites. Tachyzoite replication is a destructive process. (1) Extracellular tachyzoites actively invade host cells, surrounding themselves with host derived membrane which becomes the parasitophorous vacuole. (2) Intracellular tachyzoite in a parasitophorous vacuole. (3) Parasites replicate within the vacuole via endodyogeny. (4) Intracellular tachyzoites can spontaneously differentiate into bradyzoites. (5) Bradyzoite ‘tissue cysts’ can re-differentiate into tachyzoites at any time. (6) Egress from the host cell is an active process which destroys the host cell.

themselves in a compartment referred to as the parasitophorous vacuole that is formed from host cell plasma membrane [Striepen & Soldati, 2007]. Inside the parasitophorous vacuole, *T. gondii* divides by endodyogeny, a process whereby two daughter parasites are assembled within the mother cell [Nishi *et al.*, 2008]. Parasites undergo multiple division cycles, usually accumulating 64 to 128 parasites per host cell [Black & Boothroyd, 2000]. Tachyzoites then burst from the host cell (a process termed ‘egress’) and invade new host cells [Black & Boothroyd, 2000]. Egress is a rapid, parasite-driven event activated by a calcium signalling cascade, and leads to destruction of the host cell [McCoy *et al.*, 2012].
The bradyzoite stage corresponds to the development of chronic infection in animals. This stage can evade the host immune system and persist for years at a time. Intracellular tachyzoites will spontaneously differentiate into bradyzoites (which can in turn re-differentiate into tachyzoites at any time) (Figure 1.3) [Jerome et al., 1998]. Vacuoles containing bradyzoites are encased in a tough glycoprotein-containing cyst wall that forms from the parasitophorous vacuole membrane [Zhang et al., 2001]. Bradyzoites are refractory to the drugs currently used to treat toxoplasmosis, therefore chronic *T. gondii* infections cannot be cured [Blader & Saeij, 2009]. This is problematic for immunocompromised patients, who therefore have no long-term protection against bradyzoite cysts reactivating into tachyzoites [Alday & Doggett, 2017]. Compounding this further, anti-toxoplasma drugs can be poorly tolerated and have severe side effects [Blader & Saeij, 2009; Alday & Doggett, 2017], particularly in patients with AIDS [Luft & Remington, 1992].

### 1.1.2.2 Genetic modification of *T. gondii*

*T. gondii* is also a useful parasite from an experimental perspective. It is easy to maintain in culture and to modify genetically [Striepen & Soldati, 2007]. Many aspects of *T. gondii* biology mirror equivalent biological processes in other apicomplexans, making *T. gondii* a powerful ‘experimental model’ system for the phylum. Two broad approaches to genetic modification were used in this study. The first will be referred to as the ‘traditional recombination approach’. In this technique, linearised foreign deoxyribonucleic acid (DNA) is introduced into the parasite, which, mistaking the foreign DNA for damaged DNA, ‘repairs’ the foreign DNA by integrating it into its genome (see Methods §2.3.1). The second involves the use of the CRISPR/Cas9 system.

The adaptation of the CRISPR/Cas9 system for use in *T. gondii* [Shen et al., 2014a; Sidik et al., 2014] has greatly accelerated the rate at which genetic modifications can be performed. This includes reverse genetic approaches as are used in this study. The system allows precise, targeted changes to be made to the genome more quickly than the traditional recombination approach. Briefly, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are sequences of DNA found in bacteria and archaea. They are part of a bacterial immune system that recognises sequences in bacteriophage ribonucleic acid (RNA) that the bacterium has previously been exposed to. The CRISPR sequences encode small RNAs. When transcribed, the RNA binds to the in-
vading phage RNA, ‘guiding’ the CRISPR-associated protein (Cas) to the phage RNA. Cas, an endonuclease, cleaves the invading phage RNA, destroying it and thereby protecting the bacterium from bacteriophage infection.

Researchers can exploit the CRISPR/Cas system to genetically modify target DNA within many different cell types. To do so, DNA encoding a Cas protein and a small guide RNA that encodes the target DNA sequence is transfected into the cells of interest. The CRISPR/Cas system adapted for use in *T. gondii* uses Cas9, an endonuclease that can cleave double-stranded DNA in a site-specific manner, subsequently activating cellular DNA repair machinery (Figure 1.4). Repair by the non-homologous end joining (NHEJ) pathway results in insertions and/or deletions, a strategy that can be employed to disrupt the open reading frame of the targeted locus, thereby generating functional gene knockouts (Figure 1.4). Alternatively, if a donor template with homology to the targeted locus is supplied, the break may be repaired by homology-directed repair, which allows specific replacement mutations to be made (Figure 1.4).

### 1.2 Transporters in Apicomplexan Parasites

Through gaining a better understanding of the biology of apicomplexan parasites, progress can be made towards their control and eradication. One area of apicomplexan biology that we have a poor understanding of is the parasite’s ‘permeome’ - the channels and transporters that underpin the permeability of the cell membrane [Staines et al., 2010]. Despite their likely importance, the transport proteins of apicomplexan parasites are, in general, an under-researched area, particularly in *T. gondii* where only a handful have been characterised [Coppens, 2014]. Transporters are commonly exploited as drug targets in other diseases and, in principle, drugs targeting parasite transporters could be effective in treating parasitic diseases [Coppens, 2014; Kirk, 2004]. The knowledge gap surrounding apicomplexan transporters, and the potential to pave the way for drug development, has been a driver for the research in this thesis.

Transport proteins are ubiquitous in, and are essential to, all forms of life [Quick, 2002]. They are integral membrane proteins that mediate the translocation of molecules across biological membranes [Quick, 2002]. Transport proteins include channels and transporters [Aronson et al., 2012]. Transporters are distinct from channels in that they do not open simultaneously to both sides of the membrane, and therefore do not allow
Figure 1.4: CRISPR/Cas9 genome editing. The CRISPR/Cas9 system can be used to selectively target genes for modification. Guide RNAs homologous to the gene of interest are used to direct the Cas9 endonuclease to the desired location in the genome, at which the endonuclease will then create double stranded DNA breaks. In *T. gondii*, two pathways can be used to repair the double stranded breaks, either the non-homologous end joining pathway or a homologous repair mechanism. The former will introduce insertions or deletions at the target locus, and therefore can be used to generate functional gene knockouts (e.g. introduce frameshifts or premature stop codons in the open reading frame of the target gene). The latter repair mechanism can be used to introduce precise mutations into the gene, by providing an oligonucleotide with the desired modifications (e.g. epitope tag) and homologous flanking regions. Abbreviations: ApiAT, Apicomplexan Amino acid Transporter; HA tag, haemagglutinin epitope tag; PAM, protospacer adjacent motif; oligo, oligonucleotide; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR-associated protein 9.
free diffusion of solutes [Aronson et al., 2012]. Instead, transporters have one or more substrate binding sites which are specific to the solutes they transport. Binding sites often can interact with multiple structurally similar substrates, however they bind to each of these substrates with different affinity. This affinity can be estimated experimentally (see §6.2.2.1).

There are three major subtypes of transporter. Uniporters have a single substrate binding site. Symporters have two distinct substrate binding sites and move both substrates in the same direction. Antiporters also have two distinct substrate binding sites but move the substrates in opposite directions. Substrate is moved across the membrane in a translocation cycle. Translocation of substrate can be in either direction across the membrane but for thermodynamic reasons predominantly occurs in the energetically favourable direction, that is, in the direction of the electrochemical gradient for that substrate [Pradhan et al., 2013]. The translocation cycle involves the transporter binding substrate(s) on one side of a membrane, undergoing a conformational change which exposes the substrate to the other side of the membrane, then releasing the substrate. The transporter must undergo a second conformational change to return the substrate binding site to the original side of the membrane, before it can transport another substrate [Quick, 2002]. This second conformational change, in the absence of bound substrate is often less energetically favourable and therefore rate limiting. The presence of one or more of the transporters substrates on the trans side of the membrane can therefore sometimes accelerate transport rate. This process is known as trans-stimulation and is unique to transporters [Stein, 1986].

The essential nature of transporters stems from their diverse actions within cells. They facilitate the transport of nutrients into, and wastes out of, cells and they regulate metabolite concentrations [Quick, 2002]. They control ionic composition and maintain electro-chemical gradients [Quick, 2002]. Transporters also allow cells to respond to their environment, for example they are often activated or deactivated in response to stressors such as changes in cell pH or volume.

Cells employ multiple strategies to acquire the nutrients needed for their growth. These fall into the two broad categories that entail using either plasma membrane transmembrane transporters or endolysosomal pathways [Palm & B. Thompson, 2017]. The endolysosomal pathways eventually also rely on transmembrane transporters to release
nutrients from the membrane bound lysosomal compartments into the cytosol [Bissa et al., 2016]. Intraerythrocytic stage *Plasmodium* species can obtain nutrients via both endocytosis and transmembrane transport [Elliott et al., 2008] and both pathways are active at the same time [Divo et al., 1985; Kirk, 2001]. *T. gondii* also employs both methods of nutrient acquisition, with recent evidence suggesting that the parasite ingests and digests host cytosolic proteins [Dou et al., 2014] and that this process plays an important role in chronic infections [Di Cristina et al., 2017].

### 1.2.1 The Novel Putative Transporter Family

Due to a lack of annotated membrane transporters in *Plasmodium* species, Martin et al. [2005] carried out a bioinformatic survey of the channels and transporters encoded in the *P. falciparum* genome. One outcome of the study was the identification of five ‘orphan transporters’: transporters for which the substrate could not be predicted due to their lack of homology to known transporter families. These five proteins shared sequence and structural homology and had a common 12-transmembrane domain topology. The topology was similar to that of the Major-facilitator superfamily (MFS), and the five *Plasmodium* proteins also had a MFS-specific amino acid sequence motif in the correct structural location (between transmembrane domains two and three). Martin et al. concluded that these genes encoded a novel family of putative transporter proteins belonging to the MFS superfamily, and named them the Novel Putative Transporters (NPTs).

All *Plasmodium falciparum* NPTs have orthologues in *Plasmodium berghei* (PlasmoDB; [http://plasmodb.org/plasmo/](http://plasmodb.org/plasmo/)). Boisson et al. [2011] studied the subcellular localisation and physiological significance of one of these *P. berghei* proteins (PbNPT1). PbNPT1 localised to the parasite plasma membrane, and was important for gametocytogenesis, the first sexual stage of the parasite life cycle.

As part of a broader characterisation of 35 orphan membrane transport proteins in *P. berghei*, Kenthirapalan et al. [2016] knocked out all five PbNPTs. This study confirmed the previous results on PbNPT1, with life cycle arrest resulting from reduced development of the sexual stages of the parasite. In contrast, two other PbNPT knockouts (∆mfr2 and ∆mfr3) were able to complete the entire life cycle, but growth was severely impaired at some stages. The remaining two PbNPTs were characterised in slightly
more detail. One NPT knockout (Δmfr5) was the slowest growing in blood stages out of all their orphan transporter knockouts, growing at only one-third the rate of wild type and had defects in sexual stages. The final PbNPT (Δmfr4), had no defects in either sexual or asexual stages, but had a complete block in the life stage which colonises the salivary glands of mosquitos. These results indicate diverse rolls for NPTs in the life cycle of *P. berghei* [Kenthirapalan *et al.*, 2016].

Whilst it was known that NPT proteins have important roles, their actual function in the parasite(s) remained unknown. The first studies on the function of NPT proteins have recently been published on PbNPT1 and TgNPT1, an NPT protein in *T. gondii* with sequence homology to PbNPT1. TgNPT1 knockouts had defects in uptake of the amino acid arginine, and heterologously expressed TgNPT1 was shown to take up arginine in a Na\(^+\) independent, pH dependent manner [Rajendran *et al.*, 2017]. TgNPT1 was essential for parasite survival and virulence [Rajendran *et al.*, 2017]. Together these data indicate that TgNPT1 is critical in the uptake of arginine, an essential amino acid in these parasites. PbNPT1 was also found to function as an amino acid transporter, but had a broader substrate specificity than TgNPT1, transporting the cationic amino acids arginine, lysine, histidine and ornithine. Defects in gametocytogenesis observed in the PbNPT1 knockout confirmed the results of the earlier Boisson *et al.* study, implying that cationic amino acid uptake plays a role in gametocyte biology [Rajendran *et al.*, 2017].

These functional studies raise the possibility that the NPT proteins are a family of amino acid transporters. The substrate specificity of TgNPT1 and PbNPT1 was not entirely unexpected, as NPTs do show some, albeit low, sequence similarity to proteins from the LAT3 (or SLC43) family, a subfamily of the Major-facilitator superfamily [Parker, 2013]. The LAT3 family transports large neutral amino acids via facilitated diffusion [Babu *et al.*, 2003]. Given that NPTs are no longer putative transporters and that all the NPTs studied to date have transported amino acids, for the remainder of this thesis the family will be referred to as the Apicomplexan Amino acid Transporter family (ApiAT).

A review of what is known on the transport, synthesis and uses of amino acids in *T. gondii* and *Plasmodium* species is presented in the next section.
1.3 Acquisition and Use of Amino Acids by T. gondii and Plasmodium

Amino acids are organic molecules that are made up of an amine (-NH₂), an acidic carboxyl group (-COOH), and an organic side chain (R group) that is unique to each amino acid [Lieberman et al., 2013] (Figure 1.5A). They are vitally important compounds in all cells, where they have a plethora of uses. Amino acids are central metabolites in carbon and nitrogen metabolism, precursors for signalling molecules and neurotransmitters, constituents of phospholipids and the elementary units of proteins.
Figure 1.5: Figure legend continued over page ...
§1.3  Acquisition and Use of Amino Acids by T. gondii and Plasmodium

**Figure 1.5: General reactions in amino acid metabolism.**  
(A) General structure of an amino acid.  
(B) General transamination reaction  
(C) General oxidative deamination reaction  
(D) Reactions linking glutamine, glutamate, α-ketoglutarate, alanine and aspartate.  
Glutamine and glutamate are inter-convertible, but due to the use of glutamine in energy metabolism, flux is more likely in the glutamine to glutamate direction. Glutamate is in turn converted to α-ketoglutarate. The latter can be achieved via alanine transaminase, aspartate transaminase or glutamate dehydrogenase. These are reversible reactions and therefore can also be used to synthesise alanine, aspartate or glutamate. The changes which occur in the functional groups on each amino or keto acid are highlighted by the shaded bubbles, which are colour coded to match the enzyme which produces each change (green = glutamine synthetase, blue = alanine transaminase, orange = aspartate transaminase, yellow = glutamate dehydrogenase).  
(E) The reactions linking amino acid metabolism to the TCA cycle. Amino acids are in blue text. Pink arrows = transamination, green arrows = deamination, yellow arrows = deamination.  
**Abbreviations:** NAD, Nicotinamide adenine dinucleotide.

*T. gondii* tachyzoites divide every 6-8 hours *in vitro* [Black & Boothroyd, 2000], so in that time the parasite must double its protein content. The asexual intra-erythrocytic stage of *P. falciparum* also replicates rapidly, producing 16-22 copies of itself in approximately 48 hours [Gerald et al., 2011]. Therefore, proteinogenic amino acids, by definition the building blocks of proteins, are in high demand in these parasites. Additionally, non-proteinogenic amino acids such as γ-Aminobutyric acid (GABA) and ornithine are important metabolites in *T. gondii* and other apicomplexans [Cook et al., 2007; Teng et al., 2009; MacRae et al., 2012]. The synthesis pathways, scavenging mechanisms and uses of all proteinogenic and some non-proteinogenic amino acids in *T. gondii* are discussed in the sections below, with comparisons drawn to *Plasmodium* species.

### 1.3.1 General pathways in amino acid metabolism

Amino acid metabolism connects central carbon metabolism, nitrogen metabolism and protein synthesis. The key reactions include transamination, deamination and deamidation.

Transamination reactions are catalysed by transaminase (a.k.a aminotransferase) enzymes, and are crucial to amino acid metabolism. In these reversible reactions, the amine group is removed from one amino acid, generating its equivalent keto acid, and transferred to a different keto acid, generating a new amino acid [Lieberman *et al.*, 2007].

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3All amino acids discussed in this literature review are proteinogenic unless otherwise specified.
Acquisition and Use of Amino Acids by T. gondii and Plasmodium

All amino acids except lysine, proline and threonine can undergo transamination [Lieberman et al., 2013]. Glutamate, alanine and aspartate are the most commonly transaminated amino acids, because their keto acid equivalents are common metabolites in carbohydrate metabolism (pyruvate, $\alpha$-ketoglutarate and oxaloacetate respectively) (Figure 1.5D). The generation of $\alpha$-ketoglutarate and oxaloacetate from amino acids is an example of anaplerosis, that is, a reaction that replenishes TCA cycle intermediates [Owen et al., 2002].

Another critical reaction in amino acid metabolism is deamination, meaning the removal of an amino group. Oxidative deamination is a form of deamination that produces an ammonium ion and keto-acid (Figure 1.5C, D, E, yellow arrows). This reaction is readily reversible and classically occurs with glutamate to form $\alpha$-ketoglutarate [Lieberman et al., 2013]. The third critical reaction is deamidation, which is the removal of an amide from an R-group. Deamidation can occur on the amidic amino acids, glutamine and asparagine, to form glutamate and aspartate, respectively [Lieberman et al., 2013] (Figure 1.5D, E, green arrows).

Glutamate plays a pivotal role in amino acid metabolism, involved in both synthesis and degradation pathways. It is directly linked to central carbon metabolism via transamination or deamination, as it is the amino acid equivalent of the keto acid $\alpha$-ketoglutarate, a TCA cycle intermediate (Figure 1.5D, E). Deamidation of glutamine, present at high concentrations in human tissues and in cell culture media, generates glutamate. For these reasons, glutamate features in nearly all the metabolic pathways for amino acids discussed in this literature review [Shanmugasundram et al., 2013].

1.3.2 Overview of the amino acid auxotrophies of humans, T. gondii and Plasmodium species

Auxotrophy is the inability of an organism to synthesise a compound required for its growth [McNaught & Wilkinson, 2014]. This chapter will highlight the amino acid auxotrophies of each parasite and differences in amino acid requirements compared to the human host. Amino acids are generally classified as essential or non-essential. Essential amino acids are those for which an organism is auxotrophic, whereas non-essential amino acids are those it can synthesise [Lieberman et al., 2013]. The essential
and non-essential amino acids for human cells are listed in Table 1.1. An overview of the amino acid synthesis pathways of humans is presented in Figure 1.6A.

**Table 1.1:** The essential and non-essential amino acids in human cells. From Marks’ Basic Medical Biochemistry [2013].

<table>
<thead>
<tr>
<th>Essential</th>
<th>Non-essential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Leucine</td>
<td>Glycine</td>
</tr>
<tr>
<td>Valine</td>
<td>Serine</td>
</tr>
<tr>
<td>Methionine</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Lysine</td>
<td>Aspartate</td>
</tr>
<tr>
<td>Histidine</td>
<td>Asparagine</td>
</tr>
<tr>
<td>Threonine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Glutamate</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Proline</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
</tr>
<tr>
<td></td>
<td>Ornithine</td>
</tr>
</tbody>
</table>

1.3.2.1 *T. gondii*

It has been established experimentally that *T. gondii* is able to synthetise alanine, aspartate, glutamate [MacRae *et al.*, 2012] and ornithine [Cook *et al.*, 2007] and that these amino acids are therefore non-essential for the parasite (as in humans). Glycine, serine, asparagine, glutamine and proline are predicted to be non-essential in *T. gondii*, based on the presence of genes encoding the required synthesis enzymes in the parasite’s genome [Tymoshenko *et al.*, 2015; Chaudhary & Roos, 2005] (Figure 1.6B).

In contrast to humans, cysteine and tyrosine may be essential in *T. gondii*: the biosynthetic capability the parasite has for these amino acids remains unclear after studying genome annotations [contrast Tymoshenko *et al.*, 2015; Chaudhary & Roos, 2005]. Arginine is also non-essential in humans; and there is some experimental evidence that arginine cannot be synthesised by *T. gondii* [Fox *et al.*, 2004] (Figure 1.6B).

Of the amino acids which are essential in humans, histidine, leucine, isoleucine, valine, methionine, phenylalanine and tryptophan are also predicted to be essential in *T.*
Figure 1.6: Amino acid auxotrophies of humans, Toxoplasma gondii and Plasmodium species. Amino acids in green are ones that can be synthesised by the organisms. Those in grey are ones that cannot be synthesised by human cells or by the parasite in question. Ones that cannot be synthesised by the parasite in question, but can be synthesised by human cells are shown in red. Conversion pathways that have been experimentally demonstrated are shown with solid arrows, pathways that are only predicted to occur based on genome annotations are shown with dashed arrows. Special colours in T. gondii: Glutamine is shown in green because it can be synthesised by T. gondii, but the parasite likely requires an exogenous source for optimum growth, which has been illustrated using a red outline. The orange amino acids (cysteine and tyrosine) are ones that are non-essential in humans, for which a synthesis pathway may exist at some stage of the T. gondii lifecycle. Synthesis of cysteine and tyrosine has not been demonstrated experimentally (hence the use of broken arrows), and they require an exogenous source of cysteine and tyrosine in the tachyzoite stage. The amino acids in blue (lysine and threonine) are ones that are essential in humans but T. gondii may be able to synthesise. Synthesis of lysine and threonine has not been demonstrated experimentally (hence the use of broken arrows), and tachyzoites require an exogenous source during in vitro culture to complement any endogenous production. Abbreviations: Ala, Alanine; Arg, Arginine; Asn, Asparagine; Asp, Aspartate; Cys, Cysteine; Glu, Glutamate; Gln, Glutamine; Gly, Glycine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Phe, Phenylalanine; Pro, Proline; Ser, Serine; Thr, Threonine; Trp, Tryptophan; Tyr, Tyrosine; Val, Valine; Orn, ornithine; TCA cycle, tricarboxylic acid cycle. Figure concept from Chaudhary & Roos [2005].
T. gondii, because genes encoding the required enzymes are absent from the genome. Synthetic capability for threonine and lysine may exist in T. gondii and therefore represent synthetic capabilities not present in humans (Figure 1.6B). All but one of the genes encoding the enzymes required for threonine synthesis can be found in T. gondii’s genome [Tymoshenko et al., 2015]. However, whether these enzymes are active in threonine synthesis has not been tested. A similar situation exists for lysine, in that the genes encoding some of the enzymes for lysine synthesis are present in the T. gondii genome, but the pathway appears incomplete, and the functionality of these enzymes has not been demonstrated [Tymoshenko et al., 2015].

The terms ‘essential’ and ‘non-essential’ amino acid, as used in this literature review, refer only to whether the parasite has synthetic capability for those amino acids, and have no bearing on whether the parasites require an exogenous source of the amino acid in question. In order to test the latter, media depletion studies are often used, wherein single amino acids are removed from the growth medium and parasite growth is measured. Media depletion studies have only been performed for a few amino acids in T. gondii. These have found that the parasite requires an exogenous source of arginine [Fox et al., 2004; Rajendran et al., 2017], lysine [Rajendran et al., 2017], tyrosine [Marino & Boothroyd, 2017], phenylalanine [Parker et al., 2019] and tryptophan [Pfefferkorn et al., 1986; Sibley et al., 1994] in the growth medium. Although glutamine can be synthesised by T. gondii, it is also used by the parasite as a carbon source [MacRae et al., 2012]. Two independent studies on different strains of T. gondii both reported a 25% decrease in parasite replication when the parasites were grown in the absence of glutamine, implying that an exogenous supply of glutamine is required for optimum growth [Nitzsche et al., 2016; Lee et al., 2014].

T. gondii is routinely cultured in media with no added alanine, asparagine, asparagine, glutamate or proline, implying that an exogenous source of these amino acids is not required. The parasite’s exogenous needs for methionine, cysteine, serine, glycine, threonine, histidine, leucine, isoleucine and valine are yet to be characterised. Preliminary data from our laboratory suggests that all but serine and glycine are required in the growth medium [Rajendran, unpublished].
1.3.2.2 *Plasmodium* species

Compared to *T. gondii* and humans, *P. falciparum* has a reduced capacity to synthesise amino acids, with its genome having lost many genes for the required biochemical pathways [Chaudhary & Roos, 2005]. *De novo* synthesis of alanine [MacRae et al., 2013], glycine [Alfadhli & Rathod, 2000; Gardner et al., 2002], aspartate [Wrenger et al., 2011], asparagine [Nagaraj et al., 2015], glutamate [Gafan et al., 2001; MacRae et al., 2013], proline [Jortzik et al., 2010] and ornithine [Müller et al., 2005] has been shown experimentally in *Plasmodium* species. Finally, *Plasmodium* species are predicted, on the basis of genome analysis, to encode the enzymes for glutamine synthesis [Payne & Loomis, 2006]. Unlike *T. gondii*, which may have biosynthetic capabilities not present in humans, all of the amino acids that are known to be essential in humans are also predicted to be essential in *P. falciparum* [Payne & Loomis, 2006]. A summary is presented in Figure 1.6C.

Blood-stage malaria parasites breakdown host erythrocyte haemoglobin, a process that involves the endocytosis of red cell cytoplasm, internalising it within a specialised digestive vacuole within the parasite [Dalal & Klemba, 2015]. It has been suggested that amino acids released from haemoglobin breakdown are sufficient to supply most of the parasite’s amino acid needs [Liu et al., 2006]. In fact, *P. falciparum* effluxes large quantities of certain amino acids (alanine, leucine, valine, proline, phenylalanine, glycine, serine, threonine and histidine); these are some of the most common amino acid residues in human haemoglobin [Dalal & Klemba, 2015]. The parasite draws on the pool of amino acids released from haemoglobin breakdown [Kirk, 2001] and the fact the above amino acids are effluxed implies that the supply of these particular amino acids from haemoglobin breakdown surpasses the parasites metabolic need for them [Dalal & Klemba, 2015]. In fact, Lew et al. [2003] hypothesise that a primary purpose of haemoglobin digestion is actually to reduce osmotic contribution from haemoglobin in the host cell cytoplasm and hence protect the parasite from premature osmotic haemolysis of its host cell.

However, *P. falciparum* parasites are unable to obtain all of their amino acid needs from host protein digestion [Kirk, 2001]. Early studies found that removal of exogenous isoleucine or methionine from growth medium greatly suppressed growth of *P. falciparum* blood stages, even in the short term [Divo et al., 1985]. This may be ex-
plained by the complete absence of isoleucine, and low abundance of methionine, in adult human haemoglobin [Sherman, 1977]. In vivo these amino acids must be supplied from the plasma [Sherman, 1977]. The study found that longer term growth of *P. falciparum* also requires an extracellular source of cysteine, glutamate, glutamine, proline, and tyrosine in addition to isoleucine and methionine [Divo et al., 1985]. These amino acids are amongst the least abundant amino acids in human haemoglobin [Francis et al., 1994], with the exception of glutamate. A more recent study reported continuous blood stage growth of a strain of *P. falciparum* parasites in growth medium supplemented with isoleucine as the sole exogenously supplied amino acid [Liu et al., 2006]. The authors tested a number of isolates and found that some required both methionine and isoleucine for optimum growth, but that adding back cysteine, glutamate or glutamine did not enhance growth [Liu et al., 2006].

Despite being able to use amino acids from haemoglobin breakdown, *P. falciparum* does take up amino acids, even those for which it does not have an extracellular requirement [Divo et al., 1985; Kirk, 2001]. Metabolic labelling experiments have shown that *Plasmodium* spp. are able to take up all 20 naturally occurring α-amino acids [Sherman, 1977; Divo et al., 1985; Elford et al., 1985; Ginsburg et al., 1985; Kirk et al., 1994]. The parasite therefore likely expresses a suite of amino acid transporters on its plasma membrane [Kirk, 2001]. A number of candidate amino acid transporter proteins have been identified but not further investigated [Martin et al., 2005].

In the following sections I will summarise what is known (i.e. established experimentally or predicted from genome annotations) about the acquisition and use of each proteinogenic amino acid in *T. gondii* and *Plasmodium* species. Figure 1.6 presents a summary of amino acid auxotrophies in humans, *T. gondii* and *Plasmodium* spp. The concept for Figure 1.6 is adapted from Chaudhary & Roos [2005]. However, the content presented in the figure differs in that it represents an amalgamation of the data presented in the remainder of this chapter based, where possible, on experimental evidence (Figure 1.6, solid arrows), as well as relying on genome annotations when necessary (Figure 1.6, dashed arrows).

As can be appreciated from the colour coding in the figure, of the three species, humans have the greatest biosynthetic capability for amino acids, followed by *T. gondii* then *Plasmodium*. Of the amino acids which are normally non-essential in humans,
three of these are essential in *T. gondii* and four are essential in *Plasmodium* spp. These amino acids represent distinct auxotrophies in the parasites compared to the human host. Each of the individual amino acids is considered in detail below.

## 1.4 Alanine

Alanine is a neutral, non-polar, aliphatic amino acid which is non-essential in *T. gondii* and *Plasmodium* spp. (as in humans) [MacRae *et al.*, 2012, 2013]. This is in contrast to other apicomplexan parasites, including *Theileria* and *Cryptosporidium*, which are predicted to rely on a supply of alanine from the host [Chaudhary & Roos, 2005]. Alanine is a central player in amino acid metabolism, participating in many pathways.

### 1.4.1 Acquisition

*De novo* synthesis of alanine from carbons derived from glucose has been demonstrated in *T. gondii* [MacRae *et al.*, 2012], although the responsible enzymes have not been formally characterised. Alanine may be synthesised from pyruvate and ammonia through the action of alanine dehydrogenase (EC 1.4.1.1, TGME49_315260) or, alternatively, *T. gondii* has a gene encoding alanine transaminase (EC 2.6.1.2, TGME49_264030), an enzyme which reversibly converts pyruvate and glutamate to alanine and α-ketoglutarate. *P. falciparum* too can synthesise alanine from carbons derived from glucose [MacRae *et al.*, 2013]. Conversion of pyruvate to alanine probably contributes to the transamination reactions in *P. falciparum*’s mitochondrion necessary for converting glutamate to α-ketoglutarate [MacRae *et al.*, 2013] (c.f §1.8).

Transporters for alanine have not been identified in either *T. gondii* or in *Plasmodium*. However, it has been shown that alanine competes with both isoleucine and methionine for uptake in asexual blood-stage *P. falciparum* parasites, implying that these transporters share one or more uptake pathways [Cobbold *et al.*, 2011]. The transporter(s) making up these pathways exchange neutral amino acids across the membrane, and it has been shown that alanine can be exchanged for isoleucine or methionine [Cobbold *et al.*, 2011].
1.4.2 Uses

Alanine has been shown to be exported from \textit{T. gondii}, presumably as a waste product [MacRae \textit{et al.}, 2012]. Many organisms have a urea cycle that converts toxic ammonia formed from excess nitrogen into urea, and then excrete urea as a waste product. \textit{T. gondii} lacks a urea cycle [Chaudhary & Roos, 2005] and may use alanine as a ‘nitrogen sink’, to keep concentrations of free ammonia below toxic levels [M. McConville, personal communication]. A similar strategy is used in human muscle tissue, which exports nitrogen in the form of alanine, which travels in the bloodstream to the liver, whereupon the carbons of alanine are recycled to glucose and the amino group is disposed of in the urea cycle [reviewed in Felig, 1973].

1.5 Glycine, Serine and Cysteine

Glycine is a neutral, non-polar, aliphatic amino acid whilst cysteine and serine are neutral, polar amino acids. These amino acids share a biosynthesis pathway. All three are non-essential in humans.

1.5.1 Acquisition

\textit{Plasmodium} spp. are predicted to be auxotrophic for serine and cysteine [Chaudhary & Roos, 2005] but glycine synthesis has been shown experimentally [Alfadhli & Rathod, 2000; Gardner \textit{et al.}, 2002]. Glycine can be synthesised from serine in a single step using serine hydroxymethyltransferase (SHMT). The \textit{P. falciparum} SHMT has been expressed in \textit{E. coli} and shown to catalyze the SHMT reaction [Alfadhli & Rathod, 2000].

The \textit{T. gondii} genome contains genes encoding enzymatic pathways for glycine, serine [Chaudhary & Roos, 2005] and cysteine synthesis [Tymoshenko \textit{et al.}, 2015]. In these pathways, serine could be synthesised from 3-Phospho-D-glycerate (an intermediate of glycolysis) in three enzymatic reaction steps. Glycine could then be synthesised from serine using a homologue of PfSHMT (\textit{TgSHMT}, EC 2.1.2.1, TGME49 :234190). Serine uptake has been demonstrated experimentally in extracellular tachyzoites [Gupta \textit{et al.}, 2005], suggesting that \textit{T. gondii} has both endogenous and exogenous sources for this amino acid.
The *T. gondii* genome encodes a pathway which can react serine with homocysteine (§1.11), eventually forming cysteine (a pathway that exists in humans) although the functionality of this pathway has not been investigated. Notably, an exogenous source of cysteine appears to be essential for the *in vitro* growth of *T. gondii* (Rajendran, unpublished), so cysteine uptake may be required to complement an insufficient endogenous production.

Parasite transporters for glycine, serine or cysteine have not been identified in either *T. gondii* or *Plasmodium* spp., though it has been suggested that these three amino acids share an uptake pathway with isoleucine and leucine in *P. falciparum* [Cobbold et al., 2011].

### 1.5.2 Uses

Apart from the use of glycine, serine and cysteine in protein synthesis, serine is also used for incorporation into phospholipids in both *T. gondii* and *Plasmodium* spp. [Gupta et al., 2005; Elabbadi et al., 1997]. Cysteine is used as a sulphur donor for the biosynthesis of Fe-S clusters in *T. gondii*, a reaction catalysed by the enzyme cysteine desulphurase (EC 2.8.1.7, TGME_216170) [Feagin & Parsons, 2007]. Cysteine and glycine are also substrates for glutathione synthesis, given that glutathione is a tripeptide of glutamate, cysteine and glycine. Glutathione, an important constituent of redox metabolism, is predicted to be synthesised *de novo* in *T. gondii* using two reaction steps: gamma-Glutamylcysteine synthetase (EC 6.3.2.2, TGME49_232590) combines glutamate and cysteine into gamma-Glutamyl-cysteine which is then reacted with glycine via glutathione synthase (EC 6.3.2.3, TGME49_226800).

### 1.6 Asparagine and Aspartate

Asparagine and aspartate are two non-essential, structurally related amino acids, with asparagine having an amide group instead of a carboxyl group in its side chain. Aspartate is a negatively charged, acidic amino acid whilst asparagine is neutral and amidic.
1.6.1 Acquisition

Experimental evidence demonstrates that *T. gondii* synthesize aspartate *de novo* [MacRae *et al.*, 2012]. This is predicted to occur through the action of aspartate aminotransferase (EC 2.6.1.1, a.k.a aspartate transaminase, TGME49_248600) which reversibly reacts oxaloacetate and glutamate to form aspartate and α-ketoglutarate (Figure 1.5). It is predicted that the synthesized aspartate can produce asparagine using a (glutamine-hydrolysing) asparagine synthetase (EC 6.3.5.4, TGME49_253430).

Aspartate synthesis has been investigated in *P. falciparum*. The enzyme responsible is *P. falciparum*’s aspartate aminotransferase (*PfAspAT*) [Wrenger *et al.*, 2011]. *PfAspAT* has demonstratable *in vivo* and *in vitro* activity [Wrenger *et al.*, 2011]. *Plasmodium* spp. can then synthesize asparagine from aspartate in a single step [Nagaraj *et al.*, 2015]. The asparagine synthetase of *P. berghei* has been shown to be enzymatically active and important *in vivo* in both asexual and mosquito stages of the parasite [Nagaraj *et al.*, 2015].

The transporters for asparagine and aspartate have not been identified in either *T. gondii* or *Plasmodium* species. In *P. falciparum*, asparagine, but not aspartate, may share an uptake pathway(s) with methionine [Cobbold *et al.*, 2011].

1.6.2 Uses

Firstly, aspartate and asparagine are used in protein synthesis. Secondly, synthesis of pyrimidines, one of the essential components of DNA, requires aspartate. *T. gondii* and *Plasmodium* spp. use aspartate as substrate for the second step of their *de novo* pyrimidine biosynthesis pathways [Fox & Bzik, 2002; Gero *et al.*, 1984; Lunev *et al.*, 2016]. In this step, aspartate reacts with carbamoyl phosphate using the aspartate transcarbamoylase (ATC) enzyme (EC 2.1.3.2, TGME49_291640, PF3D7_1344800) to generate N-carbamoyl-L-aspartate. *PfATC* has been crystalised and shown to have catalytic activity [Lunev *et al.*, 2016]. *de novo* pyrimidine biosynthesis is required for *T. gondii* virulence [Fox & Bzik, 2002] and has been validated as a drug target in *P. falciparum* [Booker *et al.*, 2010; Phillips *et al.*, 2015].

A third use of aspartate is in the reaction which generates adenylosuccinate from inosine monophosphate and aspartate. Adenylosuccinate is the precursor for adenosine
monophosphate (AMP) (and therefore adenosine diphosphate (ADP) and adenosine triphosphate (ATP)).

1.7 Glutamate

Glutamate is a negatively charged, acidic amino acid, non-essential in humans, *T. gondii* and *Plasmodium* spp. Glutamate is, by some way, the most abundant amino acid in asexual blood-stage *P. falciparum* parasites, with an intracellular concentration in the millimolar range [Teng *et al.*, 2009].

1.7.1 Acquisition

In *T. gondii*, it has been demonstrated experimentally that glutamate can be synthesised *de novo* [MacRae *et al.*, 2012], however the responsible enzymes have not been characterised. Glucose-derived carbon is catabolised in the TCA cycle, generating $\alpha$-ketoglutarate [MacRae *et al.*, 2012]. To produce glutamate, it is predicted that $\alpha$-ketoglutarate combines with ammonia through the reversible action of an NAD$^+$-specific (EC 1.4.1.2, TGME49_249390) or an NADP$^+$-specific (EC 1.4.1.4, TGME49_293180) glutamate dehydrogenase (Figure 1.5D). *T. gondii* also possesses an alanine transaminase which could generate glutamate by transamination of $\alpha$-ketoglutarate and alanine (see §1.4).

There is evidence that both asexual and sexual stages of *P. falciparum* can synthesise glutamate *de novo* [MacRae *et al.*, 2013]. It is predicted that this glutamate is made from $\alpha$-ketoglutarate in a single step reaction [MacRae *et al.*, 2013]. Several enzymes catalyse this reaction. The first is aspartate aminotransferase (*Pf*AspAT) (c.f §1.6) [Wrenger *et al.*, 2011, 2012]. The genome of *P. falciparum* also includes genes coding for three glutamate dehydrogenase enzymes (EC 1.4.1.2/ EC 1.4.1.4), GDHa (PF3D7_1416500), GDHb (PF3D7_1430700) and GDHc (PF3D7_0802000) [Storm *et al.*, 2011]. GDHa has been shown to catalyse the GDH reaction [Wagner *et al.*, 1998; Werner *et al.*, 2005] and contributes most to GDH activity during intra-erythrocytic parasite development [Storm *et al.*, 2011].

The fourth enzyme that is predicted to convert $\alpha$-ketoglutarate to glutamate is an NAD(P)H-dependent glutamate synthase (GluS) (EC 1.4.1.13, PF3D7_1435300) which
§1.8 Glutamine

converts L-glutamine and α-ketoglutarate into two molecules of glutamate. Asexual blood stage \( P. \) \textit{berghei} mutants lacking GluS (PBANKA_1009500) had similar growth rates to wild type parasites [Srivastava \textit{et al.}, 2016].

Another source of glutamate for \( P. \) \textit{falciparum} is the degradation pathway of arginine, forming ornithine, then proline with glutamate as a by-product [Gafan \textit{et al.}, 2001; Jortzik \textit{et al.}, 2010]. The fact that many of the above enzymes are not essential for growth of asexual stages is possibly due to the redundancy in the many pathways of glutamate synthesis or ample supply from haemoglobin breakdown.

1.7.2 Uses

The fates of glutamate in \( T. \) \textit{gondii} and \textit{Plasmodium} species are diverse. In \( T. \) \textit{gondii}, glutamate is predicted to be used in the synthesis of proline. Glutamate is also a component of glutathione, which is predicted to be made by both parasites (§1.5). Glutamate is used by both parasites in several steps in folate biosynthesis [Kovacs \textit{et al.}, 1989].

1.8 Glutamine

Glutamine is a neutral, polar amino acid that has diverse uses in \( T. \) \textit{gondii} and \( P. \) \textit{falciparum} parasites.

1.8.1 Acquisition

In \( T. \) \textit{gondii} glutamine is predicted to be synthesised from glutamate in a reversible, single step reaction through glutamine synthetase (EC 6.3.1.2, TGME49_273490). A catalytically active glutamine synthetase from \( P. \) \textit{falciparum} (PF3D7_0922600) has been expressed in \( E. \) \textit{coli} and purified [Patel, 2015].

Scavenging of glutamine is important for the survival of \( T. \) \textit{gondii}, because apart from requiring glutamine for protein synthesis, glutamine is also used as an energy source for the parasite [MacRae \textit{et al.}, 2012]. Egressed tachyzoites utilise both glucose and glutamine under normal, nutrient-replete conditions, with glutamine uptake approximately 25% that of glucose uptake. The use of glutamine as a carbon and en-
ergy source is consistent with it being the most abundant amino acid in human blood
and human skeletal muscle [Brosnan, 2003].

The mechanism for glutamine uptake in *T. gondii* is unknown, but it has been
demonstrated that tachyzoites take up heavy isotopes of glutamine [Blume *et al.*, 2009; MacRae
*et al.*, 2012], so transporter(s) for this amino acid must be present in the parasite. The
same is true for *P. falciparum*, for which glutamine uptake has been demonstrated
[MacRae *et al.*, 2013]. The molecular identity of the transporter(s) remains elusive;
however, it has been shown that glutamine competes with methionine for uptake in *P.
falciparum*, implying that these amino acids may share an uptake pathway [Cobbbold
*et al.*, 2011].

### 1.8.2 Uses in *T. gondii*

Carbons from glutamine are catabolised in a process called glutaminolysis. In *T.
gondii*, it has been shown experimentally that glutamine feeds into the TCA cycle via
two pathways (Figure 1.7) [MacRae *et al.*, 2012]. In each pathway, the first step is
predicted to be generation of glutamate through the reversible action of the glutamine
synthetase mentioned above [Shanmugasundram *et al.*, 2013]. Glutamate can then enter
the TCA cycle in one of two ways. Firstly, it can be converted directly into a TCA cycle
intermediate, \( \alpha \)-ketoglutarate (Figure 1.7(1)). This reaction requires either glutamate
dehydrogenase, alanine transaminase or aspartate transaminase (§1.4 and §1.6) (Figure
1.5D).

The second pathway for glutamate entry into the TCA cycle of *T. gondii* is via
the ‘GABA shunt’ (Figure 1.7(2)) [MacRae *et al.*, 2012]. Gamma-aminobutyric acid
(GABA) is a non-proteinogenic amino acid which is produced from decarboxylation of
 glutamate, the first step of the GABA shunt. Extracellular tachyzoites accumulate high
levels of GABA which appears to be used as an energy reserve when carbon or amino
acid sources are limiting [MacRae *et al.*, 2012].

The enzyme which produces GABA, glutamate decarboxylase (*TgGAD*) (EC 4.1.1.15,
TGME49_280700) has been characterised in tachyzoites [MacRae *et al.*, 2012]. *TgGAD*
is the sole enzyme which can produce GABA, as *TgGAD* knockouts did not con-
tain detectable levels of GABA yet had elevated levels of the precursor glutamate
Figure 1.7: The TCA cycle and GABA shunt in *T. gondii*. Carbons derived from both glucose and glutamine are catabolised in a canonical TCA cycle. In the catabolism of glutamine, the first step is conversion to glutamate. Glutamate can then enter the TCA cycle via two pathways (numbered blue circles). (1) Directly via α-ketoglutarate, a step which involves either transamination enzymes or glutamate dehydrogenase (given in Figure 1.5). (2) via a ‘GABA shunt’. The shunt involves generation of GABA from glutamate via glutamate decarboxylase. GABA appears to function as a temporary energy store in the parasite. When parasites require energy, GABA is converted to succinate semialdehyde which can then enter the TCA cycle as succinate. Abbreviations: TCA cycle, tricarboxylic acid cycle; GABA, gamma-aminobutyric acid; NAD, Nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; GTP, guanosine-5’-triphosphate; GDP, guanosine-5’-diphosphate; Q, ubiquinone.
§1.8 Glutamine

[MacRae et al., 2012]. The enzyme was not essential for parasite survival but contributed modestly to in vivo virulence in mice [MacRae et al., 2012]. In the second step of the ‘GABA shunt’, GABA is predicted to be transaminated with α-ketoglutarate by GABA-α-ketoglutarate transaminase (EC 2.6.1.19, TGME49_269110), to form glutamate and succinate semialdehyde [MacRae et al., 2012]. This could then be converted to the TCA cycle intermediate succinate via succinic-semialdehyde dehydrogenase (SSDH, EC 1.2.1.16, TGME49_257480), thereby entering the TCA cycle. TgSSDH is known to localise to the mitochondrion [MacRae et al., 2012], but its catalytic function has not been formally investigated.

Apart from its use as (1) a proteinogenic amino acid and (2) a carbon source, glutamine is the precursor for other important biosynthesis pathways in T. gondii. Glutamine is predicted to act as the amino group donor in the first step of the hexosamine biosynthesis pathway. The product of this pathway, UDP-N-acetylglucosamine is important for N-linked glycosylation of proteins (glycoproteins) and for biosynthesis of glycolipids [Shanmugasundram et al., 2013].

Glutamine is also used in pyrimidine and purine biosynthesis. It has been shown that glutamine is involved in the first reaction in the pyrimidine biosynthesis pathway, which makes carbamoyl-phosphate via the (glutamine-hydrolysing) carbamoyl-phosphate synthase II (EC 6.3.5.5, TGME49_215260) [Fox & Bzik, 2002; Fox et al., 2004]. Glutamine is used again in the rate limiting step of the pathway by the enzyme cytidine triphosphate (CTP) synthase (CTP synthase, EC 6.3.4.2, TGGT1_299210). This enzyme converts uridine-5’-triphosphate to CTP [Narvaez-Ortiz et al., 2018]. Active recombinant TgCTPS has been purified and the enzyme appears to be essential for parasite survival [Narvaez-Ortiz et al., 2018]. Finally, glutamine is predicted to be used in purine metabolism by guanosine monophosphate synthase (EC 6.3.5.2, TGME49_230450) which synthesises guanosine monophosphate from xanthosine monophosphate [Pfefferkorn et al., 2001].

1.8.3 Uses in P. falciparum

P. falciparum, like T. gondii, may be able to use glutamine as an energy source. Glutamine is important for complete development of sexual stages and mosquito stages [Srivastava et al., 2016]. In P. falciparum, the first step of glutamine catabolism is con-
version to glutamate [Vilmont et al., 1990]. The glutamate from glutamine then enters the TCA cycle at $\alpha$-ketoglutarate [MacRae et al., 2013]. In fact, for both *P. falciparum* asexual and sexual blood stages, the majority of carbon flux through the TCA cycle is actually driven by input of carbon skeletons derived from glutamine [MacRae et al., 2013; Srivastava et al., 2016]. The *P. falciparum* TCA cycle is not essential for survival of asexual blood stages but is ultimately necessary for parasite transmission, with TCA cycle enzymes being needed in sexual stages in red blood cells and in mosquito stages [Ke et al., 2015].

Unlike *T. gondii*, *P. falciparum* does not appear to use a GABA shunt to direct glutamine-derived carbons into the TCA cycle at succinate [MacRae et al., 2013]. Instead GABA may serve a recycling function in the mitochondrion: The first step is a transamination reaction with GABA and pyruvate, forming alanine and succinate semi-aldehyde. Succinate semi-aldehyde may then be transaminated with glutamate to make $\alpha$-ketoglutarate, regenerating the GABA in the process (Figure 1.8). The net result is generation of $\alpha$-ketoglutarate and alanine from glutamate and pyruvate [MacRae et al., 2013]. However, the existence of these reactions has not been tested directly and they may not be essential in the asexual stages of the parasite [Srivastava et al., 2016].

### §1.9 Proline

Proline is a neutral, aliphatic amino acid which is non-essential in humans and *T. gondii*. It is also non-essential in *Plasmodium*, being one of the few amino acids that the species can synthesise.

#### 1.9.1 Acquisition

Whilst proline synthesis has been demonstrated experimentally in *P. falciparum*, and is predicted to occur in *T. gondii*, the mechanisms by which this is achieved differ. *T. gondii* possesses genes encoding all the enzymes required to convert glutamate to proline (4 steps using 3 enzymatic reactions). In *P. falciparum*, proline can be synthesised from ornithine (which, in turn, can be synthesised from arginine, see §1.13.2) [Jortzik et al., 2010]. It does so via two enzymes. The first is the ornithine aminotransferase, *PfOAT* (EC 2.6.1.13) which transaminates ornithine and $\alpha$-ketoglutarate ($\alpha$-KG) into glutamate-5-semialdehyde and glutamate. The former spontaneously cyclizes
Figure 1.8: The TCA cycle in *P. falciparum* and the role of GABA. Carbons derived from both glucose and glutamine are catabolised in a canonical TCA cycle. *P. falciparum* produces significant amounts of GABA, however, does not appear to possess the GABA shunt found in *T. gondii*. GABA generated by decarboxylation of glutamate may undergo transamination with pyruvate to produce alanine and succinate semi-aldehyde. *P. falciparum* does not appear to have a succinate semi-aldehyde dehydrogenase homologue and therefore cannot feed succinate semi-aldehyde into the TCA cycle as succinate [MacRae et al., 2013]. Instead, there may be a role for GABA recycling to facilitate transamination reactions in the mitochondrion via a putative glutamate/GABA transaminase (PF3D7_0608800). The net result being conversion of glutamate to α-ketoglutarate and pyruvate to alanine. Abbreviations: TCA cycle, tricarboxylic acid cycle; GABA, gamma-aminobutyric acid.
to pyrroline-5-carboxylate, then a reductase converts pyrroline-5-carboxylate into proline [Jortzik et al., 2010].

### 1.9.2 Uses

In *T. gondii*, one potential use of proline is in the synthesis of ornithine. The *T. gondii* genome harbours genes encoding the enzymes required to convert proline into ornithine, but the activity of these enzymes has not been demonstrated experimentally. One of the enzymes required is the above-mentioned ornithine aminotransferase. The *P. falciparum* homologue, PfOAT, has been expressed in *E. coli*. The structure of PfOAT has been solved and its activity investigated [Gafan et al., 2001; Jortzik et al., 2010]. Although, like most transaminases, PfOAT is a bi-directional enzyme, the study revealed that it functions mainly in the ornithine to proline direction [Gafan et al., 2001]. If TgOAT behaves in the same way, proline may not be a large source of ornithine for *T. gondii*.

### 1.10 Isoleucine, Leucine and Valine

The branched chain amino acids leucine, isoleucine and valine cannot be synthesised by either *T. gondii* or *Plasmodium* species [Chaudhary & Roos, 2005]. As essential components of proteins they are therefore essential in both parasites, just as they are in humans (Figure 1.6). Isoleucine has particular significance for intraerythrocytic *P. falciparum*, which requires isoleucine in culture medium (or plasma), unlike all other amino acids which it can source from haemoglobin breakdown (§1.3.2).

#### 1.10.1 Acquisition

Transporters for leucine, isoleucine and valine must be present in *T. gondii*, as the uptake of these amino acids has been demonstrated *in vitro*, but as yet the molecular identity of these transporters remains unknown [Limenitakis et al., 2013; Oppenheim et al., 2014].

Transport of isoleucine and leucine has been characterised extensively in *P. falciparum* [Martin & Kirk, 2007]. Isoleucine and leucine are taken up into the parasite via
the same transport system. Their uptake is saturable, and ATP-, Na\(^+\)-, and H\(^+\)-independent [Martin & Kirk, 2007]. However, the transporter(s) that comprise the system remain unidentified. The uptake system can mediate the influx of isoleucine in exchange for leucine, and likely does so \textit{in vivo}, where large amounts of leucine liberated from haemoglobin could be exchanged to speed up isoleucine uptake [Martin & Kirk, 2007].

1.10.2 Uses

The main use of leucine, isoleucine and valine in \textit{T. gondii} and \textit{Plasmodium} species appears to be in protein synthesis. The genome of \textit{T. gondii} does endode a branched-chain aminotransferase (BCAT) enzyme (EC 2.6.1.42, TGME49_297850), which has been shown to convert branched chain amino acids into their keto acids [Limenitakis et al., 2013]. \textit{T. gondii} in fact harbours an entire branched chain amino acid degradation pathway, but this pathway has been shown to be inactive in tachyzoites beyond the initial step catabolised by BCAT [Limenitakis et al., 2013]. The pathway may still be important in other stages of the life cycle.

1.11 Methionine

Methionine is a neutral, polar, sulfur-containing amino acid which is essential in humans. It is also likely to be essential in all apicomplexans [Shanmugasundram et al., 2013].

1.11.1 Acquisition

A pathway for the complete synthesis of methionine is not encoded by either the \textit{T. gondii} or \textit{P. falciparum} genome [Chaudhary & Roos, 2005]. Transport of methionine in \textit{P. falciparum} is facilitated by an equilibrative, ATP-independent transport mechanism [Cobbold et al., 2011]. The system transports a broad range of neutral amino acids and appears to be the same one that transports isoleucine and leucine, although this system has higher affinity for the isoleucine and leucine than it does for methionine [Cobbold et al., 2011]. The identity of transporter(s) underlying the system is unknown, though we do know that it functions most efficiently when exchanging one neutral amino acid
for another [Cobbold et al., 2011]. The characteristics of methionine transport in *T. gondii* have not been reported in the literature.

### 1.11.2 Uses

Apart from being a proteinogenic amino acid, methionine is a substrate for the synthesis of S-adenosyl methionine (SAM) and homocysteine. The enzymes that catalyse the synthesis of these compounds are present in both *T. gondii* and *Plasmodium* spp.

SAM is predicted to be synthesised from methionine and ATP in a single step reaction by the enzyme methionine adenosyl transferase (EC 2.5.1.6, TGME49_240690). SAM is a common co-substrate for enzymatic reactions that involve methyl group transfers, which in *T. gondii* include methylation of DNA and choline metabolism. SAM could then be converted to homocysteine, through the action of a further two enzymes. As mentioned previously, homocysteine could potentially contribute to cysteine synthesis in *T. gondii* (see §1.5). In *Plasmodium* species, methionine is used to generate SAM and homocysteine. SAM is used in the *de novo* synthesis of polyamines such as spermine and spermidine, a rare example of a biosynthetic capability that is present in *P. falciparum* yet absent in *T. gondii*. The polyamine synthesis pathway in *P. falciparum* requires a source of methionine and arginine [Müller et al., 2008].

The natural SAM analogue, sinefungin, is a potent inhibitor of *T. gondii*’s growth [Behnke et al., 2015]. Interestingly, sinefungin resistance in *T. gondii* is mediated by truncation mutations in TGME_290860 [Behnke et al., 2015], which we know to be an ApiAT protein, *TgApiAT6-2*. The proposed mechanism of sinefungin resistance is that sinefungin uptake is usually mediated by *TgApiAT6-2*, and disruption of this protein confers resistance through loss of uptake [Behnke et al., 2015]. Given that other members of the ApiAT family transport amino acids, it is possible that *TgApiAT6-2* recognises the methionine moiety present in both SAM and sinefungin, and may transport these substrates or even methionine itself.

### 1.12 Threonine

Threonine is a neutral, polar amino acid which is essential in humans. *Plasmodium* species are also auxotrophic for threonine. Unlike humans, *T. gondii* may be able to
synthesize threonine from aspartate.

### 1.12.1 Acquisition

The threonine biosynthesis pathway appears to be complete in *T. gondii* but for a single enzyme, homoserine dehydrogenase (E.C. 1.1.1.3), for which an encoding gene has not yet been identified [Tymoshenko *et al.*, 2015; Shanmugasundram *et al.*, 2013]. In their *in silico* metabolic model of *T. gondii*, Tymoshenko *et al.* suggest that, should threonine biosynthesis be active in *T. gondii*, it could represent a selective drug target, since threonine is not synthesised by the mammalian host.

### 1.12.2 Uses

*T. gondii* is rather unique in that, in addition to the normal membrane phospholipid classes, the parasite plasma membrane contains the rarely used phospholipid, phosphatidylthreonine (PtdThr) [Arroyo-Olarte *et al.*, 2015]. PtdThr is related to phosphatidylserine but uses threonine instead of serine in the head group. When the enzyme PtdThr synthase was knocked out, PtdThr was no longer present in the parasite membrane, and gliding motility, egress and host cell invasion were compromised, resulting in a defective lytic cycle [Arroyo-Olarte *et al.*, 2015]. The role of PtdThr in the parasite may therefore be related to Ca\(^{2+}\) signalling, which is upstream of the aforementioned processes [Arroyo-Olarte *et al.*, 2015].

Stable isotope labelling using \(^{13}\text{C}-\text{threonine}\) demonstrated *de novo* synthesis of PtdThr in replicating tachyzoites, consistent with exogenous threonine being imported by the parasite [Arroyo-Olarte *et al.*, 2015]. However, only about 5% of the total PtdThr content was isotope-labelled after two days of growth. The authors reported that \(^{13}\text{C}-\text{threonine}\) uptake was modest in both intracellular and extracellular tachyzoites [Arroyo-Olarte *et al.*, 2015]. The facts that threonine uptake was modest, that most threonine in PtdThr was unlabelled, and that *T. gondii* has an absolute requirement for PtdThr, provide indirect experimental evidence that *T. gondii* can synthesise threonine *de novo*. In a direct test of exogenous threonine requirements, preliminary data shows that tachyzoites do require some threonine in culture media for optimal growth [Rajendran, unpublished]. However, this does not rule out a role for threonine synthesis for parasite survival.
1.13  Arginine

Arginine is a cationic (positively charged) basic amino acid which is non-essential in humans as it is synthesised in the urea cycle. By contrast, *T. gondii* lacks the enzymes for *de novo* arginine biosynthesis [Fox *et al.*, 2004]. *T. gondii* tachyzoites are arginine auxotrophs, with arginine starvation triggering bradyzoite differentiation [Fox *et al.*, 2004]. In fact, tachyzoites require a minimum of ~50 µM arginine in culture medium for optimal growth in vitro [Rajendran *et al.*, 2017].

*P. falciparum* lacks a functional urea cycle and is therefore also an arginine auxotroph [Cobbold *et al.*, 2016; Jortzik *et al.*, 2010]. Arginine is therefore one of the few amino acids that can be synthesised by the human host but by neither of the parasites under consideration here (Figure 1.6, red boxes).

1.13.1  Acquisition

With *T. gondii* lacking the capacity to synthesise arginine, this amino acid must be scavenged from the host cell. *T. gondii*’s arginine transporter is an ApiAT protein, formally known as *Tg*NPT1 (and here renamed *Tg*ApiAT1, see §3.2.1). This selective arginine transporter is the main route for arginine uptake in vivo [Rajendran *et al.*, 2017]. *Tg*ApiAT1 is essential in vivo and under standard in vitro culture conditions, but parasite growth can be rescued if sufficient arginine is added to the culture media [Rajendran *et al.*, 2017]. This is attributed to the parasite possessing a second uptake pathway for arginine that can transport the amino acid if it is present at sufficiently high concentrations [Rajendran *et al.*, 2017]. This secondary arginine uptake route is via another ApiAT protein, *Tg*ApiAT6-1, a more general cationic amino acid transporter, which transports both arginine and lysine [Rajendran *et al.*, in preparation]. Under physiological conditions this transporter primarily takes up lysine, contributing little to arginine uptake.

Arginine uptake in *P. berghei* has been functionally characterised using reverse genetics. In parasites where *Pb*ApiAT8 (formally *Pb*NPT1) (PBANKA_020830) was knocked out, uptake of arginine was drastically decreased [Rajendran *et al.*, 2017]. Uptake of arginine via *Pb*ApiAT8 has been demonstrated in a heterologous system [Rajendran *et al.*, 2017]. *Pb*ApiAT8 has a broader range of substrates than *Tg*ApiAT1,
transporting arginine, histidine, lysine and ornithine [Rajendran et al., 2017]. \textit{P. falciparum} takes up arginine from the host erythrocyte via a membrane-potential dependent, high affinity transporter that is inhibited by other cationic amino acids [Cobbold et al., 2016]. Its molecular identity has not been definitively proven, but is likely \textit{Pf}ApiAT8, a homologue of \textit{Pb}ApiAT8 [Rajendran et al., 2017]. This is consistent with the observation that lysine, ornithine and histidine compete with arginine for uptake in \textit{P. falciparum} and also inhibit \textit{Pb}ApiAT8-mediated arginine uptake [Cobbold et al., 2016; Rajendran et al., 2017].

1.13.2 Uses

Arginine is used in protein synthesis in all organisms, and in polyamine synthesis in some organisms. Polyamines are cationic compounds with at least two primary amino groups and include putrescine, spermidine and spermine [Coppens, 2014]. The exact role of the polyamines within the two parasites is unknown, but in other eukaryotic cell types they are important for cell growth and division [Coppens, 2014; Hart et al., 2016]. It has been demonstrated experimentally that \textit{Plasmodium} species use arginine to synthesise ornithine [Müller et al., 2005], and from there polyamines, in a pathway that is indispensable to \textit{Plasmodium} spp. [Meireles et al., 2017]. Notably, \textit{P. falciparum} supplements endogenous production of polyamines by scavenging them from the host [Niemand et al., 2012]. Inhibition of polyamine biosynthesis leads to increased polyamine uptake from the extracellular medium \textit{in vitro} [Niemand et al., 2012], indicating that the parasites may use a combination of scavenge and synthesis to maintain the intracellular polyamine pool at normal levels. In liver stage \textit{Plasmodium} parasites, arginine uptake into the infected host cell is essential for parasite growth, reinforcing the idea that the parasite is auxotrophic for this amino acid [Meireles et al., 2017]. In liver stages, this arginine is primarily used to make polyamines [Meireles et al., 2017].

The biosynthesis of polyamines normally starts with converting arginine to putrescine however \textit{T. gondii} lacks the required enzymes so is incapable of doing so [Cook et al., 2007], making the parasite a polyamine auxotroph. Instead of being used in the polyamine synthesis pathway, in \textit{T. gondii}, arginine is rapidly converted into citrulline, then ornithine and carbomoyl-phosphate [Cook et al., 2007]. In contrast to \textit{Plasmodium} spp., \textit{T. gondii} lacks the enzyme arginase and therefore cannot produce ornithine directly from arginine. Whilst the activities of enzymes catalysing conversion of arginine to car-
bamoyl phosphate and ornithine have been experimentally demonstrated in *T. gondii*, the genes for those enzymes are as yet unidentified.

### 1.14 Lysine and Histidine

Lysine and histidine are cationic basic amino acids. Both humans and *P. falciparum* are unable to synthesise lysine and histidine; both amino acids are therefore essential. *T. gondii* is auxotrophic for histidine, as its genome lacks genes encoding the required enzymes [Chaudhary & Roos, 2005; Tymoshenko et al., 2015].

#### 1.14.1 Synthesis

It has been suggested that *T. gondii* is unique among the apicomplexans in that it can synthesise lysine from aspartate using the diaminopimelate pathway [Chaudhary & Roos, 2005]. The genome of *T. gondii* contains some of the genes necessary for lysine synthesis, but it remains unclear whether and, if so, when in the life cycle, lysine synthesis occurs. In tachyzoites, the last enzyme in the pathway, diaminopimelate decarboxylase (*lysA*, EC 4.1.1.20, TGME49_278740) is not essential [Birte Steinhofel, unpublished]. Tachyzoite-stage parasites require an exogenous source of lysine in growth medium *in vitro* [Rajendran et al., 2017]. These results do not demonstrate that *T. gondii* is unable to synthesise lysine, only that whatever synthesis is taking place is insufficient to meet the parasite’s demand.

#### 1.14.2 Scavenging

The uptake route for lysine in *T. gondii* has been characterised. An ApiAT family member, *TgApiAT6-1* is the main route of lysine uptake under physiological conditions [Rajendran et al., in preparation]. Access to exogenous lysine appears to be essential to *T. gondii*, as conditional knockdown of *TgApiAT6-1* is fatal [Rajendran et al., in preparation].

In *P. berghei*, lysine and histidine can be transported by the ApiAT family member, *PbNPT1* [Rajendran et al., 2017]. This transporter is the major uptake route for lysine in *P. berghei* [Rajendran et al., 2017]. However, *PbNPT1* is not essential in asexual
stages of the parasite, presumably because this life-stage can source lysine and histidine from haemoglobin breakdown.

1.15 Phenylalanine, Tryptophan and Tyrosine

Phenylalanine, tryptophan and tyrosine are all large, neutral, aromatic amino acids. In humans, phenylalanine and tryptophan are essential amino acids, but tyrosine is non-essential as it can be synthesised from phenylalanine. *Plasmodium* species are predicted to be auxotrophic for all three of these amino acids because their genomes do not encode the required enzymes [Chaudhary & Roos, 2005]. *T. gondii* is probably auxotrophic for phenylalanine and tryptophan [Marino & Boothroyd, 2017]. Asexual stages are auxotrophic for tyrosine [Wang *et al.*, 2015]. Tyrosine therefore represents a distinct auxotrophy in *T. gondii* and *Plasmodium* species compared to their human hosts (Figure 1.6).

1.15.1 Synthesis

The *T. gondii* genome encodes two aromatic amino acid hydroxylase genes (*TgAaaH1* and *TgAaaH2*, EC 1.14.16.1/EC1.14.16.2, TGME49_287510/TGME49_212740) that encode enzymes which may convert phenylalanine to tyrosine [Gaskell *et al.*, 2009], although expression levels of both enzymes are ‘negligible’ in tachyzoites [Wang *et al.*, 2015]. Both tachyzoites and bradyzoites require exogenous tyrosine for growth *in vitro* [Marino & Boothroyd, 2017]. Neither *TgAaaH1* nor *TgAaaH2* are essential in tachyzoites or bradyzoites, but both are important for the sexual reproductive cycle within the definitive feline host [Wang *et al.*, 2017]. As well as catalysing the reaction to form tyrosine, aromatic amino acid hydroxylase enzymes can use tyrosine to produce another amino acid, called L-3,4-dihydroxyphenylalanine (L-DOPA). The authors speculated that *TgAaaH1* and *TgAaaH2* are important for sexual stages because L-DOPA is a component of the oocyst cell wall [Wang *et al.*, 2017].

In prokaryotes, fungi, plants and algae, tryptophan and phenylalanine are synthesised from chorismate. Chorismate is produced by the so-called shikimate pathway. Although the *T. gondii* genome encodes a shikimate pathway, it appears to lack genes encoding the enzymes that convert chorismate into amino acids [Shanmugasundram
et al., 2013; Marino & Boothroyd, 2017]. Furthermore, experimental evidence suggests that *T. gondii* requires an exogenous source of tryptophan [Pfefferkorn et al., 1986; Sibley et al., 1994].

### 1.15.2 Scavenging

An ApiAT family member called *TgApiAT5-3* mediates uptake of tyrosine into *T. gondii* tachyzoites [Wallbank et al., 2018; Parker et al., 2019]. *TgApiAT5-3* was expressed in the *Xenopus laevis* heterologous system wherein it was shown to be an exchanger of large neutral and aromatic amino acids, transporting tyrosine, tryptophan and phenylalanine [Parker et al., 2019].

Parasites lacking *TgApiAT5-3* grow very slowly *in vitro* but their growth rate can be restored if excess tyrosine is added to growth media [Parker et al., 2019]. This result could imply the existence of an alternative, lower affinity tyrosine uptake pathway(s) in the parasites. *TgApiAT5-3* knockout parasites can also be rescued if grown in media with intermediate concentrations of phenylalanine (78 - 625 µM) and tryptophan (31 - 125 µM). This observation is consistent with the alternative tyrosine uptake pathway(s) also functioning in phenylalanine and tryptophan uptake. If *TgApiAT5-3* knockout parasites are grown at high concentrations of phenylalanine or tryptophan, uptake of tyrosine by this alternative pathway(s) is inhibited by competition with the other aromatic amino acids. Wallbank et al. [2018], who created a conditional *TgApiAT5-3* knockout using a different strategy, did not find that the growth of conditional *TgApiAT5-3* knockouts could be rescued with excess tyrosine. The reason for the discrepancy between the studies remains unclear.

*TgApiAT5-3* is important for parasite virulence in a mouse infection model [Parker et al., 2019] suggesting that the ‘high’ concentrations of phenylalanine and tryptophan are the prevailing conditions *in vivo*. The results are consistent with uptake of tyrosine into *T. gondii* parasites being mediated primarily by *TgApiAT5-3*, and the uptake of phenylalanine and tryptophan being mediated primarily by one or more alternative aromatic amino acid transporters, whose molecular identities are yet to be determined [Parker et al., 2019].
The molecular identity of the transporter(s) that allow tryptophan, phenylalanine and tyrosine to cross the plasma membrane in *Plasmodium* species are unknown.

### 1.15.3 Uses

Tryptophan is an important amino acid in the human immune response [Moffett & Namboodiri, 2003]. Interferon-gamma (IFN-γ) is a proinflammatory cytokine released by activated T-cells and other white blood cells [Moffett & Namboodiri, 2003]. In target host cells, IFN-γ is well known to induce tryptophan breakdown [Moffett & Namboodiri, 2003]. The growth of *T. gondii* in human fibroblasts is suppressed when host cells are treated with recombinant IFN-γ [Pfefferkorn *et al.*, 1986]. This parasitostatic effect of IFN-γ is likely a result of tryptophan breakdown in the host cell cytoplasm starving the parasite of this essential amino acid [Pfefferkorn *et al.*, 1986].

Rather than host-mediated tryptophan breakdown having a parasitostatic effect on *Plasmodium*, these parasites may actually induce tryptophan degradation. In a mouse model of malaria it was found that infection with *Plasmodium yoelii* resulted in systemic degradation of tryptophan via parasite-induced activation of a host enzyme that breaks down tryptophan [Tetsutani *et al.*, 2007]. If tryptophan degradation is active within immune cells themselves, T cell proliferation is suppressed [Moffett & Namboodiri, 2003]. Therefore, *Plasmodium yoelii* may induce host-mediated tryptophan breakdown to suppress the host’s immune system, thereby evading immune detection. Tetsutani *et al.* [2007] found that if host-mediated tryptophan degradation was inhibited, IFN-γ production was enhanced in malaria specific CD4+ T-cells, which also had higher rates of proliferation [Tetsutani *et al.*, 2007]. This suggests that parasite-activated tryptophan degradation normally suppresses the immune response, expediting parasite proliferation. This is supported by the observation of an increased parasite load when tryptophan-catabolism mediated immune suppression was active [Tetsutani *et al.*, 2007].

### 1.16 Summary

The human and animal diseases caused by apicomplexan parasites impose a large burden on humanity. Treatment options for many of these diseases are sub-optimal or
non-existent. Studying apicomplexan parasites is therefore, apart from being biologically interesting, medically relevant, because a better understanding of their biology can aid in drug development. Transporters can make good drug targets because they are often essential for cell function, accessible on the cell surface and druggable (able to be modulated with small-molecule drugs) [Kirk, 2001]. However, relatively little research has been done on the transporters of apicomplexan parasites [Saliba & Kirk, 2001]. Here I present a study on the Apicomplexan Amino acid transporter family (ApiAT), formerly known as the Novel Putative Transporter family. Amino acids have diverse and essential roles within cells, and the pathways for amino acid synthesis and uptake in *T. gondii* and *Plasmodium* spp. result in patterns of amino acid auxotrophy that are distinct from those of the human host.

The members of the ApiAT family studied to date are plasma membrane-localised amino acid transporters, which play essential roles in *T. gondii* and *P. berghei* development. My previous work found that ApiAT family proteins are present in other apicomplexan parasites [Parker, 2013]. However, whether the family exists in non-apicomplexan organisms remains unknown. More apicomplexan genomes remain to be searched, and the phylogenetic relationships between any new ApiAT family members are yet to be determined. Also unknown are the subcellular localisation, substrate specificity and physiological role(s) of most ApiAT-family proteins in *T. gondii*. In this PhD thesis, I set out to answer these questions.

**Specifically, I aimed to:**

1. Elucidate the phylogenetic relationships between ApiAT family proteins in apicomplexans and related organisms;
2. Determine which ApiAT proteins are expressed in *T. gondii* tachyzoites;
3. Determine the subcellular localisation of ApiAT proteins in *T. gondii* tachyzoites;
4. Create a library of functional ApiAT knockouts in *T. gondii* and determine which ApiATs are important for tachyzoite growth, including double and triple knockouts of potentially redundant genes;
5. Determine the substrate specificity of *TgApiAT2*;
6. Characterise the phenotypes of $Tg$ApiAT2 mutant parasites and determine the physiological role of $Tg$ApiAT2 within the parasites.

1.17 Chapter Overview

Chapter 2 outlines the experimental methods used in this thesis.

Chapter 3 presents a multiple sequence alignment and a phylogenetic study of ApiAT proteins. The expression and subcellular localisation of each ApiAT in $T. gondii$ is reported.

Chapter 4 presents a genetic screen where the ApiATs of $T. gondii$ were individually disrupted genetically and the growth of each the resulting mutants was characterised. Parasites with double and triple disrupted ApiAT genes were generated to assess redundancy between phylogenetically related $T. gondii$ ApiAT proteins.

Chapter 5 presents further characterisation on $Tg$ApiAT2 truncation mutants. The defect in the lytic cycle was identified. Amino acid levels in mutant and wild type parasites were measured to determine whether $Tg$ApiAT2 has a role in amino acid homeostasis and to identify candidate amino acids that may be substrates of $Tg$ApiAT2.

Chapter 6 covers $Tg$ApiAT2 expressed in a heterologous system, *Xenopus laevis* oocytes. $Tg$ApiAT2-mediated uptake of the candidate substrates identified in the previous chapter was directly tested and further candidate substrates were identified. The kinetic properties of $Tg$ApiAT2 were characterised.

Chapter 7 reports uptake experiments of $^{14}$C-labelled amino acids into wild type and $Tg$ApiAT2 mutant parasites. Deficiencies in transport of the amino acid glutamine were observed in parasites lacking $Tg$ApiAT2. This prompted a metabolomic analysis of the central carbon metabolism of $Tg$ApiAT2 truncation mutants.

Chapter 8 is an overall Discussion bringing together the results chapters, suggesting future directions for the work and summarising its contribution to the field of $T. gondii$ transporter biology.
Methods

2.1 Bioinformatic Methods

2.1.1 Genome searching

I first sought to identify orthologues of the five previously identified *Plasmodium falciparum* NPT proteins [Martin et al., 2005] in the proteomes of the apicomplexans *Plasmodium berghei*, *Toxoplasma gondii*, *Cryptosporidium parvum*, *Eimeria tenella*, *Neospora caninum*, *Babesia bovis*, and *Theileria annulata*, and the chromerids *Chromera velia* and *Vitrella brassicaformis*. Collectively I call these the ‘target species’. I performed protein Basic Local Alignment Search Tool (BLAST) searches using each *P. falciparum* NPT protein as a query sequence against each target species (using www.eupathdb.org [Aurrecoechea et al., 2017]). I examined all ‘hits’ with an E value of <10. I excluded candidates that had <8 or >15 transmembrane domains from further analysis, as this would be inconsistent with them being MFS transporters, which typically have 12 transmembrane domains [Reddy et al., 2012]. I also excluded any candidates annotated as proteins that were not transporters, or proteins annotated as members of other transporter families.

2.1.2 Reciprocal BLAST Searches

I used each remaining candidate as the query sequence for BLAST searches against the *P. falciparum* proteome, and I only accepted candidates for which the top match in the reciprocal BLAST search was one of the five *P. falciparum* NPTs. Next, I undertook BLAST searches against all organisms using the remaining candidate ApiATs as query sequences (NCBI BLAST search tool [Agarwala et al., 2017]), excluding proteins in its own genus. I only retained candidates on the potential ApiAT list if that candidate’s top hit in the all-organism BLAST searches was to proteins from apicomplexans or
My last elimination step was to retain candidates on the final ApiAT list only if their top hit amongst members of the target species was to proteins that had met all of the previous criteria. This would theoretically exclude proteins that were not descended from the common ancestor of ApiATs.

I identified sixty-six proteins that met all of the above criteria. The gene identification numbers and nomenclature of these (based on the phylogenetic groupings) are listed in Table 2.1. None of the candidate chromerid proteins met all the criteria (because they all had top hits in the NCBI BLAST search to proteins outside apicomplexans), and I therefore excluded them from the subsequent phylogenetic analyses (§2.1.4).

Table 2.1: Gene identification numbers of ApiAT proteins identified in this study

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2.1 Bioinformatic Methods

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2.1.3 Multiple sequence alignment and similarity shading

In order to generate a multiple sequence alignment of the ApiAT family, I aligned the proteins listed in Table 2.1 using Clustal Omega Version 1.2.2 [Sievers et al., 2011; Sievers & Higgins, 2018]. To create a visual representation of the alignment, I used the TeXshade package for LaTex [Beitz, 2000] (https://ctan.org/pkg/texshade), using similarity mode to shade the alignment for sequence identity. All identical residues at a position are shaded blue if 50-70% of sequences have a matching residue in that position, and purple if >70% of sequences have a matching residue at that position. A residue is shaded pink if it is a similar amino to a consensus amino acid. The groups of residues that the TeXshade package considers similar are listed in Table 2.2.

I then used the TeXshade package to present the alignment as a ‘sequence fingerprint’ [after Fröhlich, 1994], using with the ‘fingerprint’ command, also in similarity mode. In order to deal with such a large alignment, I used a texshade.sty file from the TeXshade package creator Professor Eric Beitz, which he generously provided.
Table 2.2: The groups of residues that the TeXshade program considers similar when shading in similarity mode [Beitz, 2000].

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2.1.4 Phylogenetic tree algorithm

In order to generate a maximum likelihood phylogenetic tree of the ApiAT family, I first edited the multiple sequence alignment of the 66 ApiAT protein sequences in Jalview (www.jalview.org) and I removed all poorly aligned blocks. The poorly aligned blocks that I removed included the variable sequence in the large intracellular loop between transmembrane domains 6 and 7, and the N and C terminal extensions of the proteins. After sequence editing, 464 residues were left for subsequent phylogenetic analysis.

I generated the phylogenetic tree using PHYLIP v3.69 (http://evolution.genetics.washington.edu/phylip.html) as described previously [Foth, 2007]. Firstly, I used the edited alignment file as input for the ‘seqboot’ program, which generated 300 pseudosamples of the alignment. I then used the ‘protml’ tree algorithm with a randomised order of entry and three jumbles to generate multiple phylogenetic trees from the pseudosamples. Finally, I used the program ‘consense’, which compares the groupings in the multiple phylogenetic trees and determines the most
common grouping pattern. This generated a consensus maximum likelihood tree and bootstrap values. I viewed the consensus tree using the program FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and annotated the tree using the program Inkscape (https://inkscape.org/en).

### 2.2 Culture methods

#### 2.2.1 Culturing of mammalian host cells

*T. gondii* is an obligate intracellular parasite and so, by definition, requires a host cell for replication. I obtained Human Foreskin Fibroblast (HFF) cells, a monolayer-forming adherent cell line, for use as host cells for the parasite (a gift from Holger Schülter, Peter MacCallum Cancer Centre). I cultured the HFF cells *in vitro* at 37°C in a humidified 5% CO$_2$ incubator using Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies) supplemented with 10% (v/v) bovine calf serum (Bovogen), 200 µM glutamine, antibiotics (penicillin, streptomycin and gentamicin - Sigma) and fungicide (amphotericin b). I refer to this as ‘D10 medium’. I grew HFF cells to 100% confluency before use as host cells for the parasite. I maintained the HFF cells by sub-culturing them into new flasks after trypsinising the monolayer (Typsin-EDTA, Sigma) for 1 minute at 37°C [Sigma-Aldrich, 2010].

#### 2.2.2 Culturing of *Toxoplasma gondii*

To maintain *T. gondii* tachyzoites *in vitro*, I passaged them through HFF cells, as described previously by Striepen & Soldati [2007]. I grew *T. gondii* tachyzoite cultures at 37°C in a humidified 5% CO$_2$ incubator, in a medium that will be referred to here as ‘Ed1 medium’. Ed1 medium is DMEM (Life Technologies) supplemented with 1% (v/v) fetal bovine serum, 200 µM L-glutamine, antibiotics (penicillin, streptomycin and gentamicin) and fungicide (amphotericin b).

### 2.3 Genetic Modification of Parasites

The aim of reverse genetics is to modify target genes, or their expression, and study the phenotypic consequences of this modification [Hardy et al., 2010]. Reverse genetics is a powerful tool for assessing the function of the protein that the modified gene
encodes. I used reverse genetics to determine the importance of ApiAT genes for the growth of *T. gondii* tachyzoites and also to study the function of *Tg*ApiAT2 in closer detail. A second approach which relies on genetic modification is epitope tagging. Here, a known epitope is fused to a protein of interest by modifying its encoding gene to include the epitope sequence in the open reading frame [Brizzard, 2008]. The advantage of this technique is that it makes it possible to detect proteins for which no antibody is available, because commercially available antibodies exist for the epitopes. Potential shortcomings of the approach are that the insertion of the epitope has, at least in principle, the potential to perturb the targeting of the protein to the correct location within the cell, or directly inhibit the protein’s function.

### 2.3.1 Selection of Parental Strains

I used two methods of genetic modification in this project. The first was the recently developed CRISPR/Cas9 genome editing system (Figure 1.4) [Shen *et al.*, 2014a; Sidik *et al.*, 2014]. I used this to either disrupt genes or to introduce epitope tags. The second method of genetic modification I used in this project was ‘traditional’ double crossover recombination (§2.3.4). I used this second method either to replace the promoters of genes or to introduce entire genes into the genome. I used different parental parasite strains, depending on the genetic modification system used and the desired results. To determine which parental strain to use, I considered whether the parental strain should be able to perform NHEJ (Introduction §1.1.2.2), because this would influence how the transfected parasites integrate the introduced DNA.

Like many eukaryotes, *T. gondii* preferentially uses an NHEJ pathway for the repair of double-stranded DNA breaks [Fox *et al.*, 2009]. When using the CRISPR/Cas9 system to introduce random mutations for the purposes of disrupting genes, nonhomologous end-joining is a desirable repair process (Figure 1.4). NHEJ is error prone, often causing insertions and deletions at the repair site which lead to frameshift mutations. Therefore, I used NHEJ-competent parasites when using the CRISPR/Cas9 system to create gene disruptions.

Ku80 is one of the ubiquitous eukaryotic proteins involved in NHEJ. *T. gondii* strains engineered to lack the *T. gondii* orthologue, *Tg*Ku80, are deficient in nonhomologous end-joining [Huynh & Carruthers, 2009; Fox *et al.*, 2009]. This strain, known as ∆*ku80*,
is reliant on homology-directed repair of double stranded DNA breaks, with homology-directed recombination occurring at a 300–400-fold increased efficiency compared to parasites expressing TgKu80 [Fox et al., 2009]. When using the CRISPR/Cas9 system to introduce a haemagglutinin (HA) epitope tag into a gene, it is preferable that Δku80 parasites (deficient in NHEJ) are used so that a donor DNA with homologous sequence, supplied during the transfection and also encoding a HA epitope tag, will be used to direct repair (Figure 2.1). Therefore, I used Δku80 parasites to create 3’ replacements using the CRISPR/Cas9 system.

When using the traditional homologous recombination strategy for genetic modification, I also chose to use a Δku80 strain, which increased the likelihood that the exogenous DNA integrated into the target site of the genome through homologous recombination.

### 2.3.2 Transfections into parasites

I introduced exogenous DNA into parasites by electroporation, following a standard Toxoplasma transfection protocol, as described by Striepen & Soldati [2007]. I performed transfections on parasites that had recently egressed from host cells, or on parasites that had been mechanically egressed from host cells by passage through a 26 gauge needle.
2.3.3 Cloning of CRISPR/Cas9 vector DNA and selection of genetically modified parasites

The two genetic modification strategies (CRISPR or conventional crossover recombination) required different types of vectors. The strategies used to clone each type of vector will be outlined in this section.

2.3.3.1 General cloning strategy for CRISPR/Cas9 vectors

I created the majority of the cell lines in this project using the CRISPR/Cas9 gene editing system (Introduction §1.1.2.2) [Shen et al., 2014a; Sidik et al., 2014]. To generate the CRISPR/Cas9 vectors used for 3’ replacement (§2.3.3.2) and gene disruption (§2.3.3.3), I designed forward primers which would introduce the desired single guide RNA (sgRNA) into pSAG1::Cas9-U6::sgUPRT (Addgene plasmid # 54467; [Shen et al., 2014a]), a plasmid vector already expressing Cas9-GFP. These forward primers amplify the plasmid but also contain sequence encoding the sgRNA specific to each ApiAT (§2.3.3.2, §2.3.3.3).

To introduce the sgRNA as a mutagenic insertion into the CRISPR/Cas9 plasmid, I used a Q5 Site-Directed Mutagenesis Kit (New England Biolabs #E0554S). I performed a PCR using each of the mutagenic forward primers, with a reverse primer common to each PCR reaction, called ‘Generic CRISPR Reverse’ (Table 2.3) and the circular plasmid pSAG1::Cas9-U6::sgUPRT as template DNA. This PCR process generated linear copies of the plasmid with a new insertion derived from the forward primer.

I then subjected the PCR reaction mix to a 5 minute kinase-ligase-DpnI (KLD) reaction. During this reaction, the 5’ ends of the linear PCR product are phosphorylated by the kinase and the ends are ligated together by the ligase to generate a circular DNA product. The DpnI enzyme mediates the degradation of the template DNA. I used heat shock to transform the KLD treated plasmid into chemically-competent Escherichia coli cells. I confirmed that the sgRNA had been properly incorporated into the plasmid by sequencing the plasmid DNA that I recovered from the E. coli.
Table 2.3: Forward primers used in the Q5 Site-Directed Mutagenesis reaction to generate the CRISPR 3’ replacement vectors. The sgRNA sequence is coloured blue.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’—3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApiAT3-1 3’ rep CRISPR</td>
<td>ACAGCTGAGAGATTTTTTGTTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-1 3’ rep CRISPR</td>
<td>ACCTTTTTCTCAGACGGAATTGGTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-2 3’ rep CRISPR</td>
<td>GATTTCCTACAGACGGAAGGTTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-4 3’ rep CRISPR</td>
<td>CTTCATCACTCCCACGCTCCCCGTTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-5 3’ rep CRISPR</td>
<td>AAAACAAAGCATTCAGCGGAGTGTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-6 3’ rep CRISPR</td>
<td>ATTCAAGTCATCCCTCAGCTCAAGTTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT6-3 3’ rep CRISPR</td>
<td>CACCAGACGGCTGAGAGGCTGGTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT7-2 3’ rep CRISPR</td>
<td>GAGTGAGACGCCAGAAAGGTGTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>Generic CRISPR reverse</td>
<td>AACTTGACATCCCATTTAC</td>
</tr>
</tbody>
</table>

2.3.3.2 Generating epitope tagged parasites with CRISPR/Cas9-mediated 3’ replacement

DNA encoding epitope tags can be introduced into genes to ascertain protein expression and localisation [Woodcroft et al., 2012]. The 3×HA epitope tag I used throughout this project is 27 amino acids in length [Brizzard, 2008] and this small size theoretically reduces the chance of the protein being aberrantly targeted [Woodcroft et al., 2012]. At the beginning of this project, the localisations of eight ApiAT proteins (TgApiAT3-1, TgApiAT5-1, TgApiAT5-2, TgApiAT5-4, TgApiAT5-5, TgApiAT5-6, TgApiAT6-3 and TgApiAT7-2) had not yet been determined. I used a CRISPR/Cas9 based 3’ replacement technique to introduce epitope tags into the genes encoding these eight proteins (Figure 2.1).

I designed CRISPR/Cas9 vectors with sgRNAs which targeted ApiAT proteins near their stop codon. I used the ‘ApiAT 3’ rep CRISPR’ primers (Table 2.3) as the forward primers in the Q5 Site-Directed Mutagenesis reaction (§2.3.3.1). I also designed primers with 50 base pairs of homologous sequence from either side of the stop codon of each ApiAT which I used to generate the 3×HA-containing donor DNA, the ‘repair template’ that is introduced into the genome at the target site through homologous recombination. Where possible, I mutated the protospacer adjacent motif (PAM) site in these primers to minimise further cutting of the locus after a successful 3’ replacement. I used the ‘ApiAT 3’repCRISPR’ forward and reverse primers to PCR amplify a generic 3×HA gene block (Table 2.4). This step resulted in a specific 3×HA donor DNA for each gene.
Table 2.4: Primers used to generate 3×HA encoding donor DNA. The donor DNA was designed to contain 50 bp of homologous sequence flanking either side of the stop codon in the target gene, and encode a 3×HA between the flanking sequences (see Figure 2.1). Each primer therefore contains 50 bp of homologous sequence to the target gene (parts in black). The parts in blue show where each primer binds to the generic 3×HA gene block. The sequence of the gBlock oligonucleotide encoding the 3×HA tag is also listed.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’—3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApiAT3-1 3’repCRISPR forward</td>
<td>GTGGCGGGGCTCTCGCAGCGAACCAGCGCCGACCACTGCGGTGACACTAGACGTAGCCGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT3-1 3’repCRISPR reverse</td>
<td>ACCCGAGATCGAAGCCGACGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-1 3’repCRISPR forward</td>
<td>AGCCGATACGACGACTGCGGACGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-1 3’repCRISPR reverse</td>
<td>TCCTCACCATTTTTCGTCCTGTCGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-2 3’repCRISPR forward</td>
<td>TGAAAAGAGGAGGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-2 3’repCRISPR reverse</td>
<td>CCGGCGTGTTTGGTGCTGCGGACGATGTGCTCTCAAGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-3 3’repCRISPR forward</td>
<td>CACAAAAGACCATCGCGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-4 3’repCRISPR forward</td>
<td>AAGTTGTAGCTTCATCACTCCACGTCCTGCTGCGGACGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-5 3’repCRISPR forward</td>
<td>AACTCCTTTCGCAAGCCGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-5 3’repCRISPR reverse</td>
<td>CGCGCCAGCCGAGCGGACGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-6 3’repCRISPR forward</td>
<td>GACGAACTCCTCAGCAGCTGTTACGTGGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT5-6 3’repCRISPR reverse</td>
<td>GCAGATTGTTATATCCACGAGCTTCATCTTAACATCAAAAAATCCAGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT6-3 3’repCRISPR forward</td>
<td>GAGAAGGAGGAGGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT6-3 3’repCRISPR reverse</td>
<td>CAAAGGTGGCAACTTGTTGGATGCGCTCTCCGTGTAGAGCGGACGAGCTGTTACGACGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT7-2 3’repCRISPR forward</td>
<td>TACAGCCGCGGCGCAAGAAAGAGGAGCAGGCGAAGAGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>ApiAT7-2 3’repCRISPR reverse</td>
<td>AAGGACCCGAGTTCTGCGGTGATGTGCTTGGGACGAGCTGTTACGACGAGGTAGCGGTGGTGGAAG</td>
</tr>
<tr>
<td>Generic 3×HA gBlock</td>
<td>ggtgaggtagcgcgggtggaattaccaaggtaagctcgccgagctcgtggatctctcctagtatgtggtgagtccagattagtgcggtagtcgataaaccgccccacagaagc</td>
</tr>
</tbody>
</table>
Using electroporation, I co-transfected \(\Delta ku80\) parasites with 20-50 \(\mu\)g of CRISPR 3’ replacement vector and 1-6 \(\mu\)g of 3×HA donor DNA. Two or three days later, I used fluorescence-activated cell sorting (FACS) to select GFP positive parasites, which express Cas9-GFP, and therefore have taken up the sgRNA-expressing vector. I prepared the parasites for FACS by harvesting them and filtering them through a 3 \(\mu\)m filter. I then centrifuged the parasite suspension for 10 minutes at 4°C at 1500\(\times\)g in a swing-bucket centrifuge. I resuspended the parasite pellet in 500 \(\mu\)L to 1 mL of sterile Dulbecco’s phosphate-buffered saline (PBS; Sigma). I sorted 3 or 6 GFP positive parasites directly into the wells of a pre-prepared 96 well plate with host cells using a FACS Aria I or FACS Aria II cell sorter (BD Biosciences). During sorting, some parasite cells lose viability, and I found that adding 3 or 6 parasites per well maximised the number of wells to which a single viable parasite had been added (which could be determined by examining wells for those containing single plaques 7-9 days post-sorting). After I had determined which wells contained a single parasite clone by light microscopy, I sub-cultured these samples from the 96 well plates. I screened each clone for expression of a HA tagged protein using a western blot (§2.4.1.1). The names of the cell lines I generated are listed in Table 2.9.

If I was not able to detect HA-tag expression by western blot, I extracted genomic DNA from the clone. To extract DNA, I pelleted the parasites by centrifugation at 12,000\(\times\)g for 1 minute then resuspended them in a buffer containing 0.5 mg/mL proteinase K in [5×] HF Phusion PCR buffer (NEB). I incubated the samples in a thermocycler at 60°C for 1 hour then at 95°C for a further 10 minutes. I amplified the 3’ end of the ApiAT gene of interest from the extracted genomic DNA using the ‘ApiAT 3’rep seq’ forward and reverse primers (Table 2.5) and separated DNA using agarose gel electrophoresis. I expected that the PCR product in clones that had integrated the 3×HA tag would be approximately 130 base pairs larger than the PCR product from the parental line. I sequenced the DNA from the clones with these insertions to confirm correct integration of the 3×HA tag. I used the ‘ApiAT 3’rep seq forward’ primers for the sequencing reaction (Table 2.5).
2.3.3.3 Generating parasites with CRISPR/Cas9-mediated disruption of ApiAT-encoding genes

A major aim of this project was to generate individual gene disruptions of each ApiAT protein in *T. gondii*, to provide knowledge on which ApiATs are important for growth, and to generate parasite strains for characterising the function of each ApiAT. We chose the CRISPR/Cas9 gene editing system due to the short time required to disrupt genes, making it more suitable for a semi-high-throughput knockout screen of the 16 ApiATs. I designed CRISPR/Cas9 vectors with sgRNAs that targeted the *apiAT3-1*, *apiAT3-2* and *apiAT3-3* genes in the open reading frame. My colleague designed CRISPR/Cas9 vectors to target the *apiAT1* and *apiAT2* genes, as well as the ApiAT5 subfamily, the ApiAT6 subfamily and the ApiAT7 subfamily genes. We used the ‘Generic CRISPR reverse’ primer and the ‘ApiAT CRISPR’ forward primers (Table 2.6) in the site-directed mutagenesis reaction to incorporate the sgRNA sequence (see §2.3.3.1).

To disrupt *TgApiAT2*, *TgApiAT3-1*, *TgApiAT3-2*, *TgApiAT3-3*, *TgApiAT5-1*, *TgApiAT5-2*, *TgApiAT5-3*, *TgApiAT5-4*, *TgApiAT5-5*, *TgApiAT6-2*, *TgApiAT6-3*, *TgApiAT7-1* and *TgApiAT7-2*, I used the standard electroporation protocol to introduce 20-100 µg of each CRISPR/Cas9 vector into TATi or TATi/tdTomato parasites [Striepen & Soldati, 2007]. Two to three days later, I sorted GFP positive parasites by FACS directly into a 96 well plate, then isolated clones and extracted their genomic DNA (see §2.3.3.2). I PCR amplified the ApiAT locus of interest from the extracted genomic DNA using the ‘ApiAT seq’ forward and reverse primers (Table 2.7). I sequenced the PCR products to screen for frameshift mutations at the CRISPR sgRNA target site. The names of the cell lines I generated are listed in Table 2.9.

For *TgApiAT5-1* and *TgApiAT5-3*, I did not obtain any parasites with frameshift mutations after three independent transfections, so my supervisor designed new primers (Table 2.6) and cloned CRISPR/Cas9 vectors that targeted a second site within *apiAT5-1* and *apiAT5-3*, then repeated the transfection and sequencing process.

The transfections to disrupt *TgApiAT1* and *TgApiAT6-1* and subsequent sequencing were performed by my colleague. However, I used the resultant *TgApiAT1* truncation mutant in the growth experiments reported in Chapter 4 (§4.2.2), so the primers
Table 2.5: Forward and reverse primers used to amplify the 3' end of ApiAT genes to screen for successful 3' replacement with the 3×HA tag.

<table>
<thead>
<tr>
<th>Sequence clones from . . .</th>
<th>Primer name</th>
<th>Primer sequence 5'—3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgApiAT5-1 3' CRISPR</td>
<td>ApiAT5-1 3'rep seq forward</td>
<td>CCAGGGTGACACGCGAGAGAGA</td>
</tr>
<tr>
<td>replacement transfection</td>
<td>ApiAT5-1 3'rep seq reverse</td>
<td>TCCGGTTTTTTTTCGTCCT</td>
</tr>
<tr>
<td>TgApiAT5-2 3' CRISPR</td>
<td>ApiAT5-2 3'rep seq forward</td>
<td>GCCGTCTCTGTGCTTTCTTT</td>
</tr>
<tr>
<td>replacement transfection</td>
<td>ApiAT5-2 3'rep seq reverse</td>
<td>GTGTGCTAGAAGAAGAAGACCCG</td>
</tr>
<tr>
<td>TgApiAT5-4 3' CRISPR</td>
<td>ApiAT5-4 3'rep seq forward</td>
<td>GCCTTCTCTTGTCAGCCGCT</td>
</tr>
<tr>
<td>replacement transfection</td>
<td>ApiAT5-4 3'rep seq reverse</td>
<td>ACCCGGGTTTTGATTTTGG</td>
</tr>
<tr>
<td>TgApiAT5-5 3' CRISPR</td>
<td>ApiAT5-5 3'rep seq forward</td>
<td>TGCTTCTACTGCTGTCAGGG</td>
</tr>
<tr>
<td>replacement transfection</td>
<td>ApiAT5-5 3'rep seq reverse</td>
<td>CCGCAGACGTAACCTTT</td>
</tr>
<tr>
<td>TgApiAT5-6 3' CRISPR</td>
<td>ApiAT5-6 3'rep seq forward</td>
<td>GATGGTGAGGCTGAGAAGGG</td>
</tr>
<tr>
<td>replacement transfection</td>
<td>ApiAT5-6 3'rep seq reverse</td>
<td>TACAACTACGTCGGGATGG</td>
</tr>
<tr>
<td>TgApiAT7-2 3' CRISPR</td>
<td>ApiAT7-2 3'rep seq forward</td>
<td>GACAGGGACACGAGAAGACCG</td>
</tr>
<tr>
<td>replacement transfection</td>
<td>ApiAT7-2 3'rep seq reverse</td>
<td>ACTGCCTACGTTCTGTTCA</td>
</tr>
</tbody>
</table>

Table 2.6: Forward primers used in the Q5 Site-Directed Mutagenesis reaction to generate CRISPR/Cas9 vectors targeting TgApiAT genes for gene disruption. The sgRNA sequence is coloured blue.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’—3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApiAT1 CRISPR</td>
<td>CAGTCTCTGGTTATTTTGTTAGAGACTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT2 CRISPR</td>
<td>AGTGACCCGGACTGCCAGAGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT3-1 CRISPR</td>
<td>ACCGCCACGCTGACATTTTGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT3-2 CRISPR</td>
<td>GGGGGTGGTGCTGGAGGGGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT3-3 CRISPR</td>
<td>AGGAAAGCAGTTGACAGAGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT3-1 CRISPR1</td>
<td>CCGTCTCTACAAGCCTCGGGGTTTTAGAGCTAGAAATAGCAAG (1st attempt)</td>
</tr>
<tr>
<td>ApiAT5-2 CRISPR</td>
<td>CCGTCTCTACAAGCCTCGGGGTTTTAGAGCTAGAAATAGCAAG (2nd attempt)</td>
</tr>
<tr>
<td>ApiAT3-3 CRISPR1</td>
<td>CGAACCCCAAGTGGCCAGGGTTTTAGAGCTAGAAATAGCAAG (1st attempt)</td>
</tr>
<tr>
<td>ApiAT3-3 CRISPR2</td>
<td>GGGTTCTCTCGGAGAAGCTGTTTAAGCGTAAATAGCAAG (2nd attempt)</td>
</tr>
<tr>
<td>ApiAT3-3 CRISPR2 (1st attempt)</td>
<td>CCGTCTCTACAAGCCTCGGGGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-2 CRISPR</td>
<td>AATGGGCACGCTGGGACGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT5-2 CRISPR</td>
<td>AAAGGCTGTCAGCTGCTTGGTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT6-1 CRISPR</td>
<td>GCCCTCGAGACGAGAGCCCTTTTATAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT6-2 CRISPR</td>
<td>GCCTCGGCGAAGAGACGTTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT6-3 CRISPR</td>
<td>AGCCACCATGTCGGCATTGCGTTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
<tr>
<td>ApiAT7-1 CRISPR</td>
<td>CAAATCTGGGCGTCTCGCTTTTAGAGCTAGAAATAGCAAG</td>
</tr>
</tbody>
</table>
associated with generating these cell lines are included here for completeness (Table 2.6).

2.3.4 Traditional double crossover recombination for conditional knockdown and complementation

2.3.4.1 Rationale for using a conditional knockdown system

The CRISPR-based gene disruption approach described in the previous section led to frameshift mutations in the target ApiAT genes. Although these are likely to result in functional ablation of the target protein, it is conceivable that the parasite can ‘skip’ over these frameshift mutations during protein translation. Furthermore, the resultant truncated protein could cause unexpected effects in parasites, complicating the subsequent analyses of protein function. I therefore sought an independent approach for generating mutant parasites in TgApiAT2, the protein that I characterised in-depth in Chapters 5, 6 and 7 of this thesis.

One option would be to generate a ‘knockout’ of TgApiAT2, a parasite line in which the entire apiAT2 gene is removed through homologous recombination and replaced with a selectable marker. However, a disadvantage of this approach is knockouts can, over time, adapt to loss of the target gene (e.g. by upregulating the expression of other genes). This is especially true for fitness conferring genes, whose loss places significant selection pressure on parasites to develop compensatory mutations.

Furthermore, I predicted that generating a knockout of TgApiAT2 might not be successful in the first instance, due to the large growth defect that I observed in the CRISPR-generated apiAT2 truncation mutant. It is often difficult to knockout genes such as apiAT2, the loss of which leads to large defects in parasite growth, because the selection process that is performed on transfectants requires several days or weeks of drug selection. Recovering knockout parasites is rare, as their growth is out-competed by transfectants that have integrated the drug selection marker elsewhere in the T. gondii genome, conferring drug resistance without knocking out the target gene.

This problem can be circumvented in T. gondii through the use of ‘regulatable’ expression systems which allow the conditional knockdown of targeted genes [Meissner
Table 2.7: Forward and reverse primers used to amplify sites in ApiAT genes targeted by CRISPR sgRNA to screening for CRISPR induced frameshift mutations by sequencing. If a sequencing primer is not specified, the forward primer that was used to amplify the DNA was also used to sequence it.

<table>
<thead>
<tr>
<th>ApiAT gene</th>
<th>Primer name</th>
<th>Primer sequence 5’—3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgApiAT1</td>
<td>ApiAT1 screen forward</td>
<td>GACTTTAGCCTTTTCCTCGGTCCG</td>
</tr>
<tr>
<td></td>
<td>ApiAT1 screen reverse</td>
<td>CTTTGAGTTCCAGCACTCGACTG</td>
</tr>
<tr>
<td></td>
<td>ApiAT1 3’ flank forward</td>
<td>GATCCCCGGGATGCCGGGTCCTCTAGCTCGT</td>
</tr>
<tr>
<td>TgApiAT2</td>
<td>ApiAT2 loxP forward</td>
<td>GATCGGATCCAAAATGCCGGCCTGCTCAG</td>
</tr>
<tr>
<td></td>
<td>ApiAT2 3’ flank reverse</td>
<td>GACTGCCGGCGGCCTCGACGAGACCATGAT</td>
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<tr>
<td>TgApiAT3-1</td>
<td>ApiAT3-1 Screen forward (B)</td>
<td>ATTTGTGGTTGTCGAATCG</td>
</tr>
<tr>
<td></td>
<td>ApiAT3-1 Screen reverse (B)</td>
<td>AGACAATGTGTAAGATAGTC</td>
</tr>
<tr>
<td></td>
<td>ApiAT3-1 comp forward</td>
<td>TGGACCCGGGATGCCGAACCTCTCGGAGGTTG</td>
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<td>ApiAT3-2 ORF forward</td>
<td>ATGGAGCAGAACTGCAGACCTACG</td>
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<td>ApiAT3-2 locus reverse</td>
<td>ACACCTCTCCAGCAAACGCCAAGGA</td>
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<tr>
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<td>TgApiAT3-2 seq (2)</td>
<td>GCAGGATATCTCGAACGAGATAG</td>
</tr>
<tr>
<td>TgApiAT3-3</td>
<td>ApiAT3-3 sequencing forward</td>
<td>CAGTCCGCAGTCAAGATTGCAAG</td>
</tr>
<tr>
<td></td>
<td>ApiAT3-3 3’ flank reverse</td>
<td>CAGTCCGCAGTCAAGATTGCAAG</td>
</tr>
<tr>
<td></td>
<td>ApiAT3-3 CRISPR sequencing</td>
<td>AACCTGACATGCGTCTGCCAGAAG</td>
</tr>
<tr>
<td>TgApiAT5-1</td>
<td>ApiAT5-1 seq forward</td>
<td>AGACGACACACTGCTGACCC</td>
</tr>
<tr>
<td></td>
<td>ApiAT5-1 seq reverse</td>
<td>CCAAGGGCGAGCAGAGAGG</td>
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<td>AGGCAGAAGACTCCAGAACG</td>
</tr>
<tr>
<td></td>
<td>ApiAT5-2 seq reverse</td>
<td>CGAGAAGCGAGGACTCAG</td>
</tr>
<tr>
<td>TgApiAT5-3</td>
<td>ApiAT5-2 seq forward</td>
<td>GTGAACAAAGGCAAACAGACTCC</td>
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<td>ApiAT5-2 seq forward</td>
<td>CTTCAGCTCAGTCTTCGTC</td>
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<td></td>
<td>ApiAT5-2 seq reverse</td>
<td>CGAGAAGAATCTCCACGACCTACG</td>
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<td>ApiAT5-2 seq forward</td>
<td>CCTTCCCCACTCTGAAAGATTG</td>
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<td>ApiAT5-2 seq reverse</td>
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<td>TgApiAT5-6</td>
<td>ApiAT5-2 seq forward</td>
<td>TGGGATTCTCCAGATGCTTG</td>
</tr>
<tr>
<td></td>
<td>ApiAT5-2 seq reverse</td>
<td>CGTCTAACGAGATAGG</td>
</tr>
<tr>
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<td>ApiAT6-1 seq forward</td>
<td>GATCCCCGGCGGCCACTGCTGAGGCAGAC</td>
</tr>
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<td></td>
<td>ApiAT6-1 seq reverse</td>
<td>GATCCCCGGCGGCCACTGCTGAGGCAGAC</td>
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</tr>
<tr>
<td></td>
<td>ApiAT6-2 seq reverse</td>
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</tr>
<tr>
<td>TgApiAT6-3</td>
<td>ApiAT6-3 seq forward</td>
<td>CGTGCTCTGCTCATTTACG</td>
</tr>
<tr>
<td></td>
<td>ApiAT6-3 seq reverse</td>
<td>GATGGCCATGAGGACCAG</td>
</tr>
<tr>
<td>TgApiAT7-1</td>
<td>ApiAT7-1 seq forward</td>
<td>GGCGAAGAGAAGCGGTG</td>
</tr>
<tr>
<td></td>
<td>ApiAT7-1 seq reverse</td>
<td>CCTGAAGTTCCTCTGAATCGACG</td>
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<tr>
<td>TgApiAT7-2</td>
<td>ApiAT7-2 seq forward</td>
<td>GAGCAAGTGTCTCTGCCTAGTCA</td>
</tr>
<tr>
<td></td>
<td>ApiAT7-2 seq reverse</td>
<td>CTCGGTCTCTTCGGCCTGCG</td>
</tr>
</tbody>
</table>
et al., 2002; Herm-Gotz et al., 2007; Andenmatten et al., 2013]. In regulatable systems, genetic modifications are not ‘activated’ until a specific compound is added to the parasites. I used the tetracycline regulatable system [Meissner et al., 2002] to conditionally knock down expression of TgApiAT2, by replacing the gene’s native promoter with one which can be regulated (see §5.2.3).

2.3.4.2 Bacterial expression of vectors for conventional crossover recombination

Before detailing the cloning steps involved in generating each vector (§2.3.4.3 and §2.3.4.4), I will provide a brief outline of the bacterial cloning steps that I used to generate enough plasmid vector to allow for transfection into T. gondii parasites. I electroporated the recombinant vectors into E. coli. I selected colonies expressing the vectors on nutrient agar with 100 µg/mL ampicillin. I used a standard colony PCR screening approach (using one primer specific to the insert and one specific to the plasmid backbone) to confirm whether ampicillin resistant colonies carried a copy of the plasmid with insert DNA in the correct orientation. I performed mini plasmid preparations (Promega Wizard SV miniprep kit) to extract the plasmid DNA from two positive clones. I then used a ‘Restriction Digest analysis’ for each miniprep, wherein I used restriction enzymes to digest plasmid DNA so that when the digest was run on an agarose gel the resultant DNA bands would differ by a predicted size. Finally, to obtain enough plasmid for electroporation into T. gondii, I performed a maxi plasmid preparation (Axygen AxyPrep maxi prep kit), provided that I had observed the right size bands in the restriction digest analyses.

2.3.4.3 Generating the regulatable TgApiAT2 cell line

I generated the regulatable ApiAT2 cell lines (rApiAT2) using the conventional crossover recombination approach. In my Honours project I had generated a vector called rHA3-APIAT2 designed to replace the promoter of TgApiAT2 and to introduce a 3×HA sequence at the 5’ end of the gene, tagging the N-terminus of the protein [Parker, 2013]. I used wild type T. gondii genomic DNA (RHΔhxgprt gDNA) as a PCR template with either the ‘ApiAT2 3’ flank forward/reverse’ primers to amplify a 3’ flanking region homologous to the region downstream of the start codon of apiAT2, or the ‘ApiAT2 5’ flank forward/reverse’ primers to amplify a 5’ flanking region homologous to the 5’ untranslated region (5’ UTR) of apiAT2 (Table 2.8). I digested the 3’ flank with AgeI-HF and NotI-HF and ligated it into the XmaI and NotI sites of the pPR2-HA3 vector [Katris
et al., 2014]. I assessed the success of this insertion by PCR screening and restriction digest analysis. I then ligated the 5’ flank into the ApaI and PacI sites of the vector that already contained the 3’ flank. However, I failed to generate modified parasites with the rHA$_3$-ApiAT2 vector. This failure suggested that the 3×HA tag was not being tolerated at the N-terminus of TgApiAT2.

My next strategy was to remove the 3×HA tag from the rHA$_3$-ApiAT2 vector. I used ‘TgApiAT2 loxP forward’ and ‘ApiAT2 3’ flank reverse’ primers (Table 2.8) to amplify a new 3’ flank from T. gondii genomic DNA. I used NotI and BamHI to digest the new 3’ flank insert, then I ligated it into the NotI and BglII sites of the previous rHA$_3$-ApiAT2 vector. I selected these sites as they resulted in the 3×HA tag being cut out during the restriction digest. I confirmed bacterial expression with a PCR Screen and restriction digest analysis.

I used this vector in two different transfections. The first was in work predating this project when I transfected it into a cell line in which apiAT2 had already been HA tagged, generating the rApiAT2-HA cell line (Table 2.9). The second transfection was during this project when I transfected the rApiAT2 vector into a TATi/Δku80 tdTomato cell line, generating the rApiAT2 cell line (Table 2.9). In each case, I linearised the vector with BglIII before transfection. I selected parasites by culturing them in the presence of 1 µM of pyrimethamine then cloned them out using a standard dilution cloning technique.

To knockdown expression of rApiAT2-HA or rApiAT2, I cultured parasites in the presence of 0.5 µg/mL anhydrotetracycline (ATc).
Table 2.8: Primers used to generate the regulatable rApiAT2 vector and the TgApiAT2-HA complementation.

<table>
<thead>
<tr>
<th>Research objective (cloning vector)</th>
<th>Primer name (restriction site)</th>
<th>Primer sequence 5’—3’</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Generation of regulatable rHA3·ApiAT2 cell line (pPR2-HA3)</strong></td>
<td>rHA3·ApiAT2 3’ flank forward (AgeI)</td>
<td>TCACCAAAATGGCGGCTGCTCAGGAAACCG</td>
<td>Wild type T. gondii genomic DNA (RHΔhxgprt gDNA)</td>
</tr>
<tr>
<td></td>
<td>rHA3·ApiAT2 3’ flank reverse (NorI)</td>
<td>GACTGCGGCCGCGGTCCTGCGAGAAGGACATGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rHA3·ApiAT2 5’ flank forward (ApaI)</td>
<td>TGACGGGCCGCCAGGTTCCTCAGGTTCGCGTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rHA3·ApiAT2 5’ flank reverse (PacI)</td>
<td>GCATTTAAATTAACGCAGACAAGCAGAGAAAGTC</td>
<td></td>
</tr>
<tr>
<td><strong>Generation of regulatable rApiAT2 cell line (rHA3·ApiAT2 vector in pPR2-HA3)</strong></td>
<td>rApiAT2 loxP forward (BamHI)</td>
<td>GATCAGATCAGAAATGGCGGCTGCTCAGGAAAC</td>
<td>Wild type T. gondii genomic DNA (RHΔhxgprt)</td>
</tr>
<tr>
<td></td>
<td>rApiAT2 3’ flank reverse (NorI)</td>
<td>GACTGCGGCCGCGGTCCTGCAGGAAGGACATGAT</td>
<td></td>
</tr>
<tr>
<td><strong>Generate TgApiAT2-HA complementation vector (pUgCTH3)</strong></td>
<td>TgApiAT2 loxP forward (BamHI)</td>
<td>GATCAGATCAGAAATGGCGGCTGCTCAGGAAAC</td>
<td>TATi/Δku80 genomic DNA</td>
</tr>
<tr>
<td></td>
<td>TgApiAT2 loxP reverse (AvrII)</td>
<td>GTCCTAGCACCGCAAGCGACTCTGGAC</td>
<td></td>
</tr>
</tbody>
</table>
2.3.4.4 Generating the \( Tg\text{ApiAT2-HA} \) complementation vector

Complementation of gene knockouts or knockdowns with constitutively expressed copies of the target gene is an important control to test whether defects in parasite growth or function are indeed due to the loss of the gene in question, and not due to off-target effects.

I used conventional crossover recombination to generate the complemented cell lines used in this study (Table 2.9). I amplified the open reading frame of \( Tg\text{ApiAT2} \) by PCR using the ‘\( Tg\text{ApiAT2 loxP} \)’ forward and reverse primers (Table 2.8). I used genomic DNA as the template for PCR as there are no introns in \( apiAT2 \). I digested the open reading frame insert with \( \text{BamHI} \) and \( \text{AvrII} \) and ligated it into the \( \text{BglII} \) and \( \text{AvrII} \) sites of the vector \( \text{pUgCTH3} \) [Rajendran et al., 2017]. Inserting the open reading frame in this position will add a \( 3\times\text{HA} \) tag to the C-terminus of \( Tg\text{ApiAT2} \). The promoter encoded by this vector will drive constitutive expression of \( Tg\text{ApiAT2-HA} \). I confirmed correct integration of the insert DNA with a colony PCR screen and restriction digest analysis. I refer to this vector as the \( Tg\text{ApiAT2-HA} \) complementation vector (Table 2.8). Before transfecting it into parasites, I linearised the vector with \( \text{MfeI}. \) \( \text{MfeI} \) cuts within a region of the plasmid that contains homology to \( Tg\text{UPRT} \) sequence, a gene encoding a non-essential enzyme in tachyzoites [Donald & Roos, 1995]. Linearisation of the plasmid was necessary to promote its integration into the genome, given that I was transfecting a \( \Delta ku80 \) strain (see Table 2.9).

2.4 Characterisation of genetically modified parasites

2.4.1 Protein Assays

2.4.1.1 Western Blots

I used western blots to assess whether \( \text{HA} \) tagged ApiAT proteins (§2.3.3.2) were expressed in the tachyzoite stage of \( T.\ gondii \). I also used western blots in oocyte work in order to verify that transporter protein was being made from the cRNA and was being expressed on the oocyte plasma membrane (§2.5.5 and 2.5.6).

To prepare samples from parasite work, I filtered tachyzoites which had recently lysed their host cell monolayer from host cell debris through a 3 \( \mu \text{m} \) filter, centrifuged
<table>
<thead>
<tr>
<th>Research objective</th>
<th>Cell line</th>
<th>Parental Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope tag $Tg$ApiAT3-1</td>
<td>$Tg$ApiAT3-1-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
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<td>$Tg$ApiAT5-1-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
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<td>$Tg$ApiAT5-2-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
<td>Epitope tag $Tg$ApiAT5-4</td>
<td>$Tg$ApiAT5-4-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
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<td>$Tg$ApiAT5-5-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
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<td>$Tg$ApiAT5-6-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
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<td>$Tg$ApiAT6-3-HA</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
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<td>$Tg$ApiAT7-2-HA</td>
<td>TATi/Δku80 tdTomato</td>
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<td>Disrupt $Tg$ApiAT1</td>
<td>apiAT$^{54−534}$</td>
<td>RHΔhxgprt</td>
</tr>
<tr>
<td>Disrupt $Tg$ApiAT2</td>
<td>apiAT$^{138−588}$</td>
<td>TATi tdTomato</td>
</tr>
<tr>
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<td>apiAT$^{31−599}$</td>
<td>TATi</td>
</tr>
<tr>
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<td>apiAT$^{24162−728}$</td>
<td>TATi</td>
</tr>
<tr>
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<td>apiAT$^{3184−755}$</td>
<td>TATi</td>
</tr>
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<td>apiAT$^{2239−987}$</td>
<td>TATi tdTomato</td>
</tr>
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<td>apiAT$^{3188−504}$</td>
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</tr>
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</tr>
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<td>apiAT$^{5578−575}$</td>
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</tr>
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<td>apiAT$^{620−548}$</td>
<td>TATi tdTomato</td>
</tr>
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<td>apiAT$^{24201−664}$</td>
<td>TATi tdTomato</td>
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<td>apiAT$^{3170−684}$</td>
<td>TATi tdTomato</td>
</tr>
<tr>
<td>Disrupt $Tg$ApiAT7-1</td>
<td>apiAT$^{71201−891}$</td>
<td>TATi tdTomato</td>
</tr>
<tr>
<td>Disrupt $Tg$ApiAT7-2</td>
<td>apiAT$^{24937−1027}$</td>
<td>TATi tdTomato</td>
</tr>
<tr>
<td>Complement apiAT$^{24138−588}$</td>
<td>apiAT$^{24138−588}$</td>
<td>apiAT$^{24138−588}$</td>
</tr>
<tr>
<td>Regulate $Tg$ApiAT2 expression</td>
<td>rApiAT2-HA</td>
<td>TgApiAT2-HA in TATi/Δku80</td>
</tr>
<tr>
<td>Regulate $Tg$ApiAT2 expression</td>
<td>rApiAT2</td>
<td>TATi/Δku80 tdTomato</td>
</tr>
<tr>
<td>Complement rApiAT2 cell line</td>
<td>rApiAT2/cTgApiAT2</td>
<td>rApiAT2</td>
</tr>
</tbody>
</table>
parasites at 1,500 xg for 10 min, then washed them once in PBS. I resuspended the
tachyzoites in [1×] sample buffer (0.5% [v/v] β-Mercaptoethanol in NuPage LDS sam-
ple buffer (Life Technologies)). I discuss the preparation of oocyte samples in detail in
later sections (§2.5.5 and 2.5.6).

I separated proteins using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). I boiled samples for 2-5 minutes before loading them into
pre-cast 12% Bis-Tris Novex NuPAGE gels (Life Technologies). From parasite sam-
ples I loaded the equivalent of 5\times10^6 parasites. After the electrophoresis, I transferred
the proteins from the gel to nitrocellulose membrane using a wet transfer system. I
stained the membrane with Ponceau S to check whether the transfer was successful.
I washed out the Ponceau S stain out with Blotto (4% (w/v) skim milk powder in
[1×] Tris-buffered saline (TBS)). I then blocked the membrane for at least an hour
(or overnight) in new Blotto solution to prevent non-specific antibody binding in the
subsequent western blotting.

I bound the primary and secondary antibodies consecutively by incubating mem-
branes in antibody solutions for one hour each. After each antibody incubation, I
washed the membrane twice in Blotto then twice in Tween-TBS (1×TBS with 0.05%
(v/v) Tween-20) to remove unbound antibody. For parasite samples I used rat anti-HA
(Roche) as the primary antibody and goat anti-rat Ig conjugated to horseradish perox-
idase (HRP) (Santa-Cruz Biotechnologies) as the secondary antibody. The antibody
concentration I used depended upon the ApiAT protein that I was testing. I probed all
proteins in the \( Tg \text{ApiAT5} \) subfamily and \( Tg \text{ApiAT7-2-HA3} \) with a 1:1 000 dilution of
rat anti-HA and a 1:5 000 dilution of goat-anti-rat Ig-HRP. I probed \( Tg \text{ApiAT3-1-HA3} \)
and \( Tg \text{ApiAT6-3-HA3} \) samples with a 1:3 000 dilution of rat anti-HA and a 1:10 000
dilution of goat-anti-rat Ig-HRP. To detect expression of \( Tg \text{ApiAT2-HA} \) in the comple-
mented \( apiAT2^{Δ138-588} \) cell line, I probed with rat anti-HA (1:800 dilution) and anti-rat
Ig-HRP (1:5 000 dilution). I probed preparations from oocytes with a 1:500 dilution of
rat anti-HA and a 1:5 000 dilution of anti-rat Ig-HRP (§2.5.5 and 2.5.6).

Proteomics data suggested that some ApiAT proteins may not be expressed in tachy-
zoites (www.eupathdb.org [Aurrecoechea et al., 2017]), and for some ApiATs, I
observed no signal after probing with anti-HA antibodies. I therefore stripped every
western blot membrane of antibody after I had probed with anti-for HA antibodies. To
do so, I incubated the membrane twice in stripping buffer (15g/L glycine, 1g/L SDS, 10 mL/L Tween-20 in ultrapure water, pH 2.2) at room temperature for 10 minutes. I washed the membrane twice in PBS (10 minutes each) and twice in Tween-TBS (5 minutes each). I then blocked the membrane overnight in Blotto.

This enabled me to re-probe each membrane with antibodies against a *T. gondii* cytosolic protein GRA8 [Carey *et al.*, 2000], which I used as a control to verify that protein sample was indeed present. I used mouse anti-GRA8 as the primary antibody and goat anti-mouse Ig-HRP as the secondary antibody. For the ApiAT5 subfamily, I used these at 1:80 000 and 1:2 000 dilutions respectively, but for all other ApiATs I used 1:200 000 and 1:4 000 dilutions, respectively.

To develop the western blots, I incubated the membrane in peroxide solution (Pierce ECL PlusWestern Blotting Substrate) for 5 minutes. I then placed the membrane in a photo cassette and exposed it to film in a dark-room for between 10 seconds and 3 minutes. I developed the films using an AFGA CP100 X-ray film fixer and developer. I scanned the films to obtain and digital image for presentation, and determined the molecular weight of proteins in ImageJ [Schneider *et al.*, 2012; Schindelin *et al.*, 2012].

### 2.4.1.2 Immunofluorescence assays

In order to determine the subcellular localisation of *Tg*ApiAT3-1, *Tg*ApiAT6-3 and *Tg*ApiAT7-2, I used an immunofluorescence assay (IFA). IFA is a technique in which antibodies directed against proteins are allowed to bind their targets, and secondary antibodies conjugated to fluorophores then bind to the primary antibodies. In this way, the localisation of ApiAT proteins could be determined by co-labelling with antibodies against proteins in known compartments.

I also used IFA's to distinguish invaded from non-invaded parasites during intracellular growth assays and invasion assays.

I followed standard IFA protocol as detailed by Striepen & Soldati [2007]. In brief, I grew parasites expressing the epitope-tagged ApiATs for 24 hours in HFF coated coverslips in 6-well plates. I aspirated the growth medium then fixed both host and parasite cells using 3% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS)
§2.4 Characterisation of genetically modified parasites

to preserve sub-cellular structure. I then permeabilised with a detergent (0.25% (v/v) Triton-X 100 in PBS) to allow labelling antibodies access to intracellular targets, and blocked using 2% (w/v) bovine serum albumin (BSA) in PBS to reduce non-specific binding of antibodies. I incubated coverslips in primary antibodies for 1 hour, then I washed the coverslips three times with PBS for 5 minutes each. I then incubated coverslips in secondary antibodies for one hour. I washed coverslips 3 times for 5 minutes each with PBS and once with de-ionised H₂O. I then mounted the coverslips upside-down on microscope slides using Fluorogel (an anti-fade agent, Electron Microscopy Sciences). I determined protein localisation by examining the prepared slides under excitation wavelengths of the FITC, and TRITC channels on a fluorescence microscope, depending on the fluorophore used. I took images of the parasites using a DeltaVision set-up on an inverted Olympus IX71 microscope fitted with an Olympus objective lens (UPlanSApo 100× with immersion oil of refractive index 1.14). I captured the images using a Photometrics CoolSNAP HQ2 camera. I deconvolved the images using SoftWorx software in order to computationally remove out-of-focus fluorescence. I have linearly adjusted the brightness/contrast in some images to allow for loss of brightness when printing.

To label TgApiAT3-1-HA3, TgApiAT6-3-HA3 and TgApiAT7-2-HA3, I used rat anti-HA (Roche) (1:200 dilution) as the primary antibody against the HA epitope, and I used goat anti-rat immunoglobulin (Ig) CF488A (Sigma) as the secondary antibody. To label the plasma membrane, I used rabbit anti-P30 (1:900 dilution) (a gift from John Boothroyd, Stanford School of Medicine) as the primary antibody and goat anti-rabbit Ig Alexa546 (Life Technologies) (1:500 dilution) as the secondary antibody. To label TgApiAT2-HA in the complemented apiAT2∆138−588 cell line, I used rat anti-HA (1:400 dilution) as the primary antibody and goat anti-rat Ig CF488A (1:250 dilution). To label TgApiAT2-HA in the complemented rApiAT2 cell line, I used rat anti-HA (1:200 dilution) as the primary antibody and goat anti-rat Ig Alexa488 (Life Technologies) (1:200 dilution) as the secondary antibody. To label the plasma membrane in this experiment, I used mouse anti-P30 (Abcam) (1:200 dilution) as the primary antibody and goat rat-mouse Ig Alexa546 (Life Technologies) (1:200 dilution) as the secondary antibody.
2.4.2 Growth Assays

2.4.2.1 Plaque assays

To compare the growth of ApiAT truncation mutants with their parental strains, I performed plaque assays. I followed a standard plaque assay protocol as described by Striepen & Soldati [2007]. I evaluated parasite growth under different culture conditions, using both DMEM and Minimal Amino Acid Medium (MAAM), which is a ‘homemade’ medium containing reduced amino acid concentrations compared to DMEM (Table 2.10).

The composition of amino acids in MAAM theoretically represents the minimum necessary concentration to allow for normal parasite growth in a fluorescence growth assay (FGA) (see §2.4.2.2) [Rajendran, unpublished]. The amino acid composition for MAAM was determined in our laboratory. Amino acids not present in DMEM were automatically excluded from MAAM. To determine the rest of the amino acid concentrations, my colleague made a set of media in which a single amino acid was absent from each medium. This amino acid was added back into the medium at a range of concentrations, using a serial dilution process. An FGA was used to determine the growth of wild type parasites across the concentration range [Rajendran, unpublished]. For each amino acid, the minimal concentration that was conducive to normal growth was chosen as the concentration to be used in MAAM. A comparison of the amino acid compositions and concentrations in DMEM and MAAM is provided in Figure 2.2.

I made up MAAM using the same composition of inorganic salts as present in DMEM (0.265 g/L CaCl\(_2\)·2H\(_2\)O, 0.20 g/L MgSO\(_4\)·7H\(_2\)O, 0.40 g/L KCl, 1.48 g/L NaHCO\(_3\), 6.40 g/L NaCl and 0.234 g/L NaH\(_2\)PO\(_4\)·2H\(_2\)O), a commercially available RPMI vitamin formulation (Sigma), 4.5g/L (~25mM) D-glucose, and the amino acid concentrations specified in Table 2.10. I supplemented the MAAM with 1% (v/v) dialysed fetal bovine serum (to reduce the amounts of amino acids added with the serum), antibiotics (penicillin, streptomycin and gentamicin) and fungicides (amphotericin b).

I also tested the growth of \(Tg\)ApiAT1 truncation mutants in Roswell Park Memorial Institute 1640 medium (RPMI). I supplemented the RPMI with 1% (v/v) fetal bovine serum, 200 \(\mu\)M L-glutamine, antibiotics (penicillin, streptomycin and gentamicin) and fungicides (amphotericin b).
Table 2.10: The amino acid compositions of the different growth media used in this work. The table has been colour coded on a white-red scale (0–4 000 µM) to give a visual representation of the concentrations in each medium. The concentrations of amino acids found in human plasma are included for comparison. All the values are from Trabado et al. [2017], except for cysteine, because its concentration was not reported in that study. The value for cysteine is instead from Bergström et al. [1974]. The values reported for all other amino acids were consistent between the two studies.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Human Plasma</th>
<th>RPMI</th>
<th>DMEM</th>
<th>MAAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>319.4±71.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Asparagine</td>
<td>48.4±9.29</td>
<td>379</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.26±2.34</td>
<td>150</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>46.2±21.4</td>
<td>136</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>153</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proline</td>
<td>188.3±55.0</td>
<td>174</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glycine</td>
<td>255.4±65.9</td>
<td>133</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>Serine</td>
<td>115.1±25.0</td>
<td>286</td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>63.0±10.7</td>
<td>25</td>
<td>78</td>
<td>8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>60.0±7.75</td>
<td>91</td>
<td>400</td>
<td>16</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>77.5±15.4</td>
<td>382</td>
<td>800</td>
<td>31</td>
</tr>
<tr>
<td>Leucine</td>
<td>150.1±27.7</td>
<td>382</td>
<td>800</td>
<td>31</td>
</tr>
<tr>
<td>Methionine</td>
<td>25.4±5.05</td>
<td>101</td>
<td>201</td>
<td>31</td>
</tr>
<tr>
<td>Threonine</td>
<td>127.9±28.2</td>
<td>168</td>
<td>800</td>
<td>31</td>
</tr>
<tr>
<td>Histidine</td>
<td>89.3±10.8</td>
<td>97</td>
<td>200</td>
<td>63</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>62.9±12.2</td>
<td>111</td>
<td>423</td>
<td>63</td>
</tr>
<tr>
<td>Lysine</td>
<td>197.4±30.7</td>
<td>219</td>
<td>800</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine</td>
<td>110±86</td>
<td>208</td>
<td>200</td>
<td>125</td>
</tr>
<tr>
<td>Valine</td>
<td>243.6±45.2</td>
<td>171</td>
<td>800</td>
<td>250</td>
</tr>
<tr>
<td>Arginine</td>
<td>81.4±19.3</td>
<td>1150</td>
<td>400</td>
<td>287</td>
</tr>
<tr>
<td>Glutamine</td>
<td>657.9±106.2</td>
<td>2050</td>
<td>4000</td>
<td>500</td>
</tr>
</tbody>
</table>
§2.4  Characterisation of genetically modified parasites

Figure 2.2: Graphical representation of the amino acid concentrations used in DMEM and Minimal amino acid medium (MAAM), ranked according to their concentration in MAAM. Note the split scale on the y-axis. Amino acids that are usually absent in DMEM were also omitted from MAAM. Serine and Glycine, usually present in DMEM, were omitted from MAAM. All other amino acids were represented in both media, but each at a reduced amount in MAAM.

In order to set up the plaque assays, I first aspirated growth medium from host cells, then washed flasks with 5 mL of the medium being used (DMEM or MAAM) or with PBS. I then added the appropriate growth media and 400 parasites to 25 cm$^2$ tissue culture flasks containing a confluent HFF cell monolayer. Alternatively, I used 6 well plates containing HFF monolayers to which I added 150-200 parasites per well.

I incubated to flasks and plates for 8-10 days without disturbance. After this incubation period, I aspirated the growth medium, and washed the monolayers with PBS. I fixed the host cell monolayers with 100% ethanol and stained them with crystal violet dye, as described previously [Striepen & Soldati, 2007].

I scanned the flasks and the plates to obtain digital images for presentation. To compare plaque areas from different cell lines grown in the various media, I used ImageJ...
Characterisation of genetically modified parasites

§2.4

2.4.2.2 Fluorescence growth assays

Parasite growth can be measured in a more quantitative and high-throughput way using fluorescence growth assays [Gubbels et al., 2003]. I used these assays to quantify parasite growth rates in two experiments. In the first experiment, I compared the growth of \( \text{apiAT}2^{\Delta 138-588} \) parasites and their parental line TATi/tdTomato. In the second experiment I measured growth of \( r\text{ApiAT2} \), and its parental line TATi/\( \Delta ku80 \) tdTomato, and the complemented line \( r\text{ApiAT2}/cT_g\text{ApiAT2} \), in the presence or absence of ATc. The tdTomato red fluorescent protein (RFP) that these parasites express is bright enough to be detected using a fluorescence plate reader.

To set up the fluorescence growth assays, I added 100 \( \mu \text{L} \) of phenol red-free DMEM to each well. I resuspended parasites to a concentration of 2000 parasites/100 \( \mu \text{L} \) in phenol red-free medium. In the second experiment, where ATc was required, each parasite suspension contained either 1 \( \mu \text{g/mL} \) ATc, or no ATc. I inoculated wells, in triplicate, with 100 \( \mu \text{L} \) of parasite suspension. In the second experiment this resulted in a final ATc concentration of 0.5 \( \mu \text{g/mL} \). When performing these assays, I did not use the outermost wells of the 96 well plates, in order to avoid ‘edge effects’. I included a ‘no parasite control’ in each experiment, by leaving a column of host cells on each plate uninfected (containing 200 \( \mu \text{L} \) of medium only). This allowed me to measure background fluorescence. In analysing the data, I subtracted the average fluorescence of the ‘no parasite control’ on each day from the average of the three technical replicates.
for each parasite line/condition on that day.

I took daily fluorescence measurements with a FLUOstar Optima plate reader (BMG Labtech). I performed bottom reads with an excitation wavelength of 540-10 nm and collected emission at 590 nm. I took one cycle of reads using 3×3 matrix scanning mode with 10 flashes per well. I set the gain from uninfected wells.

I calculated the growth rates using the Grofit program [Kahm et al., 2010] for R [R Core Team, 2018] in RStudio [RStudio Team, 2016].

2.4.2.3 Intracellular growth assays

I sought to determine whether the defect in the lytic cycle of \( apiAT2^{\Delta138-588} \) parasites results from a slow intracellular replication rate. I tested the intracellular growth of \( apiAT2^{\Delta138-588} \), parental TATi/ttdtomato and \( apiAT2^{\Delta138-588}/cTgApiAT2 \) parasites using an intracellular growth assay. This assay relies on fluorescence microscopy to measure the number of replication cycles that intracellular parasites undergo across a 24-hour incubation period. All of these parasite lines express RFP, which allowed me to determine the number of parasites per parasitophorous vacuole using fluorescence microscopy.

I intended to measure the intracellular growth rate of parasites that had all invaded the host cells at the same time. This way, any retardation that I observed in intracellular growth would represent a defect in the growth of the intracellular stage of the lytic cycle, and not a delay in invasion. I therefore aimed to synchronise the invasion step of the parasites. This can be achieved by suspending parasites in solutions with a high K\(^+\) concentration, which prevents the parasites from invading host cells until the solution is changed to the regular growth medium [Kafsack et al., 2004].

To obtain my parasite inoculum, I aspirated the growth medium from an infected host cell monolayer which contained predominantly intracellular tachyzoites. I washed the monolayer with 10 mL of a high K\(^+\) buffer (HPB; 125 mM KCl, 5 mM NaCl, 1 mM MgCl\(_2\), 25 mM HEPES, 20 mM glucose). I then aspirated the wash buffer and added a further 7 mL of HPB. I scraped the monolayer into this solution, which I then passed through a 26-gauge needle in order to liberate the parasites from the host cells. I filtered
the parasite suspension through a 3 μm filter to remove host cell debris, counted the parasites using a haemocytometer, then diluted the parasites in HPB to a concentration of 1.25×10^5 parasites/mL.

To prepare the host cells, I aspirated the D10 growth medium from 12 well plates containing confluent HFF cells on round coverslips. I washed each well once with 2 mL of HPB. Just prior to adding the parasites, I aspirated the wash solution, then added 2 mL of parasites in HPB (2.5×10^5 parasites) to each well. I allowed the parasites to settle for 1 hour in the incubator at 37°C. During this time the parasites settle and attach to the monolayer, but cannot invade due to the high K⁺ concentration in the buffer [Kafsack et al., 2004].

In order to initiate invasion, I aspirated the HPB, then added 2 mL of Ed1 (§2.2.2) to each of the wells. The addition of growth medium allowed the parasites to invade host cells, synchronising the invasion step. I incubated the parasites and host cells at 37°C for 4 hours. After this 4-hour period, I aspirated the Ed1 medium then washed the coverslips 3 times with 2 mL of fresh Ed1, aiming to remove any non-invaded parasites, thus leading to a largely synchronous culture.

To commence the growth assay, I added 2 mL of Ed1 to each well and grew the parasites for a further 20 hours in a humidified 5% CO₂ incubator at 37°C.

To terminate the growth assay, I aspirated the Ed1 medium and fixed the monolayers in 3% PFA in PBS for 15 minutes. Skipping the permeabilization step one would normally do for an immunofluorescence assay, I washed each well three times in PBS then blocked them in 1.5 mL 2% (w/v) BSA in PBS overnight.

To enable me to differentiate between extracellular (i.e. uninvaded) parasites, and parasitophorous vacuoles that contained a single (intracellular) parasite, I performed an IFA. Having skipped the permeabilisation step, antibodies to \textit{T. gondii} surface antigen P30 are unable to enter infected host cells, and therefore will only bind to extracellular (i.e. uninvaded) parasites. I stained the coverslips with rabbit anti-P30 (1:550 dilution) (Boothroyd Laboratory) as the primary antibody, and goat anti-rabbit Ig Alexa488 (Invitrogen) (1:450) as the secondary antibody. When I viewed the parasites under fluorescence microscopy, extracellular parasites fluoresced green in the FITC channel from
the 488-flurophore. All parasites in the experiment fluoresced red in the TRITC channel from the RFP they were expressing. Therefore, intracellular parasites only fluoresced red, whereas extracellular parasites fluoresced both red and green, allowing me to differentiate intracellular from extracellular parasites and to exclude the latter from subsequent analysis.

2.4.2.4 Invasion Assay

To determine whether TgApiAT2 contributes to T. gondii host cell invasion I performed an invasion assay on apiAT2Δ138−588, parental TATi/tdtomato and apiAT2Δ138−588 /cTgApiAT2 parasites. The invasion assay relies on fluorescence microscopy to image parasites soon after they first invade a host cell monolayer, allowing a comparison of the invasion success of each parasite strain. Each cell line expressed RFP which helped me to determine whether the parasites were intracellular or extracellular after the invasion test was complete.

The setup that I used for the invasion assay was identical to the intracellular growth assay. The protocol diverges at the invasion step, where instead of allowing the parasites to invade for 4 hours, I allowed the parasites to invade host cells for 10 minutes at 37 °C. I aspirated the E1 growth medium from the coverslips, then incubated them in 1 mL of fixative solution (3% (w/v) PFA and 0.1% (w/v) Glutaraldehyde in PBS) at room temperature for 20 minutes. I removed the fixative solution and added 2 mL of PBS to the wells, as a wash step, which I repeated twice. I blocked the coverslips using 1.5 mL 2% (w/v) BSA in PBS, overnight at 4 °C. The following day I stained the coverslips with rabbit anti-p30 antibody (Boothroyd Laboratory) and anti-rabbit Ig-Alexa 488 (Invitrogen) using the standard IFA protocol. I used fluorescence microscopy to count numbers invaded and non-invaded parasites. As the host cells had not been permeabilised, the anti-P30 antibody could not penetrate and stain invaded parasites, which I could therefore identify as parasites which were red in the TRITC channel, but did not fluoresce green in the FITC channel. Parasites which had not invaded had both red and green fluorescence in the TRITC and FITC channels, respectively. This differential labelling pattern allowed me to distinguish invaded from non-invaded parasites during counting, rather than relying on distinguishing intracellular from extracellular parasites based on their morphology, which is more subjective.
2.5 Xenopus oocyte experiments

In order to directly test the substrate specificities of \textit{TgApiAT2}, I expressed the protein in the \textit{Xenopus laevis} oocyte heterologous expression system. This allowed me to study the transporter in isolation, by comparing the phenotype of oocytes expressing \textit{TgApiAT2} to that of control oocytes that did not express the transporter.

2.5.1 Oocyte vector cloning strategy

Coding RNA (cRNA) is required in order to express proteins in oocytes. The cRNA is synthesised from a DNA template, so I first had to generate a recombinant DNA vector encoding \textit{TgApiAT2}. Using the \textit{TgApiAT2-HA} complementation vector described in Section 2.3.4.3 as a template, I amplified the \textit{TgApiAT2} open reading frame using the primers ‘\textit{TgApiAT2 oocyte forward}’ (5’- GATCggatccCCACCATGGCGGGCTGCTCAGG) and ‘\textit{TgApiAT1 oocyte reverse}’ (5’- GATCtctagaCTAAGCGTAGTCCGGGACATCGTACGGG). The primers were designed so that the resulting PCR product would include the 1×HA tag at the 3’ end of \textit{TgApiAT2-HA}, and included a Kozak sequence in the ‘\textit{TgApiAT2 oocyte forward}’ primer.

I digested the PCR product with \textit{BamHI} and \textit{XbaI}. I digested the oocyte expression vector pGEM-He-Juel \cite{Broeer1997} using the same enzymes. I then ligated the insert into the digested vector and transformed bacteria with the recombinant vector. I used a colony PCR screen to confirm correct ligation. The resulting vector was termed \textit{TgApiAT2-HA} in pGHJ. In this vector, \textit{TgApiAT2-HA} is downstream of a T7 RNA polymerase promoter to enable cRNA transcription of \textit{TgApiAT2-HA}. The resulting transcript contains 5’ and 3’ untranslated regions of the \textit{X. laevis} β-globin gene, which, along with the introduced Kozak sequence, facilitate translation of the transcript following injection into oocytes.

2.5.2 DNA precipitation

In preparation for cRNA synthesis, I linearised 5 µg of \textit{TgApiAT2-HA} in pGHJ with NotI-HF overnight at 37°C. I used a phenol-chloroform extraction to purify the linearised DNA, as follows. I topped up the restriction digest to a volume of 100 µL with trisaminomethane-hydrochloride (Tris-HCl) (pH 7.6). I then added 100 µL of phenol-chloroform, vortexed the sample for 1 minute then centrifuged it at 16,000xg
§2.5 Xenopus oocyte experiments

for 5 minutes. I transferred the aqueous layer into a new 1.5 mL tube. I precipitated the DNA by adding 1/10th the volume of 3 M sodium acetate and 2.5 times the volume of 100% (v/v) ethanol. I incubated the sample at -20°C for 3-5 hours then I centrifuged it for 20 minutes at 17,000×g. I washed the resulting DNA pellet with 1 mL of 70% ethanol and then centrifuged it for 10 minutes at 17,000×g. To dry the DNA pellet, I removed the ethanol and inverted the 1.5 mL tube for 20 minutes, then allowed it to dry in a tube rack for a further 10 minutes. I resuspended the DNA pellet in 12 µL of RNAse free dH₂O. I used half of the prepared DNA in the proceeding RNA transcription reaction (§2.5.3) and stored the other half at -20°C for future use.

2.5.3 Coding RNA (cRNA) synthesis

I generated cRNA using the T7 mMessage mMachine kit (Ambion) following the manufacturer’s instructions. Briefly, I assembled 10 µL of [2×] NTPKAP, 2 µL of the provided [10×] reaction buffer, 6 µL of the linearised DNA vector as template and 2 µL of the enzyme mix at room temperature. I incubated the reaction at 37°C for 2 hours. I then added 1.5 µL of TURBO DNAse to the reaction and I incubated it at 37°C for 15 minutes in order to destroy the template DNA.

I used a phenol chloroform extraction to purify the cRNA. I topped up the synthesis reaction to a volume of 100 µL by adding 80 µL of Tris-HCl (pH 7.4). To this I added 100 µL of phenol-chloroform, vortexed the emulsion for 1 minute, then centrifuged it for 5 minutes at 17,000×g.

I transferred the aqueous layer (approximately 90 µL) into a new 1.5 mL tube, to which I added 10 µL of Tris-HCl (pH 7.4) and re-extracted in phenol:chloroform (added 100 µL of phenol-chloroform, vortexed the emulsion for 1 minute, then centrifuged it for 5 minutes at 17,000×g). I transferred the aqueous layer of this second extraction into a new, ‘final’ 1.5 mL tube.

The two phenol:chloroform extractions resulted in two tubes comprised mostly of the chloroform layer with approximately 10 µL of aqueous layer remaining. To the chloroform layer in each tube, I added 90 µL of Tris-HCl (pH 7.4), then re-extracted in phenol:chloroform. I removed the aqueous layers of these two tubes and transferred
them into the aforementioned ‘final tube’, yielding approximately 300 µL of pooled cRNA.

To concentrate the cRNA, I precipitated it by adding 150 µL of 7.5 M ammonium acetate and 750 µL of 100% (v/v) ethanol. I vortexed the solution, then incubated it at -80°C overnight. The next morning I centrifuged the precipitation reaction at 21,000×g for 20 minutes. I washed the resulting cRNA pellet with 70% (v/v) ethanol and centrifuged it again at 21,000×g for 20 minutes. I decanted the ethanol and allowed the pellet to dry for 1 hour by inverting the tube. I then resuspended the cRNA pellet in 15-20 µL of RNase free dH₂O. Finally, to remove secondary structures from the cRNA, I placed the cRNA on ice for 10 minutes then heated it at 65°C for 3 minutes.

2.5.4 Xenopus laevis surgery and oocyte preparation

All animal research was conducted in accordance with the National Health and Medical Research Council’s Australian Code for the Care and Use of Animals for Scientific Purposes, and the Australian Capital Territory Animal Welfare Act 1992. Maintenance of Xenopus laevis and preparation of oocytes was approved by the Australian National University Animal Experimentation Ethics Committee (protocol number A2014/20).

I surgically removed X. laevis oocytes and prepared them for cRNA injection, as described previously [Bröer, 2010]. I anaesthetised frogs by submersing them for 30 minutes in 1 L of 1.5 g/L Ethyl 3-aminobenzoate methanesulfonate with 1 mM NaHCO₃ dissolved in tap water. After this time, I tested the frog’s reactions by turning the frog upside-down and gently stroking the frog’s throat. If the frog had no swallowing reaction, the anaesthesia was deemed complete. I placed each frog, belly up, on a damp paper towel on top of a tray of ice. I made a 1 cm incision through the both the skin and the muscle layer on the belly. I removed the ovary with tweezers and placed it in oocyte ringer buffer without calcium (OR₂⁻; 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.8). I closed the muscle layer incision with a single stitch of absorbable suture and closed the skin layer with two stitches of non-absorbable suture.

I then dissected the ovary into small clumps (of about 30 oocytes each) which I incubated in collagenase D (Roche Diagnostics, 1.5 mg/mL in OR₂⁻ buffer) for 2
hours at 37°C with slight agitation, then at 18°C without agitation overnight. The next morning, I washed the collagenase D from the liberated oocytes using 2 L of cold OR2\(^-\) buffer then 2 L of cold OR2\(^+\) buffer (OR2\(^-\) supplemented with 1 mM CaCl\(_2\)). I manually selected stage V and VI oocytes devoid of adhering blood vessels under a dissecting microscope for use in experiments.

I pulled microinjecting needles from boron silicate capillaries (World Precision Instruments) using a P-97 Flaming/Brown micropipette puller (Sutter Instruments). I injected half of the oocytes with 10 ng of cRNA dissolved in 15 \(\mu\)L of nuclease-free water using a Micro4™ micro-syringe pump controller and A203XVY nanoliter injector (World Precision Instruments). I did not inject cRNA into the other half of the oocytes, which I used as controls. I maintained the oocytes at 18°C in OR2\(^+\) buffer supplemented with gentamicin at 10 mg/L (Sigma). I measured the uptake of radiolabelled amino acids four days post-injection, to allow time for \(Tg\text{ApiAT2}\) to be expressed.

### 2.5.5 Oocyte whole membrane preparation

In order to determine whether injection of \(Tg\text{ApiAT2-HA}\) cRNA led to expression of a correctly sized HA tagged protein in oocytes, I collected oocyte membranes and prepared them for western blot.

I homogenised twenty-five cRNA injected and twenty-five non-injected oocytes in 1 mL of homogenisation buffer (50 mM Tris/HCl (pH 7.6), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and 1 mM cOmplete-EDTA free proteinase inhibitor cocktail (Roche Diagnostics)) by pipetting up and down with a 1000 \(\mu\)L pipette tip. I centrifuged the homogenate at 2000×g for 10 minutes at 4°C in order to pellet the cellular debris. I transferred the supernatant to a new tube, taking care not to transfer the floating lipids. I centrifuged the supernatant in an ultracentrifuge at 140 000×g for 30 minutes at 4°C in order to pellet membranes. I dissolved the resulting pellet in 30 \(\mu\)L of the aforementioned homogenisation buffer and 4% (w/v) SDS. I then diluted the dissolved pellets in [4×] western blot sample buffer in a 3:1 ratio (to yield a [1×] sample buffer) (0.5% (v/v) b-Mercaptoethanol in NuPage LDS sample buffer (Life Technologies)). I then separated protein samples by SDS-PAGE, and I detected HA tagged protein by western blotting, as described above (§2.4.1.1).
2.5.6 Oocyte surface biotinylation preparation

To confirm that TgApiAT2-HA was expressed on the plasma membrane of oocytes, I carried out a surface biotinylation assay. This assay theoretically detects only proteins that are present in oocyte plasma membrane. In contrast, the whole oocyte membrane preparation detects proteins present in any membrane, including membranes of internal organelles.

I placed ten cRNA injected (TgApiAT2 expressing) oocytes and 10 non-injected oocytes in separate disposable polypropylene culture tubes (Sigma-Aldrich). I washed the oocytes three times with 4 mL of ice-cold PBS (pH 8.0). I then incubated oocytes in 1 mL of PBS (pH 8.0) containing 0.5 mg/mL EZ-Link™Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) for 45 minutes at room temperature. During the incubation I inverted the tube every 10 minutes. After the incubation, I washed the oocytes 4 times with 4 mL ice cold PBS (pH 8.0) to remove non-bound biotin label, then transferred them to 1.5 mL tubes. I lysed the oocytes by incubating them in 1 mL of oocyte lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Triton X-100, pH 7.6) for two hours on ice. I inverted the tubes every 20 minutes to aid the lysis process. After lysis, I centrifuged the samples at 21 000×g for 15 minutes to pellet cell debris, and then transferred the supernatant into clean 1.5 mL tubes. I added 50 µL of streptavidin-coated agarose beads (Thermo Fisher Scientific) to the supernatant. I incubated the samples on a shaking platform, on slow rotation at 4°C overnight.

The following day, I centrifuged the tubes for 10 minutes at 21 000×g. I removed the majority of the supernatant, taking care not to remove any of the loosely packed beads. I washed the beads in 1 mL of oocyte lysis buffer, then I centrifuged them for 10 minutes at 21 000×g. I repeated this wash step four times. Finally, I added 10 µL of [4×] reducing western blot sample buffer (§2.4.1.1) to the 30 µL agarose bead/lysis buffer suspension (for a [1×] final sample buffer concentration). I then diluted each sample with 40 µL of [1×] western blot sample buffer in order to halve the protein concentration in the samples.

To elute proteins from the beads, I boiled the bead/sample buffer suspension at 100 °C for 10 minutes. I loaded 5-10 µL of each of the protein samples (cRNA injected or non-injected) on a 12% Bis-Tris Protein gel (Life Technologies), then followed the
regular western blot protocol (§2.4.1.1).

**2.5.7 Uptake of $^{14}$C labelled amino acids into oocytes**

In order to determine the substrate specificities of $Tg$ApiAT2, I incubated $Tg$ApiAT2-expressing and non-injected oocytes to solutions containing radiolabelled amino acids. Uptake of radiolabelled amino acids into the oocytes can be detected at the end of the experiment as radioactivity retained in the oocytes, as measured by scintillation counting. If uptake in $Tg$ApiAT2 expressing oocytes exceeds that of control oocytes, the difference can be attributed to the presence of $Tg$ApiAT2. This result would imply that the amino acid that was being tested is a substrate for the transporter.

To prepare for the $[^{14}$C] labelled amino acid uptake experiments, I transferred 10 $Tg$ApiAT2-expressing and 10 non-injected oocytes in OR2$^+$ buffer into disposable polypropylene culture tubes (Sigma-Aldrich), then incubated the oocytes and the buffer at room temperature for at least 15 minutes before proceeding to the uptake experiments.

Immediately prior to commencing the radiolabel uptake, I removed the OR2$^+$ buffer from the oocytes. Uptake experiments were performed in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM HEPES, pH 7.4). To begin the uptake, I added radioactive ‘uptake solution’ to the oocytes. The uptake solution was composed of ND96 with appropriate unlabelled substrate(s) and the radiolabelled substrate of interest (outlined in Table 2.11). I incubated the oocytes in the uptake solution for 10-30 minutes, depending on the substrate used. In time-course experiments, the time the oocytes were incubated in uptake solution for 5-80 minutes (as described in the results section).

To stop the uptake, I added approximately 3 mL of ice cold ND96 to the reaction mix. This had the effect of both diluting the radioactivity and slowing down substrate transport (due to the decrease in temperature). I aspirated the diluted uptake solution using a modified Pasteur pipette attached to a side arm flask and pump. To remove residual radiolabel, I washed the oocytes a further three times in ice cold ND96. After these 4 washes, I distributed the oocytes into a 96 well MicroBeta OptiPlate (Perkin-Elmer), with one oocyte per well. To lyse the oocytes and release the radioactivity
Table 2.11: The concentrations of unlabelled amino acids used in $^{14}$C labelled amino acid uptake experiment in *Xenopus* oocytes, and the amount of radiolabelled substrate used.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration of unlabelled amino acids</th>
<th>Radiolabelled substrate concentration ($\mu$Ci/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cRNA injection amount</td>
<td>500 $\mu$M L-alanine</td>
<td>0.7</td>
</tr>
<tr>
<td>Alanine Michaelis-Menten</td>
<td>0.1 mM L-alanine</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>1 mM L-alanine</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3 mM L-alanine</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>6 mM L-alanine</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>9 mM L-alanine</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
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<td>1.4</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>50 mM L-alanine</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>75 mM L-alanine</td>
<td>2.7</td>
</tr>
<tr>
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<td>3.2</td>
</tr>
<tr>
<td>Alanine Competition assay</td>
<td>500 $\mu$M L-alanine</td>
<td>0.7</td>
</tr>
<tr>
<td>AND</td>
<td>50mM competitor substrate</td>
<td>OR</td>
</tr>
<tr>
<td>Glutamine time-course</td>
<td>10 mM L-glutamine</td>
<td>0.95 – repeat 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.53 – repeat 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.49 – repeat 3</td>
</tr>
<tr>
<td>Isoleucine time-course</td>
<td>5 mM L-isoleucine</td>
<td>1.25 – repeat 1 and 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.95 – repeat 3</td>
</tr>
<tr>
<td>Isoleucine Michaelis-Menten analysis</td>
<td>0.5 mM L-isoleucine</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>1 mM L-isoleucine</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>2 mM L-isoleucine</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>4 mM L-isoleucine</td>
<td>1.3</td>
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<td></td>
<td>5 mM L-isoleucine</td>
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</tr>
<tr>
<td></td>
<td>7.5 mM L-isoleucine</td>
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<td>25 mM L-isoleucine</td>
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<td>30 mM L-isoleucine</td>
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<td>1.1</td>
</tr>
<tr>
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<td>1 mM L-leucine</td>
<td>1.1</td>
</tr>
<tr>
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<td>1.1</td>
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<td></td>
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<tr>
<td></td>
<td>20 mM L-leucine</td>
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<tr>
<td></td>
<td>30 mM L-leucine</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>50 mM L-leucine</td>
<td>0.9</td>
</tr>
<tr>
<td>Glutamine Michaelis-Menten analysis</td>
<td>1 mM L-glutamine</td>
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<tr>
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<td>2 mM L-glutamine</td>
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</tr>
<tr>
<td></td>
<td>5 mM L-glutamine</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>10 mM L-glutamine</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>15 mM L-glutamine</td>
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<tr>
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<tr>
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<tr>
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<td>75 mM L-glutamine</td>
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<td>Na$^+$ dependence</td>
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<tr>
<td>pH dependence</td>
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</tr>
<tr>
<td></td>
<td>OR</td>
<td>10 mM L-glutamine</td>
</tr>
</tbody>
</table>
into solution, I added 30 µL of 10% (w/v) SDS solution (in ultrapure water) to each well. I left the oocytes to lyse for at least 30 minutes and often overnight, with the plates wrapped in cling wrap to reduce evaporation. After the lysis, I added 150 µL of MicroScint-40 scintillation fluid (Perkin-Elmer) to the oocyte sample in each well.

To enable subsequent conversion of radioactivity counts to substrate amount, I collected two ‘standard’ samples. To do so, I aliquoted 10 µL of the uptake solution I had used into the wells of the plate, and added 150 µL of scintillation fluid to the standards. I sealed the MicroBeta plates with sealing film, then mixed the scintillant into the radioactive samples at 5 Hz on a shaking platform for 5 minutes. I read the plates with a $^{14}$C protocol on a Perkin-Elmer MicroBeta$^2$ 2450 microplate scintillation counter, with a 1-minute count time per well.

### 2.6 Functional characterisation of $Tg$ApiAT2 in parasites

#### 2.6.1 [$^{13}$C]amino acid labelling of extracellular *Toxoplasma gondii*

In order to determine whether $Tg$ApiAT2 has a role in amino acid homeostasis, I sought to measure the intracellular abundance of amino acids within extracellular tachyzoites. I also wished to compare the ability of parental (TATi/tdTomato) and apiAT2$^{A138−588}$ tachyzoites to take up amino acids. I chose [$^{13}$C] amino acid labelling because both the natural abundance and the uptake of several amino acids can be measured in the same experiment. I exposed parasites to 2 mg/mL [$^{13}$C]algal amino acid mix (Cambridge Isotope Laboratories) in a homemade medium (pH 7.4) containing an ‘RPMI’ vitamin mix (Sigma), 1 mM glucose, 2 mM L-glutamine and the same salt composition as DMEM (§2.4.2.1). I then used gas chromatography-mass spectrometry (GC-MS) to detect the amount of [$^{13}$C] amino acids which had entered the tachyzoites, and the intracellular abundance of amino acids.

I filtered freshly egressed TATi/tdTomato or apiAT2$^{A138−588}$ tachyzoites through a 3 µm filter to remove host cell debris. I counted the parasites, then aliquoted $1\times10^8$ parasites into a tube and pelleted them by centrifugation at 1 500×g for 30 minutes. I decanted the supernatant solution, then resuspended the parasite pellets in the [$^{13}$C]labelled
substrate solution. I transferred the parasite suspensions into 1.5 mL tubes, pre-warmed to 37°C. I incubated the parasites at 37°C in a 5% CO\textsubscript{2} incubator for 15 minutes with the lids open to allow for gas exchange. I terminated the [\textsuperscript{13}C]amino acid uptake by rapidly diluting each sample with 14 mL of ice-cold PBS.

I extracted the parasite metabolites in chloroform:methanol:water (1:3:1 v/v/v) containing 1 nmol scyllo-inositol (Sigma). I dried the aqueous phase of the extraction in a heated speedvac concentrator. I sent the dried samples for processing in the McConville Laboratory (Bio21 Institute, Melbourne). There, samples were methoxymated by treatment with 20 mg/mL methoxyamine in pyridine overnight, then trimethylsilylated by treatment with N,O-bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylsilyl for 1 hour at room temperature. Samples were analysed using GC-MS as described previously [Blume \textit{et al.}, 2015]. The fractional labelling of all detected amino acids was calculated as the fraction of the metabolite pool containing one or more \textsuperscript{13}C-atoms, after correction for natural abundance using the program DExSI, as described previously [Dagley & McConville, 2018]. Total metabolite counts were normalized to scyllo-inositol as an internal standard.

2.6.2 Parasite Metabolite extraction for ultra high performance liquid chromatography

Using GC-MS, I detected changes in the abundance of some amino acids within \textit{apiAT2}\textsuperscript{Δ138--588} parasites compared to wild type controls. As an alternative approach, I used ultra high performance liquid chromatography (uHPLC) to measure the abundances of targeted amino acids in \textit{rApiAT2-HA}, \textit{rApiAT2} and \textit{rApiAT2/cTgApiAT2} parasites grown in the absence or presence of ATc.

Three days prior to the experiment, I infected ten 25 cm\textsuperscript{2} tissue culture flasks containing confluent monolayers of HFF cells with 300 µL of \textit{rApiAT2-HA} parasites from a fully lysed parasite culture. I added ATc to five of the flasks to knockdown ApiAT2-HA expression, giving five technical replicates for each condition. Three days later I harvested the parasites by filtration through a 3 µm filter into ten pre-chilled 15 mL tubes. I pelleted the parasites by centrifugation at 1 500×g for 10 minutes at 0°C. After centrifugation, I immediately placed the tubes into a water-ice slurry to chill the parasite pellets rapidly. I aspirated the growth medium then resuspended the pellets in
1 mL of ice-cold PBS. I transferred this suspension into pre-chilled 1.5 mL tubes. I centrifuged the samples for 1 minute at 12 000×g at 0°C. I repeated this wash step two more times up to a total of three washes in 1 mL of PBS.

To extract the metabolites, I counted the parasites, pelleted them by centrifugation for 1 minute at 12 000×g at 0°C, then resuspended them in 100% methanol. I heated the samples at 65°C for 5 minutes in a heat block and then centrifuged them again for 10 minutes at 21 000×g. I transferred the supernatant into a glass HPLC vial with insert (Chromacol). I incubated the samples in a Speedvac to evaporate the methanol. The dried metabolite extracts were stored at -20°C until they were subsequently run on the HPLC machine.

I modified a previously described amino acid uHPLC detection protocol [Bröer et al., 2016]. Briefly, I resuspended dried extracts in filter sterilised 100 mM NaHCO₃ (pH 9.0) (made up fresh on the day using HPLC grade water) to a final concentration of 3×10⁷ cell equivalents/100 µL. To derivitise the amino acids present in the samples, I added 10 µL of 2,4,6-trinitrobenzenesulfonic acid (TNBSA) to each sample. I also set up amino acid ‘standards’ using a concentration of 100 µM of amino acid in 100 µL of 100 mM NaHCO₃ (pH 9.0) and 2 µL of TNBSA.

I used a Dionex Ultimate3000 uHPLC machine (Thermo Scientific) with a Kinetex 1.7 µ C18 100A column (Phenomex). I loaded 10 µL of samples/standards onto the HPLC column, which I ran at 35°C. I used 100 mM Ammonium acetate (pH 7.0) (made up fresh on the day in MilliQ, Solvent A) and Acetonitrile (Solvent B) as the mobile phase. I used a gradient of buffers as follows: A/B = 90/10% at 0 minutes increasing to 50/50% after 10 minutes and to 100% B after 12 minutes. I detected eluents at λ= 335 nm. I used the Chromeleon Chromatography Data System (Version 7.2; Thermo Scientific) to analyse the data by manually assigning peaks to alanine and by using the area under the curve to determine the amount present.

2.6.3 Radiolabel uptake assays in parasites

To determine whether TgApiAT2 functions as an amino acid transporter in parasites, I directly measured the uptake of radiolabelled amino acids across the plasma
membrane of extracellular tachyzoites. I compared the uptake rates in \( \text{apiAT}2\Delta^{138-588} \) parasites to those in parental and \( \text{apiAT}2\Delta^{138-588}/cTg\text{ApiAT}2 \) complemented parasites.

Three to five days prior to the experiment, I infected confluent monolayers of HFF cells with \( \text{apiAT}2\Delta^{138-588} \), parental or \( \text{apiAT}2\Delta^{138-588}/cTg\text{ApiAT2} \) parasites. On the day of the experiment, I harvested cells by filtration through a 3 \( \mu \)m filter. If the parasite cultures were not fully egressed from the host cells, I first filtered the supernatant through a 3 \( \mu \)m filter to remove host cell debris. I then added 10 mL of fresh Ed1 medium to the remaining cell monolayer. I scraped the monolayer into the new medium, which I then passed through a 26-guage needle to egress the parasites manually, and then filtered them through a 3 \( \mu \)m filter. I found that this technique of only needle passing the portion of parasites that were still intracellular resulted in the highest yield of parasites.

I pooled and counted the manually and naturally egressed parasites. I centrifuged the parasites at 1 500×g for 20 minutes at 4°C. I aspirated the growth medium and washed the parasites in 2 mL of PBS supplemented with 10 mM glucose, pH 7.4 (glucose-PBS). I transferred the cell suspension into 2 mL tubes and centrifuged for 1 minute at 12 000 g, then discarded the supernatant. I resuspended the parasites in glucose-PBS to a concentration of 3×10^8 cells/mL, and pooled all parasites from the same cell line. To provide technical replicates, I aliquoted the parasites into 2 mL tubes with 3×10^7 parasites per timepoint being tested.

To mimic the concentrations of amino acids that parasites might encounter during their growth, I measured the uptake of each radiolabelled substrate in the presence of a mix of unlabelled amino acids. I prepared a ‘[2×] radioactive uptake solution’ consisting of a [2×] unlabelled amino acid mix in glucose-PBS (Table 2.12), and twice the desired final concentration of radiation for the uptake assay. Specifically, for glutamine uptake, I used 0.4 \( \mu \)Ci/mL of \([^{14}\text{C}]\text{glutamine} \) and for arginine uptake I used 0.3 \( \mu \)Ci/mL of \([^{14}\text{C}]\text{arginine} \). I designed the [2×] radioactive uptake solution so that, once I added it to the parasite suspension (containing no amino acids), the uptake assay would be carried out at the desired 1× concentration.

Prior to the start of the time-course, I warmed each of the [2×] radioactive uptake solutions and the parasites suspensions to 37°C in a water bath. To start the time-
Table 2.12: The concentrations of amino acids used in ‘[2×] uptake solution’, dissolved in glucose-PBS. This solution was used during $[^{14}\text{C}]$amino acid uptake experiments on parasites.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>15.6</td>
</tr>
<tr>
<td>Phe</td>
<td>31.26</td>
</tr>
<tr>
<td>Met</td>
<td>62.5</td>
</tr>
<tr>
<td>Thr</td>
<td>62.5</td>
</tr>
<tr>
<td>Leu</td>
<td>62.5</td>
</tr>
<tr>
<td>Ile</td>
<td>62.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>125</td>
</tr>
<tr>
<td>His</td>
<td>125</td>
</tr>
<tr>
<td>Lys</td>
<td>200</td>
</tr>
<tr>
<td>Cys</td>
<td>250</td>
</tr>
<tr>
<td>Val</td>
<td>500</td>
</tr>
<tr>
<td>Arg</td>
<td>574</td>
</tr>
<tr>
<td>Gln</td>
<td>1 000</td>
</tr>
</tbody>
</table>

course, I added the [2×] uptake solution to the parasite suspension at a one to one volume. I mixed the suspension then returned it to the water bath for the duration of the time-course. Ten seconds prior to each of the designated timepoints, I removed 200 µL of the parasite suspension ($3\times10^7$ parasites) from the uptake tubes. I layered the suspension on top of 250 µL of an oil mix comprising 84% (v/v) PM-125 silicon fluid (Clearco) and 16% (v/v) light mineral oil (Sigma), which I had pre-prepared in 1.5 mL tubes. I centrifuged the parasites through the oil layer for 1 minute at 12 000×g. This stopped the uptake of radiolabel by rapidly separating the parasites from the radioactive solution (which remained above the oil layer), forming a pellet of parasites beneath the oil.

To determine the amount of unincorporated radiolabel that was ‘trapped’ between parasite cells in the pellets following centrifugation through the oil layer, I took ‘trap’ samples. For these samples, I mixed the stock parasite suspension with [2×] uptake solution then immediately centrifuged the cells down through the oil. Theoretically, by sampling immediately, any radiation present in the trap samples is likely to represent extracellular substrate, rather than substrate that was taken up by the parasites. To minimise $^{14}$C-substrate uptake during the trap measurement further, I added excess amounts of unlabelled substrate to the uptake solution in order to out-compete uptake of radiolabelled substrate. I added 20 mM of unlabelled glutamine to trap samples for
glutamine uptake experiments, and added 5 mM of unlabelled arginine to trap samples in arginine uptake experiments. When I analysed the data, I subtracted the average counts per minute (CPM) of the trap measurements from the CPM of every other sample in the time-course.

Once all timepoints and traps had been collected, I used the radiation in the solution that remained above the oil layer to take ‘standards’. I aliquoted 10 µL of the solution from five random tubes from each cell line for each substrate and I mixed this with 1.5 mL of UltimaGold™ scintillation fluid (Perkin-Elmer) in a scintillation vial. Given that the standards contain a known amount of unlabelled substrate (in pmol), I could calculate the ratio of unlabelled substrate to the CPM measured in the standards to convert the raw CPM results of the time-course samples to pmol of substrate.

After the standards had been taken, I used a pipette tip attached to a side arm flask with pump to aspirate the radiation from above the oil. I washed the tubes three times in H₂O, and I then aspirated the oil layer, leaving the parasite pellet. I observed that more oil tended to settle on the bottom of the tube after the first aspiration, so I performed a second oil aspiration for each tube.

In order to lyse the parasites and release the radiation, I incubated the pellets at room temperature in 500 µL of 0.1% (v/v) Triton X-100 for at least 30 minutes. Once the parasites were lysed, I resuspended the pellets in the lysis solution which I then transferred to scintillation vials containing 1.5 mL of UltimaGold™ scintillation fluid (Perkin-Elmer). I vortexted the scintillation vials until the scintillation fluid turned clear (~5 seconds) and then measured the radioactivity in each tube with a Perkin Elmer Tri-Carb Scintillation counter T2800, using three-minute counts.

### 2.6.4 [¹³C]glucose flux in extracellular *Toxoplasma gondii*

Earlier experiments had demonstrated that *TgApiAT2* transports glutamine, an important carbon source in *T. gondii* (Introduction §1.8). Therefore, I sought to test whether the loss of *TgApiAT2* would lead to any perturbations in the flux of heavy isotopes of glucose through central carbon metabolism. I exposed extracellular tachyzoites to [¹³C]glucose for 30 or 120 minutes, then extracted the intracellular metabolites for measurement via GC-MS.
I made a glucose and pyruvate-free medium (DMEM salt composition (§2.4.2.1), MEM amino acid solution (M5550, Sigma), MEM non-essential amino acid solution (M7145, Sigma), RPMI vitamin mix (Sigma), pH 7.4) to which I added 1 g/L $^{13}$C glucose (Cambridge Isotope Laboratories) and 4 mM unlabelled glutamine. I incubated each parasite line in this medium at 37°C in a 5% CO$_2$ incubator for 120 min with the lids left open on the tubes to allow for gas exchange.

To test whether $^{13}$C alanine was being exported into the extracellular solution during the course of the $^{13}$C glucose labelling experiment, I took supernatant samples at 0, 15, 30, 60, 90 and 120 minutes. This was of interest because earlier experiments had implicated TgApiAT2 in the export of alanine. To collect a supernatant fraction, I removed 10 µL aliquot from the incubating samples and I stored it on ice. Upon completion of the experiment, I centrifuged the aliquots at 21 000×g for 10 min at 4°C. I then transferred 5 µL of the supernatant into gas chromatography inserts containing 100 µL of 100% methanol and 1 nmol scyllo-inositol.

To harvest parasites containing the incorporated label, I terminated $^{13}$C glucose labelling reactions through rapid cooling, by adding the entire sample to 13 mL of pre-chilled PBS in a water-ice slurry. I centrifuged the samples at 3220×g for 20 minutes at 0°C. I decanted the supernatant, leaving ~200 µL of solution. I resuspended the pellets in the leftover solution, which I then transferred to pre-chilled 1.5 mL tubes. I centrifuged the samples for 10 minutes at 21 000×g at 0°C. I washed the resulting pellets once in ice-cold PBS (without resuspending) then repeated the centrifugation step. Finally, I discarded the supernatant, and kept each of the parasite pellets on ice until all other samples had been harvested in this way.

I extracted metabolites in chloroform:methanol:water (1:3:1 v/v/v) containing 1 nmol of scyllo-inositol. I vortexed the samples, sonicated them, and then centrifuged them at 21 000×g for 10 minutes. I transferred the supernatant into a new 1.5 mL tube containing 100 µL of HPLC grade water, then vortexed, sonicated and centrifuged the samples again. I transferred the aqueous solution into gas chromatography vials, then dried the samples in a SpeedVac. I resuspended the samples in 100 µL of methanol and dried them again in the SpeedVac. I stored the samples at -80°C until their shipment to Melbourne.
The GC-MS analysis was performed by Dr Martin Blume who was, at the time, based in the McConville laboratory, Department of Biochemistry and Molecular Biology, Bio21 Institute of Molecular Science and Biotechnology, University of Melbourne.
Characterisation of ApiAT family proteins: Bioinformatic analysis and studies of their expression and localisation in Toxoplasma gondii tachyzoites

3.1 Introduction

The ApiAT family was originally identified in a bioinformatic survey of candidate transporters from the malaria-causing parasite Plasmodium falciparum. Homologues have been identified in Plasmodium berghei and Toxoplasma gondii, but the full diversity of ApiAT family proteins amongst apicomplexans is poorly understood. In this chapter, I undertake a bioinformatic analysis of the ApiAT family from apicomplexan parasites, and examine the expression and localisation of the ApiAT-family proteins from T. gondii.

3.2 Results

3.2.1 Identification of ApiATs in diverse apicomplexans

To identify ApiAT-family proteins in the apicomplexan parasites T. gondii, Neospora caninum, Eimeria tenella, P. falciparum, P. berghei, Theileria annulata, Babesia bovis and Cryptosporidium parvum, Basic Local Alignment Search Tool (BLAST) searches
were used with each of the five *P. falciparum* ApiATs (NPTs) as initial query sequences ([www.eupathdb.org; Aurrecoechea et al. [2017]]). BLAST searches were also undertaken of the genomes from the chromerids *Chromera velia* and *Vitrella brassicaformis*, which are close free-living relatives of apicomplexans [Moore et al., 2008] ([www.eupathdb.org; www.blast.ncbi.nlm.nih.gov; Aurrecoechea et al. [2017]; Agarwala et al. [2017]). In addition to the previously-described five *Plasmodium* ApiAT family proteins, sixteen ApiAT family proteins were identified in both *T. gondii* and *N. caninum*, eight in *E. tenella*, nine in *T. annulata*, six in *B. bovis*, and one in *C. parvum* (Table 2.1). Candidate ApiAT family proteins were identified in chromerids, however, subsequent analyses revealed that these were more similar to MFS family proteins outside the ApiAT lineage. Therefore, they did not meet all the inclusion criteria to qualify as ApiATs, and were excluded from the subsequent phylogenetic analysis.

### 3.2.2 Sequence alignment reveals conserved regions in ApiATs

A multiple sequence alignment of the 66 ApiAT family proteins was constructed (Figure A.1, Appendix A). Figure 3.1 presents the alignment as a ‘sequence fingerprint’. This presentation (Methods §2.1.3) allows a long protein alignment to be viewed in a relatively small amount of space [inspired by Fröhlich, 1994]. Each residue is represented by a thin vertical line which has been shaded to represent the degree of conservation. All identical residues at a position are shaded blue if 50-70% of sequences have a matching residue in that position, and purple if >70% of sequences have a matching residue at that position. A residue is shaded pink if it is a similar to a consensus amino acid (as defined in Table 2.2). For example, if leucine is shaded blue/purple in a given position then any isoleucines in that same position will be shaded pink. Residues are also shaded pink if no single amino acid represents >50% of sequences but together, a group of similar amino acids (made up of leucines and isoleucines for example) exceed the 50% threshold. Non-conserved residues have been shaded grey and gaps in the sequence are white.
<table>
<thead>
<tr>
<th>TgApiAT1</th>
<th>TgApiAT2</th>
</tr>
</thead>
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</tr>
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§3.2 Results

Figure 3.1: A multiple sequence alignment of the 66 ApiAT proteins identified during this study. Reciprocal protein BLAST searches were used to identify orthologues of the five previously identified *Plasmodium falciparum* ApiAT genes. The sequences were aligned using Clustal Omega Version 1.2.2 [Sievers et al., 2011; Sievers & Higgins, 2018]. Shading for sequence identity was carried out using the TeXshade package for LaTex [Beitz, 2000]. The alignment is presented as a ‘fingerprint’. This presentation allows a long protein alignment to be viewed in a relatively small amount of space [inspired by Fröhlich, 1994]. Each residue is represented by a thin vertical line which has been shaded to represent the degree of conservation. A position is shaded blue if the number of identical residues is between 50-70% of sequences, and purple if >70% of sequences. A residue is shaded pink if it is a similar amino to a ‘consensus’ amino acid, or if no single residue represents >50% of sequences at that position but together, a group of similar residues exceed the 50% threshold. Non-conserved residues have been shaded grey and gaps in the sequence are white. The approximate locations of the predicted transmembrane domains have been represented by numbered bars.

All ApiAT proteins are polytopic proteins, and most are predicted to contain 12 transmembrane domains (www.cbs.dtu.dk/services/TMHMM/; Sonnhammer et al. [1998]), the approximate positions of which have been marked in the alignment figures. The highest sequence similarity was exhibited within these transmembrane domain regions (Figure 3.1). The multiple sequence alignment also revealed the presence of a major facilitator superfamily (MFS) signature sequence [Pao et al., 1998; Reddy et al., 2012] between transmembrane domains 2 and 3 of most ApiAT family proteins (Figure 3.2). These analyses are consistent with previous studies and protein database annotations placing members of the ApiAT family into the major facilitator superfamily of transporters [Rajendran et al., 2017; Martin et al., 2005].

3.2.3 Phylogenetic analysis of the ApiAT family

Understanding the phylogenetic relationships between proteins in a family can provide valuable insights into the evolution of members of the protein family, and may also provide predictions on functional similarities or redundancies. A maximum likelihood phylogenetic analysis revealed the presence of eleven ApiAT subfamilies, defined here as groupings that had greater than 75% bootstrap support (Figure 3.3). Several ApiAT family members from *T. annulata* did not group with any of the subfamilies (and have been named *Ta*ApiAT-ug, ‘ug’ for ‘ungrouped) and the single *C. parvum* ApiAT also did not group with any subfamily. I arbitrarily numbered the subfamilies ApiAT1-11.
Figure 3.2: Excerpt from the multiple sequence alignment showing transmembrane domains two and three and the intracellular loop between them (as marked, top). The 13 amino acid MFS signature sequence has been marked (bottom). The sequence in most MFS families is G-[RKPATY]-L-[GAS]-[DN]-[RK]-[FY]-G-R-[RK]-[RKP]-[LIVGST]-[LIM]. Of the invariant residues, most T. gondii ApiATs have the glycine in position 1, the leucine in position 3, and have the glycine in position 8. The arginine in position 9 is replaced with a proline. Other well conserved residues are the leucine in position 4, the aspartic acid in position 5 and the lysine in position 10. The last three residues in the sequence are not conserved across T. gondii ApiATs, but these are some of the more variable residues of the sequence in MFS families in general [Pao et al., 1998].
Figure 3.3: Consensus maximum likelihood tree of ApiAT family proteins. The tree was generated from a multiple sequence alignment of 66 putative ApiAT proteins from a range of apicomplexans, with 464 residues used in the analysis. Bootstrap values are depicted by black circles (>90% support), blue circles (>75-90% support), or white circles (55-75% support). Bootstrap values were generated by running the tree algorithm 300 times, and the bootstrap value is the proportion of trees in that output with the same groupings as the consensus tree above. The tree is unrooted. Groupings have been designated as a numbered ApiAT ‘subfamily’ if they had at least 70% bootstrap support (Hillis, 1993). Since some *T. annulata* proteins did not group into any subfamily with 75% bootstrap support, these proteins have been designated a ‘-ug’ label, for ‘ungrouped’. Abbreviations: *Pf*ApiAT Plasmodium falciparum, *Pb*ApiAT Plasmodium berghei, *Tg*ApiAT Toxoplasma gondii, *Cp*ApiAT Cryptosporidium parvum, *Bb*ApiAT Babesia bovis, *Ta*ApiAT Theileria annulata, *Nc*ApiAT Neospora caninum, *Et*ApiAT Eimeria tenella.
Orthologues of the ApiAT2 subfamily were present in all organisms in the study with the exception of *Cryptosporidium parvum*, although the single *C. parvum* ApiAT protein grouped with the ApiAT2 subfamily with weak support (Figure 3.3). Members of the ApiAT1, ApiAT3, ApiAT5, ApiAT6 and ApiAT7 subfamilies were restricted to coccidians (a group of apicomplexans that includes *T. gondii* and *N. caninum*), the ApiAT4, ApiAT8, ApiAT9 and ApiAT10 subfamilies were restricted to the *Plasmodium* genus, and the ApiAT11 family was restricted to the piroplasms *T. annulata* and *B. bovis* (Figure 3.3).

### 3.2.4 Expression of *T. gondii* ApiATs

Previous studies demonstrated that the *P. berghei* ApiAT8 protein localized to the periphery of the parasite, most likely to the plasma membrane [Boisson et al., 2011]. *T. gondii* ApiAT1 and ApiAT6-1 also localise to the plasma membrane [Rajendran et al., 2017; Sangaré et al., 2016]. During work pre-dating my PhD, I had determined that *TgApiAT2*, *TgApiAT3-2*, *TgApiAT3-3* and *TgApiAT5-3* are expressed in tachyzoites and localise to the periphery of the parasite, most likely to the plasma membrane [Parker, 2013; Parker et al., 2019]. *TgApiAT3-3* showed additional localisation to the trans-Golgi network [Parker, 2013; Parker et al., 2019]. *TgApiAT6-2* was detectable by western blotting but could not be detected by immunofluorescence assay (IFA), possibly because the level of expression was below the detection limits of IFAs [Parker et al., 2019]. *TgApiAT7-1* does not appear to be expressed in tachyzoites [Parker et al., 2019].

To determine the expression pattern and localisation of the remaining eight ApiAT family proteins in *T. gondii*, I used a CRISPR/Cas9 approach to edit the native locus of each target gene, introducing a hemagglutinin (HA) tag into the 3’ end of the open reading frame of *apiAT3-1*, *apiAT5-1*, *apiAT5-2*, *apiAT5-3*, *apiAT5-4*, *apiAT5-5*, *apiAT5-6*, *apiAT6-3* and *apiAT7-2* (Methods §2.3.3.2). The resulting tagged proteins were therefore expressed from their native promoters, which provides information about their levels of expression in tachyzoite stage parasites [Woodcroft et al., 2012]. The parental strains for these experiments were ∆*ku80* parasites, in which *TgKu80*, a key protein in non-homologous end joining (NHEJ) pathway, has been disrupted [Huynh & Carruthers, 2009; Fox et al., 2009]. These strains favour homologous repair (c.f §2.3.1).
Figure 3.4: Western blot on *T. gondii* members of the ApiAT family. (A) Rat anti-HA was used at 1:3000 to detect *Tg* ApiAT3-1HA3 and *Tg* ApiAT6-3HA3, with goat anti-rat Ig- horseradish peroxidase (HRP) at 1:10 000, whilst rat anti-HA was used at 1:1000 to detect *Tg* ApiAT7-2HA3, with goat anti-rat Ig-HRP) at 1:5000. The image of *Tg* ApiAT3-1HA3 shows a 2 minute exposure and the images of *Tg* ApiAT6-3HA3 and *Tg* ApiAT7-2HA3 show 1 min exposures. (B) The gel was stripped of antibodies, then mouse anti-Gra8, an antibody to a *T. gondii* dense granule protein, was used at 1:2000 dilution to detect Gra8 as a control for even gel loading. Goat anti-rat Ig-HRP was used as the secondary antibody at 1:4000. The images show a 15 second exposure. *Predicted protein mass:* *Tg* ApiAT3-1: 65.1 kDa, *Tg* ApiAT6-3: 74.3 kDa, and *Tg* ApiAT7-2: 109.9 kDa.
Western blotting indicated that \( Tg\text{ApiAT3-1}, Tg\text{ApiAT6-3}, \) and \( Tg\text{ApiAT7-2} \) proteins were expressed in tachyzoite stage parasites (Figure 3.4). A protein with an apparent molecular weight of 132.2 kDa was detected in the sample from \( Tg\text{ApiAT7-2HA3} \), larger than the predicted mass of 109.9 kDa for \( Tg\text{ApiAT7-2} \) (Figure 3.4A). Two potential reasons for the increase in apparent molecular weight are as follows. Firstly, \( Tg\text{ApiAT7-2} \) might have been post-translationally modified, for example by glycosylation, increasing its mass. Secondly, the protein may not have been fully denatured prior to electrophoresis. Since \( Tg\text{ApiAT7-1} \) is made up of approximately equal numbers of anionic amino acid residues (10.5% aspartate and glutamate) and cationic amino acid residues (9.4% arginine and lysine), an unbalance of charge on the protein is not expected to have accounted for aberrant sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) migration.

\( Tg\text{ApiAT3-1} \) has a predicted mass of 65.1 kDa, but a smaller protein with an apparent molecular weight of 58.9 kDa was detected. \( Tg\text{ApiAT6-3} \) has a predicted mass of 74.3 kDa but a protein of 68.2 kDa was detected (Figure 3.4A). This discrepancy may result from N-terminal processing of these proteins. However, ApiAT proteins do have numerous hydrophobic transmembrane helices, which can cause faster-than-predicted migration of proteins on SDS-PAGE [Rath et al., 2009].

I was unable to detect expression of the \( Tg\text{ApiAT5-1}, Tg\text{ApiAT5-2}, Tg\text{ApiAT5-4}, Tg\text{ApiAT5-5}, Tg\text{ApiAT5-6} \) proteins by western blot (Figure 3.5). To determine whether the HA tag was successfully integrated into the target locus, genomic DNA was extracted from transfectants and the 3’ end of the ApiATs of interest were sequenced. This strategy confirmed that the genes were properly tagged with in-frame 3xHA sequences (data not shown).

### 3.2.5 \textit{T. gondii} ApiATs localize to the parasite periphery

To determine the localisation of \( Tg\text{ApiAT3-1-HA3}, Tg\text{ApiAT6-3-HA3} \) and \( Tg\text{ApiAT7-2-HA3} \), I performed immunofluorescence assays. These demonstrated that \( Tg\text{ApiAT3-1} \) and \( Tg\text{ApiAT6-3} \) localized to parasite periphery, overlapping with the plasma membrane marker P30 (Figure 3.6A-B). \( Tg\text{ApiAT7-2-HA3} \) was not detected by IFA, despite being detected by western blot (Figure 3.4), possibly because its level of expression was below the detection limits of IFAs.
Figure 3.5: Western blot on tagged proteins in the ApiAT5 subfamily. TgApiAT5-3 has been tagged by a 1×HA tag and all other members of this subfamily have been tagged with 3×HA tags. All proteins were run on the same gel, transferred onto the same membrane and stained with the same antibody concentrations. (A) Rat anti-HA was used at 1:1000 to detect tagged ApiAT proteins, with goat anti-rat Ig-HRP at 1:5000, and the image shows a 3 minute exposure. A protein of 45.6 kDa is detected in the sample from TgApiAT5-3HA. (B) The gel was stripped of antibodies, then mouse anti-Gra8, an antibody to a T. gondii cytosolic protein, was used at 1:800 dilution to detect Gra8 as a control for even gel loading. Goat anti-rat Ig-HRP was used as the secondary antibody at 1:2000. The image shows a 10 second exposure. Predicted protein mass: TgApiAT5-1, 104.5 kDa; TgApiAT5-2, 93.7 kDa; TgApiAT5-3, 54.5 kDa; TgApiAT5-4, 77.0 kDa; TgApiAT5-5, 62.0 kDa; TgApiAT5-6, 60.2 kDa.
3.3 Discussion

The ApiAT protein family is found in all of apicomplexan species that were analysed. The ApiAT8 family includes the previously described cationic transporter \( \text{PbNPT1} \) [Rajendran et al., 2017], here annotated (and hence renamed) as \( \text{PbApiAT8} \). Although similar in function to the \( T. gondii \) arginine transporter \( \text{TgApiAT1} \) (previously \( \text{TgNPT1} \)), \( \text{PbApiAT8} \) and \( \text{TgApiAT1} \) appear not to be orthologous.

The ApiATs have undergone expansion in various apicomplexan lineages, including \( \text{Plasmodium} \) spp., \( T. gondii \) and piroplasms such as \( B. \text{bovis} \) and \( T. \text{annulata} \), whereas only a single representative is present in \( \text{Cryptosporidium} \) spp., an early-diverging lineage of apicomplexans [Kuo et al., 2008].

These observations are consistent with ancestral apicomplexans having a single ApiAT protein that diversified in various lineages of the phylum to encompass new and/or more discriminating amino acid substrate selectivities.

Figure 3.6: Immunofluorescence assays to determine the subcellular localisation of \( \text{TgApiAT3-1} \) and \( \text{TgApiAT6-3} \) proteins. Anti-HA has been used to detect the tagged ApiAT proteins (green in merge). Samples were co-labelled with antibodies against the plasma membrane marker P30 (red in merge). Images have been artificially coloured and brightened for printing. All scale bars are 2 \( \mu \text{m} \). (A) Subcellular localisation of \( \text{TgApiAT3-1} \) (B) Subcellular localisation of \( \text{TgApiAT6-3} \).
§3.3 Discussion

A similar expansion has been observed in the amino acid/auxin permease (AAAP) family of trypanosomatid parasites, in which fourteen AAAP genes arose from a single AAAP gene locus through a series of gene duplication events in ancestral trypanosomatids [Jackson et al., 2016]. AAAP expansion is likely to reflect an early parasitic innovation that contributed to establishing parasite dependency on the host organism, and thereby facilitating the evolution of parasitism in trypanosomatids [Jackson et al., 2016; Janouškovec & Keeling, 2016]. By contrast, much of the expansion in the ApiAT family appears to have occurred subsequent to the diversification of the major lineages in the phylum. Several subfamilies have undergone expansion within lineages. For example, the ApiAT3, 5, 6 and 7 subfamilies contain multiple members within coccidians (T. gondii, N. caninum and E. tenella), while piroplasms contain multiple ApiAT2 subfamily proteins (Figure 3.3).

Of the eleven ApiAT subfamilies that were defined, only the ApiAT2 subfamily is broadly distributed amongst the major apicomplexan lineages (Figure 3.3), suggesting its presence before these lineages diverged. The single CpApiAT groups weakly with the ApiAT2 subfamily, and given the conservation of the latter in all species of apicomplexan examined, it is plausible that an ApiAT2 like protein could represent the evolutionary origin of the family. This, coupled with the apparent importance of ApiAT2 in P. berghei, [Kenthirapalan et al., 2016] provides significant impetus for further studies on TgApiAT2. The importance and functions of TgApiAT2 will be examined further in chapters 5, 6 and 7 of this thesis.

Much of the expansion of ApiAT proteins, then, appears to have occurred subsequent to the evolution of parasitism in this phylum. An intriguing possibility is that expansion within ApiAT subfamilies is linked to expansion of these parasites into multiple different hosts organisms and cell types. Across their life cycles, apicomplexans such as T. gondii, Plasmodium spp. and piroplasms must infect different definitive and intermediate hosts and, within those hosts, infect different cell types. This may necessitate amino acid transporters with different substrate affinities and specificities. In contrast, C. parvum, the lifecycle of which can be completed within a single host and only infects cells of the intestine, has just a single ApiAT protein. Perhaps the expansion of the ApiAT family in generalist parasites might reflect their broader host range, with additional transporters assisting them in their ability to survive in diverse animal species.
Of the 16 ApiAT proteins in *T. gondii*, 10 are expressed in tachyzoites, and 6 are not [this study; Parker *et al.*, 2019; Rajendran *et al.*, 2017; Sangaré *et al.*, 2016]. Of the 6 proteins that are not expressed, 5 belong to the ApiAT5 sub-family, with *Tg*ApiAT5-3 being the only ApiAT5 subfamily member that is expressed in tachyzoites (Figure 3.5). This raises the possibility that these proteins are expressed, and function, at other stages of the life cycle. Proteomic studies identified *Tg*ApiAT5-5 in the oocyst proteome (www.toxodb.org), and it could be that the function of this transporter has particular importance at this stage of the parasite life cycle. Of the 10 *Tg*ApiAT that are expressed in tachyzoites, 8 proteins have been shown to localise to the periphery of the parasite, likely to the plasma membrane [Parker *et al.*, 2019, ; and this study]. Additionally, *Tg*ApiAT3-3 localised to the trans-golgi network [Parker *et al.*, 2019].

In summary, ten of the sixteen *Tg*ApiAT proteins are expressed in the tachyzoite stage of *T. gondii*. Those with detectable expression by IFA all localize to the periphery of the parasite, most likely to the parasite plasma membrane.
The importance of ApiAT proteins for the growth of Toxoplasma gondii

4.1 Introduction

Previous data indicate that ApiAT family proteins are important for growth in T. gondii and P. berghei [Boisson et al., 2011; Kenthirapalan et al., 2016; Rajendran et al., 2017]. A core aim of this project was to determine which of the 16 ApiATs of T. gondii were important for parasite growth. I chose to use CRISPR/Cas9 gene editing to disrupt each of the ApiAT genes in T. gondii. Similar CRISPR/Cas9-based approaches were used to disrupt other protein families in T. gondii [Shen et al. 2014b; Long et al. 2016; Cao et al. 2017; reviewed in Di Cristina & Carruthers 2018].

4.2 Results

4.2.1 CRISPR/Cas9-mediated gene disruption of fifteen of the sixteen ApiAT-encoding genes.

I used a CRISPR/Cas9-based approach [Shen et al., 2014a] to target the open reading frame of each ApiAT gene (Methods §2.3.3.3). I transfected NHEJ-competent parasite strains, in which DNA repair typically results in small insertions or deletions at the target locus, with a plasmid encoding a single guide RNA (sgRNA) targeting the desired ApiAT locus and the Cas9 endonuclease. When co-expressed, the sgRNA and Cas9 enzyme can mediate the introduction of frameshift mutations into the coding sequence of the target gene [Sidik et al., 2014]. If I did not recover frameshift mutants after the first transfection, I performed a second and, if necessary, a third transfection. In
cases where I was not able to recover frameshift mutants after three transfections, we designed a new CRISPR sgRNA and repeated the transfection process.

Overall, fifteen of the sixteen TgApiAT genes were able to be disrupted genetically using this approach (Table 4.1). Some loci proved easier to disrupt than others, with fewer transfections required to recover frameshift mutants (Table 4.2). Successful disruption of TgApiAT5-1 and TgApiAT5-3 required a second CRISPR sgRNA. In transfections using the first TgApiAT5-1 sgRNA, I suspect that the sgRNA may not have been effective at targeting the apiAT5-1 locus, as I never recovered any parasites with any sort of mutation (Table 4.2). By contrast, the first sgRNA for TgApiAT5-3 was successful in targeting the locus, as parasites with (in frame) mutations were recovered. The difficulty (i.e. multiple transfections) associated with generating this knockout are likely due to the large growth defects resulting from loss of TgApiAT5-3 (see §4.2.2).

The mutant parasites I chose to characterise all had frameshift mutations that lead to the production of truncated (and likely non-functional) proteins. I refer to each mutant on the basis of which amino acids were absent from the final protein (e.g. TgApiAT5-3

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**Table 4.1: ApiAT truncation mutants generated using CRISPR/Cas9-based genome editing**

<table>
<thead>
<tr>
<th>ApiAT</th>
<th>Parental Line</th>
<th>Clone characterised</th>
<th>Mutation</th>
<th>Position of mutation (bp from start codon)</th>
<th>Resultant mutant strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgApiAT1</td>
<td>RHΔhxgprt</td>
<td>1-G9</td>
<td>254 bp insertion</td>
<td>160</td>
<td>apiAT1Δ54-534</td>
</tr>
<tr>
<td>TgApiAT2</td>
<td>TATi/tdTomato</td>
<td>B2</td>
<td>1 bp insertion</td>
<td>411</td>
<td>apiAT2Δ138-333</td>
</tr>
<tr>
<td>TgApiAT3-1</td>
<td>TATi</td>
<td>D2</td>
<td>203 bp insertion</td>
<td>91</td>
<td>apiAT3-Δ152-755</td>
</tr>
<tr>
<td>TgApiAT3-2</td>
<td>TATi</td>
<td>B10</td>
<td>1 bp insertion</td>
<td>484</td>
<td>apiAT3-Δ138-588</td>
</tr>
<tr>
<td>TgApiAT3-3</td>
<td>TATi</td>
<td>E6</td>
<td>1 bp deletion</td>
<td>552</td>
<td>apiAT3-Δ31-599</td>
</tr>
<tr>
<td>TgApiAT5-1</td>
<td>TATi/tdTomato</td>
<td>C5</td>
<td>1 bp insertion</td>
<td>716</td>
<td>apiAT5-Δ152-397</td>
</tr>
<tr>
<td>TgApiAT5-2</td>
<td>TATi/tdTomato</td>
<td>D8</td>
<td>55 bp insertion</td>
<td>668</td>
<td>apiAT5-Δ152-255</td>
</tr>
<tr>
<td>TgApiAT5-3</td>
<td>TATi/tdTomato</td>
<td>D9</td>
<td>1 bp insertion</td>
<td>687</td>
<td>apiAT5-Δ31-599</td>
</tr>
<tr>
<td>TgApiAT5-4</td>
<td>TATi/tdTomato</td>
<td>B6</td>
<td>41 bp insertion</td>
<td>1096</td>
<td>apiAT5-Δ184-755</td>
</tr>
<tr>
<td>TgApiAT5-5</td>
<td>TATi/tdTomato</td>
<td>E3</td>
<td>2 bp insertion</td>
<td>232</td>
<td>apiAT5-Δ184-575</td>
</tr>
<tr>
<td>TgApiAT5-6</td>
<td>TATi/tdTomato</td>
<td>D5</td>
<td>1 bp deletion</td>
<td>58</td>
<td>apiAT5-Δ201-548</td>
</tr>
<tr>
<td>TgApiAT6-1</td>
<td>TATi/tdTomato</td>
<td>−</td>
<td>Two clones with a 3 bp insertion and one clone with a 3 bp deletion</td>
<td>236</td>
<td>N/A (no changes in reading frame)</td>
</tr>
<tr>
<td>TgApiAT6-2</td>
<td>TATi/tdTomato</td>
<td>D3</td>
<td>1 bp deletion</td>
<td>602</td>
<td>apiAT6-Δ201-604</td>
</tr>
<tr>
<td>TgApiAT6-3</td>
<td>TATi/tdTomato</td>
<td>E5</td>
<td>1 bp insertion</td>
<td>509</td>
<td>apiAT6-Δ201-604</td>
</tr>
<tr>
<td>TgApiAT7-1</td>
<td>TATi/tdTomato</td>
<td>C2</td>
<td>1 bp insertion</td>
<td>601</td>
<td>apiAT7-Δ152-601</td>
</tr>
<tr>
<td>TgApiAT7-2</td>
<td>TATi/tdTomato</td>
<td>B6</td>
<td>983 bp insertion</td>
<td>2810</td>
<td>apiAT7-Δ152-601</td>
</tr>
</tbody>
</table>
Table 4.2: Summary of transfections with CRISPR/Cas9 vectors targeting *T. gondii* ApiATs for disruption. The table summarises the number of clones sent for sequencing in each CRISPR/Cas9 vector transfection and the results of that sequencing. The ‘mutations without frameshift’ column refers to mutants with insertions and deletions that were in multiples of three nucleotides. The ‘mutations with frameshift’ column refers to mutants with insertions and deletions of nucleotides that were not in multiples of three. Greyed out cells indicate that the transfection was not required because a truncation mutant had already been isolated.

<table>
<thead>
<tr>
<th>Gene Transfection</th>
<th>Transfection A</th>
<th>Transfection B</th>
<th>Transfection C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of clones</td>
<td>Total No mutations Mutations without frameshift Mutations with frameshift Total No mutations Mutations without frameshift Mutations with frameshift Total No mutations Mutations without frameshift Mutations with frameshift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sent for sequencing and results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TgApiAT1 (Grown in RPMI)</td>
<td>7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT2</td>
<td>6</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT3-1</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT3-2</td>
<td>0</td>
<td>(no clones recovered)</td>
<td>3</td>
</tr>
<tr>
<td>TgApiAT3-3</td>
<td>1</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT5-1 CRISPR Site 1</td>
<td>6</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT5-1 CRISPR Site 2</td>
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<td>2</td>
<td>—</td>
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<td>2</td>
<td>—</td>
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<tr>
<td>TgApiAT5-3 CRISPR Site 2</td>
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<td>TgApiAT5-4</td>
<td>4</td>
<td>2</td>
<td>—</td>
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<tr>
<td>TgApiAT5-5</td>
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<td>—</td>
</tr>
<tr>
<td>TgApiAT5-6</td>
<td>2</td>
<td>1</td>
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<td>TgApiAT6-1</td>
<td>3</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT6-2</td>
<td>0</td>
<td>(no clones recovered)</td>
<td>6</td>
</tr>
<tr>
<td>TgApiAT6-3</td>
<td>3</td>
<td>—</td>
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<td>TgApiAT7-1</td>
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<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TgApiAT7-2</td>
<td>3</td>
<td>1</td>
<td>—</td>
</tr>
</tbody>
</table>
encodes a protein of 504 amino acids; the \( TgApiAT5-3 \) mutant is truncated from residue 188 and I refer to it as \( apiAT5-3^{\Delta 188-504} \).

Another member of the lab, Dr Esther Rajendran, found that \( apiAT1 \) could only be disrupted when parasites were grown in excess arginine. They were also unable to recover parasites with frameshift mutations at the \( apiAT6-1 \) locus despite screening 12 clones from three separate transfections of a sgRNA targeting the \( TgApiAT6-1 \) locus. Of these clones, two had a 3 bp insertion and one had a 3 bp deletion, indicating that the guide RNA was capable of targeting the \( TgApiAT6-1 \) locus (Table 4.1, Table 4.2).

### 4.2.2 Growth assays on truncation mutants identifies three family members that are important for parasite growth.

To determine which \( TgApiATs \) were important for parasite growth in human foreskin fibroblasts, I performed plaque assays (Methods §2.4.2.1) on each of the genetically disrupted \( TgApiAT \) lines. \textit{In vitro} \( T. gondii \) culturing is routinely undertaken in flasks containing adherent monolayers of mammalian cells (human foreskin fibroblasts) in Dulbecco’s modified Eagle’s medium (DMEM). As parasites undergo repeated lytic cycles, they destroy regions of the host cell monolayer within each flask, creating macroscopic zones of clearance termed plaques. In a plaque assay, the fitness of mutant parasites is assessed by comparing the size of the plaques generated by the mutant strain to the size of the plaques generated by the parental strain.

#### 4.2.2.1 Growth in commercial medium

I performed plaque assays for the \( apiAT1^{\Delta 54-534} \) strain and its parental line, \( RH \Delta hgxprt \), in both DMEM and Roswell Park Memorial Institute 1640 (RPMI) medium (Figure 4.2A). I did not observe plaques in the \( apiAT1^{\Delta 54-534} \) strain when it was grown in DMEM (containing 400 \( \mu \)M arginine) but observed normal growth of this strain when it was grown in RPMI medium (containing 1.15 mM arginine) (Figure 4.2A), replicating previous results with \( TgApiAT1 \) knockout parasites [Rajendran et al., 2017]. I measured the size of the plaques in each of three independent experiments and calculated the plaque area generated by \( apiAT1^{\Delta 54-534} \) parasites in each medium as a percentage of the plaque area of the parental line grown in DMEM. Plaque area of the \( apiAT1^{\Delta 54-534} \) strain was reduced to zero when it was grown in DMEM, which was a
statistically significant reduction compared to the plaque area generated by the parental line grown in DMEM (Figure 4.1, p<0.001, one-way ANOVA with Dunnett’s multiple comparison test). Plaque area of the apiAT1Δ54−534 strain was not significantly different from parental when grown in RPMI (Figure 4.1, p>0.05, one-way ANOVA with Dunnett’s multiple comparison test).

I performed plaque assays (in DMEM) on the other truncation mutants and compared the average plaque area in each to the average plaque area of the parental strains (Figure 4.1). The average plaque area observed for each of the ApiAT3 family truncation mutants was calculated as a percentage of the average plaque area observed for the parental TATi strain. The plaque area for all other mutants was calculated as a percentage of that observed for TATi/tdTomato strain. I observed significantly reduced plaque sizes in the apiAT2Δ138−588 and apiAT5-3Δ188−504 strains relative to that observed for the parental strain (Figure 4.1, one-way ANOVA with Dunnett’s multiple comparison test). On average, plaque area was 26±16% of parental in the apiAT2Δ138−588 strain and 26±4% of parental in the apiAT5-3Δ188−504 strain (mean±standard error). By contrast, the plaque size in the remaining 12 mutant apiAT lines when grown in DMEM was not significantly different from that seen for the parental controls (one-way ANOVA with Dunnett’s multiple comparison test).

4.2.2.2 Growth in Minimal Amino Acid Medium

Some ApiAT family proteins transport amino acids [Rajendran et al., 2017]. DMEM is a medium rich in amino acids, with most amino acids present in DMEM at concentrations higher than those present in human plasma (see Table 2.10). It is conceivable that any growth phenotypes of ApiAT mutant parasites may have been masked by the artificially high concentrations of amino acids available in DMEM. I therefore tested the growth of each of the mutant apiAT strains in a medium containing reduced concentrations of amino acids. The formulation of ‘minimal amino acid medium’ (MAAM) is outlined in Methods §2.4.2.1 (Table 2.10). In the same experiment as the one discussed in §4.2.2.1, I also performed plaque assays with each parental and mutant strain grown in MAAM.

I tested the growth of the apiAT1Δ54−534 strain and its parental strain in DMEM, RPMI and MAAM. I observed that the parental strain was able to grow in all three me-
Figure 4.1: Growth of ApiAT truncation mutants under routine culture conditions. The data comes from three independent experiments. Each data point represents the average area of the individual plaques generated by the truncation mutant. This average has been expressed as a percentage of the average area of the individual plaques generated by the parental control in the same experiment. The red bars show the mean of the three independent experiments. The plaque areas of ApiAT3 truncation mutants are expressed as a percentage of the plaque area of their parental line TATi. The plaque areas of *apiAT1*Δ54−534 in both DMEM or RPMI are expressed as a percentage of the plaque area of the parental line RHΔHX grown in DMEM. The plaque area of all other mutants is expressed as a percentage of the plaque area of TATi/tdTomato. **, p<0.01; ***, p<0.001; one-way ANOVA with Dunnett’s Multiple Comparison Test, p values shown to the right of each bar.
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Figure 4.2: Plaque assay on the TgApiAT1 truncation mutant. (A) Plaque assay on apiAT1Δ54−534 and its parental strain RHΔhxgprt, grown in DMEM, MAAM or RPMI, a medium which has previously been shown to be conducive to growth of apiAT1 knockout parasites [Rajendran et al., 2017]. 150 parasites were added per well and cultures were incubated for 9 days. All images are from the same experiment. The data is representative of three independent experiments. In all three repeats, apiAT1Δ54−534 parasites were unable to grow in DMEM but formed small plaques in minimal amino acid media, and larger plaques in RPMI. (B) The average area of the individual plaques formed by the parental strain and the apiAT1Δ54−534 strain grown in either DMEM, MAAM or RPMI, expressed as a percentage of the average area of the plaques formed by the parental strain in DMEM within each experiment. Data shows mean ± SEM from three independent experiments. n.d. = not detected. **, p<0.01; ***, p<0.001; two-way ANOVA with Bonferroni post-tests.

dia (Figure 4.2A). The area of plaques seen for the parental strain were larger in RPMI than in DMEM or MAAM, however on average, this difference was not statistically significant. Statistically, there was no significant difference in the plaque area of the parental line grown in DMEM, RPMI or MAAM (Figure 4.2B, two-way ANOVA with Bonferroni post-tests). The apiAT1Δ54−534 strain formed no plaques in DMEM but was able to grow in RPMI, and also formed plaques in MAAM (Figure 4.2A). However, the average area of the plaques formed by the apiAT1Δ54−534 strain in MAAM was significantly reduced compared to the area of the plaques it formed in RPMI (Figure 4.2B). I conclude that the formulation of MAAM is conducive to, but not optimal for, growth of the apiAT1Δ54−534 mutant.

Next, I tested the growth of the apiAT2Δ138−588 strain and its parental strain in DMEM and MAAM. The average size of the plaques formed by the parental line
was smaller in MAAM than in DMEM however this difference did not reach statistical significance (Figure 4.3A). Compared to parental parasites, the \( \text{apiAT}^2_{\Delta 138-588} \) strain was strongly attenuated in growth, with greatly reduced plaque size and numbers in both DMEM and MAAM (Figure 4.3B). The average area of the plaques formed by \( \text{apiAT}^2_{\Delta 138-588} \) parasites compared to those formed by the parental strain was significantly reduced in both media (Figure 4.3A). However, although \( \text{apiAT}^2_{\Delta 138-588} \) tended to form smaller plaques in MAAM compared to DMEM, this difference was not statistically significant (Figure 4.3A). Notably, the plaque assays for \( \text{apiAT}^2_{\Delta 138-588} \) varied somewhat across the three experiments (Figure 4.3C). In the first experiment, \( \text{apiAT}^2_{\Delta 138-588} \) formed no plaques in either media. In the second experiment small plaques were formed in both media and in the third (see Figure 4.3B) there were a few small plaques formed in DMEM but no plaques in MAAM. Overall, I conclude that \( \text{apiAT}^2_{\Delta 138-588} \) is severely attenuated in growth, and whilst culturing in MAAM reduced the growth of this strain further, the exacerbation of the growth defect of \( \text{apiAT}^2_{\Delta 138-588} \) did not reach statistical significance.

Plaque assays on truncation mutants from the ApiAT3 subfamily, and their parental strain, revealed no major differences in growth between any of the strains in MAAM (Figure 4.4A). For parasites grown in either DMEM or MAAM, there were no significant differences in the average plaque area between the mutant strains and the parental control (Figure 4.4B; two-way ANOVA with Bonferroni post-test comparing each strain to the parental strain grown in the same medium). For each strain, including the parental strain, the plaques formed in MAAM were slightly smaller than those formed in DMEM (Figure 4.4A-B). I performed a two-way ANOVA using the factors ‘strain’ and ‘medium’. Medium was a significant source of variation within the experiment, meaning that overall, culture in MAAM did reduce growth. However, in post-hoc testing, the difference in each individual strain’s average plaque area between DMEM and MAAM was not significant (Bonferroni post tests). In the ANOVA I also tested whether there was an interaction between the factors ‘strain’ and ‘medium’. This statistical test tells one whether the effect that culture in MAAM has on plaque area depends on which strain is being cultured. The interaction between the factors ‘strain’ and ‘medium’, was not significant, meaning that statistically, MAAM reduced the average plaque area in all strains to the same extent. I conclude that proteins of the ApiAT3 subfamily are not required for the growth of \( T. gondii \) in routine culture conditions or in conditions with reduced amino acids.
Figure 4.3: Plaque assay on the TgApiAT2 truncation mutant. (A) The average area of the plaques formed by the parental strain (TATi/tdTomato) and the apiAT2Δ138−588 strain grown in either DMEM or MAAM, expressed as a percentage of the average plaque area achieved by the parental strain in DMEM within each experiment. Data mean±SEM from three independent experiments. *** p<0.001; two-way ANOVA with Bonferroni post-tests. (B) Representative plaque assay on apiAT2Δ138−588 and parental TATi/tdTomato parasites grown in DMEM or MAAM. 150 parasites were added per well and cultures were incubated for 9 days. (C) Average area of the plaques formed by parental and apiAT2Δ138−588 parasites grown in DMEM or MAAM in each experiment. Bars represent the average area of the (single, i.e. non-fused) plaques in each condition and error bars represent the standard deviation in plaque area. n.d. = not detected.
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**Figure 4.4:** Plaque assay on truncation mutants from the ApiAT3 subfamily. (A) A representative plaque assay on ApiAT3 truncation mutants and their parental line (TATi), grown in DMEM or MAAM. All images are from the same experiment and are representative of three independent experiments. 150 parasites were added per well and cultures were incubated for 9 days. (B) The average area of the plaques formed by parental, \textit{apiAT3-1}\textsuperscript{Δ31-599}, \textit{apiAT3-2}\textsuperscript{Δ162-728} and \textit{apiAT3-3}\textsuperscript{Δ184-755} strains grown in either DMEM or MAAM, expressed as a percentage of the average plaque area in the parental strain in DMEM within each experiment. Data show mean±SEM from three independent experiments. In the two-way ANOVA, ‘medium’ was a significant source of variation, however in Bonferroni post-tests, there were no significant differences in the average plaque area produced by the same strain in MAAM or DMEM. The interaction between the variables ‘strain’ and ‘medium’ was not significant, and there were no significant differences between any of the strains and the TATi parental control.
In the same assay that I tested the growth of $apiAT2^{\Delta 138-588}$ in MAAM, I also tested the growth of truncation mutants from the ApiAT5, ApiAT6 and ApiAT7 subfamilies. All of these mutants were also derived from the TATi/tdTomato parental strain. I grew each strain in both DMEM and MAAM. Only the growth of $apiAT5-3^{\Delta 188-504}$ parasites appeared to be further attenuated by culturing the parasites in MAAM (Figure 4.5A). There were no other major differences in growth (Figure 4.5A-C). In both DMEM and MAAM, the only significant differences between parental and mutant strains was the reduction in plaque area in $apiAT5-3^{\Delta 188-504}$ and $apiAT2^{\Delta 138-588}$ parasites, which was significant in both DMEM and MAAM (Figure 4.6). However, as for $apiAT2^{\Delta 138-588}$ parasites, the reduction in plaque area of $apiAT5-3^{\Delta 188-504}$ parasites in MAAM compared to DMEM was not statistically significant (Figure 4.6).

Across the three replicates of the experiment, I observed a general trend of the plaque area being smaller for most strains when they were cultured in MAAM, similar to the trend in the plaque assays on the ApiAT3 truncation mutants (Figure 4.6). Again, ‘medium’ was a significant source of variation in a two-way ANOVA. In post-hoc testing, the reduction in plaque area as a result of culturing in MAAM was only statistically significant for the $apiAT5-6^{\Delta 20-548}$ strain (Figure 4.6, $p<0.05$, Bonferroni post-tests). However, post-hoc tests comparing the growth of the $apiAT5-6^{\Delta 20-548}$ strain in MAAM to the growth of the parental strain in MAAM revealed no significant differences, and this is in fact the more valid comparison (see §4.3.2).

In summary, these results indicate that $Tg$ApiAT1, $Tg$ApiAT2 and $Tg$ApiAT5-3 are required for normal intracellular growth of $T. gondii$ in standard in vitro culture conditions. The growth defects in the $apiAT2^{\Delta 138-588}$ and $apiAT5-3^{\Delta 188-504}$ strains were exacerbated in MAAM, but not significantly so. By contrast, the $apiAT1^{\Delta 54-534}$ strain grew better in MAAM. Growth in medium with reduced amino acid concentrations did not reveal any growth phenotypes in the other ApiAT truncation mutants.

4.2.3 Complementation of $apiAT2^{\Delta 138-588}$ mutants with constitutively expressed $Tg$ApiAT2 restores growth

The CRISPR/Cas9 system has, by its nature, the potential to produce off-target effects. If part of the sgRNA binds with DNA that was not the intended target, this can produce base pair mismatches in the rest of the sgRNA [Zhang et al., 2015]. This bind-
Figure 4.5: Plaque assay on truncation mutants from the ApiAT5, ApiAT6 and ApiAT7 subfamilies. A representative plaque assay on truncation mutants and parental line TATi/tdTomato, grown in DMEM or MAAM. Images are from a plaque assay representative of three independent experiments. 150 parasites were added per well and plaques were grown for 9 days. All images are from the same experiment. (A) ApiAT5 family (B) ApiAT6 subfamily (C) ApiAT7 subfamily.
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#### Figure 4.6: Plaque area of truncation mutants derived from TATi/tdTomato.  

The average area plaques formed by the parental strain (TATi/tdTomato) and the all truncation mutants derived from TATi/tdTomato, grown in either DMEM or MAAM, expressed as a percentage of the plaque area achieved by the parental strain in DMEM within each experiment. Data show mean±SEM from three independent experiments. The stars in black: ***, p<0.001; two-way ANOVA with pairwise Bonferroni post-tests comparing each mutant to the parental strain grown in the same medium. The stars in blue: *, p<0.05; two-way ANOVA, this time with Bonferroni post-tests comparing growth between the two media for each strain. There were no other significant differences in any of the post tests. *apiAT2Δ138–588* is shown for completeness but represents the same data as shown in Figure 4.3.

ing could be sufficient to allow the Cas9 protein to mediate ‘off-target’ DNA mutations [Zhang et al., 2015]. This raises the possibility that off-target effects could have contributed to the severity of growth defects in the *apiAT2Δ138–588* and *apiAT5-3Δ188–504* strains. It is also conceivable that the truncated protein products of the disrupted genes have toxic effects in the parasite. It was therefore important to test whether the growth defects in the *apiAT2Δ138–588* and *apiAT5-3Δ188–504* strains resulted specifically from the loss of these proteins.

Complementation studies have shown that the observed growth defect in the *apiAT5-3Δ188–504* strain is due to the loss of *TgApiAT5-3* [Parker et al., 2019]. To test whether growth defects I observed in the *apiAT2Δ138–588* strain were due to loss of *TgApiAT2*,
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I complemented the \textit{apiAT2^{Δ138−588}} strain with a constitutively expressed version of \textit{TgApiAT2} (Methods §2.3.4.4). The constitutively expressed version had an HA epitope tag at the C-terminus. To determine if the complemented cell line was expressing \textit{TgApiAT2-HA}, I performed a western blot, in which I detected a HA tagged protein of 57 kDa (Figure 4.7A). This is lower than the predicted 63 kDa mass of HA-tagged \textit{TgApiAT2}. However, hydrophobic membrane proteins commonly run faster than predicted on SDS-PAGE [Rath \textit{et al.}, 2009], and the mass of the overexpressed protein matches the mass observed for the \textit{TgApiAT2} protein when HA-tagged at the native locus [Parker \textit{et al.}, 2019]. This suggests that the \textit{TgApiAT2} is correctly expressed in the complemented \textit{apiAT2^{Δ138−588}} strain (which I henceforth refer to as \textit{apiAT2^{Δ138−588}/cTgApiAT2}, ‘c’ for complemented). The overexpressed protein localised to the periphery of the parasites (Figure 4.7B), a localisation that is also consistent with the localisation of the native protein [Parker \textit{et al.}, 2019].

I next used plaque assays to compare the growth of \textit{apiAT2^{Δ138−588}/cTgApiAT2} to that of parental and \textit{apiAT2^{Δ138−588}} parasites. Complementation of the \textit{apiAT2^{Δ138−588}} with a constitutive copy of \textit{TgApiAT2} restored parasite growth to parental levels (Figure 4.8). These results imply that neither off-target effects of the CRISPR/Cas9 system, nor toxic effects of the truncated \textit{apiAT2^{Δ138−588}} protein were responsible for the growth defect observed in \textit{apiAT2^{Δ138−588}} parasites.
Figure 4.8: *Complementation of the TgApiAT2 truncation mutant restores its growth.* Two independent plaque assays on the parental line, *apiAT2*Δ*138–588*, *apiAT2*Δ*138–588/cTgApiAT2* grown in Ed1 from commercial DMEM. Experiment 1 was performed in 6 well plates with 150 parasites/well and grown for 9 days. Experiment 2 was performed in T25 flasks with 400 parasites/25cm² flask and grown for 9 days.

### 4.2.4 Studies in redundancy (double and triple knockouts)

The work described above establish that most of the genes in the ApiAT family are dispensable for the growth of tachyzoite stage parasites. For some of these proteins, this may be explained by the fact that they are not expressed in tachyzoites. This applies to five of the *TgApiAT* proteins from the ApiAT5 subfamily (all save *TgApiAT5-3*) and to *TgApiAT7-1*, none of which are detectable by western blot (Figure 3.5 and Parker *et al.* [2019]).

In contrast, none of the members of the ApiAT3 subfamily are individually important for *in vitro* growth (Figure 4.4), yet all three are expressed in tachyzoites (Figure 3.4 and Parker *et al.*, 2019). There are a number of possibilities as to why these genes are non-essential. Firstly, if the encoded proteins are functional transporters, the substrate they transport might be non-essential for the growth of tachyzoites. An example of a
transporter that satisfies this description is $TgGT1$ – the major glucose transporter in the parasite [Blume et al., 2009]. Secondly, members of the ApiAT3 subfamily could be functional transporters that mediate the uptake or extrusion essential substrates, but are redundant in function with other transporters. In this scenario, the loss of an individual transporter can be overcome by the presence of other transporters that mediate the same process. An example of a transporter in this category is $TgApiAT1$; when $TgApiAT1$ is knocked out, normal growth can occur at high arginine concentrations, since parasites can take up arginine via an alternate arginine uptake pathway [Rajendran et al., 2017].

This raises the possibility that the ApiAT3 subfamily members may have redundant or overlapping functions with each other, and may therefore be collectively important for parasite growth. Consistent with this hypothesis, the three ApiAT3 proteins group together phylogenetically (Figure 3.3), and $TgApiAT3-2$ and $TgApiAT3-3$ have syntenic introns, implying that a recent duplication event gave rise to these two proteins. A direct method through which to study redundancies between proteins is to create double (or triple etc.) knockouts of the genes encoding the proteins that are suspected to be redundant.

I hypothesised that knocking out two or more members of the ApiAT3 subfamily may be ‘ synthetically lethal’, which is defined as the simultaneous perturbation of two genes resulting in cellular or organismal death [Nijman, 2011]. I used the same CRISPR/Cas9 vectors that I used to generate the original ApiAT3 subfamily truncation mutants (§4.2.1) and transfected the vectors into the $apiAT3-2^{Δ162−728}$ and $apiAT3-3^{Δ184−755}$ cell lines. After several attempts, I generated mutant parasites with double gene disruptions of $apiAT3-1/apiAT3-2$, $apiAT3-1/apiAT3-3$ or $apiAT3-2/apiAT3-3$. I was then able to disrupt $apiAT3-1$ in the double-disrupted $apiAT3-2/apiAT3-3$ parasites to generate a cell line which was disrupted at all three ApiAT3 loci. I used a plaque assay to test the growth of the double and triple ApiAT3 subfamily truncation mutants compared to their parental line, TATi, under routine culture conditions (i.e. in DMEM). All the double and triple truncation mutants had approximately equal numbers of similar sized plaques compared to the parental control (Figure 4.9). I conclude that the collective loss of the ApiAT3 subfamily proteins is not synthetically lethal, at least under routine growth conditions.
4.3 Discussion

The data presented in this chapter indicated that 15 of the 16 *T. gondii* ApiAT genes can be genetically disrupted. Three of the disrupted cell lines had significant growth defects compared to parental controls under *in vitro* culture conditions, in both complete growth medium, and in medium with reduced amino acid concentrations.

4.3.1 Consistency with other studies

4.3.1.1 Sidik et al. [2016]

While this work was underway, Sidik et al. [2016] published a genome-wide CRISPR knockout screen of all annotated *T. gondii* genes. The study assigned a ‘phenotype score’ to every gene, which correlates to how important that gene is for the fitness of tachyzoites during the lytic cycle. The authors found that genes which have previously been shown to be essential had phenotype scores less than -2.

The results of the ApiAT CRISPR/Cas9 disruption screen presented here agree with the phenotype scores for ApiAT proteins reported by Sidik et al. [2016] (cf. Table 4.3). Twelve of the ApiAT truncation mutants did not have growth defects under the conditions tested here. Of these, eleven of the disrupted genes had phenotype scores greater than -2 (and one was not tested, Table 4.3). Out of the non-essential *apiAT* genes, *apiAT7*-2 has the lowest phenotype score, at -0.93 [Sidik et al., 2016]. *apiAT6-1*, which we were unable to disrupt genetically, has a phenotype score of -5.4 [Sidik...
suggesting that the TgApiAT6-1 protein may be essential for parasite growth in vitro.

Table 4.3: Phenotype scores of T. gondii ApiAT genes. Genes with a score below -2 were fitness conferring for tachyzoites grown in vitro in the genome-wide CRISPR knockout screen performed by Sidik et al. [2016]. The results of this study are included for comparison. ‘Essential’ means the gene was unable to be knocked out, ‘Non-essential’ refers to genes that were able to be knocked out with no growth defects, and ‘Growth defect’ refers to genes that were able to be knocked out but had severe growth defects under normal culture conditions.

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene ID</th>
<th>Phenotype Score</th>
<th>Result of this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgApiAT1</td>
<td>TGGT1_215490</td>
<td>-3.91</td>
<td>Growth defect</td>
</tr>
<tr>
<td>TgApiAT2</td>
<td>TGGT1_320020</td>
<td>-4.11</td>
<td>Growth defect</td>
</tr>
<tr>
<td>TgApiAT3-1</td>
<td>TGGT1_318150</td>
<td>-0.09</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT3-2</td>
<td>TGGT1_248420</td>
<td>1.33</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT3-3</td>
<td>TGGT1_220600</td>
<td>0.93</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT5-1</td>
<td>TGGT1_248610</td>
<td>-0.75</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT5-2</td>
<td>TGGT1_205520</td>
<td>0.59</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT5-3</td>
<td>TGGT1_257530</td>
<td>-4.73</td>
<td>Growth defect</td>
</tr>
<tr>
<td>TgApiAT5-4</td>
<td>TGGT1_216710</td>
<td>0.51</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT5-5</td>
<td>TGGT1_293420</td>
<td>1.45</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT5-6</td>
<td>TGGT1_293420</td>
<td>1.45</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT5-7</td>
<td>TGGT1_240810</td>
<td>-5.4</td>
<td>Essential</td>
</tr>
<tr>
<td>TgApiAT6-1</td>
<td>TGGT1_290860</td>
<td>1</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT6-2</td>
<td>TGGT1_249580</td>
<td>0.47</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT7-1</td>
<td>TGGT1_263230</td>
<td>0.69</td>
<td>Non-essential</td>
</tr>
<tr>
<td>TgApiAT7-2</td>
<td>TGGT1_263260</td>
<td>-0.93</td>
<td>Non-essential</td>
</tr>
</tbody>
</table>

The apiAT1, apiAT2 and apiAT5-3 genes have phenotype scores of -3.91, -4.11 and -4.73, respectively [Sidik et al., 2016], consistent with the apiAT1Δ54–534, apiAT2Δ138–588 and apiAT5–3Δ188–504 truncation mutants having large growth defects (in DMEM). We were only able isolate parasites with disruptions in apiAT1 when we grew the transfectants in RPMI, a medium in which the gene is non-essential [Rajendran et al., 2017].

It is notable that we were able to isolate parasites with frameshift mutations in apiAT2 and apiAT5-3, genes which both have phenotype scores below -4, despite growing the transfectants in DMEM, a medium in which these genes (on the basis of the phenotype scores) are predicted to be essential. The isolation of these mutants may have
been a rare event, as in both cases, only one truncation mutant was isolated from multiple transfections (Table 4.2). The fact that we were able to generate these cell lines highlights the power of CRISPR/Cas9 gene editing. It is unlikely that knockouts of the \textit{apiAT2} and \textit{apiAT5-3} genes would ever be isolated using conventional homologous recombination methods, because true knockouts would be out-competed during the long drug selection process, by parasites that had integrated the drug resistance cassette at a different locus. In the CRISPR/Cas9 method, clones are isolated three days post-transfection by sorting parasites directly into 96 well plates (Methods §2.3.3), decreasing the time in which natural selection against the knockouts can occur.

4.3.1.2 Rajendran \textit{et al.} [2017]

The data presented here show that, as expected, the \textit{apiAT1}\textsuperscript{Δ54−534} strain was able to grow in RPMI, but not in DMEM. \textit{apiAT1}\textsuperscript{Δ54−534} parasites also formed plaques in MAAM, though of a significantly reduced size compared to those it formed in RPMI (Figure 4.2). Rajendran \textit{et al.} [2017] found that parasites lacking \textit{Tg}ApiAT1, an arginine transporter, could not grow in DMEM, a medium containing 400 \textmu M arginine and 800 \textmu M lysine. The authors varied the arginine concentration in the medium, and observed that the growth of Δ\textit{Tg}ApiAT1 parasites was rescued by high arginine concentrations [Rajendran \textit{et al.}, 2017]. They also observed that growth was rescued when the knockout parasites were cultured in medium containing 400 \textmu M arginine, in which the lysine concentration was reduced to below 200 \textmu M [Rajendran \textit{et al.}, 2017]. The authors concluded that Δ\textit{Tg}ApiAT1 parasites could grow in medium containing a high arginine to lysine ratio, and hypothesised the existence of a second arginine uptake pathway, that could also take up lysine. Uptake of arginine through this alternative pathway is thought to be blocked at high lysine concentrations, since lysine can compete with arginine for uptake through this pathway.

Of the media tested here the concentration of arginine is highest in RPMI (1.15 mM) followed by DMEM (400 \textmu M) then MAAM (287 \textmu M), which does not match the growth pattern I observed (plaque area in RPMI > MAAM > DMEM). This implies that the absolute concentration of arginine is not what determines whether a medium is conducive to growth of parasites in which \textit{Tg}ApiAT1 has been disrupted, supporting the results of Rajendran \textit{et al.} [2017]. The ratio of Arg:Lys in MAAM (~3:1 Arg:Lys) is intermediate between RPMI (~6:1 Arg:Lys) and DMEM (1:2 Arg:Lys) (Table 2.10).
§4.3 Discussion

The data are therefore consistent with the hypothesis that, as shown previously, for medium to be conducive to the growth of parasites lacking TgApiAT1, the ratio of the concentrations of the two amino acids is more important than the concentrations of the individual amino acids. In the previous study, only the arginine to lysine ratios were varied whilst all else was held constant [see Rajendran et al., 2017]. Here I demonstrate that this prediction holds true in a medium (MAAM) which has a completely different amino acid formulation.

4.3.2 Significance of the reduction in plaque area observed in MAAM compared to DMEM

In the plaque assays I performed on ApiAT truncation mutants, the plaque area was reduced in parasites cultured in MAAM compared to DMEM, in almost all strains. This suggests a general, rather than a strain specific, reduction in growth due to culture in MAAM. This could be a result of the decreased concentrations of amino acids in the formulation, however there were other differences that were not controlled for between the two media. The most obvious is that I supplemented MAAM with dialysed serum, but used non-dialysed serum to supplement DMEM (see Methods §2.4.2.1). Sera is the most variable component of cell culture medium [Stein, 2018; Honn et al., 1975]. Although the reduction in plaque area seen in MAAM, relative to that seen in DMEM, reached significance for the apiAT5-6Δ20−548 strain (Figure 4.6), the most valid comparison is actually between strains grown in the same medium, rather than between medium for the same strain. This is because of the confounding differences between DMEM and MAAM, such as the sera that was used. The average plaque areas of the apiAT5-6Δ20−548 strain grown in MAAM was not significantly different from the average plaque area of the parental strain grown in MAAM.

Apart from the apiAT1Δ54−534, apiAT2Δ138−588 and apiAT5-3Δ188−504 truncation mutants, none of the other mutant strains had significantly different average plaque area to that of parental parasites when culture was undertaken in DMEM. My aim of testing the growth of the truncation mutants in MAAM was to determine whether there were any growth defects that were masked by culture in DMEM. Each strain that did not show growth defects in DMEM also did not have significantly different average plaque area to that of parental parasites when its culture was undertaken in MAAM. Since the pattern was the same in both media, I conclude that there were no masked growth defects.
The significant reduction in the plaque area of the \textit{apiAT5-6}^{\Delta20−548} strain in MAAM compared to DMEM was most likely due to chance (in fact, a 5% chance, because a significance level of \(p=0.05\) was chosen).

The result that growth of the \textit{apiAT5-1}^{\Delta239−987}, \textit{apiAT5-2}^{\Delta223−856}, \textit{apiAT5-4}^{\Delta197−713}, \textit{apiAT5-5}^{\Delta178−575}, \textit{apiAT5-6}^{\Delta20−548} and \textit{apiAT7-1}^{\Delta201−891} strains was not reduced compared to parental in either medium is consistent with the observation that these proteins are not expressed in the tachyzoite stage (Figure 3.5 and Parker \textit{et al.} [2019]). Theoretically, these mutants are functionally equivalent to wild type (assuming no off-target effects from CRISPR/Cas9) so it is not expected that culture in MAAM would have had a larger effect on these strains than on the parental strains.

In the future, MAAM may need to be refined so that the growth of wild type parasites in MAAM more closely matches their growth in DMEM. Potentially, the amino acid content in MAAM was too low to support parasites during a plaque assay. The confounding differences between the two media should also be investigated. For example, using the same sera in both media may eliminate the observed differences in growth. As an alternative to MAAM, a ‘physiological’ medium could be developed that mimics the concentrations of metabolites found in human plasma, such as the ‘Human Plasma-Like Medium’ recently developed by Cantor \textit{et al.} [2017]. This may provide growth conditions that better mimic the amino acid concentrations that parasites encounter \textit{in vivo}, and may therefore more effectively uncover the importance of ApiATs for parasite growth. That said, another reason that amino acid concentrations during \textit{in vitro} parasite culture do not replicate \textit{in vivo} concentrations is that culture is undertaken in static conditions. Medium is not continuously replenished in static conditions, so amino acid concentrations are not maintained as would happen \textit{in vivo}, and may become depleted over the course of an experiment.

### 4.3.3 Variability in the growth of the \textit{TgApiAT2} truncation mutant

I observed some variability in growth of \textit{apiAT2}^{\Delta138−588} parasites between experiments (Figure 4.3C). I observed no visible plaques in one replicate, and small plaques in other replicates. It is difficult to determine the reason for the variability in growth of the \textit{apiAT2}^{\Delta138−588} strain. The growth of these parasites may depend on a factor that is not controlled for in these plaque assays. Confounding factors which were not
controlled for include the age of the host cells, and the age of the culture medium. The same stocks of DMEM and MAAM were used to set up all three experiments, which were each 15 days apart. The media were warmed each time the experiment was set up. Both the warming and the ageing of media have been shown to affect its components [Spry et al., 2013]. A better approach might have been to aliquot the media and only warm that which was used in each individual experiment.

Notably, the plaque area of the $apiAT2^{\Delta138-588}$ strain increased in successive experiments (Figure 4.3). The second and third plaque assays were performed with $apiAT2^{\Delta138-588}$ parasites that had been in continuous culture for 15 and 30 days longer (respectively) than the parasites used in the first assay. It is conceivable that the larger plaque sizes in repeats 2 and 3 resulted from parasite adaptation to the loss of ApiAT2. One way that adaption could occur through upregulation of the expression of other genes, either at the transcript or at the protein level. This could be tested by undertaking transcriptomic or proteomic analysis of pre- and post-adapted parasites to determine if particular genes or proteins were upregulated.

4.3.4 ApiAT3 family members

Double and triple gene disruption of ApiAT3 subfamily members was not synthetically lethal for the parasite. Several scenarios could explain this result. Firstly, it is possible that members of the ApiAT3 family have a redundant, but non-essential role for the tachyzoite stage of the $T. gondii$ life cycle (in the growth conditions tested). This could be the case if they are all transporting the same substrate(s), but that substrate(s) was not essential to parasite growth. A second possibility is that the three ApiAT proteins all have non-redundant, non-essential roles. They may transport three different non-essential substrates. For example, it is conceivable that ApiAT3 proteins transport one or more of the non-essential amino acids, such as alanine, aspartic acid, asparagine, proline or glutamic acid (cf. §1.3.2). As a third possibility, the ApiAT3 family proteins may be redundant with other transporters in the parasite, outside of the ApiAT3 subfamily, or even outside of the ApiAT family.

With the development of genome-wide CRISPR/Cas9 disruption screens in $T. gondii$ [Sidik et al., 2016] studies in protein redundancy can be scaled up to include the whole genome. To find which genes, if any, contribute to parasite fitness in the absence of the
ApiAT3 family transporters, the triple truncation mutant and parental parasites could be both subjected to a genome-wide genetic screen. This might identify genes that are not important for the fitness of parental parasites but become important for parasites which lack the ApiAT3 transporters. These genes might encode other transporters, or even enzymes that contribute to the synthesis of the substrate(s) that the ApiAT3 transporters scavenge.

The truncation mutants could also be used to uncover the substrates of $Tg$ApiAT3-1, $Tg$ApiAT3-2 or $Tg$ApiAT3-3. Other ApiAT proteins transport amino acids [Rajendran et al., 2017; Parker et al., 2019]. Therefore, when screening for substrates of the ApiAT3 transporters, amino acids may be a good class of compounds to start with. The uptake of several amino acids across the plasma membrane of tachyzoites can be measured in a single experiment by exposing the parasites to a mix of $^{13}$C labelled amino acids, then measuring the intracellular abundance of $^{13}$C labelled amino acids using GC-MS [see Parker et al., 2019]. By comparing amino acid uptake in wildtype parasites to that of ApiAT3 family truncation mutants, the contribution that $Tg$ApiAT3-1, $Tg$ApiAT3-2 and $Tg$ApiAT3-3 make to amino acid homeostasis could be ascertained.

It is also possible that ApiAT3 family proteins transport substrates other than amino acids. For example, the ApiAT3 proteins might transport vitamins or sugars. One way to investigate this would be test whether the ApiAT3 truncation mutants are more susceptible than parental parasites to vitamin or sugar starvation, because they have less capacity to scavenge these nutrients. To test this, the concentration of vitamins or hexoses in the growth medium could be varied, in the same way that the amino acid concentrations were in MAAM.

Although members of the ApiAT3 family are individually and collectively non-essential in DMEM, I may simply have not tested the growth of the truncation mutants under conditions in which the full-length proteins normally contribute to parasite fitness. The generation of the truncation mutants provides a tool for future studies in this area. The $\textit{in vivo}$ virulence of the double and triple knockouts could also be tested. Since the ApiAT3 proteins are localised on the parasite surface, they may have as yet unknown functions in signalling or interaction with the host immune system that are only important $\textit{in vivo}$. 
All members of the ApiAT3 subfamily are expressed in tachyzoites (Figure 3.4), however, there are a multitude of proteins expressed by tachyzoites that are not important for the growth of tachyzoites in vitro [see Sidik et al., 2016, Figure 3]. One possibility is that ApiAT3 subfamily proteins are important at other stages of the T. gondii life cycle. Notably, transcriptomics data indicates that TgApiAT3-1 mRNA is expressed in oocysts, cat endothelial stages, bradyzoites and tachyzoites. TgApiAT3-2 mRNA transcript is highly expressed in oocysts, tachyzoites and bradyzoites and is also present, at lower levels, in cat endothelial stages. TgApiAT3-3 mRNA is highly expressed in all the aforementioned stages. (ToxoDB Transcriptomics data: Fritz/Boothroyd/Gregory (oocysts), Buchholz et al. and Sibley/Gregory (bradyzoites), Gregory (tachyzoites), Hehl Lab (cat endothelial), http://toxodb.org/toxo/).

Since the ApiAT3 subfamily proteins are expressed at other life-cycle stages, they may well be important for these stages of the lifecycle. The other lifecycle stages may have different requirements for metabolites. To illustrate with some examples, bradyzoites grown in vivo require glycolysis to be active, whereas tachyzoites grown in vivo or in vitro, do not [Shukla et al., 2018]. Additionally, cat endothelial stages may have different requirements for aromatic amino acids than tachyzoites (§1.15, also see Wang et al. [2017]). To characterise which ApiATs, if any, are important for parasite growth in the definitive felid host, the growth of the sexual stages of T. gondii ApiAT truncation mutant strains in the cat intestine could be determined, as well as the yields of oocysts from mutant strains in faeces [after Wang et al., 2017]. The sporulation rate of any oocysts recovered from ApiAT truncation mutant strains could then be tested to fully examine the cat stage of the life-cycle [after Wang et al., 2017].

4.3.5 Summary

Of the ApiAT proteins in T. gondii, all but TgApiAT6-1 were dispensable in the tachyzoite stage, although functional knockout of three genes, namely apiAT1, apiAT2 and apiAT5-3, resulted in severe growth defects (Figure 4.1). Constitutive expression of ApiAT2 reversed the growth defects observed in apiAT2Δ138−588 parasites (Figure 4.8), implying that the growth defect was a result of the CRISPR-induced frameshift mutation in the native TgApiAT2 protein and not due to off-target effects of the CRISPR system.
It is important to note that the ApiAT proteins which were dispensable in tachyzoites \textit{in vitro} under the two growth conditions tested may still be important \textit{in vivo}, in other life cycle stages of the parasite, in different host types or simply under different \textit{in vitro} growth conditions. The generation of the ApiAT truncation mutants in this study will facilitate future investigations on whether these proteins are essential or important under these different circumstances.
The scavenging of amino acids from the host organism is an essential process in apicomplexan parasites. At the commencement of this project, the mechanisms by which these parasites scavenged amino acids from their hosts were poorly understood. In this thesis, I define a family of plasma membrane-localised solute transporters called the apicomplexan amino acid transporters (ApiATs), members of which function to transport amino acids into, and out of, apicomplexan cells. In this discussion chapter, I summarise my results, and place them into the broader context of apicomplexan cell physiology.

Building on the work of Martin et al. [2005], who first identified the ‘novel putative transporters’ of *P. falciparum*, I conducted a bioinformatic survey of the genomes of a further seven apicomplexan parasites and their closest free-living relatives. I found that the Novel Putative Transporters, renamed as ApiATs in this thesis, are a large family of transporters distributed throughout the apicomplexan phylum. Adding to previous work, I studied the expression and localisation of the sixteen ApiATs of *T. gondii*. The work described in this thesis, along with work published by others, collectively shows that ten of the sixteen *T. gondii* ApiATs are expressed in the tachyzoite stage, and that eight of these localise to the parasite plasma membrane [Rajendran et al., 2017; Parker et al., 2019, and this study]. I performed a CRISPR/Cas9 based gene disruption screen of *T. gondii* ApiAT proteins and characterised the growth of fifteen truncation mutants, finding that three of these are important for parasite growth. I delved deeper into the phenotype of *Tg*ApiAT2 truncation mutants, finding that three of these are important for parasite growth. I delved deeper into the phenotype of *Tg*ApiAT2 truncation mutants, finding that they have a reduced intracellular growth rate and show perturbations in amino acid homeostasis. I expressed *Tg*ApiAT2 in *Xenopus* oocytes and characterised its substrate specificities, determining that it is a neutral amino acid transporter. I measured the uptake of selected amino acids in *Tg*ApiAT2 truncation mutants, demonstrating that *Tg*ApiAT2 is the major glutamine
§8.1 The subcellular localisation of ApiAT proteins in *T. gondii*

8.1 The subcellular localisation of ApiAT proteins in *T. gondii*

To date, all of the ApiATs for which the localisation has been studied, including the *P. berghei* transporter, PbApiAT8, localise to the plasma membrane of the parasites [Boisson *et al.*, 2011; Parker *et al.*, 2019, and this study]. The plasma membrane localisation of *Tg*ApiAT1, *Tg*ApiAT5-3, *Tg*ApiAT6-1 and *Tg*ApiAT2 is consistent with the role that these transporters have in amino acid uptake (Figure 8.1) [Rajendran *et al.*, 2017; Parker *et al.*, 2019; Rajendran and Fairweather, unpublished; and this study]. The putative transporters *Tg*ApiAT3-1, *Tg*ApiAT3-2 and *Tg*ApiAT6-3 are also present on the plasma membrane (Figure 8.1, grey transporters). Plasma membrane-localised transporters have a diverse range of roles in *T. gondii* including, but not limited to, nutrient uptake [Chiang *et al.*, 1999; Blume *et al.*, 2009], waste export [Ehrenman *et al.*, 2010; Erler *et al.*, 2018] and homeostasis of ionic compositions [Arrizabalaga *et al.*, 2004]. In general, transporters also have roles in cell volume regulation and the import (or export) of xenobiotics, including drugs [Quick, 2002; Kirk, 2004]. *Tg*ApiAT6-2 and *Tg*ApiAT7-2 are expressed in tachyzoites, but their localisation has not been confirmed (Figure 8.1, yellow transporters).

*Tg*ApiAT3-3 shows a dual localisation, both to the plasma membrane and the trans-Golgi network (Figure 8.1, purple transporters). [Parker *et al.*, 2019]. It is conceivable that *Tg*ApiAT3-3 has functions in both the plasma membrane and trans-Golgi network. Equally, it is possible that the trans-Golgi network localisation reflects the trafficking route of *Tg*ApiAT3-3 to the plasma membrane. The trans-Golgi network is a major sorting compartment in *T. gondii* that directs trafficking between the Golgi and the endosomal-like compartment [Jackson *et al.*, 2013]. The endosomal-like compartment in turn is involved in the trafficking of secreted proteins [Jackson *et al.*, 2013]. It is possible that *Tg*ApiAT3-3 is normally trafficked through the trans-Golgi network, as proteins destined for the plasma membrane do traffic through this organelle in yeast and mammalian cells [Gu *et al.*, 2001].
The endosomes of eukaryotic cells contain a complex of proteins known as the ‘retromer’ [reviewed in Seaman, 2012]. The retromer is a vital element of the endosomal protein sorting machinery that is responsible for the retrieval of proteins from the endosome to the Golgi and the trans-Golgi network [Seaman, 2012]. The retromer is also required for aspects of endosome-to-plasma membrane sorting [Seaman, 2012].

Studies on the retromer complex of *T. gondii* revealed that *Tg*ApiAT6-1 is maintained at the parasite membrane by retromer-mediated endocytic recycling [Sangaré et al., 2016]. In the absence of a functioning retromer complex, *Tg*ApiAT6-1 accumulates in endocytic vesicles [Sangaré et al., 2016]. The authors also identified *Tg*ApiAT5-3 amongst the proteins which interact with the *T. gondii* retromer complex, but did not study its localisation.

These data indicate that ApiAT proteins traffic through the endosome-like compartment on their way to the plasma membrane, and that this trafficking is retromer-mediated. In mammalian cells, the trafficking of amino acid transporters from the endosome to the plasma membrane can act as an important regulator of the function of these transporters [Hyde et al., 2002; Edinger, 2007]. An intriguing possibility is that the localisation of proteins such as *Tg*ApiAT5-3, *Tg*ApiAT6-1 and *Tg*ApiAT3-3 to the endosome-like compartment and trans-Golgi network is regulated based on the availability of the substrates of these transporters.

I have previously observed *Tg*ApiAT5-3 localising either to just the plasma membrane [see Parker et al., 2019, Figure S4] or to both the plasma membrane and an unidentified subcellular compartment [see Parker et al., 2019, Figure 2]. This raises the possibility that cell surface expression of *Tg*ApiAT5-3 is a regulated process, which could involve the retromer complex. *Tg*ApiAT5-3 is the main tyrosine transporter in the parasite and an exogenous source of tyrosine is essential for the growth of tachyzoites [Parker et al., 2019]. The availability tyrosine may regulate *Tg*ApiAT5-3 surface expression. If the parasite encounters low tyrosine concentrations, it may well be advantageous to express more tyrosine transporters on the plasma membrane.

To determine whether the subcellular compartment in which *Tg*ApiAT5-3 has been observed is the endosomal-like compartment, *Tg*ApiAT5-3-HA parasites could be co-labelled with antibodies to proteins from the endosomal-like compartment. In *T. gondii*,
§8.1 The subcellular localisation of ApiAT proteins in *T. gondii*

Host?Cell

*T. gondii*

Putative amino acid transporters

TgApiAT7-2

TgApiAT6-2

TgApiAT6-3

TgApiAT3-1

TgApiAT3-2

TgApiAT3-3

Sinefungin?

Sinefungin?

trans-Golgi network

tyrosine

aromatic amino acids

Sinefungin?

TgApiAT6-2

TgApiAT7-2

Putative amino acid transporters

arginine

cationic amino acids

arginine

TgApiAT2

TgApiAT5-3

TgApiAT6-1

TgApiAT1

alanine

putative amino acid transporters

TgApiAT3-2

TgApiAT3-1

TgApiAT6-2

TgApiAT7-2

Figure 8.1: A summary of the localisation and function of *T. gondii* ApiAT proteins. Adapted from Parker *et al.* [2019]. The image depicts a tachyzoite within a host cell. The parasitophorous vacuole membrane has been shown with a dashed line to indicate that it is permeable to amino acids [Schwab *et al.*, 1994]. Grey box: putative amino acid transporters. Yellow cylinders: TgApiAT6-2 and TgApiAT7-2, for which the localisations have not been experimentally determined. ‘Sinefungin?’ refers to the possibility that TgApiAT6-2 may transport sinefungin (see §8.2). Purple cylinder: TgApiAT3-3, which shows a dual localisation to both the plasma membrane and the trans-Golgi network. Grey cylinders: Plasma membrane localised ApiATs for which the function has not been determined.
The subcellular localisation of ApiAT proteins in *T. gondii*

The trans-Golgi network and the endosomal-like compartment are in very close association, sometimes co-localising [Jackson et al., 2013]. This raises the possibility that the presence of *TgApiAT3-3* in the trans-Golgi network could represent a ‘store’ of transporter that can be mobilised to the plasma membrane when required. Again, this process could be regulated by nutrient availability, as has been observed for mammalian nutrient transporters [reviewed in Edinger, 2007].

Another possible role for *TgApiAT3-3* in the trans-Golgi network could be in the transport of the digestion products of host derived proteins. *T. gondii* possesses a plant like vacuole, also known as the vacuolar compartment [Miranda et al., 2010; Parussini et al., 2010]. Recent evidence established that *T. gondii* endocytoses host cytosolic proteins and degrades them in its endolysosomal system, including in the vacuolar compartment [Dou et al., 2014; Di Cristina et al., 2017]. The vacuolar compartment is closely associated with the endosomal-like compartment [Parussini et al., 2010; Miranda et al., 2010]. It would be interesting to determine whether *TgApiAT3-3* localises not only to the trans-Golgi network but throughout the endolysosomal system, or shows any co-localisation with the vacuolar compartment. If *TgApiAT3-3* has a transport function, it could export metabolites derived from endocytosis, such as peptides or amino acids, into the parasite cytoplasm. The existence of such transporters in the digestive vacuole of *Plasmodium* species has been proposed [Kolakovich et al., 1997; Martin & Kirk, 2004; Juge et al., 2015].

The possibilities above presume a role for *TgApiAT3-3* in the trans-Golgi network. However, the localisation may also have been artefactual. Although the 3×HA tag I used in tagging *TgApiAT3-3* adds only a small additional sequence (27 amino acids) to the resulting protein, in principle, the introduction of any sort of tag may disrupt a proteins trafficking signal motifs and lead to mistargeting [Woodcroft et al., 2012]. The possibility that the trans-Golgi network localisation of *TgApiAT3-3* may be an artefact of genetic modification could be investigated further by raising antibodies to *TgApiAT3-3*. An IFA could then be used to determine the location of the native, untagged protein.
8.2 Possible functions of non-essential \textit{Tg}ApiATs

Of the sixteen ApiATs in \textit{T. gondii}, all, with the exception of \textit{Tg}ApiAT6-1, were able to be disrupted genetically. This implies that fifteen of the \textit{Tg}ApiATs are not essential for the growth of in tachyzoites, at least in standard \textit{in vitro} culturing conditions. The disruption of three genes, \textit{apiAT1}, \textit{apiAT2} and \textit{apiAT5-3}, resulted in growth defects under normal growth conditions, meaning that a total of four out of the sixteen \textit{T. gondii} ApiATs are important for the \textit{in vitro} growth of tachyzoites under the conditions tested.

Six of the remaining 12 \textit{T. gondii} ApiAT proteins are not expressed in the tachyzoite stage, so it was not surprising that the genes encoding these proteins were able to be disrupted genetically. The final six proteins, \textit{Tg}ApiAT3-1, \textit{Tg}ApiAT3-2, \textit{Tg}ApiAT3-3, \textit{Tg}ApiAT6-2, \textit{Tg}ApiAT6-3 and \textit{Tg}ApiAT7-2, are expressed by tachyzoites, yet they are dispensable for their \textit{in vitro} growth.

\textit{Tg}ApiAT6-2 is expressed by tachyzoites, albeit at low levels (it could not be detected by IFA) Parker \textit{et al.} [2019]. My data indicating that \textit{Tg}ApiAT6-2 is a non-essential protein are consistent with the results of Behnke \textit{et al.} [2015], who showed not only that the \textit{apiAT6-2} gene could be disrupted, but that its disruption confers resistance to the anti-parasitic drug sinefungin. Behnke \textit{et al.} [2015] propose that \textit{Tg}ApiAT6-2 transports sinefungin into the parasite and that its disruption confers resistance due to loss of uptake (Figure 8.1). In trypanosomatids, an order of protozoan parasites that includes \textit{Leishmania} and \textit{Trypanosoma} species, resistance to several major drugs is mediated by loss of function mutations in transport proteins [Baker \textit{et al.}, 2012; Munday \textit{et al.}, 2014; Dewar \textit{et al.}, 2016], including loss of function mutations in an amino acid transporter [Vincent \textit{et al.}, 2010; Mathieu \textit{et al.}, 2014].

Sinefungin is a S-adenosylmethionine (SAM) analogue (Figure 8.2). SAM is an important methyl donor in numerous biochemical reactions in cells, and under physiological conditions, the role of \textit{Tg}ApiAT6-2 may be to transport SAM into parasites. Sinefungin shares an uptake system with SAM in \textit{Leishmania} [Phelouzat \textit{et al.}, 1995], via a SAM transporter, the loss of which results in sinefungin resistance [Dridi \textit{et al.}, 2010].
To test whether \(Tg\)ApiAT6-2 transports sinefungin and/or SAM, the \(Tg\)ApiAT6-2 truncation mutant that I generated could be used in radiolabel uptake assays (Methods §2.6.3). \(^{14}\text{C}\)SAM, unlike \(^{14}\text{C}\)sinefungin, is available commercially. Uptake of \(^{14}\text{C}\)SAM into wild type parasites would first need to be tested, then, if the parasites do take up SAM, its uptake in \(Tg\)ApiAT6-2 truncation mutants can be compared to that of wild type. To test the hypothesis that \(Tg\)ApiAT6-2 transports sinefungin, the uptake of \(^{14}\text{C}\)SAM uptake could be measured in the presence of unlabelled sinefungin, which if it were to inhibit the uptake of \(^{14}\text{C}\)SAM, would imply that sinefungin may share an uptake pathway with SAM.

Given the phylogenetic relationship of \(Tg\)ApiAT6-2 to other amino acid transporters, including the verified cationic amino acid transporter, \(Tg\)ApiAT6-1 [Rajendran and Fairweather, unpublished], it is conceivable that \(Tg\)ApiAT6-2 recognises the methionine moiety in SAM (Figure 8.2), and may also be a methionine transporter. There is indirect (Figure 6.4) and preliminary (Figure 6.8D) evidence that \(Tg\)ApiAT2 transports methionine. The dispensable nature of the \(apiAT6-2\) gene could be due to the presence of redundant transporter(s), such as \(Tg\)ApiAT2, in the parasite.

Alternatively, \(T. gondii\) is predicted to be able to synthesise SAM (see Introduction §1.11), so, if \(Tg\)ApiAT6-2 really does transport SAM, \textit{de novo} synthesis of SAM could account for the dispensable nature of the \(apiAT6-2\) gene. The enzyme responsible for SAM synthesis is methionine adenosyl transferase (EC 2.5.1.6) which is encoded by TGME49_240690. This gene is predicted to be fitness conferring to tachyzoites grown \textit{in vitro} [Sidik \textit{et al.}, 2016]. If in future, SAM transport via \(Tg\)ApiAT6-2 is demonstrated experimentally, this raises the possibility that the (predicted) importance of the
methionine adenosyl transferase gene is due in part to the low levels of \( Tg \text{ApiAT6-2} \) that tachyzoites likely express [Parker et al., 2019]. A future experiment would be to disrupt or conditionally knockdown the methionine adenosyl transferase gene, determine whether it is indeed important for parasite growth, and if so, test whether the overexpression of \( Tg \text{ApiAT6-2} \) rescues any growth defects.

### 8.3 The consequences of glutamine starvation

The growth phenotype of \( Tg \text{ApiAT2} \) truncation mutants was characterised in some detail. These mutants display a significantly reduced growth rate compared to parental strains (Figure 5.1). The intracellular replication rate of \( \text{apiAT2}^{\Delta^{138-588}} \) parasites was reduced relative to the parental strain (Figure 5.2) and the mutant parasites have a small but statistically significant reduction in their host cell invasion efficiency (Figure 7.7). \( Tg \text{ApiAT2} \) knockdown parasites also have a significantly reduced growth rate compared to those expressing \( Tg \text{ApiAT2} \) (Figure 5.7).

\( Tg \text{ApiAT2} \) was shown to transport alanine, glutamine, isoleucine and leucine in the \( \text{Xenopus} \) oocyte heterologous expression system (Figures 6.3 and 6.8). Transport of these substrates was inhibited by a range of neutral amino acids, suggesting that \( Tg \text{ApiAT2} \) is a broad neutral amino acid transporter (Figures 6.4 and 6.9). In parasites, \( Tg \text{ApiAT2} \) is critical for glutamine uptake as suggested by using radiolabelled amino acid uptake assays in extracellular tachyzoites (Figure 7.1). \( Tg \text{ApiAT2} \) may also contribute to alanine export, as parasites lacking \( Tg \text{ApiAT2} \) have increased intracellular alanine levels (Figures 5.3, 5.5 and 7.5). The observation of a reduced rate of appearance of \( [^{13}\text{C}] \)alanine in the extracellular medium on incubation of the parasites with \( [^{13}\text{C}] \)glucose (Figure 7.6) is consistent with \( Tg \text{ApiAT2} \) contributing to alanine export. As noted in Section 7.3.3, alternative explanations can’t be ruled out. Nevertheless, I propose that \( Tg \text{ApiAT2} \) exchanges alanine, a non-essential amino acid and potential waste product, for glutamine, an important energy source (Figure 8.1).

The severe growth defect in parasites lacking \( Tg \text{ApiAT2} \) at first seems inconsistent with previously reported findings that wild type \( T. \text{gondii} \) parasites can survive \textit{in vitro} when glutamine is completely absent from the growth medium, displaying only a modest 25% reduction in growth [Nitzsche et al., 2016; Lee et al., 2014]. However, it is
important to note that tachyzoites often reside in host cells that can synthesise glutamine [Mendez & Koshy, 2017]. In particular, glutamine synthetase is highly active in human fibroblasts cultured in vitro [Soni et al., 1991]. Human fibroblasts are often used for the in vitro culture of *T. gondii*, as they were in this study. Therefore, when residing in fibroblasts, the parasite can probably scavenge glutamine from the host cell, even when glutamine is absent from the culture medium. The fact the growth phenotype of parasites lacking TgApiAT2 is more severe than expected based on the results of Nitzsche et al. and Lee et al. might be because these studies did not capture the state of parasites which truly have no access to glutamine.

Shukla et al. [2018] recently tested whether host cell glutamine synthesis could account for the mild growth phenotypes associated with glutamine starvation. In line with the previous studies [Nitzsche et al., 2016; Lee et al., 2014], Shukla et al. [2018] observed a slight growth defect in wild type parasites grown in the absence of glutamine, which they found was reversed by supplementation of the growth medium with a cocktail of non-essential amino acids including aspartate, alanine and glutamate [Shukla et al., 2018]. Aspartate and alanine can be used to synthesise glutamate, which in turn can be used to synthesise glutamine (see Introduction §1.3.1 and Figure 1.5). Conceivably, addition of these amino acids to the growth medium facilitated increased host cell synthesis of glutamine, which the parasites then scavenged. Shukla et al. [2018] tested this hypothesis by culturing parasites in azaserine, an inhibitor of host cell glutamine synthetase [Lea & Miflin, 1975; Rowell et al., 1977] in medium with or without exogenous glutamine. In the absence of exogenous glutamine, azaserine completely inhibits the growth of wild type parasites, but has no effect in the presence of glutamine [Shukla et al., 2018].

Although these results suggest that, in the absence of host cell glutamine synthesis, an exogenous source of glutamine is essential for parasite growth, Shukla et al. [2018] did not consider the role of the parasite’s glutamine synthetase enzyme in this process. It is conceivable that, in the absence of exogenous glutamine, the parasite’s glutamine synthetase provides sufficient glutamine to sustain replication, and that the parasite’s glutamine synthetase can also be inhibited by azaserine. The TgApiAT2 truncation mutants and knockdowns add vital information to interpret the effects of glutamine starvation on *T. gondii*, as they represent, for the first time, a measure of parasite growth where these parasites have (apparently) no access to exogenous glutamine.
Having said that, further investigation is required to determine whether $ap\textit{i}A\textit{T}2^{\Delta138-588}$ parasites are unable to take up glutamine under all circumstances. Firstly, I only tested glutamine uptake into $ap\textit{i}A\textit{T}2^{\Delta138-588}$ parasites at one extracellular glutamine concentration. Increasing the concentration of glutamine might have allowed some glutamine uptake through alternate transport pathways, if they exist. Secondly, glutamine uptake was tested in the presence of a range of other amino acids (Table 2.12). Although this uptake solution was designed to be more physiological than one that contained only glutamine, the ratio in which the amino acids were present would have influenced glutamine uptake. A second test of glutamine uptake, in a different uptake solution, one in which the amino acid ratios reflect that in say, human plasma, would provide information on whether parasite glutamine uptake in $ap\textit{i}A\textit{T}2^{\Delta138-588}$ parasites is affected to the same extent under physiologically relevant uptake conditions.

Another very important potential caveat to my claim that $Tg$ApiAT2 truncation mutants represent the state of parasites with no access to exogenous glutamine is that the loss of $Tg$ApiAT2 changes the parasite’s transport capacity for multiple amino acids (Figure 5.3B), not just for glutamine. The growth phenotype of $ap\textit{i}A\textit{T}2^{\Delta138-588}$ parasites could also be due to changes in the levels of these amino acids. In particular, the raised intracellular alanine concentrations could have a detrimental effect on parasite growth. Furthermore, competition assay data from oocytes (Figure 6.4) also raises the possibility that $Tg$ApiAT2 mediates the uptake of an amino acid that is essential to the parasites; possibilities include threonine, cysteine and methionine (cf. Figure 1.6). The uptake of these three amino acids in $ap\textit{i}A\textit{T}2^{\Delta138-588}$ parasites has not been tested directly, as they were either absent from the uptake solution (cysteine), or were not detected (methionine, threonine) in the $[^{13}\text{C}]$ labelled amino acid uptake experiment (Figure 5.3B). Future research should prioritise direct uptake assays of these amino acids in $ap\textit{i}A\textit{T}2^{\Delta138-588}$ parasites, as the additional deprivation of an essential amino acid could explain the larger-than-expected growth defect from loss of glutamine transport.

My data are consistent with an exogenous source of glutamine being required for the \textit{in vitro} growth of tachyzoites. If exogenous glutamine is essential, even when glucose is available, it suggests a distinct metabolic role for glutamine, a role that cannot be filled by glucose, for example in protein synthesis. A lack of glutamine in culture medium induces a stress response in \textit{T. gondii} tachyzoites [Konrad \textit{et al.}, 2014]. Eu-
karyotic Initiation Factor 2 is a protein that is an essential component of the translation machinery of eukaryotes [Sonenberg & Hinnebusch, 2009]. The alpha-subunit (eIF2α) can be phosphorylated by several protein kinases in response to cell stress and starvation. Phosphorylation of eIF2α leads to a global inhibition of protein synthesis [Sonenberg & Hinnebusch, 2009]. The eIF2α in *T. gondii* is phosphorylated in response to glutamine starvation [Konrad *et al.*, 2014]. The levels of phosphorylated *Tg*IF2α in *apiAT2*Δ138−588 parasites or *Tg*ApiAT2 knockdown parasites could be measured to determine if they are experiencing glutamine starvation.

### 8.4 Is *Tg*ApiAT2 an exchanger?

The ApiAT family member, *Tg*ApiAT5-3, operates most efficiently under ‘exchange conditions’, that is, conditions in which its substrate is present on both sides of the membrane [Parker *et al.*, 2019]. This is because, under exchange conditions, the transporter is undergoing ‘trans-stimulation’. Trans-stimulation describes a kinetic property of transporters. It is the process by which the transport rate of a given substrate is accelerated by the presence of one or more of the transporter’s substrates on the trans side of the membrane (i.e. the opposite side of the membrane to that from which the substrate is transported; for a substrate undergoing influx into a cell the trans side of the membrane corresponds to the cytosolic side). *Tg*ApiAT2 has the characteristics of a facilitative transporter, i.e. it has so far been shown to mediate unidirectional flux of its substrates (§6.3.2). However, like *Tg*ApiAT2, *Tg*ApiAT5-3 can also mediate unidirectional flux of substrate [Parker *et al.*, 2019]. This raises the possibility that *Tg*ApiAT2 is also an exchanger. I propose a model whereby *Tg*ApiAT2 mediates uptake of the important amino acid glutamine in exchange for alanine, which is a waste product of glutamine catabolism (Figure 8.3).

To test whether *Tg*ApiAT2 also functions most efficiently under exchange conditions, the uptake of radiolabelled substrates could be measured in *Tg*ApiAT2-expressing oocytes in which the intracellular concentration of a test substrate has been increased (i.e. under trans-stimulating conditions). Uptake in these conditions can be compared to uptake when intracellular oocyte amino acid concentrations are at native levels (non-trans-stimulating conditions). To prepare oocytes with trans-stimulating conditions, *Tg*ApiAT2 expressing oocytes can be microinjected with candidate trans-stimulating
§8.4 Is TgApiAT2 an exchanger?

Figure 8.3: Model of the physiological role of TgApiAT2. A T. gondii tachyzoite is pictured. The two major energy sources for tachyzoites are glucose and glutamine. Glutamine enters through TgApiAT2 and glucose enters through TgGT1. A simplified version of central carbon metabolism showing glycolysis, the TCA cycle and glutaminolysis (catabolism of glutamine) is depicted. One of the routes of entry of glutamine into the TCA cycle is shown: via glutamate and \( \alpha \)-ketoglutarate (the other entry point is the GABA shunt, not depicted, instead see Figure 1.7). Anaplerosis via \( \alpha \)-ketoglutarate could produce alanine as a by-product, which is then exported by TgApiAT2.
§8.5 Loss of glutamine uptake, or toxic alanine concentrations?

Thus far I have shown that loss of TgApiAT2 leads to a decrease in glutamine uptake and an increase in intracellular alanine levels within the parasite. However, I have not determined which of these two effects is more detrimental to the parasites. Establishing why parasites lacking TgApiAT2 have a defect in growth could be a direction for future experiments.

All of the metabolomics experiments I performed on the apiAT2Δ138–588 strain used extracellular stage parasites. However, the metabolism of intracellular tachyzoites differs significantly from that of extracellular tachyzoites [MacRae et al., 2012]. For example, intracellular parasites catabolise more glutamine than extracellular parasites. Metabolomic analysis of intracellular apiAT2Δ138–588 parasites may reveal further differences from wild type parasites.

Extracellular apiAT2Δ138–588 parasites have a similar abundance of glycolytic and TCA cycle intermediates as the parental strain (Figure 7.5). However, the levels of TCA cycle intermediates do not necessarily reflect the rate of substrate turnover (flux) through the TCA cycle [Bowtell et al., 2007]. Therefore, a lack of glutamine uptake might still lead a to defect in energy production. This hypothesis could be tested using a luciferase-based ATP detection assay to measure the size of the intracellular ATP pool in apiAT2Δ138–588 parasites [Lin et al., 2009]. Alternatively, the ability of wild type and apiAT2Δ138–588 parasites to perform oxidative phosphorylation when provided with either glutamine or glucose as substrates could be compared by measuring mitochondrial...
Loss of glutamine uptake, or toxic alanine concentrations?

O$_2$ consumption by the parasite using a Seahorse XFe96 extracellular flux analyser [Seidi et al., 2018]. If the growth defect observed upon the loss of TgApiAT2 is due to defects in energy production, I predict that parasites lacking TgApiAT2 will synthesise less ATP and consume less oxygen.

To ascertain whether it is the alanine accumulation or the glutamine starvation that is detrimental to parasite growth in TgApiAT2 truncation mutants, one approach would be to complement apiAT2$^{Δ138−588}$ parasites with known mammalian transporters for these amino acids. For example, human ASCT1 (SLC1A4) can transport alanine, serine and cysteine, but not glutamine [Bröer, 2008]. Assuming that apiAT2$^{Δ138−588}$ parasites are able to express ASCT1 and localise this to the plasma membrane, the mammalian transporter might mediate alanine efflux. The growth of ASCT1 expressing apiAT2$^{Δ138−588}$ parasites could then be measured to assess whether the presence of the mammalian transporter can rescue their growth. The intracellular alanine abundance in ASCT1 complemented parasites could also be measured using GC-MS or HPLC. Should ASCT1 expression normalise intracellular alanine concentration, and restore parasite growth, it would suggest that the high intracellular alanine concentration is toxic to parasites. Should ASCT1 expression normalise intracellular alanine concentration, but not restore parasite growth, this would suggest that glutamine uptake is the more important aspect of TgApiAT2’s function.

If the role of TgApiAT2 in glutamine uptake is what is important for parasite growth, I could explore this further by complementing apiAT2$^{Δ138−588}$ parasites with human ASCT2 (SLC1A5), which functions like ASCT1 in transporting alanine, serine, cysteine, and threonine, but which can also transport glutamine [Bröer, 2008]. The transport mechanism of ASCT2 involves an obligatory exchange of substrate amino acids [Bröer, 2008], much like the proposed mechanism of TgApiAT2 (Figure 8.3). If ASCT1 does not complement the growth of apiAT2$^{Δ138−588}$ parasites, but ASCT2 does, it supports the hypothesis that glutamine transport is an important factor. One would also have to demonstrate that ASCT2 restores intracellular alanine levels and restores the ability of the apiAT2$^{Δ138−588}$ parasites to take up glutamine.

The suggestion of this experiment comes with the caveat that there are several reasons why it might not work. Firstly, the mammalian transporters are Na$^+$ dependent [Bröer et al., 2016], so they may function inappropriately in T. gondii, which spends
most of its lytic cycle residing in the low Na\(^+\) environment inside the host cell. Secondly, the kinetic properties of ASCT1 and ASCT2 differ from those of \(Tg\text{ApiAT2}\), as both mammalian proteins are high affinity transporters with \(K_M\) values for neutral amino acids in the \(\mu\)M range [Bröer, 2008], whereas the \(K_M\) of \(Tg\text{ApiAT2}\) for alanine and glutamine was 16.8±2.4 mM and 10.8±2.1 mM, respectively (§6.2.2.1 and §6.2.3.2).

### 8.6 Possible functions of \(Plasmodium\) ApiAT2

Of the ApiATs in \(P. falciparum\), PfApiAT2 is the most closely related to \(Tg\text{ApiAT2}\) (Figure 3.3). This raises the possibility that PfApiAT2 also functions as a neutral amino acid transporter. Isoleucine has particular importance in the asexual intraerythrocytic stage of \(P. falciparum\) [Divo et al., 1985; Liu et al., 2006]. In this stage of the parasite, isoleucine is the only amino acid that needs to be supplied in culture medium (Introduction §1.3.2). \(Tg\text{ApiAT2}\) was able to transport isoleucine in \textit{Xenopus} oocytes (Figure 6.7). This provides significant impetus to test whether the ApiAT2 in \textit{Plasmodium} species can also transport isoleucine, for example, by expressing the transporter in \textit{Xenopus} oocytes or studying isoleucine uptake into the PbApiAT2 knockouts generated by Kenthirapalan et al. [2016].

To assess whether \(Tg\text{ApiAT2}\) contributes to isoleucine uptake in tachyzoites, I attempted to measure isoleucine uptake in extracellular \(apiAT2^{\Delta138-588}\) parasites and their parental line. I found that in general, isoleucine uptake was difficult to measure in \textit{T. gondii} tachyzoites. Even in parental ‘wild type’ parasites, the uptake of isoleucine was at a much lower levels compared to the uptake of glutamine and arginine. This caused a high signal-to-noise ratio and the results were inconclusive. Further study is warranted, but the conditions under which isoleucine uptake is measured will first need to be optimised.

Isoleucine shares an uptake pathway with leucine and methionine in \(P. falciparum\) [Cobbold et al., 2011]. Uptake of these three amino acids via the pathway is inhibited by a group of neutral amino acids [Cobbold et al., 2011], similar to the amino acids that inhibit uptake of radiolabelled substrates via \(Tg\text{ApiAT2}\) (Figure 6.4). Furthermore, the uptake of \([^{14}\text{C}]\)isoleucine into isolated \(P. falciparum\) parasites is trans-stimulated by leucine [Martin & Kirk, 2007]. \(Tg\text{ApiAT5-3}\) is an exchanger [Parker et al., 2019],
and so are the other ApiAT proteins, TgApiAT1 and TgApiAT6-1 [Rajendran and Fairweather, unpublished]. This implicates exchange as the transport mechanism employed by ApiATs, a mechanism which also occurs in the uptake of isoleucine, leucine and methionine in *P. falciparum* [Martin & Kirk, 2007; Cobbold et al., 2011]. I sought to test whether the uptake of isoleucine in *P. falciparum* was mediated by one or more PfApiATs, however, my attempts to express PfApiAT2 in *Xenopus* oocytes and in *T. gondii* were not successful.

Recently, a mutagenesis screen of the *P. falciparum* genome was undertaken to identify which genes are essential in the parasite [Zhang et al., 2018]. This study revealed that the genes encoding each of the *P. falciparum* ApiATs are non-essential for the in vitro growth of the asexual blood-stage of *P. falciparum*. Loss of the apiAT2 gene did not confer a fitness cost to mutants [Zhang et al., 2018]. Given the essential nature of isoleucine uptake in asexual blood-stages of the parasite, if PfApiAT2 is an isoleucine transporter, it is unlikely to be the only isoleucine uptake pathway in the parasite.

In light of these findings, another potential role of the ApiAT2 in *Plasmodium* is in the uptake of glutamine. Glutamine is taken up by asexual red blood cell stages [MacRae et al., 2013], but this uptake is not essential for parasite growth [Liu et al., 2006], presumably because the parasite is able to obtain glutamine from the breakdown of host cell haemoglobin [Liu et al., 2006]. If PfApiAT2 represents a pathway predominantly involved in glutamine uptake, it would be consistent with the recent finding that the gene encoding PfApiAT2 is non-essential for asexual blood-stage parasite [Zhang et al., 2018]. However, glutamine metabolism (specifically the glutamine synthetase gene, see §1.8) is important for the development of oocysts in *P. berghei*, one of the parasite stages in the mosquito gut [Srivastava et al., 2016]. As a consequence, glutamine synthetase knockouts produced no sporozoites, the parasite stage that colonises the salivary glands of mosquitos [Srivastava et al., 2016]. Knockout of PbApiAT2 caused a very similar phenotype to glutamine synthetase knockouts: an attenuation of oocyst development, resulting in no sporozoite colonization of salivary glands [Kenthirapalan et al., 2016]. This raises the possibility that, like TgApiAT2, PfApiAT2 and PbApiAT2 are glutamine transporters.
In the work described in this thesis I have re-defined the original Novel Putative Transporter family of *Plasmodium* as the ‘Apicomplexan Amino Acid Transporter family’. Bioinformatic analysis revealed that the novel putative transporters have homologues in several other apicomplexan parasites. The ApiAT family shares a similar topology to, and a sequence motif with, transporter families of the major facilitator superfamily, but does not share sufficient sequence homology to be classified within one of those families. I propose that the ApiAT family should be recognised as a new family within the major facilitator superfamily. Further bioinformatic analyses will serve to examine this claim and elucidate the relationships of the ApiAT family within the major facilitator superfamily.

This work, and the work of others, has contributed to our understanding that the ApiAT family includes several plasma-membrane localised amino acid transporters. The function of each of the *T. gondii* ApiATs that is important for growth of the tachyzoite stage has now been characterised. *Tg*ApiAT1 is the parasite’s major arginine uptake pathway [Rajendran et al., 2017], *Tg*ApiAT5-3 transports aromatic amino acids and is particularly important for tyrosine uptake [Parker et al., 2019], *Tg*ApiAT6-1 is a cationic amino acid transporter [Rajendran and Fairweather, unpublished], and finally, *Tg*ApiAT2 is a broad neutral amino acid transporter with an important role in glutamine uptake and neutral amino acid homeostasis (Figure 8.1).

In characterising the function of ApiAT family members in *T. gondii*, we have made significant progress towards an understanding of amino acid acquisition in this parasite. However, the uptake pathway(s) of several essential amino acids in *T. gondii* are yet to be elucidated. This includes the transporters that mediate the uptake of the essential amino acids leucine, isoleucine, valine, threonine, methionine and cysteine. *Tg*ApiAT2 may contribute to the uptake of these amino acids, but the uptake of the latter three in the absence of the transporter has never been tested.

Apicomplexans remain a large burden for humanity, causing needless loss of animal and human life. Research into apicomplexan parasites at a molecular level provides an understanding of their basic survival strategies and allows pharmaceutical targets to be identified. Parasite transport proteins are potential targets, but remain critically
under-researched. The findings in this thesis highlight the evolutionary novelties that arise to enable parasites to scavenge essential nutrients from their hosts, as well as demonstrating the importance of amino acid scavenging, even of non-essential amino acids, for the growth of the disease-causing tachyzoite stage of *T. gondii*.
Multiple sequence alignment of ApiAT family proteins

Figure A.1: A multiple sequence alignment of the 66 ApiAT proteins identified during this study. Reciprocal protein BLAST searches were used to identify orthologues of the five previously identified *Plasmodium falciparum* ApiAT genes. The sequences were aligned using Clustal Omega Version 1.2.2 [Sievers *et al.*, 2011; Sievers & Higgins, 2018]. Shading for sequence identity was carried out using the TeXshade package for LaTeX. Residues have been shaded to represent the degree of conservation. A position is shaded blue if the number of identical residues is between 50-70% of sequences, and purple if >70% of sequences. A residue is shaded pink if it is a similar amino to a ‘consensus’ amino acid, or if no single residue represents >50% of sequences at that position but together, a group of similar residues exceed the 50% threshold. Non-conserved residues are white. Gaps in the sequence are represented by dots. The predicted transmembrane domains for the uppermost protein, *Tg*ApiAT1, have been indicated with helices and the internal and external loops marked with bars. The consensus sequence is displayed at the bottom of the alignment. In the consensus sequence, dots represent no single residue in >50% of sequences, lowercase letters indicate that the residue is present that position in 50-70% of sequences and uppercase letters indicate that the residue is present in >70% of sequences. The consensus sequence is coloured with ‘HotCold’ shading, where consensus residues are coloured with a gradient from blue representing 50% conservation to red representing 100% conservation. The location of the MFS signature sequence (between transmembrane domains two and three) has been highlighted at the bottom of the alignment.
TgApiAT1 0
TgApiAT2 0
TgApiAT3-1 0
TgApiAT3-2 0
TgApiAT5-1 MGCEPD CPAVSG 12
TgApiAT5-2 MGHWA WREELTA KMG VLSHVAFREDHVAYAK 36
TgApiAT5-3 0
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| FS.QGSGK.QRGPLSFPPRP.PLPS        |
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| SQKGT.CAS.VTSSRARSSTGREWQH       |
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