A NEW METHOD FOR THE ORTHOGONAL LABELING AND PURIFICATION OF *TOXOPLASMA GONDII* PROTEINS WHILE INSIDE OF THE HOST CELL

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Toxoplasma gondii is an obligate intracellular protozoan parasite that is estimated to infect a third of the human population. While parasite infection is usually asymptomatic in healthy individuals, it is responsible for life threatening disease in individuals with compromised immune systems. The obligate intracellular nature of the parasite makes its ability to successfully invade and interact with its host cell of paramount importance. How *T. gondii* interacts with its host cell to permit its survival and replication is still largely understood. Our knowledge of host-pathogen interaction would be strengthened if a system were available to specifically label parasite proteins in the context of an infected host cell. My thesis work in the Boyle laboratory has focused on developing such a system in *T. gondii*.

I have created a strain of *T. gondii* that expresses a mutant form of a bacterial methionyl-tRNA synthetase ($MetRS^{NLL}$), which permits methionine (Met) tRNA to be charged with the azide-containing Met analog, azidonorleucine (Anl). Any protein that incorporates Anl is susceptible to a copper-catalyzed "click" reaction, allowing affinity tags for purification or fluorescent tags for visualization to be appended to them. I show that Anl is only incorporated into parasites expressing the mutant $MetRS^{NLL}$, and that it is absent from host cell proteins. While the approach allows for the labeling of a large margin of parasite proteins, it appears that N-terminally processed proteins are only minimally labeled. This may be due to the bacterial $MetRS^{NLL}$ only being able to charge Anl to the *T. gondii* initiator $tRNA^{Met}$ and not the elongator $tRNA^{Met}$, which would result in only the N-terminal Met residue being replaced with Anl. I have begun work to try to modify the system to permit Anl to be more widely incorporated into parasite proteins. Despite the systems current limitations, it should be useful for studying proteomic changes in the parasite while inside of the host. Furthermore, the system should be readily adaptable for other Apicomplexan parasites, like *Plasmodium spp*.

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PREFACE

This thesis would not have been possible without the help of countless people. I would like to take the time to acknowledge some of them, though space will not allow me to mention everyone that helped make this a reality.

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1.0 INTRODUCTION

Toxoplasma gondii is the obligate intracellular protozoan parasite responsible for the disease toxoplasmosis in humans. It is a member of the phylum Apicomplexa, which includes important human pathogens aside from *T. gondii*. *Plasmodium spp*. is one of the most noteworthy examples, being the causative agent of malaria. In 2012, *Plasmodium spp.* were estimated to be responsible for over 600,000 deaths, the vast majority being children in Africa [1]. While *T. gondii* infection is not responsible for as many deaths in humans, it has been estimated that close to a third of the human population is infected with *T. gondii*, with infection rates as high as 70% in some geographic regions [2]. Infection in humans is generally asymptomatic in individuals with healthy immune systems, though it can cause severe encephalitis in patients with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome or individuals taking immunosuppressive drugs for organ transplants. Also, if a woman first becomes infected with *T. gondii* during pregnancy, it can lead to hearing loss, blindness, and even death in the developing fetus [3,4].

1.1 HOST RANGE

T. gondii not only infects humans, it is able to infect virtually all warm-blooded animals. The genus *Toxoplasma* only has a single species (*gondii*), which is able to infect all the aforementioned organisms. This is unique compared other apicomplexans, which have much more restricted host ranges [5]. The *Plasmodium* genus has over 200 species which differ in the vertebrate hosts that they can infect. All *Plasmodium* species have an obligate two host life cycle, alternating between a mosquito vector and a vertebrate host. Malaria affects a wide range of vertebrates other than humans, including reptiles, birds, rodents, and non-human primates, though there are different *Plasmodium* species for each of these vertebrate hosts [6]. The reasons for the disparity in host ranges between *T. gondii* and other apicomplexans is currently unknown [5,7].

1.2 LIFE CYCLE

Like most apicomplexans, *T. gondii* has a complex life cycle with both asexual and sexual stages [8]. It can reproduce asexually in all warm-blooded animals (the intermediate host), but is known to sexually reproduce in the intestines of felines, including domestic cats and larger wild cats like lions and tigers (the Felidae family; the definitive host).

1.2.1 Asexual reproduction

The asexual phase of the parasite's life is divided into two distinct stages (Fig. 1). Acute infection is characterized by a quickly growing form of the parasite known as the tachyzoite. Tachyzoites replicate within host cells inside a vacuole composed mainly of the host cell plasma membrane, dividing every 6 to 8 hours until the infected host cell lyses due to parasite buildup [9]. The released parasites then go on to infect neighboring cells [10]. The tachyzoite stage is the most experimentally tractable stage of *T. gondii*'s life cycle, as it can be easily maintained in the laboratory in cultured cell lines serving as host cells. During an infection, the majority of the tachyzoites are killed by an interferon-gamma (IFN-γ)- dependent immune response, though in some tissues, mostly the central nervous system and muscle, the surviving tachyzoites differentiate into slower growing bradyzoites. The vacuole that the parasites grow inside is modified to become a cyst wall. Each cyst contains hundreds of bradyzoites and is largely non-immunogenic, persisting for the remainder of the host organism's life, marking the chronic stage of infection [11]. Bradyzoites will occasionally reactivate and convert back into tachyzoites, but the immune system is able to prevent them from disseminating to the rest of the host. It is this reactivation that poses a threat to immunocompromised individuals.

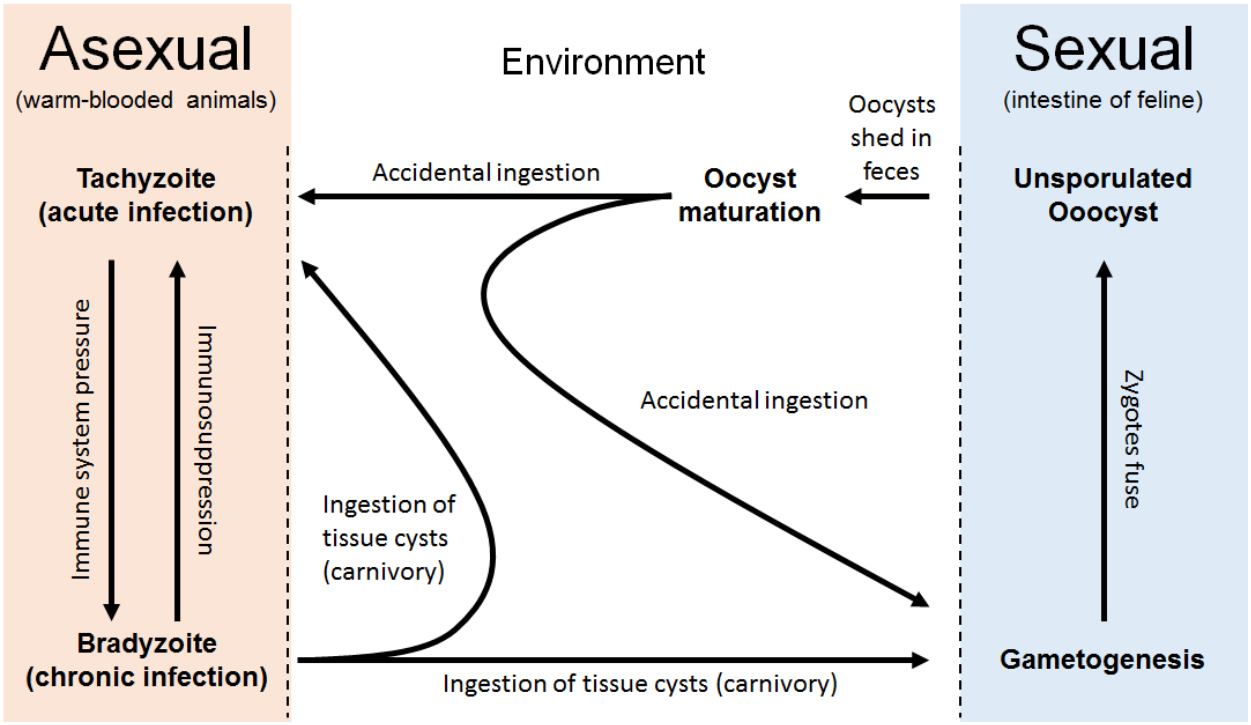

Figure 1. The life cycle of *T. gondii*

The asexual phase of *T. gondii*'s life cycle can take place in any warm-blooded animal. Acute infection is initiated in two primary ways, 1) accidental ingestion of environmentally stable oocysts from cat feces, or 2) ingestion of tissue cysts from chronically infected intermediate hosts via carnivory. In either instance, parasites from these vessels infect the new host's intestinal epithelium and differentiate into the fast growing tachyzoite, marking the acute stage of the infection. The host immune system is able to eliminate the majority of the tachyzoites, though some differentiate into bradyzoites, which reside in cysts in the host's nervous system and musculature. The development of these tissue cysts marks the chronic stage of infection. If a chronically infected host is devoured by another intermediate host, the bradyzoites are released and they mount a new infection, reverting back to tachyzoites. If the new host is a feline, the sexual stage of the parasite's life cycle is initiated in the feline's intestine, leading to the production of unsporulated oocysts which are shed in the feline's feces. The unsporulated oocysts mature in the outside environment, and if ingested by a warm-blooded animal, can initiate an acute infection. Image created by Gregory Wier.

1.2.1.1 Transmission of T. gondii in its asexual form

The asexual bradyzoite form of *T. gondii* can be passed between intermediate hosts via carnivory. If a chronically infected organism is eaten by another animal (i.e. a chronically infected mouse is eaten by a hawk, or a human eats undercook meat from an infected lamb), the cyst walls of latent tissue cysts from the devoured organism are dissolved by proteolytic enzymes in the stomach of the carnivore, releasing bradyzoites, which mount an infection in the intestinal epithelium [8]. In this way, *T. gondii* can be transmitted from various hosts asexually, never needing to transition to the sexual stage for dissemination. This is at odds with other apicomplexans, which have obligate two-stage life cycles and must undergo sexual reproduction before infecting a new intermediate host.

1.2.2 Sexual reproduction

The sexual stage of the parasite's life cycle is solely confined to the intestines of felines, culminating with the cat shedding oocysts in its feces [12]. When a cat ingests tissue cysts from a chronically infected organism, bradyzoites are released into the small intestine, where they infect the intestinal epithelial cells. After undergoing multiple rounds of asexual reproduction in the intestinal epithelial cells, sexual reproduction begins with the formation of male and female gametocytes [13,14]. The male gametocyte gives rise to numerous flagellated microgametes, while the female gametocyte gives rise to a single macrogamete. The microgametes use their flagella to swim and fertilize the macrogamete, forming a zygote. After the formation of the zygote, an oocyst wall forms around it. These oocysts are released into the environment in the cat's feces in an

immature, “unsporulated” form [14]. It is in the outside environment that the oocysts fully mature, where they remain stable and highly infectious for months to years. If these oocysts are ingested by a warm-blooded animals, they can initiate an infection, leading to the reproduction of the parasite asexually. Due to the intractability of working with infected felines, this stage of *T. gondii*’s life cycle has received considerably less attention than the asexual stages.

1.3 MEANS OF HUMAN INFECTION

There are four primary ways of humans acquiring a *T. gondii* infection, 1) ingesting tissue cysts from previously infected intermediate hosts by eating raw or undercooked meat, 2) ingesting food or drink contaminated with oocysts from the feces of felines, 3) receiving an organ transplant or a blood transfusion from an infected individual, or 4) via transmission from mother to fetus if the mother becomes infected with *T. gondii* during her pregnancy.

1.4 TREATMENTS FOR *T. GONDII* INFECTION

Treatments for *T. gondii* infection are only available for the acute stage of infection using antifolate combination therapy (pyrimethamine and sulfadiazine), which can trigger severe allergic reactions in some patients [15]. The chronic stage of *T. gondii*’s life cycle is refractory to current drugs. Because of this, the need for new and more effective pharmaceuticals is high. This need has prompted researchers to devote considerable effort towards understanding the unique morphological features of *T. gondii* and other apicomplexans in order to serve as potential drug targets. One of the most promising targets is a distinct set of morphological features for which the Apicomplexan phylum derives its name, the apical complex.

1.5 UNIQUE STRUCTURAL FEATURES OF *T. GONDII*

As with other Apicomplexan parasites, the obligate intracellular life cycle of *T. gondii* makes host cell invasion of paramount importance to parasite viability. The cell types which different Apicomplexans are able to invade is varied, from erythrocytes to macrophages. Despite variation in host-cell type, the modes of invasion are very well conserved. Successful host cell invasion is accomplished with the aid of the apical complex, a unique assembly of structural and secretory components at the apical end of the organisms. The apical complex not only provides the structure to shape the parasites, but directs a collection of secretory organelles which play an essential role in attachment to and invasion of the host cell [16-18]. At the apical end of the complex is the conoid (Fig. 2), a hollow barrel of twisted microtubules which presently has no ascribed

function, though it is known to extend and protract when the parasite is extracellular [19]. The conoid sits atop an apical polar ring that nucleates a collection of 22 microtubules that migrate about two thirds of the way down the parasite (Fig. 2) [16,20]. These microtubules connect with a structure known as the Inner Membrane Complex (IMC), which provides structural rigidity and shape to the parasite. The IMC is composed of two parts 1) a collection of flattened membrane sacs known as alveoli, which lie just below the parasites' plasma membrane [21], and 2) a network of intermediate filaments to provide support for the alveoli [22]. Together, the plasma membrane and the a IMC are known as the pellicle [23].

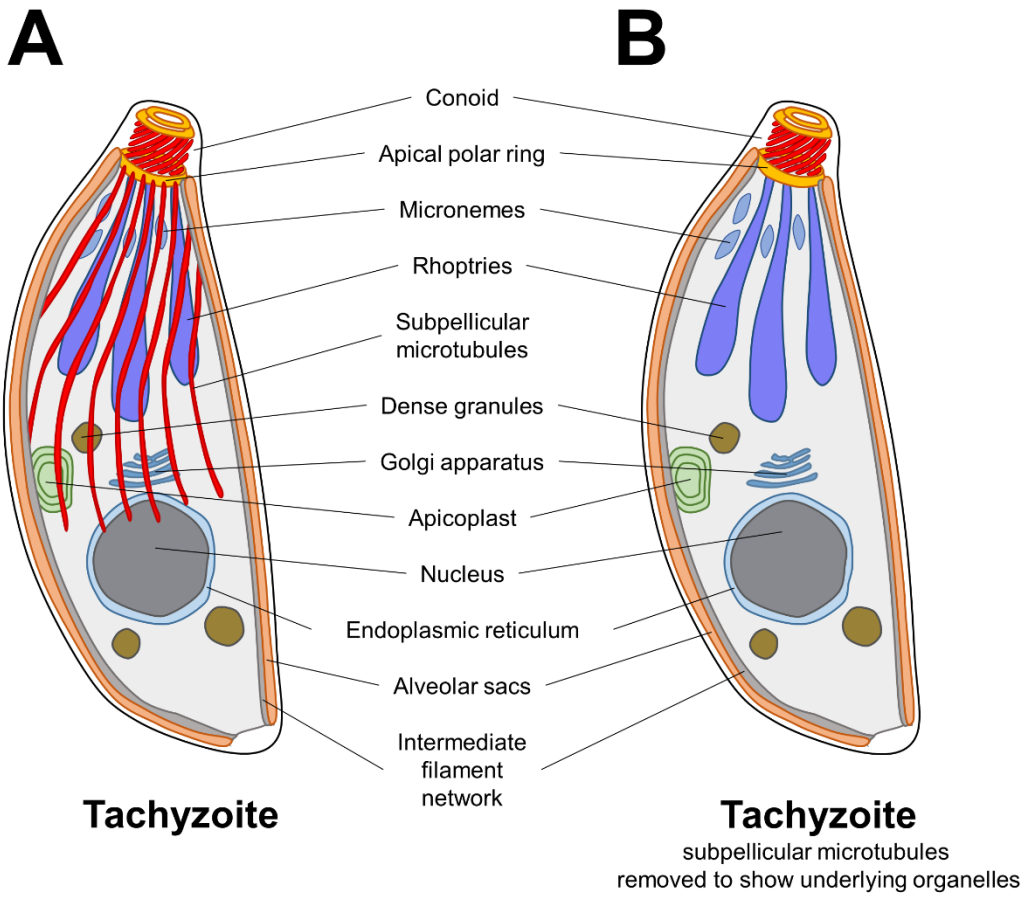

Figure 2. Structural features of a *T. gondii* tachyziote

A) Diagram of the tachyzoite phase of *T. gondii* with notable features denoted. B) An identical image to that in A, with the subpellicular mircotubules removed to more clearly show the underlying structures. Image created by Gregory Wier.

1.5.1 Secretory organelles

Beyond the structural components of the apical complex, it houses two types of secretory organelles, the micronemes and the rhoptries (Fig. 2B). *T. gondii* uses these organelles to secrete effector proteins to assist in parasite motility and host cell invasion [24-26]. The micronemes are the smallest of the secretory organelles, located at the very apical end of the parasite. They are generally thought to be the first to discharge their contents during invasion [27]. The rhoptries are larger, bulb-shaped organelles, which primarily discharge their contents after the micronemes. There are two classes of proteins housed within the rhoptries, differentiated by their position within the organelles: 1) those positioned at the neck of organelle (rhoptry neck proteins; RONs), and 2) those positioned in the posterior bulb (rhoptry bulb proteins; ROPs). Several of the RONs are known to interact with microneme proteins during invasion, while most ROPs are involved with later stages of invasion and survival of the parasite after invasion [28]. While not part of the apical complex, *T. gondii* also has another set of secretory organelles known as dense granules. These are more generally located in the cytoplasm of the parasite (Fig. 2), and play a role in modulating the host cell to make it a more suitable environment for parasite survival after successful invasion [25,27].

1.6 GLIDING MOTILITY AND HOST CELL INVASION

T. gondii and other Apicomplexans do not have cilia or flagella, and instead use gliding motility for both locomotion and host cell invasion [29,30]. Gliding motility and host cell invasion are both driven by an actin-myosin motor anchored in the parasite's pellicle. The parasite secretes a collection of adhesive microneme proteins to its surface which associate with receptors on the host cell. The myosin motor pulls along polymerized actin in the pellicle, engaging the adhesive microneme proteins bound to host cell receptors, applying a rearward force to propel the parasite forward (Fig. 3) [30].

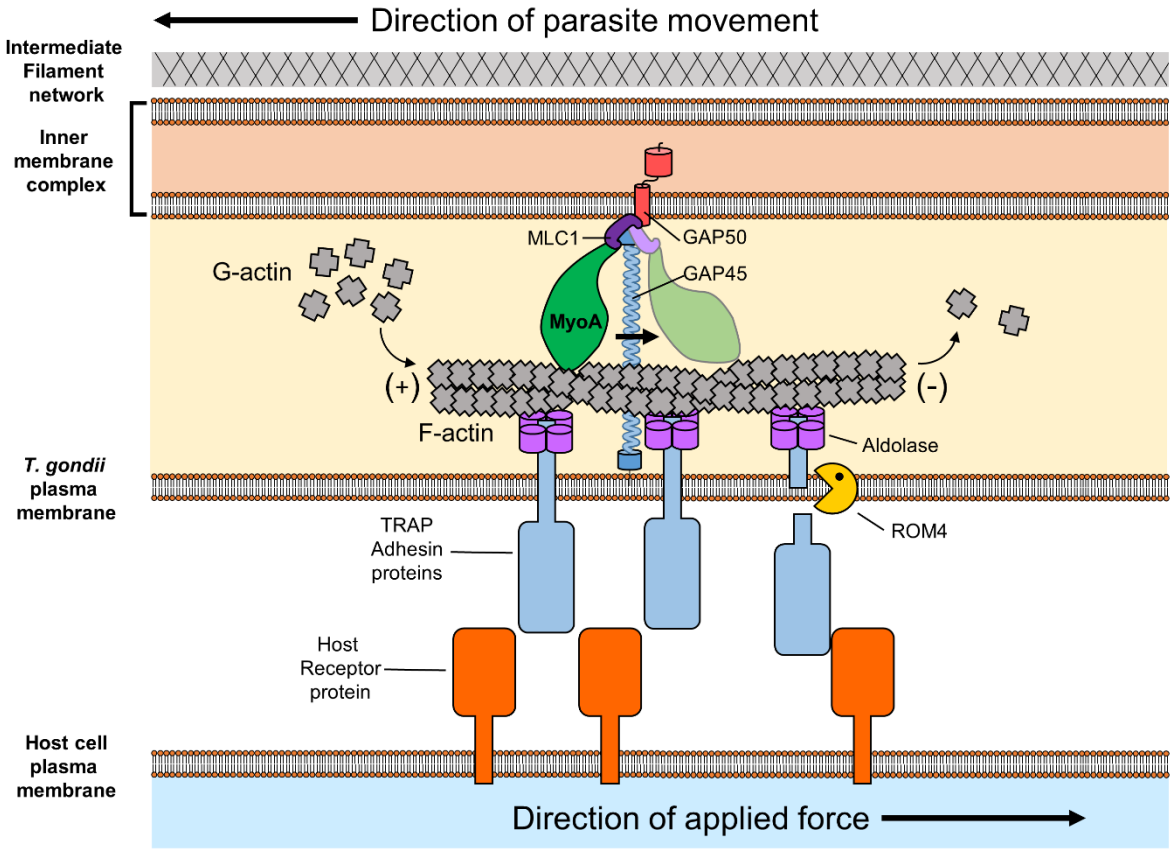

Figure 3. Gliding motility in *T. gondii* and other Apicomplexan parasites

Gliding motility in *T. gondii* is powered by the myosin motor protein MyoA, which is anchored to the IMC by association with GAP50. Membrane-bound secreted microneme proteins from the TRAP adhesion family bind to receptors on the host cell plasma membrane, and associate with short segments of F-actin via an interaction with the glycolytic enzyme aldolase. MyoA pulls along the F-actin, pushing the TRAP adhesion proteins backwards and thrusting the parasite forward. Membrane-bound rhomboid-like proteases like ROM4 cleave the bound TRAP adhesion proteins in their transmembrane domain, permitting the parasite to proceed forward. Image created by Gregory Wier.

1.6.1 Actin-myosin motor

The first clues suggesting that gliding motility required actin polymerization came from the observation that parasites were unable to move or invade in the presence of cytoclasin D. Cytoclasin D is a compound which disrupts microfilament formation by binding to monomeric, globular actin (G-actin) and preventing it from polymerizing into filamentous actin (F-actin) [31]. The actin-myosin motor complex that *T. gondii* uses to power gliding motility is anchored into the IMC [32]. *T. gondii* only has a single actin gene, though it has multiple genes for myosin. The encoded myosin motor proteins have atypically short necks and tails, constituting a class of

myosins only found in Apicomplexans [33]. Of these multiple myosin genes, Myosin A (MyoA) has been specifically implicated in both locomotion and invasion in *T. gondii* [34,35]. MyoA is associated with its myosin light chain (MLC1) and two gliding-associated proteins (GAP45 and GAP50) [36,37]. GAP50 is a integral membrane glycoprotein that is responsible for anchoring MyoA to the IMC [36]. While the function of GAP45 is currently unknown, it is anchored to the parasite's plasma membrane via N-terminal myristoylation, and to the IMC by its C-terminal end associating with GAP50 [37].

1.6.2 The generation of force

To generate force, the IMC-anchored MyoA pulls along short segments of F-actin. F-actin, in turn, is linked to the cytoplasmic tails of membrane-bound microneme proteins that contact the extracellular environment and bind to receptors on the host cell plasma membrane (Fig. 3) [38-40]. In *Plasmodium spp*. these micronemal proteins are members of the thrombospondin-related anonymous protein (TRAP) adhesin family, with notable orthologs in *T. gondii,* including micronemal protein 2 (MIC2) [41-43]. These TRAP adhesin proteins do not contact with F-actin directly, rather they are linked via the tetrameric glycolytic enzyme aldolase. The force of the myosin pulling along the F-actin pushes the TRAP adhesin proteins rearward, driving the parasite forward. Disengagement of these TRAP proteins with host cell receptors is required for the parasite to be propelled forward, a task which is accomplished by proteolyticly cleaving the TRAP proteins at their transmembrane domains (Fig. 3). Understanding of this process has only recently come to light with the identification of some of the proteolytic enzymes involved, including rhomboid-like proteases (ROM4) [44,45].

1.6.3 Host invasion

Host cell invasion is a parasite-driven process distinct from host-mediated endocytosis, taking between 10 and 20 seconds (Fig. 4). Much of the same machinery utilized for gliding motility is also employed for host cell invasion. Secreted micronemal proteins allow the parasite to traverse the host cell, and then situate its apical end to the host plasma membrane, whereupon it discharges effector proteins from the rhopties into the host cell [27]. These rhoptry proteins aggregate with the microneme protein Apical Membrane Antigen 1 (AMA1) to form tight ring-shaped connection between the parasite and the host plasma membrane, known as the moving junction [27,28]. The moving junction acts as a stable support for the parasite to actively force its way

into the cell using the same myosin motor employed for gliding motility [46]. The rhoptry proteins that make up the moving junction are all rhoptry neck proteins (RONs), which are secreted as a complex of RON2, RON4, RON5 and RON8 [47]. AMA1 is a type 1 transmembrane protein that sits in the parasites' plasma membrane, and makes a direct connection to RON2, which is anchored into the host plasma membrane [47]. RON 4/5/8 are all soluble proteins that are thought to localize to the cytoplasm of the host cell, potentially connecting to the host cell's cytoskeleton. RON5 is important for the organization of the RON proteins in the moving junction, as its deletion prevents RON2 and RON4 from localizing properly, and prevents the parasites from being able to invade host cells [48].

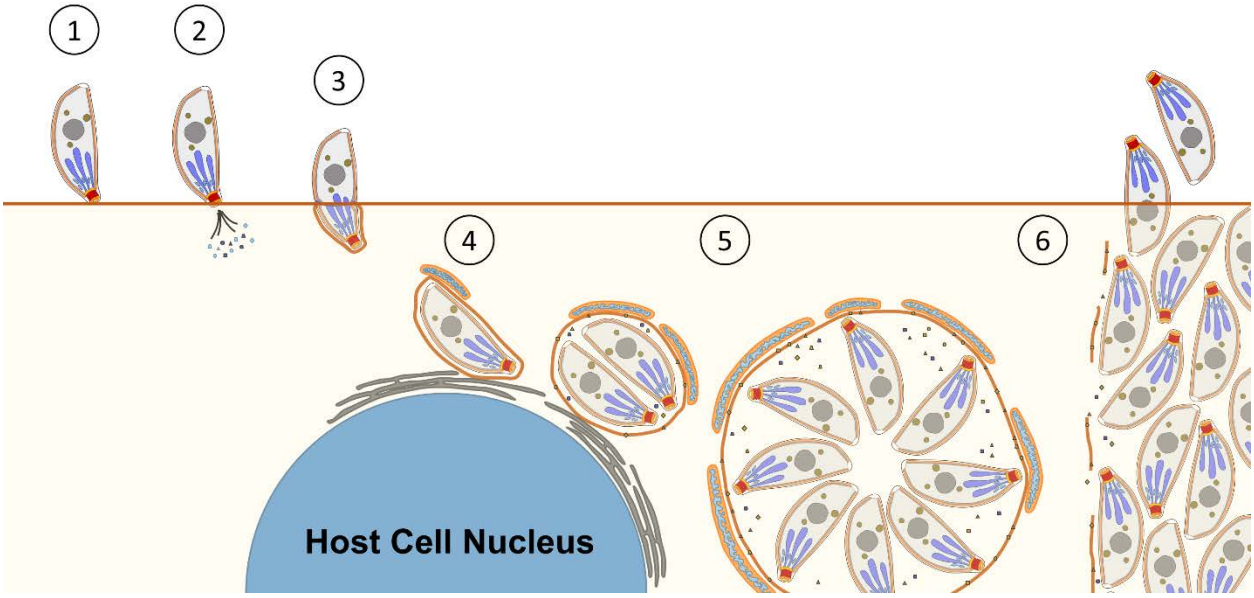

Figure 4. *T. gondii* invading a host cell

Host cell invasion is initiated when the parasite apically associated with the plasma membrane of the host cell (1). The parasite secretes a collection of effector proteins directly into the host cell (2), which help the parasite force its way into the host cell, coating itself in the host plasma membrane, forming what is known as the parasitophorous vacuole (3). The parasitophorous vacuole forms a close association with the host cell's endoplasmic reticulum and mitochondria (4). The parasite grows and divides inside of the parasitophorous vacuole (5), before egressing from the host cell. Image created by Gregory Wier.

As per its name, the moving junction "moves" down the length of the parasite towards its posterior, with the host plasma membrane following in its wake, enveloping the parasite as it pushes its way into the cell. This process creates a vacuole in which the parasite can grow and divide inside, known as the parasitophorous vacuole (PV) [24,26]. In addition, the moving junction

serves as a means to filter the host plasma membrane proteins, selectively determining what host proteins remain in the PV [49]. This exclusion process is thought to be the primary reason why the PV does not fuse with endosomes or lysosomes of the host [50-52]. This is at odds with a parasite that has been coated with antibodies and is phagocytosed and degraded in the lysosome [46,51]. After the completion of the invasion process, the parasite continues to secrete proteins from the rhoptries and the dense granules, which deposit themselves in the PV and the PV membrane. After numerous rounds of replication inside the host cell, the parasites escape from the infected host cell, resulting in its death (Fig. 4). The released parasites are then able to mount new infections to neighboring cells. Successful escape of intracellular parasites is known to be dependent on an increase in $[Ca^{2+}]$ in the parasite cytoplasm, which follows a decrease in $[K^{+}]$ in the cytoplasm of the host cell [53,54]. The increase in $[Ca^{2+}]$ leads to an activation of parasite motility, which is needed for successful escape [53].

1.7 PROTEIN TRAFFICKING IN *T. GONDII* AND PROTEIN PROCESSING

Proper protein trafficking is especially important for the viability of *T. gondii* and other Apicomplexans, as all of the proteins that comprise the parasites' secretory organelles must travel through it before reaching their respective organelles. In general terms, the secretory pathway constitutes all of the steps in which a cell transports proteins to destinations beyond their initial site of translation, the cytoplasm. Despite its name, not all proteins that travel through the secretory pathway are destined to be secreted into the extracellular environment, as membrane bound proteins and organellar proteins must pass through it, as well. The endoplasmic reticulum (ER) is the heart of the secretory pathway, as secreted proteins are sent there first to fold properly and then to be forwarded to their destinations, often transitioning to the Golgi apparatus, before being transferred to its final destination in vesicles. The ER in *T. gondii* is contiguous with the nuclear membrane, and the parasites only have a single Golgi apparatus stack [55,56].

1.7.1 Transport into the endoplasmic reticulum

Proteins traveling through the secretory pathway are usually co-translationally translated into the ER. Most secretory proteins have a short hydrophobic string of amino acids at their N-terminus called the signal peptide which, after emerging from the ribosome, is bound by the signal-recognition particle (SRP). This halts translation until SRP/ribosome complex is guided to an SRP receptor on the ER membrane. Translation resumes, stringing the nascent protein through a

translocon and into the ER, whereupon the signal peptide is removed by signal peptidase [57]. The components for this process are conserved in eukaryotes, and virtually all of them are found in *Plasmodium falciparum* and *T. gondii* [56,58]. After entering the ER, the protein finishes folding and is then free to be transferred to other compartments in the cell. In eukaryotes, proteins traveling between the ER and the Golgi apparatus bud off from their respective organelle in COPI/COPII-coated vesicles, the components of which are present in *T. gondii* [59,60].

1.7.1.1 Transport of membrane proteins into the endoplasmic reticulum

If the protein is destined to be inserted into a membrane, that membrane association is first established while being co-translationally translated into the ER, with its orientation determined by specific topogenic sequences [61]. These topogenic sequences are fall into two categories, 1) a stop-transfer sequence and 2) a signal-anchor sequence. Stop-transfer sequences code for a short, hydrophobic alpha helical segment of amino acids, and are present in many single pass membrane proteins. In addition to a stop-transfer sequence, these proteins have a signal peptide, which directs them to the ER. When the stop-transfer sequence is reached during co-translation, the protein stops being imported into the ER lumen. In most cases, the protein is laterally transferred into the membrane while translation is still occurng. Transmembrane proteins of this sort are considered Type I transmembrane proteins, with a single transmembrane domain, and their N-terminal region facing the ER lumen [61]. Proteins with signal-anchor sequences do not have signal peptides, but are directed to the ER by virtue of their internal signal-anchor sequence, which forms a hydrophobic alpha helical segment. Despite functioning like a signal peptide to guide the protein to the ER, signal peptidase does not cleave the protein. Much like with the stop-transfer sequence, the hydrophobic alpha helix is what anchors the protein in the membrane. Type II and III transmembrane proteins are of this sort, differing in the facing of the N- and C-terminal portions of the protein. Type II transmembrane proteins have the N-terminus facing the cytoplasm and the C-terminus facing the ER lumen, and Type III have the opposite [61]. There are many proteins which cross the membrane more than a single time, including ion channels. These proteins are grouped together as Type IV transmembrane proteins, and accomplish this feat by alternating between stop-transfer and signal-anchor sequences [61].

1.7.1.2 Glycosylphosphatidylinositol (GPI) anchored proteins

Not all membrane proteins are anchored there with a hydrophobic alpha helical segment. Post translational modifications, in the form of glycosylation, also play a role in tethering *T. gondii* proteins to membranes. *T. gondii* parasites are coated with a collection of developmentally regulated surface antigens (SAGs) that are attached to the parasites' plasma membrane via glycosylphosphatidylinositol (GPI) anchors [62,63]. In fact, the vast majority of *T. gondii* plasma membrane proteins are GPI-anchored SAG proteins [63]. GPI-anchored proteins have a signal peptide and a C-terminal stop-transfer sequence that initially anchors them into the membrane. After being inserted into the ER membrane by virtue of the stop-transfer sequence, the protein is cleaved adjacent to the stop-transfer sequence on the luminal face, releasing the protein into the ER lumen. The released protein is then attached to a preformed GPI membrane anchor, once again linking the protein to the membrane [61,64]. With the GPI anchor, the protein is more free to move around in the ER membrane than if it was attached via a transmembrane domain. Beyond surface antigens, the micronemal subtilisin-like serine protease TgSUB1 is GPI anchored [65]. TgSUB1 is secreted from the micronemes during invasion and is involved in the processing of the adhesive microneme protein complexes responsible for host cell attachment in gliding motility and invasion [66].

1.7.2 Dense granule proteins

After a protein has been transferred to the Golgi apparatus, it is free to be transported to any number of destinations, including the parasite's secretory organelles, the rhoptries, micronemes, and dense granules. The default destination of a soluble protein that is transferred through the secretory pathway is the dense granules, which are post-Golgi vesicles [67]. This notion comes from the observation that when a signal peptide is recombinantly appended to a soluble protein, it is transported to the dense granules [55,56]. Proteins bound for the micronemes and rhoptries have additional sorting sequences which direct them to their respective organelles. Experimentally removing these signals from Rhoptry protein 1 (ROP1) and Microneme protein 3 (MIC3) divert the proteins to the dense granules [68,69]. Many of the presently characterized dense granule proteins are type I transmembrane proteins that deposit themselves in the membrane of the PV (PVM), contributing to the maintenance of the vacuole [70]. With the exception of the processing

of the signal peptide, most dense granule proteins do not undergo any other processing, which is at odds with proteins bound for the micronemes and rhoptries, which are extensively processed [56].

1.7.3 Microneme proteins

The majority of *T. gondii* microneme proteins are soluble proteins which aggregate into complexes inside of the ER, before being transported to the micronemes. There are three primary microneme protein complexes: 1) MIC1/MIC4/MIC6 [68], 2) MIC3/MIC8 [71], and 3) MIC2/M2AP [72]. Many of these are soluble proteins that bind to receptors on the host cell (MIC1, MIC4, and MIC3). Each of the complexes features a single type I transmembrane protein which anchors the complex to *T. gondii*'s plasma membrane (MIC6, MIC8, MIC2). Apart from supplying a membrane anchor, these transmembrane proteins often have conserved amino acid motifs in their cytoplasmic tails which are needed for proper microneme targeting [40]. In the case of MIC6, the cytoplasmic domain has two tyrosine-containing motifs, SYHYY and EIEYE, which are required for it to be transported to the micronemes [73]. Beyond having targeting sequences, some microneme proteins are originally synthesized as propeptides which require proteolytic maturation to be properly targeted to the micronemes. As an example, MIC2 is the transmembrane protein in the MIC2/M2AP complex, though processing of a propeptide sequence in its binding partner, M2AP, is needed for the complex to sort properly to the micronemes. Deletion of this propeptide sequence in M2AP arrests the complex in a post-Golgi compartment [74,75].

Aside from proteolytic processing involved in sorting microneme proteins to the micronemes, extensive protein processing occurs after the proteins have been secreted to the parasite's surface [76]. In particular, there are three proteases active at the surface of *T. gondii*, known as Microneme Protein Proteases 1, 2, and 3 (MPM1, 2, 3) [76]. Upon secretion to the parasite's surface, several of the soluble microneme proteins are processed to expose adhesive domains needed to allow them to tightly bind to host cell receptors [77]. This processing is believed to be facilitated by MPP2 and MPP3, though the identities of both MPP2 and MPP3 are currently unknown. MPP1 is responsible for intramembrane cleavage of the transmembrane domains of the MIC proteins anchored to the parasite's plasma membrane. This causes the release of the adhesive microneme complexes bound to the host cell receptors, a process that is needed for efficient parasite motility and host cell invasion [45]. The identity of MMP1 is believed to be the rhomboid-like protease ROM4 [78].

1.7.4 Rhoptry proteins

Knowledge pertaining to the processing of rhoptry proteins is not as well defined as that for microneme proteins. Processing of rhoptry proteins is known to take place even while the organelle is being formed, with Rhoptry protein 1 (Rop1) being synthesized as a proprotein which is processed during the biogenesis of the organelle [79]. Other rhoptry proteins that are synthesized as proproteins are processed during their trip through the secretory pathway on their way to the mature rhoptry organelles [80]. The majority of rhoptry protein processing occurs while the protein transitions through the secretory pathway, and not after being secreted from the parasite, as is the case with many microneme proteins. Treatment of *T. gondii* with inhibitors to subtilisin (SUB) serine proteases results in the disruption of rhoptry formation and a block in parasite replication, implying that proteases of the subtilase family might be involved in rhoptry processing [81]. *T. gondii* has 12 subtilase genes, though most have not been subjected to extensive study. *T. gondii* subtilisin 2 (TgSUB2) is a rhoptry protein itself, one that is believed to play a role in the processing of proprotein forms of rhoptry proteins. There is a predicted TgSUB2 cleavage site in Rop1. Additionally, TgSUB2 co-immunoprecipitates with Rop1, suggesting that TgSUB2 might be responsible for Rop1 processing [81,82]. Toxopain-1 is another rhoptry protease (cathepsin B-like) which is believed to play a role in the processing of rhoptry proteins, as treatment with cathepsin-B inhibitors impedes rhoptry formation and parasite invasion [83].

1.8 DEVELOPING A STRATEGY TO LABEL PARASITE PROTEINS IN THE CONTEXT OF THE HOST CELL

While our understanding of how *T. gondii* is able to invade a host cell and grow inside it has made considerable progress since the discovery of the parasite in 1908 [84], there is still much to learn. How the parasite co-opts the host cell to permit is survival is still poorly understood. The parasitophorous vacuole in which the parasite grows acts as the interface between the host and the parasite, but beyond identifying some of the parasite proteins deposited in the vacuolar membrane, our knowledge is lacking. Designing a system to specifically label parasite proteins while *T. gondii* grows inside of the host cell could be instrumental to shedding light on some of these questions. My work in the Boyle lab has focused on developing such a system, one which utilizes a nonstandard amino acid to specifically label *T. gondii* proteins.

The use of noncanonical amino acids for the labeling nascent proteins has been receiving increased traction in present years [85]. In particular, the methionine (Met) analog azidohomoalanine (Aha) has been utilized because it has an azide functional group that can undergo a copper(I)-catalyzed cycloaddition "click" reaction with any alkyne-containing molecule [86,87]. This provides the opportunity to use click chemistry to append a fluorescent tag for visualization, or affinity tag for purification, to any Aha-containing protein. Aha is similar enough in size to Met (Fig. 5A, B) that it is charged to $tRNA^{Met}$ by a wild-type methionyl-tRNA synthetase (MetRS), such that it can be utilized with virtually any cell line [88-91]. However, in the case of *T. gondii* infecting a host cell, Aha would get incorporated in both parasite and host proteins, making it ineffective for specifically labeling parasite proteins.

To get around this problem, I have created a strain of *T. gondii* that expresses a mutant *E. coli* methionyl-tRNA synthetase ($MetRS^{NLL}$) that is able to charge $tRNA^{Met}$ with the Met analog, azidonorleucine (Anl) [86]. Anl is similar to Aha in that it also has an azide functional group, but its larger size prevents it from being recognized by a wild type MetRS (Fig. 5A). This means that Anl can only be incorporated into the proteins of cells that express the mutant form of the enzyme. So, in the case of the *T. gondii* strain expressing the mutant *E. coli* $MetRS^{NLL}$ (the Tg-$MetRS^{NLL}$ strain), Anl can be incorporated into nascent parasite proteins, but not those of the infected host cell.

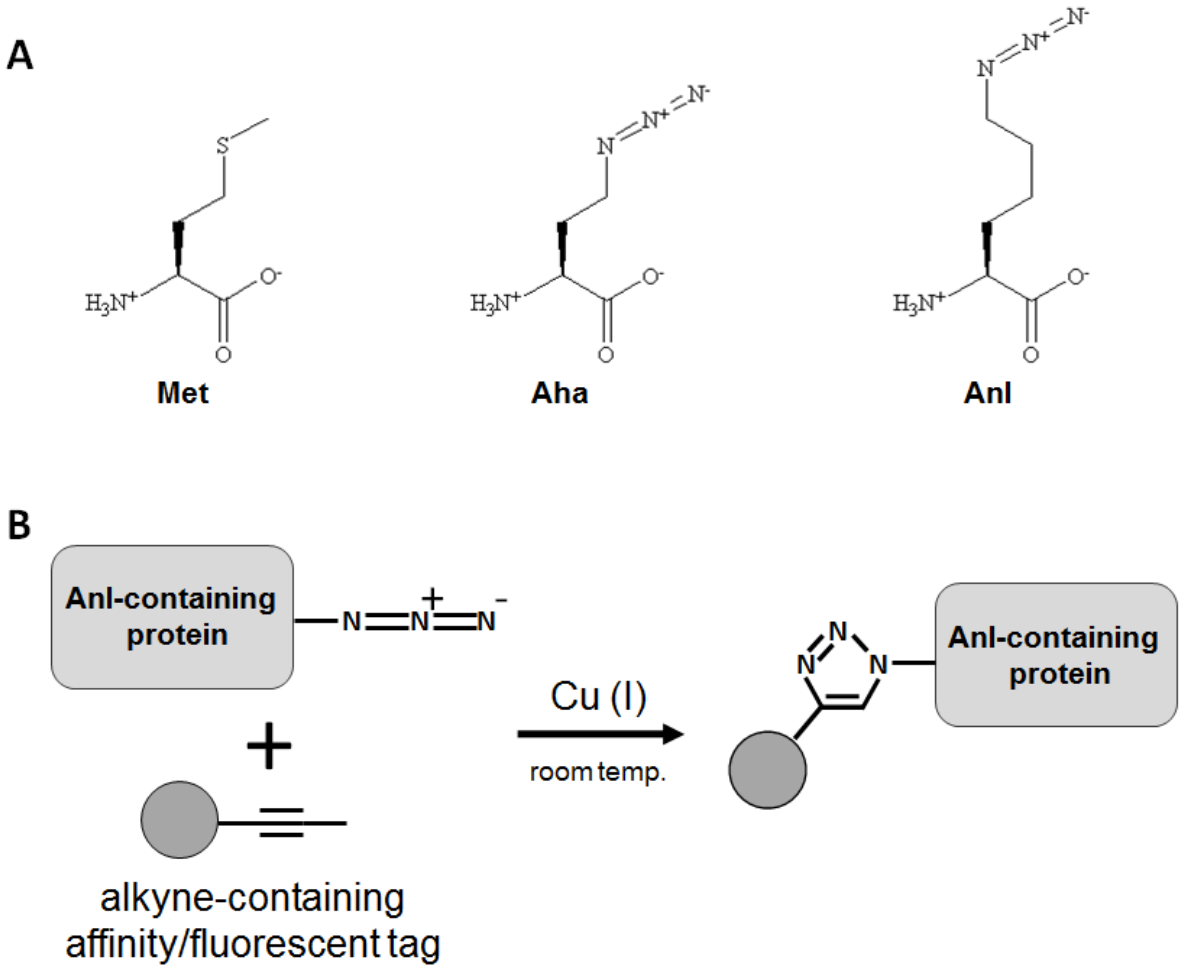

Figure 5. Methionine analogs with azide functional groups can be modified via click chemistry

A) The structures of methionine (Met), azidohomoalanine (Aha), and azidonorleucine (Anl). B) A reaction scheme for the click chemistry reaction between and Anl (or Aha)-containing protein and an alkyne-containing molecule. Image created by Gregory Wier.

1.9 DESCRIPTION OF THE UPCOMING CHAPTERS

In the following chapters, I will discuss my efforts towards implementing the MetRS^{NLL}/Anl labeling system in *T. gondii*. Chapter 2 consists of my published paper documenting the creation of the MetRS^{NLL}-expressing *T. gondii* strain and its initial characterization, where I show the bacterial MetRS^{NLL} is functional in *T. gondii* [92]. Chapter 3 describes the additional work that I have done to advance the project beyond what was published. I discuss the mass spectrometry studies that I conducted to help elucidate what Met residues are capable of incorporating Anl. I describe ongoing work to engineer *T. gondii*'s MetRS to recognize

Anl and charge it to both of the parasite's $tRNA^{Met}$. Furthermore, I discuss ongoing work to express a mutated version of murine MetRS in *T. gondii*, which is capable of charging Anl to both $tRNA^{Met}$ in mammalian cells. Chapter 4 summarizes my work with the $MetRS^{NLL}$/Anl labeling system in *T. gondii* and offers suggestions of the steps that should be taken to see the project continue in the future. Chapter 5 contains all of the relevant materials and methods used for the $MetRS^{NLL}$/Anl labeling project. Finally, the appendices describe additional projects which I have worked on during my time in the Boyle lab which do not fit with the overall scope of the thesis.

2.0 A NEW METHOD FOR THE ORTHOGONAL LABELING AND PURIFICATION OF *TOXOPLASMA GONDII* PROTEINS WHILE INSIDE OF THE HOST CELL

What follows is a replication of my first author paper in mBio, published March 10, 2015 [92]. I wrote the paper in its entirety, and have included it here largely unmodified, with some minor changes to fit with the formatting of the thesis and to improve its cohesion with the remainder of the document. Furthermore, the Materials and Methods section of the paper was incorporated into Chapter 5 of the thesis.

2.1 INTRODUCTION

In the past several years, the use of noncanonical amino acids (NCAAs) to label newly synthesized proteins has expanded rapidly [85,90,93-95]. Numerous methionine (Met) surrogates have been developed that can be activated by wild-type methionyl-tRNA synthetases (MetRS) and incorporated into cellular proteins. Several such surrogates carry reactive functional groups that allow attachment of affinity tags for protein purification or fluorescent probes for visualization. Among the most commonly used NCAAs are homopropargylglycine (Hpg) and azidohomoalanine (Aha) [88-91], both of which are susceptible to the copper(I)-catalyzed variant of the Huisgen 1,3-dipolar cycloaddition reaction, known commonly as “click chemistry” [96-98] (Fig. 6A, B). Aha- or Hpg-containing proteins can be specifically labeled with alkyne or azide tags for visualization or purification followed by identification by mass spectrometry.

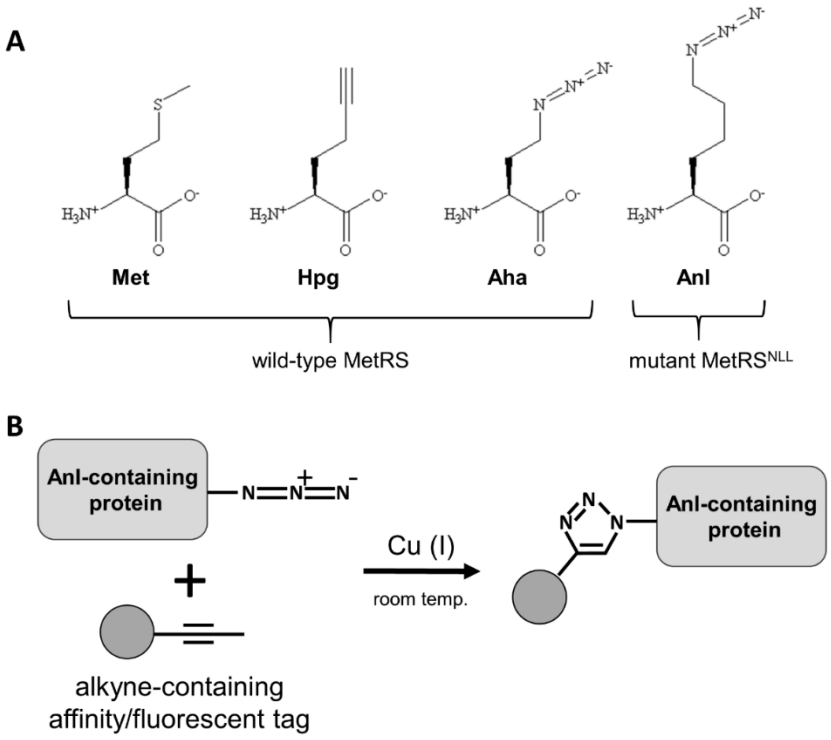

Figure 6. Azidonorleucine is a methionine analog that is susceptible to modification via click chemistry

A) The structures of methionine (Met) and methionine analogs: homopropargylglycine (Hpg), azidohomoalanine (Aha), and azidonorleucine (Anl). They can all be charged to $tRNA^{Met}$ by the wild-type MetRS, with the exception of Anl, which can be utilized only by a mutant synthetase like *E. coli* $MetRS^{NLL}$. B) Reaction scheme of a click cycloaddition between a protein that has incorporated Anl and an alkyne conjugated to an affinity/fluorescent tag.

While this is a powerful approach, it does not allow monitoring of individual cell types in mixed cell populations, as all cell types incorporate the NCAA. This limitation prompted the development of cell-selective systems for NCAA incorporation. In 2009, Ngo and coworkers used random mutagenesis on active site residues to create a mutant version of *Escherichia coli* MetRS (the $MetRS^{NLL}$ strain) that activates the Met analog azidonorleucine (Anl), which is not utilized by the wild-type MetRS (Fig. 6A) [86,99]. This was accomplished by mutation of three highly conserved residues in the Met-binding pocket of the enzyme (L13N, Y260L and H301L; $MetRS^{NLL}$) [100,101]. *E. coli* expressing $MetRS^{NLL}$ (Ec:$MetRS^{NLL}$) were able to use Anl in protein synthesis, whereas wild-type *E. coli* were unable to do so. Furthermore, when Ec:$MetRS^{NLL}$ was grown in co-culture with other bacterial strains or with mammalian cells, Anl was detected only

in Ec:MetRS^{NLL} proteins [86]. Because MetRS^{NLL} has a higher specificity constant (k_{cat}/K_m) for Anl than for Met, the growth medium does not have to be depleted of Met for effective labeling [99].

Such a system could be extremely useful to study the interaction between the human parasite *Toxoplasma gondii* and its host. *T. gondii* is an obligate intracellular protozoan parasite of the phylum Apicomplexan (of which the causative agent of malaria, *Plasmodium* spp., is part) and is estimated to infect up to a third of the human population [102]. For this purpose, we have developed a *T. gondii* strain that heterologously expresses an HA-tagged version of *E. coli* MetRS^{NLL}, and show that it is fully functional.

2.2 RESULTS AND DISCUSSION

In order to implement the MetRS^{NLL}/Anl system in *T. gondii*, we heterologously expressed an HA-tagged version of *E. coli* MetRS^{NLL} in a type II strain of *T. gondii* (Me49Δ*HPT*:*Luc* [103]). Successful expression of the bacterial gene was confirmed by comparing lysates of human foreskin fibroblasts (HFFs) infected with either wild type (WT) *T. gondii* or the MetRS^{NLL}-expressing strain (the Tg-MetRS^{NLL} strain) via western blotting (Fig. 7A). A prominent signal (at the expected 64 kD size) is present only in the Tg-MetRS^{NLL} strain-infected sample, confirming that *E. coli* MetRS^{NLL} can be successfully expressed in *T. gondii*.

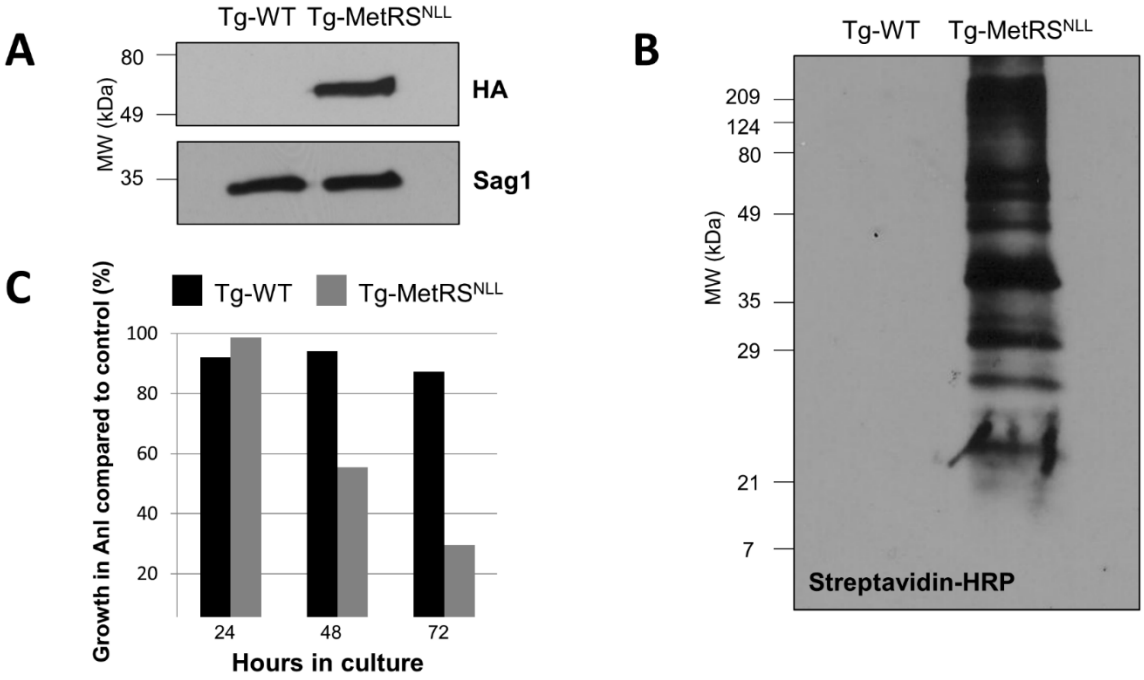

Figure 7. The bacterial MetRS^{NLL} is expressed and functional in *T. gondii*

A) *Toxoplasma gondii* is able to express the *E. coli*-derived MetRS^{NLL} under control of the *T. gondii* GRA1 promoter. An HA-tagged version of MetRS^{NLL} was expressed in a type II strain of *T. gondii* (Me49Δ*HPT*:*Luc*) under the control of the *T. gondii* GRA1 promoter. MetRS^{NLL} expression was confirmed by anti-HA western blotting. Anti-Sag1 antibody served as a loading control. B) Incorporation of Anl into proteins is limited to MetRS^{NLL}-expressing parasite strains. Lysates of Anl treated human foreskin fibroblasts (HFFs) infected with either the Tg-MetRS^{NLL} strain or WT *T. gondii* were subjected to click chemistry in the presence of biotin-alkyne. Successful biotinylation was assessed by probing western blots with streptavidin-HRP. C) The number of *T. gondii* parasites is reduced after 48 hours (h) of Anl exposure. The effects of growing the Tg-MetRS^{NLL} strain in 1 mM Anl over a period of 3 days were assessed by a luciferase assay. Data are represented as the percent of growth in Anl compared to non-exposed controls. Growth of the Tg-MetRS^{NLL} strain is reduced after 48 and 78 h of Anl-exposure. Quantitative data are consistent with multiple qualitative observations of growth differences between the Tg-MetRS^{NLL} strain and WT *T. gondii* in Anl.

2.2.1 The Tg-MetRS^{NLL} strain is able to incorporate Anl into proteins

To determine if the Tg-MetRS^{NLL} strain is able to incorporate Anl into nascent proteins, monolayers of HFFs were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain in the presence of 1 mM Anl for 24 hours (h). It is important to note that Met-free medium was not used, such that synthesis of new proteins is not dependent on the incorporation of Anl. The growth medium was supplemented with additional Anl after 24 h, and after a total of 48 h of growth in Anl, the infected HFFs were solubilized and subjected to click chemistry using a biotin-conjugated alkyne [104,105]. Anl-incorporation was confirmed via western blotting, demonstrating that the *E. coli* MetRS^{NLL} is functional in *T. gondii*, and that it is limited to parasites that express MetRS^{NLL} (Fig. 7B). Furthermore, a wide variety of proteins > 21 kD are labeled only in MetRS^{NLL}-expressing parasites. Taken together, these biotinylation data show that MetRS^{NLL} is functional in *T. gondii*, and is the first demonstration of the utility of this system in any protozoan pathogen. Moreover, it is likely that this system would be operational in other closely related apicomplexans, including *Plasmodium spp*. and *Cryptosporidum spp*.

It should be noted that at longer exposure times, background streptavidin-HRP labeling is visible around 35, 45, 65, and 130 kD in both the WT and MetRS^{NLL}-expressing samples (data not shown). Biotin is a cofactor that is covalently attached to a number of carboxylase enzymes, such as apicoplast-located acetyl coenzyme A (acetyl-CoA) carboxylase [106,107] and the mitochondrion-located pyruvate carboxylase in *T. gondii*. These naturally biotinylated proteins are known to be

visible on streptavidin-HRP blots [106,108], so they should be visible regardless of Anl-incorporation. The 35 kD band is the most prominent, which may correspond to a subdomain of the multisubunit acetyl-coA carboxylase [106], which is prone to proteolysis in rapeseed *Brassica napus* [109]. The 130 kD band likely corresponds to pyruvate carboxylase, as it agrees with its predicted size.

2.2.2 The effects of Anl-incorporation on parasite growth and protein stability

Incorporation of Anl into nascent proteins may lead to protein misfolding or instability, which could be toxic or lethal to replicating parasites. It is important to note that while the functional group of Anl is slightly larger than that of Met (Fig. 6A), both amino acids are nonpolar, suggesting that replacement of Met with Anl may not have a strongly disruptive effect on protein folding. Studies using a similar azide-containing methionine analog, Aha (Fig. 6A), that is capable of being charged to $tRNA^{Met}$ by a wild-type MetRS, found that the tag was non-toxic to the growing cells and did not significantly affect protein stability or promote degradation [88,90,110-112]. The majority of these studies were conducted using short pulse times (2-4 h [88,110,112], up to 24 h [111]), so it remains to be seen whether prolonged exposure to Aha affects cell growth. While Anl is very similar in structure to Aha (Fig. 6A), it is slightly larger, and therefore cannot be charged to $tRNA^{Met}$ by a wild type MetRS. To assess the effects of Anl-incorporation, we 1) examined how prolonged Anl-exposure affected parasite growth and 2) globally addressed whether Anl-incorporation affects protein stability.

2.2.3 Examining the effect of prolonged Anl-exposure on parasite growth

The effect of Anl-exposure on parasite growth was tested by growing either WT *T. gondii* or the Tg-$MetRS^{NLL}$ strain in the presence or absence of 1 mM Anl over a 72 h time period, and performing luciferase assays to quantify parasite replication rate. After 24 h of growth in 1 mM Anl, luciferase-derived signal intensity was similar between Anl-exposed and control parasite lysates (Fig. 7C). However, noticeable differences in parasite numbers were observed in the $MetRS^{NLL}$-expressing strain after 48 h and 72 h of Anl exposure (55%, and 30% compared to wild type controls, respectively; Fig. 7C). These data suggest that incorporation of Anl leads to a decrease in parasite number, particularly after prolonged Anl exposure. Since this experiment was only performed once, no statistical analysis could be performed to give credence to the findings. However, a reduction in parasite number after treating the Tg-$MetRS^{NLL}$ strain with Anl was consistently observed in all subsequent labeling experiments over 48 hours. Without additional

experiments, it is not possible to say whether this reduction in parasite numbers is due to a change in growth rate, or whether it is a result of increased parasite death.

Despite the reduced parasite numbers, parasites exposed to Anl for 48 h were still able to effectively invade host cells and replicate normally after Anl removal (Fig. 11, discussed later in text). Parasites exposed to Anl for 72 h were also able to successfully invade host cells, as well (data not shown). This indicates that Anl-incorporation did not have long-term effects on parasite physiology and that parasites subjected to 72 h of Anl-exposure could still be used in downstream experiments. While the exact mechanism of the reduction of parasite numbers is unknown, prolonged Anl-exposure may result in destabilization or misfolding of *T. gondii* proteins, leading to reduced parasite growth rate or increased parasite death. Regardless, Anl-exposed parasites are fully capable of initiating new infections, indicating that key secretory proteins involved in invasion remain functional after Anl-exposure.

The reasons for the reduced parasite numbers after 48 h and 72 h of Anl exposure are unclear. The Anl-exposed *T. gondii* parasites could be experiencing toxicity associated with aberrant folding of Anl-containing proteins although, compared to published studies with Anl [113] and particularly Aha [88,110,112], the 48 h pulse time greatly exceeds typical analog pulse times. To avoid complications of reduction in parasite number, we advise using Anl pulse times less than 24 h. Importantly, if the system were to be used for pulse-chase experiments, the Anl pulse time would likely be considerably shorter than 24 h, such that prominent reductions in parasite numbers would not be observed. Indeed, pulse-chase studies utilizing Aha as a Met-analog use 2- 4 h pulse times to achieve substantial labeling [88,110,112].

2.2.4 Assessing the stability of Anl-containing proteins

An Anl pulse-chase experiment was used to globally examine the stability of Anl-containing proteins. HFFs were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain and pulsed with Anl for 2 h. As expected, a biotinylated signal is only present in the Tg-MetRS^{NLL} strain-infected samples (Fig. 8A, lanes 5-8). The signal intensity is the highest directly after the pulse, with the intensity dropping off steadily over the 6 h chase time (Fig. 8A, C). Despite the declining signal intensity, after the 6 h pulse there is still a sizable amount of labeled protein, with the signal appearing to stabilize between 3 and 6 h. These data suggest that many Anl-labeled parasite proteins remain stable for at least 6 h. From these data, the average half-life of Anl-

containing proteins is approximately 7 h. Pulse-chase studies with [^{35}S]methionine in mammalian cells have shown that approximately 30% of newly synthesized proteins are degraded by the proteasome with a half-life of <10 minutes, mainly consisting of proteins that do not adopt their native fold due to translational errors [114]. It remains to be determined whether a similar margin of proteins is degraded in *T. gondii* within this time frame. The possibility still exists that Anl-incorporation has a strong destabilization effect on some proteins, leading to their rapid degradation by the proteasome, though those would be missed by the pulse-chase experiment presented here.

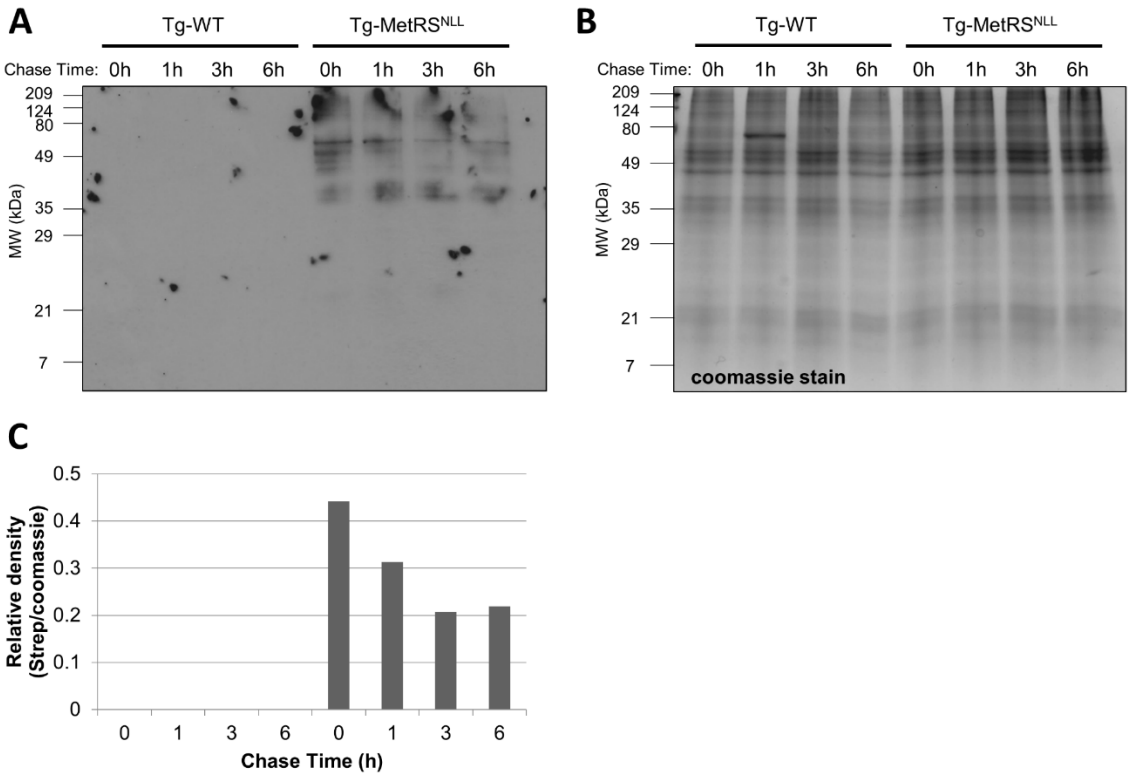

Figure 8. Anl-labeled proteins are still present 6 h after being pulsed with Anl

A) HFFs were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at an MOI of 3 and grown for 48 h before being pulsed with 1mM Anl for 2 h. Following the 2 h pulse, Anl was washed away and the infected HFFs were harvested at different time points after the pulse (0, 1, 3, 6 h). Samples were biotinylated with click chemistry using biotin-alkyne. Successful biotinylation was assessed by western blotting and probing with streptavidin-HRP. A biotinylation signal is only visible in the MetRS^{NLL}-expressing strain. The signal intensity is strongest at the 0 h time point, dropping steadily over a period of 6 h. B) A coomassie stained SDS-PAGE gel identical to the one used to

prepare the western blot in A), confirming equal protein loading. C) Quantification of the data from B). The experiment was conducted at least 2 times with similar results.

2.2.5 Optimal Anl concentration for efficient incorporation into nascent proteins

To optimize the MetRS^{NLL}/Anl system in *T. gondii*, Anl-incorporation was examined at concentrations ranging from 0.1 to 4 mM over a 48 h time period (Fig. 9A, B). The levels of Anl-incorporation with 0.5 mM and 1.0 mM Anl were similar, though there is considerably less labeling with 0.1 mM Anl compared to 0.5 mM and 1.0 mM (Fig. 9A, B). Based on visual inspection, parasite numbers were similar in 0.1 mM, 0.5 mM, and 1.0 mM Anl. However, it was apparent that replication was clearly reduced for parasites growing in 4.0 mM Anl. The reduction in parasite number was confirmed by the observed reduction in HA-tagged MetRS^{NLL} on the western blot (identical "cell equivalents" were loaded for each sample; Fig. 9A). While overall Anl incorporation is higher in 4.0 mM Anl than in other tested Anl concentrations (Fig. 9B), the effect on the parasites may outweigh this benefit when large numbers of parasites are required for downstream experiments. However, this concentration may be useful in cases where parasite number is less important than overall Anl-incorporation. Taken together, these data suggest that 1.0 mM Anl is a suitable concentration for maximizing Anl-incorporation while avoiding reductions in parasite number.

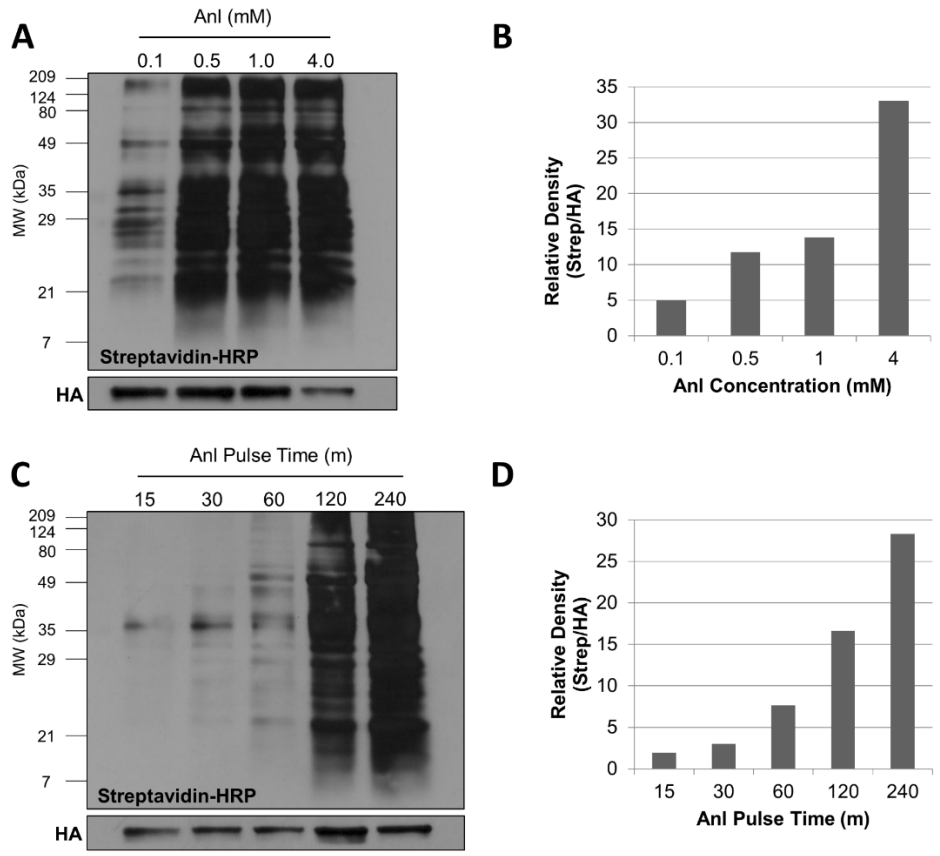

Figure 9. 1 mM Anl is an ideal concentration for labeling, and Anl pulse times as short as 1 hour exhibit labeling

A) Growing parasites in 1 mM Anl allows high levels of incorporation while limiting negative effects on parasite growth. HFFs were infected with the Tg-MetRS^{NLL} strain at an MOI of 3 and either 0.1, 0.5, 1, or 4 mM Anl was added to the growth medium at the time of infection. Cells were harvested after 48 h of growth. The samples were biotinylated with click chemistry using biotin-alkyne. Successful biotinylation was assessed by western blotting and probing with streptavidin-HRP. Anti-HA western blotting for the HA-tagged MetRS^{NLL} served as a loading control. B) Quantification of the data from A). The experiment was conducted at least 2 times with similar results. C) Anl is incorporated into a substantial number of proteins just 1 h after being added to growth medium. HFFs were infected with the Tg-MetRS^{NLL} strain at an MOI of 3 and after 2 days of growth, 1 mM Anl was added to the growth medium. Samples were collected at time points following Anl addition: 15 m, 30 m, 60 m, 120 m, and 240 m. The samples were biotinylated with click chemistry using biotin-alkyne. Western blotting with streptavidin-HRP was used to confirm successful labeling. D) Quantification of the data from C). The experiment was conducted at least 2 times with similar results.

2.2.6 Anl is incorporated into a substantial number of proteins 1 hour after being added to growth medium

Knowing that prolonged growth in Anl slows reduces parasite numbers, we wanted to determine the amount of time parasites needed to be exposed to Anl for incorporation to be detectable. To address this question, HFFs were infected with the Tg-MetRS^{NLL} strain and allowed to grow for 48 h (such that the HFFs were well-infected and would lyse the monolayer within 12 h). After the 48 h of growth, the growth medium was supplemented with 1 mM Anl for varying amounts of time (15, 30, 60, 120, and 240 m). Anl was then washed from the monolayers and samples were harvested for click chemistry with biotin-alkyne. Western blotting was used to compare the levels of Anl-incorporation at the different Anl pulse times (Fig. 9C, D). Anl-incorporation can be detected with just a 30 m Anl pulse and after an hour of Anl-exposure, a substantial number of proteins have incorporated Anl, based on the extensive biotinylation banding pattern that is observed. As expected, the level of Anl-incorporation increases with longer Anl pulse times, with the 4 h pulse time exhibiting the strongest signal (Fig. 9C, D).

2.2.7 Is Anl-incorporation specific to parasite proteins?

The utility of this approach for studying an intracellular parasite like *T. gondii* depends on the specificity of Anl-incorporation into parasite proteins. To determine if Anl-incorporation is specific to *T. gondii* proteins compared to host cell proteins, HFFs infected with WT *T. gondii* or the Tg-MetRS^{NLL} strain were treated with Anl for 48 h, solubilized, and subjected to click chemistry with biotin-alkyne. The clicked samples were then used for neutravidin affinity purification. Anl-containing proteins were successfully purified (Fig. 10A), but only from the HFFs infected with the Tg-MetRS^{NLL} strain. To assess whether host cell proteins were labeled and purified, an identical blot to the one in Fig. 10A was probed with an antibody against human GAPDH (Fig. 10B). A distinct signal at the expected 37 kD size is present in the input and unbound lanes of both the WT *T. gondii* and the Tg-MetRS^{NLL} strain samples, though it is absent from the elutions. The absence of a signal from the Tg-MetRS^{NLL} strain elutions strongly suggests that host cell proteins are not incorporating Anl, and are therefore not being biotinylated and purified. To confirm the presence of parasite proteins in the Tg-MetRS^{NLL} strain elutions, an identical blot to the hGAPDH blot was probed with an anti-HA antibody to detect the parasites' HA-tagged MetRS^{NLL} (Fig. 10C). A distinct signal is seen for the HA-tagged MetRS^{NLL} in both the input,

unbound, and eluted samples, but only in the Tg-MetRS^{NLL} strain samples (at the expected 64 kD size). The signal intensity in the elution is approximately 30-40% of that of the input, giving a general estimation of the level of Anl-incorporation. The prominent signal seen in the unbound sample likely applies to the HA-tagged MetRS^{NLL} that did not incorporate Anl, and therefore was not biotinylated and purified. These data strongly suggest that host proteins are not labeled with Anl during exposure *in vitro*, which is consistent with the fact that they have not been engineered to express *E. coli* MetRS^{NLL}. These data also suggest that the majority, if not all, of the biotinylated proteins seen in the blot from Figure 10A correspond to Anl-labeled parasite proteins.

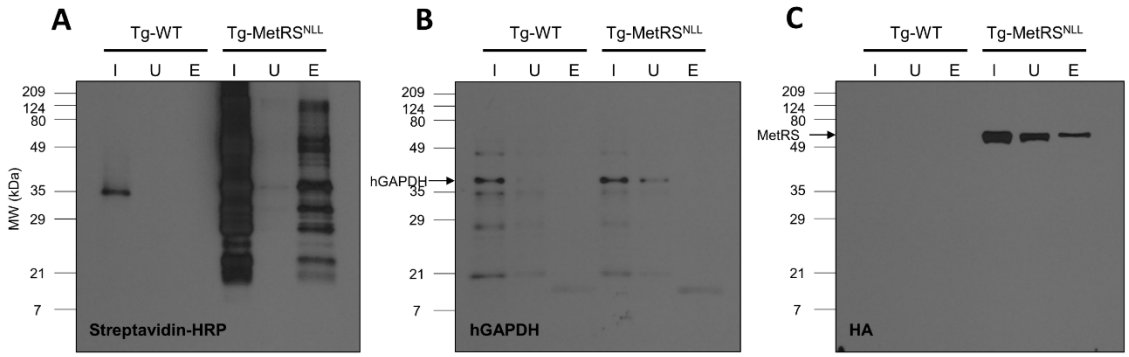

Figure 10. Anl-incorporation is limited to parasite proteins

A) Neutravidin purification of lysates of HFFs that had been infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain and grown in the presence of 1 mM Anl for 2 days. The samples were biotinylated with click chemistry using biotin-alkyne and incubated with neutravidin resin for 1 h. Biotinylated samples were eluted by boiling in SDS sample buffer. Labeling is only present in the Tg-MetRS^{NLL} strain samples, where it is visible in the input (I) and elutions (E), and largely absent from the unbound (U) fraction. B) An identical blot to the one in A) was probed with an anti-human GAPDH antibody to see if host cell proteins were purified with neutravidin. A signal for hGAPDH is present in the input and, to a lesser extent, the unbound samples, but is absent from the elutions, suggesting that only parasite proteins are labeled. C) An identical blot to the one in A) was probed with an anti-HA antibody to identify the HA-tagged MetRS^{NLL}. A prominent signal is seen in both the input and elution lanes of the Tg-MetRS^{NLL} strain samples only. This suggests that the biontinylated proteins from A) are *T. gondii* proteins.

2.2.8 Parasites can be labeled *in situ* using click chemistry

The MetRS^{NLL}/Anl system should allow for Anl treated parasites to be labeled *in situ* by attaching a fluorescent tag to Anl-containing proteins using click chemistry [88,113,115]. To test this in *T. gondii*, we infected HFFs with WT *T. gondii* or the Tg-MetRS^{NLL} strain parasites that had been

pre-treated with 1 mM Anl for 48 h. Six hours post-infection, parasites were fixed and permeabilized. Infected HFFs were then subjected to click chemistry using an Alexa Fluor 594-conjugated alkyne. Tg-MetRS^{NLL}-expressing parasites stained brightly with Alexa Fluor 594 in the parasite cytoplasm, while WT *T. gondii* parasites exposed to Anl did not. This further confirms that Anl is not incorporated into host cell proteins (Fig. 11B, F). The infected HFFs were co-stained with the secretory protein Gra7 (Fig. 11C, G), which exhibited intense parasite staining as well as host cell cytosolic punctate staining, which is characteristic of this *T. gondii* protein [116]. Importantly, we did not observe any such punctate staining in the Alexa Fluor 594 channel (Fig. 11F, H), suggesting that secreted proteins like Gra7 either 1) are not tagged with Anl, 2) incorporate Anl but are highly unstable and therefore undetectable, 3) are not properly secreted from the parasite when they have incorporated Anl, or 4) are processed in a manner that removes all Anl-containing residues. We addressed this further in the experiments described immediately below.

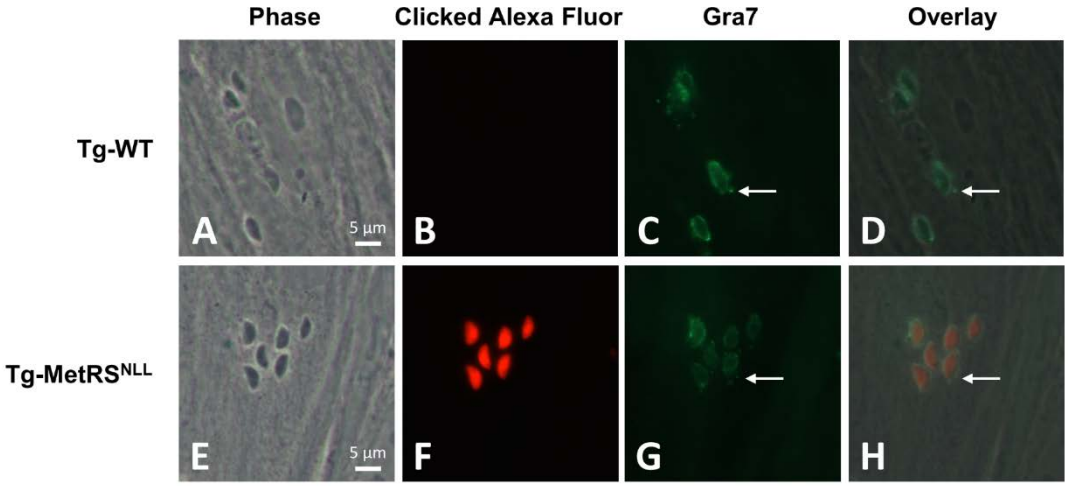

Figure 11. Anl treated parasites can be labeled *in situ* using click chemistry

WT *T. gondii* and the Tg-MetRS^{NLL} strain were grown in the presence of Anl for 48 h, before being used to infect HFFs for 6 h. After fixation and permeabilization, a click reaction was performed on the samples using Alexa Fluor 594-alkyne (**B**, **F**). Additionally, the parasites were stained for the secreted protein Gra7 using a mouse Ab followed by Alexa Fluor 488 Goat Anti-Mouse IgG (**C**, **G**). The punctate Gra7 staining emanating from around the parasites (white arrows, **C**, **G**) is not stained with the clicked Alexa fluorophore (**F**), suggesting secreted proteins are not labeled. The channels have been overlaid in **D** and **H**. 100x magnification was used for all images.

2.2.9 Anl is only minimally incorporated into secreted parasite proteins

A study by Ngo *et al*. showed that mammalian cells expressing the bacterial MetRS^{NLL} only incorporated Anl at the N-terminal Met position in nascent proteins and not internal positions [113]. This was attributed to an incompatibility between the bacterial MetRS^{NLL} and one of the two mammalian tRNA^{Met}, the elongator tRNA^{Met} (described further below). If this were also the case in *T. gondii*, secretory proteins would not be labeled, since the signal peptide of secreted proteins is cleaved during protein processing, effectively removing the N-terminal Anl residue if it had been incorporated. The system would also bypass most mitochondrial proteins, which have N-terminal presequences that are removed upon successful entry into mitochondria [117]. Furthermore, most apicoplast proteins would be missed, as they have a distinct N-terminal bipartite signal consisting of a classical secretory signal peptide that is removed, followed by a transit peptide needed for import into the apicoplast [118,119]. To determine if this was a limitation in the *T. gondii*/MetRS^{NLL}/Anl system, parasites were grown in the presence of 1 mM Anl for 2 days, solubilized and proteins were biotinylated with click chemistry. The biotinylated samples were then purified with neutravidin resin and run on a western blot probing for the secreted *T. gondii* protein Rhoptry protein 7 (Rop7; Fig. 12B) and biotinylated proteins with streptavidin-HRP (Fig. 2-7A). The prevalent signal in both the input and elution samples on the streptavidin-HRP blot (Fig. 12A) implies that a sizeable number of parasite proteins incorporate Anl, allowing them to be biotinylated and purified. However, the absence of a signal in the elution on the Rop7 blot (Fig. 12B) implies that Rop7 cannot be purified, potentially because it 1) does not incorporate Anl, 2) any incorporated Anl gets removed via processing, or 3) incorporation destabilizes the protein causing it to be degraded.

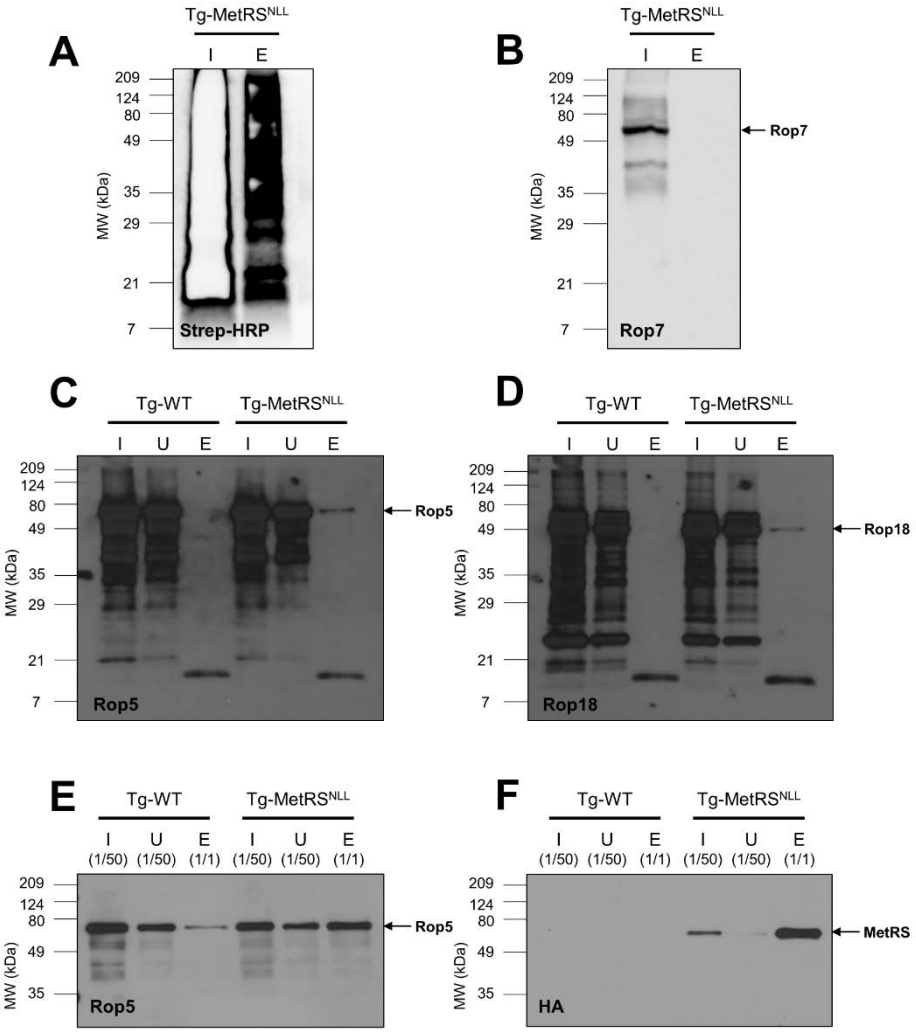

Figure 12. Proteins from *T. gondii* parasites grown in the presence of Anl can be biotinylated with click chemistry, though secreted rhoptry proteins are minimally labeled

A) A western blot of a neutravidin purification of biotinylated parasite proteins, probed with streptavidin-HRP. HFFs were infected with the Tg-MetRS^{NLL} strain in the presence of 1 mM Anl, and solubilized after 2 days of growth. Anl-containing proteins were biotinylated with click chemistry and purified with neutravidin resin. There is a strong signal in both the input (I) and elution (E), suggesting that a sizeable number of proteins were successfully purified. Note: the large amount of biotinylated protein in each lane resulted in a rapid depletion of the chemiluminescent substrate, hence the lack of signal in the internal portion of the input lane. B) An identical blot to the one in A), probed for the secreted parasite rhoptry protein 7 (Rop7). The absence of a signal for Rop7 in the elution sample suggests that it did not incorporate Anl, and therefore was not biotinylated. C) A western blot of a neutravidin purification of biotinylated proteins from HFFs infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain and probed with an anti-rhoptry protein 5 (Rop5) antibody. The blot was overexposed, revealing a signal for Rop5 in the elution of the Tg-MetRS^{NLL}

strain sample. D) The blot from C) was stripped and reprobed with and anti-rhoptry protein 18 (Rop18) antibody. As with C), the blot was overexposed, revealing a signal for Rop18 in the elution of the Tg-MetRS^{NLL} strain. E) The samples used to prepare C) and D) were used for a new blot, where the input and unbound samples were diluted 50 fold and the elution samples were undiluted. The blot was probed with an anti-Rop5 antibody, revealing Rop5 signal in both the WT *T. gondii* and the Tg-MetRS^{NLL} strain elutions. F) The blot from E) was stripped and reprobed with an anti-HA antibody to identify the HA-tagged MetRS^{NLL}.

To investigate whether other secreted proteins could be purified, additional neutravidin purifications were performed, using HFFs infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain, and probed for the secreted parasite proteins Rhoptry protein 5 (Rop5; Fig. 12C) and Rhoptry protein 18 (Rop18; Fig. 12D). Interestingly, a signal is visible in the Tg-MetRS^{NLL} strain elutions only when the blots are overexposed (Fig. 12C, D). In both cases, the clear disparity between signal intensities in the input/unbound and the elutions implies that both Rop5 and Rop18 are very minimally labeled. This is at odds with the purified HA-tagged MetRS^{NLL} (Fig. 10C), which suggests around 30-40% of the HA-tagged MetRS^{NLL} population is labeled and purified. If both the input and unbound samples are diluted by a factor of 50 and the elution samples remains undiluted, a Rop5 western blot reveals that there is purified Rop5 in the elutions from both the WT *T. gondii* and the Tg-MetRS^{NLL} strain samples (Fig. 12E). The Rop5 signal in the WT *T. gondii* elution is perplexing, though it may correspond to Rop5 protein that nonspecifically adheres to the resin used in the purification. Importantly, the Rop5 signal seen in the Tg-MetRS^{NLL} strain elution is approximately 2x the intensity of the WT *T. gondii* elution, implying that there was some enrichment beyond the background level seen in the WT *T. gondii* sample. The Rop5 blot was stripped and reprobed with an anti-HA antibody, revealing a strong signal for the HA-tagged MetRS^{NLL} only in the Tg-MetRS^{NLL} strain elution sample (Fig. 12F), a signal considerably stronger than that of the eluted Rop5. These neutravidin purification data are consistent with the lack of any host cell cytoplasmic labeling in the *in situ* tagging experiments described in Figure 11. While it would be expected that the cytoplasmic signal from Anl-tagged (and therefore Alexa Fluor 594-labeled) proteins would be observed, we did not observe any evidence that Anl-tagged proteins could be found in multiple known locations for *T. gondii* secreted proteins (primarily the host cell nucleus, as well as in cytoplasmic puncta). It has been well-established that multiple rhoptry and dense granule proteins can be found in these host cell locations.

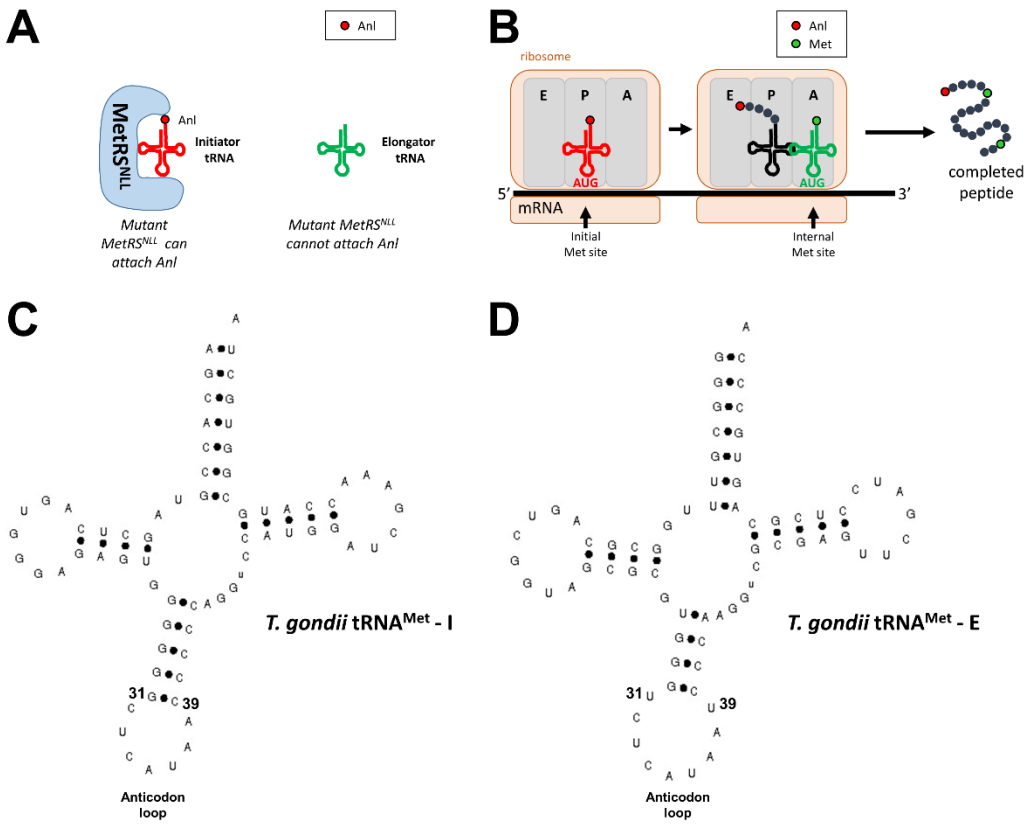

Figure 13. The bacterial MetRS^{NLL} cannot charge Anl to *T. gondii* elongator tRNA^{Met}

A) Bacterial MetRS (wild type or the NLL mutant) cannot charge eukaryotic elongator tRNA^{Met}. B) A diagram of the ribosome reading mRNA to synthesize a protein. Since the initiator tRNA^{Met} can be charged with Anl, it gets incorporated into the protein. The elongator tRNA^{Met} cannot be charged with Anl, so all of the internal Met sites stay as Met. C) The secondary structure prediction of *T. gondii* initiator tRNA^{Met} (gene models: TGME49_269820, TGME49_248915, TGME49_248925). Bases 31 and 39 form a Watson-Crick base pair in the anticodon loop. D) The secondary structure prediction of *T. gondii* elongator tRNA^{Met} (gene models: TGME49_212750, TGME49_202895, TGME49_257140). Bases 31 and 39 cannot form a Watson-Crick base pair, increasing the size of the anticodon loop. The models in C and D were generated with tRNAscan-SE 1.21 using sequences from the *T. gondii* Me49 strain from ToxoDB.org.

The most likely explanation for the minimal labeling of secretory proteins is that only the initiator methionine is replaced with Anl, while internal Met residues are not. This stems from the fact that there are two tRNA^{Met} in prokaryotes and eukaryotes, the initiator tRNA^{Met}, which carries the first Met, and the elongator tRNA^{Met}, which carries all of the internal Met residues [120]. Normally, MetRS is responsible for attaching Met to both the initiator and elongator tRNA^{Met}. However, a *bacterial* MetRS cannot attach Met (or Anl) to a *eukaryotic* elongator tRNA^{Met}, only

an initiator $tRNA^{Met}$ (Fig. 13A) [120-123]. The reason for this inability was suggested to be due to a difference in the size of the anticodon loop of the eukaryotic elongator $tRNA^{Met}$ [124], which is one of the key determinants for $tRNA^{Met}$ binding to the MetRS [125]. The initiator and elongator $tRNA^{Met}$ from *E. coli* both have a base pair between residues 31 and 39, which forms a 7 base anticodon loop. In *T. gondii* (and most other eukaryotes), the anticodon loop is 7 bases in the initiator $tRNA^{Met}$ (Fig. 13C), but is predicted to be 9 bases in the elongator $tRNA^{Met}$, due to a lack of base pairing between residues 31 and 39 (Fig. 13D). Making a single nucleotide change to introduce a Watson-Crick base pair between residues 31 and 39 in rabbit elongator $tRNA^{Met}$ was enough for the $tRNA^{Met}$ to be aminoacylated by a bacterial MetRS [124]. Taken together, these data suggest that the *E. coli* $MetRS^{NLL}$ system is similarly incapable of replacing internal Met residues with Anl, and therefore proteins processed via the secretory pathway would be undetectable in the Tg-$MetRS^{NLL}$ strain. The inability to identify Rop7 in pulldowns and the *in sit*u data in Figure 11 are consistent with this explanation. The small amount of labeled and purified Rop5 and Rop18 may correspond to newly synthesized protein that has not yet fully transitioned through the secretory pathway to the rhoptries. Another possible explanation for the low labeling of secreted proteins is that they are more susceptible to misfolding than other classes of proteins upon Anl incorporation, and are more readily degraded by quality control machinery.

If only the N-terminal Met residue can be replaced by Anl, the label could still be removed by methionine aminopeptidases. These enzymes are responsible for removing the N-terminal Met residue from cytoplasmic proteins that are not otherwise processed [126]. This could add further complications to effectively using the $MetRS^{NLL}$/Anl system. When Ngo *et al*. expressed the bacterial $MetRS^{NLL}$ in mammalian cells, they found that a N-terminal Anl residue could be removed by methionine aminopeptidase, though the rate of removal was at least three times slower than that of Met [113]. This suggests that the bacterial $MetRS^{NLL}$/Anl system will still be useful for examining methionine aminopeptidase targets, but the labeling may be slightly reduced due to this processing. *T. gondii* has at least two putative methionine aminopeptidases, so this form of N-terminal processing needs to be accounted for when utilizing the $MetRS^{NLL}$/Anl system in the parasite. GAPDH is a known target of methionine aminopeptidase-2 (MetAP2) in human cells [127], which could account for the inability to purify hGAPDH from Anl-labeled samples (Fig. 10). However, the Anl-containing N-terminal residue would be expected to be detectable at some level

in labeled cells given the predicted decrease in processing rate associated with N-terminal Anl residues [113]. Other host cell proteins that are not targets of aminopeptidases should be examined in future studies to further confirm the orthogonality of the *T. gondii* Anl system. .

Which specific Met residues can be replaced by Anl will be addressed further in future studies using mass spectrometry and genetics will be used to mutate the endogenous *T. gondii* MetRS to incorporate Anl.

2.3 SUMMARY

Combining click chemistry and the incorporation of azide/alkyne-containing NCAAs into nascent proteins is finding increasing use in the study of protein synthesis and degradation. A combination of Aha-incorporation/click chemistry and ribosomal RNA (rRNA)-targeted fluorescence *in situ* hybridization (FISH) has been used to monitor protein synthesis in diverse microbial populations [115]. Zhang and colleagues used Aha-incorporation to monitor the degradation of long-standing proteins via autophagy [128]. These examples are all using a NCAA which can be incorporated into virtually any protein, as the NCAAs are recognized by wild type aminoacyl-tRNA synthetases. This poses a problem when trying to use the system for studying specific cell types in a varied population, in that all the cell types can incorporate the NCAA. To surmount this problem, we have employed a system that was developed by Ngo *et al*., using a mutant form of methionyl-tRNA synthetase ($MetRS^{NLL}$) in *T. gondii*, allowing the azide-containing Met analog, azidonorleucine (Anl), to be incorporated in nascent parasite proteins [86,99]. This system is highly effective at labeling nascent proteins in *T. gondii*, and is orthogonal, labeling proteins only in $MetRS^{NLL}$-expressing parasites. The Tg-$MetRS^{NLL}$ strain is a new tool to study novel aspects of parasite protein expression while parasites are growing inside of infected host cells, and strains and plasmids will be given freely upon request. However, our data also show that, in *T. gondii*, proteins which are N-terminally processed, like those destined for secretory organelles like the dense granules and the rhoptries, are only very minimally labeled. This method could potentially be used to determine which secreted proteins, and apicoplast/mitochondria-located proteins, are not N-terminally processed. We will address the mechanism for this labeling specificity, as well as how to label N-terminally processed parasite proteins with Anl, in future studies. A study in the pathogenic bacteria *Yersinia enterocolitica* used the $MetRS^{NLL}$/Anl system to identify proteins that it secreted in HeLa cells [129], providing evidence that the system should be

applicable to studying/identifying secreted proteins in apicomplexan parasites, like *T. gondii*, if system can be modified to label N-terminally processed proteins.

2.4 ACKNOWLEDGMENTS

We would like to acknowledge Frank Truong and David A. Tirrell from the Division of Chemistry and Chemical Engineering at the California Institute of Technology for generously providing the *E. coli* $MetRS^{NLL}$ gene cloned into the pQE-80L vector. This work was funded by grants R21AI110351, R21AI093906 (National Institutes of Health), and a Pew Scholarship from the Pew Charitable Trusts to J.P.B..

3.0 FURTHER ADVANCEMENTS OF THE METRS^{NLL}/ANL SYSTEM

3.1 INTRODUCTION

While it is evident that the MetRS^{NLL}/azidonorleucine (Anl) labeling system is functional in *T. gondii*, more work is necessary to better characterize the system, and to improve upon it such that it more completely labels *T. gondii* proteins. It is likely that the *T. gondii* strain expressing the bacterial MetRS^{NLL} (the Tg-MetRS^{NLL} strain) is only able to charge its initiator tRNA^{Met} with Anl, and not its enlongator tRNA^{Met}. As such, only the Met residue that initiates protein synthesis can be substituted with Anl, and all internal Met residues cannot be replaced with Anl. The minimal labeling of secreted parasite proteins supports this notion (Section 2.4.9), as many secreted proteins are subjected to N-terminal processing during their transition through the secretory pathway, effectively removing the Anl tag if it had been added. Moreover, even proteins not destined for the secretory pathway may be subject to elimination of the N-terminal residue by aminopeptidases, further decreasing the efficiency of the system. More conclusive data is needed to confirm that it is only the initial Met residue that can incorporate Anl. Presupposing this notion to be the case, there is the possibility that *T. gondii*'s MetRS gene could be mutated to code for a version of protein which is able to recognize Anl. Since this would be utilizing the parasite's own MetRS, it is likely that it would be able to effectively bind both the initiator tRNA^{Met} and the elongator tRNA^{Met}, ensuring that Anl would be incorporated at all Met positions.

In this section, I will describe the additional efforts that have I have made to further characterize the MetRS^{NLL}/Anl labeling system, and those to improve the system. Specifically, I will describe 1) the mass spectrometry experiments that I performed, 2) my efforts to mutate *T. gondii*'s MetRS to charge tRNA^{Met} with Anl, and 3) my efforts to express a mutated version of the murine MetRS in *T. gondii*, one capable of charging Anl to both eukaryotic tRNA^{Met}.

3.2 MASS SPECTROMETRY

While it has been confirmed that Anl is successfully incorporated into parasite proteins in the Tg-MetRS^{NLL} strain (Section 2.4.1, 2.4.7, 2.4.9), the specific identity of the majority of the labeled proteins remains unknown. Furthermore, which Met residues are replaced by Anl is unknown, though it is likely that only the N-terminal Met residue can incorporate the analog (Section 2.4.9) [113]. To address these outstanding questions, I have conducted a series of mass spectrometry experiments, aimed at identifying specific proteins that incorporate Anl, and at what

Met positions the analog is incorporated. I have conducted several mass spectrometry experiments with a numerous collaborators, though they have been largely inconclusive. I performed mass spectrometry analysis on four different sample types: 1) infected host cell lysates which were treated with Anl, 2) purified parasites which were grown in the presence of Anl, 3) neutravidin-purified Anl-containing proteins, and 4) immunopurified HA-tagged $MetRS^{NLL}$.

In the present section, I will describe how Anl is recognized via mass spectrometry, followed by a summary of the mass spectrometry experiments performed on the aforementioned sample types.

3.2.1 Differentiating Anl from Met using mass spectrometry

Met differs from Anl by the addition of C, H, N_3, and the loss of S, differences that are readily detectable via mass spectrometry by an increase in molecular mass (Fig. 14). Mass spectrometry data analysis software, like Mascot, can be programed to specifically look for this change in mass at Met residues [130]. The conversion of Met to Anl is marked by a monoisotopic mass increase of 23.04 Da (Fig. 14). Some elements have natural isotopes that are present at low abundance in nature, which contribute to the mass of identified protein fragments. The monoisotopic mass is the mass of a molecule without the contribution of any of these stable isotopes. Many mass spectrometers have the resolving power to differentiate between the monoisotopic variant of a peptide, and those which feature various stable isotopes. Complicated molecules like peptides feature a large number of elements, such that stable isotopes can be highly represented. Based on the abundance of particular isotopes in nature, the contributions of each can be estimated for a molecule, and an average mass value for a particular molecule can be determined [131]. In the case of the conversion of Met to Anl, the average mass increase is 22.97 Da.

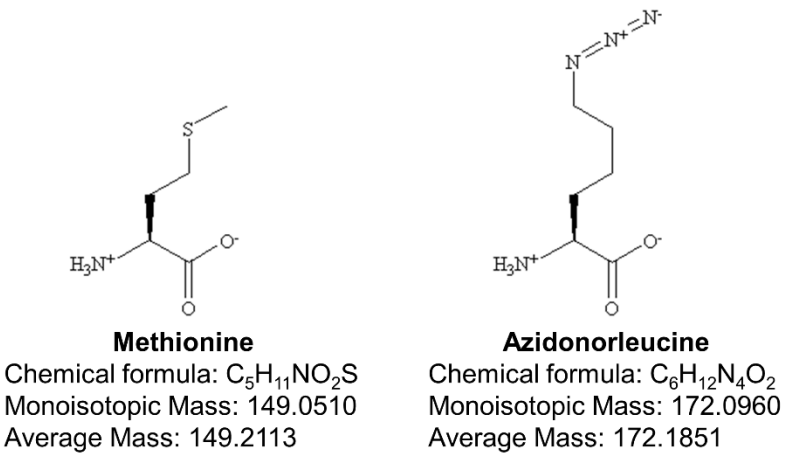

Figure 14. The structures of methionine and azidonorleucine

3.2.2 Analysis of infected host cell lysates which were treated with Anl

As the simplest means to look for Anl-incorporation into parasite proteins, I grew parasites in human foreskin fibroblasts (HFFs) in the presence of Anl and sent solubilized samples of the infected host cells for mass spectrometry analysis. Since whole cell lysates were used in these experiments, host cell proteins would be identified alongside of parasite proteins, ideally demonstrating that the incorporation of Anl was specific to parasite proteins.

Specifically, HFFs were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain and grown in the presence of Anl for 2 days. This time period allowed sufficient time for parasite replication and incorporation of Anl into nascent proteins. The WT *T. gondii* strain served as a negative control, as it did not express the bacterial MetRS^{NLL}, such that it should not be able to incorporate Anl into proteins. After the 2 days of growth, the infected HFFs were washed with PBS to remove unincorporated Anl. The HFFs were then solubilized directly with the RIPA buffer (prepared from Sigma-Aldrich's Mammalian Cell lysis kit). RIPA buffer is a lysis buffer that is commonly used for protein solubilization, though it mild enough to maintain the biological activity of most proteins and their binding interactions 132. Due to the mild nature of the lysis buffer used, it is likely that not all proteins were fully solubilized and that some host and parasite proteins were missed. After solubilization, the samples were centrifuged at 12,000xg for 10 minutes to pellet the cellular debris. The supernatant was removed and sent for mass spectrometry analysis.

I prepared two sets of samples in this fashion. For the first sample (02.28.12), 7 T25 culture flasks of HFFs were infected per strain (WT *T. gondii* and the Tg-MetRS^{NLL} strain). After 2 days of growth in Anl, the infected HFFs were combined, based on strain, in a total of 1ml of RIPA buffer. The protein concentration in the samples was low, as determined with Life Technologies' EZQ Protein Quantitation Kit (WT *T. gondii* ~0.94 µg/ul and Tg-MetRS^{NLL} strain ~0.99 µg/ul). For the second sample (07.09.12), 8 T25s of HFFs were used per strain, and the samples were prepared as the first, with the exception that after solubilizing the samples in RIPA buffer, they were centrifuged in an ultracentrifuge at 100,000xg for 45 minutes. In this instance, the samples were considerably more concentrated (WT *T. gondii* ~5.75 µg/ul and Tg-MetRS^{NLL} strain ~5.57 µg/ul). These samples were sent to Dr. Philip C. Andrews at the University of Michigan Medical School for analysis.

I performed a click reaction on a small portion of the second set of samples to see if any of the proteins had successfully incorporated Anl. The click reaction was performed on both the WT *T. gondii* and the Tg-MetRS^{NLL} strain samples, using Alexa Fluor 488 alkyne. This should result in appending a fluorescent tag to any proteins which incorporated Anl. The clicked samples were run on an SDS-PAGE gel and imaged on a fluorescent gel reader. As expected, there is a fluorescent signal seen in the Tg-MetRS^{NLL} strain samples, but it is largely absent from the WT *T. gondii* samples (Fig. 15A). This confirmed the notion that Anl-labeled proteins were amongst the sample sent for mass spectrometry analysis, but the relatively weak signal compared to its coomassie stained counterpart (Fig. 15B) suggests that a large margin of the proteins in the sample did not incorporate Anl, or that click labeling with the fluorescent tag was not very efficient.

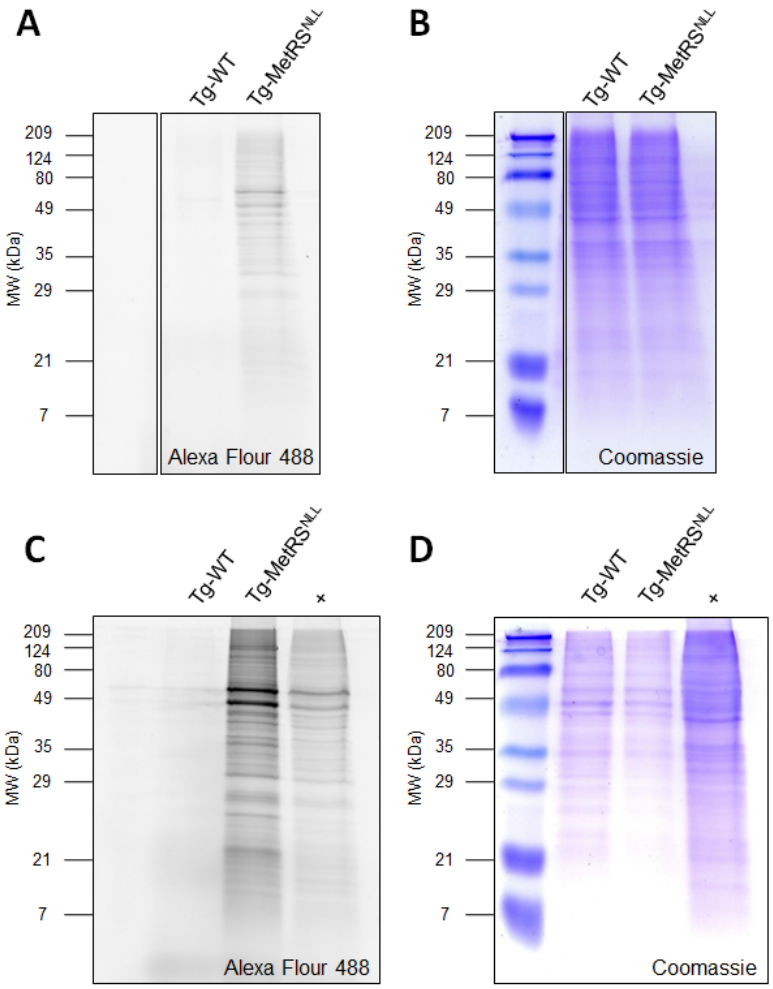

Figure 15. The protein samples sent to Dr. Philip Andrews' laboratory for mass spectrometry analysis contain proteins which have incorporated Anl

A) Fluorescent gel of lysates of Anl treated, *T. gondii*-infected HFFs sent to Dr. Phillip Andrews' laboratory for mass spectrometry analysis. HFFs were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at a MOI of 3 in the presence of 1 mM Anl. After 2 days of growth, the HFFs were washed with PBS to remove excess Anl, and were then solubilized in RIPA buffer. A portion of the samples were subjected to click chemistry with Alexa Fluor 488 and then run on an SDS-PAGE gel. The gel was analyzed on a fluorescent gel reader. The gel was cropped to remove lanes not pertinent to the experiment. B) A coomassie stain of the gel from A. C) Fluorescent gel of purified parasite samples isolated from Anl treated, *T. gondii*-infected HFFs, samples which were sent to Dr. Phillip Andrews' laboratory for mass spectrometry analysis. HFFs were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at a MOI of 3 in the presence of 1 mM Anl. After 2 days of growth, the HFFs were washed with PBS to remove excess Anl, and the parasites were isolated from the HFFs via needle passage. Parasites were purified by filtration through a 5.0 µm filter and were then solubilized in RIPA buffer. A portion of the samples were subjected to click chemistry with Alexa Fluor 488 and then run on an SDS-PAGE gel. A sample prepared from HFFs infected with the Tg-MetRS^{NLL} strain in the presence of Anl served as a positive control. D) A coomassie stain of the gel from C.

3.2.2.1 Results

Of these two sets of samples, I received data from only the first set. The samples were trypsinized and subjected to MS/MS analysis with a 4800 Plus MALDI TOF/TOF Analyzer. The peptide fragments were assigned to their respective proteins by comparing the masses to a database containing predicted trypsinized products of all human and *T. gondii* proteins. From these data, there were 105 distinct proteins represented from the WT *T. gondii* samples, and 242 from the Tg-MetRS^{NLL} strain samples, many of which were covered by multiple peptide fragments. Both samples featured similar protein profiles, including prominent surface antigens, secreted rhoptry proteins, and metabolic enzymes like glyceraldehyde 3-phosphate dehydrogenase and phosphofructokinase. Anl was only found in two peptide fragments from the Tg-MetRS^{NLL} strain samples. One of these was the host cell protein Sister chromatid cohesion protein DCC1, and the other was a hypothetical parasite protein on chromosome III. In both of these cases, the Met residue replaced with Anl was an internal Met residue. For the hypothetical *T. gondii* protein, the replaced Met residue was near the C-terminus of the protein.

Of the 105 distinct proteins from the WT *T. gondii* samples, only 28 of them were represented with peptide fragments containing Met. Most of these were only represented by a single fragment. Only one of the peptide fragments featured the initiating N-terminal Met residue (polynucleotide 5'-hydroxyl-kinase). Of the 242 distinct proteins from the Tg-MetRS^{NLL} strain samples, only 26 of them were represented with peptide fragments containing Met, none of which were the N-terminal Met residue.

In the infected host cell lysates, between 80-86% of the peptide fragments were from host cell proteins. Due to the extremely small number of proteins that were identified with Anl, it seems unlikely that either of the identifications are legitimate. Performing mass spectrometry on purified Anl-treated parasites, as opposed to infected host cell lysates, would help to increase sequence coverage of *T. gondii* proteins.

3.2.3 Analysis of purified parasites which were grown in the presence of Anl

The second set of samples that were subjected to mass spectrometry analysis where of purified parasites. Ideally, by focusing the analysis specifically on parasite proteins, the percent protein coverage would increase, as the samples would largely be devoid of host proteins.

For these experiments, HFFs were infected with parasites in the presence of Anl, and instead of solubilizing the HFFs directly, the cells were syringe lysed by needle passage to release the parasites. The lysed host cell mixture was then passed through a 5.0 µm syringe-driven filter to remove host cell debris. Parasites are small enough to pass through the 5.0 µm pores in the filters. The released parasites were pelleted via centrifugation at 800xg and then solubilized in RIPA buffer. This method removed the majority of host cell proteins from the sample, such that the majority of the peptide fragments analyzed were of parasite origin.

I prepared three sets of samples in this fashion. The first two sets were sent to Dr. Andrews' laboratory for analysis. The first sample (06.14.12) was prepared by infecting 10 T25 culture flasks of HFFs per strain (WT *T. gondii* and the Tg-MetRS^{NLL} strain). Low protein concentrations prompted me to prepare a second sample (07.18.12) using 15 T25 culture flasks of HFFs per parasite strain. Protein concentrations were considerably higher in this instance, as determined by the EZQ Protein Quantitation Kit (WT *T. gondii* ~2.98 µg/ul and Tg-MetRS^{NLL} strain ~1.28 µg/ul). To confirm Anl incorporation in the samples, a click reaction was performed on the samples using Alexa Fluor 488 alkyne, and the samples were run on an SDS-PAGE gel and analyzed for fluorescence. There is a prominent fluorescent signal in the Tg-MetRS^{NLL} strain sample, but not in the WT *T. gondii* sample (Fig. 15C). A comparison of the coomassie stained signal with the fluorescent signal (Fig. 15C, D) suggests that a large portion of the protein in the Tg-MetRS^{NLL} strain sample contains Anl. No mass spectrometry data were obtained from these two samples due to experimental failure during sample processing. The third set of samples was prepared using 10 T25 culture flasks of HFFs per parasite strain and was sent to Dr. Louis M. Weiss at the Albert Einstein College of Medicine, who has considerable experience with *T. gondii* proteomics [133-136].

3.2.3.1 Results

From the mass spectrometry analysis of the samples sent to Dr. Weiss, a total of 1279 distinct proteins were identified, each being represented by a minimum of 2 peptides. The vast majority of these proteins were of parasite origin (978), with 60% being common between both samples and 27% and 13% being unique to WT *T. gondii* and the Tg-MetRS^{NLL} strain, respectively. Amongst these proteins, 24 were predicted to have incorporated Anl, though 8 were human proteins, such that 3% of the identified human proteins incorporated the analog. Of the 16 remaining *T. gondii* proteins with Anl, 5 of them exhibited poor percent protein coverage, 2 of

which were not tryptic digests calling them into further question (Table 1). Of the 11 *T. gondii* proteins which featured Anl, some fragments contained Anl at multiple Met residues. Furthermore, none of the replaced Met residues were the initiating Met residue.. The majority of the proteins that incorporated Anl are not predicted to have signal peptides. Of the 1,279 proteins identified between the two samples, only 11 proteins could be confidently assigned to have incorporated Anl, indicating that Anl was incorporated at very low levels (1%).

Some of the identified peptides were from highly abundant parasite proteins (heat shock protein 90 and fructose-1,6-bisphosphate aldolase) which might not have been fully removed from the enrichment procedure. As a means to help differentiate from contaminating proteins in future studies, the Anl pulse could be performed simultaneously with a pulse of heavy lysine ([$^{13}C_6$, $^{15}N_2$]lysine). Only proteins that incorporated the heavy lysine would be considered as legitimate hits, with those lacking the isotopic label representing proteins synthesized before the pulse, and likely corresponding to proteins that were non-specifically purified [113].

The incorporation of Anl at internal Met positions is at odds with the findings of Ngo and co-workers when they expressed the bacterial MetRS^{NLL} in mammalian cells [113]. However, the extremely low number of proteins featuring Anl, both in host and parasite proteins, suggests that the identified Anl-containing fragments might have been misidentified. A way to help determine this would be to do a virtual tryptic digest of the proteome of an organism not used in the experiment, potentially a strain of yeast, and compare the identified mass values to the virtual digest. Since there should be no yeast proteins in the sample, ideally no Anl-containing yeast proteins should be identified. If some are identified, and this number is similar to the number of Anl-containing host cell/parasite proteins assigned, it would suggest they were background noise and were misidentified.

Table 1. Anl-containing proteins from a mass spectrometry analysis of purified parasite samples

	Protein name	% coverage	Unique peptides (#)	Signal peptide	Anl-containing fragment (Anl denoted by lowercase "m")	Notes
01	heat shock protein 90, putative	38%	32	Yes	LPENQPNIYYAAGESAEQLmKAPEmQIFLKK	N-terminus not represented
02	fructose-1,6-bisphosphate aldolase	37%	21	No	mSGYGLPISQEVAK	N-terminus not represented
03	ribosomal protein RPS6	30%	8	No	QGFPmmQGVLVNHR	N-terminus not represented
04	prolyl-tRNA synthetase	22%	13	No	LADEELLGPmLDVSKGSVTPLAAm	N-terminus not represented
05	bifunctional dihydrofolate reductase-thymidylate synthase	19%	10	No	FGCTmRYSLDQAFPLLTTK	N-terminus not represented
06	protein phosphatase 2C domain-containing protein	15%	6	No	QTmEDAHIATPSLR	N-terminus not represented
07	sarco/endoplasmic reticulum Ca2+-ATPase	14%	10	No	QADIGVAmGIAGTEVAK	N-terminus not represented
08	AMP-binding enzyme domain-containing protein	14%	10	No	IFTIVYTSGTTGNPKGVmmSNR	N-terminus not represented
09	rhoptry metalloprotease toxolysin TLN1	13%	19	Yes	HLmHSRVTAPSNVLASVQSSR	N-terminus not represented
10	Inner Membrane Complex Protein 17	12%	10	No	QQmEEDTEATEVEKK	N-terminus not represented
11	cysteine-tRNA synthetase	12%	7	Yes	YIFHmLKIFGVVEDDEDNMAYVK	N-terminus not represented
12	hypothetical protein	5%	6	No	NISLEQANALVSKCmHNm	Chrom. VI, N-terminus not represented
13	hypothetical protein	4%	3	No	QMQGWDmVQKEGK & LEKYQEATEEAEKELEKm	Chrom. X, questionable based on MS/MS, N-terminus not represented

14	hypothetical protein	2%	1	No	MTAGKEGAGm	N-terminal peptide, Not tryptic peptide fragment
15	hypothetical protein	2%	4	No	LQNQGAKSEDPmGSEKQK	Chrom. XII, N-terminus not represented
16	DEAD/DEAH box helicase domain-containing protein	1%	1	No	TIAMKTQSENTADDPLDAFm	N-terminus not represented, questionable based on MS/MS, Not tryptic peptide fragment

3.3.4 Analysis of neutravidin-purified Anl-containing proteins

Based on the limited number of peptide fragments containing Anl in the purified parasite samples, I decided to specifically isolate Anl-containing proteins by biotinylating them with click chemistry and purifying them with neutravidin resin. Then, rather than trying to elute the biotinylated proteins from the resin, I sent the resin to the Weiss laboratory to be trypsinized directly. The biotin-neutravidin interaction is extremely strong, such that the only means of eluting the bound protein is by boiling the resin in sodium dodecyl sulfate (SDS) sample buffer. Unfortunately, the addition of SDS renders the samples incompatible for mass spectrometry analysis [137]. Barring non-specific binding to the resin, this method should limit the proteins analyzed to those that actually incorporated Anl.

To prepare the samples for analysis, 3 T25 culture flasks of HFFs were infected per strain (WT *T. gondii* and the Tg-MetRS^{NLL} strain) in the presence of 1 mM Anl. After 2 days of growth, the parasites were released from the HFFs by needle passage and pelleted via centrifugation. The pelleted parasites were solubilized in RIPA buffer and centrifuged at 12,000xg for 10 minutes to pellet the cellular debris, whereupon the supernatant was saved for click chemistry. After biotinylating any Anl-containing proteins with click chemistry, the samples were incubated with neutravidin resin for 2 hours. The resin was washed with PBS, and then sent to the Weiss laboratory to be trypsinized and analyzed.

Trypsinizing the biotinylated proteins should generate a collection of peptide fragments for identification by mass spectrometry, though the fragments actually containing Anl would not be identified, as they should be biotinylated and still bound to the resin (assuming that 100% of the

Anl residues are biotinylated from the click reaction). Any of the identified peptide fragments could have been from a biotinylated protein, or from a protein which nonspecifically bound to the neutravidin resin. Performing the experiment with both WT *T. gondii* and the Tg-MetRS^{NLL} strain was done to help differentiate between these two possibilities. Since the WT *T. gondii* do not express the bacterial MetRS^{NLL}, they should not be able to incorporate Anl into their proteins and, therefore, cannot be biotinylated. So, any parasite proteins identified from the WT *T. gondii* samples would be the result of nonspecific binding to the resin. A protein identified only in the Tg-MetRS^{NLL} strain samples, and not the WT *T. gondii* samples, would be a strong candidate for an Anl-containing protein.

The large majority of the proteins identified in the experiment were human proteins. Of the *T. gondii* proteins that were identified, none of them were solely confined to samples from the Tg-MetRS^{NLL} strain. In fact, very few of the identified parasite proteins were from the Tg-MetRS^{NLL} strain. Why this was the case is uncertain, though if the experiment were to be repeated, more parasites should be used to generate the samples to increase the concentration of parasite proteins being handled. Some of the identified *T. gondii* proteins included actin, heat shock protein 70 (HSP70), and nitrate reductase. These are highly abundant proteins, so it is likely that they were not specifically purified, and are remnants that were not completely removed in the purification procedure.

Future analyses of neutravidin purified samples could be improved by biotinylating the Anl-containing proteins with a cleavable biotin probe. This would allow the samples to be eluted from the resin without resorting to the harsh conditions of boiling the resin in SDS sample buffer. This would simplify the elution samples, and proteins that non-specifically bound to the resin would likely not be eluted. Ngo and co-workers used a enrichment probe where the biotin moiety was separated from the clickable moiety via a disulfide bond. Reduction of the disulfide bond allowed for bound samples to be efficiently eluted [113].

3.3.5 Analysis of immunopurified HA-tagged MetRS^{NLL}

For the final set of mass spectrometry experiments, I wanted to immunopurify a cytoplasmic *T. gondii* protein and send it for mass spectrometry analysis. Since cytoplasmic proteins are not sent through the secretory pathway, they do not have N-terminal signal peptides

to be removed. If Anl is only incorporated at the N-terminal position, it would still be present in these cytoplasmic proteins, so long as it was not removed by a aminopeptidase. The logical choice for a cytoplasmic protein to purify was the HA-tagged MetRS^{NLL}, as I had already confirmed it could be successfully purified (Section 2.4.7). Since the protein is HA-tagged, I could be purified with a anti-HA antibody. The purified protein sample could be run on an SDS-PAGE gel, and the respective band excised from the gel for mass spectrometry analysis.

For the immunopurifications, I infected HFFs with the Tg-MetRS^{NLL} strain in either the presence or absence of Anl. After 2 days growth, the infected monolayers were washed to remove excess Anl and the parasites were isolated by needle passage and filtering with 5.0 µm syringe-driven filters. The parasites were solubilized in TEN buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl) containing RIPA detergents (1% Triton-X100, 0.5% Deoxycholate, 0.01% SDS, and EDTA-free protease inhibitors). Insoluble material was removed by centrifugation, and the supernatant was used for immunopurification. The HA-tagged MetRS^{NLL} was purified with an anti-HA monoclonal IgG1 antibody followed by incubation with Protein G Plus Agarose. After washing, the bound protein was removed by elution in either: 1) 0.1 M triethylamine (pH 11.5), or 2) by boiling the resin in 100 µl of 1x SDS sample buffer.

A western blot probing for the HA-tagged MetRS^{NLL} confirmed that the purification was successful (Fig. 16A), though an analogous coomassie stained gel revealed that there were three prominent protein bands in the purification (Fig. 16B). The three distinct bands ran between 49-80 kDa (Fig. 16B). The HA-tagged MetRS^{NLL} is estimated to be 64 kDa, suggesting that the middle band is the one of interest. Regardless, each was sent for identification via mass spectrometry. For this purpose, the immunopurified samples were run on separate SDS-PAGE gels, one for the Anl treated samples (Fig. 16D) and one for non Anl treated samples (Fig. 16C), and each band was excised with a clean razor blade. The samples were run in duplicate, such that the excised bands could be sent to the Weiss laboratory for analysis and to Dr. Tatiana Laremore at the Proteomics and Mass Spectrometry Core Facility at The Huck Institutes of the Life Sciences, Pennsylvania State University.

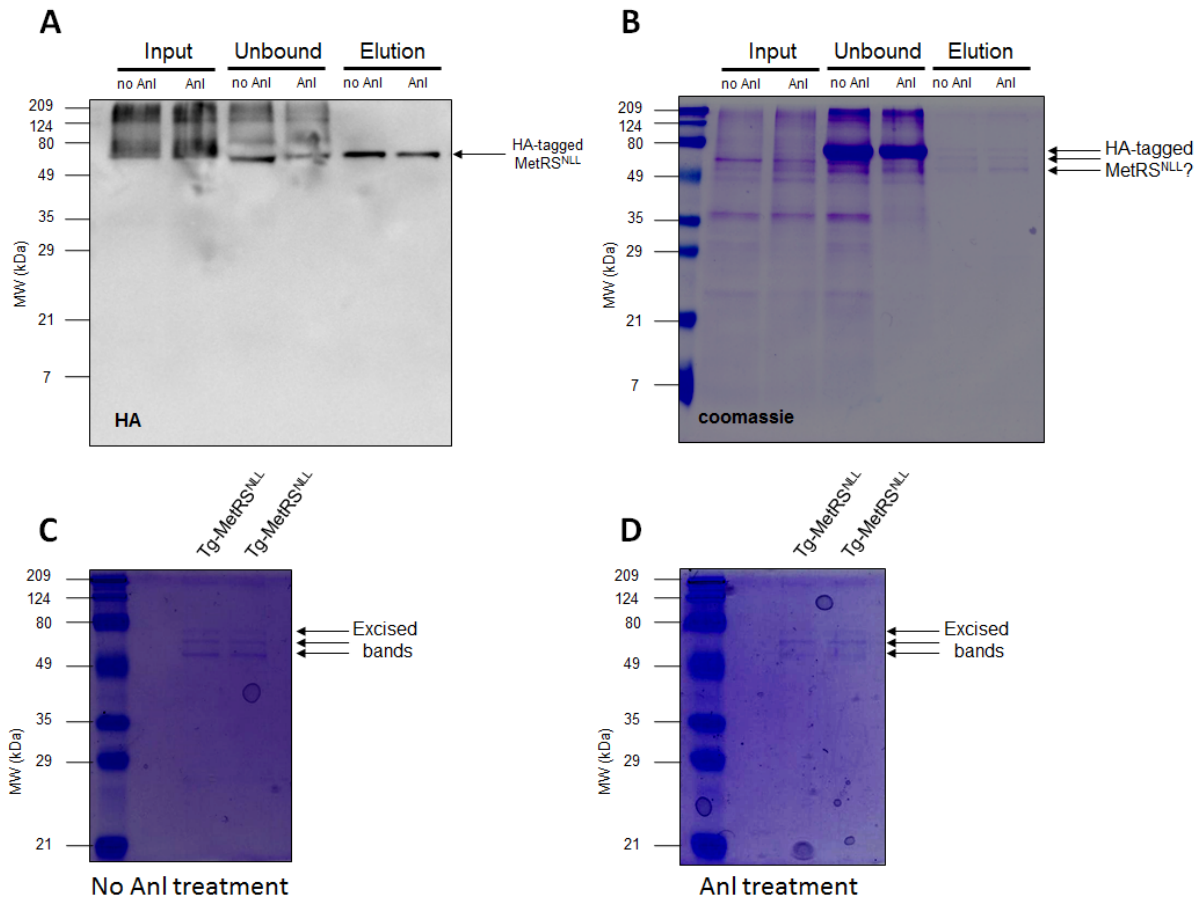

Figure 16. The HA-tagged MetRS^{NLL} was immunopurified and sent for mass spectrometry analysis

A) Western blot of the HA-tagged MetRS^{NLL} from parasites of the Tg-MetRS^{NLL} strain. HFFs were infected with the Tg-MetRS^{NLL} strain at a MOI of 3 in the presence or absence of 1 mM Anl. After 2 days of growth, the parasites were isolated and solubilized in TEN buffer. The HA-tagged MetRS^{NLL} was immunopurified with an anti-HA monoclonal antibody followed by incubation with Protein G resin, and eluted by boiling in SDS loading dye. The eluted samples were run on an SDS-PAGE gel alongside of "input" samples taken before the purification, and "unbound" samples of the protein which did not stick to the resin. The SDS-PAGE gel was transferred to nitrocellulose and probed with an anti-HA-HRP antibody. B) Coomassie stained version of a gel identical to the one used to prepare the western blot in A. Note: The brightness has been reduced by 30%, and contrast increased by 47% to make the bands more visible. C) Coomassie stained SDS-PAGE gel of the immunopurified HA-tagged MetRS^{NLL} from the non Anl treated samples. Three protein bands were excised for mass spectrometry analysis. The samples were run in duplicate. Note: The brightness has been reduced by 30%, and contrast increased by 47% to make the bands more visible. D) An identical gel to the one from C, using the Anl treated samples instead.

With the samples sent to the Weiss laboratory, the HA-tagged MetRS^{NLL} was identified amongst the peptide fragments, though the percent coverage of the protein was low, such that the N-terminal fragment was not identified. Some of the identified peptide fragments contained internal Met residues, though none of them had incorporated Anl.

For the samples sent to Dr. Laremore, three *T. gondii* proteins were identified in both the Anl treated and non-treated samples: the HA-tagged MetRS^{NLL} (64 kDa), elongation factor 1-alpha (49 kDa), and Calmodulin (16 kDa). Anl was not found in any of the three proteins. In the case of the non-treated sample, 4.62% of the HA-tagged MetRS^{NLL} was represented by 2 unique peptides. Coverage was better for the Anl treated sample, with 55.95% coverage from 19 unique peptides (Fig. 17). None of the peptide fragments corresponding to the HA-tagged MetRS^{NLL} were the N-terminal fragment. As such, Anl could have been incorporated N-terminally, but it was missed because no N-terminal fragments were identified. The HA-tagged MetRS^{NLL} contains 18 Met residues, including the N-terminal Met. Seven of the identified fragments had a Met residue. In order to assure that the N-terminal peptide fragment is identified, more concentrated samples are needed to increase the number of peptide fragments that are identified from the samples.

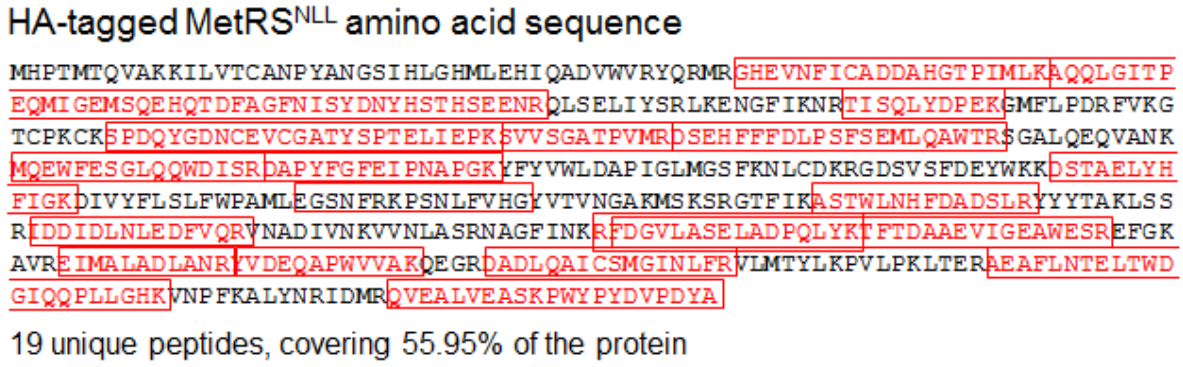

Figure 17. The identified tryptic fragments of the HA-tagged MetRS^{NLL}

The sequence of the HA-tagged MetRS^{NLL} with identified tryptic fragments highlighted in red, and surrounded by red boxes. Methionine residues are in bold.

3.3.5.1 Additional HA-tagged MetRS^{NLL} purifications

Based on the incomplete coverage of the HA-tagged MetRS^{NLL} from the first two mass spectrometry studies, I decided to prepare additional samples, increasing the amount of parasites

utilized. In the first set of HA purifications, each well in the SDS-PAGE gel contained approximately $5x10^6$ parasites/well. In a new experiment, I doubled the number of T25s infected per strain, as a means to get a larger number of parasites to isolate the HA-tagged $MetRS^{NLL}$. After isolating the parasites and doing the purification, the approximate number of parasites was $3.05x10^6$ parasites/well when run on an SDS-PAGE gel, a lower concentration than that from the first experiment. Despite this, I still excised bands and sent them to Dr. Laremore for analysis. Interestingly, when the immunopurified samples were run on the SDS-PAGE gel and coomassie stained for excision, there was only a single band present, as opposed to the three bands seen in the previous experiment.

I sent Dr. Laremore six gel slices for analysis: two excised bands of the HA-tagged $MetRS^{NLL}$ from parasites grown in the presence of Anl, two excised bands of the HA-tagged $MetRS^{NLL}$ from parasites grown in the absence of Anl, and two excised bands of biotinylated HA-tagged $MetRS^{NLL}$. Biotinylating the Anl residues would change the mass of the Anl residue, increasing it by 553.32 mass units when compared to Met.

In all of the excised bands, the major identified protein was bovine serum albumin (BSA), swamping out all of the other signals. The HA-tagged $MetRS^{NLL}$ was identified in most of the samples, but with a limited number of unique peptides (between 2 and 7), such that the percent coverage of the protein never rose above 17%. None of these fragments corresponded to the N-terminal fragment, and none of them contained Anl, or biotin-conjugated Anl. Interestingly, a few other *T. gondii* proteins were represented by peptide fragments, including some rhoptry proteins (ROP5, 7, 8), a few heat shock proteins (HSP70, 90), and a putative elongation factor-1 alpha.

The BSA contamination is peculiar, particularly since it was not a problem in the first set of immunopurified samples analyzed. The growth medium for HFFs is supplemented with fetal bovine serum, potentially explaining were it could have come from. Before isolating the parasites, the infected host cells were washed three times with PBS, before isolating the parasites and solubilizing them in TEN buffer. Dr. Laremore informed me that from her experience, BSA has a tendency to stick to resin. As such, any BSA remaining from the preparation of the parasites could have stuck to the resin, and ended up in the mass spectrometry analysis.

3.2.5 Conclusions

The mass spectrometry studies that I conducted were designed to help determine what proteins incorporated Anl, and at what Met positions. Conclusive answers were not drawn for any of those queries. The mass spectrometry data collected by the Weiss laboratory from purified parasite samples suggested that Anl was incorporated at internal Met positions in both host and parasite proteins, but only at a very low level. The percent incorporation was low enough to call into question the accuracy of the peptide assignments. Additional mass spectrometry studies will need to be performed in order to determine what proteins can incorporate Anl. Performing all of the parasite preparations on a larger scale would supply higher protein concentrations at the onset of the mass spectrometry experiments, hopefully leading to better protein coverage. Different subcellular fractions could be isolated from Anl treated parasites (membrane fraction [134], rhoptry fraction [138], etc.) to have a more focused screen for the incorporation of Anl into a particular set of proteins.

Specifically immunopurifying the HA-tagged $MetRS^{NLL}$ from Anl treated parasites was used to determine what Met residues could incorporate Anl. However, percent coverage was not high enough to identify an N-terminal peptide from the protein. Immunopurifications should be performed on additional parasite proteins that are not thought to be processed. A good candidate would be the click beetle luciferase that is expressed in the Tg-$MetRS^{NLL}$ strain, which the lab already has antibodies against. More vigorous washes should be performed to minimize BSA contamination issues. Furthermore, the HFFs could be grown in a minimal growth medium, not supplemented with fetal bovine serum.

3.3 MUTATING *T. GONDII* METRS TO CHARGE $TRNA^{MET}$ WITH ANL

Mutating the *T. gondii* MetRS gene to encode a version of the enzyme that can accept Anl, and charge it to both *T. gondii* $tRNA^{Met}$, might allow Anl to be readily incorporated in parasite proteins. This would replace the need to employ the bacterial $MetRS^{NLL}$ in *T. gondii*, and would foreseeably result in an increase in the number of parasite proteins that are Anl-labeled. To recognize Anl, the bacterial MetRS has three amino acid changes in the methionine binding pocket of the enzyme (L13N, Y260L and H301L; hence the name $MetRS^{NLL}$) [100]. These three mutated residues are conserved in methionine tRNA synthetases from bacteria to humans [101]. In fact, the Met binding pocket of the enzyme is one of the few portions of the enzyme that is well conserved,

as assessed by mapping conservation to the crystal structure of the *E. coli* enzyme (Fig. 18). In *T. gondii*, the three amino acids correspond to I284, Y507, and H537. The first of the three residues is isoleucine (Ile) in *T. gondii*, as opposed to leucine (Leu) like most other organisms examined. Since Ile and Leu are both similarly sized, hydrophobic amino acids, the change is not likely to cause a functional change. Mutating these three residues to correspond to the mutations in the bacterial MetRS^{NLL} may allow the *T. gondii* protein to charge Anl onto both *T. gondii* tRNA^{Met}.

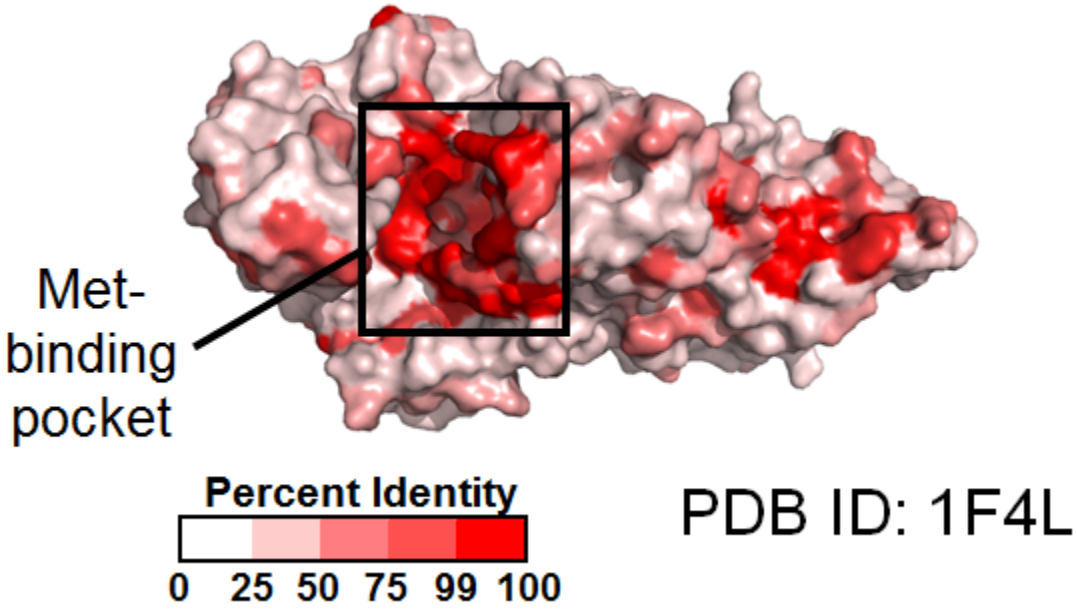

Figure 18. *E. coli* MetRS crystal structure with sequence conservation of MetRS mapped to the surface

The sequence conservation of MetRS was mapped to the surface representation of the crystal structure of the enzyme from *E. coli*.

3.3.1 Amplifying the *T. gondii* MetRS gene

The coding sequence of *T. gondii* MetRS was amplified from cDNA using primers which added attB1 and attB2 sites on either side of the gene (Table 2) to be inserted into the Gateway pDONR221 vector (Life Technologies, Carlsbad, CA) by a BP Clonase II reaction. This was prepared simply as a means to have a version of the unmutated *T. gondii* MetRS gene in a bacterial vector (pDONR-TgMetRS) to use as a template for future amplification reactions, as opposed to resorting to using cDNA.

Table 2. Primers used for mutating the *T. gondii* MetRS and for amplifying the mutant murine MetRS

Primer name	Sequence (5′-3′)	Purpose
For_MetRS_B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTTCTGCCGTTCTGGATTGC	Forward primer for amplifying the TgMetRS gene. Adds attB1 site for cloning into pDONR221 vector via a BP Clonase II reaction.
Rev_MetRS_B2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGTCGTCCTGACATCTCCC	Reverse primer for amplifying the TgMetRS gene. Adds attB2 site for cloning into pDONR221 vector via a BP Clonase II reaction.
For_A_TgMetRS_SbfI	GCTACCTGCAGGTCCACAATGACCGGCTCTTCTCC	Forward primer for Fragment A of TgMetRS. Not properly in frame of promoter in pGra-HA-HPT. **Do not use**.
New_For_A_TgMetRS_SbfI	GCTACCTGCAGGgaTCCACAATGACCGGCTCTTCTCC	New forward primer for Fragment A of TgMetRS. Has a SbfI restriction site. Properly in frame of promoter in pGra-HA-HPT.
Rev_A_TgMetRS	CGTGTAGTTGTTGGCCGTGG	Reverse primer for Fragment A of TgMetRS. Introduces T851A nucleotide change. Results in I284N substitution in translated protein.
For_B_TgMetRS	CCACGGCCAACAACTACACG	Forward primer for Fragment B of TgMetRS. Introduces T851A nucleotide change. Results in I284N substitution in translated protein.
Rev_B_TgMetRS	CCGTTGCTGAGTAGAGGTTGG	Reverse primer for Fragment B of TgMetRS. Introduces T1519C, A1520T nucleotide changes. Results in Y507L substitution in translated protein.
For_C_TgMetRS	CCAACCTCTACTCAGCAACGG	Forward primer for Fragment C of TgMetRS. Introduces T1519C, A1520T nucleotide changes. Results in Y507L substitution in translated protein.
Rev_C_TgMetRS	GCCAAATCACCGTGAGAAACC	Reverse primer for Fragment C of TgMetRS. Introduces A1610T nucleotide change. Results in H537L substitution in translated protein.
For_D_TgMetRS	GGTTTCTCACGGTGATTTGGC	Forward primer for Fragment D of TgMetRS. Introduces A1610T nucleotide change. Results in H537L substitution in translated protein.
Rev_D_TgMetRS_SbfI	GCTACCTGCAGGAAGCGCGAGAGAAAGGCG	Reverse primer for Fragment D of TgMetRS. Has a SbfI restriction site. **Do not use**. This adds the same restriction site as the forward primer for Fragment A. A new restriction site is needed.
Rev_D_TgMetRS_BspHI	GCTATCATGAAAGCGCGAGAGAAAGGCG	Reverse primer for Fragment D of TgMetRS. Has a BspHI restriction site. Properly in frame of HA tag in pGra-HA-HPT.

For_TgMetRS_Q5	CCTTGACCAACCTCTACTCAGCAACG	First of two primers for introducing the T1519C, A1520T nucleotide changes to the TgMetRS gene via Q5 site-directed mutagenesis.
Rev_TgMetRS_Q5	CATCGAACCAGACGTACATGACATG	Second of two primers for introducing the T1519C, A1520T nucleotide changes to the TgMetRS gene via Q5 site-directed mutagenesis.
For_mMetRSg_SbfI	GCTACCTGCAGGGAAGACTGTTCGTGAGCGAG	Forward primer for amplifying the mutated murine MetRS (mMetRS^{G}) gene from a vector provided by the Tirell lab. Has a SbfI restriction site. Properly in frame of promoter in pGra-HA-HPT.
Rev_mMetRSg_BspHI	GCTATCATGACTTTTTCTTCTTGCCTTTAGGA	Reverse primer for amplifying the mutated murine MetRS (mMetRS^{G}) gene from a vector provided by the Tirell lab. Has a BspHI restriction site. Properly in frame of HA tag in pGra-HA-HPT.

3.3.2 Introducing nucleotide changes into the *T. gondii* MetRS gene

Before devising the nucleotide changes that needed to be introduced for the appropriate amino acid substitutions (I284N, Y507L, H537L), I looked into the literature to determine which codon for each of the substituted amino acids was most commonly utilized in *T. gondii* [139]. I found that CTC was the most commonly used codon for leucine (L), and that AAC was most common for asparagine (N). For I284N, only a single nucleotide change was needed (T851A). For Y507L, two nucleotide changes were needed (T1519C, A1520T). And for H537L, only a single nucleotide change was needed (A1610T).

Originally, I planned to use Splicing by overhang extension (SOE) PCR to introduce all four of the nucleotide changes. This utilized four pairs of primers to create overlapping fragments of the gene, designated fragment A through D, which could be combined via SOE PCR (Fig. 19). Successfully amplifying and purifying fragment C proved difficult due to its small size. To get around this, I used the B forward primer and the C reverse primer (Table 2) to create a larger BC fragment. Doing this bypassed two of the nucleotide changes (T1519C, A1520T), however. Fragments A, BC, and D were successfully combined using SOE PCR, creating a version of the *T. gondii* MetRS gene with two of the mutations (T851A, and A1610T). This was inserted into the pGra-HA-HPT expression plasmid [140], in frame of a C-terminal HA tag and a highly active *T. gondii* promoter (GRA1 [141]). The final two nucleotide changes (T1519C, A1520T) were

introduced using New England Biolab's Q5 site-directed mutagenesis kit and two specially designed primers (Table 2). After two attempts to transfect the expression vector (pGra-HA-HPT-TgMetRS^{NLL}) into *T. gondii* and being unable to detect any HA-tagged protein in the transfected populations, I discovered that the gene was not properly in frame of the dense granule promoter in the pGra-HA-HPT expression vector. I designed a new forward primer (New_For_A_TgMetRS_SbfI, Table 2) to amplify the mutated gene out of the current pGra-HA-HPT-TgMetRS^{NLL} vector, which should correct the problem when inserted into pGra-HA-HPT.

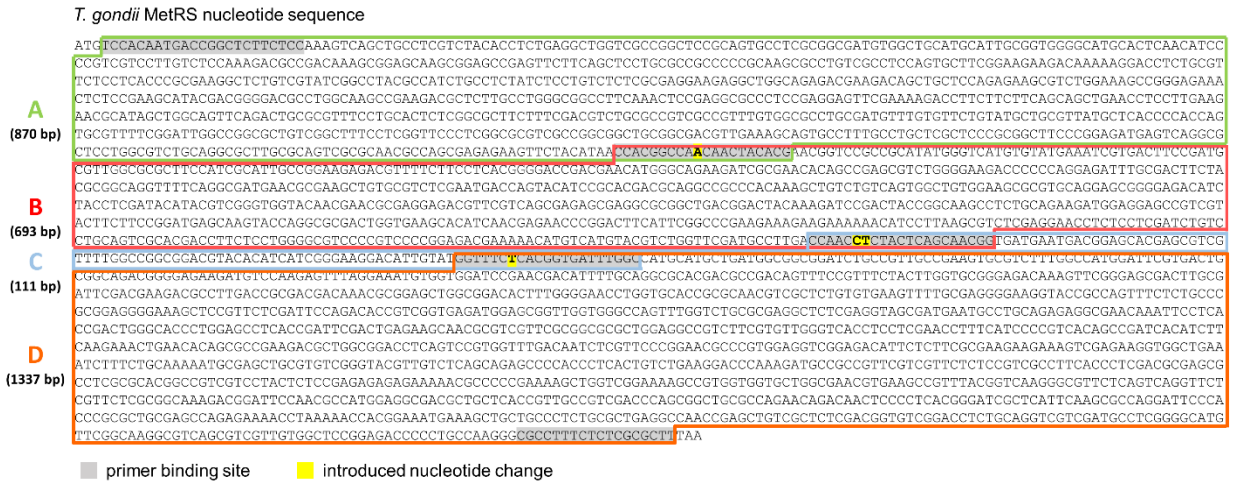

Figure 19. Engineering four nucleotide changes into *T. gondii*'s MetRS gene may allow the enzyme charge Met tRNA with Anl

Four nucleotide changes (T851A, T1519C, A1520T, and A1610T) were engineered into *T. gondii*'s MetRS gene. SOE PCR was used to generate four gene fragments (A though D) and then combine them. Each fragment is delineated by a colored box. The specific nucleotides that were mutated from their original residues are highlighted in yellow. The binding sites for the primers used to generate the fragments are highlighted in grey.

Once the new pGra-HA-HPT-TgMetRS^{NLL} vector is prepared, inserting it into parasites will be a relatively simple task, as *T. gondii* is a very genetically tractable organism. The mutated TgMetRS^{NLL}'s ability to charge tRNA^{Met} with Anl can be tested on the transfected parasite population, before isolating stable clones. The transfected population can be grown in the presence of Anl and the parasites can be solubilized and biotinylated with click chemistry. The presence of biotinylated proteins on a western blot would provide evidence that the TgMetRS^{NLL} was functional.

3.4 EXPRESSING A MUTATED MURINE METRS IN *T. GONDII*, CAPABLE OF CHARGING ANL TO BOTH EUKARYOTIC TRNA^{MET}

In February (2015), David Tirell, who first developed the MetRS^{NLL}/Anl system, sent us an expression vector with a mutated version of the murine MetRS which, when expressed in mammalian cells, resulted in the incorporation of Anl at internal Met residues. While these data are as yet unpublished, a publication is forthcoming.

Interestingly, there is only one amino acid substitution in the protein. The substitution is a leucine to a glycine in the Met binding site of the enzyme. It happens to correspond to the same active site leucine that was mutated in the *E. coli* MetRS to permit the binding of Anl. The lack of a functional group on glycine likely provides additional space in the Met binding site of the enzyme, allowing the bulkier Anl to fit inside.

I have designed primers to amplify the mutant murine MetRS (mMetRS^{G}) out if the mammalian expression vector, adding restriction sites for inserting it into the pGra-HA-HPT *T. gondii* expression vector. Once the gene is inserted into the pGra-HA-HPT expression vector, it can be transfected into parasites. Since the bacterial MetRS^{NLL} is functional in *T. gondii*, it seems very likely that the MetRS from a fellow eukaryotic organism would be functional.

4.0 CONCLUSIONS AND FUTURE DIRECTIONS

My time in the Boyle laboratory has largely been spent designing a system for specifically labeling *T. gondii* proteins while the parasite grows inside of a host cell. Developing such a system could have a profound impact on better understanding how *T. gondii* interacts with the host cell to permit its survival. Initially, the project was formulated as a means to better characterize the cohort of proteins that the parasite secretes into the host cell during the invasion process. A pioneering study attempting to identify the components of the *T. gondii* secretome utilized a 1% ethanol solution to artificially trigger secretion *in vitro* [142]. While the approach is a powerful one, it does not allow the study of secretion in the more natural environment of the host cell. Most conventional strategies for the metabolic labeling of proteins utilize radiolabeled amino acids or, more recently, the incorporation of reactive nonstandard amino acids which can be modified for identification or purification [90]. When studying a system that involves two cell types, like *T. gondii* infecting a host cell, these methods result in the labeling of both cell types. To circumvent this problem, I developed a strain of *T. gondii* that expresses a mutated form of *E. coli* methionyl-tRNA synthetase ($MetRS^{NLL}$) that permits a methionine analog, azidonorleucine (Anl), to be charged to $tRNA^{Met}$, and subsequently incorporated into nascent proteins [86]. The azide functional group on Anl allows proteins which have incorporated the analog to be tagged fluorescently or with a affinity tag by a copper-catalyzed "click" chemistry reaction [87].

The majority of my efforts in the Boyle laboratory have been spent characterizing the $MetRS^{NLL}$/Anl labeling system in *T. gondii*. The bacterial $MetRS^{NLL}$ is successfully expressed in *T. gondii*, and is functional, allowing for the incorporation of Anl into a plethora of proteins. The incorporation of Anl is limited to the *T. gondii* strain that expresses the mutant MetRS (the Tg-$MetRS^{NLL}$ strain). Prolonged growth in the presence of Anl reduces parasite numbers of the Tg-$MetRS^{NLL}$ strain, but does not affect that of a WT *T. gondii* strain. Despite this, the Anl treated parasites are able to successfully mount new host cell invasions, suggesting that Anl incorporation does not have an overly deleterious effect on the parasites. Most applications of the $MetRS^{NLL}$/Anl labeling system would only require short pulses of Anl, such that prolonged growth in Anl would not be necessary.

While a large number of parasite proteins appear to incorporate Anl, secreted parasite proteins look to only be minimally labeled. This may be due to an incompatibility between the

bacterial MetRS^{NLL} and the two *T. gondii* tRNA^{Met} [113]. Early work examining the ability of a bacterial MetRS to substitute for its eukaryotic counterpart found that it could only append Met to the eukaryotic initiator tRNA^{Met}, and not the elongator tRNA^{Met} [120-123]. If this is the case with the *E. coli* MetRS^{NLL} being expressed in *T. gondii*, then only the initiator tRNA^{Met} could be charged with Anl, permitting only the N-terminal Met residue to be replaced with Anl. Since most proteins that travel through the secretory pathway have their N-terminus processed, those proteins would no longer maintain the Anl label. Indeed, when Ngo and coworkers tried expressing the bacterial MetRS^{NLL} in mammalian cells, they found that Anl was only found at the N-terminus and that N-terminally processed proteins were not labeled [113].

To help determine whether the incorporation of Anl was confined solely to the N-terminus, or whether it could replace internal Met residues, I collaborated with a number of laboratories on mass spectrometry studies. The majority of these studies proved to be inconclusive, though one conducted with Dr. Louis Weiss' laboratory implied that Anl could be incorporated at internal Met positions in both parasite and human proteins. The percentage of proteins incorporating Anl was extremely low, casting doubt on whether the peptide assignments were legitimate. Mass spectrometry studies on the immunopurified HA-tagged MetRS^{NLL} did not reveal that it incorporated Anl, though percent coverage of the protein by identified peptides never rose above 50%. Furthermore, the N-terminal fragment was never identified.

In order for the project to move forward, more mass spectrometry studies will be needed to concretely determine what Met residues can incorporate Anl. Doing analyses on solubilized parasite samples is not the ideal means to get at the question, as the complexity of the samples prevents obtaining a large number of reads from any particular protein. If the incorporation of the analog is infrequent, and all of the present mass spectrometry studies suggest that it is, more inclusive coverage of individual proteins is what is needed. For this reason, immunopurifying specific proteins from Anl treated parasites and individually subjecting them to mass spectrometry analysis will be essential. Despite not have good luck with the experiments with the HA-tagged MetRS^{NLL}, I still think that it is a good choice. The protein is cytoplasmic, so it should not be N-terminally processed, leaving the N-terminus intact in case that is the only Met residue capable of being replaced by Anl. It should be noted that this residue could still be removed by methionine aminopeptidases. The HA-tagged MetRS^{NLL} has an additional 17 Met residues beyond the

initiating Met, which is a sizeable number for a 563 amino acid protein. Another potential candidate for immunopurification is the click beetle luciferase that is expressed by the parasite. Like the HA-tagged $MetRS^{NLL}$, it is an ectopically expressed protein, but it is cytoplasmic and the laboratory already has an antibody against it.

If the $MetRS^{NLL}$/Anl labeling system in its present format cannot label proteins which are N-terminally processed, then it will not be useful for comprehensively characterizing the *T. gondii* secretome. Though, it could identify those secreted proteins that are not N-terminally processed. Indeed, not all proteins that migrate through the secretory pathway are N-terminally processed. Membrane bound proteins with signal anchor sequences do not have signal peptides, and are guided to the membrane by the hydrophobic alpha helical signal anchor.

The most promising means to move the project forward involve creating new parasite strains with a mutated eukaryotic version of the MetRS. Mutating *T. gondii*'s MetRS to recognize Anl and charge it to $tRNA^{Met}$ would likely ensure that Anl is incorporated at all Met residues. The high level of conservation of the Met binding site in MetRS suggests that mutating the same three residues from the *E. coli* MetRS in the *T. gondii* enzyme might permit it to bind Anl [101]. Even if that is unsuccessful, expressing the recently acquired mutated murine $MetRS^{G}$ in *T. gondii* might also allow more complete incorporation of Anl into *T. gondii* proteins. If a bacterial MetRS was functional in *T. gondii*, the MetRS from a fellow eukaryotic organism will doubtlessly be functional, as well. Since the incorporation of Anl results in a reduction of parasite numbers after prolonged Anl exposure, the possibility exists that when Anl is more widely incorporated into *T. gondii* proteins, there could be more profound effects. If this is the case, adjusting the concentration of Anl used in the labeling studies might remedy the problem.

While some additional work is still needed to fully characterize the $MetRS^{NLL}$/Anl labeling system in *T. gondii*, it is poised to make a significant impact our understanding of host/pathogen interaction. Furthermore, in its present form, the system should also be functional in other Apicomplexan parasites, including *Plasmodium spp*. If any of the modifications of the system prove effective at increasing the levels of Anl-incorporation in parasite proteins, those modifications should be readily applicable to other parasite systems, as well.

5.0 MATERIALS AND METHODS

5.1 HOST CELL CULTURE

All assays were performed in human foreskin fibroblasts (HFFs), which were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine (Life Technologies, Rockville, MD), 50 μg/mL each of penicillin (Life Technologies, Rockville, MD) and streptomycin (Life Technologies, Rockville, MD). The cells were maintained at 37°C in 5% CO_2.

5.2 EXPRESSION OF *E. COLI* METRS^{NLL} IN *T. GONDII*

The *E. coli* MetRS^{NLL} gene used for creating the MetRS^{NLL}-expressing *T. gondii* strain (the Tg-MetRS^{NLL} strain) was cloned into the pQE-80L bacterial expression vector. The version of the MetRS gene housed within the vector encodes a truncated form of the protein, shortened from 677 amino acids to 548. The missing C-terminal amino acids have a role in MetRS dimerization, though their absence should have minimal effects on successful incorporation of Anl into proteins, as monomeric MetRS is functionally indistinguishable from the native dimeric form [143]. The gene was PCR amplified from the vector using primers with unique restriction sites (Forward Primer, NsiI site underlined: gtta<u>atgcat</u>cctactatgactcaagtcgcga; Reverse Primer, NcoI site underlined: gcta<u>ccatgg</u>tttagaggcttccagtgcttcaacc). Using the added restriction sites, the PCR product was cloned into the pGra-HA-HPT expression plasmid [140], in frame with a C-terminal HA tag and under the control of a highly active dense granule protein promoter (GRA1 [141]). The pGra-HA-HPT vector includes the parasite hypoxanthine-xanthine-guanine phosphoribosyl transferase (HPT) gene which is used to select for stable incorporation of the vector into the genome of a ΔHPT *T. gondii* strain when grown in the presence of mycophenolic acid (MPA) and xanthine [144,145]. The pGra-HA-HPT-MetRS^{NLL} vector was transfected into the type II parasite strain Me49Δ*HPT*:*Luc* and successful transformants were selected for using MPA (25 μg/ml; Sigma-Aldrich, St. Louis, MO) and xanthine (50 μg/ml; Sigma-Aldrich, St. Louis, MO) [144,145]. Drug resistant clones were obtained by limiting dilution in 96-well plates. A MetRS^{NLL}-expressing strain (the Tg-MetRS^{NLL} strain) was generated with this process, along with a MPA-resistant strain which lacked the MetRS^{NLL} gene. This strain served as a wild type control for the experiments, as it was prepared under the same conditions but does not express the MetRS^{NLL}.

5.3 WESTERN BLOT ANALYSIS

To probe for expression of the HA-tagged MetRS^{NLL}, HFFs infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain were lysed in 1x sodium dodecyl sulfate (SDS) sample buffer and resolved on 8% SDS-polyacrylamide gels. The samples were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). After the transfer, the membranes were blocked in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) and 5% Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) or 5% non-fat dry milk for 1 h. After blocking, the membrane was incubated in anti-HA-peroxidase (Roche, Mannheim, Germany) antibody at a 1:4000 dilution for 30 m. After incubation, the blot was washed 3 times with TBST and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). To test for equal loading, the blot was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL), and reprobed with Mouse anti-TgSAG1 (GenWay) at a 1:4000 dilution for 1 h. After three washes with TBST, the blot was incubated with Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:4000 dilution for 1 h. After washing the blot 3 times with TBST, it was developed using SuperSignal West Pico Chemiluminescent Substrate.

5.4 INCORPORATION OF AZIDONORLEUCINE (ANL) INTO PARASITES

A confluent monolayer of HFFs in a T25 culture flask was infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at a multiplicity of infection (MOI) of 3, and supplemented with 1 mM Anl (from a 100 mM stock in H_20). In some instances, the medium was supplemented with additional Anl 24 h after initial infection. After 48 h of growth, the infected monolayer of HFFs was washed with Phosphate Buffered Saline (PBS; Fischer Scientific, Pittsburgh, PA) and scraped from the T25 culture flask in 3 ml of PBS. The infected HFFs were centrifuged at 800xg for 10 m, and solubilized in 100 µl of 2% SDS in PBS, followed by boiling for 5 m. The samples were diluted to 0.5% SDS with PBS including cOmplete EDTA-free protease inhibitors (Roche Diagnostics; Indianapolis, IN).

5.5 CLICK CYCLOADDITION REACTION

Cycloaddition reactions were performed on the cell lysates in a final volume of 50 µl of 50 µM biotin-alkyne (Invitrogen Molecular Probes, Eugene, OR) or 10 µM Alexa Fluor 594-alkyne (Invitrogen Molecular Probes, Eugene, OR), along with 1 mM tris(2-carboxyethyl)phosphine (TCEP; Sigma-Aldrich, St. Louis, MO), 100 mM tris-(benzyltriazolylmethyl)amine ligand

(TBTA; Sigma-Aldrich, St. Louis, MO), and 1 mM copper(II) sulfate ($CuSO_4$; Sigma-Aldrich, St. Louis, MO). This mixture was incubated at room temperature for 2 h with rotation [104,105]. When using Alexa Fluor 594-alkyne, samples were obscured from light during the incubation. Samples were used directly for SDS-PAGE analysis or neutravidin purification.

5.6 DETERMINING THE OPTIMAL ANL CONCENTRATION FOR EFFICIENT INCORPORATION INTO NASCENT PROTEINS

HFFs were infected with the Tg-MetRS^{NLL} strain and the growth medium was supplemented with varying concentrations of Anl (0.1 mM, 0.5 mM, 1.0 mM, or 4.0 mM) at the time of infection. After 48 h of growth, the infected monolayer was washed to remove excess Anl and the infected HFFs were solubilized in 2% SDS/PBS and diluted to 0.5% SDS to facilitate click chemistry. The samples were biotinylated with click chemistry using biotin-alkyne and analyzed via western blot, probing with streptavidin-HRP.

5.7 LUCIFERASE GROWTH ASSAYS

WT *T. gondii* and the Tg-MetRS^{NLL} strain were derived from the same parental parasite line, which expresses a soluble form of firefly luciferase. To determine the effects of Anl on parasite growth, 10000 parasites were used to infect confluent HFF monolayers in a 96-well plate in triplicate. Cells were exposed to either 1 mM Anl or vehicle (water) 16 h post-infection, and processed for bioluminescence at 24, 48 and 72 h post-Anl exposure. Cells were lysed using 1X luciferase lysis buffer (Promega, Madison, WI) and frozen at -80°C until analysis. Twenty µL of each sample was added to a Greiner Cellstar® 96-well plate with an opaque bottom, and 50 µL of luciferase assay reagent (Promega, Madison, WI) was added to each well immediately prior to analysis on a Centro XS^3 LB 960 microplate luminometer. Luminescence was measured for 10 s in each well with no delay between each. For each strain (WT *T. gondii*, the Tg-MetRS^{NLL} strain) data were converted to percent of control by dividing the signal in the presence of Anl by the signal in its absence.

5.8 NEUTRAVIDIN PURIFICATION

A confluent monolayer of HFFs in a T25 culture flask was infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at MOI 3, and supplemented with 1 mM Anl . After 48 h of growth, the infected monolayers were washed 3 times with PBS to remove unincorporated Anl. The monolayers were scraped from their culture flasks, and syringe lysed by passage through 25 gauge

(g) and 27g needles in 3 ml of PBS including cOmplete EDTA-free protease inhibitors (Roche Diagnostics; Indianapolis, IN). The samples were centrifuged at 800xg for 10 m, and solubilized in 100 µl of 2% SDS in PBS, followed by boiling for 5 m. The samples were diluted to 0.5% SDS with PBS including cOmplete EDTA-free protease inhibitors (Roche Diagnostics; Indianapolis, IN). The samples were subjected to click chemistry with biotin-alkyne. Half of the sample was saved as an input and the rest was used for neutravidin purification. 200 µl of well-mixed neutravidin agarose resin (Thermo Scientific, Rockford, IL) was used for each purification, and was equilibrated according to manufacturer's instructions. The samples were incubated with the equilibrated resin for 2 h at room temperature with rotation. After incubation, the samples were centrifuged at 2500xg for 1 m and the supernatant was saved as an "unbound" sample. The resin was washed 3 times with PBS, one time with PBS with 0.2% SDS, and a final time with PBS. The bound samples were eluted by boiling for 5 m in 200 µl of 1x SDS sample buffer. The eluted samples were then used directly for SDS-PAGE analysis followed by western blotting. The mouse monoclonal anti-ROP7 1B10 antibody was generously provided by Peter Bradley (University of California, Los Angeles; [146]), and used at a 1:1000 dilution. The rabbit anti-Rop5 antibody [147] and rabbit anti-Rop18 antibody [148] were generously provided by David Sibley (Washington University School of Medicine).

5.9 *IN SITU* LABELING OF PARASITES USING CLICK CHEMISTRY

A confluent monolayer of HFFs in a T25 culture flask was infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at MOI 3, and supplemented with 1 mM Anl. After 48 h of growth, the infected monolayers were washed 2 times with PBS to remove unincorporated Anl. The monolayers were scraped from their culture flasks, and syringe lysed by passage through 25g and 27g needles. The Anl treated parasites were then used to infect HFF-seeded coverslips at MOI5 for 6 h. The cells were washed once with PBS and then were fixed for 20 m at room temperature in 4% electron microscopy grade paraformaldehyde (prepared from 16% stock). The coverslips were washed twice with PBS, and then blocked with PBS supplemented 5% BSA and 0.1% Triton X-100 for 1 h. After fixation and blocking, a click reaction was performed on the samples using Alexa Fluor 594-alkyne for 1 h. The coverslips were washed 2 times with PBS to remove excess Alexa Fluor 594-alkyne. Additionally, the coverslips were incubated with anti-Gra7 monoclonal mouse antibody at a dilution of 1:2000 for 1 h, washed 3 times with PBS, and incubated with Alexa

Fluor 488 Goat Anti-Mouse IgG at a dilution of 1:2000 for 1 h. After 3 PBS washes, the coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). The monoclonal antibody to Gra7 was generously provided by Peter Bradley (University of California, Los Angeles; [146]).

5.10 SAMPLE PREPARATION FOR MASS SPECTROMETRY

Human foreskin fibroblasts (HFFs) were infected with either WT *T. gondii* or the Tg-MetRS^{NLL} strain at a MOI of 3 in the presence of 1 mM Anl. 8 to 15 T25 culture flasks of HFFs were infected per strain. After 2 days of growth, the infected host cells were washed two times with 4 ml of PBS to remove unincorporated Anl. Sample preparation diverges at this point, depending on whether 1) infected host cell lysates were required, or 2) purified parasite samples are required.

5.10.1 Infected host cell lysates

After washing the infected HFFs with PBS, they were combined in 1 ml of RIPA buffer prepared from Sigma-Aldrich's Mammalian Cell lysis kit. The samples were incubated at room temperature for 15 minutes with shaking before being centrifuged at either 12,000xg for 10 minutes, or 100,000xg for 45 minutes, to pellet the cellular debris. The supernatant was frozen and sent for mass spectrometry analysis

5.10.2 Purified parasites

After washing the infected HFFs with PBS, the parasites were isolated by passage through 25g and 27g needles in 3 ml of PBS. The lysatse were passed through 5.0 µm syringe-driven filters to remove cell debris, followed by centrifugation at 800xg to pellet the parasites. The pelleted parasites were resuspended in 300 µl of RIPA buffer and incubated with shaking at room temperature for 15 minutes, before centrifugation at 12,000xg to pellet insoluble material. The supernatant was frozen and sent for mass spectrometry analysis.

5.11 IMMUNOPURIFICATION OF HA-TAGGED METRS^{NLL}

To prepare samples for the immunopurification of the HA-tagged MetRS^{NLL}, 10-15 T25s were infected with the Tg-MetRS^{NLL} strain at a MOI of 3. 1 mM Anl was added to the growth medium of half of the T25 culture flasks of infected HFFs, the others served as no treatment controls. After 48 hours of growth, the infected monolayers were washed twice with 3 ml of PBS. The infected HFFs were removed from the culture flasks with cell scrapers, and combined in a total of 3 ml of PBS, with separate samples based on Anl treatment. Parasites were released from the HFFs by

passing the combined samples through 25g and 27g needles, and host cell debris was removed with a 5 μm syringe-driven filter. After counting parasites, the samples were centrifuged at 800xg for 10 minutes to pellet the parasites. The pelleted parasites were resuspended in 1 ml of TEN buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl) containing RIPA detergents (1% Triton-X100, 0.5% Deoxycholate, 0.01% SDS, and cOmplete EDTA-free protease inhibitors (Roche Diagnostics; Indianapolis, IN)). The mixture was incubated on ice for 30 minutes before centrifugation at 3,000xg for 20 minutes to remove any insoluble material. The supernatant was saved as the sample to be used for immunopurification. 500ul of the sample was saved as an input, and the remainder was incubated with a 1/1000 dilution of anti-HA (clone 3F10) monoclonal rat IgG1 antibody (Roche, Mannheim, Germany) for 4 hours at 8^{o}C with gentle agitation. After incubation with the anti-HA antibody, the sample was added to 50 μl of Pierce Protein G Plus Agarose (Life Technologies, Carlsbad, CA) which was equilibrated according to the manufacturer's protocol. The samples were incubated with rotation at 4^{o}C for 2 hours, before being centrifuged at 800 xg for 1 minute. The supernatant was saved as an "unbound" sample. The resin was washed three times with 500 μl of TEN buffer before elution. The bound samples were eluted in one of two ways, 1) with 0.1 M triethylamine (pH 11.5), or 2) by boiling the resin in 100 μl of 1x SDS sample buffer. For the 0.1 M trimethylamine elution, there were five successive elutions which were combined. For each elution, 50 μL of 0.1 M trimethylamine was added to the resin and incubated with gentle agitation at room temperature for 5 minutes. The resin was centrifuged at 800xg for 1 minute and the supernatant was carefully removed as the elution. The trimethylamine was neutralized by adding an equal volume of 1.0M Tris, pH 6.8. The five neutralized elutions were combined, and concentrated by TCA precipitation. The immunopurified samples were run on SDS-PAGE gels and coomassie stained. Bands were excised with clean razor blades and sent for mass spectrometry analysis.

APPENDIX A THE ROP16 ORTHOLOG IN *HAMMONDIA HAMMONDI* IS FUNCTIONAL IN *T. GONDII*

I worked briefly on a project looking at orthologs of *T. gondii* effector proteins in *Hammondia hammondi*, *T. gondii*'s closest extant relative [149,150]. Since *H. hammondi* is not able to be grown in cell culture, we have heterologously expressed the orthologs in *T. gondii* and looked for phenotypic changes. In particular, I was working with secreted rhoptry kinase, Rop16, which is known to phosphorylate and activate members of the signal transducer and activator of transcription (STAT) signaling pathway, namely STAT3 and STAT6 [151]. The various differing strains of *T. gondii* are known to differ in their ability to activate STAT3/6. There are three predominant strain types (I, II, and III), with Types I and III being able to activate STAT3/6, and Type II strains are unable to do so [140]. When expressing *H. hammondi*'s Rop16 (HhRop16) in a type II strain of *T. gondii* (*T. gondii* Type II:HhRop16), there was an increase in the levels of phosphorylated Stat6 in the host nucleus [152].

Since the *T. gondii* Type II:HhRop16 still has its endogenous copy of the Rop16 ($TgRop16_{II}$), there is a possibility that the increase in phosphorylated Stat6 is due to dosage effects. In order to investigate this possibility, I created a Type II *T. gondii* clone which expressed a second copy of $TgRop16_{II}$, driven by the same promoter as the HhRop16 (*T. gondii* Type II:$TgRop16_{II}$). I compared how this strain activated Stat6 in infected host cells to WT Type II *T. gondii* and *T. gondii* Type II:HhRop16. I found that both the WT Type II *T. gondii* and *T. gondii* Type II:$TgRop16_{II}$ were similar in their inability to activate Stat6, but *T. gondii* Type II:HhRop16 significantly increased levels of activated Stat6 (Fig. 19). This supports the notion that *H. hammondi* has a functional ortholog of Rop16.

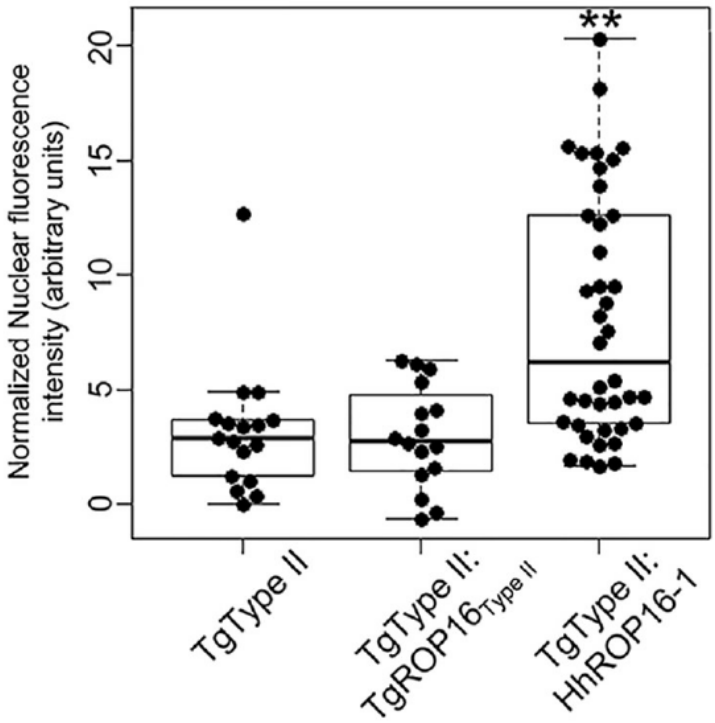

Figure 20. The H. hammondi ortholog of ROP16 increases levels of pSTAT6 in the host cell nucleus

HFFs were infected with either WT Type II *T. gondii*, Type II *T. gondii* expressing a second copy of TgRop16II, or Type II *T. gondii* expressing HhRop16. Phosphorylated Stat6 levels in the nuclei of HFFs was examined by immunofluorescence, subtracting cytoplasmic fluorescent signal from nuclear fuorescent signal. At least 16 randomly selected infected HFFs were chosen per strain. **, P <0.001 compared to WT Type II *T. gondii*. Note: The image used in this figure is a slightly modified version of that which appears in the published version of the work [152].

APPENDIX B MITOCHONDRIAL STUDIES USING THE SEAHORSE ANALYZER

I began a collaborative project with Dr. Bennett Van Houten investigating the effect that *T. gondii* infection has on host cell mitochondria using a Seahorse XF^{e} Mitochondrial Analyzer. The parasitophrous vacuole of some strains of *T. gondii* is known to associate host mitochondria upon invasion, though the biological significance for this association is not presently known. A Seahorse XF^{e} Mitochondrial Analyzer monitors oxygen consumption in the analyzed cells to assess levels of oxidative phosphorylation taking place, and monitors lactate production as an indicator for levels of glycolysis. Cells are grown in specially designed 24 well plates which are loaded into the Seahorse analyzer.

I was able to perform three Seahorse analysis of HFFs infected with *T. gondii*. The Seahorse 24 well plates were seeded with HFFs a week before the analysis, allowing suitable time for them to become confluent. A day before the analysis, they were infected with *T. gondii*. After 3 experiments with extremely variable results, I discovered that the viability of the HFFs in outer wells of the plates was compromised due to evaporation of growth medium. This was likely the reason for the inconsistent results. If additional Seahorse experiments are performed, special care should be made to ensure no growth medium evaporates while the HFFs are allowed to grow to confluence. Before infecting any of the cells with *T. gondii*, it is imperative that each well is examined to confirm the health of the cells. If any of the infected cells are not healthy and confluent, the results will not be consistent.

APPENDIX C CLICK REACTION

Below is a detailed protocol for performing the copper(I)-catalyzed "click" cycloaddition reaction.

Stock solutions needed:

TCEP (tris(2-carboxyethyl)phosphine), Sigma-Aldrich, Prod. No. C4706

- prepare 50x stock in distilled water, *make fresh for each experiment*
    - to make 1 ml of 50x stock: 14.3 mg in 1 ml of distilled water

TBTA (tris-(benzyltriazolylmethyl)amine ligand), Sigma-Aldrich, Prod. No. 678937

- prepare 17x solution in DMSO and t-Butanol (1:4)
    - to make 17x stock: 0.045 g in 10 ml DMSO and 40 ml t-Butanol

$CuSO_4$ (copper (II) sulfate), Sigma-Aldrich, Prod. No. C1297

- prepare 50x solution in distilled water
    - to make 50x stock: 0.339 g in 50 ml distilled water

Biotin-alkyne (PEG4 carboxamide-propargyl biotin), Invitrogen, Cat. No. B10185

- prepare 4 mM solution in DMSO

Setting up a 50 µl reactions:

Reaction Component	Final Conc.	Vol. of stock
TCEP (50x)	1 mM	1.0 µl
TBTA (17x)	100 µM	2.9 µl
CuSO4 (50x)	1 mM	1.0 µl
Biotin-alkyne (4 mM)	50 µM	0.6 µl
Azide labeled sample	-	45 µl

1. Combine reaction components in a 1.5 ml eppendorf tube and gently vortex.
2. Allow samples to incubate at room temperature for at 1 hour with rotation.
3. After the incubation is completed, the click reaction is finished. There is nothing done to stop the reaction. For temporary storage, the samples stored in the -20^{o}C freezer, or -80^{o}C freezer for long term storage.

APPENDIX D ANL-LABELING EXPERIMENT, NEUTRAVIDIN PURIFICATION

Below is a detailed protocol for performing an Anl-labeling experiment, followed by a neutravidin purification.

Strains utilized:

1. WT *T. gondii* (Me49ΔHpt:Luc + MetRS *clone 2*)
2. Tg-MetRS^{NLL} strain (Me49ΔHpt:Luc + MetRS *clone 12*)

I. Grow parasites in the presence of azidonorleucine (Anl) for 48 hours in human foreskin fibroblasts (HFFs)

1. Obtain a T25 culture flask of HFFs and check for the confluence of the cells under the microscope. Remove the media and replace it with 4ml of cDMEM. *Note: Depending on how many parasites you want to collect in the end, you may need to infect multiple T25s per strain and combine them isolating the parasites during step 6-7.*
2. Infect the HFFs with parasites at the multiplicity of infection (MOI) of 3.
    a. Add 1 mM Anl to the T25 culture flask (from a 100 mM stock solution, stored at -80ºC).
3. Allow parasites to grow for 48 hours in a 37ºC incubator with CO_2.
4. After 48 hours of grow, check to confirm that the HFFs are well infected.
5. Wash the infected HFFs with 3ml of PBS. 2x.
6. Scrape off the HFF monolayer from the T25 culture flask in 3ml of PBS with protease inhibitors (cOmplete EDTA-free protease inhibitors, prepare a 50x stock). Lyse infected HFFs by passage through a 25g and 27g needle. Count parasites.
7. Transfer solution to a 15 ml centrifuge tube and centrifuge at 800xg for 10 minutes.
8. Aspirate off the supernatant and resuspend the pelleted parasites in 100 µl of 2% SDS in PBS. At this point, the sample can be transferred into a 1.5 ml Eppendorf tube.
9. Transfer the samples to a 95ºC heat block for 5 minutes to help solubilize the parasites. *Note: Condensation can build up and cause the lids to pop open during this step.*
10. Dilute the samples to 0.5% SDS with PBS with protease inhibitors (292ul of PBS + 8ul of 50x protease inhibitor stock). You should now have 400 µl of each sample, ready for click chemistry.

II. Click reaction

For a detailed explanation of preparing the stock solutions for the reaction, see Appendix C.

Performing a 50 ul reaction (1x) is standard, where 45 µl of your azide-labeled sample is utilized. In this case, we have 400ul of sample. If we want to use all for the click reaction: 45/400 = 8.9

Reaction Component	Final Conc.	Vol.of stock (1x)	Vol. of stock (8.9x)
TCEP (50x)	1 mM	1.0 µl	8.9 µl
TBTA (17x)	100 µM	2.94 µl	26.17 µl
$CuSO_4$ (50x)	1 mM	1.0 µl	8.9 µl
Biotin-alkyne (4 mM)	50 µM	0.6 µl	5.34 µl
Azide-labeled sample	-	45 µl	400 µl

1. Combine reaction components in a 1.5 ml eppendorf tube and gently vortex.
2. Allow samples to incubate at room temperature for 1 hour with rotation.
3. Samples are now ready for neutravidin purification. There is nothing done to stop the reaction.

III. Neutravidin Purification

200 µl of each sample will be used from the purification, and the other 200 µl saved as the input.

Equilibrate the neutravidin resin:

1. Obtain 200ul of well mixed neutravidin resin per sample.
2. Centrifuge at 2500xg for 1 minute. Remove and discard the supernatant.
3. Equilibrate the resin by adding 500 µl of 0.5% SDS in PBS. Centrifuge at 2500xg for 1 minute. Remove and discard the supernatant. Repeat 2x.

Incubation with the neutravidin resin:

1. Add 200 µl of sample to the resin (leaving the other 200 µl for the input)
2. Put the samples on a rotator at room temperature for 2 hours.
3. After incubation, centrifuge the samples at 2500xg for 1 minute. Remove and save the supernatant as an “unbound” sample.

4. Wash the resin by adding 1 ml of PBS. Centrifuge at 2500xg for 1 minute. Remove and discard the supernatant. Repeat 2x.
5. Add 1 ml of 0.2% SDS in PBS. Centrifuge at 2500xg for 1 minute. Remove and discard the supernatant.
6. Wash 1 final time with 1ml of PBS. Centrifuge at 2500xg for 1 minute. Remove and discard the supernatant.

Elution of bound sample:

1. Elute sample from the resin by adding 200 µl of 1x SDS dye. Boil for 5 minutes on a heat block at 95ºC.
2. Centrifuge at 2500xg for 1 minute. Collect the supernatant as the elution (200 µl).
3. Repeat the above 2 steps for a second elution sample.

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