

# New Method for the Orthogonal Labeling and Purification of *Toxoplasma gondii* Proteins While Inside the Host Cell

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**ABSTRACT** *Toxoplasma gondii* is an obligate intracellular protozoan parasite that is capable of causing severe disease in immunocompromised humans. How *T. gondii* is able to modulate the host cell to support itself is still poorly understood. Knowledge pertaining to the host-parasite interaction could be bolstered by developing a system to specifically label parasite proteins while the parasite grows inside the host cell. For this purpose, we have created a strain of *T. gondii* that expresses a mutant *Escherichia coli* methionyl-tRNA synthetase (MetRS<sup>NLL</sup>) that allows methionine tRNA to be loaded with the azide-containing methionine analog azidonorleucine (Anl). Anl-containing proteins are susceptible to a copper-catalyzed “click” reaction to attach affinity tags for purification or fluorescent tags for visualization. The MetRS<sup>NLL</sup>-Anl system labels nascent *T. gondii* proteins in an orthogonal fashion, labeling proteins only in MetRS<sup>NLL</sup>-expressing parasites. This system should be useful for nonradioactive pulse-chase studies and purification of nascently translated proteins. Although this approach allows labeling of a diverse array of parasite proteins, secreted parasite proteins appear to be only minimally labeled in MetRS<sup>NLL</sup>-expressing *T. gondii*. The minimal labeling of secreted proteins is likely a consequence of the selective charging of the initiator tRNA (and not the elongator methionine tRNA) by the heterologously expressed bacterial MetRS.

**IMPORTANCE** Studying how *T. gondii* modifies the host cell to permit its survival is complicated by the complex protein environment of the host cell. The approach presented in this article provides the first method for specific labeling of *T. gondii* proteins while the parasite grows inside the host cell. We show that this approach is useful for pulse-chase labeling of parasite proteins during *in vitro* growth. It should also be applicable during *in vivo* infections and in other apicomplexan parasites, including *Plasmodium* spp.

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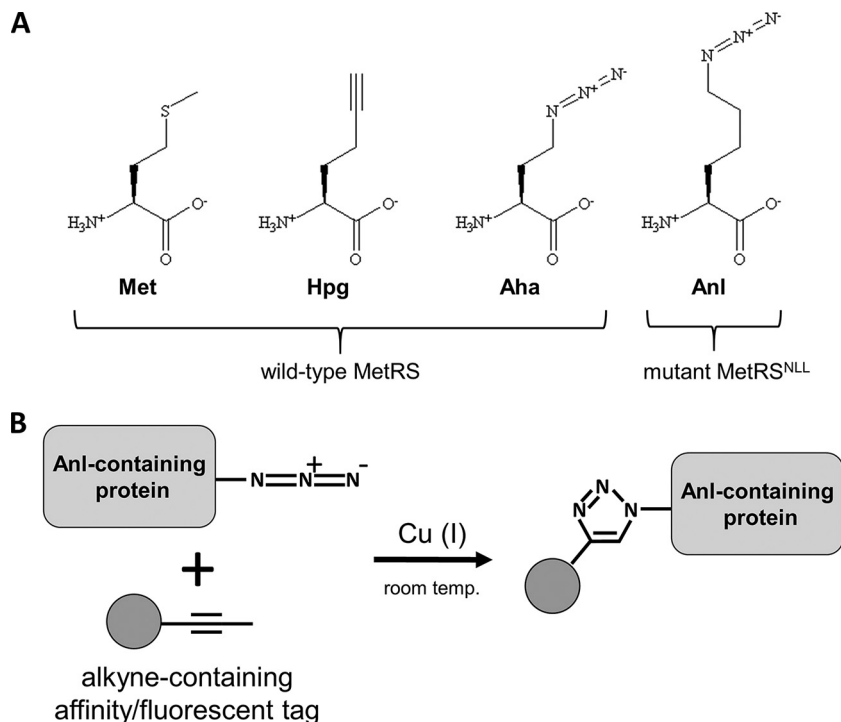
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In the past several years, the use of noncanonical amino acids (NCAAs) to label newly synthesized proteins has expanded rapidly (1–5). Numerous methionine (Met) surrogates have been developed that can be activated by wild-type (WT) methionyl-tRNA synthetases (MetRS) and incorporated into cellular proteins. Several such surrogates carry reactive functional groups that allow the attachment of affinity tags for protein purification or fluorescent probes for visualization. Among the most commonly used NCAAs are homopropargylglycine (Hpg) and azidohomoalanine (Aha) (3, 6–8), both of which are susceptible to the copper(I)-catalyzed variant of the Huisgen 1,3-dipolar cycloaddition reaction, known commonly as “click chemistry” (9–11) (Fig. 1A and B). Aha- or Hpg-containing proteins can be specifically labeled with alkyne or azide tags for visualization or purification followed by identification by mass spectroscopy.

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 NCAAs. This limitation prompted the development of cell-selective systems for NCAA incorporation. In 2009, Ngo and coworkers (12) used random mutagenesis on active site residues to create a mutant version of *Escherichia coli* MetRS (MetRS<sup>NLL</sup>) that activates the Met ana-

log azidonorleucine (Anl), which is not utilized by the wild-type MetRS (Fig. 1A) (13). This was accomplished by mutation of three highly conserved residues in the Met-binding pocket of the enzyme (L13N, Y260L, and H301L; leading to MetRS<sup>NLL</sup>) (14, 15). *E. coli* expressing MetRS<sup>NLL</sup> (the Ec-MetRS<sup>NLL</sup> strain) was able to use Anl in protein synthesis, whereas wild-type *E. coli* was unable to do so. Furthermore, when the Ec-MetRS<sup>NLL</sup> strain was grown in coculture with other bacterial strains or with mammalian cells, Anl was detected only in Ec-MetRS<sup>NLL</sup> proteins (12). Because MetRS<sup>NLL</sup> 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 (13).

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 (16). For this purpose, we have developed a *T. gondii* strain that heterologously expresses a hemagglutinin (HA)-tagged version of *E. coli* MetRS<sup>NLL</sup> (the Tg-MetRS<sup>NLL</sup> strain) and show that it is fully functional.



**FIG 1** (A) The structures of methionine (Met) and methionine analogs: homopropargylglycine (Hpg), azidohomoalanine (Aha), and azidonorleucine (Anl). They can all be charged to tRNA<sup>Met</sup> by the wild-type MetRS, with the exception of Anl, which can be utilized only by a mutant synthetase like *E. coli* MetRS<sup>NLL</sup>. (B) Reaction scheme of a click cycloaddition between a protein that has incorporated Anl and an alkyne conjugated to an affinity/fluorescent tag.

## RESULTS AND DISCUSSION

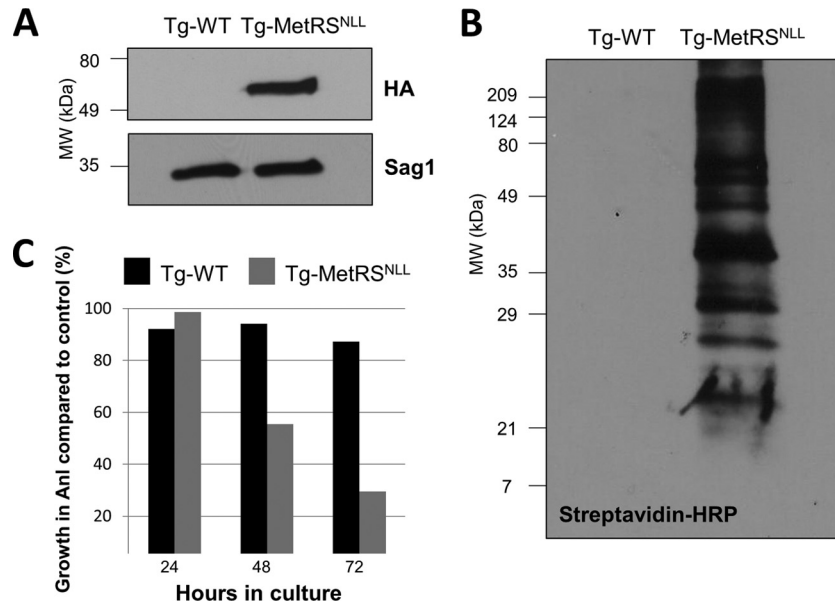
In order to implement the MetRS<sup>NLL</sup>-Anl system in *T. gondii*, we heterologously expressed an HA-tagged version of *E. coli* MetRS<sup>NLL</sup> in a type II strain of *T. gondii* (Me49ΔHPT:Luc strain) (17). Successful expression of the bacterial gene was confirmed by comparing lysates of human foreskin fibroblasts (HFFs) infected with either WT *T. gondii* or the MetRS<sup>NLL</sup>-expressing strain (Tg-MetRS<sup>NLL</sup> strain) via Western blotting (Fig. 2A). A prominent signal (at the expected 64-kDa size) is present only in the Tg-MetRS<sup>NLL</sup> strain-infected sample, confirming that *E. coli* MetRS<sup>NLL</sup> can be successfully expressed in *T. gondii*.

**The Tg-MetRS<sup>NLL</sup> strain is able to incorporate Anl into proteins.** To determine if the Tg-MetRS<sup>NLL</sup> strain is able to incorporate Anl into nascent proteins, monolayers of HFFs were infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> 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 was 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 (18, 19). Anl incorporation was confirmed via Western blotting, demonstrating that the *E. coli* MetRS<sup>NLL</sup> is functional in *T. gondii* and that it is limited to parasites that express MetRS<sup>NLL</sup> (Fig. 2B). Furthermore, a wide variety of proteins of >21 kDa are labeled only in MetRS<sup>NLL</sup>-expressing parasites. Taken together, these biotinylation data show that MetRS<sup>NLL</sup> 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 *Cryptosporidium* spp.

It should be noted that at longer exposure times, background streptavidin-horseradish peroxidase (HRP) labeling is visible around 35, 45, 65, and 130 kDa in both the WT and MetRS<sup>NLL</sup>-expressing samples (data not shown). Biotin is a cofactor that is covalently attached to a number of carboxylase enzymes, such as the apicoplast-located acetyl coenzyme A (acetyl-CoA) carboxylase (20, 21) and the mitochondrion-located pyruvate carboxylase in *T. gondii*. These naturally biotinylated proteins are known to be visible on streptavidin-HRP blots (20, 22), so they should be visible regardless of Anl incorporation. The 35-kDa band is the most prominent, which may correspond to a subdomain of the multi-subunit acetyl-CoA carboxylase (20), which is prone to proteolysis in rapeseed (*Brassica napus*) (23). The 130-kDa band likely corresponds to pyruvate carboxylase, as it agrees with its predicted size.

**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. 1A), 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. 1A), which is capable of being charged to tRNA<sup>Met</sup> by wild-type MetRS, found that the tag was nontoxic to the growing cells and did not significantly affect protein stability or promote degradation (3, 6, 24–26). The majority of these studies were



**FIG 2** (A) *Toxoplasma gondii* is able to express the *E. coli*-derived MetRS<sup>NLL</sup> under control of the *T. gondii* GRA1 promoter. An HA-tagged version of MetRS<sup>NLL</sup> was expressed in a type II strain of *T. gondii* (Me49 $\Delta$ HPT:Luc strain) under the control of the *T. gondii* GRA1 promoter. MetRS<sup>NLL</sup> 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<sup>NLL</sup>-expressing parasite strains. Lysates of Anl-treated human foreskin fibroblasts (HFFs) infected with either Tg-MetRS<sup>NLL</sup> or wild-type *T. gondii* parasites were subjected to click chemistry in the presence of biotin-alkyne. Successful biotinylation was assessed by probing Western blots with streptavidin-HRP. A prominent signal is seen with the Tg-MetRS<sup>NLL</sup> strain, but no signal is seen with WT *T. gondii*. (C) Growth of *T. gondii* is reduced after 48 h of Anl exposure. The effects of growing the Tg-MetRS<sup>NLL</sup> strain in 1 mM Anl over a period of 3 days were assessed by a luciferase assay. Data are represented as the percentage of growth in Anl compared to that in nonexposed controls. Growth of the Tg-MetRS<sup>NLL</sup> 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<sup>NLL</sup> and WT *T. gondii* strains in Anl.

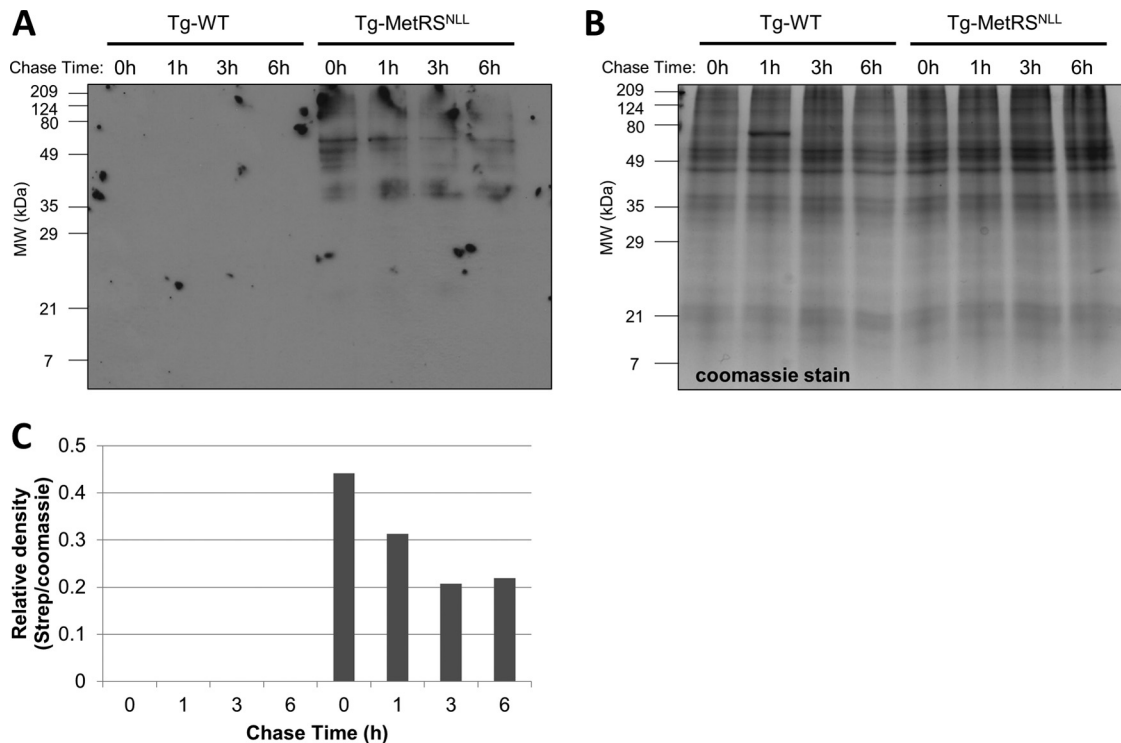
conducted using short pulse times (2 to 4 h [6, 24, 26] and up to 24 h [25]), so it remains to be seen whether prolonged exposure to Aha affects cell growth. While Anl is very similar in structure to Aha (Fig. 1A), it is slightly larger and therefore cannot be charged to tRNA<sup>Met</sup> by a wild-type MetRS. To assess the effects of Anl incorporation, we (i) examined how prolonged Anl exposure affected parasite growth and (ii) globally addressed whether Anl incorporation affects protein stability.

**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<sup>NLL</sup> strain in the presence or absence of 1 mM Anl over a 72-h time period and performing luciferase assays to quantify the parasite replication rate. After 24 h of growth in 1 mM Anl, luciferase-derived signal intensities were similar between Anl-exposed and control parasite lysates (Fig. 2C). However, noticeable growth differences were observed in the MetRS<sup>NLL</sup>-expressing strain after 48 h and 72 h of Anl exposure (55% and 30% compared to wild-type controls, respectively; Fig. 2C). Based on these data, it is evident that incorporation of Anl slows parasite growth, particularly after prolonged exposures. Despite this, parasites exposed to Anl for 48 h were still able to effectively invade host cells and replicate normally after Anl removal (see Fig. 6, discussed later in the text). Parasites exposed to Anl for 72 h were also able to successfully invade host cells (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 Anl-driven growth defects is not known, prolonged Anl exposure

on parasite growth may result in destabilization or misfolding of *T. gondii* proteins, leading to a reduced parasite growth rate. 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 slowed growth of *T. gondii* 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 (27) and particularly Aha (6, 24, 26), the 48-h pulse time greatly exceeds typical analog pulse times. To avoid complications of the growth rate reductions, 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 significant growth defects would not be observed. Indeed, pulse-chase studies utilizing Aha as a Met analog use 2- to 4-h pulse times to achieve substantial labeling (6, 24, 26).

**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<sup>NLL</sup> strain and pulsed with Anl for 2 h. As expected, a biotinylated signal is present only in the Tg-MetRS<sup>NLL</sup> strain-infected samples (Fig. 3A, lanes 5 to 8). The signal intensity is the highest directly after the pulse, with the intensity dropping off steadily over the 6-h chase time (Fig. 3A and C). Despite the declining signal intensity, after the 6-h pulse there is still a sizeable amount of labeled protein, with the signal appearing to stabilize between 3 and 6 h. These data suggest that many Anl-



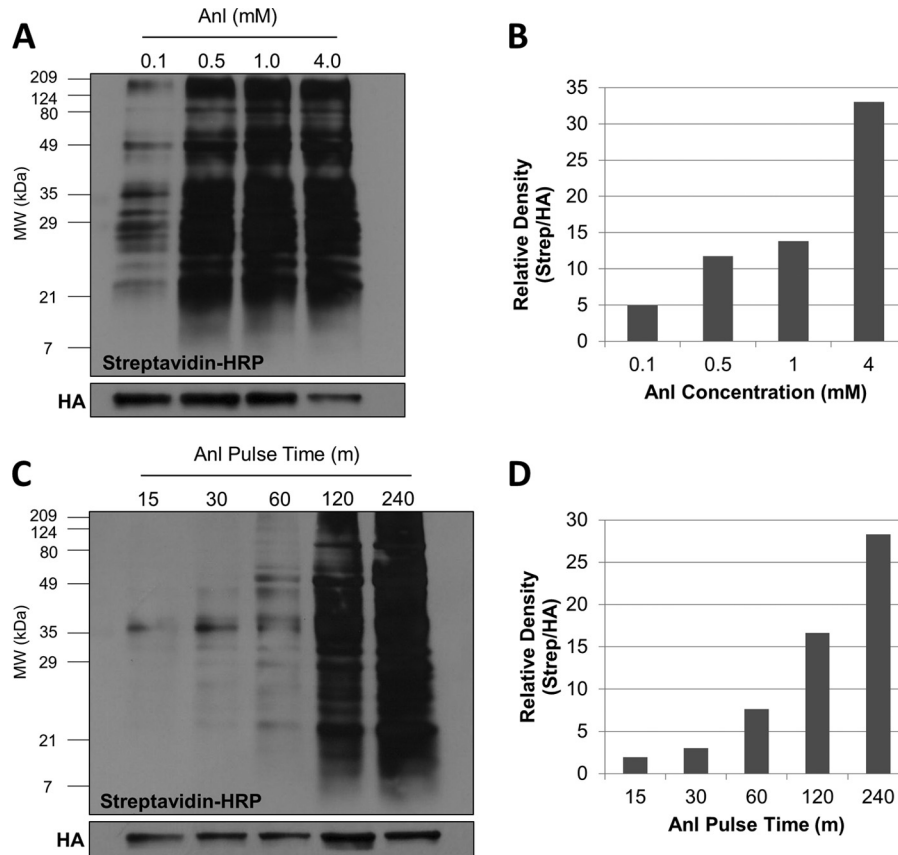
**FIG 3** Anl-labeled proteins are still present 6 h after being pulsed with Anl. (A) HFFs were infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> strain at a multiplicity of infection (MOI) of 3 and grown for 48 h before being pulsed with 1 mM Anl for 2 h. Following the 2-h pulse, Anl 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 visible only in the MetRS<sup>NLL</sup>-expressing strain. The signal intensity is strongest at the 0-h time point, dropping steadily over a period of 6 h. (B) Coomassie-stained SDS-PAGE gel identical to the one used to prepare the Western blot in panel A, confirming equal protein loading; (C) quantification of the data from panel B. The experiment was conducted at least 2 times with similar results.

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 [<sup>35</sup>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 min, consisting mainly of proteins that do not adopt their native fold due to translational errors (28). 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.

**Optimal Anl concentration for efficient incorporation into nascent proteins.** To optimize the MetRS<sup>NLL</sup>-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. 4A and 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 than with 0.5 mM and 1.0 mM (Fig. 4A and B). Based on visual inspection, parasite growth was similar in 0.1 mM, 0.5 mM, and 1.0 mM Anl. However, it was apparent that replication was reduced for parasites growing in 4.0 mM Anl. The reduction in parasite number was confirmed by the observed reduction in HA-tagged MetRS<sup>NLL</sup> on the Western blot (identical “cell equivalents” were loaded for each sample; Fig. 4A). While overall Anl incorpo-

ration is higher in 4.0 mM Anl than in the other tested Anl concentrations (Fig. 4B), 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 keeping the negative effects on parasite growth to a minimum.

**Anl is incorporated into a substantial number of proteins 1 h after being added to growth medium.** Knowing that prolonged growth in Anl slows parasite growth, 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<sup>NLL</sup> strain and allowed to grow for 48 h (such that the HFFs were well infected and would lyse the monolayer within 12 h). After 48 h of growth, the growth medium was supplemented with 1 mM Anl for various amounts of time (15, 30, 60, 120, and 240 min). 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. 4C and D). Anl incorporation was detected with just a 30-min Anl pulse, and after an hour of Anl exposure, a substantial number of proteins incorporated Anl, based on the extensive biotinylation banding pattern that was observed. As expected, the level of Anl incorporation

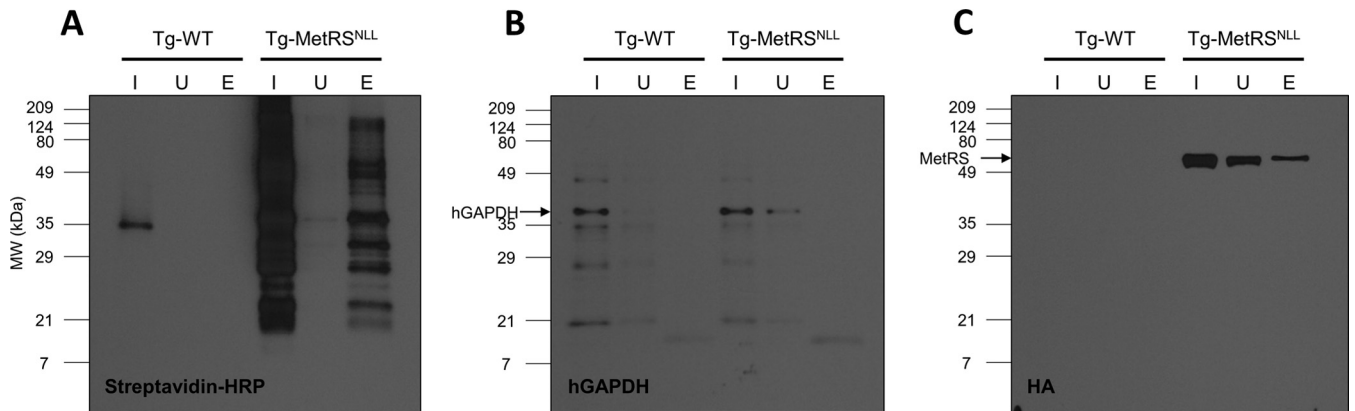


**FIG 4** (A) Growing parasites in 1 mM Anl allows high levels of incorporation while limiting negative effects on parasite growth. HFFs were infected with the Tg-MetRS<sup>NLL</sup> strain at an MOI of 3, and either 0.1, 0.5, 1, or 4 mM Anl was added to the growth medium at the time of infection. Cells 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<sup>NLL</sup> served as a loading control. Both 0.5 mM and 1.0 mM Anl result in similar levels of labeled protein, whereas 0.1 mM Anl yields considerably less labeling. Tg-MetRS<sup>NLL</sup> strain growth is greatly reduced in 4 mM Anl, as indicated by the less-intense HA-tagged MetRS<sup>NLL</sup> band. (B) Quantification of the data from panel 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 the growth medium. HFFs were infected with the Tg-MetRS<sup>NLL</sup> 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, 30, 60, 120, and 240 min. The samples were biotinylated with click chemistry using biotin-alkyne. Western blotting with streptavidin-HRP was used to confirm successful labeling. Anl is incorporated into proteins just 30 min after addition to the growth medium, and after 1 h of growth, a prevalent banding pattern is present, implying incorporation into a wide range of proteins. (D) Quantification of the data from panel C. The experiment was conducted at least 2 times with similar results.

increased with longer Anl pulse times, with the 4-h pulse time exhibiting the strongest signal (Fig. 4C and D).

**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<sup>NLL</sup> 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. 5A) but only from the HFFs infected with the Tg-MetRS<sup>NLL</sup> strain. To assess whether host cell proteins were labeled and purified, a blot identical to the one in Fig. 5A was probed with an antibody against human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) (Fig. 5B). A distinct signal at the expected 37-kDa size is present in the input and unbound lanes of both the WT *T. gondii* and Tg-MetRS<sup>NLL</sup> strain samples, though it is absent

from the elutions. The absence of a signal from the Tg-MetRS<sup>NLL</sup> strain elution 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<sup>NLL</sup> strain elution, a blot identical to the hGAPDH blot was probed with an anti-HA antibody to detect the parasites' HA-tagged MetRS<sup>NLL</sup> (Fig. 5C). A distinct signal is seen for the HA-tagged MetRS<sup>NLL</sup> in both the input, unbound, and eluted samples, but only in the Tg-MetRS<sup>NLL</sup> strain samples (at the expected 64-kDa size). The signal intensity in the elution is approximately 30 to 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<sup>NLL</sup> 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<sup>NLL</sup>. These data also suggest that the majority, if not all, of the biotinylated



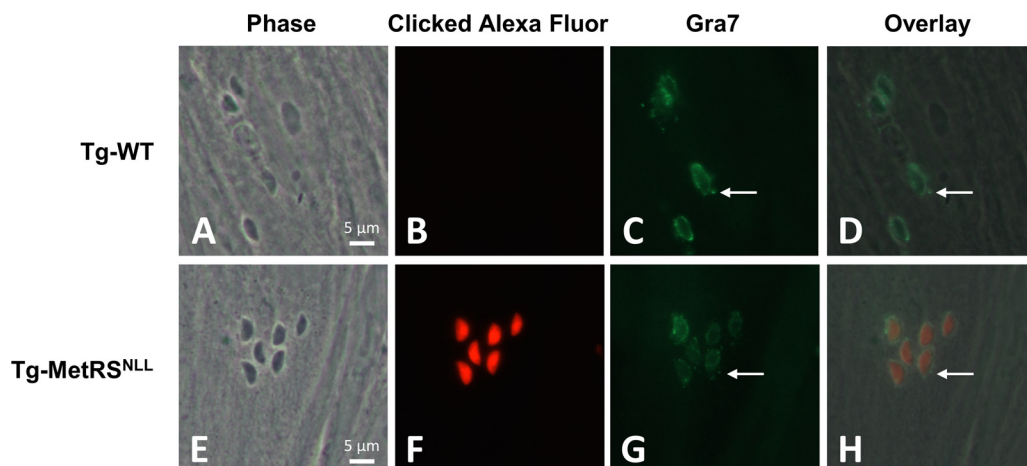
**FIG 5** Anl incorporation is limited to parasite proteins. (A) NeutrAvidin purification of lysates of HFFs that had been infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> strain and grown in the presence of 1 mM Anl for 2 days. The samples were biotinylated with click chemistry using biotin-alkyne and incubated with NeutrAvidin resin for 1 h. Biotinylated samples were eluted by boiling in SDS sample buffer. Labeling is present only in the input (I) and elution (E), and is largely absent from the unbound (U) fraction. (B) An identical blot to the one in panel 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 panel A was probed with an anti-HA antibody to identify the HA-tagged MetRS<sup>NLL</sup>. A prominent signal is seen in both the input and elution lanes of the Tg-MetRS<sup>NLL</sup> strain samples only. This suggests that the biotinylated proteins from panel A are *T. gondii* proteins.

proteins seen in the blot from Fig. 5A correspond to Anl-labeled parasite proteins.

**Parasites can be labeled *in situ* using click chemistry.** The MetRS<sup>NLL</sup>-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 (6, 27, 29). To test this in *T. gondii*, we infected HFFs with WT *T. gondii* or Tg-MetRS<sup>NLL</sup> parasites that had been pretreated with 1 mM Anl for 48 h. Six hours postinfection, parasites were fixed and permeabilized. Infected HFFs were then subjected to click chemistry using an Alexa Fluor 594-conjugated alkyne. Tg-MetRS<sup>NLL</sup> 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. 6B and F). The infected HFFs were costained for the secreted parasite Dense granule

protein 7 (Gra7) (Fig. 6C and G), which exhibited intense parasite staining as well as host cell cytosolic punctate staining, which is characteristic of this *T. gondii* protein (30). Importantly, we did not observe any such punctate staining in the Alexa Fluor 594 channel (Fig. 6F and H), suggesting that secreted proteins like Gra7 (i) are not tagged with Anl, (ii) incorporate Anl but are highly unstable and therefore undetectable, (iii) are not properly secreted from the parasite when they have incorporated Anl, or (iv) are processed in a manner that removes all Anl-containing residues. We addressed this further in the experiments described immediately below.

**Anl is only minimally incorporated into secreted parasite proteins.** A recently published study showed that mammalian cells expressing the bacterial MetRS<sup>NLL</sup> incorporated Anl only at the N-terminal Met position in nascent proteins and not internal



**FIG 6** Anl-treated parasites can be labeled *in situ* by using click chemistry. WT *T. gondii* and the Tg-MetRS<sup>NLL</sup> strain were grown in the presence of Anl for 48 h, before being used to infect HFFs for 6 h. (B and F) After fixation and permeabilization, a click reaction was performed on the samples using Alexa Fluor 594-alkyne. (C and G) Additionally, the parasites were stained for the secreted protein Gra7 using a mouse Ab followed by Alexa Fluor 488 goat anti-mouse IgG. The punctate Gra7 staining emanating from around the parasites (C and G, white arrows) is not stained with the clicked Alexa fluorophore (F), suggesting secreted proteins are not labeled. The channels have been overlaid in panels D and H. Magnification at  $\times 100$  was used for all images.

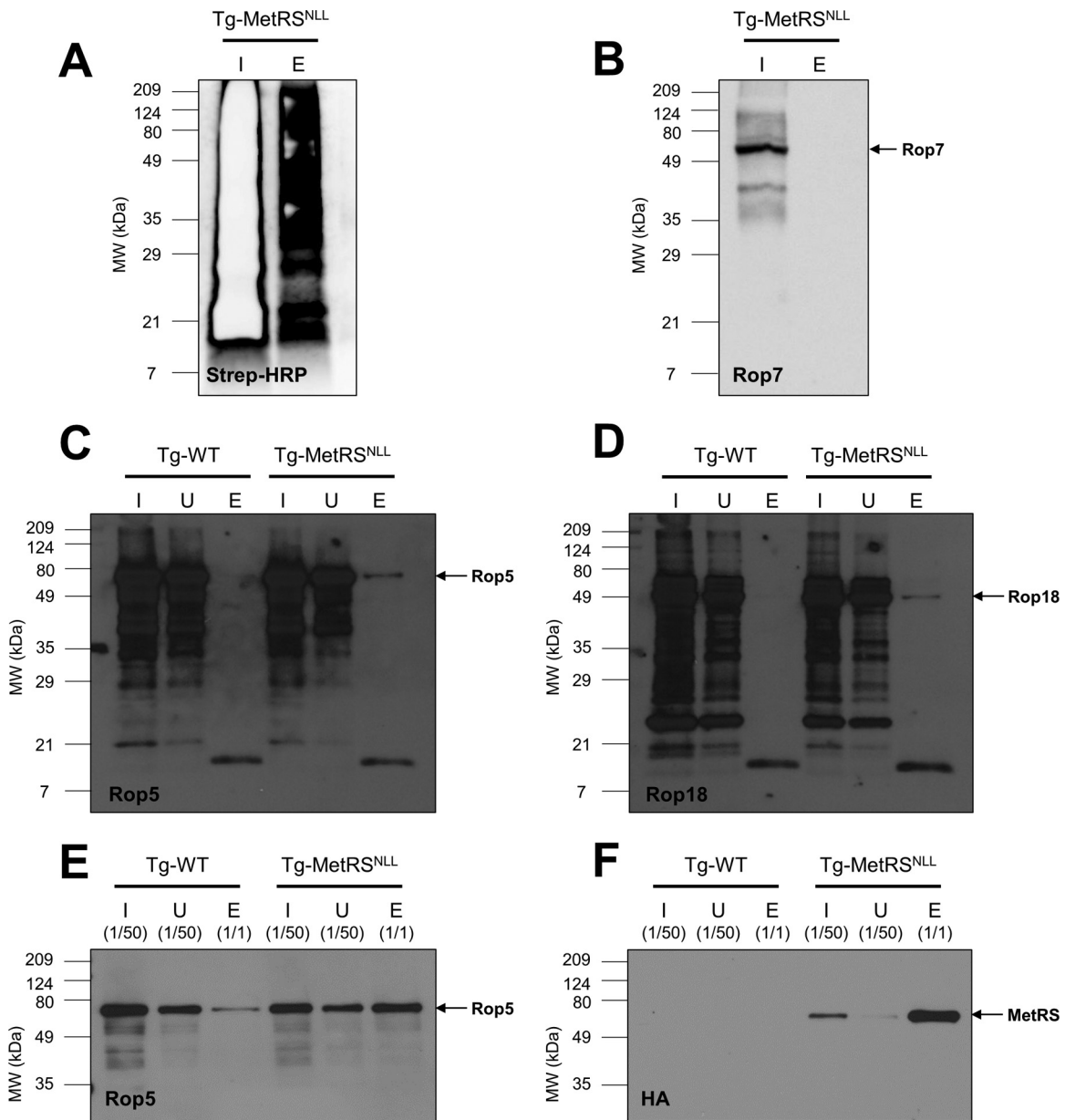
positions (27). This was attributed to an incompatibility between the bacterial MetRS<sup>NLL</sup> and one of the mammalian Met tRNAs, the elongator tRNA (described further below). If this were also the case in *T. gondii*, secretory proteins would not be labeled, since the signal peptide of secreted 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 the mitochondria (31). Furthermore, most apicomplast 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 apicomplast (32, 33). To determine if this was a similar limitation in the *T. gondii*-MetRS<sup>NLL</sup>-Anl system, parasites were grown in the presence of 1 mM Anl for 2 days, solubilized, and 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* rhostry protein 7 (Rop7) (Fig. 7B) and biotinylated proteins with streptavidin-HRP (Fig. 7A). The prevalent signal in both the input and elution samples on the streptavidin-HRP blot (Fig. 7A) 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. 7B) implies that Rop7 cannot be purified, potentially because (i) it does not incorporate Anl, (ii) any incorporated Anl gets removed via processing, or (iii) incorporation destabilizes the protein, causing it to be degraded.

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<sup>NLL</sup> strain and probed for the secreted parasite proteins Rop5 (Fig. 7C) and Rop18 (Fig. 7D). Interestingly, a signal is visible in the Tg-MetRS<sup>NLL</sup> strain elution only when the blots are overexposed (Fig. 7C and D). In both cases, the clear disparity between signal intensities in the input/unbound samples and the elutions implies that both Rop5 and Rop18 are very minimally labeled. This is at odds with the purified HA-tagged MetRS<sup>NLL</sup> (Fig. 5C), which suggests around 30 to 40% of the HA-tagged MetRS<sup>NLL</sup> population is labeled and purified. If both the input and unbound samples are diluted by a factor of 50 and the elution samples remain undiluted, a Rop5 Western blot reveals that there is purified Rop5 in the elutions from both the WT *T. gondii* and Tg-MetRS<sup>NLL</sup> strain samples (Fig. 7E). The Rop5 signal in the WT *T. gondii* elution is perplexing, though it may correspond to Rop5 protein that non-specifically adheres to the resin used in the purification. Importantly, the Rop5 signal seen in the Tg-MetRS<sup>NLL</sup> strain elution is approximately 2 times 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<sup>NLL</sup> only in the Tg-MetRS<sup>NLL</sup> strain elution sample (Fig. 7F), 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 Fig. 6. 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 (primar-

ily the host cell nucleus, as well as in cytoplasmic puncta). It has been well established that multiple rhostry and dense granule proteins can be found in these host cell locations.

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 Met tRNAs in prokaryotes and eukaryotes, the initiator tRNA, which carries the first Met, and the elongator tRNA, which carries all of the internal Met residues (34). Normally, MetRS is responsible for attaching Met to both the initiator and elongator tRNAs. However, a bacterial MetRS cannot attach Met (or Anl) to a eukaryotic elongator Met tRNA, only to an initiator tRNA (Fig. 8A) (34–37). The reason for this inability was suggested to be due to a difference in the size of the anticodon loop of the eukaryotic elongator Met tRNA (38), which is one of the key determinants for Met tRNA binding to the MetRS (39). The initiator and elongator Met tRNAs 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 Met tRNA (Fig. 8C) but is predicted to be 9 bases in the elongator Met tRNA, due to a lack of base pairing between residues 31 and 39 (Fig. 8D). Making a single nucleotide change to introduce a Watson-Crick base pair between residues 31 and 39 in rabbit elongator Met tRNA was enough for the tRNA to be aminoacylated by a bacterial MetRS (38). Taken together, these data suggest that the *E. coli* MetRS<sup>NLL</sup> system is similarly incapable of replacing internal Met residues with Anl in *T. gondii* and therefore proteins processed via the secretory pathway would be undetectable in the Tg-MetRS<sup>NLL</sup> strain. The inability to identify Rop7 in pulldowns and the *in situ* data shown in Fig. 6 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 rhotries. 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. We will address this further in future studies using mass spectrometry to identify Anl-containing proteins and using genetics to mutate the endogenous *T. gondii* MetRS to incorporate Anl.

**Overall summary.** Combining click chemistry and the incorporation of azide/alkyne-containing NCAs into nascent proteins is finding increasing use in the study of protein synthesis and degradation. A combination of Aha incorporation/click chemistry and rRNA-targeted fluorescence *in situ* hybridization (FISH) has been used to monitor protein synthesis in diverse microbial populations (29). Zhang and colleagues used Aha incorporation to monitor the degradation of long-standing proteins via autophagy (40). These examples are all using an NCA which can be incorporated into virtually any protein, as the NCAs 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 NCA. To surmount this problem, we have employed a system that was developed by Ngo et al. (12), using a mutant form of methionyl-tRNA synthetase in *T. gondii*, allowing the azide-containing Met analog, azidonorleucine, to be incorporated in nascent parasite proteins (13). This system is highly effective at labeling nascent proteins in *T. gondii* and is orthogonal, labeling proteins only in MetRS<sup>NLL</sup>-expressing parasites. The Tg-

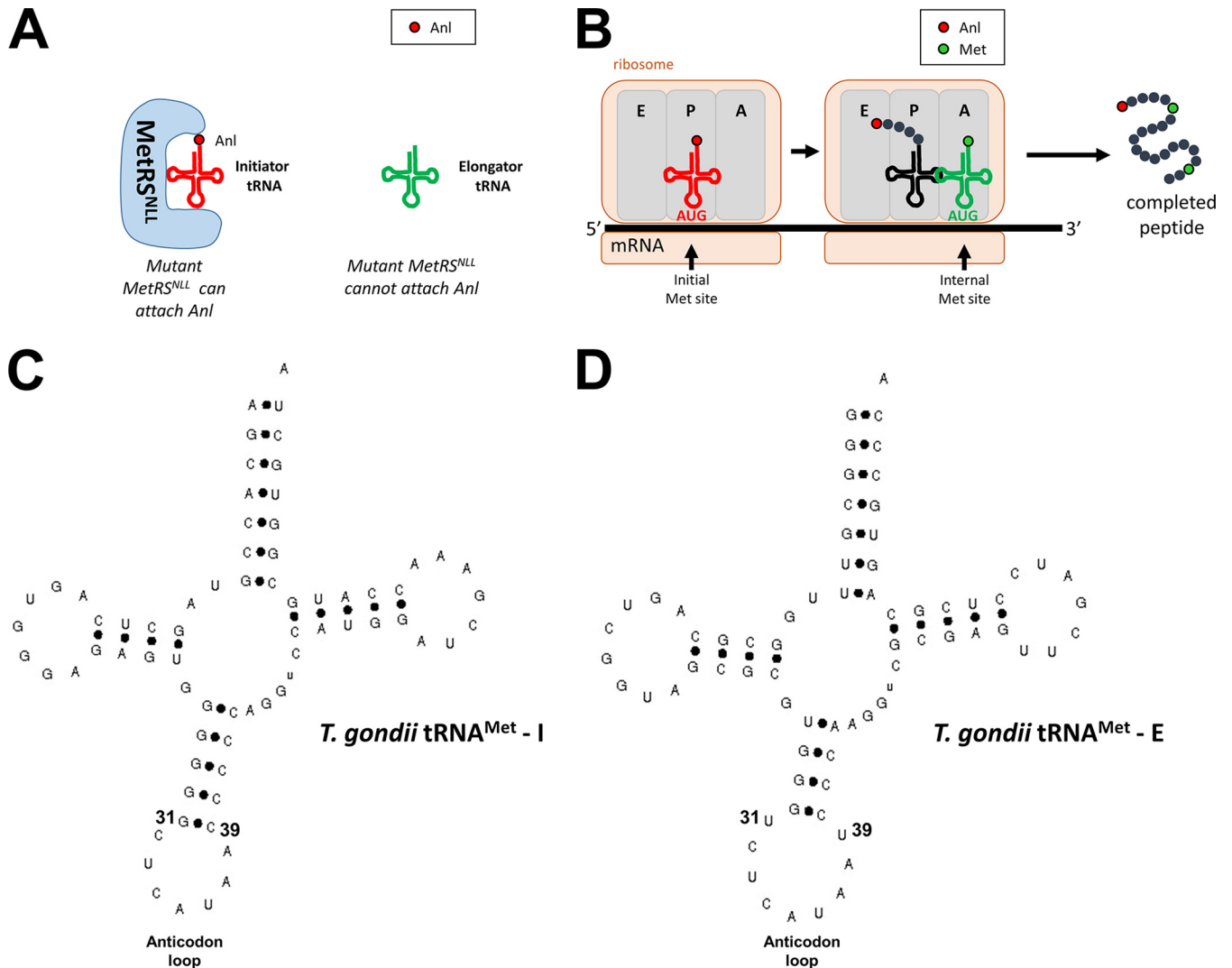


**FIG 7** Proteins from *T. gondii* parasites grown in the presence of Anl can be biotinylated with click chemistry, though secreted rophtry proteins are minimally labeled. (A) Western blot of a NeutrAvidin purification of biotinylated parasite proteins, probed with streptavidin-HRP. HFFs were infected with the Tg-MetRS<sup>NLL</sup> 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 that 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) Blot identical to the one in panel A, probed for the secreted parasite rophtry 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) Western blot of a NeutrAvidin purification of biotinylated proteins from HFFs infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> strain and probed with an anti-rophtry protein 5 (Rop5) antibody. The blot was overexposed, revealing a signal for Rop5 in the elution of the Tg-MetRS<sup>NLL</sup> strain sample. (D) The blot from panel C was stripped and reprobed with anti-Rop18 antibody. As in panel C, the blot was overexposed, revealing a signal for Rop18 in the elution of the Tg-MetRS<sup>NLL</sup> strain. (E) The samples used to prepare panels 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 Tg-MetRS<sup>NLL</sup> strain elutions. (F) The blot from panel E was stripped and reprobed with an anti-HA antibody to identify the HA-tagged MetRS<sup>NLL</sup>.

MetRS<sup>NLL</sup> strain is a new tool to study novel aspects of parasite protein expression while parasites are growing inside 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 organ-

elles like the dense granules and the rophtries, are only very minimally labeled. This method could potentially be used to determine which secreted proteins, and apicoplast/mitochondrion-located proteins, are not N-terminally processed. We will address the mechanism for this labeling specificity, as well as how to label





**FIG 8** (A) Bacterial MetRS (wild type or the NLL mutant) cannot charge eukaryotic elongator Met tRNAs. (B) A diagram of the ribosome reading mRNA to synthesize a protein. Since the initiator Met tRNA can be charged with Anl, it gets incorporated into the protein. The elongator Met tRNA cannot be charged with Anl, so all of the internal Met sites stay as Met. (C) Secondary structure prediction of *T. gondii* initiator Met tRNA (gene models TGME49\_269820, TGME49\_248915, TGME49\_248925). Bases 31 and 39 form a Watson-Crick base pair in the anticodon loop. (D) Secondary structure prediction of *T. gondii* elongator Met tRNA (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 panels C and D were generated with tRNAscan-SE 1.21 using sequences from the *T. gondii* Me49 strain from ToxoDB.org.

N-terminally processed parasite proteins with Anl, in future studies. A recent study in the pathogenic bacterium *Yersinia enterocolitica* used the MetRS<sup>NLL</sup>-Anl system to identify proteins that it secreted in HeLa cells (41), providing evidence that the system should be applicable to studying/identifying secreted proteins in apicomplexan parasites, like *T. gondii*, if the system can be modified to label N-terminally processed proteins.

## MATERIALS AND METHODS

**Host cell culture.** All assays were performed in human foreskin fibroblasts (HFFs), which were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine (Life Technologies, Rockville, MD), and 50  $\mu$ g/ml each of penicillin (Life Technologies, Rockville, MD) and streptomycin (Life Technologies, Rockville, MD). The cells were maintained at 37°C in 5% CO<sub>2</sub>.

**Expression of *E. coli* MetRS<sup>NLL</sup> in *T. gondii*.** The *E. coli* MetRS<sup>NLL</sup> gene used for creating the MetRS<sup>NLL</sup>-expressing *T. gondii* strain (Tg-MetRS<sup>NLL</sup> 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 (42). The MetRS gene was PCR amplified from the vector using primers with unique restriction sites (forward primer, NsiI site underlined, GTTAATGCATCCTACTATGACTC AAGTCGCGA; reverse primer, NcoI site underlined, GCTACCATGGTT TAGAGGCTTCCAGTGCTTCAACC). Using the added restriction sites, the PCR product was cloned into the pGra-HA-HPT expression plasmid (43), in frame with a C-terminal HA tag and under the control of a highly active dense granule protein promoter (GRA1) (44). 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  $\Delta$ HPT mutant *T. gondii* strain when grown in the presence of mycophenolic acid (MPA) and xanthine (45, 46). The pGra-HA-HPT-MetRS<sup>NLL</sup> vector was transfected into the type II parasite strain Me49 $\Delta$ HPT:Luc, and successful transformants were selected for using MPA (25  $\mu$ g/ml; Sigma-Aldrich, St. Louis, MO) and xanthine (50  $\mu$ g/ml; Sigma-Aldrich, St. Louis, MO) (45, 46). Drug-resistant clones were obtained by limiting dilution in 96-well plates. A MetRS<sup>NLL</sup>-expressing strain (the Tg-MetRS<sup>NLL</sup> strain) was generated with this process, along with an MPA-resistant strain which lacked the MetRS<sup>NLL</sup> gene. This strain (WT *T. gondii*) served as a wild-type control for the experiments, as it was prepared under the same conditions but does not express MetRS<sup>NLL</sup>.

**Western blot analysis.** To probe for expression of the HA-tagged MetRS<sup>NLL</sup>, HFFs infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> strain were lysed in 1 $\times$  sodium dodecyl sulfate (SDS) sample buffer and resolved on 8% SDS-polyacrylamide gels. The samples were transferred to a 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% nonfat dry serum for 1 h. After being blocked, the membrane was incubated in anti-HA-peroxidase (Roche, Mannheim, Germany) antibody at a 1:4,000 dilution for 30 min. 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-Tg-SAG1 (GenWay) at a 1:4,000 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 1:4,000 dilution for 1 h. After the blot was washed 3 times with TBST, it was developed by using the SuperSignal West Pico chemiluminescent substrate.

**Incorporation of Anl into parasites.** A confluent monolayer of HFFs in a T25 culture flask was infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> strain at a multiplicity of infection (MOI) of 3 and supplemented with 1 mM Anl (from a 100 mM stock in H<sub>2</sub>O). 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; Thermo Fisher Scientific, Pittsburgh, PA) and scraped from the T25 culture flask in 3 ml of PBS. The infected HFFs were centrifuged at 800  $\times$  g for 10 min and solubilized in 100  $\mu$ l of 2% SDS in PBS, followed by boiling for 5 min. The samples were diluted to 0.5% SDS with PBS, including cComplete EDTA-free protease inhibitors (Roche Diagnostics, Indianapolis, IN).

**Click cycloaddition reaction.** Cycloaddition reactions were performed on the cell lysates in a final volume of 50  $\mu$ l of 50  $\mu$ M biotin-alkyne (Invitrogen Molecular Probes, Eugene, OR) or 10  $\mu$ M 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<sub>4</sub>; Sigma-Aldrich, St. Louis, MO). This mixture was incubated at room temperature for 2 h with rotation (18, 19). 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.

**Determining the optimal Anl concentration for efficient incorporation into nascent proteins.** HFFs were infected with the Tg-MetRS<sup>NLL</sup> strain, and the growth medium was supplemented with various 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 blotting, probing with streptavidin-HRP.

**Luciferase growth assays.** WT *T. gondii* and the Tg-MetRS<sup>NLL</sup> 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, 10,000 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 postinfection and processed for bioluminescence at 24, 48, and 72 h after Anl exposure. Cells were lysed using 1 $\times$  luciferase lysis buffer (Promega, Madison, WI) and frozen at  $-80^{\circ}$ C until analysis. A total of 20  $\mu$ l of each sample was added to a Greiner Cellstar 96-well plate with an opaque bottom, and 50  $\mu$ l of luciferase assay reagent (Promega, Madison, WI) was added to each well immediately prior to analysis on a Centro XS<sup>3</sup> LB 960 microplate luminometer. Luminescence was measured for 10 s in each well, with no delay between each well. For each strain (the WT *T. gondii* and Tg-MetRS<sup>NLL</sup> strains), data were converted to the percentage of the control by dividing the signal in the presence of Anl by the signal in its absence.

**NeutrAvidin purification.** A confluent monolayer of HFFs in a T25 culture flask was infected with either WT *T. gondii* or the Tg-MetRS<sup>NLL</sup> strain at an MOI of 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 and 27-gauge needles in 3 ml of PBS, including cComplete EDTA-free protease inhibitors (Roche Diagnostics, Indianapolis, IN). The samples were centrifuged at 800  $\times$  g for 10 min and solubilized in 100  $\mu$ l of 2% SDS in PBS, followed by boiling for 5 min. The samples were diluted to 0.5% SDS with PBS, including cComplete 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. Two hundred microliters of well-mixed NeutrAvidin agarose resin (Thermo Scientific, Rockford, IL) was used for each purification and was equilibrated according to the 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 2,500  $\times$  g for 1 min, 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 min in 200  $\mu$ l of 1 $\times$  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) (47) and used at a 1:1,000 dilution. The rabbit anti-Rop5 antibody (48) and rabbit anti-Rop18 antibody (49) were generously provided by David Sibley (Washington University School of Medicine, St. Louis, MO).

**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<sup>NLL</sup> strain at an MOI of 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 25-gauge and 27-gauge needles. The Anl-treated parasites were then used to infect HFF-seeded coverslips at an MOI of 5 for 6 h. The cells were washed once with PBS and then were fixed for 20 min 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 with 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-Gr7 monoclonal mouse antibody at a dilution of 1:2,000 for 1 h, washed 3 times with PBS, and incubated with Alexa Fluor 488 goat anti-mouse IgG at a dilution of 1:2,000 for 1 h. After 3 PBS washes, the coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). The monoclonal an-

tibody to Gra7 was generously provided by Peter Bradley (University of California, Los Angeles) (47).

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