Human African trypanosomiasis (HAT), also known as African sleeping sickness, is caused by *Trypanosoma brucei*, a protozoan parasite transmitted by the tsetse flies of genus *Glossina* (1). HAT is endemic in over 36 countries and threatens over 60 million people within sub-Saharan Africa. Few drugs are available to treat HAT, and their use is complicated by limited efficacy that depends on both the subspecies and the development stage of the parasite (2, 3). Treatment of late-stage disease is particularly problematic, and while the recent introduction of nifurtimox-eflornithine combination therapy (NECT) has improved treatment outcomes (4), more-effective drugs that combat all forms of the disease are still badly needed.

Alliances among academic and industry partners have emerged with the goal of exploiting the molecular-target approach to antiparasitic drug discovery (5, 6). An open-access resource (tdtrargets.org) was developed to improve prioritization of potential drug targets for major pathogens (7, 8). The premise of this undertaking is the idea that gene products that have been exploited for the treatment of human disease are more likely to represent druggable targets than those for which no information is available (9). Druggable targets were further defined as those that bind small drug-like molecules with high potency, resulting in disease-modifying outcomes. These reported computational approaches utilized the availability of inhibitors for homologs of a gene and the drug-like properties of these inhibitors to assign targets a druggability score.

The translation apparatus, including ribosomes, specific elongation and initiation factors, and aminoacyl-tRNA synthetases (aaRSs), represents one of the major pathways targeted by commercial antibiotics (10–14). The aaRSs are essential participants in the protein translation mechanism, catalyzing the esterification of specific amino acids and their corresponding tRNAs (15–18) (Fig. 1). Two classes of aaRSs have been described, each of which is in turn divided into three additional subclasses. These classes are distinguished by different structural folds and by the site of esterification (class I enzymes esterify the 2'-hydroxyl of the adenine ribose whereas class II enzymes esterify the 3'-hydroxyl of the ribose). Inhibitors of aaRSs with both antibacterial and antifungal activity have been reported, and while mupirocin (used as a topical antibiotic) is currently the only aaRS inhibitor in clinical use, many others are in various stages of discovery and development (11–13) (see Table 1). Recent examples of the identification of aaRS inhibitors targeting parasitic protozoa have also been described, including the discovery that cladosporin targets *Plasmodium falciparum* LysRS, providing a potential lead for malaria drug discovery (19). The targeted aaRSs are diverse, spanning both different amino acid specificities and different enzyme classes. Thus, the aaRSs appear to be broadly appealing targets for the development of new antimicrobial agents.

*Trypanosomatids* encode a full complement of both class 1 and class 2 aaRSs; bioinformatics analysis of *Leishmania major* (20) and *T. brucei* (21) identified 25 and 24 genes encoding canonical aaRS homologs, respectively, covering all key amino acids. Additionally, they encode three multiple-aaRS (MARS) complex-associated proteins (MCPs). The MCPs share sequence identity with aaRS noncatalytic domains or with aaRS editing domains but do

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**Genetic Validation of Aminoacyl-tRNA Synthetases as Drug Targets in *Trypanosoma brucei***

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not have aminoclaylation activity (21). Both cytosolic translation and mitochondrial translation require aaRSs. Trypanosomatids, unlike other eukaryotes, do not code for tRNA molecules in their mitochondrial genome; instead, mitochondria import their tRNAs and aaRSs from the cytosol (13, 22, 23). Almost all aaRSs in the T. brucei genome are single copy and thus must serve dual roles in the cytosol and mitochondria; TrpRS, LysRS, and AspRS are three notable exceptions where separate cytosolic and mitochondrial genes have been identified (24–26). Several T. brucei aaRS genes have been reported to be essential in parasites in either the insect stage (procyclic) or the mammalian stage (21, 25–30). Several groups have also reported the identification of MetRS, IleRS, and LeuRS inhibitors with antitrypanosomal activity (30–35). Crystal structures have been solved for many of the aaRSs across several species (15), including the structures of HisRS (36) and TrpRS (37) from T. brucei, which will aid in understanding how to target these enzymes for HAT drug discovery. Finally, we recently showed that a number of aaRSs from T. brucei associate into a MARS complex (21). However, despite these extensive efforts, a number of T. brucei key aaRSs remain uncharacterized.

Since aaRSs have been successful drug targets in other species, we undertook a comprehensive study to identify essential aaRSs in T. brucei with the goal of demonstrating their potential utility as a new class of targets in antitrypanosomal drug discovery. The present study focused on the genetic validation of eight uncharacterized T. brucei aaRSs by RNA interference (RNAi) gene knockdown in the infectious mammalian bloodstream-form (BSF) parasites. Genes were selected both for high druggability scores and to encompass examples from each of the major aaRS classes. We show here that all eight newly characterized genes are essential for mammalian BSF parasites. There was a partial correlation between the extent of RNAi knockdown and the effects on cell growth, morphology, cell cycle, and cellular DNA. A heterologous expression system and an enzyme assay were developed for threonyl-tRNA synthetase (ThrRS) that ready the target for future work in a drug discovery program.

**MATERIALS AND METHODS**

TriTrypDB accession numbers for genes analyzed in this study. Putative aaRS genes were selected based on their annotation in TriTrypDB, and the following were studied: T. brucei (22.5.1090) (Tb927.5.1090), arginyl-tRNA synthetase (ArgRS) (Tb927.11.1990), asparaginyl-tRNA synthetase (AsnRS) (Tb927.4.2310), phenylalanyl-tRNA synthetase alpha (PheRSα) subunit (Tb927.11.14120), PheRS subunit (Tb927.11.2360), tryptophanyl-tRNA synthetase (TrpRS-1) (Tb927.3.5580), valyl-tRNA synthetase (ValRS) (Tb927.6.4480), and glutamyl-tRNA synthetase (GluRS) (Tb927.6.4590).

**Growth of T. brucei BSF in vitro cultures.** BSF 427 single-marker (SM) cells were cultured in HMI-9 media supplemented with 10% fetal bovine serum as described previously (38–40). Cells were maintained at exponential growth (between 10^4 and 10^7 cells/ml) and RNAi was induced at a cell density of 2 × 10^6/ml by addition of tetracycline (Tet) (1 μg/ml) to the media. Cell numbers were monitored by hemocytometer, with the exception that the data collected for glutamyl-tRNA synthetase (GluRS) were obtained by particle counting, which also detects cell debris. All growth data were collected in biological triplicate, with the exception of the PheRSα and GluRS data, which were collected in duplicate. To determine the 50% effective concentration (EC_{50}) for borrelidin, the dose-response data were fitted to the log(inhibitor) versus response—variable slope (4-parameter) equation using GraphPad Prism.

**Generation of cell lines containing inducible RNAi constructs.** RNA interference (RNAi) cell lines were generated using gateway vector (TryptRNAiGate) for the following genes: AsnRS (Tb927.4.2310), PheRSα subunit (Tb927.11.14120), PheRSβ subunit (Tb927.11.2360), and ValRS (Tb927.6.4480). A 400- to 500-bp region of each gene was selected using RNAi software (41). PCR products were generated using High Fidelity Platinum Taq DNA polymerase (Invitrogen) and the following forward and reverse primer pairs: for AsnRS, 5’-(GGGGCTACATTTGCTGTTGCT)-3’ and 5’-(CAATGTTCCACCCGCGCTAC)-3’; for PheRS subunit, 5’-(TTTATTGCTCAGGTGACCAG)-3’ and 5’-(GGGGCCACGCTTACACTCA)-3’; for PheRS β subunit, 5’-(AATGGTGCACGGATGTCAG)-3’ and 5’-(TGATTGCAAGTGGTGGCAAG)-3’; for and ValRS 5’-(GGGTACACAGGAAAGTGTTAG)-3’ and primer 5’-(AATGTGCACGGGCTACTGC)-3’. Gel-purified PCR fragments were then incubated with pCR8/GW/TOPO vector (supplied ready to use) for 5 min at room temperature, transformed into Top10 E. coli, and plated on spectinomycin (100 μg/ml)-containing LB plates as described in the Gateway PENTR vectors instruction manual (Invitrogen Life Technologies). Sequence-verified plasmids were then used in the LR recombination reaction with pTryptRNAiGate destination vector to generate stem-loop constructs, and the parasite transfections were performed as previously described (38, 39). Inducible RNAi plasmids for ArgRS (Tb927.11.1990), ThrRS (Tb927.5.1090), TrpRS-1 (Tb927.3.5580), and GluRS (Tb927.6.4590) were generated using a pQuadra system (42). Briefly, an ~400-bp fragment of the gene was selected using RNAi software and amplified by PCR, using the following forward and reverse primer pairs, all containing added BstXI sites at the 5’ end: for ArgRS, 5’-(TACAAATGTTGAGGCCCTTGTTGACCTTTTCGCG)-3’ and 5’-(ATACATGAGTTGTGTTTCTGGCAGCTGAGA)-3’; for ThrRS, 5’-(ATAAAGTGTAGGATTTTAATGGATCTCGCGC)-3’ and 5’-(ATACATGATTGTGCCGCCTCTCGTTCGAGA)-3’; for TrpRS-1, 5’-(ATAAAGTGTAGGATTTTAATGGATCTCGCGC)-3’ and 5’-(ATACATGATTGTGCCGCCTCTCGTTCGAGA)-3’; for GluRS, 5’-(ATACATGATTGTGCCGCCTCTCGTTCGAGA)-3’ and 5’-(ATACATGATTGTGCCGCCTCTCGTTCGAGA)-3’. Ligation with BstXI-digested PCR fragments and pQuadra 3 plasmids generated the RNAi vectors, containing inverted repeats of the PCR product separated by a spacer region. Transfection of NotI-linearized constructs into the SM cell line (43) and selection of transgenic cell lines (44) were carried out as described previously.

**Quantitative real-time PCR analysis.** Quantitative real-time PCR was performed using SYBR green Mastermix (Bio-Rad). Total RNA was extracted from parasites using TRIzol reagent (Invitrogen) and an RNAse mimi kit (Qiagen), treated with DNeasy II (Worthington), and reverse transcribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems). An approximately 100-bp fragment of each gene was amplified using SYBR green Mastermix on a CFX96 real-time system (Bio-Rad). Reaction conditions maintained according to the manufacturer’s instructions were as follows: 95°C for 1 min and 40 cycles of 95°C for 3 s and 60°C for 30 s, followed by a continuous melting curve (55°C to 95°C in 0.5°C increments). Data were normalized to that of a constitutively expressed gene (TERT) with TERT as the internal standard (primers are previously described (38)). Ligation with BstXI-digested PCR fragments and pQuadra 3 plasmids generated the RNAi vectors, containing inverted repeats of the PCR product separated by a spacer region. Transfection of NotI-linearized constructs into the SM cell line (43) and selection of transgenic cell lines (44) were carried out as described previously.

**T. brucei Aminoacyl tRNA Synthetases**
### TABLE 1

| Product Gene ID | Gene names | Class | Localization | MARS complex-associated proteins (MCP) 1 1, 2, and 3 (conditional null) | No. of BSF RNA reads | Druggability | Chemical validation | Structure(s) available |
|-----------------|------------|-------|--------------|-------------------------------------------------|----------------------|---------------|---------------------|-----------------------|
|                |            |       |              | (conditional null)                                |                      |               |                     |                       |
| Tb927.6.700     | AlaRS      | IIc   | C/M          | Y                                                 | 589.1                | 0.2           | Severe              | (21)                  |
| Tb927.11.1990   | ArgRS      | Ic    | C/M          | Y (Homo sapiens)                                  | 302.2                | 0.6           | Severe              | (79)                  |
| Tb927.4.2310    | AsnRS      | IIb   | C/M          | Y                                                  | 566.3                | No data       | Severe              | (60)                  |
| Tb927.10.1260   | AspRS-2    | IIb   | M            |                                                   |                      |               | Microcin C (63)     |                       |
| Tb927.6.1880    | AspRS-1    | IIb   | C            |                                                   | 98.3                 | 0.2           | Microcin C (63)     | Y (Homo sapiens) (50) |
| Tb927.6.950     | CysRS      | Ia    | C/M          | Y                                                  | 517.0                | No data       | No                  |                       |
| Tb927.9.5210    | GlnRS      | Ic    | C/M          |                                                   | 345.0                | 0.8           | Glu-KPA (E. coli) (64) | Y (Saccharomyces cerevisiae) |
| Tb927.6.4590    | GluRS      | Ic    | C/M          | Y (Saccharomyces cerevisiae)                      | 465.1                | 0.3           | Moderate            | Yes                   |
| Tb927.11.9640   | GlyRS      | IIc   | C/M          | Y                                                  | 302.4                | No data       | Yes                 |                       |
| Tb927.6.2060    | HisRS      | IIa   | C(M?)        | Y (T. brucei)                                      | 100.0                | 0.6           | Severe (36)         | (58)                  |
| Tb927.10.9190   | IleRS      | Ia    | C/M          |                                                   | 1000.2               | 0.9           | Reveromicin (S. aureus) (65); muporicin (S. enterica) (66); NSC70422 (T. brucei) (21); PLD-118 (icofungipen) (C. albicans) (67); SB-203207 (Streptomyces) (68, 69) | Y (Homo sapiens) Severe (21) |
| Tb927.11.3730   | LeuRS      | Ia    | C/M          |                                                   | 320.1                | 0.7           | Granaticin (B. subtilis) (67); Moderate (21) | No |
| Tb927.8.1600    | LysRS-1    | IIb   | C/M          |                                                   | 400.2                | No data       | Cladosporin (Plasmodium falciparum) (19) | Y (Homo sapiens, Plasmodium falciparum) Severe (21) |
| Tb927.6.1510    | LysRS-2    | IIb   | M            |                                                   | 105.9                | No data       | No                  |                       |
| Tb927.11.14120  | PheRS      | IIc   | C/M          |                                                   | 283.1                | 0.6           | Ochratoxin A and B (hepatoma tissue culture) (71); ethanolamine-based inhibitors (Staphylococcus aureus) (72) | Moderate 57 No |
| Tb927.11.2360   | PheRS      | IIc   | C/M          |                                                   | 248.2                | No data       | Severe              | Yes                   |
| Tb927.10.12890  | ProRS      | IIa   | C/M          |                                                   | 859.4                | 0.8           | Halofuginone (Plasmodium) (73) | Y (Homo sapiens) Severe (21) |
| Tb927.11.7170   | SerRS      | IIa   | C/M          |                                                   | 308.5                | No data       | SB-217452 (rat, S. aureus) (74) | Y (Saccharomyces cerevisiae, Homo sapiens, Escherichia coli) Severe |
| Tb927.5.1090    | ThrRS      | IIa   | C/M          |                                                   | 372.0                | 0.6           | Borrelidin (fungi, bacteria) (53, 55, 56, 57); T. brucei (this work) | Y (Saccharomyces cerevisiae, Homo sapiens, Escherichia coli) Severe |
| Tb927.3.5580    | TrpRS-1    | Ib    | Y            |                                                   | 168.4                | 0.2           | Chuangxinmycin (bacteria) (73); indolmycin (Streptomyces) (73) | Y (T. brucei, Plasmodium falciparum) severe 59 No |
| Tb927.8.2240    | TrpRS-2    | Ib    | M            |                                                   | 117.4                | 0.2           | Y (Plasmodium falciparum) | Severe (conditional null) (27) No |
| Tb927.7.3620    | TyrRS      | Ib    | C/M          |                                                   | 200.0                | 0.6           | 3-Aryl-4-arylaminofuran-2(5H)-ones (bacteria) (75); SB-219383 (Micromonospora sp.) (76) | Y (Leishmania major) No |
| Tb927.6.4480    | ValRS      | Ia    | C/M          |                                                   | 402.0                | 0.9           | S-Adenosylhomocysteine (77) | Moderate 63 No |
| Tb927.7.2400    | MCP1       |        |              |                                                   | 221.2                | No data       | Unknown             |                       |
| Tb927.8.5330    | MCP2       | C     |              |                                                   | 80.6                 | Moderate      | (21)                |                       |
| Tb927.10.1250   | MCP3       |        |              |                                                   | 68.7                 | Unknown       |                       |                       |

**Notes:** Gene names and druggability scores (tdrtargets.org) were taken from the indicated web sites. Enzymes evaluated in this study are shown in bold type. Loss-of-fitness data from the RNAi screen performed by Alsford et al. (3) are summarized for comparison. BSF RNA reads are scaled read counts (median normalized; median is 100) taken from reference 78. MCP complex-associated proteins (MCP) 1 1, 2, and 3 are also shown, although they do not have aaRS activity. C, cytoplasm; M, mitochondrion; C/M, both cytoplasm and mitochondrion; Glu-KPA, glutamyl-beta-ketophosphonate-adenosine; Y, yes. Numbers in parentheses refer to publications cited.
AG)-3' and reverse primer 5'-(GCGGGTTTGGAAATGAAAGA)-3' for PheRSs subunit, forward primer 5'- (GAGGACCACATTGCGA GCA)-3' and reverse primer 5'-(AGAAGGCTCTCCCTCCTTG)-3' for PheRS subunit, forward primer 5'-(AGAAAGATCAAGGTAAGC)-3' and reverse primer 5'-(TACATCAACCAAGCAAGC)-3' for VaRS, forward primer 5'-(CGCTATGGTCTGTCAAGCT)-3' and reverse primer 5'-(TCAAATACCCA TATGCTAGGA)-3' for TrpRS-1.

Cell morphology analysis. Log-phase cells were collected by centrifuging at 3,500 rpm for 10 min in a 15-mL tube. Cells were resuspended in freshly made 4% paraformaldehyde (PFA), fixed at room temperature for 45 min, washed with phosphate-buffered saline (PBS; pH 7.4), and resuspended in 50 to 100 μL PBS. Cells (20 μL) were placed on a poly-L-lysine-coated slide, spread, and dried for 1 h. For nuclear (N) and kinetoplast (K) DNA, the cells were applied to coverslips with Vectashield mounting medium containing DAPI (4',6-diamidino-2-phenylindole; hard set) and analyzed by fluorescence confocal microscopy (Leica TCS SP5) at a magnification of ×63 and an excitation wavelength of 350 nm and by bright-field differential interference contrast (DIC). Both uninduced and Tet-induced cultures were examined at multiple time intervals. The percentage of morphological anomalies (number of nuclei, kinetoplasts, and phenotypic forms) was determined by counting 150 cells.

Flow cytometric analysis. For cell cycle analysis, BSF T. brucei was inoculated at 10⁵ parasites/ml and the cultures were harvested at 0, 2, and 3 days after plating. Cells were washed with PBS (pH 7.4) fixed in 70% ethanol and resuspended in PBS containing 10 μg/ml RNase A and 10 μg/ml propidium iodide (PI; Sigma). After incubation for 30 min at 37°C, PI-stained cells were analyzed by flow cytometry as described previously (47, 48). Histograms were analyzed for populations with 2C, 4C, and >4C as a measurement of relative DNA content.

Immunofluorescence localization assay. The gene encoding ThrRS (Tb927.5.1090) was amplified by PCR from genomic DNA of T. brucei strain Listser 427 using specific primers forward 5'- (CCCAAGCTTATGGCCACGGCTTGG-3') and reverse 5'- (CAAGATCTGAAAGGACCGA TCAATTATATC)-3' and cloned into HindIII and BamHI sites of pCMV-TK (Novagen). The construct was transformed into E. coli Rosetta (2DE3) strain pLYS S (Novagen), and protein expression was induced with 0.1 mM IPTG (iso-propyl-β-D-thiogalactopyranoside). E. coli was grown in LB media and harvested, and a lysate was prepared with Bugbuster reagent (Novagen). Proteins were purified using Ni²⁺ magnetic beads (Millipore), dialyzed against 50 mM Tris (pH 7.4)–140 mM NaCl, and kept at 4°C (with 0.05% sodium azide) until use.

Aminoacylation spectrophotometric assays. Amino acylation assays were performed as previously described (50). Briefly, T. brucei tRNAThr was synthesized by in vitro transcription from a PCR product template that produced a T7 promoter followed by the tRNAThr sequence (Tb927.8.2858) and the CCA sequence. The tRNA Thr sequence template was produced by PCR using primers that overlap the tRNAThr sequence: A forward 5'- (TAATACGACTCTATAGGGCGCGCTGAGCAAGC)-3', B reverse 5'- (TGGAGGCCGCGCCGAGAG)-3', C forward 5'- (GGCTCGTGA CAGACCTGACGCTGGTCGTTCTAAAC)-3', and D reverse 5'- (AGGGCCGCCGAGAATTGAACTCGGGACCGCTGTTTACACAG CAGTGCAC)-3'. The standard reaction ingredients, unless otherwise stated, were: aminoacyl buffer (30 mM HEPES buffer [pH 7.4], 140 mM NaCl, 30 mM KCl, 40 mM MgCl₂), 1 mM diithiothreitol (DTT), 200 μM ATP, 2 μM inorganic pyrophosphatase (Sigma-Aldrich), 10 mM L-Thr (Sigma-Aldrich), 8 μM T. brucei tRNA Thr, and 0.4 μM recombinant ThrRS. The aminoacylation reactions (35 μl total volume each) were performed in clear, flat-bottom, 96-well plates (Costar 96-well standard microplates), and the reaction mixtures were incubated for 30 min at 37°C. Liberated inorganic phosphate was detected by addition of 100 μl of malachite green (Echelon Biosciences) and incubated for 30 min at room temperature. Absorbance was measured at 620 nm using a SpectraMax M2 microplate reader (Molecular Devices). Reactions without enzyme or without L-Thr were performed as background controls, and data from reactions without L-Thr were subtracted from the measurements. For time course experiments, aliquots of 10 μl were withdrawn from an 80-μl reaction mixture at different time points (0, 2.5, 5, 10, 20, 30, and 60 min) and mixed with 10 mM EDTA on ice to stop the reaction. For ThrRS inhibition with borrelidin, a reaction mixture containing ThrRS (0.4 μM) was mixed with 5 μl of borrelidin at 10 to 10,000 nM in a 50-μl volume and incubated for 30 min at 37°C. Reactions were stopped as described above. All data are shown for three replicates as the means ± the standard errors. To determine the 50% inhibitory concentration (IC₅₀) for borrelidin, the dose-response curves were fitted to the log(inhibitor) versus response—variable slope (4-parameter) equation using GraphPad Prism with the slope restrained to 1.0.

RESULTS

Gene selection. Enzymes were selected for analysis of essentiality in T. brucei based on two criteria: first, we were interested in genes with high druggability scores; second, we sought to study a representative sample from each of the major aaRS classes and subclasses (Table 1). Of the selected genes, ThrRS, ArgRS, AsnRS, ValRS, and GluRS are single-copy genes that have dual cytosolic and mitochondrial localizations based on a reported proteomic analysis (21, 51). In contrast, two TrpRSs are encoded in the T. brucei genome, and the cytosolic enzyme was analyzed in this study. Two subunits of PheRS were studied; the alpha subunit consists of the catalytic core, whereas the beta subunit consists of DNA and ATP binding sites. Druggability scores were obtained from tdrtarget s.org and showed good predicted druggability for ThrRS, PheRS, ArgRS, and ValRS (≥0.6 based on a range from 0 to 1, with 1 representing the most druggable targets [7, 8]), suggesting that, if essential, they would be strong candidates for drug discovery programs. Druggability data are not available for AsnRS, and TrpRS-1 and GluRS score below the 0.6 threshold for good druggability.

Aminoacyl tRNA synthetases are essential for BSF T. brucei growth. To test the selected aaRSs for essentiality in T. brucei BSF parasites, we established eight stable transgenic cell lines that allow Tet-inducible RNAi-mediated ablation of enzyme expression. Stem-loop RNAi constructs were generated using either a Gateway or a pQuadra cloning system for enzymes ThrRS, AsnRS, PheRSs, and PheRSβ belonging to class II tRNA synthetases and ArgRS, TrpRS-1, ValRS, and GluRS belonging to class I aaRSs.
FIG 2 Effect of RNAi-induced knockdown of ThrRS in BSF T. brucei. Independent RNAi cell lines generated in BSF cells for ThrRS were analyzed for growth and for cell cycle progression following RNAi induction. (A) Growth curves for three clonal lines with or without Tet (data represent the means, and error bars represent the ranges). Cell numbers were monitored by hemocytometer, and cell number was calculated as the product of cell density and the total dilution. (B) Real-time qPCR analysis of uninduced cells and cells treated with Tet for 24 h and 48 h relative to TERT as the normalization standard. Analyses were run in triplicate, and the error bars represent the root mean square deviation (RMSD). (C) Genome analysis by microscopy: distributions of nuclei (N) and kinetoplasts (K). Cells were grown with or without Tet for 48 h, stained with DAPI, and examined by fluorescence microscopy. A total of 150 cells were counted per condition. (D and E) Flow analysis of cells minus (D) or plus (E) Tet for 48 h and stained with propidium iodide (PI). PI fluorescence (FL3-Area) is plotted versus cell count. The tallest peak in panel D represents G0/G1 (48%), the span between peaks represents the S phase (4.59%), and the second tallest peak represents G2/M (27.6%). (F) Confocal imaging of representative cells before and after Tet induction (48 h).

Figures 2, 3, 4, 5, and 7 show the characterization of each of the aaRS knockdowns, while Fig. 8 depicts the characteristics of the parental line for comparative purposes. For all 8 of the targeted genes, induction of RNAi led to significant growth defects within 2 to 3 days, showing that all 8 genes are essential for parasite viability (Fig. 2A, both panels A of Fig. 7, and Table 2). These growth defects correlated with decreased mRNA levels ranging from 55% to 85% as determined by RT-PCR analysis using TERT as a control (Fig. 2B and Table 2). Knockdown of four genes (ThrRS, AsnRS, PheRSβ, and ArgRS) led to rapid cell death, and no evidence of live cells could be detected past day 6. Knockdown of the remaining genes (PheRSα, TrpRS-1, ValRS, and GluRS) stopped growth but did not completely lyse the cells even though the level of mRNA knockdown was similar to the level seen with those genes that showed a more robust cell death effect. Of note, however, changes in protein levels may not necessarily track with the mRNA levels, and since protein levels were not measured, it remains possible that protein levels decreased more significantly in the cell lines that showed the more robust effects.

Morphology and flow cytometric analysis of T. brucei BSF cells after aaRS knockdown. To explore the consequences of gene knockdown beyond the observed growth arrest, the RNAi cell lines and both Tet induced and uninduced control cells and control wild-type SM cells were stained with PI and DNA content was analyzed by flow cytometry. Cell morphology and numbers of nuclei (N) and kinetoplasts (K) were additionally analyzed by confocal microscopy following staining with DAPI. Cells were scored as “normal” when they contained 1K1N or 2K1N/2K2N and “abnormal” when they contained a different genome composition. The majority (90% to 94%) of the control populations had a normal phenotype, and most contained 1K1N, while a lesser fraction of dividing cells (2K1N or 2K2N) was observed (Fig. 8A and B). Based on flow cytometry, 53% of the cells were in G0/G1 phase, 4% in S phase, and 26% in G2/M phase.

Dramatic morphological effects and cell cycle changes were observed for the ThrRS, ArgRS, and AsnRS RNAi cell lines and, to a lesser extent, for PheRSβ, where morphological abnormalities were observed as early as 16 h after Tet induction (Fig. 2 to 5 and Table 2). Flow cytometry suggested that knockdown of ThrRS led to an accumulation of cells with low DNA content (Fig. 2D and E). This corresponded to the appearance of both 0N1K cells (4%) and 1N0K cells (3%) (quantitated by confocal microscopy) (Fig. 2C). There was also an increase in the number of 2N2K cells suggestive of a block in cytokinesis (Fig. 2C). Phenotypically, a large percentage of Tet-induced ThrRS RNAi cells were rounded, had detached flagella, and lacked motility (Fig. 2F). Knockdown of ArgRS led to rapid cell death and to an increase in the number of rounded cells
with detached flagella (Fig. 3F), but no significant change in DNA content was seen (Fig. 3D and E). Knockdown of AsnRS led to increases in abnormal complements of nuclei and kinetoplasts. Both confocal microscopy and flow cytometry identified increased populations of 0N1K (up to 20%), 1N0K, and 1N2K cells (Fig. 4C to E) suggestive of a block in entering S phase. A significant percentage of Tet-induced AsnRS RNAi cells were either spherical or round with either detached flagella or no flagella.
After knockdown of PheRS, the DNA content was normally distributed (Fig. 5C to E, bottom panel) but cells were somewhat enlarged (Fig. 5F, bottom panel).

Milder but notable effects on the cell cycle and cell morphology were observed for PheRSΔ knockdown (Fig. 5). Knockdown of PheRSΔ led to a strong increase in populations of cells, with 4C and 8C DNA content suggesting a block in cytokinesis (Fig. 5D and E, top panel), though cell morphology looked fairly normal (Fig. 5F, top panel). Knockdown of TrpRS-1 and ValRS did not alter cell morphology (data not shown), and while knockdown of these genes reduced the rate of cell growth, the remaining cells had a normal distribution of DNA content (Fig. 6 and 7). Thus, RNAi
knockdown of any one of the eight aaRSs resulted in morphological and/or cell division abnormalities and growth inhibition and/or death. There was a partial correlation between the degree of RNAi knockdown and the morphological and growth defects, with those lines where knockdown was more pronounced (73% to 84%) showing greater growth defects, with the exception that AsnRS cell lines showed a decrease in mRNA of only about 60%, which was sufficient to lead to cell lysis (Table 2).

ThrRS enzymatic activity. Based on the strong cell death phenotype observed for ThrRS knockdown, we decided to characterize this protein in greater detail. Recombinant ThrRS was expressed and purified from E. coli, and we used a coupled-enzyme assay to evaluate the enzymatic activity by following the generation of pyrophosphate via conversion to inorganic phosphate, which was then detected by malachite green (50). ThrRS acylated tRNA\textsuperscript{Thr} in a time- and enzyme concentration-dependent manner, demonstrating that the T. brucei ThrRS gene encodes a functional enzyme (Fig. 9A and B). The assay remained linear over 30 min, which was taken as representing the steady-state phase of the reaction, and this time point was used for subsequent studies. Enzyme concentrations above 0.25 μM led to a loss in the linear dependence on enzyme activity, thus, 0.25 μM enzyme was used in subsequent studies. The specific activity of the enzyme under these conditions was 1.4 min\textsuperscript{-1}, which is comparable to published values for the yeast enzyme (k\textsubscript{cat} = 2.8 min\textsuperscript{-1} [52]).

To provide chemical validation of ThrRS as a drug target in T. brucei, we tested the reported ThrRS inhibitor borrelidin against both the T. brucei ThrRS enzyme and, for activity, the BSF T. brucei cells in vitro. Borrelidin is a natural-product polyketide that is reported to have broad-spectrum antimicrobial and anticancer activity (53–57). Borrelidin was a potent inhibitor of T. brucei ThrRS (IC\textsubscript{50} = 0.066 μM), and this result is consistent with the finding that the amino acids in the putative borrelidin binding pocket are conserved in both T. brucei and the bacterial enzymes that are sensitive to the compound (53) (see Fig. S1 in the supple-
ment material). *T. brucei* BSF cell growth was also inhibited (EC$_{50}$ = 2.2 μM) (Fig. 9C), with parasites being about 30-fold less sensitive to the compound than the purified recombinant enzyme. This difference may relate to poor uptake of borrelidin by the parasites, but we cannot rule out potential off-target effects of borrelidin on the parasites.

**Cell localization of ThrRS.** In order to determine the localization of the ThrRS enzyme, we prepared transgenic cell lines allowing inducible expression of ThrRS with three V5 tags at the C terminus. Cells were stained with DAPI (nuclei) and MitoTracker (mitochondria) to mark those compartments along with anti-V5 antibody. A Tet-inducible diffuse staining of the tagged *T. brucei* ThrRS was observed, consistent with a cytosolic localization (Fig. 10). However, the merged image also showed evidence for mitochondrial staining as marked by the appearance of yellow along the cell edges in the position of the mitochondrion. This result is consistent with the *T. brucei* ThrRS gene encoding a dual-purpose aaRS that functions in both the cytoplasm and mitochondria and is in good agreement with our prior mass spectrometry data (21).

**DISCUSSION**

The aaRSs are validated antimicrobial drug targets with high drug-gability scores (11–13). In order to systematically evaluate the potential of this family of enzymes as targets for drug discovery in *T. brucei*, we assessed the essentiality in the mammalian infective stage of examples from each major aaRS class and subclass (Table 1) by RNAi-based gene expression knockdown. RNAi resulted in growth inhibition for each of them, which became evident between days 1 and 4, depending on the cell line (Table 2). The cell lines also exhibited differences in morphology and DNA content (revealed by flow cytometry profiles) after RNAi induction. Thus, aaRSs from each class are essential for normal growth of mammalian stage *T. brucei*, and our data specifically provide genetic validation for seven key aaRSs as potential drug targets in this parasite. Analysis of recombinant *T. brucei* ThrRS demonstrated that the enzyme had catalytic efficiency similar to that of enzymes from other species and that it was also inhibited by borrelidin, a natural-product inhibitor of bacterial ThrRS. Borrelidin also inhibited parasite growth, providing preliminary chemical validation for this target.

To date, including the data reported here, 16 of the 24 aaRSs have been shown to be essential in BSF *T. brucei* (Table 1). Additionally, the aaRSs were examined as part of a genome-wide RNAi fitness screen in BSF cells (58). Of the genes we studied here, AsnRS, PheRS β, ArgRS, and GluRS were important for fitness in that screen, but knockdown of PheRSα, ValRS, ThrRS, or TrpRS-1 did not lead to reduced fitness. The level of RNA knockdown was not quantified in the screen, illustrating the importance of detailed analysis to obtain conclusive results. In the genome-wide RNAi screen, it may be that for highly essential genes, such as the aaRSs, leaky expression of RNAi constructs even in the absence of Tet led to selection of parasites containing mutations in the inducible T7 promoter. If this occurred, RNAi would no longer be expressed, leading to normal growth of cells and a false read of nonessentiality. That study also assessed the essentiality of the three aaRSs that localize to the mitochondrion, and none appeared essential in BSF parasites. However, RNAi showed that AspRS and TrpRS-1 were both essential in the insect (procyclic) stage (23, 24, 25) and analysis of a conditional null of mitochondrial TrpRS-2 in BSF parasites showed it to be essential (27). Thus, mitochondrial aaRSs and hence mitochondrial protein synthesis are also essential in the mammalian stage parasites, which is important for drug discovery, since the mitochondrial aaRSs are more divergent from eukaryotic enzymes (21).

While all of the aaRSs appear essential, they may not have equal potential as drug targets as implied by the differences in responses to RNAi expression knockdown. These included the time course.

**TABLE 2 Description of *T. brucei* BSF RNAi knockdown phenotypes analyzed in this study**

| Figure | aaRS | % KD (24 h) | Time to growth defect (h) | Cell morphology (at 48 h) | DNA content by FACS and microscopy |
|--------|------|-------------|--------------------------|--------------------------|----------------------------------|
| 2      | ThrRS| 84          | 48                       | Round up and lyse         | Increase in ON, 0K, and 2K cells |
| 3      | ArgRS| 79          | 16                       | Round up and lyse         | As wild type                     |
| 4      | AsnRS| 60          | 16                       | Round up and lyse         | Increase in ON, 0K, and 2K cells |
| 5      | PheRS-α| 57        | 48                       | Normal                    | As wild type                     |
| 5      | PheRS-β| 73        | 24                       | Enlarge and lyse          | Increase in ON, 0K, and 2K cells |
| 6      | TrpRS-1| 59        | 24                       | Normal and lyse           | As wild type                     |
| 7      | ValRS| 63          | 48                       | Normal                    | As wild type                     |
| 7      | GluRS| ND          | 48                       | Normal                    | ND                               |

*FACS, fluorescence-activated cell sorter analysis; KD, knockdown; ND, not done.*
of growth effects, the effect on cellular morphology, and the effect on the DNA flow cytometric profile. The data show that, of the eight genes studied here, knockdown of ThrRS, ArgRS, AsnRS, and PheRSβ led to the most rapid cell death phenotypes and to morphological and cell cycle changes. The different responses to RNAi knockdown may be due to differences in the levels of the remaining mRNAs and the proteins but might also reflect differing cellular requirements for each enzyme, including relative catalytic efficiency levels and the relationship of substrate concentrations to the enzyme $K_m$. In addition, the cellular dependence on each enzyme may be affected by whether it functions in cytosolic and/or mitochondrial compartments, whether it is integrated within the MARS complex, and whether or not the enzyme has any alternative functions in cellular processes in addition to protein synthesis. While the degree of mRNA reduction and the extent of growth and morphological abnormalities do not entirely correlate, for the gene knockdowns that showed the most severe phenotypes (ThrRS, ArgRS, PheRS, and AsnRS), the mRNA levels at 24 h were reduced by 60% to 84%, while for those with more moderate effects (PheRSα, TrpRS-1, and ValRS), mRNA levels were reduced by 57% to 63% (Table 2). However, the data show in all cases that only a partial knockdown of the target is required to cause growth reductions, suggesting that even partial reduction in enzyme activity with inhibitors could affect parasite growth. This strengthens the potential of these aaRSs to be successfully targeted for drug discovery.

In assessing the potential effects of the MARS complex on essentiality, knockdown of one enzyme in the MARS complex could potentially lead to destabilization of the complex structure and to changes in the abundance of additional complex aaRSs. However, our analysis did not find any indication that knockdown of enzymes that are in the complex led to more-severe growth phenotypes than knockdown of those that are not. Knockdown of ArgRS, PheRSβ, and ThrRS, which are not part of the complex, caused severe growth effects, as also observed for TrpRS-1 and other aaRSs in the complex (21). Finally, it has been reported that aaRSs can have alternative roles in cell function, including the production of dinucleotide polyphosphates, which can act as second messengers (59), amino acid sensing (60), and transcriptional regulation (61). Thus, the involvement of an enzyme in a cellular process other than translation could lead to an enhanced response to gene knockdown with respect to growth and cell cycle changes in comparison to genes that are involved only in translation. Overall, it seems likely that several factors, including the degree of mRNA loss, the cellular requirement of a certain amount of aaRS, its cellular location, and perhaps other potential functions of the aaRS, contribute to the phenotype and could be considered when choosing a particular aaRS as a drug target based on essentiality data.

Finally, essentiality is only one consideration in evaluating whether or not an enzyme will be a good drug target. Both the druggability of the target and the likelihood of achieving selectivity versus the human host also need to be considered. Of the genes targeted in this current study, four (ThrRS, ArgRS, PheRS, and ValRS) have high (>0.6) druggability scores (Table 1), and among these, severe growth and morphological changes were observed upon knockdown of ThrRS and ArgRS, suggesting that the latter two enzymes have a strong potential to be successfully targeted. Furthermore, chemical validation also strengthens the case for a particular target, and our data showing that borrelidin inhibited *T. brucei* ThrRS and parasite growth provide preliminary chemical validation of the target, though at this stage, direct proof that the

![FIG 9 Characterization of ThrRS expression and activity. (A) ThrRS activity assays for recombinant protein purified from *E. coli*. Data represent dependence of PO₄²⁻ formation versus time. (B) Dependence of PO₄²⁻ formation on ThrRS concentration. Two moles of PO₄²⁻ are formed for every mole of tRNAThr that is acylated. (C) Dose response of *T. brucei* BSF growth and recombinant ThrRS activity versus concentration of borrelidin, a known ThrRS inhibitor. For inhibition of parasite growth, the EC₅₀ = 2.2 µM (1.8 to 2.5); for inhibition of ThrRS activity, the IC₅₀ = 0.066 µM (0.020 to 0.22), where values in parentheses represent the 95% confidence interval.](image)

![FIG 10 Subcellular localization analysis of *T. brucei* BSF expressing C-terminal V5-tagged ThrRS. Cells were fixed in 4% paraformaldehyde and stained with FITC-conjugated anti-V5 monoclonal antibodies. Mitochondria were stained with MitoTracker (MT), and DNA was stained with DAPI. Differential interference contrast (DIC) is shown. Images were merged as indicated.](image)
borrelidin growth effects are a consequence of ThrRS inhibition is lacking. Since ThrRS was found to localize in the cytoplasm and mitochondrion, it is possible that inhibition of cell growth may be a consequence of protein synthesis inhibition in both cellular compartments.

With regard to selectivity, comparison of the *T. brucei* ThrRS amino acid sequence with the human enzyme sequence shows that the two enzymes are highly conserved (see Fig. S1 in the supplemental material). This is also the case for the *E. coli* enzyme, and yet a recent study showed that differences in amino acid sequence in second-shell residues could be exploited to identify bisubstrate analogs of Thr and adenine that were highly (>300-fold) selective for the bacterial enzyme (62). X-ray structure analysis showed that the replacement of Leu361 for Cys361 in the bacterial enzyme created an enlarged binding pocket that allowed inhibitors to adopt a conformation when bound to the *E. coli* enzyme that was different from that seen when they were bound to the human enzyme. Examination of the putative Thr/ATP binding sites in *T. brucei* ThrRS shows that while the *T. brucei* enzyme, like the human enzyme, has a Leu residue at position 361 (TTbThrRS Leu 409), other amino acid differences in second-shell residues are present that might be exploited for the identification of selective inhibitors (see Fig. S1). The borrelidin binding site, which is adjacent to the substrate binding site, was explored in bacterial aaRSs by site-directed mutagenesis, identifying six key residues that are conserved in enzymes that are inhibited by the compound (53). These residues are conserved between the *T. brucei* and human enzymes, suggesting that it may be hard to selectively target this site. However, structural data are not available to fully map the borrelidin binding site, making it difficult to determine if species-selective binding might also be possible at this site.

In summary, we demonstrate here that seven aaRSs, one of which has two subunits, are essential in the infectious mammalian form of *T. brucei* and we previously showed that AlaRS, ProRS, LeuRS, LysRS, and IleRS are also essential in the mammalian infective stage of the parasite (21, 30). In addition to our genetic validation of these 13 aaRS genes, others have shown an additional 5 to be essential in this relevant mammalian stage of the life cycle. That mRNA knockdown of only 60% to 70% was sufficient for cell viability and, in several cases, cell death suggests that an aaRS is essential in this relevant mammalian stage of the life cycle.

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