Genetic validation of *Trypanosoma brucei* glutathione synthetase as an essential enzyme

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Running title: *T. brucei* glutathione synthetase
Abbreviations. HAT, human African trypanosomiasis; TbGS, *Trypanosoma brucei* glutathione synthetase; γ-GCS, γ-glutamylcysteine synthetase; TryS, trypanothione synthetase; TR, trypanothione reductase; ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; DHODH, dihydroorotate dehydrogenase; BSF, Blood stream form; cDKO, conditional double knockout *T. brucei* cell line.
Abstract

Human African trypanosomiasis (HAT) is a debilitating and fatal vector borne disease. Polyamine biosynthesis is the target of one of the key drugs (eflornithine) used for the treatment of late stage disease suggesting the pathway might be exploited for the identification of additional drug targets. The polyamine spermidine is required in trypanosomatid parasites for formation of a unique redox cofactor termed trypanothione, which is formed from the conjugation of glutathione to spermidine. Herein we characterize recombinant T. brucei glutathione synthetase (TbGS) and show that depletion of TbGS in blood form parasites using a regulated knockout strategy leads to loss of trypanothione and to cell death as quantified by FACS analysis. These data suggest that >97% depletion of TbGS is required before trypanothione is depleted and cell growth arrest is observed. Exogenous glutathione was able to partially compensate for the loss of TbGS suggesting that parasites are able to transport intact glutathione. Finally, reduced expression of TbGS leads to increased levels of upstream glutathione biosynthetic enzymes and decreased expression of polyamine biosynthetic enzymes providing evidence that the cells cross regulate the two branches of the trypanothione biosynthetic pathway to maintain spermidine and trypanothione homeostasis.
Introduction

*Trypanosoma brucei* is the causative agent of human African trypanosomiasis (HAT), a vector-borne illness endemic in 36 African countries (1-4). The World Health Organization (WHO) estimates that 30,000 people are currently infected, while millions in the region remain at risk of contracting the disease (5). HAT is nearly always fatal if untreated, however, current drug therapy is limited by toxicity and difficult treatment regimens. Eflornithine, a key drug for treatment of late stage HAT, is a suicide inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC), thus establishing the polyamine biosynthetic pathway as an essential and targetable pathway in these parasites (6). Polyamines are positively charged, flexible hydrocarbons that are essential for growth and proliferation of eukaryotic cells, with roles in transcription, translation, chromatin structure and ion channel function (7-10). Trypanosomes have evolved to use the polyamine spermidine in an additional role through conjugation with two molecules of glutathione to form the essential redox cofactor trypanothione (76, N1, N8-bis(glutathionyl)-spermidine) (11).

Trypanothione synthesis occurs via four ATP dependent enzymatic steps beginning with the conjugation of L-Cys to L-Glu by γ-glutamyl cysteine synthetase (γ-GCS)(12) (Fig. 1). The product of this reaction (γ-GC) is then ligated to Gly by glutathione synthetase (GS) to form the thiol peptide glutathione that is used to maintain redox homeostasis in most cells. The third and fourth steps are catalyzed by trypanothione synthetase (TryS), which conjugates glutathione to spermidine forming N1-glutathionylspermidine and subsequently adds a second molecule of glutathione to N1-glutathionylspermidine to form trypanothione (13, 14). TyrS also contains an amidase domain and can catalyze the breakdown of trypanothione to glutathione and spermidine.
Trypanothione is required for growth and proliferation of trypanosomatids and is necessary to maintain cellular redox balance, replacing the role of glutathione found in mammalian cells (12, 15). The presence of two novel enzymes in the trypanosomatids required for trypanothione synthesis (TryS) and reduction (trypanothione reductase (TR)) has led to interest in identifying inhibitors of these enzymes for drug discovery efforts, though none have yet advanced beyond hit identification or early lead optimization (16-21). With the exception of GS, the biosynthetic enzymes for both the polyamine and trypanothione biosynthesis have been characterized and shown to be essential in *T. brucei* using genetic methods (14, 22-25). A crystal structure of *T. brucei* GS (*Tb*GS) has been reported and shows conservation of the overall fold when compared to the human GS protein, and a similar active site composition (26). However, no kinetic analysis of the *T. brucei* enzyme has been reported and genetic studies on its role and essentiality are lacking.

In mammalian cells, glutathione biosynthesis is regulated primarily by modulation of γ-GCS expression, the first step in the pathway (27). While *T. brucei* γ-GCS has been characterized and shown to be essential for growth in *T. brucei*, gene knockdown did not lead to compensatory changes in the levels of the polyamine biosynthetic enzymes and no evidence for cross regulation between the polyamine and trypanothione arms of the pathway was found (22, 28, 29). Regulation has been shown to occur within the *T. brucei* polyamine biosynthetic pathway where gene knockdown or inhibition of S-adenosylmethionine decarboxylase (AdoMetDC), an essential enzyme in spermidine biosynthesis, led to increased protein expression of both the AdoMetDC regulatory subunit prozyme and ODC (23, 24, 30, 31). The AdoMetDC regulatory protein prozyme is itself also an activator of AdoMetDC as heterodimer formation with prozyme (an inactive paralog of AdoMetDC) increases enzymatic activity by
three orders of magnitude. *T. brucei* deoxyhypusine synthase, an enzyme that uses spermidine as a substrate to modify an essential elongation factor eIF5A also requires activation by an inactive paralog (32). Despite these advances in understanding polyamine pathway regulation in *T. brucei*, key questions remain about how the glutathione branch of the pathway is regulated, and how or if the polyamine and glutathione pathways show evidence for cross regulation.

Herein we investigate the function and regulation of *TbGS*, including steady-state kinetic analysis of the recombinant enzyme and characterization of a *TbGS* knockout in *T. brucei* blood stream form (BSF) cells. We show that *TbGS* has similar catalytic activity to other characterized GS homologs, and we demonstrate through the use of a *TbGS* conditional knockout (cDKO) cell line that *TbGS* is essential for trypanothione biosynthesis and for parasite growth. Furthermore, we show that loss of *TbGS* protein leads to an increase in γ-GCS protein and a decrease in the levels of AdoMetDC prozyme. Thus, similarly to the other polyamine and trypanothione biosynthetic enzymes, *TbGS* is essential to *T. brucei* growth and it appears that *TbGS* protein levels are linked to the regulatory mechanisms that control expression levels of both polyamine and glutathione/trypanothione biosynthetic enzymes.
Materials and Methods

Gene ID numbers

The TriTrypDB gene ID numbers are as follows: GS (Tb927.7.4000), γ-GCS (Tb927.10.12370), TryS (Tb927.2.4370), TR (Tb927.10.10390), ODC (Tb927.11.13730), AdoMetDC (Tb927.6.4410 and Tb927.6.4460), AdoMetDC prozyme (Tb927.6.4470), SpdSyn (Tb927.9.7770) and DHODH (Tb427.05.3830).

T. brucei cell culture

Blood stream form (BSF) *T. brucei* strain 90-13 was cultured in HMI-9 media with 10% FCS at 37°C and 5% CO₂ with the appropriate antibiotics (G418 (2.5 μg/ml), hygromycin (5 μg/ml) or phleomycin (2.5 μg/ml) as required) (33). When required, Tet (1 μg/ml) was added to cultures fresh daily. Cells were split every 1 - 3 days to maintain log phase growth (10³-10⁶ cells/ml) and cell densities were calculated with a hemocytometer (Fisher). Growth curves represent total cell number, which is a product of cell density and total dilution.

Genomic DNA Purification

Genomic DNA was isolated from *T. brucei* 427 BSF cells (1.0 x 10⁸) harvested by centrifugation and washed 3x with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2.0 mM KH₂PO₄, pH 7.4). Pellet was resuspended in 500 μl DNA lysis buffer (100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 60 μg/ml RNase A), incubated for 30 min at 37°C, proteinase K (5 μl of 20 mg/ml) was added and the mixture incubated at 55°C overnight. DNA was extracted by phenol/chloroform/ethanol precipitation.

Cloning of the TbGS construct for recombinant expression in *E. coli*

The TriTryp database contains a single gene annotated as *TbGS* (Tb927.7.4000). The *TbGS* gene was synthesized and cloned into pUC57 by Genscript.
from the plasmid to obtain a product flanked with BsaI and XbaI restriction sites. The PCR product was digested with BsaI/XbaI and subcloned into BsaI-linearized pE-SUMO(Kan) (Life Technologies), generating the pE-SUMO-\textit{Tb}GS construct for expression of an N-terminally tagged His\textsubscript{6}-SUMO-\textit{Tb}GS.

\textit{Protein expression and purification of SUMO-\textit{Tb}GS from} \textit{E. coli}

The pE-SUMO-\textit{Tb}GS construct was transformed into T1 phage resistant \textit{E. coli} BL21(DE3) competent cells for protein expression. Protein expression was induced at A\textsubscript{600} of 0.5 with isopropyl \textbeta-D-1-thiogalactopyranoside (200 \textmu M, Sigma) for 18 h at 14°C. Cells were harvested by centrifugation (1000 \texttimes g for 0.5 h). Pellet was resuspended in Buffer A (25 mM Hepes pH 7.5, 300 mM NaCl, 1 mM MgCl\textsubscript{2}, 5% glycerol) and supplemented with protease inhibitor mix (1 \textmu g/ml Leupeptin, 2 \textmu g/ml Antipain, 10 \textmu g/ml Benzamidine, 1 \textmu g/ml Pepstatin, 1 \textmu g/ml Chymostatin, 200 \textmu M PMSF). Cells were lysed by high-pressure disruption (EmulsiFlex-C5, Avestin), the lysate was clarified by centrifugation (15,000 \texttimes g for 0.5 h), and protein was purified by Ni\textsuperscript{2+} affinity chromatography (HiTrap Chelating HP column, GE Life Sciences) using an AKTA FPLC (GE Life Sciences). The column was washed with Buffer A plus 10 mM imidazole before protein elution with Buffer A plus 250 mM imidazole. Fractions were analyzed by SDS-PAGE and those containing His\textsubscript{6}-SUMO-\textit{Tb}GS were pooled and dialyzed against Buffer A (16h, 4°C). To remove the His\textsubscript{6}-SUMO tag, Ulp1 (20 \textmu g/ml) was added and the mixture was incubated at 30° for 2 h. Protein was concentrated (Centricon-30, Millipore) and \textit{Tb}GS was separated from the tag with a second Ni\textsuperscript{2+}-affinity purification. \textit{Tb}GS protein fractions (flow-through) were pooled and dialyzed against storage buffer (25 mM Hepes pH 8.0, 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 1mM DTT, 5% glycerol). Protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 60.0 mM\textsuperscript{-1}cm\textsuperscript{-1} (computed using ProtParam, ExPASy,
Swiss Institute of Bioinformatics). The final yield was 0.8 mg of TbGS per liter of bacterial culture. Ulp1 was expressed and purified as previously described (31, 32). All purification steps were performed at 4°C.

Steady-state kinetic analysis of TbGS activity

TbGS activity was measured using a previously described ATP-coupled assay (34). All assays were performed at 37°C on a Synergy H1 Hybrid Reader (BioTek) using UVStar 96-well half-area plates (Greiner). Total reaction volume was 100 μl, containing 100 mM Tris-HCl (pH 8.2), 50 mM KCl, 20 mM MgCl₂, 250 μM NADH, 5 mM phosphoenolpyruvate, 10 U lactate dehydrogenase, 10 U pyruvate kinase and substrates. The assay was shown to be linearly dependent on the TbGS concentration (range 10 - 50 nM). TbGS concentrations for substrate titration experiments were 15 and 25 nM. Unless otherwise stated, substrate concentrations were fixed at 3 mM γ-GC (BAC Chemicals), 30 mM Gly, or 10 mM ATP. For substrate titrations, the varied substrate concentrations were as follows γ-GC (1 μM – 1 mM), Gly (100 μM – 100 mM), and ATP (10 μM – 10 mM). Reactions were monitored by absorbance at 340 nM and rates were fitted to the Michaelis-Menten equation using GraphPad Prism to determine $K_m$ and $k_{cat}$ values.

Cloning of the TbGS RNAi construct

The TbGS RNAi stem loop construct was generated according to previous methods from the pLEW100 and pJM326 vectors (35). Using primers containing the appropriate restriction enzyme sites (Table A1) a TbGS gene fragment (nucleotides 1-440) was PCR-amplified from T. brucei 427 genomic DNA and subcloned into pLEW100 in the forward direction (using XbaI and MluI) and into pJM326 in the reverse direction (using HindIII and NheI). The TbGS gene along with the stuffer region was excised from pJM326 using HindIII and XbaI and ligated into pLEW100 containing the first TbGS RNAi section. The construct generated contained the two
fragments of the *TbGS* gene separated by the stuffer region in opposing directions under the transcriptional control of a Tet-regulated promoter. These elements were flanked by a ribosomal DNA sequence, allowing for homologous recombination into the multi-copy ribosomal locus of *T. brucei*. Sequencing confirmed that the correct construct was obtained.

**Cloning of GS allelic replacement constructs**

The *TbGS* knockout cell lines were generated using plasmids and protocols that have been previously described for gene disruption in *T. brucei* (23, 36, 37). Briefly, two approximately 300 bp segments of the *TbGS* 5' and 3' UTR (corresponding to nucleotide -387 to -61 and 1745 to 2135, respectively where base 1 represents the ATG start codon and base 1668 represents the TAA stop codon) were PCR amplified using primer sets containing the appropriate restriction enzymes (Table A1) and the resultant PCR fragment was cloned into TOPO-Blunt (Life Technologies). The UTRs were sequentially subcloned into the pLEW13 vector first by introducing the 5' UTR region using NotI/MluI followed by the 3' UTR region using XbaI/StuI. The resulting pLEW13-*TbGS* knockout construct A (KO-A) contained the T7 polymerase and neomycin resistance cassette sandwiched between the *TbGS* UTRs. To generate the pLEW90-*TbGS* knockout construct B (KO-B), the *TbGS* KO-A vector was digested with XhoI/SwaI to remove T7 polymerase and neomycin resistance cassette, and the pLEW90 vector was digested with XhoI/StuI to liberate the Tet repressor and Hygromycin resistance cassette (SwaI/StuI are blunt end endonucleases). The TetR and Hygromycin resistance cassette were ligated into the pLEW13-*TbGS* KO-A forming the pLEW90-*TbGS* KO-B vector.

**Cloning TbGS Tet-regulatable *T. brucei* expression construct**

To generate the C-terminal FLAG-tag *TbGS* *T. brucei* expression construct, the gene was PCR amplified using primers with appropriate restriction enzymes (Table A1), digested with...
HindIII/KpnI, and ligated into HindIII/KpnI linearized pT7-FLAG-MAT to generate the pT7-
GS-FLAG-MAT bacterial expression vector. The GS-FLAG insert was obtained by PCR
amplification from this vector, digested with BamHI/HindIII and ligated to pLEW100v5 vector,
creating the C-terminal FLAG-tag ectopic expression vector.

Generation of T. brucei transgenic cell lines

The pLEW100-TbGS stemloop RNAi construct was transfected as previously described
(23). Briefly, the pLEW100-TbGS stem loop RNAi vector was linearized by EcoRV (80 μg) and
transfected into log phase T. brucei 90-13 bloodstream form (BSF) cells using the Amaxa
Nucleofector system. Phleomycin resistant cells that integrated the construct into rRNA locus
were selected and clonal lines were obtained through limited dilution. To induce RNAi
knockdown of the TbGS gene, 1 μg/ml of Tet was added every 24 h during experiments.

To generate the TbGS double knockout (DKO) cell line, log phase bloodstream 427 cell
lines were transfected with the NotI linearized pLEW13-TbGS KO-A vector. After establishing
a TbGS single knockout (SKO) cell line with neomycin/G418, glutathione (80 μM) was added to
the medium and parasites were transfected with the NotI linearized pLEW90-TbGS KO-B
vector. The resulting transfectants were established in medium containing glutathione,
neomycin, and hygromycin. Analysis demonstrated that these clones retained a copy of the wild-
type allele of the gene.

To generate the TbGS conditional double knockout (cDKO) cell line, TbGS SKO cells
were transfected with Tet-regulated TbGS ectopic expression vector and selected in the presence
of phleomycin. The resulting cell lines, single knockout with inducible TbGS (SKO+iGS), were
investigated for expression of a FLAG-tagged TbGS protein. After expression of the ectopic
copy of TbGS had been established, parasites were transfected with the pLEW90-TbGS KO-B
vector in the presence of Tet to generate the *TbGS* cDKO cell line. Limited dilution was used to obtain clonal lines and parasites were maintained in medium that contained Tet, hygromycin, neomycin, and phleomycin.

*Collection of *TbGS* cDKO growth curves*

*TbGS* cDKO growth was evaluated by first washing cells twice with Tet-free media followed by plating into fresh media at a density of 1-5 x 10^4 cells/ml with or without Tet. Cells were counted on the days indicated and split into fresh media every 24 - 48 h, dependent on growth.

*Western blot analysis*

Parasites (~10^8) were harvested by centrifugation (1900 x g for 10 min) and washed 3x with cold (4°C) PBS pH 7.4. Pellets were resuspended in protein lysis buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol, and protease inhibitor mix (described above). Cells were lysed by three freeze/thaw cycles and clarified by high-speed centrifugation using a bench top centrifuge. Protein concentration was determined by a colorimetric protein assay (BioRad).

Protein obtained from samples were separated by a 12% SDS-PAGE gel and transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham) using a wet transfer at 4°C for one hour using 100 V. Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) overnight at 4°C. Primary rabbit *TbGS* polyclonal antibodies were generated under contract by Proteintech Group Inc (Chicago, IL) from recombinant His_6*-TbGS* protein isolated as above. Antibodies against polyamine and trypanothione biosynthetic enzymes were used as previously described (22, 23, 25, 38, 39). Dilutions for primary antibodies in 5% Milk in TBS-T were: α-*TbGS* – 1:2,500, α-
TbDHODH – 1:10,000, α-FLAG – 1:1,000 (Sigma), α-TbAdoMetDC – 1:2,500, α-Tbprozyme – 1:5,000, α-TbODC – 1:10,000, α-LdSpdSyn – 1:1,000, α-TbTryS – 1:1,000, and α-Tbγ-GCS – 1:10,000. The TbTryS antibody was a gift from Dr. Alan Fairlamb (University of Dundee) and the LdSpdSyn antibody was a gift from Dr. Buddy Ullman (Oregon Health and Science University). Secondary antibodies (anti-rabbit, anti-rat (TryS), and anti-mouse (FLAG)) were used at 1:10,000 dilution in 5% nonfat milk TBS-T. For detection of protein by multiple antibodies, parallel gels were loaded with 20-40 μg total protein per well, transferred, and each membrane was sectioned and probed with a different antibody. Each membrane was used to assess protein levels of two biosynthetic pathway proteins along with the DHODH loading control. Protein levels were visualized by chemiluminescence detected when incubating the membrane with ECL HRP substrate (ThermoFisher) and exposing to film (Fisher).

Bands on western blots were quantified using ImageJ software. High resolution scans of film were loaded into ImageJ and analyzed correcting for background using an area of the corresponding film that was signal-free. Each band was quantified as a ratio to the DHODH loading control, and the value was compared to the protein levels expressed on day 0.

**Intracellular polyamine determination**

Analysis of polyamines was done as previously described (23). For analysis of intracellular polyamines, 1 x 10⁷ cells were harvested by centrifugation (3000 rpm. for 10 min), washed twice with 1 ml of PBS pH 7.4 and then resuspended in 25 μl polyamine lysis buffer (100 mM MOPS, pH=8.0, 50 mM NaCl, 20 mM MgCl₂). Cells were lysed by 3 freeze/thaw cycles followed by acid precipitation of proteins by addition of 7.5 μl of 40% trichloroacetic acid (TCA) and incubation on ice for 10–15 min. Cell debris was removed by centrifugation (20000 x g). Supernatant was fluorescently labeled using AccQ-Fluor reagent (6-aminoquinolyl-n-
hydroxysuccinimidyl, Waters) by incubating 5 µl of sample supernatant with 20 µl labeling reagent and 75 µl borate buffer at 55°C for 10 min. Polyamines were separated by HPLC using a Waters AccQtag (3.9x150mm) column run on a Beckman System Gold HPLC with a Rainin Dynamax Fluorescent detector. Peaks were separated using a previously described linear gradient with eluent A containing 450 mM sodium acetate pH 4.95, 17 mM triethylamine (TEA), 0.01% sodium azide, and eluent B containing 60% acetonitrile and 0.01% acetone (23, 24, 40). The gradient was as follows: 0% B for 5 min, 20% B 45 min, 50% B 5 min, 100% B 3 min, 0% B over 1 min, 0% B for 10 min. Polyamines were identified by retention time and quantified by peak area in comparison to standards run under the same conditions.

Intracellular thiol determination

Reduced intracellular thiols were determined as described with slight modification (41, 42). Briefly, cells (1 x 10⁸) were harvested by centrifugation (3000 rpm, 10 min at RT) and washed twice with pre-warmed HMI-9 medium lacking fetal bovine serum (FBS), β-mercaptoethanol, and L-Cys. A final wash was done with cold (4°C) PBS pH 7.4 and cells were pelleted by centrifugation (3000 rpm for 5 min). Cell pellets were immediately resuspended in 150 µl ice cold 5% TCA (trichloroacetic acid) in 10 mM HCl and incubated on ice for 5-10 min. Denatured proteins and cell debris was removed by centrifugation at 4°C. The supernatant was extracted four times with 450 µl ice-cold diethyl ether to remove excess TCA and 345 µl of 40 mM HEPPS (3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid) pH 8.0 containing 4 mM DTPA (diethylene triamine pentaacetic acid) was added to the extractant.

To derivatize thiols in the extracted supernatant, 5 µl of 200 mM monobromobimane was added for a final concentration of 2 mM and vortexed. Samples were incubated at RT for 10-15
min in the dark. To stop the labeling reaction and prevent oxidation of samples, 2.5 µl of 5 M MSA (methanesulfonic acid) was added. Samples were stored at -80°C until analysis by HPLC. HPLC analysis used a Phenomenex Kinetex 2.6 µ C18 column with a constant flow rate of 1.25 ml/min at room temperature using gradient mixtures of eluent A (0.25% camphorsulphonic acid (CSA) pH 2.64) and eluent B (0.25 % CSA pH 2.64 and 25% 1-propanol both) as follows: 0% eluent B for 1 min, 0% to 5% eluent B over 0.5 min, 5% eluent B for 4.5 min, 5% to 12.5% eluent B over 29 min, 12.5% to 35% eluent B over 12 min, 35% to 60% eluent B over 2 min, 60% eluent B for 4 min, then 60% eluent B to 0% eluent B over 1 min. The column was re-equilibrated for 20 min between samples using the same flow rate and 0% eluent B. Thiols were identified by retention time and quantified by reduced standard’s peak area.

Evaluation of the anti-trypanosomal activity of BSO

Parasites were grown with or without Tet for 2 d, quantified, and diluted to 1 x 10³ cells/ml. Parasites were plated into 96 well plates (Greiner) with 200 µl/well. BSO concentrations were varied from 0 to 3 mM using 10 different concentrations and incubated with parasites for 72 h. Cell viability was measured using CellTiter-Glo (Promega), an ATP/luminescence assay. BioTek Synergy H1 Hybrid reader measured cell viability in the form of luminescence. Data were fitted by nonlinear regression to the log (agonist) v. response (four parameter fit) in GraphPad Prism to determine EC₅₀ for 3 replicates at each concentration. Final data represent the average of three independent experiments and the standard error of the mean is reported. The P value was calculated using a two-tailed unpaired t test with equal SD using GraphPad Prism.

FACS analysis/Live/Dead cell assay
This protocol was based on previously published methods (43) and optimized for *TbGS*
cDKO cell lines. Cells (~10^6) were harvested by centrifugation (3000 rpm for 10 m) and washed
using pre-warmed (37°C) PBSG (PBS plus 10 mM glucose, pH 7.4). Cells were then
resuspended in 500 μl PBSG and calcein and ethidium homodimer were added to final
concentrations of 50 nM (Life Technologies). Cells were incubated for 15-20 min at 37°C and
then analyzed immediately by FACS. Samples were analyzed on a Beckman Coulter FACS
Calibur flow cytometer set to measure forward and side scatter to determine cell size and
morphology as well as fluorescence using filters 530/40 (calcein) and 692/40 (ethidium). Data
obtained were analyzed using FlowJo software with single-stained cells used for compensation
of spectral overlap within the software. Eflornithine treated cells were used to determined FACS
 gating for unhealthy populations while gating for healthy cells was determined by unstained
proliferating cells.
Results

Recombinant expression and steady-state kinetic analysis of TbGS

*T. brucei* GS was expressed as a His6-SUMO-tagged fusion protein in *E. coli*, purified by Ni\(^{2+}\)-agarose affinity column chromatography and the N-terminal fusion was then removed by the protease Ulp1. The purified recombinant protein was tested for GS activity using an ATPase-coupled assay. The reaction was well modeled by simple Michaelis-Menten kinetics. *TbGS* catalyzed the synthesis of glutathione with a *k*\(_{cat}\) between 7.0 – 7.3 s\(^{-1}\) (Table 1), which is comparable to previously published eukaryotic GS enzymes from humans, plants and *T. cruzi* (44-46). The apparent *K*\(_{m}\) (*K*\(_{m-app}\)) values were measured for each of the three substrates at fixed concentrations of the other substrates (Table 1). *K*\(_{m-app}\) for γ-GC, glycine, and ATP were similar to reports for other GS homologs.

Evaluation of the essentiality of TbGS by RNA interference (RNAi)

In order to decipher the role of *TbGS* in the trypanothione biosynthesis pathway, a Tet-inducible *TbGS* RNAi stem loop BSF cell line was generated and the effects of gene knockdown were studied. Upon addition of Tet (1 μg/ml), the *TbGS* RNAi stem-loop was expressed and *TbGS* protein levels were reduced by 80-90% based on western blot analysis (Fig. 2). Because trypanothione biosynthesis enzymes were previously shown to be essential in *T. brucei* (22, 25), we anticipated cells would display a growth phenotype upon reduction of *TbGS* protein levels. However, *TbGS* RNAi cell lines continued to grow at the same rate as wild-type cells (Fig. 2) and the reduced intracellular thiol levels as measured by HPLC remained unchanged (Fig. A1). Three independent clones were evaluated and all showed the same phenotype. Our previous studies showed that the addition of glutathione to the cultures was sufficient to rescue the RNAi knockdown of γ-GCS (22) so to rule out the possibility that glutathione in the medium was
leading to rescue of cell growth we analyzed glutathione levels using HPLC and verified that medium did not contain glutathione (data not shown). These results suggested that even though the \(TbGS\) protein levels were reduced, \(TbGS\) was not sufficiently depleted by the RNAi mechanism to yield a phenotype.

Evaluation of the essentiality of \(TbGS\) by gene knockout

To generate a more robust gene knockdown that would allow investigation of the role of \(TbGS\) in trypanothione biosynthesis, we generated \(TbGS\) knockout cell lines. \(TbGS\) is a single copy gene, but \(T. brucei\) is a diploid organism, requiring that any knockout approach target both alleles. Initially we attempted to knock out both alleles of the \(TbGS\) gene in the absence of a regulatable copy of the gene, but in the presence of exogenous glutathione (80 \(\mu\)M). A cell line was selected that exhibited resistance to both selectable markers used to target the \(TbGS\) gene (neomycin and hygromycin). However, Southern blot showed that the cells retained the \(TbGS\) gene along with the 2 resistance genes, and thus it was not possible to select cells with a knockout of \(TbGC\) under these conditions (Fig. A2). This phenomenon has been previously described in \(Leishmania\) and is suggestive of an essential gene (47). Therefore, in order to investigate the role of \(TbGS\) a conditional double knockout (cDKO) was generated by replacement of the first allele with the neomycin resistance gene, followed by insertion of a Tet-regulatable ectopic copy of \(TbGS\) into the ribosomal repeat region of the genome and then knockout of the second \(TbGS\) allele with the hygromycin resistance gene. Cultures were grown in the presence of Tet to maintain \(TbGS\) expression during cloning and selection of the cDKO cell line.

The effects of \(TbGS\) depletion on growth of the cDKO cell line were studied. Removal of Tet from the media of \(TbGS\) cDKO cells led to reproducible growth arrest that began on day 4.
and lasted through day 6, after which growth resumed at rates similar to the +Tet controls (Fig. 3A). TbGS protein levels were decreased to less than 3-4% of control levels by day 2 and were undetectable (<99%) by day 4 (Fig. 3A). However, small amounts of TbGS expression (3-4% of control levels) could again be detected starting on day 8, corresponding to the point where cell growth resumed (Fig. 3A). These data suggest that cells escaped Tet regulated control of TbGS expression 7-8 days after Tet removal leading to re-expression of the gene. Furthermore these data suggest >97% of TbGS must be depleted to maintain cell growth arrest.

We next tested the effects of adding either exogenous glutathione or glutathione ethyl ester to determine if the cell growth effects caused by loss of TbGS expression could be reversed. Glutathione ethyl ester was shown to be more readily transported into some mammalian cells than glutathione (46). Exogenous glutathione at 80 uM did not rescue the growth defect that occurred upon TbGS depletion consistent with the finding that we could not generate the DKO cell line in the absence of the Tet regulated copy of TbGS. We found that concentrations of glutathione and glutathione ethyl ester above 1 mM were toxic to the cells so additional rescue experiments were carried out using the maximum tolerated concentration of 1 mM. Glutathione at 1 mM, but not glutathione ethyl ester, was able to partially rescue the cell growth phenotype that occurred upon Tet removal and loss of TbGS expression (Fig. 4). Cells grown with glutathione ethyl ester (1 mM) did show slightly cell numbers compared to control cells but growth rates were comparable suggesting that toxicity of the ester did not contribute to the lack of rescue.

**TbGS knockout leads to trypanothione but not polyamine depletion**

Intracellular thiol and polyamine levels were measured at different time points after Tet withdrawal from the TbGS cDKO cell lines (Fig. 3C and 3D). While polyamine levels remained...
relatively unchanged, trypanothione levels were depleted by 50% on day 3 and by 100% on day 4, corresponding to the time point where cells stopped growing. Trypanothione levels returned to normal by days 8 - 10 corresponding to the point where TbGS protein was again detected and cell growth resumed. Glutathionylspermidine levels were 3-4% of trypanothione levels, and were depleted with a similar time course to trypanothione after Tet removal (data not shown). Thus, the observed growth arrest correlates with both depletion of the TbGS protein and the complete loss of the reduced trypanothione and glutathionylspermidine pools, supporting the conclusion that TbGS is an essential protein required for trypanothione production.

*Effects of TbGS knockout on expression of polyamine and trypanothione biosynthetic enzymes*

The levels of other polyamine and trypanothione biosynthetic pathway proteins were measured by western blot to determine if depletion of TbGS protein led to any potential regulatory responses (Fig. 3B and Fig. A3). While no changes were observed in SpdSyn, TryS or AdoMetDC protein levels, γ-GCS protein levels were increased (2-3-fold) and AdoMetDC prozyme and ODC levels were reduced on days 4 and 5 (3-fold for prozyme and 1.5 fold for ODC compared to day 0, and up to 6-fold for prozyme from the peak at day 3). The observed changes in expression correlated with the time point where trypanothione levels were most reduced. The observed trends were reproducible as analyzed for three independent biological replicates, though the effects were not always observed on the same day after Tet withdrawal.

*TbGS cDKO cells have increased sensitivity to a pathway inhibitor*

To determine of knockdown of TbGS sensitized cells to inhibitors of glutathione biosynthesis, the TbGS cDKO cells were treated with the γ-GCS inhibitor buthionine sulfoximine (BSO). TbGS cDKO cells were grown without Tet for 2 days to deplete GS and trypanothione before incubation with various levels of BSO. The EC50 for BSO was increased 3-
fold in the absence of Tet relative to the control *Tb*GS cDKO cells that were grown in the presence of Tet, which was shown to be a statistically significant effect (Fig. 4A). After resumption of growth around day 8, cells displayed similar EC$_{50}$ values to control cells.

*Re-expression of *Tb*GS in the cDKO *Tb*GS cell line was not the result of genetic mutation*

To assess if parasites had acquired a genetic mutation leading to loss of Tet-regulation, *Tb*GS cDKO parasites capable of growing 10 days after removal of Tet were used to generate clonal lines using limited dilution. These cell lines were then grown in media containing Tet for 3 days prior to removal of Tet to monitor the effects on cell growth (Fig. A4). Re-cloned parasites underwent a period of stalled growth after the removal of Tet similar to the original clonal lines suggesting that escape from Tet-regulation did not result from an inheritable genetic mutation.

*FACS analysis of cDKO cells provides evidence for multiple populations*

We investigated the possibility that clonal cDKO cell lines contained multiple populations with variable responses to Tet withdrawal that would suggest that not all cells in the population experienced the same level or kinetics of *Tb*GS depletion. A previously described live/dead cell based fluorescence activated cell sorting (FACS) assay (43) was used to evaluate the *Tb*GS cDKO cell line for mixed populations of live versus dead or dying cells. Ethidium and calcein staining were used for the analysis. Ethidium enters cells with compromised membranes and becomes fluorescent upon binding to nucleic acid indicating dead or dying cells. Calcein is a cell permeable non-fluorescent compound that is cleaved by non-specific esterases upon crossing the cell membrane to produce a fluorescent compound that is retained within the cell. Cells that are positive for calcein have retained esterase activity, and calcein is considered to be a live cell marker.
Cells were analyzed using side-scatter (SSC) and forward-scatter (FSC) followed by FACS analysis of calcein and ethidium fluorescence in the FL1 and FL3 channels, respectively (Fig. 5). Control cells contained 94% calcein only positive cells, consistent with a healthy growing cell population, 5% cells double labeled with ethidium/calcein, which we define as unhealthy, and there was no evidence for completely dead cells (ethidium-positive only) (Fig. 5A and Fig. A6). Cells treated with the anti-trypanosomal drug eflornithine (200 µM) for 24 h on the other hand showed an increase in cells with higher side-scatter, and an increase in cells staining positive with ethidium suggesting they were dead or in the process of dying (Fig. 5A). Eflornithine treated cells were used to define the gating regions between the unhealthy and healthy populations of cells.

To analyze and compare GS cDKO cells by FACS at different time points after Tet withdrawal, three staggered growth curves were set up where Tet was removed at 24 h intervals allowing up to three different time points to be analyzed together by FACS on the same day. The growth curves from the study were identical with growth arrest occurring 4 d after Tet withdrawal (Fig. A5). As the TbGS cDKO cells progressed into growth arrest (days 3-5 after Tet withdrawal), a new population of cells was observed on the FSC v. SSC plot (quadrant marked as unhealthy). This new population fell within the same region as control dead parasite cells that had been treated with eflornithine and that stained positive for ethidium. When the two populations were analyzed for ethidium and calcein staining (gated populations), the unhealthy population showed a mixture of cells that were either ethidium positive alone (dead) or stained positive for both ethidium and calcein, suggesting cells with damaged membranes that still contained esterase activity. The percentage of ethidium or dual ethidium/calcein cells increased on day 3 and peaked on days 5 after Tet withdrawal (Figs. 5B and 5C). On day 5 ~75% of the
cells were in the dead or dying population, corresponding to the time point of most significant growth arrest and to greatest trypanothione depletion. These data confirm that that TbGS is essential for parasite survival. However, 25% of cells were not yet compromised enough by trypanothione depletion to stain with ethidium in this analysis. These data suggest that Tet withdrawal from the GS cDKO cells results in minimally two cell populations, one that is dead/dying and the other that shows delayed response to GS knockdown, allowing these cells time to upregulate GS expression and escape selective pressure.
Polyamine and trypanothione biosynthesis has been shown to be a key pathway for proliferation and infectivity of the African trypanosome, *T. brucei* (6). Knockdown of pathway proteins by gene disruption using RNAi leads to growth arrest, and several inhibitors of polyamine biosynthesis have been shown to have anti-trypanosomal activity, the most important to date being the ODC inhibitor efornithine that is currently a front-line treatment for late stage *T. brucei gambiense* infection. The polyamine and glutathione pathways are highly regulated in eukaryotic cells, however, the regulatory mechanisms that have been observed in other eukaryotes do not seem to be present in *T. brucei*. Instead, polyamine biosynthesis is uniquely regulated by the presence of a novel regulatory subunit of AdoMetDC, which activates the enzyme and also appears to be translationally regulated (23, 30, 31). Less is known about how the glutathione branch of the trypanothione biosynthetic pathway is regulated. Herein we show that *TbGS* encodes a functional GS enzyme that is essential for growth of mammalian blood form parasites, and we demonstrate that knockdown of *TbGS* leads to compensatory effects on the expression levels of other pathway enzymes.

Knockout of the *TbGS* gene in mammalian blood stage parasites led to >99% reduction in *TbGS* protein levels, cell growth arrest and complete depletion of trypanothione pools. These data show that like other enzymes in the pathway, *TbGS* is essential for parasite growth and confirm that trypanothione is an essential cofactor. The observed increase in sensitivity to the γ-GCS inhibitor BSO is also consistent with increased oxidative stress in the cells after depletion of *TbGS*. As was observed for knockout of TR (48), knockout of *TbGS* led to an initial stalling of growth but eventually parasites were able to escape the selective pressure leading to re-expression of *TbGS* and growth restoration. Cells that escaped growth arrest were recloned and
retained sensitivity to Tet withdraw and GS depletion suggesting that escape did not result from
a genetic mutation in the Tet promotor. Instead, epigenetic differences between different
subpopulations of the TbGS cDKO cells may lead to cell survival. Similarly, in the TR knockout
line gene deletions or nucleotide mutations were not identified, and growth restoration was
postulated to be due to epigenetic effects on TR expression (48). In another example, in the
conditional knockout of UDP-Glc-4'-epimerase, deletion of the TetR gene was responsible for
the growth restoration phenotype (49).

FACS analysis using live cell and dead cell markers was used to provide insight into the
mechanism of escape and regrowth that occurred after TbGS knockdown. These data suggest that
at least two cell populations arise after TbGS expression is shut off by Tet withdrawal. At days 4
and 5 after loss of GS expression the cells are in a transition. While most of the TbGS-depleted
cells (75%) stain with ethidium homodimer by day 4, suggesting that they have compromised
membranes and are committed to cell death, a significant proportion of cells (25%) do not. Thus,
at least by this marker of cell death, 25% of the cells appear not to be severely compromised by
depletion of TbGS and trypanothione pools. This result could be explained by differences in the
kinetics of TbGS depletion in the two cell populations, the result of a stochastic process or
epigenetic differences. If trypanothione depletion in this subset of cells is delayed, they may not
have experienced trypanothione depletion for long enough to lead to cell damage. While FACS
analysis provides data about individual cell populations, the growth curve analysis and
trypanothione measurements can show only average results. Trypanothione levels on day 3 are
reduced by about 50%. That could be interpreted to mean that all cells had lost half their
trypanothione content, or it could reflect an average value between a cell population that was
fully depleted of trypanothione and a second population that lags behind and still retains a
substantial amount. It may take at least 24 h of trypanothione depletion before cells become ethidium-positive, so only cells that reached complete depletion faster (by day 3) have been irreversibly damaged before TbGS expression is upregulated, trypanothione pools are restored and growth reversal occurs. For the fraction of cells where onset of trypanothione depletion is slower they will have more time for an epigenetic event to cause upregulation of GS expression before irreversible damage has been done. Our data suggest that upregulation of GS by only a few percent would be enough to restore trypanothione biosynthesis to the parasites leading to normal growth. These data highlight the importance of considering individual cell populations and not just an average response in order to fully understand the mechanism and function of analyzed genes.

In previous studies, we demonstrated that the first enzyme in the glutathione biosynthetic pathway γ-GCS was essential for parasite growth (22). In these studies we also showed that exogenously added glutathione was able to rescue cell growth arrest that occurred after γ-GCS knockdown by RNAi. The ability of glutathione to similarly rescue the TbGS double knockout suggests that like in yeast (27, 50) glutathione may be transported into the parasite intact, though there is no obvious homolog of the yeast-like glutathione transporter in the T. brucei genome. These data are in contrast to the glutathione transport mechanism that has been described in mammalian cells, which use a transpeptidase transporter mechanism that couples glutathione cleavage to cysteinyl-glycine and γ-glutamyl with conjugation of the γ-glutamyl moiety to an amino acid, and subsequent uptake of the respective products of both reactions (27, 50). Notably the glutathione levels (1 mM) that were required to rescue growth after TbGS knockdown are higher than is present in human plasma (range 4 – 40 uM) (www.hmdb.ca,(15, 20)) so parasites would be unable to overcome inhibition of GS through uptake of exogenous glutathione in the
blood. However, quantitation of protein expression levels in the TbGS cDKO and RNAi lines suggests that >97% depletion of TbGS is required before trypanothione is depleted and cells begin to die, showing that a small molecule inhibitor of TbGS would need to nearly fully inhibit the enzyme. This is in contrast to ODC and AdoMetDC where ~80% inhibition is sufficient (23, 30).

Regulatory control of the polyamine biosynthetic pathway in *T. brucei* appears to center around the AdoMetDC regulatory subunit prozyme, which responds to decarboxylated AdoMet depletion by upregulation of prozyme expression and to a lesser extent ODC (23, 30). Interestingly, knockdown of TbGS led to a decrease in prozyme and ODC protein and to an increase in γ-GCS protein. These data suggest that the cells respond to trypanothione depletion by increasing levels of the first enzyme required for glutathione synthesis in a classical feedback mechanism, though whether the metabolic trigger is glutathione or trypanothione levels remains unknown. The concomitant decrease in levels of two key polyamine biosynthetic enzymes appears to be a cell response to limit buildup of polyamine intermediates in the situation where trypanothione is no longer a sink for spermidine flux. Spermidine levels remain unchanged after depletion of TbGS, consistent with this result. In our previous study, we showed that decarboxylated AdoMet levels were inversely proportional to AdoMetDC prozyme levels, suggesting that elevated decarboxylated AdoMet leads to reduced prozyme expression (30). SpdSyn from human and *Plasmodium* have been shown to be feedback inhibited by methylthioadenosine, a reaction product of SpdSyn chemistry (51, 52) and both spermidine and methylthioadenosine have been demonstrated to bind SpdSyn by crystallographic studies (53). Thus, a working model that explains the available data suggests when *T. brucei* cells no longer require spermidine for trypanothione biosynthesis, spermidine or methylthioadenosine might
feedback inhibit SpdSyn, leading to build up of dcAdoMet and to down regulation of prozyme expression. Down regulation of ODC would then also be needed to maintain constant putrescine levels, since in this model putrescine flux into spermidine would be decreased. Direct studies with *T. brucei* SpdSyn would be required to confirm this hypothesis.

In conclusion *TbGS* is an essential protein in *T. brucei* and our studies suggest that >97% inhibition of the protein will be required to lead to cell growth arrest. We find that glutathione is unable to rescue the *TbGS* cDKO cell line at physiological concentrations, providing a path forward for exploring *TbGS* as a potential drug target in the parasite. We also find that knockdown of *TbGS* leads to an increase in γ-GCS, suggesting that γ-GCS may be the key regulatory control point for glutathione biosynthesis. Additionally, prozyme and ODC levels decrease, providing additional evidence that the AdoMetDC prozyme is the key regulatory protein in the polyamine biosynthetic pathway in *T. brucei* and providing evidence for cross talk between the polyamine and glutathione branches of the pathway.
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Table 1. Steady-state kinetic parameters of recombinant TbGS

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<tr>
<th></th>
<th>γ-GC</th>
<th>Glycine</th>
<th>ATP</th>
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<tr>
<td></td>
<td>kcat (s⁻¹)</td>
<td>Km,app (mM)</td>
<td>kcat/Km,app</td>
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<tr>
<td>T. brucei</td>
<td>7.1 ± 0.1</td>
<td>0.040 ± 0.004</td>
<td>1.8x10⁵</td>
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<td>A. thaliana</td>
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<td>0.039</td>
<td>3.2x10⁵</td>
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Error represents the standard error for n=6. Units for kcat/Km,app are M⁻¹ s⁻¹. Published values from a(11); b(45); c(44).
Figure Legends

Figure 1. Trypanothione biosynthetic pathway in *T. brucei*. GS, glutathione synthetase; γ-GCS, γ-glutamylcysteine synthetase; TryS, trypanothione synthetase; TR, trypanothione reductase; ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase.

Figure 2. *Tb*GS RNAi growth curve. RNAi was induced using a Tet-inducible stem loop vector. Tet was added every 24 h to maintain knockdown. Protein levels were evaluated by western with DHODH as a loading control. Data are shown for three replicates and error bars represent the standard error of the mean, though because of the log plot they are typically not visible.

Figure 3. Analysis of *Tb*GS cDKO cell line. A. Growth curve of the *Tb*GS cDKO cell line in the presence and absence of Tet. For the +Tet group, Tet was added daily to maintain expression of the ectopic copy of *Tb*GS protein. Cell number is represented as the product of cell density and total dilution. Three clonal lines were evaluated with triplicate data collected for each. A representative data set for one line is displayed. B. Representative western blots of selected polyamine and glutathione biosynthetic enzymes. For the prozyme antibody * marks the specific prozyme band, whereas the band above arises from non-specific labeling. DHODH was used as a loading control. C. Intracellular polyamine levels measured by HPLC. Data for 3 biological replicates, each in quadruplicate, (n = 12 in total) is displayed. D. Intracellular reduced trypanothione levels measured by HPLC. Data for 3 biological replicates, each in triplicate, (n = 9 in total) is displayed. Error bars represent the standard error of the mean.

Figure 4. Effects of glutathione biosynthetic inhibitors and glutathione on the GS cDKO line. A. Knockdown of GS increases sensitivity to BSO. The EC<sub>50</sub> of BSO was measured on cells.
grown in the presence (EC$_{50} = 270 \pm 14$ uM) and absence of Tet (EC$_{50} = 88 \pm 25$ uM). Cells were grown without Tet for 2 days to deplete GS and trypanothione before incubation with various levels of BSO. EC$_{50}$ values were measured independently three times and were calculated in each case from triplicate data points. The plot represents the mean of the three experiments and error bars are the standard error of the mean. The P values were calculated using the student test. **B. Metabolic rescue of growth in the GS cDKO cell line.** Glutathione (1 mM) or glutathione ethyl ester (1 mM) were added to parasite cultures simultaneously to the removal of Tet and the effects on cell growth were monitored. The +Tet control is included as a comparator. Data were collected in triplicate and errors represent the standard error of the mean.

**Figure 5. FACS analysis of TbGS cell line.** **A.** Analysis of control TbGS cDKO cells (+Tet) in the absence and presence of efornithine (200 µM) using forward scatter v. side scatter to observe cell shape and morphology and FL1 v. FL3 channels to observe calcein or ethidium positive cells, respectively. **B.** Bar Graph summarizing the effects of TbGS knockdown (-Tet) on the percentage of cells that are either calcein positive (white bar), ethidium positive (grey bar) or calcein and ethidium double positive (hatched bar). Numbers were obtained from ungated fluorescent populations where unstained cells set quadrants. Data represent the average and standard deviation of the mean for n=3 independent experiments **C.** Representative samples of FACS data obtained from TbGS cDKO cells on days 3, 5 and 7 minus Tet. Ungated populations forward v. side scatter plots are shown on the left while the ungated fluorescent populations are shown in left center plots. Within the fluorescent population plots, the upper left quadrant represents cells staining with ethidium only, the lower right quadrant represents cells staining with calcein only, and the upper right quadrant represents cells that stain with both ethidium and calcein. The populations that were gated by their forward versus side scatter plot (gates shown in
first plot) are shown in the two right hand columns. The health/unhealthy gates were determined by healthy proliferating cells and cells that had been treated with drug (eflornithine) for 24 h where the majority of cells were dead (stained with ethidium only).