A *Trypanosoma brucei* ORFeome-based Gain-of-Function Library reveals novel genes associated with melarsoprol resistance

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24 **LIST OF ACRONYMS AND ABBREVIATIONS**

- 25 ORF – Open reading frame
- 26 RIT – RNA-Interference Targeted
- 27 GOF – Gain-of-Function
- 28 RFU – Relative fluorescence units
- 29 DOX – doxycycline
- 30 SM – Single marker
- 31 LP – Landing pad
- 32 NGS – Next generation sequencing
- 33 GSH1 – γ -glutamylcysteine synthetase
- 34 TR – Trypanothione reductase
- 35 T(SH)₂ – Trypanothione
- 36 GSH2 – glutathione synthetase
- 37 ODC – Ornithine decarboxylase
- 38 TXN – Tryparedoxin
- 39 RR – Ribonucleotide reductase

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ABSTRACT

Trypanosoma brucei is an early branching protozoan that causes Human and Animal African Trypanosomiasis. Forward genetics approaches are powerful tools for uncovering novel aspects of Trypanosomatid biology, pathogenesis, and therapeutic approaches against trypanosomiasis. Here we have generated a *T. brucei* ORFeome consisting of over 90% of the targeted genome and used it to make an inducible Gain-of-Function library for broadly applicable forward genetic screening. Using a critical drug of last resort, melarsoprol, we conducted a proof of principle genetic screen. Hits arising from this screen support the significance of trypanothione, a key player in redox metabolism, as a target of melarsoprol and implicate novel proteins of the flagellum and mitochondria in drug resistance. This study has produced two powerful new genetic tools for kinetoplastida research, which are expected to promote major advances in kinetoplastida biology and therapeutic development in the years to come.

INTRODUCTION

Trypanosomatids are a major parasitic lineage that include the African trypanosomes, American trypanosomes, and *Leishmania* spp. (family *Trypanosomatidae*, order *Kinetoplastida*), which collectively cause death and disease in millions of people living in tropical and sub-tropical regions (1). There are no vaccines against this family of parasites and the limited number of anti-Trypanosomatid drugs present ongoing challenges of host toxicity, complex treatment regimens, and burgeoning drug resistance (2). These evolutionarily ancient eukaryotes have highly divergent genomes from well-established model organisms with more than 50% of open reading frames (ORFs) annotated as hypothetical (3). Trypanosomatid parasites appear to have diverged from a shared ancestor around 100 million years ago, yet have retained a high proportion of orthologous gene clusters and chromosomal synteny. Of the 9,068 genes in the *Trypanosoma brucei* (African trypanosome) genome, 6158 are orthologous with both *Trypanosoma cruzi* (American trypanosome) and *Leishmania major* (3). Many of the nonsyntenic regions of the genome appear to be directly associated with organism specific aspects of parasitism, such as surface antigen variation, leaving many biological functions highly conserved among kinetoplastida parasites (3). Trypanosomatids share essential subcellular structures, such as the flagellum, glycosomes (specialized organelles of glycolysis), and the kinetoplast (a network of mitochondrial DNA). While reverse genetics based on well-established models can promote discrete advances, forward genetics approaches have the potential to uncover important aspects of kinetoplastida biology shared among orthologous genes.

T. brucei, the causative agent of Human African trypanosomiasis (HAT), has traditionally been the most genetically tractable of the Trypanosomatid parasites. Discoveries made in *T. brucei* have provided critical understanding to the biology of other kinetoplastida as well as providing insights into eukaryotic evolution (4-7) and uncovered broadly significant biological processes, such as GPI-anchors and mitochondrial RNA-editing (8-10). For the past decade a whole-genome interfering-RNA (RNAi) knock-down library has been the primary forward-genetics tool in *T. brucei* resulting in identification of essential genes and genes associated with drug resistance and pathogenesis, to name a few (11-13). A strength of the RNAi library and associated RNA-Interference Targeted (RIT)-seq approaches is the identification of genes that result in a loss-of-fitness phenotype (14). However, knockdown strategies may fail when screening for a cellular event that requires a particular gene to be turned on rather than turned off. Furthermore, they preclude identification of essential genes associated with key processes (14). A Gain-of-Function library approach may be more effective in the identification of genes whose expression is critical to the initiation of the event. For example, Gain-of-Function screens have been employed to identify drug targets, as well as resistance mechanisms (15-17), and in areas of basic biology have been critical for discoveries in the areas of chromosome segregation and cell cycle, signal transduction, transcriptional regulation, cell polarity, and stem cell biology (18). Thus, Gain-of-Function screens are a powerful tool that can be used as a complement to loss-of-function screening to uncover new biology in diverse areas.

Traditional methods of inducible gene library formation by cDNA synthesis and cloning are not viable for *T. brucei* as most gene expression regulation in kinetoplastida

occurs post-transcriptionally, with 5' and 3'UTRs playing a major role in determining steady state levels of their associated transcripts (19). Existing overexpression libraries for *T. brucei*, generated by whole-genome fragmentation methods have demonstrated the usefulness of this approach, for example, in the identification of the benzoxaborole drug target (polyadenylation specificity factor, CPSF3), despite their somewhat limited ability to control individual ORF expression (20-23). In addition, generation of an ORFeome provides a tool that can be applied to the downstream generation of multiple whole-genome methodologies including yeast 2-hybrid libraries, tagging libraries, inducible expression libraries for Gain-of-Function studies, and complementation for loss-of-function mutant phenotypes (24-27). A *T. brucei* ORFeome would permit production of an inducible Gain-of-Function library, which is expected to be an ideal design for the identification of novel gene functions based on observable phenotypes in large scale screens.

African trypanosomiasis is transmitted by the tsetse vector and characterized by a stage one bloodstream infection (though other tissues are implicated (28)) and a stage two central nervous systems (CNS) infection, which is invariably fatal if left untreated. HAT is predominantly caused by *T. b. gambiense*, whose stage two infections can be treated by NECT (nifurtimox/eflornithine combination therapy) (2) and fexinidazole (29). To a lesser extent HAT is caused by *T. b. rhodesiense*, which rapidly progresses to stage two infection and host death. Melarsoprol, an arsenical compound, remains the only drug available to treat stage two *T. b. rhodesiense* infections despite treatment challenges, high levels of host toxicity, and increasing reports of drug resistance (30). Following melarsoprol uptake by the parasite-specific P2 adenosine

transporter (*AT1* gene) and aquaglyceroporin transporter (*AQP2* gene), intracellularly metabolized melarsoprol interacts with trypanothione (30). Trypanothione is a kinetoplastida specific form of glutathione required for thiol based redox metabolism (31). While the inactivation of trypanothione by melarsoprol has been demonstrated, and effects on cellular redox metabolism are known, it is has remained unclear if melarsoprol has more than one intracellular target and if inactivation of trypanothione is responsible for cell lysis (30).

In this study we sought to produce a whole-genome *T. brucei* ORFeome and use it to create a Gain-of-Function library for genetic screens. Following PCR amplification and cloning, the resulting ORFeome includes more than 90% of the targeted *T. brucei* ORFs (>6500 of 7245 ORFs targeted). The ORFeome was then introduced into bloodstream *T. brucei* (*Lister427*) to produce the *T. brucei* Gain-of-Function (GoF) Library. Because of melarsoprol's clinical significance and unknown aspects of its functionality, it was selected for a proof of principle GoF library genetic screen. Following the isolation of melarsoprol survivor populations from the induced GoF library, we identified 57 genes that were significantly overrepresented, which included an anticipated biosynthetic precursor of trypanothione. In addition, we identified sets of genes associated with gene expression, proteins predicted to localize to the mitochondria, and others that localize to regions of the flagellum, whose association with melarsoprol have not been reported previously. Collectively, these findings support the usefulness of the broadly applicable *T. brucei* ORFeome developed herein, the essential functionality of the resulting GoF library, and provide new insights into mechanisms of melarsoprol-based cell killing and genes whose induced expression can

promote drug tolerance. Thus, we have generated two genetic tools and a novel dataset, which are all posed to provide new insights into Trypanosomatid biology, pathogenesis, and aid in the development of novel therapeutics.

RESULTS

Generation of a Trypanosoma brucei ORFeome

T. brucei and others in the class kinetoplastida primarily regulate gene expression post-transcriptionally based on transcript stability mediated by 5' and 3' UTRs (19). Thus, previous production of overexpression libraries, based on whole-genome fragmentation and cloning (20,21), cannot ensure that individual ORFs are similarly activated upon induction. To mitigate these problems, we produced a *T. brucei* ORFeome using PCR amplification, which ensures the placement of each ORF under the same inducible gene regulation system. ORF start and stop sites for all *T. brucei* genes were obtained from available *TREU927* ribosomal profiling data for 9200 genes (32). The targeted *T. brucei* ORFeome filtered out 1956 ORFs that were unsuitable in size (<100 bp or > 4500 bp), an undesired product (annotated as ribosomal genes, VSGs, ESAGs, pseudogenes, or hypothetical unlikely), or if they were known multidrug resistant channels (including MRPA whose overexpression is known to cause melarsoprol resistance (33)). PCR primers for the resulting 7245 targeted ORFs were designed *in silico* with matched melting temperatures and *attB1* cloning sites were added to both forward and reverse primers for subsequent Gateway cloning. The resulting 7245 primer pairs for ORFeome amplification were synthesized and

resuspended in 21 separate 384-well plates that were organized by ORF size and gene annotations as either 'known' or 'hypothetical' (Table 1 and SUP. 1).

Each gene of the ORFeome was PCR amplified in 384-well format from *Listeria* 427 genomic DNA. The general quality of the PCR reactions was assessed by the addition of SYBR green and measurement of the resulting Relative Fluorescence Units (RFU) (FIG. 1A). Based on the SYBR green assessment, the initial PCR reactions resulted in the successful amplification of 94% of the ORFeome (6820/7245 ORFs) (FIG. 1B & SUP. 2). Most PCR plates resulted in $\geq 90\%$ amplification and only three of the 384-well plates between 80-90%. To increase our coverage, we attempted to reamplify 429 failed PCR reactions and succeeded in producing 228 products resulting in a final total of 7039 PCR products amplified (97.2% of the targeted genome).

The PCR reactions from each 384-well plate were pooled (10 μ l from each well) into 21 corresponding PCR product pools, irrespective of the SYBR result, which maintained the product size range associated with each plate (Table 1). Each resulting size-sorted PCR pool was run on agarose gels and gel purified prior to gateway cloning (FIG. 1C). Thus, the DNA bands visualized on gels in Figure 1C represent hundreds of similarly sized PCR products arising from the same PCR plate, which were gel extracted prior to gateway cloning. Gateway cloning occurred in two steps: 1) introduction of ORFs into a general pENTR vector, which can function as a useful repository for the ORFeome, and 2) transfer of ORFs into a pDEST vector designed for ORF integration into the *T. brucei* rDNA spacer region under the rRNA promoter and tetracycline inducible gene regulation (FIG. 1D and SUP. 3 pSUN6 vector map). For the purpose of this study, we refer to the entire set of cloned ORFs as the pENTR

ORFeome and the resulting set of pDEST (pSUN6 derived) libraries ready for transfection into the *T. brucei* genome as the pTrypLib ORFeome.

Sequencing, assessment, and final coverage of the *T. brucei* ORFeome

The resulting *T. brucei* pENTR and pTrypLib ORFeome harboring plasmids were each pooled and prepared for Illumina sequencing by tagmentation. In order to assess which of the 7245 targeted ORFs were not present in the pENTR and pTrypLib ORFeome harboring plasmids, we aligned the sequencing reads to the trypanosome genome, removed PCR duplicates, and counted the number of reads corresponding to each targeted gene. Because we knew that some of the targeted genes were highly similar or duplicated, we aligned the reads under two modes, one that required unique alignments and one that allowed multiple alignments. Both datasets were then assessed to determine how many genes were ‘missing’ from each library; defined as any targeted gene with zero aligned reads. The analysis of uniquely aligned reads determined that 1,845 genes were missing from the pENTR library and 2,593 ORFs were missing from the pTrypLib ORFeome. For reads that were multiply aligned, 1,656 ORFs were missing from the pENTR library and 2,420 ORFs were missing from the pTrypLib ORFeome (Figure 2A – pENTR_1, pDEST_1).

Our analysis indicated that many of the ‘missing’ ORFs were positive by SYBR analysis following PCR and showed appropriately sized bands when randomly selected missing ORFs were run on agarose gels (approx. 100 PCR products visualized, data not shown). Thus, we predicted that our DNA input was exceeding the functionality of the gateway system in these cloning stages and that the largest losses arose during the

formation of the pENTR ORFeome. To address this limitation, PCR products representing each missing ORF were isolated from the original PCR plates (using a Perkin-Emer Janus Automated Workstation to select 2450 PCR products) and used to produce 8 additional size-sorted ORF sub-pools (SUP. 4 – Table of cloning pools including ‘MISS_1-8’), which were gel purified and gateway cloned. These 8 new plasmid pools were then combined with the previous pools and sequenced by tagmentation, as described above.

Our final ORFeome analysis indicated that 457 and 292 ORFs were missing after both rounds of cloning in the pENTR vector using unique or multiply aligned reads, respectively. Gateway cloning into the final pDEST vector to form the pTrypLib ORFeome resulted in 636 and 442 missing ORFs using unique or multiple alignments, respectively (FIG. 2A – pDEST_ Final and SUP. 5 – pENTR and pDEST missing ORFs). Thus, the final pTrypLib ORFeome contains between 6,609 and 6,803 (depending on the type of analysis used) *T. brucei* ORFs, resulting in the successful inclusion of 91-94% of the targeted ORFs.

The resulting sequencing data were used to assess both the overall coverage of ORFs in the library and if specific ORF characteristics (length or annotation) resulted in biases during ORFeome generation. First, we analyzed the coverage of each ORF in the pTrypLib ORFeome. For ORFs that were present in the plasmid libraries (based on reads aligned), we analyzed the count distribution in the sequenced pDEST plasmids for both uniquely and multiply aligned reads. This analysis showed that most ORFs resulted in $\log_2(\text{RPKM})$ values between 0 and 10 (FIG. 2B and SUP. 6). Thus, the number of poorly represented ORFs ($\text{RPKM} < 1$) is 195 for uniquely aligned reads and

369 for multiply aligned reads, representing 3% or 5% of all ORFs in the library, respectively. The $\log_2(\text{RPKM})$ value was then plotted against each corresponding ORF length to determine if gene length affected their representation in the ORFeome (SUP. 6). No strong correlation was observed between ORF length and coverage in the pTrypLib ORFeome, with a best fit line showing a small negative slope for both unique and multiply aligned reads (-.000673 and -.000778, respectively). Thus, in general, shorter ORFs are not significantly more highly represented than longer ORFs.

Next, we sought to determine if there were patterns in the properties of genes missing from the pTrypLib ORFeome. Histograms of ORF length were generated for targeted genes, and compared to histograms of ORF lengths that were missing from the pDEST library. The distribution of ORF lengths in the targeted library was similar to those missing from the the pDEST library. These results indicated the successful cloning of the ORFeome into pDEST and that the resulting libraries were not skewed toward larger or smaller ORFs (FIG. 2C). Thus, we have generated both pENTR ORFeome and pTrypLib ORFeome genetic tools that will broadly support progress in Trypanosomatid research.

Gain-of-Function library generation from the inducible pTrypLib ORFeome

The pTrypLib ORFeome contains more than 6,500 *T. brucei* ORFs ready for transfection into the genome using targeting to the rDNA spacer regions by homologous recombination. To increase the frequency of transfection and ensure integration into a single rDNA spacer we employed the established method of generating a Landing Pad (LP) parental cell line that targets recombination to a single iteration of the rDNA spacer

repeats. The stable LP cell line is then transfected with pRPa-Sce* harboring the ISceI enzyme and restriction site directed for recombination in the targeted rDNA spacer (FIG. 3A) (12). Thus, following ISceI DNA double-stranded break induction by doxycycline (Dox), transfected ORFs are recombined into the targeted rDNA spacer resulting in more consistent transcription induction among library incorporated ORFs.

Validating inducible ORF transcription from a whole-ORFeome transfection would be challenging to detect due to the overall complexity of the resulting cell populations (harboring approx. 6,500 ORFs in multiple copies). Thus, prior to transfecting the TrypLib ORFeome we sought to verify that the transcription of individual ORFs within this library would be inducible and highly transcribed. Toward this goal we generated a subset 'mini-library', in which one pDEST bacterial pool was plated (SUP. 2, Plate name – "2_Known", ORF range 849-1056 bp) and six random individual colonies isolated, plasmid DNA prepared, and sequenced to identify the ORF harbored in each pDEST vector (FIG. 3B). The resulting plasmids were pooled equimolarly, transfected into *T. brucei*, recovered as a single population of parasites (the ORFeome 'mini-library'), and then grown with or without Dox induction for 12 or 24 hours prior to RNA extraction. The relative level of four ORFs were determined using RT-qPCR with a forward oligo specific to pDEST (*attB1* site) and a reverse oligo specific to individual ORF. Because these oligo pairs can amplify the ORFs in the ORFeome library (exogenous) and not endogenous ORF, we can measure the inducibility of our ORFeome library. Levels of ORF transcripts were compared between uninduced and Dox induced culture. All ORFs showed increased transcript levels following Dox induction at 12 and 24 hours, 3 of the 4 ORFs resulted in approximately 10-30-fold

increased after 12 hours and 50-600-fold increased after 24 hours (FIG. 3B). Thus, the overall strategy of ORFeome exogenous transcription induction from pTrypLib cloned ORFs is viable.

We then generated a doxycycline inducible *T. brucei* Gain-of-Function (GoF) library from the pTrypLib ORFeome by transfection of 360 million cells (LP parent following pRPa-Sce incorporation of IScel cassette) and selected in blasticidin. After 72 hours of selection mock transfected cells were dead and approx. 60 million cells survived transfection (FIG. 3C – red line), which were then propagated to 3 billion cells over 3 days to generate the *T. brucei* GoF library. Assessment of the resulting GoF libraries by Illumina sequencing requires a specialized procedure to ensure that only the introduced pTrypLib ORFs are sequenced and not the endogenous gene copies. Illumina sequencing libraries are prepared using a gateway *attB1* site specific forward oligo, during PCR enrichment, and a similar custom sequencing primer complementary to the *attB1* site upstream of the introduced ORF. Thus, the resulting sequencing reads primarily correspond to the 5' ends of the introduced ORF (SUP. 7A – Sequencing strategy). Immediately following transfection and recovery in blasticidin, the *T. brucei* GoF library consisted of 5,818 ORFs and then between 4,269 and 4,360 ORFs when thawed and propagated for 3 days. The GoF 'INPUT' library, arising from 3 days of library propagation, represents the full complement of ORFs present before a screening condition is applied (FIG. 3D). It is unclear if the apparent loss of 1,459 genes arose through an artifact associated with a relatively low number of NGS reads returned from those samples or a true loss of content between library transfection and the subsequent thawing of frozen library.

Identification of overrepresented GoF library ORFs in a melarsoprol survivor screen

Melarsoprol remains the only drug available for the treatment of second stage *T. b. rhodesiense* infection, yet it is highly toxic and difficult to administer (30). In addition, there have been increased reports of melarsoprol resistance and treatment failures in sub-Saharan Africa since the 1990s, only some of which have been explained by transporter mutations (34-37). Since melarsoprol has been widely studied, with both known and unknown genes associated with its cytotoxicity and treatment failures, it was selected for a *T. brucei* GoF library proof of principle genetic screen.

To identify ORFs whose induced expression promote survival in the presence of lethal doses of melarsoprol, we tested three concentrations of drug on the LP parental cell line. Similar to previous reports, we observed that *T. brucei* LP cells died after 3 days in 35 nM, 5 days in 26 nM, and 7 days in 17 nM melarsoprol (17 nM is approximately two times the standard EC50) (FIG. 4A) (11). We predicted that the progression of cell killing might be a critical factor in design of the GoF Library melarsoprol resistance screen. That is cells that die too quickly might not have the opportunity to successfully activate ORFs that confer resistance. In fact, when a genetic screen was attempted in 35 nM melarsoprol, the death of the culture was delayed by one day in both uninduced and induced GoF library cells when compared with parental landing pad, yet no survivor population emerged (FIG. 4B – Red dashed and dotted lines overlap). In contrast, when cells harboring the GoF library were treated with 17 nM melarsoprol, uninduced cells (no Dox), took almost 4 days longer to die than the LP

parental line (preliminary test, data not shown). Thus, a GoF forward-genetic screen was prepared in 17 nM melarsoprol.

The experimental steps for the 17 nM melarsoprol GoF genetic screen, including 24 hour pre-induction of all Dox treated cultures (Day -1), are shown at the bottom of Figure 4C. A freezer vial containing 100 million GoF library harboring cells was thawed and propagated for less than 3 days before splitting it into triplicate samples for the following conditions: GoF 'INPUT' (grown for 3 days without melarsoprol selection), GoF 17 nM No Dox (uninduced library maintained in melarsoprol), and GoF 17 nM +Dox (Dox induced library maintained in melarsoprol) (FIG. 4C). The parental cell line (LP) was maintained in melarsoprol alongside GoF library cultures and were below the limit of detection (FIG. 4 – grey dashed line at 1×10^2 cells/mL) on day 4 with complete cell death observed from day 5 until the completion of the screen on day 10. In contrast, uninduced (No Dox) GoF Library harboring cells showed partially delayed melarsoprol killing, with counts below detection at day 5, but some observable surviving cells in culture until day 8, after which all cells appeared dead (FIG. 4C – Blue dotted line). The melarsoprol treated, Dox induced GoF library was distinct from LP parent by day 3, and by day 5 populations of survivors emerged and grew similarly well in all three induced GoF (+Dox) biological replicates (FIG. 4C – Blue dashed line). At day 7 the biological replicates of induced (+Dox) GoF library melarsoprol survivor cultures were split into media with and without Dox during continued treatment with 17nM melarsoprol (FIG. 4C – Green lines). The uninduced (no Dox) cultures died rapidly over the next 2 days (dotted green line), whereas the induced GoF library, once again, survived and grew robustly (dashed green line) in the presence of melarsoprol. Thus, survival in the

presence of melarsoprol was dependent on GoF library induction. Biological triplicate cultures of melarsoprol survivors were harvested from both GoF 'MEL-1x' and GoF 'MEL-2x' (resulting from the day 7 split) on day 9 and day 10 respectively (FIG. 4C – blue and green dashed lines, red circles indicate the date of harvest). Genomic DNA from biological triplicate cultures of untreated INPUT (day 3, black circle), GoF 'MEL-1x', and GoF 'MEL-2x' cultures (9 cultures total grown to ~1 million cells per mL 200 mL each) was prepared for NGS analysis.

To identify the pTrypLib ORF incorporated into the GoF library harboring cells recovered from each replicate and condition (INPUT, GoF MEL-1x, and GoF MEL-2x), genomic DNA was fragmented and prepared for ORFeome specific Illumina sequencing (shown in SUP. 6). During PCR enrichment INPUT samples were prepared in two ways: long elongation time (INPUT1, 75 seconds) and shorter elongation time (INPUT2, 20 seconds). Sequencing data was obtained in biological triplicate from the two sets of INPUT libraries (no melarsoprol treatment) and the two sets of melarsoprol selected parasites (MEL-1x and MEL-2x). We performed principle component analysis on the sequencing data from both unique and multiple alignment datasets (FIG. 4D). The PCA analysis shows two clearly separated clusters for untreated and melarsoprol treated samples, with most biological replicates clustering proximal to one another. Melarsoprol treated samples were distinct from INPUT and show more variation between samples (FIG. 1D). In order to check whether ORF length influenced the level of coverage in the genomic INPUT libraries, we analyzed whether ORF representation in the INPUT libraries correlated with ORF length. We observed, at best, a weak negative association between gene length and normalized read count (slopes of -.00042 and -.00045 for

unique and multiple alignment analysis, respectively), indicating that ORF representation in the library is largely independent of ORF length (SUP. 8)

We reasoned that any gene whose induction contributed to melarsoprol resistance should be overrepresented in libraries generated from melarsoprol survivor populations. The designation 'overrepresented' indicates that the normalized read count for a particular ORF in the presence of melarsoprol is higher in libraries arising from doxycycline induced parasites harboring the GoF library (MEL-1x and MEL-2x) when compared with the untreated GoF library (INPUT), which suggests that overrepresented genes have enhanced survival in the presence of drug. To determine the fold change that represents a valid difference between melarsoprol treated and untreated conditions, we compared each of the three biological replicates of INPUT2 to one another and counted the number of ORFs with a 1.5, 2.0, or 4.0-fold in normalized read count (FIG. 5A – INPUT2 replicates compared pairwise). By evaluating the biological variation between similarly treated replicates we found that while many ORFs varied in normalized read count by greater than 1.5-fold between replicates (more than 300), very few ORFs varied by greater than 4-fold (FIG. 5A, similar results obtained from INPUT 1, data not shown). Thus, we used a 4-fold change in normalized read count between melarsoprol treated and untreated samples as the minimum threshold for identifying an ORF as overrepresented (a 'hit') in this study.

In order to identify ORFs overrepresented in melarsoprol survivor populations, we aligned reads from each of the 12 samples (3 reps of each: INPUT1, INPUT2, MEL-1x, and MEL-2x) to the genome, and then calculated the number of reads that fell within the first 100bp of each of the ORFeome targeted ORFs (SUP. 7B – Example read

alignments). DESeq was used to normalize read counts between libraries (38). We performed this analysis for uniquely aligned reads and multiple alignments. DESeq was used to identify genes that were ‘differentially expressed’ between melarsoprol selected samples and INPUT samples with a p adjusted value of < 0.05 (FIG. 5B) (38). In this context, ‘differential expression’ simply refers to an ORF being overrepresented or underrepresented in the melarsoprol selected samples. To be considered as a potential hit, we required a minimum normalized read count of 5 in INPUT samples to avoid ORFs being called as overrepresented that were simply poorly covered. To identify putative hits, we then asked which of the differentially represented ORFs with a p adjusted value < 0.05 had a normalized read count > 4 -fold higher in 3 biological replicates of melarsoprol treated populations (MEL-1x and MEL-2x) compared to untreated populations (INPUT1 and INPUT2). Four different comparisons were analyzed using this pipeline: INPUT1 vs. Mel-1x, INPUT1 vs. Mel-2x, INPUT2 vs. Mel-1x, and INPUT2 vs. Mel-2x (FIG. 5B). Figure 5C shows a volcano plot of DESeq generated significance values compared with fold change for the comparison between INPUT2 and MEL-1x. After hits had been called for each individual comparison, we identified the hits common between all 4 comparisons for both uniquely and multiply aligned reads (FIG. 5D and SUP. 9 – Tables of all comparisons). These analyses resulted in the identification of 57 overrepresented ORFs (uniquely aligned) in the GoF melarsoprol survivor populations compared to INPUT populations. In the comparison of INPUT2 vs. MEL-1x depicted in a volcano plot, we observe that these 57 ORFs common to all the comparisons were among the most highly overrepresented genes and with some of the lowest P adjusted values determined by DESeq (FIG. 5C – blue

dots). Similar results were obtained for all comparisons between melarsoprol selected and input samples. We conclude that our analysis is reasonably robust at identifying the most consistent, statistically significant, highly overrepresented genes in melarsoprol treated samples compared to untreated samples.

The 57 genes overrepresented in melarsoprol survivor populations consist of 25 genes annotated as conserved hypothetical and among the genes with annotations most have only putative functional assignments (SUP. 10). Among the top 5 overrepresented hits we identified that the gene encoding γ -glutamylcysteine synthetase (GSH1, *Tb927.10.12370*) was 191-fold higher among melarsoprol survivors when compared with input control. GSH1 is required for the biosynthesis of trypanothione, a known target of melarsoprol, and its overexpression has been demonstrated to promote melarsoprol resistance in laboratory studies (33). This finding supports the role of trypanothione as one of the major intracellular targets of melarsoprol and the usefulness of the GoF library in the direct identification of drug targets. Using available functional annotations (tritypdb.org), we found that 15 of the 57 overrepresented hits are either known or implicated in gene expression regulation. It is not clear at this time if this is a true aspect of melarsoprol functionality or whether this is an artifact arising from the nature of the GoF library; namely increased transcript stability and translation of diverse proteins having a nonspecific effect on cell viability in the presence of drug. Only through further GoF library validation will we know if gene expression is truly associated with melarsoprol resistance. We then utilized publicly available resources (tritypdb.org and tryptag.org) to develop additional categories based on subcellular localization (from proteomic and microscopic studies) and published protein functions. We found that the

majority of overrepresented genes encoded proteins that localize to the mitochondria and kinetoplast (10 genes, shown in Table 2) or the flagellum (9 genes, shown in Table 3) (SUP. 10 – Hits listed with categories). The prevalence of mitochondrial and flagellar proteins arising from the melarsoprol GoF screening may implicate previously unrecognized melarsoprol drug targets or pathways of drug tolerance.

Overexpression of genes identified in melarsoprol GoF screen result in increased drug tolerance

GoF library induction in the presence of melarsoprol resulted in the isolation of survivor populations harboring a consistent set of overrepresented genes. The growth environment arising from the induced expression of many genes is likely distinct from cell populations overexpressing only one gene and may influence population survival through the secretion of unknown factors or other events. To determine if the overexpression of individual genes identified among the 57 overrepresented hits can contribute to melarsoprol resistance, we cloned a subset of genes in to the pLEW100v5-BSD vector, which permits Dox inducible gene expression in a manner similar to the GoF library itself. Bloodstream form *T. brucei* (SM) were transfected with linearized plasmids harboring individual genes for overexpression and, following their recovery were, assayed for their effects on melarsoprol sensitivity by alamarBlue assays. The gene encoding γ -glutamylcysteine synthetase (*Tb927.10.12370*, GCS1), which is responsible for the biosynthesis of glutathione used to form trypanothione, was among the most overrepresented genes (190-fold), is essential, and its overexpression has been linked to resistance in previous studies (39,40). As anticipated induced

overexpression of *GSH1* resulted in an increase in the EC50 of melarsoprol of almost 2-fold (FIG. 6A – SM = EC50 7.5 nM and OE *GSH1* = 14.5 nM) suggesting that increased expression of components of this pathway can produce more trypanothione and diminish the efficacy of melarsoprol. The trypanothione pathway (FIG. 6B) and its implications for melarsoprol resistance are covered in more detail in the discussion.

We also found that the overexpression of the hypothetical conserved gene *Tb927.7.2780* (overrep. 322-fold) resulted in a 2-fold shift in EC50 (FIG. 6C – OE = EC50 15.1). The protein encoded by *Tb927.7.2780* is a predicted post-transcriptional activator associated with mRNA stability or increased translation (41) and is annotated as an essential gene based on RIT-seq analysis (42). Whether melarsoprol resistance associated with *Tb927.7.2780* arises from direct effects on gene expression of known pathways, such as trypanothione biosynthesis enzymes, or more general effects on global gene regulation remains to be determined. A similar shift in EC50 was also observed upon overexpression of *Tb927.9.15020* (FIG. 6C – OE = EC50 14.0), which encodes a hypothetical protein of unknown function (overrep. 30.1-fold) that we categorized as flagellar (axonemal) based on TrypTag.org localization data (images shared ahead of online deposition). It is unclear at this time how flagellar proteins may participate in melarsoprol resistance, discussed in more detail below. Multiple genes encoding mitochondrially localized proteins were cloned for similar overexpression analyses, but resulted in assay challenges arising from either growth defects, inability to reduce alamarBlue, or both. Because cell viability in alamarBlue assays is reported based on the reduction of the active reagent (resazurin) by mitochondrial redox metabolism, it is not a surprise that the effects mitochondrial proteins could not be

assayed in this manner and may indicate their association with the trypanothione redox pathway itself. The role of mitochondrial proteins in melarsoprol tolerance will be the subject of future investigations. Together these results show that genes identified in melarsoprol GoF screening can promote drug resistance upon overexpression, support the significance of the trypanothione pathway, and implicate new aspects of kinetoplastida biology in melarsoprol resistance.

DISCUSSION

The forward genetics tools generated here address an urgent need to extend genomic functional characterization in *T. brucei* and its kinetoplastida relatives. More than 30 years of genetic and biochemical studies in Trypanosomatids, 10 of which included the extensive use of an RNAi-based loss-of-function library, have produced key discoveries in parasitology and basic biology. Yet, with the functions of more than 50% of Trypanosomatid encoded genes largely unknown, many mysteries remain unsolved and very few functional pathways have been delineated. To unlock potential discoveries in Trypanosomatids, here we have generated two powerful tools for forward genetics and unbiased studies based on the *T. brucei* genome (*TREU927*): an ORFeome consisting of over 6500 ORFs and an inducible Gain-of-Function library harbored in *T. brucei* parasites (*Lister427*) for genetic screening. To validate the functionality of the *T. brucei* GoF library, we conducted a genetic screen using the troublesome drug melarsoprol and identified both an anticipated target as well as novel genes that may reveal new facets for melarsoprol's mechanism of action.

528 Melarsoprol has a complex history dating back to early uses of arsenic treatment
529 of HAT, treatments which are of specific significance because of their ability to cross the
530 blood-brain barrier and treat second stage infections (30). *In vivo* melarsoprol is rapidly
531 metabolized to other trypanocidal metabolites, including melarsen oxide, which interacts
532 with trypanothione to form MeIT, a potent inhibitor of trypanothione reductase (TR) (11).
533 Yet, the precise mechanism of melarsoprol cell killing has remained unclear and the
534 complex forms of metabolized melarsoprol may have additional intracellular targets.
535 Melarsoprol drug resistance and treatment failures have been on the rise since the
536 1990s and while most resistance has been connected to mutations in aquaglyceroporin
537 transporters, numerous treatment failures remain unexplained (30). The trypanothione
538 biosynthetic precursor, γ -glutamylcysteine synthetase (GCS1), was among the most
539 overrepresented genes in melarsoprol survivors and is known to result in melarsoprol
540 resistance upon laboratory overexpression (39,40). Upon deeper excavation of the
541 melarsoprol GoF screen data, we observed that a number of genes upstream and
542 downstream of T(SH)₂ with enriched reads in melarsoprol survivors, but did not meet
543 the criteria to be called overrepresented (SUP. 11). For instance, both of the T(SH)₂
544 biosynthetic precursors ornithine decarboxylase (ODC, *Tb927.11.13730*) and
545 glutathione synthetase (GSH2, *Tb927.7.4000*) were partially overrepresented in some
546 cultures of melarsoprol survivors (but not all). Downstream from T(SH)₂, we saw
547 increased levels of trypanothione reductase (TR, *Tb927.10.10390*), a trypanothione
548 dependent peroxidase (*Tb927.7.1140*), and ribonucleotide reductase (RR,
549 *Tb927.11.7840*), in some survivor cultures. The generation of dNTPs by the action of
550 RR has known effects on kDNA replication (43) and melarsoprol treatment was recently

shown to stall cell cycle (44), which might suggest inhibition of DNA synthesis as a mechanism of cell killing. Collectively these findings may further support the trypanothione pathway as a primary target of melarsoprol-based cell killing.

Among the 57 significantly overrepresented genes approximately 45% are annotated as hypothetical. The majority of overrepresented genes with functional predictions are associated with gene expression (15 genes) with the largest groups associated post-transcriptional regulation (6 genes) or splicing factors (5 genes). Notably, one of the overrepresented genes associated with post-transcriptional gene expression regulation (*Tb927.10.1490*, MKT1L) was also a high significance gene in melarsoprol RNAi screening (45). For overrepresented genes without functional annotations we focused on the cellular localization of the encoded proteins (tritypdb.org and tryptag.org) and identified a preponderance of proteins that localize to either the mitochondria (11 genes) or flagellum (9 genes) (FIG. 7). The flagellar proteins have diverse localizations in procyclic form (insect stage) parasites, that include localization to the flagellar pocket and along the body of the organelle (axonemal and paraflagellar rod, specifically). Since the melarsoprol transporter AQP2 is specifically localized to the flagellar pocket in bloodstream form parasites (46), one could speculate that overexpression of a protein that interferes with drug transport in this region might enhance resistance. Two of the overrepresented flagellar genes, one axonemal (*Tb927.5.4150*) and one localized to the hook complex (*Tb927.1.1020*), were associated with a loss of fitness in RIT-seq screens, which is indicative of their essentiality. It will be the subject of future studies to determine how flagellar proteins might contribute to drug resistance or if they are viable drug targets.

Connections between T(SH)₂ and mitochondrial redox reactions have been widely established (31) and the anti-Trypanosomatid drugs pentamidine and nifurtimox have been linked to specific mitochondrial functions (44,47). Yet, the data arising from the GoF screen described herein represent the first genetic links between melarsoprol and the mitochondria. Furthermore, since the majority of the GoF proteins that localize to the mitochondria are hypothetical or have only putative functionality, the 11 mitochondrial genes identified here have the potential to uncover novel biology in the mitochondria and kinetoplast. Among them, the best studied gene encodes β -ketoacyl-ACP reductase (*Tb927.2.5210*, 23-fold overrep.), a key member of the Type II Fatty Acid Biosynthesis (FAS) pathway in the mitochondria. Interestingly, lipoic acid, whose biosynthesis arises from Type II FAS, is an alternative redox active low molecular mass thiol alternative to T(SH)₂ (31,48). Further studies will be required to determine if overexpression of β -ketoacyl-ACP reductase and other novel mitochondrial proteins promote melarsoprol resistance either through their redox potential or other functions in the mitochondria.

The functionality of the *T. brucei* ORFeome could be extended to generate additional genetic tools, such as, yeast two-hybrid libraries, tagging libraries, and in dominant negative genetic screening approaches (22,26,49,50). Based on the conservation of orthologous gene clusters among kinetoplastida (3), we also predict the ORFeome could be used in other Trypanosomatids to generate orthologous Gain-of-Function libraries and other tools. The vast majority of genes overrepresented in melarsoprol survivor populations (~80%, see SUP. 13) are conserved among sequenced kinetoplastida genomes. This supports the use of these tools to broadly

expand our understanding of gene functions in this family of parasites. Notably, approximately 25% of all genes overrepresented in melarsoprol survivors were identified as resulting in loss-of-fitness in RIT-seq screening (26-23% depending on life cycle stage) (SUP. 12 – RIT-seq compared), which validates the usefulness of the GoF library tool in identifying essential genes, an acknowledged limitation of RNAi based screening (14,51). Additionally, we found genes overrepresented in the melarsoprol GoF screen that were also identified in nifurtimox and melarsoprol RNAi based screens (SUP. 12 – RIT-seq compared) (51). Thus, we see the GoF library as a powerful new tool, complementary to RNAi knock-down approaches, to expand our understanding of drug targets and pathways of resistance. In future drug resistance screens, it will be useful to determine if certain sets of genes are commonly overrepresented in survivor populations and contrast that with genes that appear uniquely associated with an individual treatment. The tools and discoveries arising from this study are expected to support broad advances in basic biology, pathogenesis, pathways of drug resistance, and the discovery of new therapeutic targets in Trypanosomatid parasites.

MATERIALS AND METHODS

Oligo design and PCR amplification of the ORFeome

In order to design oligos for the ORFeome, we used gene annotations generated from ribosome profiling data (32). We first filtered out genes that were either < 100 bp in size or > 4,500bp in size. We then filtered out any genes annotated as pseudogenes, hypothetical protein unlikely, VSG, ISG, ESAG, GRESAG, or ribosomal protein. Following this procedure, we used custom python scripts to design forward and reverse primers. If the first 30bp of the ORF had a T_m >55, this primer was used. Otherwise, a nucleotide was added one at a time until the primer had a T_m of greater than 55. A reverse primer was designed using the same procedure for the reverse complement of the last 30 nucleotides. Many genes in the *T. brucei* genome have identical beginnings and ends, and we did not wish to order duplicate sets of primers that were identical to each other. For this reason, only one primer pair was kept for duplicated or highly similar genes, and a record was made of any pair that was eliminated based on duplication. All excluded ORFs are listed in SUP. 14. Finally, the ATT cloning sequences 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT were added to the forward and reverse primers, respectively.

Each 384-well plate containing mixed forward and reverse primer pairs were diluted to 10 mM in primer dilution plates. PCR plates were filled with a master mix for KOD Hotstart (Novagen #71086) PCR reactions containing Lister427 gDNA according to manufacturer's specifications and primer pairs specific to each ORF were transferred into the associated well number for each plate by TECAN Freedom EVO 150 pipetting

instrument. PCR reaction conditions followed manufacturers specifications for 25 μ L reactions with annealing temperature of 55°C and elongation times ranging from 10-60 seconds depending on the anticipated lengths of products in each plate. Following PCR reaction completion SYBR Green I (Invitrogen S-7563) was added to each well at 1:10 PCR volume for a final concentration of 10x SYBR and relative fluorescence units (RFU) were measured on a BioTek Synergy H4 Hybrid Multi-Mode Microplate reader in comparison to a standard dilution of known DNA concentration. The resulting PCR reactions from all wells of each 384-well plate were then pooled prior to agarose gel separation and DNA extraction. PCR reactions called 'negative' based on SYBR (less than 10,000 RFU) were revisited by isolating primer pairs from original 384-well plates, using a Perkin-Emer Janus Automated Workstation, into two new PCR plates (SUP. 2 – NEG_PICKS_#1 and NEG_PICKS_#2), which were then amplified using KOD Hotstart specifications and extracted from agarose gels as described. The resulting extracted DNAs from each PCR product pool were utilized in subsequent gateway cloning reactions.

Gateway cloning and plasmids

The pENTR library was generated by cloning each size-sorted PCR product pool into pENTR 221 Gateway Entry vector according to manufacturer's specifications (<https://www.thermofisher.com/us/en/home/life-science/cloning/gateway-cloning.html>) and transformed into One Shot *ccdB* Survival Competent Cells by electroporation. The resulting transformants were plated on large LB plates, assessed for efficiency of transformation, and bacterial colonies isolated from plates into LB liquid, which was split

for maxi preps of plasmid and storage at -80°C in glycerol stocks. A *T. brucei* specific pDEST vector, pSUN6 (map shown in SUP. 3) was generated by introducing a *ccdB* gateway cassette into a pLEW type vector (52) for incorporation into the *T. brucei* genome based on rDNA spacer homology, blasticidin selection, and ORF transcription from an rDNA spacer promoter repressed by two tetracycline operators. Pools of pENTR plasmids harboring size-sorted ORF populations were combined with pSUN6 in LR clonase reactions and transfected into *ccdB* Survival Competent Cells following manufacturers guidelines as described. The resulting transformants were plated on large LB plates, assessed for efficiency of transformation, and bacteria and DNA isolated as described for pENTR steps above. The resulting extracted DNAs from both pENTR and pDEST ORFeome gateway cloning steps were assessed by NGS, described below. Following the initial assessment of ‘missing’ ORFs from both pENTR and pDEST cloning reactions, ‘missing’ PCR products were isolated from original plates, using a Perkin-Emer Janus Automated Workstation, to generate 8 new pools of size sorted PCR reactions (SUP. 4), which underwent the same series of pENTR and pDEST (pSUN6) cloning reactions described above and subjected to NGS analysis. The final set of ORFeome NGS validated (below) pDEST vectors are pooled to generate a single pTrypLib ORFeome for introduction into the *T. brucei* genome.

***T. brucei* cell lines and transfections**

Cell lines were generated from *Lister427* bloodstream-form trypanosomes derived from the ‘single marker’ (SM) line (53) and maintained in HMI-9 medium (54) under appropriate drug selection when indicated. A landing pad (LP) cell line was

generated using plasmids gifted to us by the Alsford Lab and validated for inducible gene expression, prior to transfection with pRPaSce* as described (12,51). LP parasites harboring the I-SceI restriction site and enzyme in the targeted rDNA spacer are doxycycline induced to permit ISceI cutting prior to pTrypLib ORFeome transfection by AMAXA Nucleofector (55). To generate the *T. brucei* GoF library described here, four 100ml flasks grown to ~1 million cells/mL were AMAXA transfected in four separate transfection reactions, which were then pooled into a single cell population in 500 mL of HMI-9 containing blasticidin and recovered in a large roller flask (FIG. 3C). An additional four transfections were completed in parallel with TE (Mock) to compare outgrowth with GoF Library transfection. The resulting blasticidin recovered GoF Library population was expanded to an 800 mL culture at ~1 million cells per mL and saved in aliquots of 100 million cells per vial for future genetic screens. Cells were also sampled prior to freezing for NSG analysis ('GoF library', described below) and after freeze thaw (INPUT1).

Single gene overexpression cell lines were generated by cloning ORFs of interest into pLEW100v5-BSD (<https://www.addgene.org/27658/>), which following validation were digested with NotI and transfected into SM by AMAXA.

Quantitative PCR assessment of individual ORF induction

Individual cloned ORFs were selected randomly from pDEST colonies plated on LB originating from the pool '2_known', ORFs confirmed by traditional DNA sequencing, and DNAs arising from 6 individual ORF harboring pDEST vectors were transfected into LP harboring pRPaSce* by AMAXA as described above. This generated a 'Mini-Library' following transfection and recovery, which was split into No Dox and +Dox conditions for

24 hours, RNA extracted, and cDNA prepared by Superscript III (ThermoFisher #18080044), prior to qPCR analysis. Quantitative PCR data was produced on a Bio-Rad CFX96 Real-Time PCR Detection System with iTaq Universal SYBR Green Supermix (Bio-Rad, #1725121) reagent using a forward primer unique to cloned ORFeome genes (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT) and reverse primer to each gene analyzed: *Tb927.8.2230* (PRIMER: 5'-CACGGTTTTTGCCCATTCGT), *Tb927.1.4830* (PRIMER: 5'-ATTTTTGCCGAAGCGCTTGA), *Tb927.10.12940* (PRIMER: 5'-CCGTGATTCCCTGTCGACAT), and *Tb927.11.15810* (PRIMER: 5'-CACCACCCGATGTACGGTAG). With these primer combinations only transcripts arising from the exogenous ORFeome copy were measured with and without Dox induction (FIG. 3B).

Next-generation Illumina sequencing of the *T. brucei* ORFeome and Gain-of-Function libraries

Libraries for the pENTR and pDEST (pTrypLib) ORF plasmid libraries were prepared for next generation Illumina sequencing using tagmentation kits from Illumina (Nextera XT kit) according to the manufacturer's instructions. Sequencing was performed either on an Illumina HiSeq 2500 or an Illumina MiSeq. Reads were trimmed for quality using trim galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) using this command: trim_galore --nextera --stringency 3. Reads were aligned to the Tb927v5.1 genome using bowtie (56) requiring unique alignments: bowtie --best --strata -t -v 2 -a -m 1, or allowing multiple alignments: bowtie --best --strata -t -v 2 -a -m 10. RPKM values were

calculated using SeqMonk from Babraham Bioinformatics
(<http://www.bioinformatics.babraham.ac.uk/projects/seqmonk>).

For quantification of introduced ORFs following transfection into the *T. brucei* to generate the GoF library, genomic DNA was isolated from 100-200 million transfected parasites and fragmented by sonicating water bath (Bioruptor). The NEBNext Ultra II DNA library prep kit was then used to prepare libraries for high throughput sequencing according to the manufacturer's instructions with the following exception: a custom universal primer containing the *ATT* cloning site was used for 5' indexing and PCR enrichment (5'- AATGATACGGCGACCACCGAGATATATAACAAGTTTGTACA AAAAAGCAGGCTATG). A custom sequencing oligo (5'-GGGACAAGTTTGTAC AAAAAAGCAGGCTATG) is loaded on to the Illumina sequencing platform to sequence only GoF library containing reads. Analysis and raw counts were calculated using SeqMonk from Babraham Bioinformatics (<http://www.bioinformatics.babraham.ac.uk/projects/seqmonk>). Sequencing reads were trimmed for quality and aligned to the genome using the same parameters described above. Raw counts were calculated for reads aligning exclusively to the first 100 bp of the ORF using SeqMonk from Babraham Bioinformatics. DESeq2 was used to calculate the normalized read counts between sequencing libraries (38).

Melarsoprol GoF genetic screening

For melarsoprol selective screening, GoF library cells were seeded for each condition at 1×10^5 cell/ml, induced with doxycycline (1 µg/mL) 24 hours (when appropriate), and grown in HMI-9 medium containing Dox (when appropriate) plus melarsoprol at 17 nM

or 35 nM melarsoprol (BoC sciences, CAS 494-79-1). Melarsoprol stocks were diluted in DMSO.

Bioinformatic analysis platforms for ORF identification and hit calling pipeline

In order to call which genes were overrepresented in the melarsoprol selected libraries, we exclusively counted reads that fell within the first 100bp of the gene. DESeq (38) was then used to identify genes that were ‘differentially expressed’ in the melarsoprol selected libraries with a multiple testing corrected p value of less than 0.05. Using normalized read counts, we then calculated the fold change between each pair of melarsoprol selected and unselected replicates (3 total) for every statistically significant gene called by DESeq, provided that gene had a normalized read count of 5 or greater in the minimally propagated samples. If all 3 replicates showed a > 4 fold change for a particular gene, that gene was considered significantly overrepresented. Because we had two sets of replicates for unselected libraries and two sets of replicates for melarsoprol selected libraries, we ran the pipeline described above for all 4 comparisons of selected and unselected sets of replicates. Only those genes that were called as hits in all 4 comparisons were reported in the final hit list.

EC50 determination by alamarblue

For EC50 determination, induced and uninduced cells were plated across a melarsoprol dilution series and assessed viability after 72 hours using Alamarblue (ThermoFisher) as previously described (57). All experiments were performed in biological triplicate.

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FIGURES AND FIGURE LEGENDS

Figure 1. Generating a *T. brucei* ORFeome. A) Assessment of PCR amplification by SYBR Green Relative Fluorescence Units (RFU). Each dot represents an individual PCR reaction, each color represents one of the 21, 384-well plates, graph is of SYBR Relative Fluorescence Units (RFU) vs. 384-well plate position. B) Percent of PCR positive wells (SYBR assessment) for each of the 21, 384-well plates, from the first time amplified. C) Agarose gel bands from each of the 21, 384-well PCR plates pooled prior to gel extraction and cloning, from the first time amplified, compared to 10 Kb ladder DNA ladder. D) ORFeome cloning strategy: *attB1* site addition to *T. brucei* ORFs during

PCR amplification, Gateway cloning into pENTR to generate the pENTR ORFeome, and Gateway cloning into *T. brucei* specific pDEST type vector (pSUN6, shown in detail in SUP. 3) to generate the complete pTrypLib ORFeome.

Figure 2. Assessment of pENTR and pDEST (pTrypLib) plasmid libraries. A) Bar graph showing the number of targeted ORFs with zero detectable aligned reads from

1041 the first round of cloning (pENTR_1 and pDEST_1) and after both rounds of cloning
 1042 (pENTR_Final and pDEST_Final) using analysis generated from both uniquely and
 1043 multiply aligned reads. B) Histogram showing the distribution of normalized read counts
 1044 for each ORF in the pooled pDEST (pTrypLib) plasmid libraries (Uniquely aligned reads
 1045 shown, SUP. 6 both uniquely and multiply aligned reads). C) Histograms showing the
 1046 distribution of ORF lengths for the target gene list (red) and the set of ORFs with zero
 1047 detectable aligned reads after both rounds of cloning (labeled as missing). Analyses
 1048 from unique (dark blue) and multiply (light blue) aligned reads are shown. Inset graph,
 1049 target ORF lengths have been left out to better visualize the lengths of the missing
 1050 ORFs.

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Figure 3. Generation and validation of the *T. brucei* GoF library. A) Transfection of pTrypLib ORFeome into parental Landing Pad (LP) cell line harboring pRPa-Sce* plasmid for I-SceI induced enzymatic cleavage of a single rDNA spacer site (12). B) Generation of a Mini-library GoF library, 6 ORFs. Procedure shown in flow diagram on the left. Transcript level of four GoF library encoded ORFs (exogenous) measured by RT-qPCR following 12 and 24 hours of doxycycline induction compared to uninduced cells (No Dox). C) Generation of the pTrypLib ORFeome based GoF library. Procedure shown in flow diagram on the left. Graph indicates the recovery of GoF library harboring cells (red line) compared to mock transfection (blue line) in blasticidin. D) Assessment of the number of ORFeome genes present in the GoF library following initial transfection

(GoF library), in INPUT1 (following freeze thaw and minimal propagation), and INPUT2, which used an alternative NGS protocol.

Figure 4. Isolation of melarsoprol survivor populations from a GoF screen. A)

Growth of Landing Pad (LP) parental cell line in 17 nM (blue line), 26 nM (purple line),

1071 35 nM (red line), or no (black line) melarsoprol treatment over 14 days. B) GoF library
 1072 screen in 35 nM melarsoprol treatment: LP cell line (solid red line), uninduced GoF
 1073 library (red dotted line), induced GoF library (red dashed line); dotted and dashed lines
 1074 overlap. C) GoF genetic screen in 17 nM melarsoprol is shown as a time course at the
 1075 bottom and graph of cell growth: INPUT, untreated GoF library harboring cells grown for
 1076 3 days (black line), LP parental cell line (solid blue line), uninduced GoF library (blue
 1077 triangles on dotted line), induced GoF library (blue circles on dashed line), harvested on
 1078 Day 9 (red circle on blue line) to produce MEL-1x. On day 7, biological triplicates from
 1079 induced GoF library (blue circles on dashed line) were split into two sets of triplicate
 1080 samples, both with 17 nM melarsoprol, one of which was not further induced (green
 1081 triangles on dotted line). The other continued to be induced (green squares on dashed
 1082 green line) and was harvested on Day 10 to produce MEL-2x (red circle indicates
 1083 harvest). D) Principle Component Analysis (PCA) comparing INPUT libraries (1 and 2)
 1084 with libraries arising following 2 weeks of continuous melarsoprol selection (MEL-1x and
 1085 MEL-2x).
 1086

Figure 5. Identification of significantly overrepresented ORFs in melarsoprol GoF survivor populations. A) The number of genes with a fold change > 1.5, 2, or 4-fold is given for all three replicates of INPUT2: Replicate 1 vs. Replicate 2, Replicate 2 vs. Replicate 3, and Replicate 1 vs. Replicate 3. B) Hit calling pipeline to identify genes overrepresented in melarsoprol survivor populations. Following read count normalization and alignment to the first 100 bp of each gene in every sample, DESeq was used to filter for differential representation using a P adjusted cutoff of <0.05. Each melarsoprol treated replicate was compared with each untreated INPUT replicate and those with 4-fold or greater change of melarsoprol selected over unselected in all three comparisons were called as preliminary hits. Preliminary hits were determined for INPUT1 vs. MEL-1x, INPUT2 vs. MEL-1x, INPUT1 vs. MEL-2x, and INPUT2 vs. MEL-2x. The final list of 57 hits represent genes common to all four comparisons. C) Volcano plot showing the $-\log_{10}(\text{P adjusted value})$ vs. $\log_2(\text{Fold Change in normalized counts})$

for the comparison of melarsoprol selected MEL-1x/INPUT2) for each ORF in the targeted library. Red dots represent fold changes >4 with a P adjusted significance of <0.05 and blue dots represent overrepresented ORFs common to all four comparisons described in B. D) Venn Diagram illustrates the significantly overrepresented genes common to each comparison and shared among all comparisons, resulting in 57 overrepresented hits identified in melarsoprol survivor populations compared to input populations.

Figure 6. Melarsoprol sensitivity following gene overexpression. AlamarBlue assays were used to measure the EC50 over a range of melarsoprol concentrations (2,

1112 7, 10, 15, 20, 25, and 50 nM) for both parental cell line (SM, blue line) and following Dox
1113 induction of genes identified in the melarsoprol GoF screen (A, C, D red lines labeled
1114 OE). A) Effect of γ -glutamylcysteine synthetase (*Tb927.10.12370*, GSH1)
1115 overexpression (red line) on the EC50 of melarsoprol (shown in text on the graph). B)
1116 The associated trypanothione (T(SH)₂) pathway with GSH1 is also depicted, in which
1117 the green oval is the overrepresented hit GSH, yellow ovals indicate pathway
1118 components that were modestly effected but did not meet the statistical cutoffs for GoF
1119 hit calling (SUP. 11), and genes encoding enzymes in white ovals were not changed in
1120 the GoF screen. The effects of melarsoprol on T(SH)₂ are also depicted as well as the
1121 outcomes of this pathway (panel B). Data panels C) and D) show the effects of
1122 *Tb927.7.2780* and *Tb927.9.15020* overexpression on the EC50 of melarsoprol,
1123 respectively. All alamarBlue assays were done in biological triplicate for both uninduced
1124 (No Dox, not shown) and induced (+Dox) conditions. Data shown in panel A, C, and D
1125 (red lines) are single experimental representative of all Dox induced biological
1126 replicates.
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Figure 7. Categories of overrepresented genes in melarsoprol GoF screen survivor populations. An illustrative diagram of a *T. brucei* cell is shown with relevant features highlighted. Boxes of gene numbers indicate major functional or localization categories for the genes overrepresented in melarsoprol survivor populations.

Table 1 - ORFeome 384-well oligo plates

Plate name	Min length (bp)	Max length (bp)	# ORFs per plate
0_hypothetical	102	375	373
0_known	147	591	315
1_hypothetical	375	522	374
1_known	594	849	365
2_hypothetical	522	666	372
2_known	849	1056	363
3_hypothetical	669	822	382
3_known	1056	1287	367
4_hypothetical	822	993	372
4_known	1287	1524	348
5_hypothetical	993	1155	365
5_known	1524	1857	342
6_hypothetical	1158	1365	375
6_known	1857	2337	362
7_hypothetical	1368	1635	378
7_known	2340	3504	376
8_hypothetical	1635	1953	381
9_hypothetical	1953	2508	381
10_hypothetical	2508	3501	381
hypothetical_last	3504	4488	135
known_last	3507	4497	138

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1134 **Table 1. 384-well oligo plates.** Plate names, ORF size ranges, and number of oligo
1135 pairs per plate are indicated.

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Table 2 - Melarsoprol GoF Hits with Mitochondrial Localization

GENE ID	CATEGORY	DESCRIPTION	LOCALIZATION (TrypTag.org)	FOLD CHANGE	P ADJUSTED DESEQ
<i>Tb927.11.590</i>	Mitochondrial*	hypothetical protein, conserved	Strong mitochondrial signal, kinetoplast	350	1.81E-39
<i>Tb927.10.12050</i>	Mitochondrial*	hypothetical protein, conserved	kinetoplast, mitochondrion	31	2.16E-34
<i>Tb927.9.7200</i>	Mitochondrial*	hypothetical protein, conserved	kinetoplast, mitochondrion	31	5.61E-28
<i>Tb927.9.7080</i>	Mitochondrial & ER ^o	hypothetical protein, conserved	cytoplasm (patchy, points)	23	1.14E-23
<i>Tb927.2.5210</i>	Mitochondrial#	3-oxoacyl-ACP reductase, putative	mitochondrion, kinetoplast (strong)	23	2.16E-34
<i>Tb927.11.2910</i>	Mitochondrial*	phosphoglycerate mutase, putative (iPGAM)	mitochondrion (75%), kinetoplast (75%)	23	6.79E-05
<i>Tb927.9.5220</i>	Mitochondrial ^o	conserved protein	endocytic, cytoplasm(weak)	19	2.60E-21
<i>Tb927.10.6850</i>	Mitochondrial#	Mitochondrial ribosomal protein S18, putative	cytoplasm(reticulated)	16	3.68E-10
<i>Tb927.8.7790</i>	Mitochondrial ^o	zinc finger domain, LSD1 subclass, putative	ND	13	7.88E-22
<i>Tb927.11.5600</i>	Mitochondrial#	Archaic Translocase of outer membrane 14 kDa subunit	mitochondrion	8	9.21E-11
<i>Tb927.3.5190</i>	Mitochondrial+	hypothetical protein, conserved	mitochondrion	7	8.48E-21

Key: + From localization data, ^o Proteomic data, * both localization and proteomics, or # published

Table 2. Melarsoprol GoF hits with Mitochondrial localization.

Table 3 - Melarsoprol GoF Hits with Flagellar Localization

GENE ID	CATEGORY	DESCRIPTION	LOCALIZATION	FOLD CHANGE	P ADJUSTED DESEQ
<i>Tb927.11.11435</i>	Flagellar+	dynein light chain lc6	Strong flagellum axoneme signal	120	1.30E-26
<i>Tb927.9.15020</i>	Flagellar+	hypothetical protein, conserved	Weak axoneme signal	31	4.45E-20
<i>Tb927.11.1810</i>	Flagellar+	Ring finger domain containing protein, putative	flagellar pocket (ring)	16	1.50E-18
<i>Tb927.10.390</i>	Flagellar*	hypothetical protein, conserved	Flagellum Matrix Proteome (BSF)	14	2.78E-12
<i>Tb927.5.4150</i>	Flagellar*	hypothetical protein, conserved	paraflagellar rod	13	6.29E-06
<i>Tb927.7.710</i>	Flagellar*	heat shock 70 kDa protein, putative (HSP70)	cell tip (anterior), cytoplasm, flagellar cytoplasm	10	3.15E-26
<i>Tb927.10.9060</i>	Flagellar +	hypothetical protein, conserved	basal body	8	5.86E-10
<i>Tb927.1.1020</i>	Flagellar +	leucine-rich repeat-containing protein	hook complex	8	1.61E-10
<i>Tb927.6.3980</i>	Flagellar +	hypothetical protein, conserved	Flagellum, axoneme	6	3.88E-10

Key: + From localization data, ^o Proteomic data, * both localization and proteomics, or # published

Table 3. Melarsoprol GoF hits with Flagellar Localization.

SUPPLEMENT LEGENDS

Supplement 1. Table of oligo pairs from all 21, 384-well plates. The sequences of all 7245 oligo pairs used to generate the *T. brucei* ORFeome are included with respect to their plate “name” and well position in each plate.

Supplement 2. Table of ORFeome amplification tracking. Table indicates the percent of PCR amplifications that were scored as positive (based on SYBR assessment) and the number of missing ORFs in reference to each oligo plate. Sub-tables show the reamplification of “NEG_PICKS” in two addition PCR plates, which recovered an additional 228 positive PCR products and the final total of 7044 positive PCR reactions for an overall success rate of 97.2%.

Supplement 3. Map of *T. brucei* specific pDEST vector, pSUN6. Critical features of plasmid map are indicated, including: rDNA spacer homology, T7 terminators, Blastidicin resistance cassette, rRNA promoter, two tetracycline operators, attR site for pENTR recombination, and the 3’ GPEET and 5’ Aldolase (long) UTRs.

Supplement 4. Table of all final ORF pools used to make the pENTR ORFeome. Following the first pENTR_1 and pDEST_1 ORFeome assessments, ORFs missing from libraries were identified and reselected from original 21 PCR plates to form an additional 8, size sorted pools. The original 21 pools, 2 pools of PCR reamplifications (NEG_PICKS) and 8 ‘missing’ ORF pools (1-8_MISS) collectively result in 31 pools for pENTR cloning. The size ranges and total number of ORFs per pool are indicated.

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1181 **Supplement 5. Tables of all missing genes from final pENTR and pDEST**

1182 **(pTrypLib) ORFeomes.** Consists of four sub-tables for all the ORFs identified as

1183 missing from either pENTR or pDEST by analysis of uniquely or multiply aligned reads:

1184 MISSING_pENTR_UNIQUE, MISSING_pENTR_MULTIPLE,

1185 MISSING_pDEST_UNIQUE, AND MISSING_pDEST_MULTIPLE.

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1187 **Supplement 6. Assessment of the pTrypLib ORFeome.**

1188 A) Histograms showing the distribution of normalized read counts in the pTrypLib

1189 ORFeome in analyses using uniquely aligning reads (left) and allowing multiple

1190 alignments (right). B) The length of each ORF in the pTrypLib Orfeome was plotted

1191 against the normalized read count of the ORF. A best fit line was calculated using linear

1192 regression (shown in white). For uniquely aligned reads this was $y = 7.623751 -$

1193 $0.000673x$ and for multiply aligned reads this was $y = 7.306181 - 0.000778x$.

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1195 **Supplement 7. Sequencing strategy and results from transfected libraries.**

1196 A) Library preps from pTrypLib transfected parasites proceed by first fragmenting

1197 genomic DNA, ligating illumina adaptors, and amplifying library introduced ORFs using

1198 a primer complementary to complementary to the *attB1* Gateway cloning sequence and

1199 the standard Illumina barcoded reverse primer. Sequencing proceeds using a custom

1200 forward primer complementary to the *attB1* Gateway cloning sequence. B) Red

1201 rectangles in the first row represent annotated genes from a section of the *T. brucei*

1202 chromosome 5. Bars in subsequent rows represent reads that align to the genes in the

1203 first row. Most reads align to the first 100bp of the gene, as expected from the library
1204 prep and sequencing strategy.

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1206 **Supplement 8. Assessment of coverage in the GoF INPUT library.** A) Histograms
1207 showing the distribution of normalized read counts in the sequencing libraries from
1208 INPUT 1 in analyses using uniquely aligning reads (left) and allowing multiple
1209 alignments (right). B) The length of each ORF in the pTrypLib Orfeome was plotted
1210 against the normalized read count for the INPUT1 sequencing library. A best fit line was
1211 calculated using linear regression (shown in white). For uniquely aligned reads this was
1212 $y = 6.046291 - 0.000423x$ and for multiply aligned reads this was $y = 6.083779 -$
1213 $0.000452x$.

1214

1215 **Supplement 9. Tables of all data pertaining to genes overrepresented in**
1216 **melarsoprol survivor populations.**

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1218 **Supplement 10. Table of categorization and localization of genes overrepresented**
1219 **in melarsoprol survivor populations.**

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1221 **Supplement 11. Table of trypanothione pathway genes and modest effects**
1222 **observed in melarsoprol GoF survivor populations.**

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1224 **Supplement 12. Table of melarsoprol GoF hits compared with RIT-seq genetic**
1225 **screens.**

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1227 **Supplement 13. Table conservation of the 57 genes overrepresented in**

1228 **melarsoprol survivors among other kinetoplastida.**

1229

1230 **Supplement 14. Table of genes not included in ORFeome design.**

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