Published in final edited form as:

Chem Biol Drug Des. 2011 September ; 78(3): 454–463. doi:10.1111/j.1747-0285.2011.01156.x.

High Throughput Analysis of an RNAi Library Identifies Novel Kinase targets in *Trypanosoma brucei*

Zachary B. Mackey¹, Kyriacos Koupparis^{1,2}, Mari Nishino^{1,3}, and James H. McKerrow^{1,*}

¹Department of Pathology, University of California, San Francisco, and Sandler, Center for Drug Discovery

²BMS Graduate Program

³TETRAD Graduate Program

Abstract

New drugs are needed to treat Human African Trypanosomiasis because the currently approved treatments are toxic or limited in efficacy. One strategy for developing new drugs involves discovering novel genes whose products can be targeted for modulation by small molecule chemotherapeutic agents. The *Trypanosoma brucei* genome contains many genes with the potential to become such targets. Kinases represent one group of genes that regulate many important cell functions and can be modulated by small molecules, thus represent a promising group of enzymes to screen for potential therapeutic targets. RNAi screens could help identify the most promising kinase targets, but the lack of suitable assays represents a barrier for optimizing the use of this technology in *T. brucei*. Here we describe an RNAi screen of a small RNAi library targeting 30 members of the *T. brucei* kinome utilizing a luciferase-based assay. This screen both validated the luciferase-based assay as a suitable method for conducting RNAi screens in *T. brucei*, and also identified 2 kinases (CRK12 and ERK8) that are essential for normal proliferation by the parasite.

Keywords

Trypanosoma brucei; HTS; RNAi; ATP-bioluminescence; target

Introduction

Human African Trypanosomiasis (HAT) in sub-Saharan Africa accounts for about 1.5 million Disability-Adjusted Life Years (DALYs), a metric that describes disease-caused loss of health and productivity (1, 2). There are two forms of HAT, a slow chronic disease caused by *Trypanosoma brucei gambiense* in western Africa, and an acute disease caused by *T. brucei rhodesiense* in eastern African. Both diseases are fatal if untreated. The 4 drugs currently approved for treating HAT are inadequate as therapies due to toxicity, poor efficacy or difficulty of administration. For example, the arsenic derivative melarsoprol causes encephalopathy in 5%–10% of patients, 10%–70% of this group die from melarsoprol (3–6). Pentamidine and suramin are not effective against the cerebral stages of the parasite. Although Eflornithine is effective against the *T. brucei gambiense* form of HAT, its administration requires IV administration in a hospital setting. These factors all point to the great need for more effective chemotherapy against HAT.

*Correspondence: James H. McKerrow, QB3, Byers Hall 508B, Mission Bay Campus, University of California San Francisco, San Francisco, CA 94158-2330, Phone: 415 476-2940, Fax: 415 476-3165, jmck@cgl.ucsf.edu.

One approach to new therapies for HAT is to discover novel gene products in the clinically relevant bloodstream form (BSF) of the parasite that can be modulated by small molecules. A promising group of such genes is the protein kinases. Protein kinases catalyze the covalent transfer of the gamma phosphate group from adenosine triphosphate (ATP) to the hydroxyl group of serine, threonine or tyrosine located on a specific protein substrate. Phosphorylation of a substrate protein can redirect its overall function, causing it to alter key biological functions, including cell cycle control and signal transduction. Protein kinases thus act as key biochemical switches that affect the physiology of cells. Modulating their activity has the potential to treat many diseases, even in cases where discriminating kinase targets might present a challenge. Gleevec (imatinib) is an important example of one such protein kinase inhibitor utilized in the treatment of certain types of leukemias (7–10). A benefit of studying the biological role of protein kinases in *T. brucei* includes discovery of novel drug targets to treat HAT.

RNA interference (RNAi) technology is a powerful strategy for identifying and understanding BSF *T. brucei* kinases that are biologically important targets (11). *T. brucei* is particularly well suited for this approach to drug discovery because the organism is amenable to chemical-genomic studies and tolerates molecular manipulations like RNAi. In *T. brucei*, RNAi has been an invaluable tool for understanding the biological roles of many of the parasite's genes and is also an ideal methodology for scanning groups of genes to identify essential drug targets. Here we describe an RNAi screen of 31 previously uncharacterized kinase genes in BSF *T. brucei* utilizing a 96-well high throughput screening format. The assay uses a luciferase-based system that is widely used in small-molecule screens (12).

Methods

Culturing of *T. brucei*

The transgenic bloodstream form *T. brucei* clone 90-13 was a gift from the laboratory of George A. M. Cross. Bloodstream parasites were incubated in 5% CO₂ at 37 °C in HMI-9 medium containing 10% fetal bovine serum, 10% Serum Plus (Omega Scientific), 1× penicillin/streptomycin with 5.0 µg/ml hygromycin B, and 2.5 µg/ml G418.

PCR amplification of partial cDNA fragments for 31 kinases

Each of the PCR products for the kinases was amplified from 2 µg of *T. brucei* genomic DNA. The conditions for PCR amplification were 98 °C for 30 sec, (98 °C 10 sec, 50 °C 10 sec 72 °C 10 sec) ×25 cycles plus 72 °C for 5 min (Phusion polymerase, NEB). The kinase PCR products and the pZJM vector both were prepared for ligation by double digestion with *Hin*DIII/*Xho*I. Ligation reactions were performed using the Rapid ligation kit from Roche. Ligation products were transformed into competent *E. coli* and plated onto LB-Ampicillin agar plates. *E. coli* clones containing the pZJM-kinase RNAi constructs were propagated in LB-Ampicillin growth medium.

Preparation of *T. brucei* Genomic DNA

T. brucei genomic DNA was prepared from 5 × 10⁸ trypanosomes grown in HMI-9 medium. The trypanosomes were pelleted by centrifugation and resuspended with 300 µl of PBS in an Eppendorf tube. SDS was added to the re-suspended trypanosomes to a final concentration of 0.5%, followed by 10 units/µl protease K and 1 unit/µl RNase A. The tube was incubated at 55 °C for 3 hours. After incubation, the lysed solution was extracted with phenol:chloroform:isoamyl alcohol and centrifuged in PhaseLock tubes (Qiagen). The genomic DNA pellet was obtained from the aqueous portion by precipitation with isopropyl alcohol and centrifugation. The dried DNA pellet was re-suspended in TE buffer at pH 7.5.

Stable transfection of *T. brucei* 90-13 clones with pZJM-kinase RNAi constructs

Each RNAi plasmid was linearized with Not I restriction endonuclease and precipitated with ethanol. The dried pellet was re-suspended in H₂O to 1.0 µg/µl. 10 µg of linearized plasmid was used in each transfection. Transfections of the plasmids were conducted with the Lonza Nucleofector using the X01 setting. The selection of stable *T. brucei* RNAi clones was done by limiting dilutions and conducted in medium containing 2.5 µg/ml of phleomycin. Individual clones were selected and expanded. Aliquots of individual clones were cryogenically preserved for future reference. The clones remaining in culture were counted with a Beckman Multisizer 3.

T. brucei Proliferation/Viability Screen

All *T. brucei* kinase RNAi clones were maintained in complete HMI-9 selection medium containing 2.5 µg/ml G418, 5.0 µg/ml of HygB, 2.5 µg/ml of Phleomycin at 37°C with 5% CO₂ as previously described. RNAi screens were carried out in 96-well format. Each RNAi clone was counted using a Beckman-Coulter Multisizer 3. RNAi clones were then diluted in selection medium to 1.0 × 10⁵ trypts/ml. After dilutions, 100 µl of both control and induced RNAi clones were transferred into 96-well plates. Clones were induced with tetracycline (100 ng/ml) for 48 hours. After induction, proliferation and viability of both control and induced clones were measured using CellTiter Glow reagent (Promega).

Northern Blot Analysis of *T. brucei* RNA

T. brucei total RNA was purified from 10⁸ parasites using TRIzol reagent (Invitrogen). 15 micrograms of total RNA were loaded on a 1.2% agarose-formaldehyde gel and resolved by electrophoresis at 50 V for 2.5 h. After electrophoresis, the RNA was transferred to polyvinylidene difluoride membrane and cross-linked to the membrane with Stratalinker (Stratagene). ³²P-Labeled cDNA probes were generated using the random primed labeling kit (Amersham Biosciences). The probes were denatured at 100 °C and hybridized to the blot overnight at 42 °C in buffer containing 50% formamide, 5× SSC, 4× Denhardt's solution, 0.1% SDS, and 0.1% sodium pyrophosphate.

Data Analysis

The quality of the HTS assay was assessed by calculating Z_{prime} and Z_{factor} using equations 1 and 2 (13, 14). Compounds having statistically significant parasite inhibition ($Z_{\text{score}} > 1.96$, $p < 0.05$) in both screens were declared hits (14). The Z_{score} was calculated using equation 3.

$$Z_{\text{prime}} = 1 - \frac{(3 \times SD_{\text{high}} + 3 \times SD_{\text{low}})}{(\text{mean}_{\text{high}} - \text{mean}_{\text{low}})} \quad (1)$$

$$Z_{\text{factor}} = 1 - \frac{(3 \times SD_{\text{compound}} + 3 \times SD_{\text{low}})}{(\text{mean}_{\text{compound}} - \text{mean}_{\text{low}})} \quad (2)$$

$$Z_{\text{score}} = \frac{(X - X_{\text{mean}})}{(SD_X)} \quad (3)$$

Results

Construction of RNAi Library

RNAi is a powerful general tool for elucidating the relationship between a gene product and its cellular function. Specifically applied to BSF *T. brucei*, the method is useful for identifying and validating novel gene products as potential therapeutic drug targets. Ideally, a genome-wide RNAi screen of the organism could provide a framework for cataloging all the potential gene targets in *T. brucei*, but the lack of suitable screening assays for BSF parasites has made it impractical to undertake large-scale projects. As a proof of concept, we have tested a screening assay utilizing a small RNAi library directed against members of the *T. brucei* kinome to determine the suitability of this approach for screening RNAi libraries in HTS format. Utilizing the primers listed in table I, we PCR-amplified 31 kinases from *T. brucei* genomic DNA. PCR products ranged in size from 300–800 bp (data not shown). Each of the PCR products was digested with *Hind*III and *Xho*I, then subcloned into the parental vector pZJM that was digested with the same restriction endonucleases (11). This cloning strategy resulted in the production of a library consisting of 31 RNAi vectors that were subsequently used to transform the *T. brucei* 90-13 strain. The transformations resulted in the generation of 30 stable phleomycin-resistant RNAi clones. Table II lists the diversity and the Gene Database identifiers of the clones. One clone, the K13 plasmid targeting the Tb11.01.4230 kinase, resisted multiple attempts to generate a stable phleomycin-resistant phenotype and was accordingly dropped from the RNAi library. The pZJM parental vector was utilized as a positive control because it contains a partial cDNA for α -tubulin; silencing of the α -tubulin message by RNAi causes a lethal phenotype in *T. brucei* (11).

Table III describes the 170 protein kinase genes in the latest tabulation of the *T. brucei* kinome, including 156 kinase genes predicted by the genome project and 14 additions based on proteomic work reported by Nett et al. (15–18). Cross-species comparison with the human kinome revealed that the *T. brucei* kinome contains fewer total kinases, lacks three groups entirely (RGC, TK and TKL), and is over-represented by the CMGC kinase group. The majority of the kinase clones in the library reported here belong to the CMGC kinase group. This result is not surprising given that this group represents 28% of the total protein kinases in the *T. brucei* kinome. Only the CRK1 homolog, Tb10.70.7040, which was amplified by the **K8** primer set, has been previously characterized in BSF parasites (19). The remaining 30 kinases have not been characterized in BSF parasites prior to this study. Of the kinases in the RNAi library reported here, 13 appear to be unique to *T. brucei* and other kinetoplastids, while the remainder appears to have homologues in other model systems.

Luciferase-based RNAi Screen

The kinome represents an ideal target environment to search for physiologically important genes that are also potentially druggable. Proliferation was the phenotypic characteristic used to determine the importance of a kinase for normal *T. brucei* physiological function. Proliferation as a phenotype is simple to score and tractable to the several uncomplicated assays. AlamarBlue has become a method of choice for measuring viability in *T. brucei* in response to HTS drug screens (20, 21). We chose a comparable luciferase-based assay. This assay measures ATP-bioluminescence that is generated in response to the oxidation of luciferin by luciferase in the presence of cellular ATP. Here the intensity of light in the bioluminescence reaction is proportional to the amount of ATP released from viable trypanosomes. Just as the fluorescence intensity in the alamarBlue assay is proportional to the number of viable cells in the reaction mixture, the light signal generated from the luciferase-based assay is also proportional to the number of viable cells in the reaction mixture. We chose the CellTiter Glo® viability assay (Promega Madison, WI) as the reference luciferase-based assay for measuring proliferation in the RNAi clones. Screening

the RNAi library involved transferring the clones to 96-well plates. We conducted a pair-wise comparison between each clone and its tetracycline-induced counterpart as an initial strategy for screening the library. Each of the clones was plated into a row of a 96-well plate such that control and induced clones were paired together in adjacent rows. Proliferation of the clones growing in the 96-well plates was measured utilizing the luciferase-based assay as outlined in figure 1.

The output of the assay was the Relative Light Unit (RLU), a value proportional to the amount of proliferation. RLU values from the wells were obtained with a luminometer with plate-reading capability. We calculated the mean RLU (RLU_{Mean}) of both control and induced RNAi clones, then compared the RLU_{Mean} of each control clone to its induced counterpart. Induced clones with t -values calculated from the Student's t -test greater than 7.64 indicated significant differences in the RLU_{Mean} values, with probability p -values less than .00001.

The RLU_{Mean} values of both α -tubulin RNAi control clones were demonstrably higher than those of the tetracycline-induced α -tubulin RNAi clones. To determine if these differences were statistically significant, we compared the RLU_{Mean} values of the control vs. induced clones using Student's t -test. The p -values were 1.6×10^{-12} for α -tubulin clone 1 and 2.5×10^{-13} for α -tubulin clone 2. Both p -values were less than the .00001 cutoff, suggesting that induction of RNAi against α -tubulin significantly affected normal proliferation in *T. brucei*. The luciferase-based assay performed as expected, detecting large differences in proliferation in response to silencing of the well-characterized *T. brucei* gene α -tubulin (11). After observing the data from the α -tubulin clones, we used the same assay to examine the 30 kinase RNAi clones.

The first set of kinase plates was read 3 days post-induction. RLU values were collected from each of the RNAi clones and the RLU_{Mean} values were plotted as histograms. The mean percent coefficient of variation for each row was about 10% when averaged over all plates, suggesting that the clones were evenly distributed among the plates. While the majority of the 30 clones tested in the screen were not affected by RNAi after 3 days post-induction, several clones had RLU_{Mean} values that changed noticeably in response to RNAi induction by tetracycline. Figure 2 shows the RLU_{Mean} for each of the 30 clones screened by the luciferase assay that were tested over a period of 9 days. These changes in the RLU value represented instances in which the proliferation of several clones was affected in response to RNAi.

Individual Student's t -tests were conducted for all control and induced pairs to obtain probabilities and t -scores. The t -test analyses revealed that 9 of the 30 kinase RNAi clones had RLU_{Mean} values with differences that were statistically significant. The 9 kinases that appeared to influence normal proliferation at day 3 post-induction were those amplified by primer sets **K5** ($p = 2.27 \times 10^{-09}$), **K6** ($p = 5.59 \times 10^{-06}$), **K8** ($p = 1.01 \times 10^{-08}$), **K10** ($p = 1 \times 10^{-05}$), **K12** ($p = 3.12 \times 10^{-08}$), **K16** ($p = 8.28 \times 10^{-06}$), **K22** ($p = 5.72 \times 10^{-06}$), **K23** ($p = 1.61 \times 10^{-07}$) and **K24** ($p = 1.07 \times 10^{-08}$) (see Figure 2, Day 3 column). In addition to clone K10, the following clones K9, K15, and K26 also appeared to have had increased proliferation in response to RNAi. However after 3 days post-induction, only **K10** had an increase in proliferation that was statistically significant.

Kinases targeted in this screen were chosen based on the lack of information about their biological function in *T. brucei*. This screen was designed to identify potential kinase targets that would diminish or arrest trypanosome proliferation upon silencing by RNAi. Five clones identified from the pair-wise screen were selected for further studies because they showed significant differences in RLU_{Mean} values over the 3 time points (Figure 2 Day 3,

Day 6 and Day 9 columns). These clones **TbK5**, **TbK6**, **TbK8**, **TbK24** and **TbK16** had constructs that targeted kinases homologous to the cdc2-related kinases (CRKs) *CRK12*, *CRK8*, *CRK1*, the extracellular signal regulated kinase (ERK) ERK8, and a kinetoplastid-specific kinase, Tb927.7.4090, respectively. The RLU_{Mean} values of the induced RNAi clones were significantly lower than those of their respective controls. Reproducible detection of a significantly lower rate of proliferation for each time point suggested that silencing any of these 5 kinases would be sufficient to affect normal *T. brucei* proliferation.

Table IV lists the clones that were expanded in flasks to follow up the results of pair-wise comparisons determined by the luciferase assay in 96-well format. The expanded clones were sub-cultured in complete HMI-9 medium with or without tetracycline and counted daily over a period of 9 days. Figure 3 demonstrates that 2 of the 5 kinases that were re-tested, CRK12 and ERK8, had strong growth phenotypes and appeared to be essential for normal growth and proliferation in *T. brucei*. The 3 remaining kinases CRK1, CRK8 and Tb927.7.4090 had subtle growth phenotypes, suggesting that they were not essential for normal proliferation over the same time period.

In cell-based small-molecule HTS assays, assayed plates include reference controls that define a dynamic range for the experiment. Bioactive hits are identified by wells with readout signals shifted away from the mean of signals from the general sample population (14). The same methodology applied to a library of RNAi clones in an HTS format should reveal wells containing clones in which essential genes have been silenced. To test this notion, we screened the same 30 RNAi clones in an HTS format similar to that used to screen small-molecule libraries against cultured *T. brucei* (12). Overall, the RNAi screen was easy to perform in the HTS format. The replicated RLU values for the 30 clones plotted in figure 4A demonstrate that the majority of the clones had similar RLU values even after day 9 post-induction. The mean Z_{factor} for the screen was 0.2, indicating a well-performing assay that could be suitably extended to a full-scale HTS (14). To identify clones with signals that shifted away from the mean, the RLU_{Mean} for each clone was calculated and plotted as a separate scatter plot depicted in figure 4B. Induced clones in which the Z_{score} was greater than 1.96 ($p < 0.05$) were considered to have had an essential kinase silenced by RNAi. The Z_{score} for both CRK12 and ERK8 fell below that threshold, as did both α -tubulin positive controls. Those 2 RNAi clones were expanded in culture flasks for examination by northern blot analysis. The analysis demonstrated that the mRNAs for both of these clones were silenced by RNAi (figure 5).

Discussion

Several technical advances have brought improvements to the ability to screen RNAi libraries in BSF *T. brucei* since the chromosome 1 RNAi project was reported (22). That study required a small consortium of labs to score each RNAi clone for many complex phenotypes associated with knocking down all of the *T. brucei* genes on chromosome 1 (22). One improvement to screening RNAi libraries in BSF parasites included the development of a more efficient transformation protocol (23). Improved transformation efficiency has now made it feasible to construct RNAi plasmid libraries that cover the entire *T. brucei* genome, a necessity for genome-wide screens. Recently, a genome-wide RNAi library was created and transfected into BSF parasites employing the more efficient protocol. The library was transfected in plasmid pools sufficient to give 9-fold genome coverage (24). In that study, the strategy was to induce the RNAi clones with tetracycline while selecting them in the presence of melarsoprol or eflornithine. That functional genomic strategy was suitably designed as a gain-of-function screen aimed at identifying genes implicated in drug uptake or resistance.

The HTS assay described in the current study was designed to identify genes essential for the parasite to proliferate normally. Identifying proliferation-deficient clones in response to gene silencing represented an efficient way to screen for potential targets in BSF parasites. Kinases are attractive targets because of their druggable properties. They are also important regulators of physiological pathways in *T. brucei*. To identify potential kinase targets, we constructed a small RNAi library consisting of 31 previously uncharacterized kinases and used them to generate 30 stable RNAi clones. One clone was removed from the library because no stable clone could be made from it despite several attempts. The promoters of the pZJM vector can “leak” producing low background levels of dsRNA (11, 25, 26). The fact that this clone could not be made may indicate that the mRNA message being targeted by this RNAi construct has some important physiological function. It should be possible to productively examine this RNAi construct with an RNAi vector system with tighter promoter regulation (27).

Pair-wise analysis of the library revealed several kinases that appeared to be important for normal proliferation. The kinases identified from that analysis included three *cdc2*-related kinases, CRK1, CRK 8 and CRK 12, one extracellular signal-related kinase, ERK 8, and one uncharacterized kinase unique to kinetoplastids, Tb927.7.4090. Upon more detailed analysis of each clone identified from the pair-wise comparison, we discovered that only two of the kinases were essential for normal proliferation.

Screening the same RNAi library in an HTS format however only identified two kinases, ERK8 and CRK12, that were essential for normal proliferation in trypanosomes. Further analysis of these two clones after RNAi by northern blot analysis revealed that the mRNA messages of these two kinases were indeed silenced after RNAi induction, validating them as essential for normal proliferation in *T. brucei*. This represents the first study to identify *TbERK8* as an essential gene in BSF *T. brucei*. *TbERK8* is homologous to the human *ERK8* gene. There are three subfamilies of mitogen-activated protein kinases (MAPKs); the ERKs, c-jun N-terminal kinases (JNK) and the p38 Map kinases. The human ERK 8 represents the latest member of the ERK subfamily of MAPK to be discovered (28), thus little is known about the functions of this kinase in comparison to the other ERK family members. In mammalian cells, Erk8 activity becomes rapidly up-regulated in response to DNA single-strand breaks caused by hydrogen peroxide. Its activity is also up-regulated by single-strand breaks caused by other agents such as MMS, however at a reduced rate (29, 30). It was recently demonstrated that in human cells ERK8 regulates proliferation and DNA repair through a proliferating cell nuclear antigen (PCNA) mechanism (31). It interacts with PCNA through a PCNA-interacting protein box (PIP) sequence aa 297-QALQHPYVQRFH-308. Interaction between the human Erk8 and PCNA is required in order to maintain the stability of PCNA in the cell, preventing PCNA from interacting with the E3 ligase HDM2 (31). TbErk8 also contains a putative PIP box at aa 293-TAEQALEHPYVAAFH-306. However, further studies are needed to determine if TbErk8 interacts with TbPCNA through its putative PIP box.

Several *cdc2*-related kinases (CRKs) in *Trypanosoma brucei* function analogously to their homologues in other eukaryotes as master regulators of cell cycle progression (32, 33). For example, cyclins E1 and CRK1 regulate G1/S transition and the complexes of cyclin E1 and CRK3 as well as that of cyclin B2 and CRK3 control passage of the G2/M boundary during cell cycle progression (34, 35). Like these other CRKs, it is possible that CRK12 may also be involved in cell-cycle regulation. This may explain why silencing its gene product by RNAi resulted in reduced proliferation in trypanosomes. Further studies of ERK8 and CRK12 will be necessary to confirm these hypotheses and further elucidate their function in *T. brucei*.

Now that these two kinases have been identified as potential targets, we will take advantage of small-molecule libraries to further characterize them.

In addition to identifying two potential targets, we have tested and validated a luciferase-based assay for its suitability in screening *T. brucei* RNAi libraries. Here, we demonstrated that a single-step luciferase-based assay was sufficient to screen a small RNAi library. Three clones K19, K23, and K24 showed a significantly lower growth rate than the other clones present in the screen under un-induced conditions using a pair-wise comparison. The lower RLU reading for K19 occurred because this clone grew slower than the other clones present on plate 5. A plausible explanation for the slow growth rate of K19 was that the RNAi targeting vector was misincorporated in the genome. In this scenario, the vector integrates into a genomic locus and disrupts a gene that is important for normal proliferation. The reduced growth rates of clones K23 and K24 on plate 6 appeared to be caused by loading errors in the primary plate on day 3. The RLU values of these two un-induced clones were comparable to values of the other clones on plate 6 by days 6 and 9, indicating that these two clones grew normally under control conditions. These same three clones were also screened in an HTS format that included the entire set RNAi clones. The performance of the un-induced clones in the HTS format was comparable to their performance in the pair-wise format (data not shown). When RNAi was induced in this screening format, only the RLU values of K5 and K24 shifted away from the mean significantly. The RLU value of K19 shifted away from the mean, but its Zscore was less than 1.96 once again indicating that it was not significant enough to be counted as a hit. These results suggest that the luciferase-based assay was unaffected by small changes in experimental methods or parameters during both formats. Overall, we conclude that the HTS format was more robust for screening RNAi libraries than the pair-wise comparison.

The larger implication of this project is the potential for the assay to be scaled up to screen a genome-wide RNAi library consisting of the roughly 9,000 genes that make up the *T. brucei* genome. Identifying every essential *T. brucei* gene will be critical for prioritizing the targeting environment of this parasite. Having a suitable HTS assay in hand eliminates a barrier that has been a critical bottleneck in *T. brucei* drug discovery, and can provide the HAT community with the ability to select the best druggable targets from the complete repertoire of genes.

Acknowledgments

This work was supported by the Sandler Foundation and 5U01AI075641 - 04 from the NIAID.

Literature Cited

1. Sassi F. Calculating QALYs, comparing QALY and DALY calculations. Health Policy Plan. 2006; 21:402–408. [PubMed: 16877455]
2. King CH, Bertino AM. Asymmetries of poverty: why global burden of disease valuations underestimate the burden of neglected tropical diseases. PLoS Negl Trop Dis. 2008; 2:e209. [PubMed: 18365036]
3. Blum J, Burri C. Treatment of late stage sleeping sickness caused by *T.b. gambiense*: a new approach to the use of an old drug. Swiss Med Wkly. 2002; 132:51–56. [PubMed: 11971197]
4. Burchmore RJ, Ogbunode PO, Enanga B, Barrett MP. Chemotherapy of human African trypanosomiasis. Curr Pharm Des. 2002; 8:256–267. [PubMed: 11860365]
5. Nok AJ. Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human African trypanosomiasis. Parasitol Res. 2003; 90:71–79. [PubMed: 12743807]
6. Kennedy PG. Human African trypanosomiasis of the CNS: current issues and challenges. J Clin Invest. 2004; 113:496–504. [PubMed: 14966556]

7. Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, Lydon NB. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res.* 1996; 56:100–104. [PubMed: 8548747]
8. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med.* 1996; 2:561–566. [PubMed: 8616716]
9. O'Dwyer ME, Druker BJ. STI571: an inhibitor of the BCR-ABL tyrosine kinase for the treatment of chronic myelogenous leukaemia. *Lancet Oncol.* 2000; 1:207–211. [PubMed: 11905636]
10. Capdeville R, Buchdunger E, Zimmermann J, Matter A. Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. *Nat Rev Drug Discov.* 2002; 1:493–502. [PubMed: 12120256]
11. Wang Z, Morris JC, Drew ME, Englund PT. Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J Biol Chem.* 2000; 275:40174–40179. [PubMed: 11013266]
12. Mackey ZB, Baca AM, Mallari JP, Apsel B, Shelat A, Hansell EJ, Chiang PK, Wolff B, Guy KR, Williams J, McKerrow JH. Discovery of trypanocidal compounds by whole cell HTS of *Trypanosoma brucei*. *Chem Biol Drug Des.* 2006; 67:355–363. [PubMed: 16784460]
13. Sui Y, Wu Z. Alternative statistical parameter for high-throughput screening assay quality assessment. *J Biomol Screen.* 2007; 12:229–234. [PubMed: 17218666]
14. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.* 1999; 4:67–73. [PubMed: 10838414]
15. Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Bohme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Alsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivens A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Larke N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G, Peacock CS, Peterson J, Quail MA, Rabinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van Aken S, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, et al. The genome of the African trypanosome *Trypanosoma brucei*. *Science.* 2005; 309:416–422. [PubMed: 16020726]
16. El-Sayed NM, Myler PJ, Blandin G, Berriman M, Crabtree J, Aggarwal G, Caler E, Renauld H, Worthey EA, Hertz-Fowler C, Ghedin E, Peacock C, Bartholomeu DC, Haas BJ, Tran AN, Wortman JR, Alsmark UC, Angiuoli S, Anupama A, Badger J, Bringaud F, Cadag E, Carlton JM, Cerqueira GC, Creasy T, Delcher AL, Djikeng A, Embley TM, Hauser C, Ivens AC, Kummerfeld SK, Pereira-Leal JB, Nilsson D, Peterson J, Salzberg SL, Shallom J, Silva JC, Sundaram J, Westenberger S, White O, Melville SE, Donelson JE, Andersson B, Stuart KD, Hall N. Comparative genomics of trypanosomatid parasitic protozoa. *Science.* 2005; 309:404–409. [PubMed: 16020724]
17. Parsons M, Worthey EA, Ward PN, Mottram JC. Comparative analysis of the kinomes of three pathogenic trypanosomatids: *Leishmania major*, *Trypanosoma brucei* and *Trypanosoma cruzi*. *BMC Genomics.* 2005; 6:127. [PubMed: 16164760]
18. Nett IR, Martin DM, Miranda-Saavedra D, Lamont D, Barber JD, Mehlert A, Ferguson MA. The phosphoproteome of bloodstream form *Trypanosoma brucei*, causative agent of African sleeping sickness. *Mol Cell Proteomics.* 2009; 8:1527–1538. [PubMed: 19346560]
19. Tu X, Wang CC. Pairwise knockdowns of cdc2-related kinases (CRKs) in *Trypanosoma brucei* identified the CRKs for G1/S and G2/M transitions and demonstrated distinctive cytokinetic regulations between two developmental stages of the organism. *Eukaryot Cell.* 2005; 4:755–764. [PubMed: 15821135]

20. Raz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) in vitro. *Acta Trop.* 1997; 68:139–147. [PubMed: 9386789]
21. Ahmed SA, Gogal RM Jr, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [³H]thymidine incorporation assay. *J Immunol Methods.* 1994; 170:211–224. [PubMed: 8157999]
22. Subramaniam C, Veazey P, Redmond S, Hayes-Sinclair J, Chambers E, Carrington M, Gull K, Matthews K, Horn D, Field MC. Chromosome-wide analysis of gene function by RNA interference in the african trypanosome. *Eukaryot Cell.* 2006; 5:1539–1549. [PubMed: 16963636]
23. Burkard G, Fragoso CM, Roditi I. Highly efficient stable transformation of bloodstream forms of *Trypanosoma brucei*. *Mol Biochem Parasitol.* 2007; 153:220–223. [PubMed: 17408766]
24. Schumann Burkard G, Jutzi P, Roditi I. Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. *Mol Biochem Parasitol.* 175:91–94. [PubMed: 20851719]
25. Wirtz E, Hoek M, Cross GA. Regulated processive transcription of chromatin by T7 RNA polymerase in *Trypanosoma brucei*. *Nucleic Acids Res.* 1998; 26:4626–4634. [PubMed: 9753730]
26. Alibu VP, Storm L, Haile S, Clayton C, Horn D. A doubly inducible system for RNA interference and rapid RNAi plasmid construction in *Trypanosoma brucei*. *Mol Biochem Parasitol.* 2005; 139:75–82. [PubMed: 15610821]
27. Alsford S, Kawahara T, Glover L, Horn D. Tagging a *T. brucei* rRNA locus improves stable transfection efficiency and circumvents inducible expression position effects. *Mol Biochem Parasitol.* 2005; 144:142–148. [PubMed: 16182389]
28. Abe MK, Saelzler MP, Espinosa R 3rd, Kahle KT, Hershenson MB, Le Beau MM, Rosner MR. ERK8, a new member of the mitogen-activated protein kinase family. *J Biol Chem.* 2002; 277:16733–16743. [PubMed: 11875070]
29. Klevernic IV, Martin NM, Cohen P. Regulation of the activity and expression of ERK8 by DNA damage. *FEBS Lett.* 2009; 583:680–684. [PubMed: 19166846]
30. Erster O, Seger R, Liscovitch M. Ligand interaction scan (LIScan) in the study of ERK8. *Biochem Biophys Res Commun.* 399:37–41. [PubMed: 20638370]
31. Groehler AL, Lannigan DA. A chromatin-bound kinase, ERK8, protects genomic integrity by inhibiting HDM2-mediated degradation of the DNA clamp PCNA. *J Cell Biol.* 190:575–586. [PubMed: 20733054]
32. Li Y, Li Z, Wang CC. Differentiation of *Trypanosoma brucei* may be stage non-specific and does not require progression of cell cycle. *Mol Microbiol.* 2003; 49:251–265. [PubMed: 12823826]
33. Tu X, Wang CC. The involvement of two cdc2-related kinases (CRKs) in *Trypanosoma brucei* cell cycle regulation and the distinctive stage-specific phenotypes caused by CRK3 depletion. *J Biol Chem.* 2004; 279:20519–20528. [PubMed: 15010459]
34. Van Hellemond JJ, Neuville P, Schwarz RT, Matthews KR, Mottram JC. Isolation of *Trypanosoma brucei* CYC2 and CYC3 cyclin genes by rescue of a yeast G(1) cyclin mutant. Functional characterization of CYC2. *J Biol Chem.* 2000; 275:8315–8323. [PubMed: 10722661]
35. Hammarton TC, Clark J, Douglas F, Boshart M, Mottram JC. Stage-specific differences in cell cycle control in *Trypanosoma brucei* revealed by RNA interference of a mitotic cyclin. *J Biol Chem.* 2003; 278:22877–22886. [PubMed: 12682070]

A

B

Figure 1. Flow of luciferase-based RNAi Screen

31 partial cDNA fragments were PCR amplified from genomic DNA and subcloned into RNAi plasmids. The plasmids were linearized with NotI and transfected into *T. brucei* 90-13 to make stable RNAi clones A). Each of the *T. brucei* RNAi clones was counted then diluted with HMI-9 and transferred to 96-well plates so that each well contained 10^4 clones. Each row contained 12 replicates of RNAi clones that were maintained in either control medium or induction medium containing 100 ng/mL tetracycline, allowing 4 RNAi clones to be tested per plate. At the appropriate time point (days 3, 6 or 9 post-induction), luciferase reagent was added to the plates and RLU values were obtained with read with a luminometer B).

Figure 2. Pair-wise comparison of RNAi clones

Mean RLU (RLU_{Mean}) values of control vs. tetracycline induced RNAi clones as graphed in histograms. Bars in the histogram represent the RLU_{Mean} from an entire row of RNAi clones. Each plate contains the data from 4 clones in which the control and tetracycline-induced RLU_{Mean} was evaluated in a pair-wise manner. Replicas were made from each plate prior to reading and then assayed on every third day for nine days, as represented by each column (Day 3, Day 6, Day 9). The mean and standard deviations were calculated for each row. The Student's *t*-test was used to determine which RNAi clones had significant differences in the RLU_{Mean} in response to tetracycline induction. Clones in which the RLU_{Mean} values were significantly different are represented by the hatched bars.

Figure 3. Pair-wise counting of RNAi clones

The 5 RNAi clones that showed a significant change in growth rate over all 3 reads were each expanded into individual flasks and counted every day for 9 days. The numbers were plotted as a line graph with the $\log_{10}$ cell number plotted on the y-axis. For each clone, the closed diagrams (● ▲ ◆) represent the respective clones growing under control conditions whereas open diagrams (○ △ ◇) represent the clones being induced with tetracycline at 100 ng/ul. TbK5 and TbK24 represent the clones with strong growth phenotypes after RNAi induction A). TbK6, TbK8 and TbK16 represent the clones with subtle growth phenotypes after RNAi induction B). The values on the graph represent the averages of 3 separate experiments.

A

B

Figure 4. HTS assay format for RNAi library

Each of the wells tested were inoculated with 10^4 RNAi clones in medium containing 100 ng/ μL tetracycline. Duplicated screens of the RNAi library were conducted on each 96-well plate. The data points graphed on the scatter plot represent the log-transformed RLU values for each of the replicated RNAi clones tested in the screen. A). The RLU_{Mean} values for all of the RNAi clones with SD were calculated and then graphed as a separate scatter plot B). The Z_{score} at 1.96 below the mean RLU depicted by the dashed line represents the cut-off point for the $p = 0.05$.

Figure 5. Northern blot analysis of K5 and K24

The two proliferation-deficient clones identified from screening the 30 RNAi clones in HTS format were further analyzed by northern blot analysis. Total RNA was isolated from control or tetracycline-induced clones after 48 hours post induction. Labeled gene specific probes were hybridized to the blots and visualized by autoradiography. CRK12 A). ERK8 B).

Table I

PCR Primers used to amplify kinases.

Primer Set	5'-Sequence-3'
K1 f	TGCGACACAGTGAAGCTTGGTTCTGGTAGTAG
K1 r	TATATCTCGAGCCGACGATGGAAGGCCTTGT
K2 f	GAATACTCAGATGTGAGCGAGCACGTAGATCG
K2 r	GCGCGAAGCTTAGCTTTCCTGTAAACCATTTTC
K3 f	GTCGAAAGCTTGATCCGTACGTTCCGCTGTTA
K3 r	GTATTCTCGAGCTGAGACGTCTGGAAGGATCA
K4 f	GTGACCGCAAGCTTCGTCGTATCATGTTTCAG
K4 r	CATATCTCGAGCTTCTTCTCACGCACCTCAT
K5 f	GATATCTCGAGATAAGTGAGGGCACGTACGGT
K5 r	CATATGCCGTTGGGAAAGCTTCATCAAGGTCA
K6 f	GTAATATAAGCTTGTCACAGCCCACCTTCGC
K6 r	TATATCTCGAGTTGGGCTCAAGCTGCAGCATC
K7 f	ATCGAGTAAGCTTGGCGAAGGAACCTATGGAG
K7 r	TAGATCTCGAGTGCCACGTCGTGAGAGTACTT
K8 f	CGCAGAAGCTTTTACGAGCGGCTTCAGAAGAT
K8 r	ATATACTCGAGGTATGGGTGCTCAATGCCTG
K9 f	GCGAGAAGCTTGAAGTGACAAGCTGAAGGAAG
K9 r	ATGCACTCGAGTTCCACAGTGTAGAAGATCC
K10 f	GCGCGAAGCTTTACGATGTTGTTGTCGATAGG
K10 r	TATATCTCGAGGAAAAATGGGTGCTGCAGCGC
K11 f	GCGCGAAGCTTTTCTTGTGGTACACAATGGG
K11 r	TATATCTCGAGGTCTTCATCCTGCGGATCGCT
K12 f	GCGACAAGCTTTTTCGTTTTGTCATCGGCACC
K12 r	GCGCCAGTCTCGAGCTAGTTCAGCAAAAATAC
K13 f	TGCATAAGCTTGTCGTTTCGGGAAGCAAACGTG
K13 r	GCGCGCTCGAGATGATTGAAAGGACCATGTTT
K14 f	ATAGTAAGCTTAGCACAAACGCAATGGTGCCG
K14 r	GCGAGCTCGAGCCACAGATCTGTTGAATACAT
K15 f	GACTGAAGCTTTTTCATACGGGCGCTTGGTGA
K15 r	ATAGTCTCGAGGAGCTCCAGTCTGCAATCAT
K16 f	GCGTGAAGCTTAATGGGGATTACTTTCGTGCG
K16 r	TATGTCTCGAGCACTGCGCTCGATCATATCGT
K17 f	GCGAGAAGCTTGTAATTTGCGAGTTCCCCGTA
K17 r	TACGTCTCGAGTTTCGTACCAACAACCTGCTGCA
K18 f	GCGGTAAGCTTAATCAACAGCGTCTGTAGAGG
K18 r	GCGCGCTCGAGACTATTAGACTGGATATCTGA
K19 f	TACGTAAGCTTGCGCCTTGATGGTTGTCGTGT
K19 r	ATAGTCTCGAGTTGGCCCTCATGCACGTTAGA
K20 f	GCGGTAAGCTTTCGGAACAATGGGATGAACTC

Primer Set	5'-Sequence-3'
K20 r	GCGGTCTCGAGTACACATGTGTTTCCTTGTAGT
K21 f	GCGACAAGCTTCGTACGAGATGGACGAGATAA
K21 r	CAGTCCTCGAGCAAGTCAATCACTTTCAGTGC
K22 f	GCGCGAAGCTTTTGTGAACTTGATTTTCGAGGC
K22 r	ATATACTCGAGACGGCGCGACACGTACCATT
K23 f	ATAGTAAGCTTCACCATCTCTGAGGGTCGGAC
K23 r	CGCGCCTCGAGTTTGTCTAACTGGTTGTAGAA
K24 f	GACGTAAGCTTTCGCGCAACGAATGACAAGGA
K24 r	TATATCTCGAGATACGGGTGTTCAAGCGCCTG
K25 f	GACGTAAGCTTGGAAGGTGCACCATCCTAACA
K25 r	ATATACTCGAGACTGTCGTAGTGGCGTTGGAG
K26 f	CGCGAGAAGCTTGCTGACTTTGGTCTCGTTAA
K26 r	GCGTGCTCGAGCTTTTGTATCGGAATTCGAA
K27 f	CGCGCAAGCTTAGCGATCAAGTGCATGAGAAA
K27 r	ATATACTCGAGCGCATTGGAGAGGTCCTCAA
K28 f	ATAGTAAGCTTGGCTGGGGTGAGTTCAGCACT
K28 r	TATATATCTCGAGCGATCCTTGTTCCGGAGGC
K29 f	GACGTAAGCTTCTTTCGCGTAAAACCCGGAAC
K29 r	GCGCGCTCGAGTGGTTTTAGATCCGTATGAAT
K30 f	GACGTAAGCTTCTTCTCCGGGTAAATCGCGT
K30 r	GCACGTCTCGAGGAAGTTTAAAGTGAGGTTCC
K31 f	TACGTAAGCTTTGCGTGAGGTTGCGATGATGC
K31 r	TACGTCTCGAGAGTTTGCCCATGTCCAATTCG

Table II*T. brucei* kinases with Gene database annotations.

RNAi Clone	Kinase Type	GeneDB ID
TbK1	S_TKc	Tb11.02.3010
TbK2	PKc_like Super-family	Tb10.329.0030
TbK3	STKc_CCRK	Tb927.7.1900
TbK4	STKc_CDK2_like	Tb927.7.7360
TbK5	CRK12	Tb11.01.4130
TbK6	CRK8	Tb11.02.5010
TbK7	CRK4	Tb927.8.5390
TbK8	CRK1	Tb927.10.1070
TbK9	CRK6	Tb11.47.0031
TbK10	CRK10	Tb927.3.4670
TbK11	CRK11	Tb927.6.3110
TbK12	CRK3	Tb927.10.4990
*TbK13	PKc	Tb11.01.4230
TbK14	CDC-like kinase 1 isoform 2	Tb11.01.4250
TbK15	CDC like kinase 4	Tb927.3.1610
TbK16	S/T PKc	Tb927.7.4090
TbK17	S/T PKc	Tb927.7.3880
TbK18	S/T PKc	Tb927.5.1650
TbK19	S/T PKc	Tb10.61.1520
TbK20	S/T PKc	Tb10.100.0230
TbK21	S/T PKc	Tb11.02.0640
TbK22	Protein kinase,	Tb10.6k15.0770
TbK23	MAPK ERK1/2 like	Tb927.8.3770
TbK24	ERK8	Tb927.10.5140
TbK25	MAPK9 S/T PKc	Tb10.61.1850
TbK26	Putative MAPK S/T PKc	Tb927.3.690
TbK27	Putative MAPK S/T PKc	Tb09.211.0960
TbK28	S/T PKc	Tb927.7.960
TbK29	SRPK	Tb927.6.4970
TbK30	PKc	Tb11.01.0380
TbK31	MAPK 3	Tb927.8.3550

Table III*T. brucei* Kinome

	AGC	CAMK	CKI	CMGC	RGC	STE	TK	TKL	Other	NEK	Total
<i>T. brucei</i>	22	28	5	47	0	28	0	0	20	20	170
% of Kinome	13	16	3	28	0	16	0	0	12	12	
<i>H. sapiens</i>	82	95	12	68	5	61	91	48	6	10	478
% of Kinome	17	19	3	14	1	13	19	10	1	2	

Table IV

Replication deficient clones identified from pairwise comparison.

RNAi Clone	Type of Kinase	GeneDB ID
TbK5	CRK12	Tb11.01.4130
TbK6	CRK8	Tb11.02.5010
TbK8	CRK1	Tb927.10.1070
TbK16	S/T PKc	Tb927.7.4090
TbK24	ERK8	Tb927.10.5140