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Citation for published version:

Digital Object Identifier (DOI):
10.1073/pnas.1305404110

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Proceedings of the National Academy of Sciences (PNAS)

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Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes

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Viability of the tsetse fly-transmitted African trypanosome Trypanosoma brucei depends on maintenance and expression of its kDNA, the mitochondrial genome of this parasite and a putative target for veterinary and human antitrypanosomatid drugs. However, the closely related animal pathogens T. evansi and T. equiperdum are transmitted independently of tsetse flies and survive without a functional kinetoplast for reasons that have remained unclear. Here, we provide definitive evidence that single amino acid changes in the nuclearly encoded F1F0-ATPase subunit γ can compensate for complete physical loss of kDNA in these parasites. Our results provide insight into the molecular mechanism of compensation for kDNA loss by showing F0-independent generation of the mitochondrial membrane potential with increased dependence on the ADP/ATP carrier. Our findings also suggest that, in the pathogenic bloodstream stage of T. brucei, the huge and energetically demanding apparatus required for kDNA maintenance and expression serves the production of a single F1F0-ATPase subunit. These results have important implications for drug discovery and our understanding of the evolution of these parasites.

MALAVARIAN TRYpanosomes are extracellular protist parasites that cause important diseases in humans (human African trypanosomiasis) and their livestock (nagana). They predominantly infest the blood but, depending on the (sub)species, also other organs, such as the CNS. Transmission typically occurs through the saliva of blood-sucking insect vectors during feeding. The lifecycle of African trypanosomes, such as Trypanosoma congolense, T. b. brucei, T. b. gambiense, and T. b. rhodesiense, is fully dependent on cyclical development in the tsetse fly vector and highly complex (1). Thus, these parasites are restricted to areas inhabited by the tsetse fly (i.e., sub-Saharan Africa).

However, some pathogenic trypanosome species have adapted to efficient tsetse-independent transmission, abandoning any developmental stages associated with that vector, and therefore, they were able to escape from the African tsetse belt. T. evansi is transmitted mechanically by biting flies when the insect’s blood meal on an infected host is interrupted and a second host is bitten with trypanosome-contaminated mouth parts shortly thereafter (2). T. evansi infects various mammalian animals, including livestock, and it is the pathogenic trypanosome with the widest geographical distribution. The disease caused by this parasite, therefore, has many different names but is known as surra in large parts of Asia. The second species, T. equiperdum, causes a sexually transmitted horse disease called dourine and predominantly infects genital tissues (2). T. evansi and T. equiperdum are morphologically indistinguishable from each other and T. b. brucei, and their status as independent species has been questioned (3, 4).

Mitochondrial DNA (mtDNA) of trypanosomatids is organized as the kinetoplast (kDNA), a gigantic network of concatenated, circular DNA molecules (5). The second key feature distinguishing T. evansi and T. equiperdum from T. b. brucei is that they are dyskinetoplastic [DK; i.e., lacking all (akinetoplast or kDNAγ) or critical parts (kDNAΔγ) of their mitochondrial DNA] (6). T. brucei kDNA contains two types of molecules. The ~23-kb maxicircle, present in ~20–50 copies, contains a typical set of rRNA and protein-coding genes, most of which encode subunits of respiratory chain complexes (6). Most trypanosomatid mitochondrial mRNAs require a unique form of posttranscriptional editing before they can be translated into functional proteins (7). The second kDNA component is a highly diverse set of thousands of ~1-kb minicircles, which encode guide RNAs required for editing. Maintenance and expression of kDNA require numerous essential enzymes and have been suggested as a target for existing and novel drugs for T. brucei and other trypanosomatids (8). Indeed, antitrypanosomatid therapeutics, such as pentamidine and ethidium bromide, have been shown to directly affect kDNA (9, 10). T. equiperdum strains typically have retained their maxicircle—in some cases with substantial deletions—but have lost their minicircle diversity (4, 6). T. evansi strains do not have a maxicircle and either show minicircle homogeneity or are akinetoplast. Consequently, both species are incapable of functional mitochondrial gene expression.

T. evansi and T. equiperdum cannot develop in the tsetse fly, probably because ATP production in that environment requires oxidative phosphorylation (11) and, therefore, the capacity to express numerous mitochondrial genes. They can only survive as bloodstream forms (BFs), which produce ATP exclusively through glycolysis; however, they still require a mitochondrion, because it hosts other essential activities (12–14). A key process underpinning mitochondrial function is the maintenance of an electrochemical potential, Δψm, across the inner mitochondrial membrane (15). BF T. brucei, which lacks the proton pumping respiratory complex III and IV, generates Δψm using the mitochondrial F1F0-ATP synthase complex functioning in reverse to pump protons from the matrix into the intrermembrane space (16–18). Subunit a of the membrane-embedded F0 part is critical for proton translocation (Fig. S1A) and kDNA-encoded, and its pre-mRNA requires substantial RNA editing (19). DK trypanosomes are incapable of expressing subunit a, because they lack either the gene itself or most, if not all, guide RNAs. The puzzling fact that these organisms are viable was hypothesized to involve compensatory mutations in the nuclearily encoded F1 subunit γ (Fig. S1 B–D) that enable F0γ-independent Δψm generation (4, 17).

We tested this hypothesis by generating BF T. b. brucei that express mutated γ subunits and investigating their response to kDNA loss. Our results show that a single amino acid change in subunit γ fully compensates for complete loss of kDNA and provide insight into the molecular mechanism of this compensation. This finding has important consequences for our understanding of

Author contributions: S.D., M.K.G., and A.C.S. designed research; S.D., M.K.G., C.E.D., and A.C.S. performed research; S.D., M.K.G., C.E.D., and A.C.S. analyzed data; and S.D., M.K.G., and A.C.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freedly available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. KF293288).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305404110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1305404110

PNAS | September 3, 2013 | vol. 110 | no. 36 | 14741–14746
the mitochondrial function in these organisms, their evolution, and the suitability of kDNA as a drug target.

Results

We first investigated the significance of the F_{1,γ} L262P mutation identified in the *T. b. brucei* 164DK cell line (17). These cells had lost their kinetoplast after several months of in vivo selection with acriflavine (Acr), a DNA-intercalating compound (20). (Table 1 lists the mutations investigated, and Table S1 lists the trypanosome cell lines and strains used in this study). We first determined whether this mutation confers Acr resistance in a standard 3-d drug sensitivity assay (21). We combined introduction of a single ectopic subunit γ allele bearing the L262P mutation (γL262P) into *T. b. brucei* 427 with a single (sKO) or double KO (dKO) of the endogenous γ subunit, resulting in cell lines sKO + γL262P and dKO + γL262P. Cell lines expressing an ectopic WT allele (γWT) were generated as controls. We then compared the Acr sensitivity of these cell lines with parental *T. b. brucei* 427, *T. evansi* Antat 3/3 (which has the A281del mutation) (Table 1), and *T. b. brucei* 164DK (the source of the L262P mutation).

For *T. b. brucei* 164DK, the average EC_{50} value for Acr was sevenfold greater than for the *T. b. brucei* 427 WT strain (Fig. 1 A). *T. evansi* Antat 3/3 had an intermediate EC_{50} value. Strains expressing γL262P showed considerable resistance, similar to the resistance level of the 164DK cell line, in which this mutation had originally been identified. Although the EC_{50} for sKO cells was slightly lower than for dKO cells, this result was not statistically significant (P value = 0.062; unpaired two-tailed Student’s t test). In contrast, cells expressing the ectopic γWT retained the 427 parental sensitivity. These results show that the L262P mutation is sufficient to confer a level of Acr resistance that is similar to the one observed for *T. b. brucei* 164DK.

Next, we investigated if F_{1,γ} mutations identified in DK trypanosomes (Table 1) enable long-term survival of parasites in the presence of 20 nM Acr, a concentration well above the 3-d EC_{50} for WT cells. Trypanosomes expressing only γWT showed a severe growth defect by 48 h, and no live cells were seen by microscopy after 72 h (Fig. 1B and Fig. S2B). Conversely, γL262P-expressing trypanosomes continued to proliferate without an apparent lag period that would have indicated selection for additional mutations or adaptations and at a rate similar to the laboratory-induced *T. b. brucei* 164DK cells. Microscopy and PCR assays showed that the Acr-treated γL262P-expressing cells rapidly became kDNA^- (Fig. 1 C and D and Fig. S2 C and D).

Dramatically different Acr sensitivities were observed for *T. b. brucei* expressing F_{1,γ} with mutations identified in field isolates of *T. evansi* and *T. equiperdum* from various geographical areas (Table 1). Cells expressing γM282L behaved like WT and were dead by 72 h (Fig. 1B and Fig. S2B); in contrast, the γA273P-expressing cells continued to grow like γL262P-expressing cells (Fig. 1B). The result for γA281del-expressing cells (the mutation present in the vast majority of field isolates; see Table 1) was more complex. Although some clones died as quickly as the negative controls, other clones persisted, and a small number of live cells were still observed after 7 d (Fig. 1B and Fig. S2B, asterisks). After transfer to Acr-free medium, these cells recovered, and when reexposed to Acr, no lag in growth was observed (Fig. 1E), suggesting that they had undergone a secondary adaptation. Like the γL262P-expressing cells, all cell lines that had survived Acr treatment were kDNA^-.

Expression of functional F_{1,γ}-ATPase was broadly equivalent (Fig. S3), ruling out variations in expression level as an explanation for the differing Acr sensitivities. The antibody that we used detects the 15 kDa N-terminal β-barrel domain of the α subunit, which in trypanosomatids, is proteolytically cleaved off from the rest of the polypeptide but remains associated with the F_{1} complex (22). Interestingly, the β-barrel domain seems to have been lost or altered in *T. evansi* Antat 3/3, because here, the 15 kDa band was absent (Fig. S3A, lane 8). Whether this absence is a general feature of the *T. evansi* and *T. equiperdum* F_{1,γ}-ATPase and if it is of functional significance remain to be investigated. Loss of the domain is not a direct consequence of kDNA loss, because the dKO + γL262P cells retained the domain after Acr treatment (Fig. S3A, compare lane 2 with lane 4). Growth rates for sKO or dKO cells expressing a mutated γ subunit in the absence of Acr were very similar to WT (Fig. S2 A and B), suggesting that, under standard culture conditions and within the time frame observed, the mutated subunits were fully functional. The γA281del-expressers seemed to not be adapted as well to life without kDNA as cells expressing γL262P or γA273P, which was indicated by the slightly reduced growth rate in Acr-free medium (Fig. S4).

### Table 1. ATPase subunit γ sequence variations tested in this study

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Source (origin, host, year of isolation)</th>
<th>Source for mutation; isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L262P</td>
<td><em>T. b. brucei</em> 164DK (USA, mouse, 1971)</td>
<td>17; 20</td>
</tr>
<tr>
<td>CTT → C</td>
<td><em>T. equiperdum</em> BoTat1.1 (Morocco, horse, 1924)</td>
<td>4; 3</td>
</tr>
<tr>
<td>A273P</td>
<td><em>T. equiperdum</em> BoTat1.1 (Morocco, horse, 1924)</td>
<td>4; 3</td>
</tr>
<tr>
<td>GCCG → CG</td>
<td><em>T. equiperdum</em> BoTat1.1 (Morocco, horse, 1924)</td>
<td>4; 4</td>
</tr>
<tr>
<td>A281del</td>
<td><em>T. equiperdum</em> ATCC30019 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td>TCTGCTATG → TCT → ATG</td>
<td><em>T. equiperdum</em> ATCC30023 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. equiperdum</em> ATCC30019 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. equiperdum</em> ATCC30023 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. equiperdum</em> ATCC30019 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. equiperdum</em> ATCC30023 (France, horse, 1903)</td>
<td>This work; 3</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> Antat 3/3 (South America, capybara, 1969)</td>
<td>This work; 51</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> C13 (Kenya, camel, 1982)</td>
<td>This work; 51</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> CPOgz1 (China, water buffalo, 2005)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> E110 (Brazil, capybara, 1985)</td>
<td>This work; 44</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> E9/CO (Columbia, horse, 1973)</td>
<td>This work; 51</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> S5143M (Philippines, water buffalo, 2006)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> S573M (Philippines, water buffalo, 2006)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB805 (China, water buffalo, 1985)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB807 (China, water buffalo, 1979)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> STIB810 (China, water buffalo, 1985)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> Stock Kazakh (Kazakhstan, camel, 1995)</td>
<td>This work; 4</td>
</tr>
<tr>
<td></td>
<td><em>T. evansi</em> KETRI2479 (Kenya, camel, 1981)</td>
<td>This work; 4</td>
</tr>
<tr>
<td>M282L</td>
<td>ATG → T</td>
<td>4; 51</td>
</tr>
<tr>
<td>A281del</td>
<td>ATG → T</td>
<td>4; 51</td>
</tr>
</tbody>
</table>

In all strains investigated, the L262P and A273P mutations are homozygous, whereas the A281del and M282L mutations are heterogeneous.
These results suggested that strains of *T. evansi* and *T. equiperdum* with an A281del or A273P mutation depend on these mutations to be viable. To test this suggestion further, we inducibly expressed either γWT or γL262P in *T. evansi* Antat 3/3 (these parasites are heterozygous for the A281del mutation; see Table 1). Expression of γWT caused a strong growth defect after 48 h, whereas expression of γL262P had no effect (Fig. 2A), suggesting that replacement of sufficient endogenous (A281del-mutant) γ subunits in the ATP synthase complex with WT subunits severely impairs the viability of these DK cells. This observation also suggested that the L262P mutation from the laboratory-induced DK strain of *T. b. brucei* and the A281del mutation present in *T. evansi* Antat 3/3 are, at least to some extent, functionally interchangeable. In another experiment, we induced expression of an ectopic γL262P allele in *T. b. brucei* 427 cells and then treated the cells with 20 nM Acr. As predicted, these cells were resistant to Acr and rapidly lost their kinetoplast. Subsequent repression of the γL262P allele forced these DK cells to rely on endogenous γWT alone and caused a severe growth defect after 24 h (Fig. 2B). In contrast, the growth rate of cells with maintained expression of γL262P remained constant. These results confirm that, after expression of a mutated F_{γ} subunit has permitted loss of kDNA, the DK trypanosomes now depend on continued expression of this mutated subunit to remain viable. Because specific F_{γ} mutations are able to compensate for kDNA loss, all genes exclusively involved in kDNA biogenesis or expression would be predicted to become dispensable. RNA editing ligase 1 (REL1) is essential in the kinetoplast mRNA editing process and its knockdown lethal (23). To determine whether the L262P mutation can compensate for REL1 loss, the γL262P gene or the γWT control was constitutively expressed in *T. b. brucei* 427 engineered for inducible REL1 RNAi. Contrasting with γWT cells, γL262P-expressing trypanosomes showed no growth effect after RNAi-mediated knockdown of REL1 (Fig. 3). Interestingly, γL262P-expressing *T. brucei* was not viable after ablation of mitochondrial topoisomerase II (Top2mt), a protein essential for kDNA replication (24). To validate this observation, we ablated Top2mt expression in *T. evansi* Antat 3/3 and observed a growth defect after 48 h (Fig. S5A). Secondary effects resulting from faulty kDNA replication (for example, potentially toxic accumulation of kDNA) are unlikely to be responsible, because the Antat 3/3 strain used for this study is kDNA− (Fig. S5B) (17), probably as a result of long-term in vitro culture (25). The most likely explanation for the growth defect in *T. evansi* is, therefore, that Top2mt has an additional important function outside of its role in kDNA replication. Nevertheless, together with the experiments investigating chemically induced kDNA loss, these genetic data show that specific point mutations of ATPase γ are fully sufficient to compensate for loss of kDNA or its gene expression.

The proton-translocating function of the membrane-embedded F_{0} part of the ATP synthase involves subunit a, which is thought to be the only ATPase subunit encoded in the kinetoplast (Fig. S1A). To test directly whether a mutated ATPase γ is necessary for generating ΔΨm in kDNA− cells, we measured ΔΨm in BF trypanosomes over the course of Acr treatment. For cells expressing only γWT, ΔΨm decreased after 24 h and was completely abolished after 48 h (Fig. 4A), preceding cell death by at least 24 h. In contrast, ΔΨm of the γL262P-expressing trypanosomes was not affected by Acr-induced kDNA loss (Fig. 4B). The slight decrease in ΔΨm during Acr exposure is probably the result of kDNA-independent Acr toxicity, because no difference in ΔΨm could be observed after removal of Acr from the medium (Fig. S6). The current model for ΔΨm generation in BF trypanosomes proposes that increased ATP hydrolysis by a mutated γ subunit facilitates the electron exchange of cytosolic ATP/ADP: for mitochondrial ATP/ADP:O4− exchange by the ADP/ATP carrier (AAC) (17, 26). We measured sensitivity of our cell lines to oligomycin, an inhibitor of the coupled F_{0}F_{c}-ATPase, and the AAC inhibitor bongkrekic acid. Trypanosomes expressing mutant ATPase γ showed similar levels of oligomycin resistance before and after Acr-induced kDNA loss.
activities, even in kDNA T. brucei by the AAC (17, for T. brucei for an AAC/F mutations on F cells. Such a supercomplex might L262P muta-
T. evansi for ATP was also suggested as part in mtDNA and F cells. In support of the γ mutations (32), and the effects of T. brucei T. equiperdum T. equiperdum mutations in yeast in the presence of a functional F this change in sensitivity was irrespective of the presence or absence (solid line and closed circles) of Tet was determined. Cumulative cell growth in the presence (dashed line and open circles) or ab-
Fig. 2. Viability of DK trypanosomes depends on expression of a mutated Fγ. (A) Cumulative growth of T. evansi with Tetracycline (Tet) -inducible ectopic expression of a WT (circles) or L262P-mutated (squares) subunit γ (dashed lines and open symbols, + Tet; solid lines and closed symbols, −Tet). (B) A Tet-in-
ducible γL262P was expressed in T. b. brucei cells, and kDNA loss was triggered by exposure to 20 nM Actr for 7 d. The culture was split, and expression of γL262P in one subculture was repressed by transfer to Tet-free medium (0 h). Cumulative cell growth in the presence (dashed line and open circles) or absence (solid line and closed circles) of Tet was determined.

(Fig. 4C), suggesting that these mutations fully uncouple the F1 and FO activities, even in kDNA+ cells. In support of the proposed importance of the AAC for ΔΨm generation in the absence of a proton-pumping F0F1-ATPase, cells expressing γL262P showed increased sensitivity to bongkrekic acid. Again, this change in sensitivity was irrespective of the presence or absence of kDNA (Fig. 4D), sustaining the hypothesis that, even in the presence of a functional FO, the mutation results in a complete switch to the alternative mode of ΔΨm generation.

Discussion

The apparently conflicting observations that BF T. brucei de-

pends on kDNA for survival (23, 27, 28), whereas the closely related species T. evansi and T. equiperdum as well as certain laboratory-generated T. brucei strains are viable, despite the loss of all or critical parts of their kDNA (6), have generated a puzz-

ling conundrum. In this report, we show that specific single amino acid mutations in the nuclearly encoded γ subunit of the mitochondrial ATP synthase complex are fully sufficient to com-
pensate for loss of kDNA-encoded gene products in the BF par-

asite. The underlying biochemical mechanism involves uncoupling of the F1 and FO parts of the enzyme and increased dependence on a highly active AAC. Some field isolates seem to depend on additional adaptations that remain to be identified.

Four different candidate mutations have been identified to date (Table 1). We found that the L262P and A273P mutations are fully sufficient to permit normal growth of BF parasites in the absence of kDNA. Neither chemically induced loss of the kinet-
oplast nor loss of kDNA expression by RNAi resulted in any lag in growth that would have indicated the requirement for additional adaptations. This finding also rules out a potential kDNA replica-
tion checkpoint for cell cycle completion (29). More than 40 y after the generation of the only surviving DK T. b. brucei strain (20), its viability can now be explained. Likewise, the A273P mutation present in certain field isolates of T. equiperdum strains is fully sufficient to explain why these strains are viable, despite the lack of intact kDNA or its expression. The change in kDNA expression by RNAi –tet (Tet). (D) What is the biochemical mechanism that enables BF trypanosomes to generate a ΔΨm in the absence of a functional FO part? Our study shows that introduction of an FγL262P mutation results in oligomycin resistance and, consequently provides direct evidence that it uncouples the F1 and FO ATPase, cells expressing the mutant γ shows an increased importance for the role of the AAC in these cells. There is evidence in yeast and Leish-

mania for an AAC/F0-ATPase supercomplex (33, 34), which might explain the apparent preference for the ATPase as the source of ADP3− in mtDNA+ cells. Such a supercomplex might generate localized zones of increased ΔΨm and, in association with the protein import machinery, exploit them for protein im-
port, similar to the supercomplex of Tim21 and respiratory comp-
exes III and IV reported for yeast (35). A number of pro-
tists, including many important parasites, lack classical mitochondria and instead, harbor related organelles classified as hydrogen-

osomes or mitosomes that usually lack an organellar genome. How these organelles generate a ΔΨm is unclear (36, 37), and try-
panosomes promise to be an excellent experimental system to further investigate this important problem in cell biology.

How did T. evansi and T. equiperdum evolve from T. brucei, and can the findings reported here help to address this question?
likely that isolates sharing the same mutation also share the same ancestor. Importantly, the largest group, characterized by the A281del mutation, contains isolates from Africa, Asia, and South America collected during a span of 100 y, and it contains both T. evansi and T. equiperdum isolates. Either these T. evansi isolates are T. evansi isolates that had been misclassified (3) or many isolates of these two species are descendants of the same evolutionary event (43, 44). Based on these considerations, we would propose that, contrary to what was proposed recently (4), the extant strains seem to be the result of a limited rather than large number of independent evolutionary events. It has been suggested that T. evansi/T. equiperdum are analogous to yeast petite mutants (4, 38), and, indeed, the mechanism that allows mtDNA loss in petite-negative yeast and T. brucei is strikingly similar (17). However, it is important to stress that the ability to survive without a mitochondrial genome is only one distinctive feature of these parasites, because the other one, efficient tsetse-independent transmission, has had such dramatic epidemiological consequences.

Maintenance and expression of kDNA has been suggested as a target for existing and novel antitrypanosomatid drugs (8–10). In fact, the Acr compound used for kDNA elimination in the present study was originally developed with the aim of finding a cure for sleeping sickness (45). The fact that single nucleotide changes can make the parasite completely independent of kDNA and its expression suggests that this target needs to be treated with caution, at least for T. brucei. However, a few considerations are important. First, an uncoupled F0F1 enzyme will be incapable of proton gradient-driven ATP synthesis, which is thought to be required for survival in the tsetse vector because of the low abundance of glucose in the insect’s midgut (11, 46). The mutation would, consequently be expected to lock the parasite in the mammalian host and prevent spread of resistant parasites. Second, other pathogenic trypanosomatids, including T. congolense, T. cruzi, and Leishmania spp., do not seem to be able to circumvent the need for functional kDNA, which therefore, remains a highly promising target in those parasites.

Another important conclusion from our study is that ATP synthase subunit a seems to be the sole kDNA product ultimately required for viability of BF T. brucei. A subunit of the mitochondrial ribosome, RPS12, may be encoded in kDNA (47), and it has been reported that the product of an alternatively edited mRNA, AEP-1, is required for kDNA maintenance (48); however, these proteins would also become dispensable, along with subunit a, in the presence of a compensatory ATP synthase γ-mutation. The same is true for the numerous nuclearly encoded proteins required exclusively for maintenance and expression of kDNA (5, 7, 49). Thus, a single amino acid mutation in BF T. brucei makes a large number of otherwise essential proteins redundant. The compensatory mutations reported in this paper offer an attractive tool for their identification and characterization.

Materials and Methods
Materials, Trypanosome Strains, and Culture Conditions. Details on materials can be found in SI Materials and Methods. All experimental work and culturing were carried out with BF trypanosomes only. ATP synthase γ (Tb927.10.180) genetic manipulations in T. b. brucei were conducted on the Lister 427 strain, except for inducible expression and RNAi experiments, which used the 247 single marker cell line (50). Inducible expression in T. evansi was conducted in a cell line expressing T7RNAP and TETR (17). Cell lines T. evansi Antat 3/3 (51) and T. b. brucei DK 164 (20) were included in growth experiments for comparison. SI Materials and Methods has details on culturing, plasmid construction, and transfection. See Table S2 for oligonucleotides.

Alamar Blue Dose–Response Assay. The Alamar Blue assay was performed essentially as described (21) with minor modifications. Briefly, test compound was doubly diluted in 100 μL Hirumi-modified Iscove’s medium 9/10% (vol/vol) PBS in a 96-well plate; an equal volume of medium containing BF trypanosomes was added to give a final density of 5 × 10⁵ cells/mL. The plate was incubated at 37 °C and 5% (vol/vol) CO₂ for 72 h, after which 20 μL 0.5 mM resazurin sodium salt in PBS were added to each well; then, the plate was incubated for another 4 h. Fluorescence was measured in a plate reader with excitation and emission filters of 544 and 590 nm, respectively. 

The correlation between loss of a functional kinetoplast and an oligomycin-sensitive F1F0-ATPase on the one hand and loss of fly transmissibility on the other hand was first recognized by Opperdoes et al. (38). The temporal order of the two critical events in the evolutionary history of these parasites—acquisition of a propensity for efficient tsetse-independent transmission (the basis of which is still mysterious) and occurrence of compensatory changes that allowed kDNA loss—has been debated elsewhere (39–42). However, regardless of whether the γ subunit mutation occurred before or after adaptation to nontsetse transmission, it is evident that it was a key event and, thus a valuable marker for tracing that history. Each mutation in Table 1 is specifically correlated with other genetic polymorphisms (42), and it is, therefore,
values were derived from a variable slope (four parameter) nonlinear regression using Prism 5 software (GraphPad).

DAPI Staining. Trypanosomes were washed in PBS, settled onto poly-1-lysine coated slides, and fixed in 3% (wt/vol) formaldehyde for 10 min before treating with excess cold methanol for at least 30 min. Slides were then rehydrated in PBS and mounted in glycerol containing 1 μg/mL DAPI before imaging using a Leica SPW525 confocal laser microscope (blue diode laser at 405-nm wavelength) with Volocity version 5.2 image analysis software (PerkinElmer).

PCR and Western Analyses. Details are given in SI Materials and Methods.

Acknowledgments. The authors thank Cindy Gibson, Julius Lukel, and Ken Stuart for sharing parasite DNA or strains; Keith Gull and Ken Stuart for antibodies; Sinclair Cooper for help with minicircle annotation; and Marilyn Parsons and Keith Matthews for helpful comments on the manuscript. This work was funded by Medical Research Council Grant G0600129 (to A.C.S.) and National Institutes of Health Grant R01AI69057 (to A.C.S.).