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Pharmacological Inhibition of the Vacuolar ATPase in Bloodstream-Form *Trypanosoma brucei* Rescues Genetic Knockdown of Mitochondrial Gene Expression

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1 Pharmacological inhibition of the vacuolar ATPase in bloodstream form *Trypanosoma*
2 *brucei* rescues genetic knockdown of mitochondrial gene expression

3

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5

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9

10 Running head: Vacuole-mitochondrion crosstalk in *T. brucei*

11

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13 M.M. and C.S.B. contributed equally to this work

14

15 [Abstract]

16 Trypanosomatid parasites cause diseases in humans and livestock. It was reported that
17 partial inhibition of the vacuolar ATPase (V-ATPase) affects dependence of *Trypanosoma*
18 *brucei* on its mitochondrial genome (kDNA), a target of the anti-trypanosomatid drug
19 isometamidium. Here we report that V-ATPase inhibition with bafilomycin A1 (BafA)
20 provides partial resistance to genetic knockdown of mitochondrial gene expression. BafA
21 does not promote long-term survival after kDNA loss, but in its presence, isometamidium
22 causes less damage to kDNA.

23

24

25 Trypanosomatid parasites cause a range of debilitating or fatal diseases in humans and
26 animals, typically transmitted by insect vectors (1, 2). Important diseases include Chagas
27 disease, caused by *Trypanosoma cruzi*, various forms of leishmaniasis, caused by
28 *Leishmania* spp, Human African Trypanosomiasis (also known as sleeping sickness),
29 caused by *T. brucei* subspecies *T. b. gambiense* and *T. b. rhodesiense*, and the livestock
30 disease nagana, caused by *T. vivax*, *T. congolense* and, less frequently, *T. b. brucei*.

31

32 Trypanosomatids belong to the clade *Kinetoplastea*, flagellated protists characterised by
33 their unusual mitochondrial DNA, called the kinetoplast or kDNA. *T. brucei* kDNA is a
34 concatenated network of dozens of 23-kb maxicircles (the equivalent of mitochondrial DNA
35 in other eukaryotes) and thousands of ~1-kb minicircles that encode guide RNAs (gRNAs).
36 The latter 'guide' post-transcriptional editing of most maxicircle-encoded mRNAs, a
37 process that is essential for generating functional transcripts (3–5). Maintenance and
38 expression of kDNA are essential in both the mammalian bloodstream form (BF) and the
39 insect stage of *T. brucei* (3), and interference with kDNA maintenance is involved in the
40 mode of action of anti-trypanosomatid drugs such as ethidium bromide (EtBr) and

41 isometamidium chloride (ISM) (6–8). However, BF *T. brucei* appear to require only a single
42 mitochondrial gene product for survival, subunit *a* of the F_o moiety of the F_1F_o -ATP
43 synthase (although translation of the subunit *a* mRNA requires another kDNA-encoded
44 protein, subunit RPS12 of the mitochondrial ribosome). In that stage of the life cycle this
45 complex operates in reverse, as an ATP-driven proton pump, to generate the mitochondrial
46 membrane potential (9–11). Mutations in the nuclearly encoded γ subunit of the ATP
47 synthase, such as L262P, can fully compensate for loss of kDNA in BF *T. brucei* (12) and
48 result in a substantial decrease of ISM sensitivity (7, 13). The mechanism of compensation
49 is not fully understood but appears to involve uncoupling of F_1 from F_o and altered kinetics
50 (11, 12).

51

52 Recently, it was reported that perturbation of the vacuolar ATPase (V-ATPase) affects
53 mitochondrial ATPase function and kDNA dependence in trypanosomes. V-ATPase is
54 essential in *T. brucei*, but sub-lethal inhibition of the complex by low-efficiency RNAi or with
55 the V-ATPase inhibitor bafilomycin A1 (BafA) permitted survival for at least 3 days in the
56 presence of normally lethal concentrations of ISM (14). The physiological mechanism of
57 compensation remained obscure. We decided to investigate the effects of sublethal
58 concentrations of BafA on kDNA dependence over longer time scales. All methods were
59 performed as described previously (7, 12) unless specified.

60

61 We first investigated whether V-ATPase inhibition affects dependence of BF *T. brucei* on
62 RNA editing. RNA Editing Ligase 1 (REL1) is a key component of the *T. brucei* editosome
63 and its knock-down is lethal (15, 16). Expression of an ATP synthase γ subunit with an
64 L262P mutation fully rescues from this phenotype (12). If partial inhibition of the V-ATPase
65 by BafA renders cells impervious to kDNA loss, then treatment with the drug should also
66 rescue from the growth phenotype observed upon knock down of REL1.

67

68 We used a REL1 conditional knock-out cell line (REL1-cKO), where an ectopic copy of the
69 REL1 gene is under control of a tetracycline (Tet)-inducible promoter and both
70 endogenous REL1 alleles have been deleted (15). After removal of Tet from the medium,
71 cKO-REL1 cells exhibited a rapid and severe growth defect, with growth ceasing
72 completely after 96 hours (Fig. 1A, dashed black curve), and no live cells being visible
73 under the microscope at later time points, as observed before (15). The presence of 8 nM
74 or 10 nM BafA alleviated the growth defect, with cells continuing to proliferate 168 hours
75 after Tet removal (Fig. 1A and B, dashed cyan and blue curves and columns, respectively)
76 despite REL1 being below the detection limit in a western blot assay (Fig. 1C and D; all
77 image acquisitions and analyses were performed digitally with Li-Cor Odyssey or C-Digit
78 systems). Lower concentrations of BafA did not alleviate the growth defect caused by
79 REL1 depletion (Fig. 1A, green curves) while higher BafA concentrations caused a severe
80 growth defect even in the presence of Tet (Fig. 1A, pink curves). We note that the range of
81 concentrations in which rescue occurred was narrow and varied slightly between
82 experiments and BafA stocks (data not shown). To investigate if BafA affected knockdown
83 of RNA editing itself, we assessed levels of the F_1F_0 ATPase subunit Tb2. The stability of
84 this protein depends on presence of the kDNA-encoded F_0 subunit *a*: in the absence of
85 functional kDNA (and thus subunit *a*), the level of Tb2 is substantially reduced (Fig. 1E and
86 F) (17). We found that even in the presence of 8 nM or 10 nM BafA, knockdown of REL1
87 reduced levels of Tb2 to at least the same extent as loss of kDNA (Fig. 1C). We conclude
88 that the rescue effect provided by BafA appears to reduce dependence on a functional
89 F_1F_0 -ATPase, as suggested by Baker et al. (14).

90

91 Next, we investigated if treatment with BafA would rescue from cell death caused by kDNA
92 loss, induced either genetically or pharmacologically. To induce kDNA loss genetically, we

93 repressed expression of TAC102, an essential component of the tripartite attachment
94 complex and thus required for kDNA segregation during cell division (18). As expected,
95 Tet-induced TAC102 knockdown in the published RNAi cell line (18) caused a severe
96 growth defect and kDNA loss (Fig. 2). In contrast to the RNA editing knockdown
97 experiment, however, we did not observe any rescue of either phenotype by incubation
98 with BafA (Fig. 2A and D).

99

100 To induce kDNA loss pharmacologically, we treated BF *T. brucei* cells with 0.1 nM ISM. At
101 this concentration, ISM causes kDNA loss but only a very minor growth defect in kDNA-
102 independent cells, as demonstrated by transgenic expression of an F₁ subunit γ allele with
103 the L262P mutation (Fig. 3A, green triangles and lines; see also reference (7)). In the
104 absence of BafA, kDNA-dependent cells showed a severe growth defect: cells complete
105 stopped proliferating after 168 hours (Fig. 3A, black squares and lines), with few, if any,
106 surviving cells visible by microscopic inspection after 240 hours or later. In contrast, in the
107 presence of 10 nM or 15 nM BafA cells continued to proliferate, albeit at much reduced
108 rates (Fig. 3A, blue and orange symbols and lines). The difference in cumulative growth
109 after 313 hours (the endpoint of the experiment) was reproducible and statistically
110 significant (Fig. 3B). At 8 nM BafA the rescue effect was less pronounced (Fig. 3A, cyan
111 triangles and lines, experiment terminated after 192 hours), while 20 nM BafA caused a
112 severe growth defect even in the absence of ISM, as observed before (Fig. 3A and Fig.
113 1A, pink circles and lines, experiment also terminated after 192 hours). In contrast to what
114 we had found for genetic induction of kDNA loss, we observed that BafA afforded
115 significant protection from kDNA loss caused by ISM. After 96 hours of exposure to ISM,
116 about 70% of control cells had lost their kDNA, as observed by staining with 4',6-
117 diamidino-2-phenylindole (DAPI) and microscopy (Fig. 3C; 0K1N, cells with 1 nucleus but
118 no kinetoplast). In cultures where we added 10 nM or 15 nM BafA to the growth medium

119 the fraction of 0K1N cells present at 96 hours was reduced to ~50% and ~30%,
120 respectively (Fig. 3C). In cells where a kinetoplast was visible (1K1N cells), quantitation of
121 kDNA by measuring relative fluorescence compared to the nucleus confirmed that
122 exposure to ISM for 96 hours caused a significant reduction in the amount of organellar
123 DNA, presumably an intermediate stage to complete kDNA loss (Fig. 3D). Incubation with
124 15 nM BafA ISM provided highly significant protection from this effect (Fig. 3D). Taken
125 together, the experiments with kDNA loss induced genetically or by ISM treatment suggest
126 the following: pharmacological inhibition of the V-ATPase with BafA cannot protect *T.*
127 *brucei* from the lethal effects of complete loss of kDNA. Instead, BafA reduces kDNA
128 damage and loss caused by ISM.

129

130 Finally, we induced kDNA loss with an alternative pharmacological regime, exposure to 10
131 nM EtBr. Like ISM, EtBr is a compound from the phenanthridine class that, at low
132 concentrations, causes kDNA loss but affects growth of kDNA-independent cells only
133 mildly (Fig. 3E, green triangles and lines represent growth of cells expressing a γ subunit
134 with the L262P mutation; see also references (7, 8)). We found that simultaneous
135 incubation with 15 nM BafA resulted in a reproducible rescue from cell death caused by
136 treatment with EtBr (Fig. 3E and F), although the effect was much less pronounced
137 compared to ISM, and very few surviving cells were apparent after 168 hours (the endpoint
138 of this experiment).

139

140 These results pose two important questions. First, why did we observe robust rescue by
141 BafA of growth defects caused by knockdown of RNA editing, but not of growth defects
142 caused by inhibition of kDNA maintenance? To our present knowledge both processes
143 serve the same goal in BF *T. brucei*, namely the production of F₁F₀ subunit a (12). One
144 potential explanation is that partial inhibition of V-ATPase makes the parasites less

145 dependent on proton pumping by the F_1F_0 ATPase, for example by changing the pH
146 balance between cellular compartments, but it does not make the parasites completely
147 independent from it. A similar scenario has been proposed for yeast cells lacking
148 mitochondrial DNA (19). The inducible gene expression system that we used in our study
149 to knock down REL1 is unlikely to achieve complete repression in the absence of inducer
150 (20), presumably resulting in some residual expression of REL1 and, consequently,
151 subunit *a*. In contrast, interference with kDNA maintenance will result in an 'all or nothing'
152 response in affected cells, i.e. cells that lose kDNA will completely lose subunit *a* and
153 F_1F_0 -ATPase proton pumping activity. We must note, however, that REL1 in repressed
154 cells was below the detection limit of our western blot analysis, and F_0 subunit Tb2 in
155 these cells was reduced to levels comparable to what we observe in cells depleted of
156 kDNA. Testing this hypothesis will require development of more sensitive and highly
157 quantitative methods for detection of intact F_1F_0 -ATPase. Second, why was BafA more
158 effective in rescuing the cells from the effects of ISM compared to the related EtBr? Our
159 results provide indirect evidence that V-ATPase inhibition by BafA reduces mitochondrial
160 uptake of ISM. How ISM and EtBr enter the mitochondrion is unknown, although efficient
161 uptake of at least ISM appears to depend on the mitochondrial membrane potential (13).
162 Our results suggest that the mitochondrial uptake mechanisms for ISM and EtBr may not
163 be identical, and that V-ATPase inhibition affects uptake of ISM more than uptake of EtBr.
164 Resolving this question will require development of methods to specifically measure
165 mitochondrial uptake of these compounds.
166
167 In summary, our results indicate complex effects of sublethal inhibition of the V-ATPase on
168 *T. brucei* that affect both the degree of dependence on kDNA-encoded products as well as
169 mitochondrial uptake of kDNA-intercalating trypanocides. Our study corroborates an
170 intriguing link between vacuolar and mitochondrial ATPase function that begs further

171 investigation. Furthermore, we suggest that pharmacological inhibition of V-ATPase
172 function can be a useful research tool in the study of otherwise lethal perturbations of
173 mitochondrial gene expression in BF *T. brucei*, but it should be noted that any experiment
174 will require careful titration of inhibitors.

175

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178 and wrote the paper; C.S.-B., M.M. and J.S.G. performed the research. We thank David
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186

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240

241 **Figure legends**

242

243 **Figure 1. (A)** Cumulative growth curves of *T. brucei* REL1-cKO BF cells cultured in the presence (filled
244 symbols, solid lines) and absence (open symbols, dashed lines) of 1 µg/ml tetracycline (Tet; required for
245 expression of REL1) and at varying concentrations of BafA. Each data point is the average of at least six
246 separate growth curves; error bars indicate SD. **(B)** Comparison of cumulative cell numbers (panel A) after
247 168 hours at 0 nM (n=6), 8 nM (n=8) and 10 nM (n=6) BafA. Statistical significance of differences was
248 assessed with the Wilcoxon rank sum test; p<0.001 (***) for -Tet 0 nM BafA vs. -Tet 8 nM BafA and vs. -Tet
249 10 nM BafA. **(C)** Western blot of samples taken at 0, 8 and 10 nM BafA after 168 hours, probed with a REL1
250 antibody. The same blot was probed with antibodies for Tb2, to assess levels of intact F₁F₀-ATPase complex
251 (the asterisk indicates a cross-reacting protein), and for EF-1α (Millipore), as a loading control. **(D)**

252 Quantification of Western blot signals, taking the average of two replicates (one shown in panel C) and
253 indicating relative protein levels under non-induced (-Tet) compared to induced (+Tet) conditions for each
254 BafA concentration (normalized to EF-1 α). **(E)** Western blot of samples from *T. brucei* BF cells expressing an
255 ATPase subunit γ allele with the L262P mutation, taken after 3 and 7 days of culturing in the presence of 10
256 nM EtBr to remove kDNA. Cells grown in the absence of EtBr were used as controls. The blot was probed
257 with antibodies for F₁F₀-ATPase subunit Tb2 (the asterisk indicates a cross-reacting protein, see panel C)
258 and for EF-1 α as loading control. **(F)** Relative quantification of the Tb2 western blot signals shown in panel C
259 (normalized to EF-1 α).

260

261 **Figure 2. (A)** Cumulative growth curves of *T. brucei* TAC102 RNAi BF cells cultured in the absence (filled
262 symbols, solid lines) and presence (open symbols, dashed lines) of 1 μ g/ml Tet and at varying
263 concentrations of BafA. Each data point is the average of 2 growth curves; error bars indicate SD. **(B)**
264 Western blot of samples taken at 0, 8 and 10 nM BafA after 168 hours, probed with antibodies for TAC102
265 and EF-1 α . **(C)** Quantification of Western blot signals, taking the average of two replicates (one shown in
266 panel B) and indicating relative protein levels under RNAi-induced (+Tet) to non-induced (-Tet) conditions
267 for each BafA concentration (normalized to EF-1 α). **(D)** Loss of kDNA (OK1N cells) assessed by DAPI staining
268 and microscopy after 168 hours of culturing.

269

270 **Figure 3. (A)** Cumulative growth curves for *T. brucei* BF cells cultured in the absence (filled symbols, solid
271 lines) and presence (open symbols, dashed lines) of 0.1 nM ISM and at varying concentrations of BafA. In
272 parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation was also grown in the
273 absence of presence of 0.1 nM ISM (green triangles and lines). Each data point is the average of two growth
274 curves, error bars indicate SD. **(B)** Comparison of cumulative cell numbers (panel A) after 313 hours.
275 Statistical significance of differences was assessed with the Student unpaired T-test; 0.01<p<0.05 (*) for 0
276 nM BafA +ISM vs. 10 nM BafA +ISM; p<0.001 (***) for 0 nM BafA +ISM vs. 15 nM BafA +ISM. **(C)** Loss of
277 kDNA (OK1N cells) assessed by DAPI staining and microscopy after 96 hours of culturing. Statistical

278 significance of differences was assessed with the Student unpaired T-test; $0.01 < p < 0.05$ (*) for WT +ISM 0
279 nM BafA vs. WT +ISM 10 nM BafA or vs. WT +ISM 15 nM BafA. **(D)** The relative amount of kDNA in 1K1N
280 cells after 96 hours of culturing was assessed by DAPI staining and quantitation of kinetoplast vs. nucleus
281 fluorescence intensity. Statistical significance of differences was assessed with the Mann-Whitney test;
282 $p < 0.001$ (***) for -ISM 0 nM BafA (n=26) vs. +ISM 0 nM Baf A (n=35); $p < 0.001$ (***) for +ISM 0 nM BafA vs.
283 +ISM 15 nM BafA (n=56). **(E)** Cumulative growth curves of *T. brucei* BF cells cultured in the absence (filled
284 symbols, solid lines) and presence (open symbols, dashed lines) of 10 nM EtBr and at varying
285 concentrations of BafA. In parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation
286 was also grown in the absence of presence of 10 nM EtBr (green triangles and lines). Each data point is the
287 average from four separate growth curves, error bars indicate SD. **(F)** Comparison of cumulative cell
288 numbers (panel E) after 168 hours. Statistical significance of differences was assessed with the Wilcoxon
289 rank sum test; $0.01 < p < 0.05$ (*) for +EtBr 0 nM BafA vs. +EtBr 15 nM BafA.
290

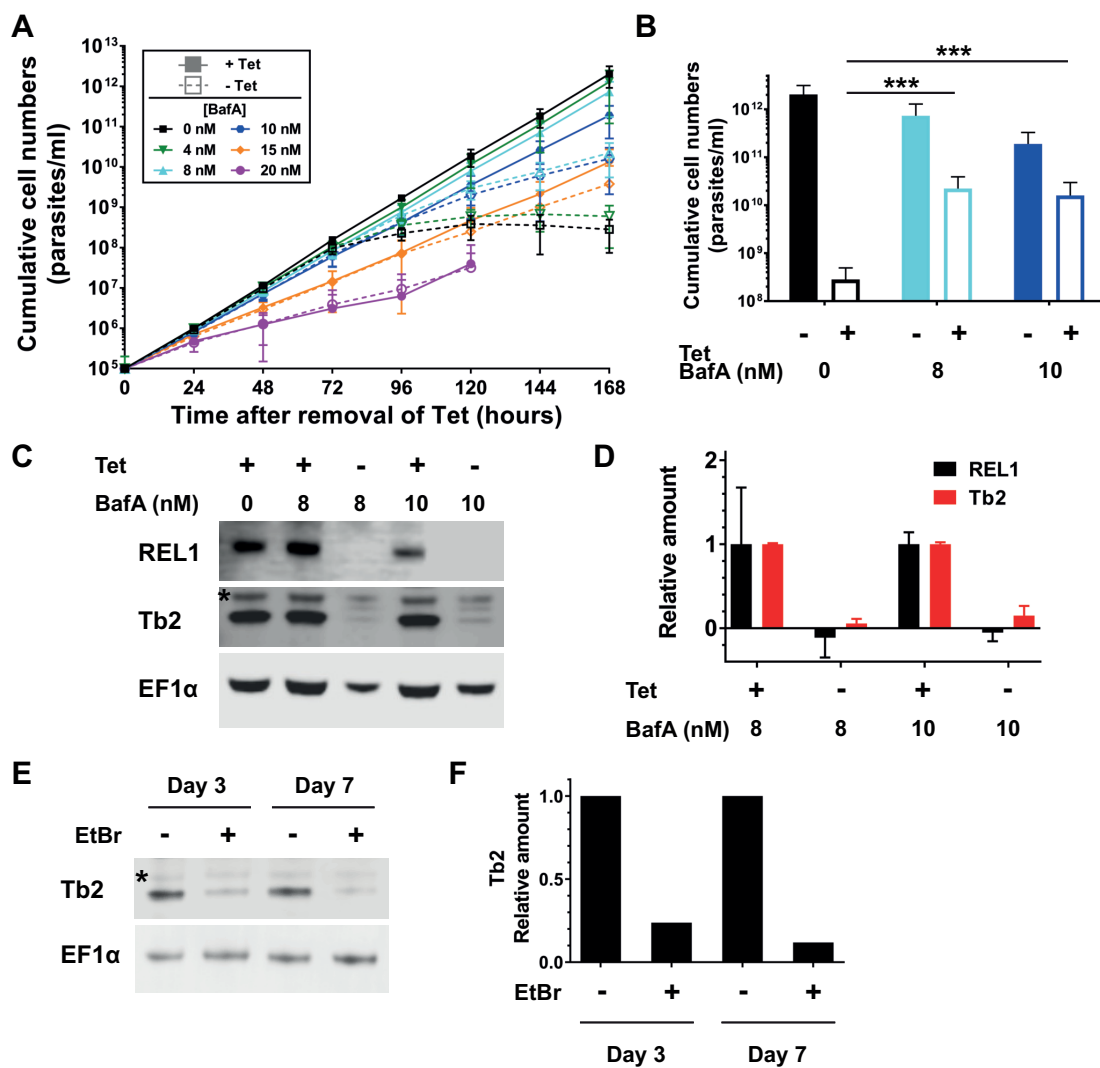


Figure 1. (A) Cumulative growth curves of *T. brucei* REL1-cKO BF cells cultured in the presence (filled symbols, solid lines) and absence (open symbols, dashed lines) of 1 μ g/ml tetracycline (Tet; required for expression of REL1) and at varying concentrations of BafA. Each data point is the average of at least six separate growth curves; error bars indicate SD. (B) Comparison of cumulative cell numbers (panel A) after 168 hours at 0 nM (n=6), 8 nM (n=8) and 10 nM (n=6) BafA. Statistical significance of differences was assessed with the Wilcoxon rank sum test; $p < 0.001$ (***) for -Tet 0 nM BafA vs. -Tet 8 nM BafA and vs. -Tet 10 nM BafA. (C) Western blot of samples taken at 0, 8 and 10 nM BafA after 168 hours, probed with a REL1 antibody. The same blot was probed with antibodies for Tb2, to assess levels of intact F_1F_0 -ATPase complex (the asterisk indicates a cross-reacting protein), and for EF-1 α (Millipore), as a loading control. (D) Quantification of Western blot signals, taking the average of two replicates (one shown in panel C) and indicating relative protein levels under non-induced (-Tet) compared to induced (+Tet) conditions for each BafA concentration (normalized to EF-1 α). (E) Western blot of samples from *T. brucei* BF cells expressing an ATPase subunit γ allele with the L262P mutation, taken after 3 and 7 days of culturing in the presence of 10 nM EtBr to remove kDNA. Cells grown in the absence of EtBr were used as controls. The blot was probed with antibodies for F_1F_0 -ATPase subunit Tb2 (the asterisk indicates a cross-reacting protein, see panel C) and for EF-1 α as loading control. (F) Relative quantification of the Tb2 western blot signals shown in panel A (normalized to EF-1 α).

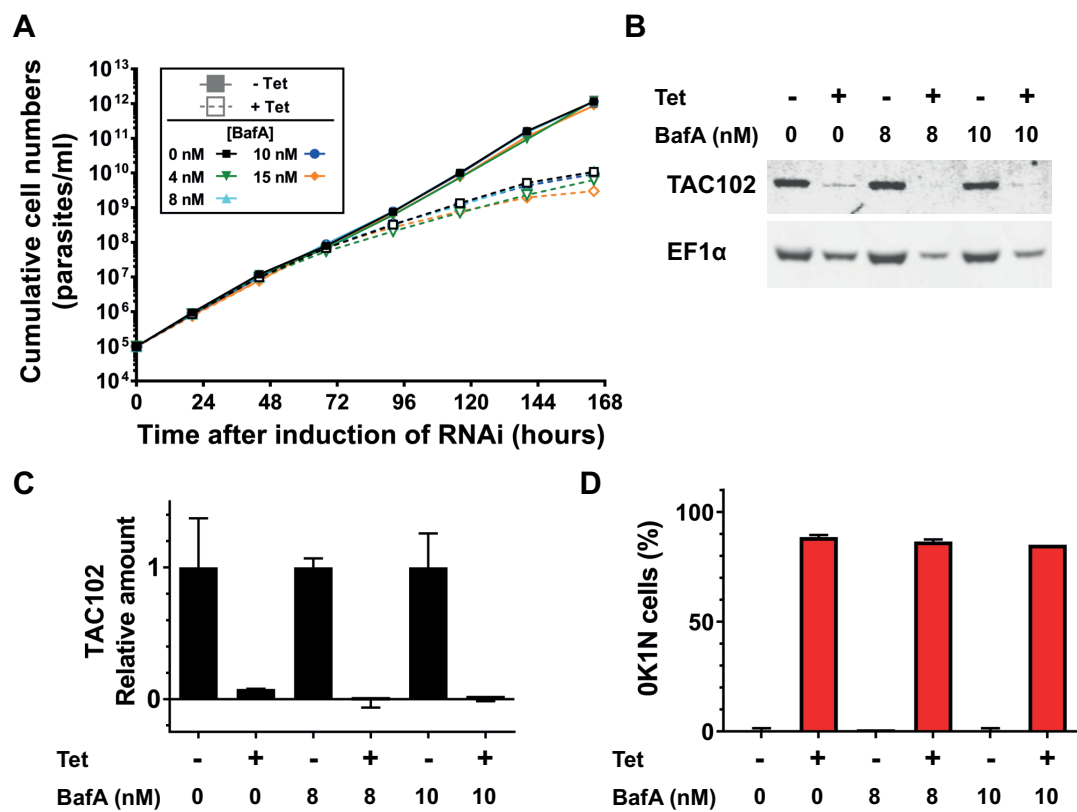


Figure 2. (A) Cumulative growth curves of *T. brucei* TAC102 RNAi BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 1 μ g/ml Tet and at varying concentrations of BafA. Each data point is the average of 2 growth curves; error bars indicate SD. (B) Western blot of samples taken at 0, 8 and 10 nM BafA after 168 hours, probed with antibodies for TAC102 and EF-1 α . (C) Quantification of Western blot signals, taking the average of two replicates (one shown in panel B) and indicating relative protein levels under RNAi-induced (+Tet) to non-induced (-Tet) conditions for each BafA concentration (normalized to EF-1 α). (D) Loss of kDNA (OK1N cells) assessed by DAPI staining and microscopy after 168 hours of culturing.

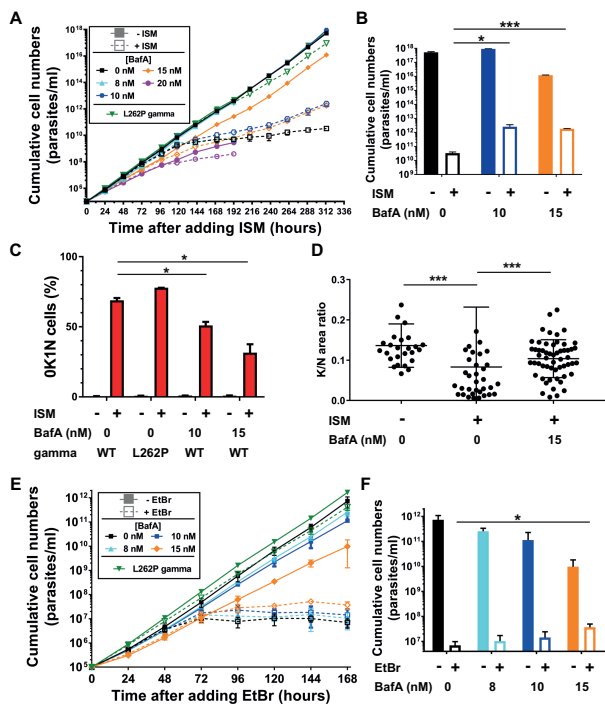


Figure 3. (A) Cumulative growth curves for *T. brucei* BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 0.1 nM ISM and at varying concentrations of BafA. In parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation was also grown in the absence of presence of 0.1 nM ISM (green triangles and lines). Each data point is the average of two growth curves, error bars indicate SD. (B) Comparison of cumulative cell numbers (panel A) after 313 hours. Statistical significance of differences was assessed with the Student unpaired T-test; $0.01 < p < 0.05$ (*) for 0 nM BafA +ISM vs. 10 nM BafA +ISM; $p < 0.001$ (***) for 0 nM BafA +ISM vs. 15 nM BafA +ISM. (C) Loss of kDNA (OK1N cells) assessed by DAPI staining and microscopy after 96 hours of culturing. Statistical significance of differences was assessed with the Student unpaired T-test; $0.01 < p < 0.05$ (*) for WT +ISM 0 nM BafA vs. WT +ISM 10 nM BafA or vs. WT +ISM 15 nM BafA. (D) The relative amount of kDNA in 1K1N cells after 96 hours of culturing was assessed by DAPI staining and quantitation of kinetoplast vs. nucleus fluorescence intensity. Statistical significance of differences was assessed with the Mann-Whitney test; $p < 0.001$ (***) for -ISM 0 nM BafA (n=26) vs. +ISM 0 nM BafA (n=35); $p < 0.001$ (***) for +ISM 0 nM BafA vs. +ISM 15 nM BafA (n=56).

Figure 3. (continued)

(E) Cumulative growth curves of *T. brucei* BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 10 nM EtBr and at varying concentrations of BafA. In parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation was also grown in the absence of presence of 10 nM EtBr (green triangles and lines). Each data point is the average from four separate growth curves, error bars indicate SD. (F) Comparison of cumulative cell numbers (panel E) after 168 hours. Statistical significance of differences was assessed with the Wilcoxon rank sum test; $0.01 < p < 0.05$ (*) for +EtBr 0 nM BafA vs. +EtBr 15 nM BafA.