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Short technical report

Depletion of the extracellular-signal regulated kinase 8 homolog in *Trypanosoma brucei* in vivo reduces its virulence in a mouse target validation study

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ABSTRACT

Trypanosoma brucei sub-species are vector borne kinetoplastid parasites that cause the potentially lethal disease Human African trypanosomiasis. The target-based therapy for curing this parasitic disease relies on one drug, Eflornithine. The roles of mitogen-activated protein kinases in regulating key cellular processes in eukaryotic cells such as proliferation, stress response and differentiation plus their druggability make them attractive targets for therapeutic exploitation. The extracellular-regulated kinase 8 homolog in *T. brucei* (TbERK8) is a MAPK that is required for the parasite to proliferate normally in culture. We examined the importance of TbERK8 for permitting *T. brucei* to thrive in mice. Here we show that depleting TbERK8 in vivo negatively affected the virulence of *T. brucei* reducing its ability to progress to lethal infections or cause significant pathology in mice, which validates it as an attractive target.

Human African trypanosomiasis is a lethal disease caused by sub-species of the vector-borne parasite *Trypanosoma brucei*. The primary treatments for this disease include Melarsoprol and Eflornithine [1–3]. The latter of these drugs acts on ornithine decarboxylase (ODC), which is the only validated therapeutic target for *T. brucei*. Such a dearth of targets highlights the great need for a continual push to validate new genes whose products can be therapeutically exploited to kill the parasite. Mitogen-activated protein kinases (MAPKs) are an evolutionarily conserved superfamily of serine/threonine protein kinases. They are integral parts of signaling cascades in eukaryotes that regulate key cellular processes including stress responses, differentiation, apoptosis, and cell proliferation. The core of the MAPK signaling cascade consists of a three-tiered kinase module in which the MAPK kinase kinases (MEK kinases) represent the first level. MEKKs are serine/threonine kinase that phosphorylate and activate their substrates known as the MAPK kinases (MEKs). The MEKs are dual specific kinases that phosphorylate threonine and tyrosine residues of a conserved Thr-X-Tyr (T-X-Y) motif within the activation loop of the MAPK. Upon phosphorylation of both activation loop residues, MAPKs becomes fully activated to affect downstream effector proteins. Tb927.10.5140 encodes the extracellular-signal regulated kinase 8 homolog in *T. brucei* (TbERK8) also referred as MPK2 [4] and MPK6 [5]. It was originally characterized from screening a RNA interference (RNAi) library to define kinases that were essential for survival in cultured bloodstream form *T. brucei* [6]. TbERK8 has been implicated in having a role in *T. brucei* DNA replication based on its ability to phosphorylate the proliferating cell nuclear antigen homolog of *T. brucei* (TbPCNA) [7]. Because knocking down TbERK8 makes the parasite more susceptible to the genotoxic agent methyl methanesulfonate (MMS), it has also been implicated as having a role in the repair of *T. brucei* DNA damage [4]. The small-molecule inhibitor AZ960 was recently shown to be a potent inhibitor of TbERK8 that also has *T. brucei* bioactivity [8]. Such studies with RNAi and small molecules inhibitors have provided clues to the important role of TbERK8 in sustaining proliferation and survival in cultured *T. brucei*. However, the importance of TbERK8 in the pathobiology of *T. brucei* and its potential as a therapeutic target is less well understood. Only through studies done with in vivo models can the role of TbERK8 in pathogenies and its validity as a therapeutic target be assessed. In this study, we silenced TbERK8 in *T. brucei* infected into mice to examine how its depletion in vivo would affect the course of parasite infection and pathogenicity. We made the K24 RNAi construct using the pZJM vector system, which allows expression of double-strand RNA under the control of opposing tetracycline-inducible promoters [9]. The pZJM-K24 RNAi construct encodes DNA corresponding

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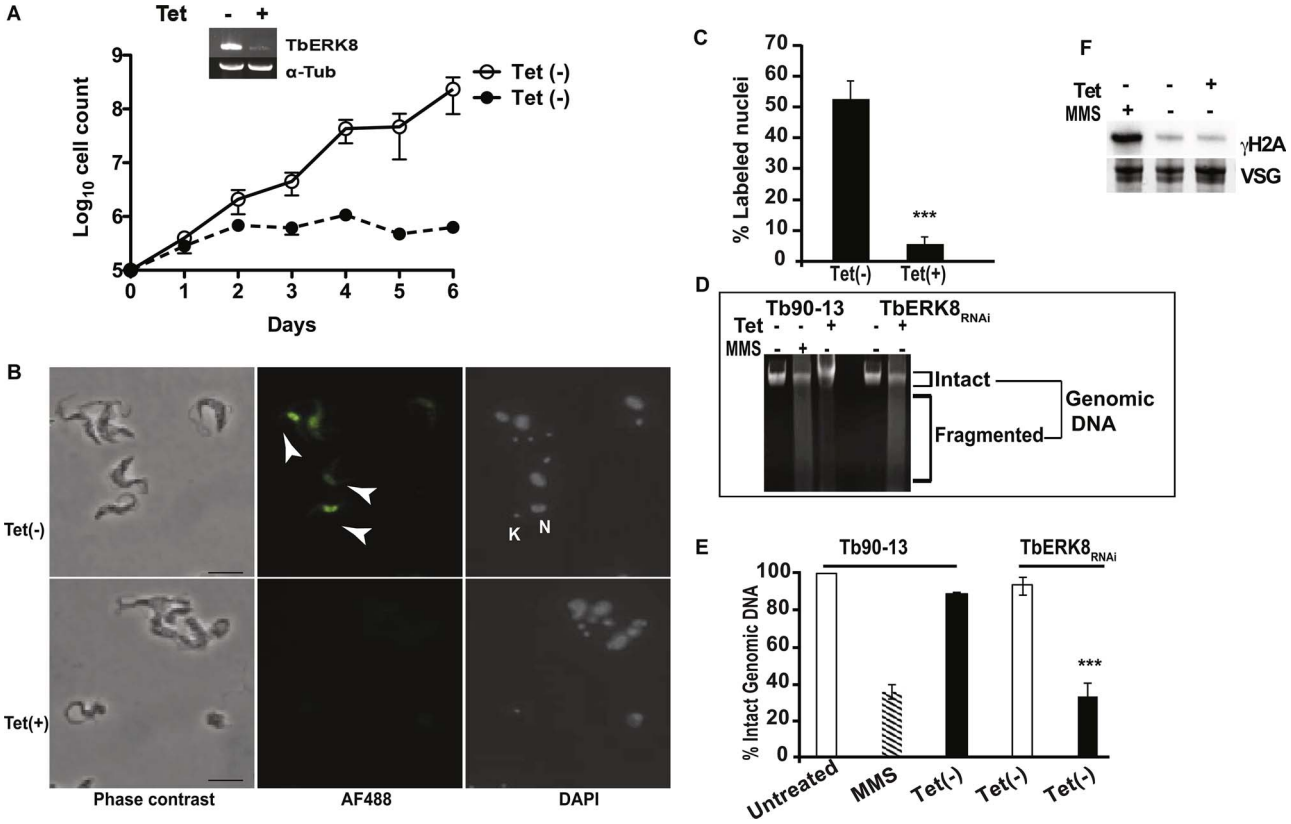

Fig. 1. Depletion of TbERK8 mRNA in bloodstream form *T. brucei* prevents DNA replication. (A) Mean growth curve was generated from three independent stably transfected with the K24 TbERK8$_{RNAi}$ construct to compare non-induced Tet(−) to tetracycline induced Tet(+) *T. brucei* over 6 days with semiquantitative RTPCR demonstrating knockdown of TbERK8 mRNA after 48 h induction (α-tubulin loading control). (B) Examination by fluorescence microscopy of control (Tet(−)) and induced ((Tet(+))TbERK8$_{RNAi}$ *T. brucei* strains after 2 h pulse with EdU to visualize the effects that depleting TbERK8 mRNA had on the synthesis of DNA (AF488 row). White arrows point to faintly and intensely labeled nuclei, which were all counted as positive. Nuclear DNA staining is verified with 4′,6-Diamidino-2-Phenylindole, Dihydrochloride staining (DAPI row). (C) Percent of AF488-labeled nuclei after 2 h pulse with EdU showing significant reduction in the percent of labeled nuclei after TERK8mRNA depletion. Bar graphs shows mean percent of nuclei from 3 independent samples with error bars showing standard deviations. (D) Alkaline gel assay showing effects of silencing TbERK8 on genomic integrity. (E) Bar graphs showing the mean percent with standard deviation of intact genomic DNA after 48 h TbERK8 depletion (Tet(+)) from experiments done in triplicates. (F) *T. brucei* 90-13 Lane 1 with exposure to MMS or TbERK8$_{RNAi}$ harvested after 48 h growth (Tet(−)) lane 2 or (Tet(+)) lane 3. Lysates were resolved by SDSPAGE, transferred to PVDF membrane and prepared for immunoblot. γH2A was detected by anti-γH2A antiserum. Below StainFree ® of VSG was used as the loading control.

to nucleotides 79–901 of the TbERK8 coding region, originally used in the RNAi screen that identified TbERK8 as being essential for normal growth in cultured *T. brucei* [6]. The bloodstream form 90–13 *T. brucei* strain was electroporated with this plasmid construct to generate the TbERK8$_{RNAi}$ strain. This strain was maintained in complete HMI-9 medium [10] supplemented with 100 units of penicillin, 100 µg of streptomycin and, 2.5 µg/mL of phleomycin 2.5 µg/mL of G418 and 5 µg/mL Hygromycin B. Before the cultured strain was injected into mice, we first re-verified that tetracycline induction (1.0 µg/mL) for 48 h resulted in silencing the expression of TbERK8 mRNA. We isolated total RNA from both tetracycline-induced and non-induced parasites by using TRIzol reagent (ThermoFisher, Waltham, MA) according to manufacturer's protocol. We performed semi-quantitative PCR with gene specific primers to demonstrate that TbERK8 mRNA depletion occurred in this strain after 48 h induction with tetracycline (top insert Fig. 1A). We also compared the growth curve of induced and non-induced parasites for 6 days and observed that the tetracycline-induced TbERK8$_{RNAi}$ parasites had a growth defect upon tetracycline induction. Beginning from 24 h post-induction, flow cytometry analysis provided evidence that the growth in cultured *T. brucei* induced by tetracycline began to slow in comparison to that of the non-induced population (S1). By day 6 post-induction, the difference in the mean number of control versus induced parasites was nearly a 1000-fold as indicated by the growth curve in Fig. 1A. We recently demonstrated that TbERK8 was able to phosphorylate the replication factor TbPCNA in vitro and in *T. brucei* [7], implicating it having a role in DNA replication. To test if DNA replication was affected in *T. brucei* after depleting TbERK8 in cultured parasites, we pulsed the TbERK8$_{RNAi}$ strain for 2 h with 20 µM of the thymidine analog 5-Ethynyl-2′-deoxy-uridine (EdU) (Fig. 1B). This thymidine analog incorporate into synthesized DNA and is detected by cycloaddition of Alexafluor 488-azide (AF488) through click chemistry [11]. When the non-induced *T. brucei* were pulsed with EdU, they efficiently incorporated the analog into their genomic DNA as expected. After labeling the non-induced *T. brucei* with AF488, we detected fluorescents in 52% of their nuclei (Fig. 1C). The tetracycline-induced group of cultured *T. brucei* were pulsed labeled with EdU as done with the controls. Analysis of these parasites by fluorescence microscopy revealed that about 5% had AF488-labeled nuclei. This difference between the percentage of AF488-labeled nuclei was significant *p-value* < 0.001) and clearly demonstrated that the ability of *T. brucei* to synthesize DNA was negatively affected by silencing TbERK8. We examined the genomic DNA of the TbERK8$_{RNAi}$ strain directly for DNA strand breaks using the alkaline gel assay (Fig. 1D). This approach involved resolving 10 µg of genomic DNA from control or induced parasites in an agarose gel containing alkaline electrophoresis buffer [12]. The degree of fragmentation was quantified by dividing the intensity of ethidium bromide fluorescence from intact genomic DNA by that from fragmented DNA. In Fig. 1E, we calculated that about 30% of the genomic DNA in the induced parasites remained intact after being resolved in alkaline gels, indicating that these parasites had significantly more DNA strand breaks than the control parasites. We used antibodies against γH2A from *T. brucei* [13] that were obtained from