Characterising the unusual function of thioredoxin 2 – an essential mitochondrial protein in *Trypanosoma brucei*

Introduction: Trypanosomatids possess a unique redox metabolism based on the trypanothione/trypanothione reductase system. The majority of reactions are mediated by tryparedoxin, the main oxidoreductase found in the parasite cytosol, however maintenance of the thiol-redox homeostasis in the mitochondrion is not yet fully understood. The *Trypanosoma* genome also encodes two thioredoxin-type proteins, Trx1 and Trx2. Previous studies showed that Trx1 functions as a conventional oxidoreductase [1] whereas the role of Trx2, a protein unique to Kinetoplastids, is currently unknown.

Results: We report that Trx2 is a mitochondrial protein (Fig. 1A) and is essential for proliferation of *T. brucei* parasites. Depletion of Trx2 in procyclic cells caused a proliferation defect which was overcome after approximately 8 days of RNAi induction (Fig. 1Bi). Remarkably this was not due to the reappearance of the protein (Fig. 1Bii) suggesting that this occurred via a compensatory mechanism involving the overexpression of a protein with a similar function to Trx2. In bloodstream cells, the effect of Trx2 RNAi on cell proliferation was minor (Fig. Ci), and the protein abundance appeared to be much lower than in procyclic cells (Fig. Cii).

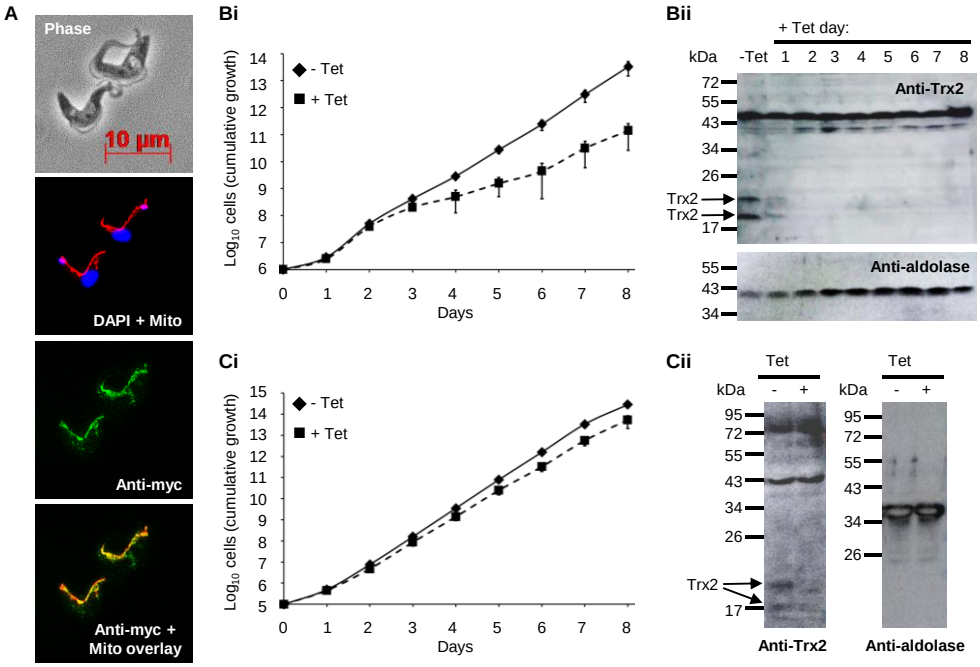

Figure 1. Trx2 is an essential mitochondrial protein in bloodstream and procyclic form *Trypanosoma brucei*. (A) Localization of Trx2: Immunofluorescence microscopy of bloodstream *T. brucei* 2T1 cells overexpressing Trx2^{6MYC} using anti-myc antibodies overlapped with MitoTracker (Mito) staining of the mitochondrion, indicating mitochondrial localization of Trx2. The kinetoplast and nucleus were stained with the DNA intercalating dye, DAPI. For RNAi knockdown, a stem loop construct targeting Trx2 was assembled in the pHD678 vector and transfected into *T. brucei* wild type 449 cells. (B) RNAi knockdown of Trx2 in procyclic *T. brucei*: Cumulative growth analysis following RNAi induction using 1 µg/ml tetracycline resulted in a growth defect (mean ± standard deviation from three independent cell lines), however after approximately 8 days of RNAi induction, parasites were able to adapt to depletion of Trx2 (Bi). Western blot using anti-Trx2 antibodies showed that after 48 hrs of RNAi induction the protein is no longer detectable (Bii). (C) RNAi knockdown of Trx2 in bloodstream *T. brucei*: Cumulative growth analysis following RNAi induction using 1 µg/ml tetracycline resulted in a minor growth defect (mean ± standard deviation from three independent cell lines) (Ci). Western blot using anti-Trx2 antibodies confirmed depletion of Trx2 in bloodstream parasites following RNAi induction using 1 µg/ml tetracycline for 5 days (Cii). Aldolase served as loading control for western blots (Bii, bottom panel and Cii, right panel).

Interestingly, Trx2 did not behave as a typical thioredoxin but appeared to exhibit chaperone activity. This was demonstrated by the insulin reduction assay; the recombinant protein inhibited, rather than accelerated protein precipitation of the insoluble insulin β-chain (Fig. 2, dark blue line).

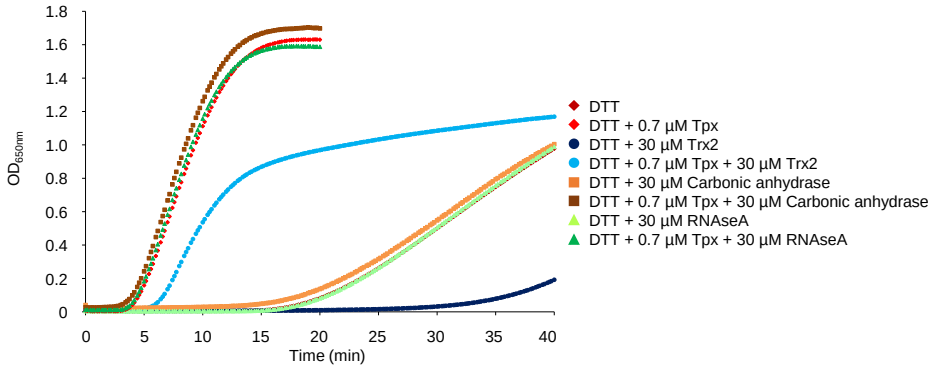

Figure 2. Trx2 exhibits chaperone activity. Insulin aggregation following reduction was monitored by the increase in light scattering at 650 nm at 30°C. Each assay mixture contained 131 µM insulin and 679 µM DTT. Tryparedoxin (Tpx) (light red line) was used as a positive control for oxidoreductase activity, which catalyzed the reduction of insulin disulfides by dithiothreitol (DTT) and resulted in an increased optical density. Carbonic anhydrase (light orange line) and RNaseA (light green line) were used as positive controls for chaperone activity which slowed down aggregation of insulin. Trx2 (dark blue line) inhibited aggregation of insulin. When Tryparedoxin was added to Trx2 (light blue line), carbonic anhydrase (dark orange line) or RNaseA (dark green line), insulin aggregation was observed. Dark red line represents 131 µM insulin and 679 µM DTT.

Conditional knock-out cells expressing a mutant Trx2 lacking all five cysteine residues were viable and proliferative (Fig. 3). We demonstrated that, similarly to wild type recombinant Trx2, the 5-cysteine mutant recombinant protein also inhibited precipitation of insulin in the reduction assay (data not shown), indicating that the cysteine residues are not necessary for the activity of Trx2. These findings suggest that the primary role of *T. brucei* Trx2 may be a chaperone function that is independent of its thiol redox activity. Further studies to unravel this chaperone activity, specifically to determine the role that Trx2 may play in protein folding, are currently ongoing.

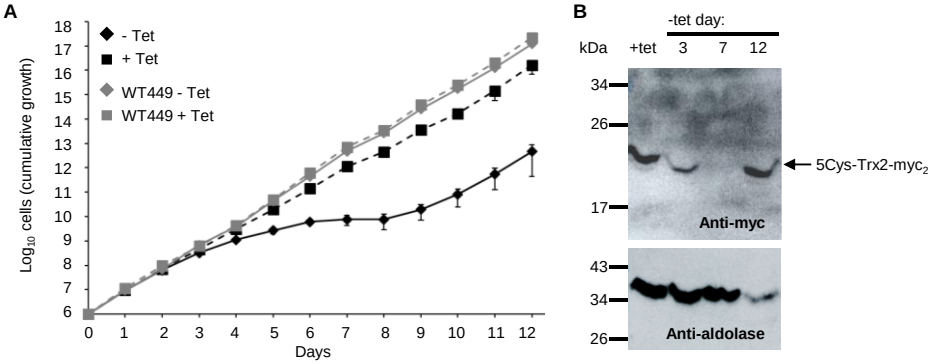

Figure 3. Cysteine residues are not required for proliferation of procyclic *Trypanosoma brucei*. (A) Conditional knock-out cell lines expressing the mutant protein, 5-Cys-Trx2^{2MYC}, were generated in procyclic *T. brucei* 449 cells using the pHD1700 vector. Cell lines cultured in the presence of 1 µg/ml tetracycline (dashed black line) showed a minor proliferation defect in comparison to wild type cells (grey lines). Upon removal of tetracycline, cells exhibited a significant growth defect until approximately day 10, when cells resumed proliferation; this was explained by re-expression of the 5-Cys-Trx2^{2MYC} detected in cells harvested on day 12 following removal of tetracycline using anti-myc antibodies (B, top panel). Aldolase served as loading control for western blot (B, bottom panel). Cumulative growth analysis was performed using three independent cell lines (mean ± standard deviation).

Reference: [1] Schmidt, A., Clayton, C.C, Krauth-Siegel, R.L. 'Silencing of the thioredoxin gene in *Trypanosoma brucei brucei*.' Molecular and Biochemical Parasitology 2002; 125:207-210.