Functional characterization of the methionine sulfoxide reductase repertoire in *Trypanosoma brucei*

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*Running title: methionine sulfoxide reductases from *Trypanosoma brucei*

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Abstract

To combat the deleterious effects that oxidation of the sulfur atom in methionine to sulfoxide may bring, aerobic cells express repair pathways involving methionine sulfoxide reductases (MSRs) to reverse the above reaction. Here, we show that *Trypanosoma brucei,* the causative agent of African trypanosomiasis, expresses two distinct trypanothione-dependent MSRs that can be distinguished from each other based on sequence, sub-cellular localisation and substrate preference. One enzyme found in the parasite's cytosol, shows homology to the MSRA family of repair proteins and preferentially metabolises the *S* epimer of methionine sulfoxide. The second, which contains sequence motifs present in MSRBs, is restricted to the mitochondrion and can only catalyse reduction of the *R* form of peptide-bound methionine sulfoxide. The importance of these proteins to the parasite was demonstrated using functional genomic-based approaches to produce cells with reduced or elevated expression levels of MSRA, which exhibited altered susceptibility to exogenous H_2O_2. These findings identify new reparative pathways that function to fix oxidatively damaged methionine within this medically important parasite.

Keywords

Trypanosoma brucei, methionine, tryparedoxin, trypanothione, recombinant protein expression, RNA interference, GFP

Introduction

The Trypanosomatida represent an order of parasitic protozoa belonging to the Class Kinetoplastidea [1]. They are responsible for several infections in humans with *Trypanosoma brucei* and *Trypanosoma cruzi* causing human African trypanosomiasis (HAT) and Chagas disease, respectively. In addition, more than 20 *Leishmania* species can trigger a spectrum of pathologies collectively termed leishmaniasis. Spread by the hematophagous habits of insect vectors, these pathogens are endemic throughout tropical and sub-tropical regions of the world and cause more than 55,000 deaths per year (http://www.dndi.org/).

All organisms living in an aerobic environment are exposed to a range of reactive oxygen species (ROS) primarily generated as by-products of respiration. These can readily react with various macromolecules leading to formation of other toxic metabolites and/or damage to the target. One target that ROS have a considerable effect on are proteins, resulting in oxidation of certain residues such as cysteine, histidine, tyrosine, and methionine (Met), which can reducing equivalents into change and, in some cases, modification of protein function [2, 3]. Oxidation of Met produces methionine sulfoxide (MetSO) that exists as a mixture of two epimers, methionine-(S)-sulfoxide (Met(*S*)O) and methionine-(R)-sulfoxide (Met(*R*)O). To combat the potentially deleterious effect of these diastereomers, cells express several unrelated enzymes, known as methionine sulfoxide reductases (MSRs), that catalyse reduction of MetSO back to Met [4, 5]. These distinct activities can be distinguished based on sequence and substrate specificity. Metabolism of both free and protein-bound Met(*S*)SO is performed by methionine sulfoxide reductase A (MSRA) [6, 7] while reduction of free or protein-bound Met(*R*)O is mediated by free methionine-(R)-sulfoxide reductase (fRMSR) or methionine sulfoxide reductase B (MSRB), respectively [8-10]. The activity of these enzymes is generally driven by a thioredoxin/thioredoxin reductase-dependent redox cascade that facilitate transfer of reducing equivalents from NADPH to the MSR, although other molecules such as

metallothionein or glutaredoxin, may also act as source of reductant [8, 11-14]. The importance of these pathways has been demonstrated as organisms lacking MSR(s) are more susceptible to oxidative stress, often have a shortened life span and, in the case of bacterial pathogens, reduced virulence [7, 15-19].

In contrast to their mammalian hosts, trypanosomatids lack a raft of enzymes including Cu/Zn- and Mn-superoxide dismutase (SOD), catalase, selenium-dependent glutathione peroxidase, glutathione reductase and thioredoxin reductase, activities that help maintain the redox balance in many other eukaryotic cells. Instead, they express alternative mechanisms that fulfil the above activities, with many of these proposed as potential targets for chemotherapeutic intervention. For example, removal of superoxide anions by trypanosomes and leishmanial parasites is exclusively catalysed by Fe-SOD, enzymes normally found in bacteria, lower eukaryotes and the chloroplasts of plants, while maintenance of the intracellular thiol redox homeostasis is centred upon the trypanosomatid-specific molecule trypanothione [20-22]. In a pathway analogous to the glutathione (GSH)/glutathione reductase system, trypanothione is maintained in its reduced, dihydrotrypanothione ($T(SH)_2$) form by the activity of a NADPH-dependent flavoprotein trypanothione reductase (TR) [23, 24]. $T(SH)_2$ then drives a series of two component cascades, facilitating flux of reducing equivalents into tryparedoxins (TXNs), GSH or ascorbate which in turn reduce various peroxidases, reductases, glyoxalases and transferases [25, 26].

One recipient of the electron flux *via* the $T(SH)_2$/TXN cascade is MSRA, with analysis of the *T. cruzi* and *Leishmania major* homologues revealing its role as an anti-oxidant enzyme within the parasite [27, 28]. Here, we report the dissection of two MetSO metabolising pathways expressed by *T. brucei* focusing on the biochemical properties, subcellular localisation, and functional importance of its MSRA and MSRB complement.

Materials and Methods

Parasites.

Bloodstream form (BSF) *T. brucei* SMB and 2T1 trypomastigotes that constitutively express the tetracycline repressor protein were grown at 37ºC under a 5% (v/v) CO_2 atmosphere in modified Iscove's medium containing 2.5 μg ml-1 G418 (SMB) or 1 μg ml^{-1} phleomycin (2T1) [29-31]. Transformed parasites were maintained in this growth medium supplemented with 2.5 μg mL^{-1} hygromycin. DNA and total RNA were extracted from parasites using the DNeasy® Tissue and RNeasy® mini kits (Qiagen), respectively. *T. brucei* genes that encode for Tb*msrA* and Tb*msrB* were identified from the TriTrypDB (http://tritrypdb.org/tritrypdb/) database [32]: TriTrypDB Gene ID Tb927.8.550 and Tb927.11.11930.1 for *TbmsrA* and *TbmsrB*, respectively.

Protein purification.

DNA fragments containing the full length coding sequence of *TbmsrA* and a version of *TbmsrB* lacking its 5' (1-130 bp) region were amplified from *T. brucei* genomic DNA using the primer combinations TbMSRA-1/TbMSRA-2 or TbMSRB-1/TbMSRB-2 (Table 1), respectively. The products were digested with BamHI/HindIII (*TbmsrA*) or BglII/HindIII (*TbmsrB*) and cloned into the BamHI/HindIII sites of the expression vector pTrcHis-C (Invitrogen).

Protein expression and purification were conducted as previously described [27]. Overnight cultures of *E. coli* BL21 (DE3) transformed with the expression plasmid were diluted 1/100 in Terrific Broth (12 g l^{-1} peptone, 24 g l^{-1} yeast extract, 4 mL l^{-1} glycerol, 2.3 g l^{-1} KH_2PO_4, 12.5 g l^{-1} K_2HPO_4, pH 7.0) supplemented with 100 μg ml^{-1} ampicillin and grown to exponential phase at 37ºC with aeration. Expression of the HIS-tagged recombinant protein was induced with 0.5 mM IPTG, followed by incubation at 25 °C. After 4 hours, cells were harvested and bacterial pellets stored at −20 °C. Purification of recombinant protein was

performed using a Ni^{2+}–HiTrap column (GE Healthcare). Briefly, the bacterial pellet was resuspended in binding buffer (20 mM Tris.HCl, pH 7.5, 10 mM imidazole and 400 mM NaCl) and disrupted by sonication. The lysate was centrifuged (10,000g, 30 min) to remove cell debris. The resultant crude extract was loaded onto a Ni^{2+}–HiTrap column (1 ml) previously equilibrated with binding buffer. After washing with 15 bead volumes of binding buffer plus 30 mM imidazole, the recombinant protein was eluted with elution buffer (20 mM Tris.HCl, pH 7.5, 400 mM NaCl, 300 mM imidazole). Purified enzyme fractions were pooled, concentrated by ultrafiltration, and stored at -80 °C in 20 mM Tris.HCl pH 7.5; 100 mM NaCl and 10% (v/v) glycerol.

Enzyme activity.

MSR activity was measured by monitoring NADPH oxidation at 340 nm by means of a coupled assay that guaranteed the regeneration of TXNI to its reduced form [27]. All enzyme assays were performed at 30 °C using a Multiskan Ascent one-channel vertical light-path filter photometer (Thermo Electron Co.). The reaction mixture (final volume of 50 µl) contained (unless otherwise specified) 100 mM Tris.HCl, pH 7.5, 2 mM EDTA, 300 µM NADPH, 2 U ml^{-1} TcTR, 100 µM $T(SH)_2$ (Bachem), 10 µM TcTXNI, and the respective MSR included in a specific range of concentrations (0.5–3.5 µM TbMSRA or TbMSRB). TcTXN1 was used in these assays as it was readily available in our laboratory and was assumed to function in an equivalent manner to its *T. brucei* counterpart (TcTXNI and TbTXN1 share 62 % identity [33]). Reactions were started by the addition of 5 mM MetSO substrate (racemic N-acetyl MetSO (N-AcMetSO) (Bachem), racemic L-MetSO (Sigma-Aldrich), L-Met(*S*)SO, L-Met(*R*)SO or N-Acetyl Met(*R*)SO: The enantiomers were prepared as previously described [34]). Addition of EDTA into the reaction did not affect TbMSRB activity and as such was included in assays to minimise heavy metal mediated thiol oxidation.

For TbMSRB steady-state kinetic analysis, the assay was performed using 20–2500 μM N-AcMet(*R*)SO and 0.5–20 μM TcTXNI. Kinetic data were plotted as initial velocity (μM min^{-1}) versus substrate concentration (μM). The kinetic constants were acquired by fitting the data with a nonlinear least-squares formula and the Michaelis–Menten equation using the program Origin 7.0. Kinetic constants were the means of at least three independent sets of data, and they were reproducible within ± 10%.

Yeast complementation.

In vivo MSR activity of *T. brucei* enzymes was checked using the triple *msr* mutant GY202 (Δ*msrA* Δ*msrB* Δ*fRmsr*) [18]. The *Saccharomyces cerevisiae* GY202 strain was transformed with parental plasmid p425GPD, p425GPD–Tb*msrA* or p425GPD–Tb*msrB* and selected for leucine prototrophy on Yeast Nitrogen Based (YNB)-agar medium supplemented with L-Met [35]. To perform the complement assay, each recombinant clone was cultivated on YNB-agar medium supplemented with L-Met, a L-MetSO racemic mix, L-Met(*S*)SO-or L-Met(*R*)SO (all 100 μM) at 30 °C until growth was visualized.

RNA interference.

Fragments corresponding to internal sequences of *TbmsrA* (491 bp) and *TbmsrB* (426bp) were amplified from *T. brucei* genomic DNA using the primers TbMSRA-3/TbMSRA-4 or TbMSRB-3/TbMSRB-4 (Table 1), respectively. The products were digested with BamHI/XhoI (*TbmsrA*) or BglII/XhoI (*TbmsrB*) and cloned into the BamHI/XhoI sites of the vector $p2T7^{Ti}$ [36]. In this vector, the inserted DNA is flanked by two opposing T7 promoters with each promoter under the control of a tetracycline operator. Constructs were linearised with *Not*I, electroporated into *T. brucei* SMB parasites and transformants selected using hygromycin [37]. Induction of RNA interference (RNAi) was initiated by adding 1 μg ml^{-1} tetracycline to the

culture. To demonstrate down regulation of the Tb*msrA* or Tb*msrB* transcript, cDNA generated using the Superscript® VILO™ cDNA synthesis kit (ThermoFisher Scientific) from total RNA extracted from cells induced to undergo RNAi for 48 hours was subject to qPCR using the QuantiTect SYBR® Green PCR kit (Qiagen) and the primer combinations TbMSRA-7/TbMSRA-2 or TbMSRB-7/TbMSRB-2 (Table 1), respectively. All reactions were performed in triplicate on two independently generated cDNA samples. From the resultant sigmoidal curves, the cycle threshold (CT) value was determined and normalized against standardized control (Tb*tert*; primer combination TbTERT-R and TbTERT-F) amplified in parallel [38] using the comparative CT method [39].

Trypanosomal epitope tagging vectors.

The full length coding sequences of *TbmsrA* and *TbmsrB* were amplified from genomic DNA using the primer combinations TbMSRA-5/TbMSRA-6 or TbMSRB-5/TbMSRB-6 (Table 1), respectively. The products were digested with HindIII/XbaI and cloned into the corresponding sites of the vectors pRPa$^{C\text{-}GFP}$ or BSF-9e10 [31, 40]. The cloning was carried out such that the sequences coding for the green fluorescence protein (GFP) or 9E10 epitope from the human c-myc protein were inserted in-frame at the 3' end of the *Tbmsr*-derived DNA fragment. The *Asc*I digested constructs were introduced into *T. brucei* 2T1 and transformants selected using hygromycin. Expression of the recombinant protein in the parasite was initiated by adding 1 μg ml^{-1} tetracycline to the culture.

Localisation.

BSF trypanosomes expressing TbMSRB-GFP were suspended at 5 x 10^6 cells ml^{-1} in medium containing 100 nM MitoTracker Red (Molecular Probes) and incubated at 37^{o}C for 5 min. Cells were washed twice in phosphate buffered saline (PBS), fixed in 2% (w/v)

paraformaldehyde/PBS then washed again in PBS. Aliquots of the cell suspension (10^5 cells) were then air dried onto microscope slides. Parasite DNA was stained with Vectashield containing 200 pM 4, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Ltd) and slides were viewed using a Leica SP5 confocal microscope. BSF trypanosomes expressing TbMSRA-GFP were treated similarly except the MitoTracker Red step was omitted.

Peroxide sensitivity experiments.

T. brucei BSF parasites were seeded at 1 x 10^4 ml^{-1} in 200 µl growth medium containing different concentrations of H_2O_2 and tetracycline (1 µg ml^{-1}), where appropriate. After incubation at 37°C for 3 days 2.5 µg resazurin (Sigma Aldrich) was added to each culture and the plates incubated for a further 8 hours. Cell densities were determined by monitoring the fluorescence of each culture using a Gemini Fluorescent Plate Reader (Molecular Devices (UK) Ltd, Wokingham, UK) at an excitation λ = 530 nm, emission λ = 585 nm and a filter cut off at 550 nm, and EC_{50} values established using the non-linear regression tool on GraphPad Prism (GraphPad Software Inc.).

Results

Trypanosoma brucei express functional methionine sulfoxide reductases.

Reduction of the MetSO stereoisomers to Met is mediated by distinct MSRs. Analysis of the *T. brucei* genome database [32] identified two hypothetical genes that encode for two such activities. One 543 bp open reading frame (Tb927.8.550; designated as Tb*msrA*) located on chromosome 8 has potential to be translated into a 20 kDa protein related to peptide MSRs, a family of enzymes that mediate metabolism of Met(*S*)SO [6, 7]. The second 423 bp open reading frame (Tb927.11.11930.1; designated as Tb*msrB*) located on chromosome 11, has potential to encode for a 16 kDa protein that has homology to SelR enzymes which catalyse

reduction of Met(*R*)SO [8]. The two *T. brucei* MSRs share no homology but do have approximately 60% identity to their trypanosomal and leishmanial orthologues (Figure 1). When compared to other sequences, TbMSRA exhibits similar % identities (35-40%) to counterparts from plants, humans, fungi and bacteria while TbMSRB has higher identity to SelR proteins of plant (~48%) and fungal (~38%) origin relative to bacterial and human (both ~25%) enzymes. Based on sequence, TbMSRA is composed of a single peptide methionine sulfoxide reductase (PF01625) domain that contains a characteristic GCFWG motif [5]. In MSRAs from other organisms, cysteine (Cys13 in TbMSRA) at this site plays a key role to catalyse reduction of MetSO, with a second conserved cysteine (Cys171 in TbMSRA) found towards the carboxyl terminal helping maintain the catalytic cysteine in its reduced, active form (Figure 1A). Further searches using localisation prediction algorithms (*e.g.* PSORT II) indicates that TbMSRA lacks any classical sub-cellular localisation signals, suggesting that this enzyme is mostly likely found in the parasite's cytoplasm. Similarly, TbMSRB is composed of a single SelR (PF01641) domain containing several conserved cysteines [5]. MSRBs from other organisms also contains several conserved cysteine (or selenocysteine) residues. These are involved in oxidoreductase activity (Cys117 and Cys171 in TbMSRB), with the latter cysteine (or selenocysteine) catalysing substrate reduction and the former functioning to maintain the catalytic cysteine in its reduced, active form, or zinc co-factor binding (Cys99, Cys102, Cys145 and Cys148 in TbMSRB) (Figure 1B). Here, localisation prediction algorithms (e.g. PSORT II, iPSORT and TargetP) suggest that the amino terminal of this enzyme, characterized by the presence of hydrophobic and basic amino acids and a lack of acidic residues, may function as a mitochondrial targeting signal.

To investigate whether the trypanosomal proteins can function as MSRs, the DNA sequences encoding for their catalytic domains were cloned into pTrcHis-C (Invitrogen) and expressed in *E. coli*. In this system, the recombinant enzymes were tagged at their amino

terminus with a histidine-rich sequence and an epitope detectable with the anti-Xpress monoclonal antibody (Invitrogen). For TbMSRA, expression of the full length gene generated soluble recombinant protein. In contrast, the only construct that gave functional TbMSRB was a deletion derivative in which the recombinant protein lacked the amino terminal, mitochondrial targeting extension (Figure 1B). After induction with isopropyl β-D-thiogalactoside, these constructs generated ~24 and ~21 kDa proteins corresponding to HIS-tagged TbMSRA and TbMSRB respectively, proteins that could be readily purified after one round of affinity chromatography on a nickel-HiTRAP column (Figure 2A).

Previous studies on trypanosomal MSRA revealed that their activity is driven by a TXN-dependent cascade, with NADPH being the source of reducing equivalents [27]. Using a coupled assay that guaranteed regeneration of TXN, the activity of TbMSRA and TbMSRB was monitored by following NADPH oxidation at 340 nm (Figure 2B). Under the conditions employed, both enzymes were able to reduce N-AcMetSO confirming that they exhibited MSR activities, with the rate of substrate reduction being dependent upon TbMSR concentration (Figure 2B). When any of the constituents (TR, $T(SH)_2$, TXN or TbMSRA/TbMSRB) of the pathway were missing, no activity was observed. TbMSRA was able to metabolise N-AcMetSO more readily than TbMSRB with TbMSRA exhibiting an apparent k_{cat} >110-fold higher than that of TbMSRB for this substrate: TbMSRA and TbMSRB display apparent k_{cat} values of 12.3 and 0.11 min^{-1}, respectively, towards N-AcMetSO.

TbMSRs display different MetSO isomer specificity.

To determine TbMSRA or TbMSRB specificity, their activity was followed using different MetSO stereoisomers as substrate and compared to reactions using a MetSO racemic mix (Figure 2C). In agreement with MSRs from other organisms [5], TbMSRA could only metabolise L-Met(*S*)SO while TbMSRB was specific for towards L-Met(*R*)SO.

To investigate substrate specificity further and provide additional evidence that the two *T. brucei* enzymes do function as MSRs, the Tb*msrA* and Tb*msrB* DNA sequences in pTrcHis-C were transferred into the yeast expression vector p425-GPD. The resultant plasmids were transformed into a *S. cerevisiae* strain lacking three MSR enzymes and growth of the modified yeast on YNB agar plus dextrose agar supplemented with Met or different MetSO monitored (Figure 3). On medium containing L-Met, the growth of all fungal line was supported. In contrast, only strains expressing TbMSRA or TbMSRB could grow on medium where L-MetSO racemate was the sole source of L-Met. When using L-Met(*S*)SO or L-Met(*R*)SO supplemented YNB, only *S. cerevisiae* transformed with the TbMSRA could grow on the former medium whereas only yeast expressing TbMSRB displayed strong growth on the latter.

Previous work revealed that TbMSRA activity could be readily saturated by TXN and L-Met(*S*)SO [27]. Here, we showed that free L-Met(*R*)SO was not efficiently metabolised by TbMSRB indicating that this was not the physiological substrate for this enzyme (Table 1). Instead TbMSRB displayed Michaelis-Menten type kinetics towards TXN and N-AcMet(*R*)SO although metabolism of this particular substrate *via* this pathway was extremely low (k_{cat} of 0.085 min^{-1}) (Figure 2D). Comparison of K_m values suggests that the rate limiting step within this pathway may be the interaction of TbMSRB with N-AcMet(*R*)SO (TbMSRB has a K_m of 1.8 ± 0.3 μM towards TXN and a K_m of 429 ± 58 μM towards N-AcMet(*R*)SO). These low kinetic values are typical for those reported for bacterial, plant and mammalian MSRBs [41-43] while the ability of the parasite enzyme to metabolise N-AcMet(*R*)SO indicates that it can effectively reduce protein bound Met(*R*)SO: this form of MetSO is often used as substrate to test for peptide bound MSR activity [44].

TbMSRs are targeted to different cellular localisations.

The subcellular location of the trypanosomal MSRs was examined by expressing GFP-tagged versions of each enzyme in BSF parasites. The DNA sequences encoding for TbMSRA and TbMSRB were amplified then cloned in-frame and upstream of the GFP gene in a trypanosomal vector that facilitated tetracycline inducible gene expression. The localisation constructs were electroporated into *T. brucei* and recombinant parasites selected.

To induce expression of the tagged protein, cells were incubated in the presence of tetracycline for 48 hours. The parasites were then examined by western blotting using an antibody against GFP (Figure 4A), with each extract containing a single band of the expected size (bands of ~48 and ~45 kDa were observed in TbMSRA-GFP or TbMSRB-GFP containing lanes), or were fixed and examined by confocal microscopy (Figure 4C and D). For *T. brucei* induced to express tagged TbMSRA, a fluorescence signal was observed throughout the main body of the cell but absent from the nucleus indicating that this protein is found in the parasite's cytoplasm (Figure 4C). In contrast, for parasites expressing TbMSRB-GFP, a lattice-like structure spread throughout the cell and reminiscent of the pattern reported for proteins that localise to the parasite's large, single mitochondrion, was observed (Figure 4D). To confirm this, cells were co-stained with the mitochondrial dye, MitoTracker. When the images were superimposed, a pattern of co-localization (yellow staining) was noted indicating that TbMSRB was located in the same compartment as MitoTracker. When cells expressing untagged GFP were analysed, fluorescence was detected throughout the whole cell (Figure 4B). The above localisation patterns were observed by immunofluorescence studies using parasites expressing TbMSRA or B variants tagged at their carboxyl terminal with the 9E10 epitope from the human c-myc protein (see below).

Functional Analysis of TbMSRs in *T. brucei*.

To assess the importance of MSR activity to BSF *T. brucei*, an RNAi-based approach was employed. DNA fragments corresponding to the central regions of Tb*msrA* and Tb*msrB* were amplified and cloned into the vector p2T7^{Ti}. The RNAi constructs were transformed into *T. brucei* and recombinant parasites selected.

The effect of inducing RNAi was examined by following the cell density of tetracycline-treated cultures over a 4 day period and compared against untreated controls (Figure 5A). In the absence of RNAi, all recombinant cell lines were found to grow at roughly the same rate as wild type control cells. For cells undergoing RNAi targeting the *TbmsrA* transcript, no significant difference in rate of growth was observed over the initial 24 hour period (Figure 5B). However, over the following 24 hours, a dramatic and reproducible reduction in the cell density was observed in all replicates and clones tested, with this correlating to ~75% fall in the *TbmsrA* transcript, as judged by qPCR. Further, the presence of cell debris was noted in these cultures, suggestive that a proportion of the parasite population had undergone lysis, while any remaining viable cells exhibited reduced movement. From 48 hours onwards, an outgrowth of viable parasites were observed in all replicates, a type of reversion previously observed when targeting transcripts important to the growth of BSF *T. brucei* [36, 45, 46].

In contrast, cells undergoing RNAi targeting the *TbmsrB* transcript continued to grow throughout the entire period albeit with a reduced rate: over the first 24 hours, the growth rate of tetracycline-treated cells was roughly half that of untreated cultures with this falling further to approximately 20% in the next 24 hours (Figure 5B). Over the subsequent 48 to 72 hours, an outgrowth of viable parasites were observed.

Alteration of MSR expression levels in other organisms has shown that they play an important role in protecting cells from exogenous oxidants [7, 15, 19, 27, 28]. Here, we investigated whether down-regulation of the trypanosomal MSR transcripts altered parasite susceptibility to H_2O_2. Tetracycline induced and non-induced cells harbouring the Tb*msrA* or

Tb*msrB* RNAi constructs were grown in the presence of oxidant and the effective compound concentration that inhibits parasite growth by 50% (EC_{50}) determined (Figure 5C). For cells expressing reduced levels of TbMSRB, no significant difference in parasite sensitivity was observed: the EC_{50} values ranged from 210 to 225 μM. In contrast, recombinant *T. brucei* induced to undergo RNAi targeting the Tb*msrA* transcript were approximately 2-fold more susceptible to H_2O_2 than controls: the EC_{50} of non-induced RNAi parasites was 204.7 ± 9.4 μM in comparison to tetracycline treated cells that exhibited an EC_{50} of 111.4 ± 13.5 μM.

As parasites with reduced levels of Tb*msrA* are more susceptible to oxidative stress, we tested whether MSR overexpression leads to resistance. Tb*msrA* and Tb*msrB*, minus their STOP codon, were amplified then cloned in-frame and upstream of the DNA sequence encoding for the 9E10 epitope from the human c-myc protein in a trypanosomal vector that facilitated tetracycline inducible gene expression. The constructs were introduced into *T. brucei* and recombinant parasites selected. To verify expression of the tagged TbMSRs, cell extracts generated from parasites cultured in the presence of tetracycline for 48 hours were examined by western blotting using an antibody against c-myc epitope (Figure 6A). A single band was observed in lanes where TbMSRA-9e10 (~21 kDa) or TbMSRB-9e10 (~20kDa) expression had been induced. Tetracycline treated and untreated *T. brucei* were then grown in the presence of H_2O_2 and EC_{50}s determined (Figure 6B). For cells expressing TbMSRB-9e10, no significant difference in parasite sensitivity was observed. When these studies were extended to TbMSRA, parasites expressing the c-myc-tagged enzyme were 1.5-fold more resistant to H_2O_2 than controls: the EC_{50} of non-induced parasites was 212.9 ± 7.7 μM in comparison to tetracycline induced cells that exhibited an EC_{50} of 320.4 ± 2.9 μM.

Discussion

Throughout its life cycle, *T. brucei* is continually exposed to ROS. To combat their detrimental effects, this parasite expresses a series of novel protection strategies that collectively constituent this pathogen's oxidative defence system. As these mechanisms are distinct from those of its mammalian host [21, 22, 47-49], with several components being essential for growth of the medically relevant parasite stage, they are viewed as potential targets for development of new HAT chemotherapies [21, 36, 49, 50]. Here, we report the characterisation of two additional components of the trypanosomal oxidative defence system, showing that *T. brucei* expresses two structurally unrelated MSRs (TbMSRA and TbMSRB) that can be distinguished on the basis of their sequence, substrate specificity and sub-cellular localisation.

Although functionally related, TbMSRA and TbMSRB share no significant sequence homology with this diversity conferring difference in substrate specificities. In the case of TbMSRA, the presence of a GCFWG motif in its sequence coupled with it being only able to metabolise the S epimer of MetSO establishes this as a member of the peptide methionine sulfoxide reductase, or MSRA, family of antioxidant proteins (Figures 1A, 2 and 3). In contrast, TbMSRB contains a domain structure that places it in the SelR, or MRSB, group of methionine sulfoxide reductases, with enzymatic and phenotypic screening showing that this parasite oxidoreductase can only mediate conversion of peptide-bound Met(*R*)SO to Met (Figures 1B, 2 and 3).

Based on sequence and substrate specificity, the two parasite enzymes are typical members of the MSRA or MRSB clades although they are different in relation to the pathways that facilitate their reduction. In many instances, the concerted action of thioredoxin reductase, thioredoxin and MSR act as intermediaries to shuttle reducing equivalents from NADPH to MetSO [11]. As trypanosomes lack thioredoxin reductase this pathway cannot operate in *T. brucei*. In the case of TbMSRA, its *in vitro* activity is dependent upon TR and trypanothione.

Using NADPH as electron donor, these parasite specific factors fulfil the role of 'thioredoxin reductase', functioning to maintain TXN in its reduced state [51], with reducing equivalents then transferred onto Met(*S*)SO to form Met *via* the trypanosomal MSR (Fig. 2D) [27]. Steady state kinetics indicate that the rate limiting step of this pathway appears to be the TbMSRA mediated reduction of free Met(*S*)SO to Met, a trait noted for MSRAs from other organisms [27, 52-54]. As all components of this cascade are widely distributed throughout the parasite cytosol (Fig. 4C) [47, 55] and that TbMSRA exhibits reasonable kinetics towards both TXN and free Met(*S*)SO, it is hypothesized that the above pathway functions within *T. brucei*, acting to repair damaged Met, specifically free Met(*S*)SO, at this particular subcellular site. Intriguingly, our yeast complementation studies indicate that TbMSRA (and TbMSRB) can utilize non-trypanothione redox cascades as source of reducing equivalents suggesting that the activity of these enzymes within the trypanosome may be driven by an alternative system to that described above. This is analogous to the situation seen with other tryparedoxin-dependent enzymes, including the tryparedoxin peroxidases, which can exploit different thioredoxin/thioredoxin-like molecules as electron donor [48, 56]. We postulate that the trypanosomal MSRs are able to interact with the yeast thioredoxin system and complement for the appropriate fungal mutation to produce the observed growth phenotype.

In contrast, the pathway that operates to maintain TbMSRB in its reduced state is unclear. Biochemical studies on recombinant enzyme has shown that the TR/T$[SH]_2$/TXN system can support TbMSRB activity resulting in slow turnover of N-AcMet(*R*)SO (Fig. 2). The kinetic values exhibited by the parasite protein to both TXN and MetSO are on par with those noted for other MSRBs, with the rate of N-AcMet(*R*)SO reduction limited by the enzyme/substrate interaction [8, 57]. Such observations, coupled with the MSRBs preference to bind and metabolise protein-bound Met(*R*)SO, has led to the idea that free MetSO may not be the major physiological substrate for these reductases [8] and may account for the fact that some

organisms have evolved other free Met(*R*)SO metabolising activities (*e.g.* fRMSRs), with turnover of this particular substrate taking place at an appreciably faster rate than MSRBs [9, 10]. Further, the components that support the trypanothione-dependent, TbMSRB reduction cascade are not present at the same cellular site: TR and TbMSRB are restricted to the cytosol and mitochondrion, respectively, while TXNI is apparently found across both sites (Fig. 4B) [47, 55]. By implication, either TbMSRB activity is supported by an unidentified pathway found entirely within the parasite mitochondrion or the TR/$T[SH]_2$/TXN redox cascade is split across different cellular compartments, with comparative analysis indicating that the thiol constituent being the transferable factor. In other eukaryotic cells, GSH is maintained at high (mM) levels in the mitochondrial lumen even though this organelle lacks the biosynthetic machinery to make this tripeptide. Instead, cytosolic pools of GSH are transferred into mitochondria *via* several transporters which may include dicarboxylate and 2-oxoglutarate carriers [58-60]. If an equivalent $T[SH]_2$ translocation mechanism(s) does function in trypanosomes then given the unique properties of the parasite specific thiol, any transport system would be mechanistically distinct from that which operates in the transfer of GSH and as such would be of particular interest as a target(s) for chemotherapy. Once in the mitochondrion, $T[SH]_2$ can then facilitate transfer of reducing equivalents *via* TXN and TbMSRB to Met(*R*)SO. Intriguingly, data released as part of the TrypTag project [61] indicates that trypanosomes express a classical thioredoxin (Tb927.9.3370 on TriTrypDB [32]) which can be readily reduced by $T(SH)_2$ [62, 63], present throughout the parasite mitochondrion. If correct, this may also function as an intermediary in shuttling reducing equivalents from $T[SH]_2$ to TbMSRB.

Our data shows that TbMSRA and TbMSRB are located in the *T. brucei* cytoplasm and mitochondrion respectively, a distribution also observed with the S. cerevisiae counterparts (Figure 4) [64]. Why trypanosomes and yeast target these two enzymes to only these sites is

unclear given that mammalian cells possess multiple isoforms of each enzyme type at different sub-cellular sites (mammalian cells express cytosolic, mitochondrial and nuclear versions of MSRA and MSRB while an isoform of the latter is also present in the endoplasmic reticulum [65-67]). Taking into consideration their substrate specificity, the specific localisation displayed by TbMSRA and TbMSRB raises a number of interesting questions such as how is free or protein bound Met(S)SO metabolised in parasite organelles and how is free or protein bound Met(R)SO detoxified at non-mitochondrial sites. Part of this may be attributed to uncharacterised activities (*e.g. T. brucei* appears to have potential to encode for a cytoplasmic fRMSR (Tb927.5.1250 on TriTrypDB)) or could reflect the importance of various MetSO forms in different compartments of the cell (*e.g.* it may be favourable to export free MetSO epimers out of the mitochondrion into the cytoplasm to facilitate conversion back to Met, rather than carrying out this repair within the organelle).

MSRs represent key components in an organism's oxidative defence armoury, functioning in processes such as bacterial and protozoal virulence [7, 28, 68-70] and ageing [16, 17, 71]. To evaluate the importance of trypanosomal enzymes to *T. brucei*, functional genomic approaches were used to generate parasite lines expressing altered levels of each reductase. Using RNAi, each enzyme was shown to be important but not essential for the growth of bloodstream form parasites (Fig. 5). For trypanosomes engineered to express reduced levels of the Tb*msrA* transcript, the stalling of parasite growth was accompanied by an increased susceptibility to exogenous H_2O_2 with the reciprocal phenotype observed in cells engineered to over express this enzyme. In contrast, the reduction in cell growth observed when targeting the Tb*msrB* or the elevated expression of this reductase in *T. brucei* did not affect parasite sensitivity to H_2O_2. The observed susceptibility phenotypes suggest that the amount of exogenous oxidant added to cultures is sufficient to promote MetSO formation in targets found in the parasite's cytosol, damage that can be readily repaired by TbMSRA. However, these

peroxide levels are not sufficient to cause significant Met oxidation in the trypanosomal mitochondrion, presumably because this oxidant is detoxified by the various tryparedoxin peroxidase systems expressed by *T. brucei* [47, 49].

In summary, we have demonstrated that *T. brucei* expresses cytosolic and mitochondrial methionine sulfoxide reducing pathways which together constitute a new arm of this parasite's oxidative reparative defence system. As the redox cascades associated with these activities rely upon trypanosome-specific factors and that the activity of the terminal reductase is important for pathogen growth, these mechanisms may have potential as chemotherapeutic targets.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Table

Table 1. Oligonucleotides used in this study. The sequences in lower case italics correspond to restriction sites incorporated into the primers to facilitate cloning.

Function	gene	Primer name	sequence (5' to 3')
Protein expression	*TbmsrA*	TbMSRA-1	aaa*ggatcc*TGAACCCAAATGCTGTTGCTA
		TbMSRA-2	ggg*aagctt*CCATTCACCAGTAGAGACGGT
	Tb*msrB*	TbMSRB-1	aaa*agatct*TGACACACTGCGCAAGTAAGA
		TbMSRB-2	aaa*aagctt*TTACTTCTCGGATTGAAAACG
RNAi	Tb*msrA*	TbMSRA-3	aaa*ggatcc*ACTTTTGCTGCAGGTTGCTT
		TbMSRA-4	aaa*ctcgag*AATACCCATTGGGGTTTTCC
	Tb*msrB*	TbMSRB-3	aaa*agatct*GCCCTCTTATTTTTCCTGCC
		TbMSRB-4	aaa*ctcgag*CGTTCGTTAGGTGGTGGATT
Localisation	Tb*msrA*	TbMSRA-5	aaa*aagctt*ATGAACCCAAATGCTGTTGCT
		TbMSRA-6	aaa*tctaga*CCAGTAGAGACGGTGTGCACA
	Tb*msrB*	TbMSRB-5	aaa*aagctt*ATGCGCAGCAGGAACCTGTCC
		TbMSRB-6	aaa*tctaga*CTTCTCGGATTGAAAACGAAT
qPCR	Tb*msrA*	TbMSRA-7	TAGTAGTGCTAAGGTTGTAAC
	Tb*msrB*	TbMSRB-7	ACGGAAATTTTATGCAATGCG
	Tb*tert*	Tbtert-F	AGGAACTGTCACGGAGTTTGC
		Tbtert-R	GAGCGTGTGACTTCCGAAGG

Figure legends

Figure 1. Sequence analysis of *T. brucei* methionine sulfoxide reductases. (A). Alignment of MSRA sequences from *T. brucei* (AAZ12826), *T. cruzi* (EAN83377), *Leishmania major* (CAJ07082), *Saccharomyces cerevisiae* (NP_010960), *Escherichia coli* (WP_044721421), *Arabidopsis thaliana* (NP_56893) and *Homo sapiens* (NP_036463). Residues that are highly or moderately conserved are highlighted in black or grey, respectively. The catalytic (C13; triangle) and recycling (C171; diamond) cysteines are highlighted [5]. (B). Alignment of MSRB sequences from *T. brucei* (XP_829255), *T. cruzi* (XP_817746), *Leishmania major* (XP_001684552), *Saccharomyces cerevisiae* (NP_009897), *Escherichia coli* (AE016761_217) and *Arabidopsis thaliana* (NP_193915). Residues that are highly or moderately conserved are highlighted in black or grey, respectively. The putative amino terminal mitochondrial targeting sequence in the *T. brucei* MSRB sequence (boxed), the catalytic (C171; triangle), recycling (C117; diamond) and zinc binding (C99, C102, C145 and C148; asterisk) cysteines are all highlighted [5]. For both TbMSRA and B, the arrows corresponds to the primers used for expression of the recombinant protein (Experimental Procedures).

Figure 2. *T. brucei* expresses functional methionine sulfoxide reductases. (A). Coomassie-stained SDS-PAGE gel (15%) containing size standards (in kDa; lane M) and purified, recombinant TbMSRA (lane 1) and TbMSRB (Lane 2). (B). MSR activity of TbMSRA or TbMSRB was followed by monitoring NADPH oxidation at 340 nm using a coupled assay that maintained tryparedoxin (10 μM) to its reduced form (see panel E). Reactions were carried out in 100 mM Tris-HCl pH 7.5, 2 mM EDTA buffer containing NADPH (300 μM), trypanothione reductase (2 U ml^{-1}) and $T(SH)_2$ (100 μM), to generate reduced tryparedoxin, and different concentrations of TbMSRA (0.05-3.5 μM) or TbMSRB (4-270 μM). Assays were initiated by addition of N-AcMetSO (5 mM) to reactions. (C). The MetSO isomer specificity of TbMSRA

and TbMSRB (20 μM) was performed using L-Met(*S*)SO or N-AcMet(*R*)SO (both 5 mM) as substrate (see Materials and Methods). The data are presented as mean % activity values (± standard deviations) derived from three independent experiments relative to assays performed using racemic L-MetSO (5 mM) as substrate. (D). Proposed scheme for the metabolism of Met(*R*)SO *via* a tryparedoxin-mediated TbMSRB pathway. Tryparedoxin (TXNI) acts as a redox shuttle between trypanothione and TbMSRB. Trypanothione disulfide (TS_2) is converted to dihydrotrypanothione ($T[SH]_2$) at the expense of NADPH by the activity of trypanothione reductase (TR) with "red" and "oxi" representing the reduced and oxidized form of proteins/substrates, respectively. The interactions of TbMSRB with TXNI (reaction I) and Met(*R*)SO (reaction II) are indicated. Kinetic parameters of recombinant TbMSRs is shown in the associated Table. To study *Reaction I*, the interaction between TXNI with TbMSRB, TbMSR activity was assayed as described in panel B using different concentrations of TXN1 (0.3-20 μM) and a fixed concentration of N-AcMet(*R*)SO (2.5 mM). For *Reaction II*, the interaction between TbMSRB with N-AcMet(*R*)SO, TbMSR activity was assayed as described in panel B using a fixed concentration of TXN1 (10 μM) and different concentrations of N-AcMet(*R*)SO (40-2500 μM). *Data relating to TbMSRA activity taken from Arias et al 2011 [27] .

Figure 3. Yeast complementation assay demonstrates substrate preference of *T. brucei* MSRs. The growth of *S. cerevisiae* GY202 triple mutant (*ΔmsrA ΔmsrB ΔfRmsr*) transformed with plasmids that facilitate expression of Tb*msrA* (*msrA*) or Tb*msrB* (*msrB*) on YNB agar the vectorplus dextrose agar supplemented with Met or different MetSOs was compared against control strains transformed with empty vector (p425 GPD).

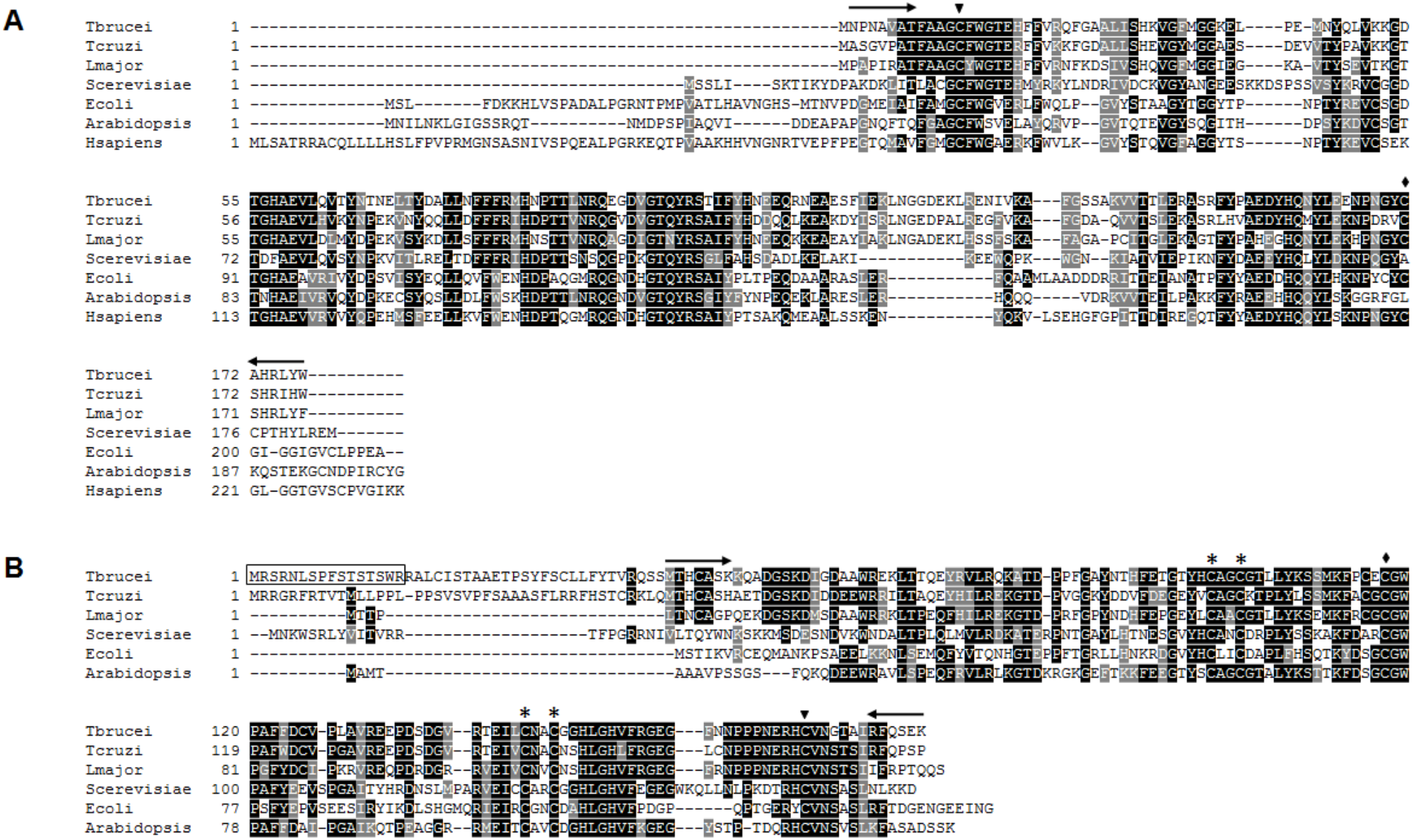
Figure 4. Localisation of TbMSRs in bloodstream form *T. brucei*. (A). Expression of GFP tagged TbMSRs was examined by probing a blot containing cell lysates from *T. brucei* wild type (lane 1) and TbMSRA-GFP or TbMSRB-GFP expressing cells (lanes 2 and 3 respectively) using an anti-GFP antibody (upper panel). Protein from 1 x 10^7 cells was loaded in each track and equal loading verified by Coomassie staining (lower panel). (B). *T. brucei* expressing untagged GFP. The blue spots correspond to the nuclear (N) and mitochondrial (K) genomes of a trypanosome expressing GFP alone. (C). *T. brucei* cells expressing TbMSRA-GFP (panel 2) were co-stained with DAPI (panel 1) with the merged signals (panel 3) and phase image (panel 4) shown. (D). *T. brucei* cells expressing TbMSRB-GFP (panel 2) were co-stained with DAPI (panel 1) and Mitotraker (TM; panel 3). The merged GFP/DAPI/Mitotraker signals (panel 4) and phase image (panel 5) are shown, with the yellow pattern in panel 4 revealing the co-localisation on TbMSRB-GFP and Mitotraker. Scale bar in B, C and D = 5 μm.

Figure 5. Phenotypic analysis of RNAi cell lines. (A). The Tb*msrA* or Tb*msrB* transcript levels from non-induced cells (dark grey) and trypanosomes induced for 48 hours to undergo RNAi (light grey) was assessed by qPCR and compared against the expression level of a standardized control (Tb*tert*). The relative fold difference, as judged by $2\text{-}(\Delta\Delta C_T)$ from reactions performed in triplicate ± standard deviation, was plotted as a measure of the relative expression level. The difference in relative expression levels between the non-induced and induced lines was judged to be statistically significant (P <0.01), as assessed by the Student's *t* test. The Tb*msrA* or Tb*msrB* mRNAs were both ~3.5-fold lower in the corresponding RNAi line relative to controls. (B). Growth of cells induced to undergo RNAi (dashed lines) targeting the Tb*msrA* or Tb*msrB* transcript was compared against non-induced cultures (solid line). The growth patterns shown relate to a single clone. Two other clones analysed in parallel exhibited the same profiles. (C). The BSF RNAi-TbmsrA and RNAi-TbmsrB lines were grown for 24

hours in the presence of tetracycline (1 μg ml^{-1}) (square, dotted line), seeded at 1 x 10^4 ml^{-1} and then exposed to various concentrations of H_2O_2 (12.5–1000 μM): where applicable, RNAi induction was maintained in peroxide treated cultures by addition of fresh tetracycline to the growth medium. After 3 days at 37°C, resazurin (2.5 μg) was added to each culture and used to determine cell density (Experimental Procedures). Untreated (circles, solid line) and wild type parasites were analysed in parallel. From the resultant dose response curves, the EC_{50} of each line towards H_2O_2 was calculated. All data points are means for experiments performed in quadruplicate ± standard deviation. The difference in susceptibility to H_2O_2 displayed by the tetracycline-treated and untreated RNAi-TbmsrA cells as judge by EC_{50} values was statistically significant ($P < 0.0001$), as assessed by Student's *t* test.

Figure 6. Overexpression of TbMSRs in bloodstream form *T. brucei*. (A). Expression of -myc (9e10) tagged TbMSRs was examined by probing a blot containing cell lysates from *T. brucei* wild type (lane 1) and TbMSRA-9e10 or TbMSRB-9e10 expressing cells (lanes 2 and 3 respectively) using c-myc (9e10) anti-serum (upper panel). Protein from 1 x 10^7 cells was loaded in each track and equal loading verified by Coomassie staining (lower panel). (B). The *T. brucei* Tb*msrA*-9e10 and Tb*msrB*-9e10 lines were grown for 24 hours in the presence of tetracycline (1 μg ml^{-1}) (diamond, dotted line), seeded at 1 x 10^4 ml^{-1} and then exposed to various concentrations of H_2O_2 (50–500 μM). After 3 days at 37°C, resazurin (2.5 μg) was added to each culture and used to determine cell density (Experimental Procedures). Untreated (circles, solid line) and wild type parasites were analysed in parallel. From the resultant dose response curves, the EC_{50} of each line towards H_2O_2 was calculated. All data points are means for experiments performed in quadruplicate ± standard deviation. The difference in susceptibility to H_2O_2 displayed by the tetracycline-treated and untreated Tb*msrA*-9e10 cells as judge by EC_{50} values was statistically significant ($P < 0.0001$), as assessed by Student's *t* test.

Figure 1.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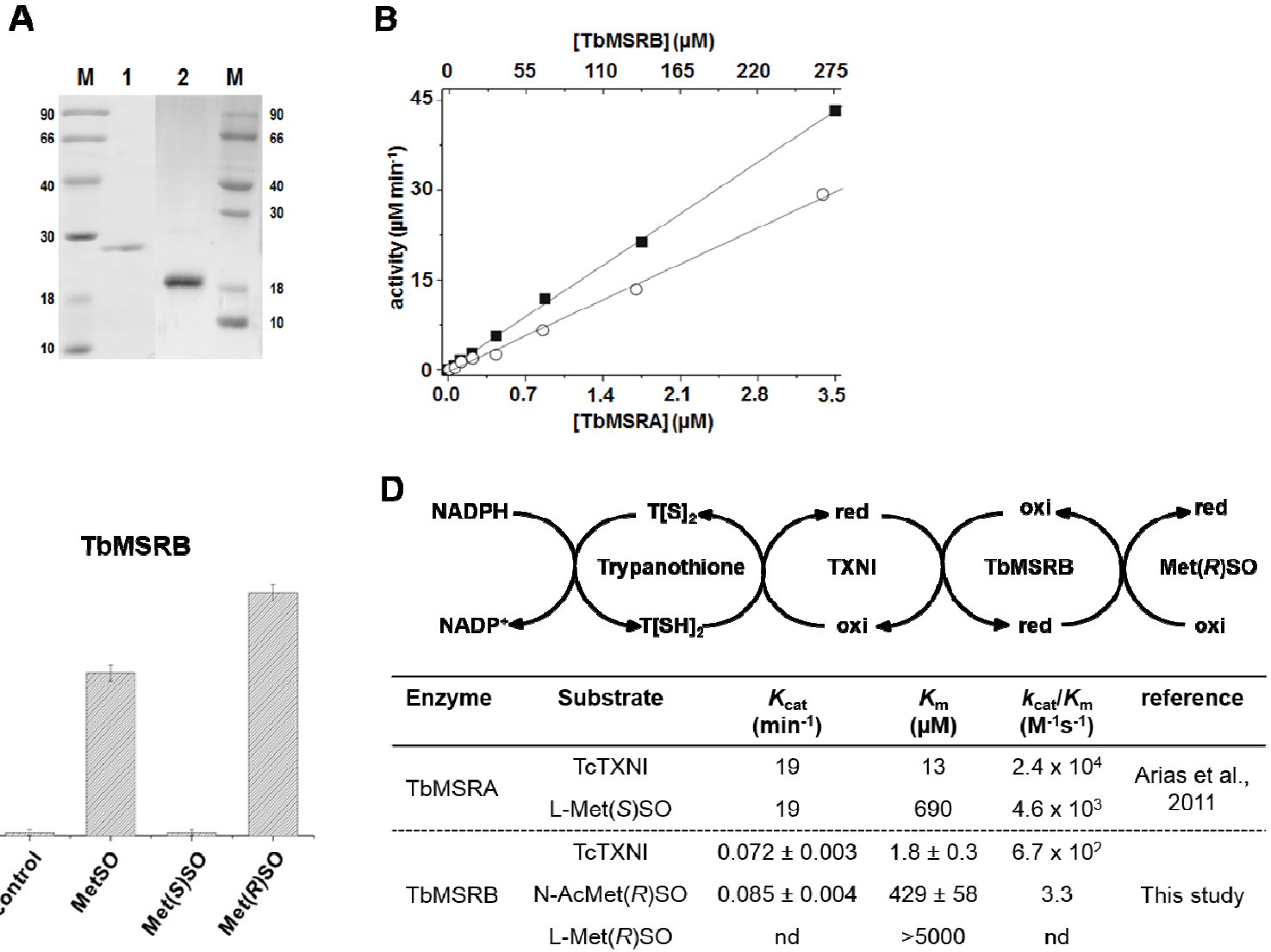
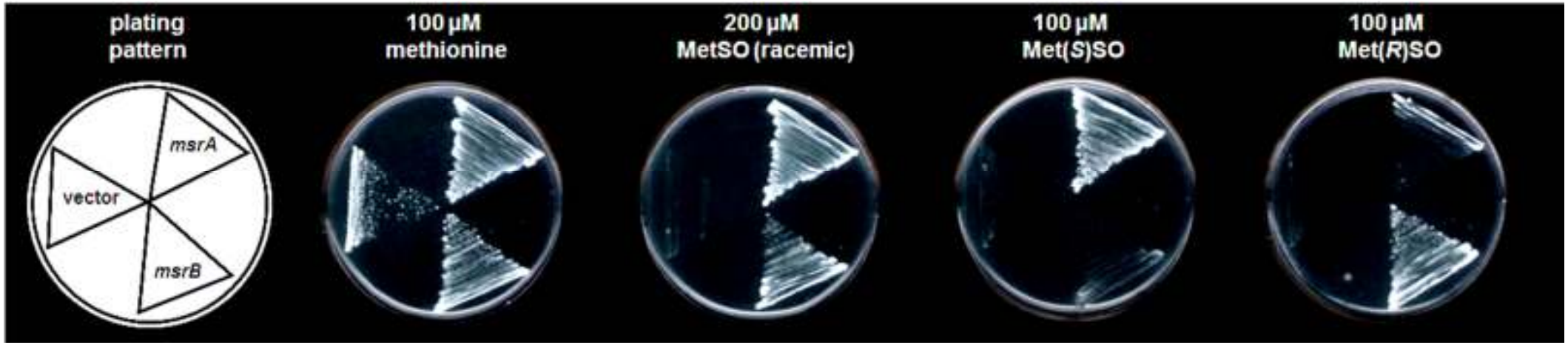
Tcruzi
Lmajor
Scerevisiae
Ecoli
Arabidopsis
Hsapiens
B
Tbrucei
Tcruzi
Lmajor
Scerevisiae
Ecoli
Arabidopsis

Figure 2.



Enzyme	Substrate	K_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	reference
TbMSRA	TcTXNI	19	13	2.4×10^4	Arias et al., 2011
	L-Met(*S*)SO	19	690	4.6×10^3	
TbMSRB	TcTXNI	0.072 ± 0.003	1.8 ± 0.3	6.7×10^2	This study
	N-AcMet(*R*)SO	0.085 ± 0.004	429 ± 58	3.3	
	L-Met(*R*)SO	nd	>5000	nd	

Figure 3.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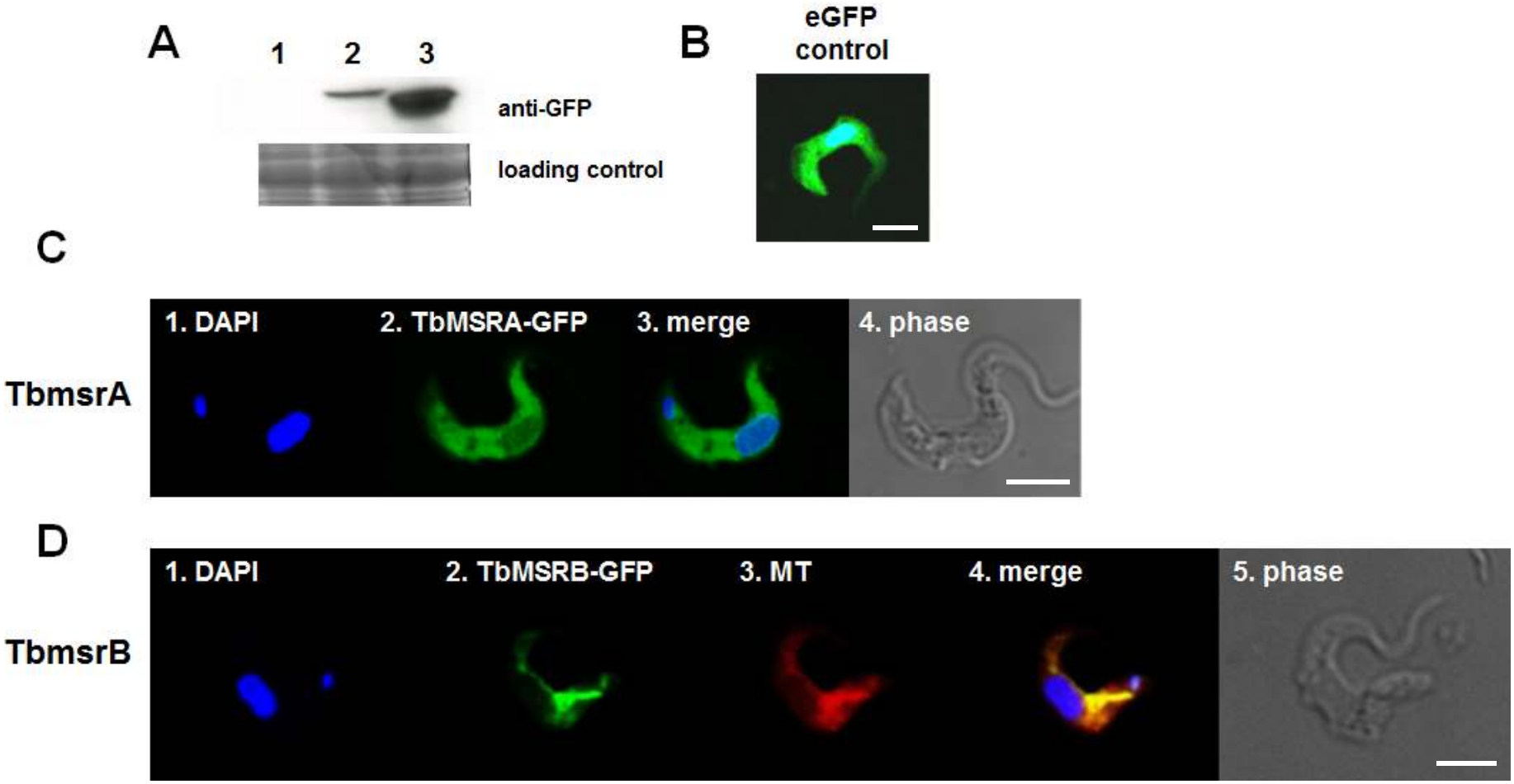
100 µM Met(S)SO
100 µM Met(R)SO
msrA
vector
msrB

Figure 4.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3
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B
eGFP
control
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1. DAPI
2. TbMSRA-GFP
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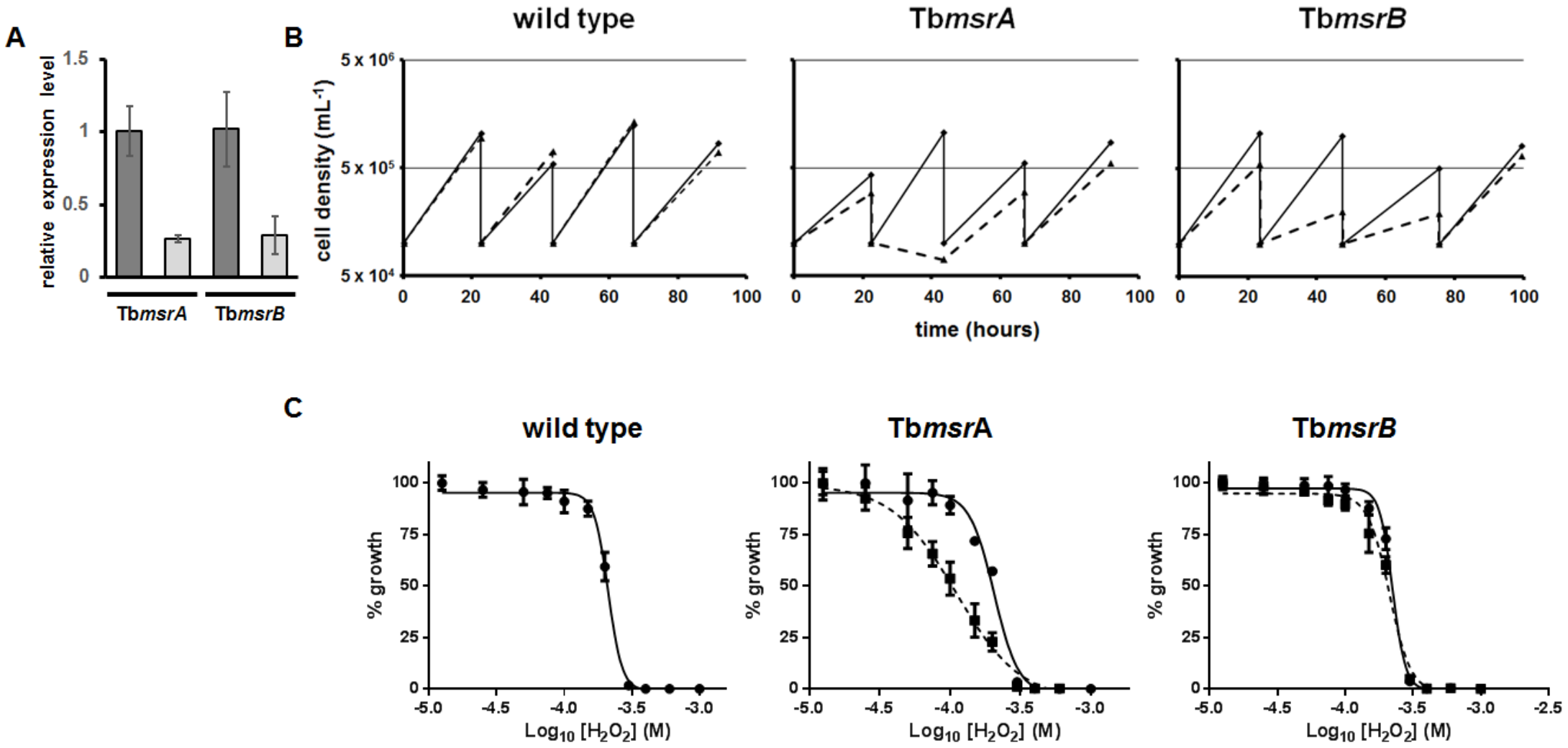
4. phase
D
TbmsrB
1. DAPI
2. TbMSRB-GFP
3. MT
4. merge
5. phase

Figure 5.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0.5
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TbmsrA
TbmsrB
B
wild type
TbmsrA
TbmsrB
cell density (mL-1)
5 x 10^6
5 x 10^5
5 x 10^4
0
20
40
60
80
100
time (hours)
C
wild type
TbmsrA
TbmsrB
% growth
100
75
50
25
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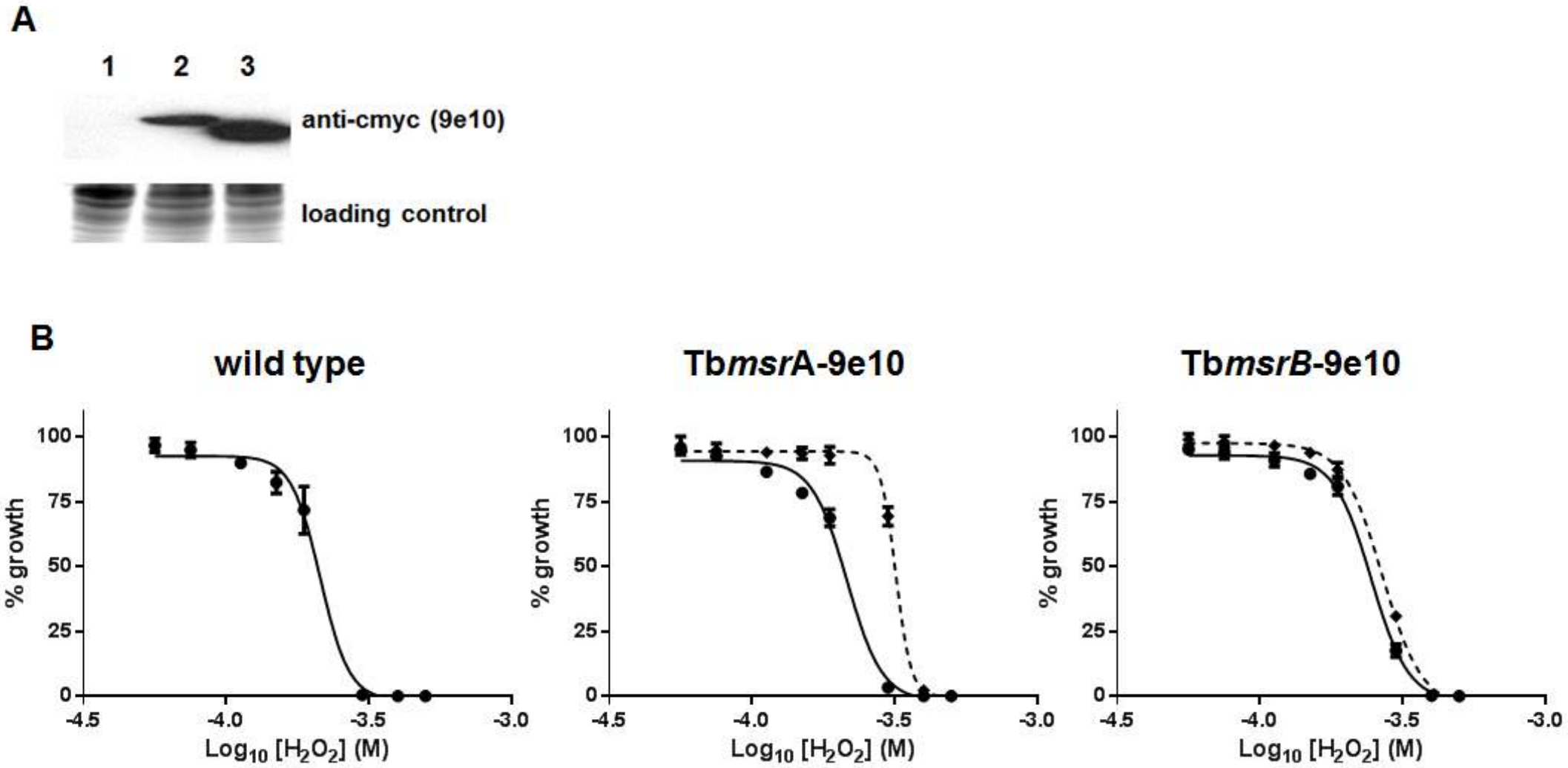
-3.5
-3.0
-2.5
Log10 [H2O2] (M)

Figure 6.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2
3
anti-cmyc (9e10)
loading control
B
wild type
TbmsrA-9e10
TbmsrB-9e10
% growth
100
75
50
25
0
-4.5
-4.0
-3.5
-3.0
Log10 [H2O2] (M)