Dissertation

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Dissecting the physiological roles of dithiolic glutaredoxins in *Trypanosoma brucei*

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Summary

Glutaredoxins (Grxs) are small ubiquitous oxidoreductases. *Trypanosoma brucei,* the causative of African sleeping sickness, contains two dithiol glutaredoxins. Previous studies had shown that RNA interference against Grx2 resulted in a proliferation defect of the procyclic insect form whilst depletion of Grx1 did not show any phenotype in both the mammalian bloodstream and procyclic cells.

The aim of my thesis was to investigate the physiological roles of Grx1 and Grx2 in more detail. For this purpose, I generated bloodstream cell lines lacking both alleles of Grx1 or Grx2. Under normal culture conditions, the mutant cells did not show any growth defect, however, remarkably, displayed an improved proliferation compared to wildtype cells when cultured at elevated temperature. Immunofluorescence microscopy of DAPI-stained cells showed in the wildtype population a significantly higher proportion of parasites with at least two kinetoplasts and two nuclei when compared to the knockout cell lines. The two glutaredoxins cannot substitute for each other. In bloodstream cells there was no upregulation of either protein when the other one was absent. Treatment of recombinant Grx1 with GSH/diamide resulted in the glutathionylation of its non-active site third cysteine accompanied by a three-fold decrease in activity to reduce glutathione-mixed disulfides. Procyclic Grx1-deficient cells could also be obtained. The protein proved to be dispensable even in the presence of different stresses. However, in the case of Grx2, the second allele could only be replaced in cells possessing an inducible ectopic copy of either wildtype or C34S Grx2, but not the C31S/C34S mutant. Both types of cell lines displayed leaky expression of the ectopic copy when the second *grx2* allele was knocked out, suggesting an essential role for this protein in procyclic cells as the system was tightly controlled in parasites still having one *grx2* allele. These results show that Grx2 has a redox function in procyclic cells requiring at least the N-terminal active site cysteine. *In vitro* assays with wildtype and C34S Grx2 suggested that the protein is mainly involved in deglutathionylation reactions. I also showed that Grx1 and Grx2 are fully in the dithiol state in bloodstream and procyclic cells, respectively, and return to their reduced states within 5 min after the removal of an oxidative stress.

Zusammenfassung

Glutaredoxine (Grxs) sind kleine ubiquitäre Oxidoreduktasen. *Trypanosoma brucei,* der Erreger der afrikanischen Schlafkrankheit, besitzt zwei Dithiol-Glutaredoxine. Vorherige Studien zeigten, dass RNA Interferenz gegen Grx2 zu einem Wachstumsdefekt in der prozyklischen Insektenform führt, während die Depletierung von Grx1 keinen phenotypischen Effekt hatte weder in Säuger-Blutstrom noch in prozyklischen Zellen.

Ziel meiner Doktorarbeit war die detaillierte Untersuchung der physiologischen Rolle von Grx1 und Grx2. Zu diesem Zweck wurden Blutstromform-Zelllinien, denen beide Allele von Grx1 oder Grx2 fehlten, hergestellt. Unter Standard-Kulturbedingungen zeigten beide mutierte Zelllinien keinen Wachstumsdefekt, auffallend war jedoch, dass diese im Vergleich zum Wildtyp ein verbessertes Wachstum bei erhöhter Temperatur aufwiesen. Die Immunfluoreszenz-Mikroskopie DAPI-gefärbter Zellen ließ einen signifikant höheren Anteil an Zellen mit mindestens zwei Kinetoplasten und zwei Zellkernen in der Wildtyp-Population im Vergleich zu den defizienten Zelllinien erkennen. Die beiden Glutaredoxine können sich nicht gegenseitig ersetzen. In Blutstromform-Zellen kam es zu keiner Hochregulierung des einen bei Fehlen des anderen Proteins. Die Behandlung von rekombinantem Grx1 mit GSH/Diamid führte zu einer Glutathionylierung des dritten Cysteins außerhalb des aktiven Zentrums zusammen mit einer dreifach verminderten Aktivität, Glutathion-gemischte Disulfide zu reduzieren. Prozyklische Grx1-defiziente Zellen konnten ebenfalls hergestellt werden. Das Protein war selbst unter unterschiedlichsten Stressbedingungen nicht essentiell. Im Falle des Grx2 hingegen konnte das zweite Allel nur in Zellen ersetzt werden, die eine induzierbare ektopische Kopie von entweder Wildtyp oder C34S Grx2, nicht aber die C31S/C34S Mutante, besaßen. Beide Zelllinien exprimierten die ektopische Kopie bereits ohne Induktion, sobald das zweite *grx2* Allel ausgeschaltet wurde. Dies deutet auf eine essentielle Rolle des Proteins in prozyklischen Zellen hin, da das System in Parasiten, die noch ein *grx2* Allel besaßen, streng kontrollierbar war. Diese Ergebnisse weisen darauf hin, dass Grx2 eine Redoxfunktion in prozyklischen Zellen besitzt, die zumindest das N-terminale Cystein des aktiven Zentrums benötigt. *In vitro* Assays mit Wildtyp und C34S Grx2 legen nahe, dass das Protein hauptsächlich an Deglutathionylierungen beteiligt ist. Ebenso konnte ich zeigen, dass Grx1 und Grx2 sowohl in Blutstromform als auch in prozyklischen Zellen vollständig als Dithiol vorliegen und innerhalb von 5 min nach Entfernung eines oxidative Stresses wieder in ihren reduzierten Zustand zurückkehren.

Contents

List of abbreviations

AMS	4-Acetamido-4'-Maleimidylstilbene-2,2'-Disulfonic Acid Disodium Salt
bp	Base pairs
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DTT	1,4 - Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
GSH	Glutahione
GSSG	Glutathione disulfide
h	Hour
HRP	Horseradish peroxidase
KO	Knockout
mA	Milliampere
min	Minute
NEM	N-Ethylmaleimide
^{o}C	Degree Celsius
PBS	Phosphate buffered saline
rcf	Relative centrifugal force
rpm	Revolutions per minute
SDS	Dodecylsulfate-Na-salt
SKO	Single knockout
TAE	Tris-acetate-EDTA
TCA	Trichloroacetic acid
tet	Tetracycline
TXNPx	2-Cys-peroxiredoxin-type tryparedoxin peroxidase
V	Volt
WT	Wildtype

1 Introduction

1.1 Trypanosomes and African sleeping sickness

Human African Trypanosomiasis (sleeping sickness) is caused by *Trypanosoma brucei* species transmitted by tsetse fly of the genus *Glossina* (Brun et al., 2010). The disease is fatal if left untreated and mainly affects the poor and rural areas of sub-Saharan Africa (Brun et al., 2010). Currently, the number of cases are between 50 000 – 70 000 (Brun et al., 2010). The protozoan parasites are kinetoplastids which live and multiply extracellularly in the blood and tissue fluids of mammalian hosts. Kinetoplastids are flagellated protozoans that contain mitochondrion DNA called kinetoplast in their single mitochondrion (Stuart et al., 2008). *Trypanosoma brucei rhodesiense* (*T.b. rhodesiense*) and *Trypanosoma brucei gambienses (T.b. gambienses)* are the only ones that infect humans (Roditi and Lehane, 2008). *T.b. rhodesiense* causes the acute form of the disease endemic in East Africa whilst *T.b. gambienses* causes the chronic form of the disease in West and Central Africa (Migchelsen et al., 2011). Over 95 % of cases of sleeping sickness are due to *T.b. gambienses* (Jones and Avery, 2015). There are two stages of the disease. In the first stage, the parasites live in the blood and lymph system and the symptoms include itching, headache and fever (Steverding, 2010). In the second stage, the parasites invade the central nervous system and symptoms are of neurological and endocrinical disorders (Steverding, 2010). *Trypanosoma brucei brucei*, which is a closely related parasite, infects livestock, but not humans and is often used in laboratory studies (Roditi and Lehane, 2008). Animals can be infected by other trypanosomes which are transmitted by tsetse flies that include *T. congolenses*, *T. vivax* and *T. simiae* (Roditi and Lehane, 2008).

1.2 Life cycle of *Trypanosoma brucei*

Trypanosoma brucei proliferate in mammalian hosts (bloodstream form) and in the midgut of the tsetse fly (procyclic form). Metacyclic trypomastigotes (growth-arrested form) are delivered to the mammals through the bite of an infected tsetse fly (Langousis and Hill, 2014). The metacyclic forms are adapted

to survive upon entering the mammals (MacGregor et al., 2012). When in the bloodstream, the metacyclic trypomastigotes differentiate into the long slender forms that then multiply by binary fission. At high density in bloodstream, the long slender forms differentiate to non-proliferative short stumpy forms which are ready for retransmission upon biting by the tsetse fly (Matthews, 2005). In the midgut of the tsetse fly, the stumpy-form of the parasites then differentiate to procyclic trypomastigotes which then resume the multiplication within the parasite (Langousis and Hill, 2014) and long slender forms die (Dyer et al., 2013). Tryanosomes migrate to the proventriculus of the tsetse fly where they transform to long trypomastigotes which would then divide asymmetrically to form long and short epimastigotes (Dyer et al., 2013). The generalized life cycle of *T. brucei* is shown in Figure 1.

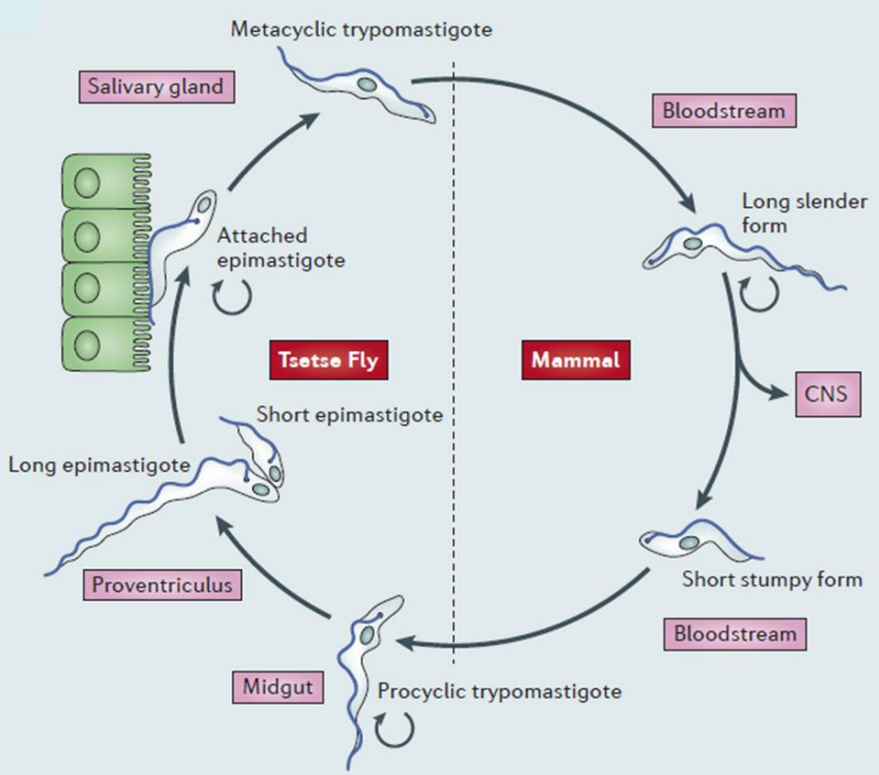


Figure 1. Life cycle of *T. brucei*. Trypanosomes can multiply both in the tsetse fly and mammalian host. Shown are the different stages of the parasites in the tsetse fly or mammals. The long slender form multiplies within the mammalian host. In a density dependent manner, these parasites transform into short stumpy forms, ready for uptake by the tsetse fly. Here the short stumpy forms differentiate into proycyclic trypomastigotes which then multiply in the midgut of the tsetse fly. In the proventriculus, the procyclic trypomastigotes undergoes restructuring to form epimastigotes. The epimastigotes, when at salivary glands, eventually divide to form the infectious metacyclic trypomastigotes which are adapted to survive within the mammalian host (Langousis and Hill, 2014).

Trypanosomes are constantly attacked by the immune system, but they survive long-term due to antigenic variation of the variant surface glycoprotein (VSG) that involves the stochastic switching to a new VSG that has a varied coat (Morrison et al., 2009). There are about 10^7 identical VSG molecules on the surface of the parasites that create a coat that prevents detection of invariant antigens (Stockdale et al., 2008). When in the tsetse fly, the parasites differentiate to procyclic form by losing their VSG and replacement by procyclins which are glycoproteins characterized by internal Glu-Pro (EP) or Gly-Pro-Glu-Glu-Thr (GPEET) repeats (Roditi and Lehane, 2008). The procyclins protect the parasites from proteases in the midgut of the tsetse fly (Roditi and Lehane, 2008).

1.3 Current drugs for African sleeping sickness

The current drugs against the two stages of African sleeping sickness are summarized in Table 1.

Table 1. Current drugs for African sleeping sickness

	Drug	**Mode of action**
T.b. rhodesienses sleeping sickness		
stage 1	Suramin	inhibits many enzymes through electrostatic interactions (Denise and Barrett, 2001)
stage 2	Melarsoprol	interacts with thiols, targets might include trypanothione (Denise and Barrett, 2001)
T.b. gambienses sleeping sickness		
stage 1	Pentamidine	interacts tightly with kinetoplast though there might be other targets (Denise and Barrett, 2001)
stage 2	Melarsoprol	as mentioned above
	Eflornithine	inhibitor of ornithine decarboxylase (Delespaux and de Koning, 2007)
	Nifurtimox	production of radicals after one-electron reduction of its nitro group (Denise and Barrett, 2001)

1.4 Unique redox metabolism of trypanosomes

Unlike other organisms, trypanosomatids lack catalase, glutathione reductase (GR) and thioredoxin reductase (TrxR) (Berriman et al., 2005).Glutathione (GSH)/GR and thioredoxin (Trx)/TrxR systems maintain the intracellular thiol homeostasis in most organisms, in trypanosomes this metabolism is based on the dithiol trypanothione and trypanothione reductase (TR) (Comini et al., 2013). Glutathione is made up of L-glutamate, L-glycine and L- cysteine in a two-step process. The first step involves the ligation of L-glutamate and L-cysteine to form y-glutamylcysteine and is catalyzed by y-glutamylcysteine synthetase. Glutathione synthetase then catalyses the reaction between y-glutamylcysteine and glycine to form glutathione (Manta et al., 2013). Trypanothione is synthesized by conjugation of two glutathione molecules by a spermidine bridge in two ATP-dependent processes catalyzed by trypanothione synthetase (TryS) (Leroux et al., 2013). Trypanothione is kept reduced by an NADPH-dependent trypanothione reductase (TR)(Comini et al., 2013). Transfer of reducing equivalents from trypanothione can be mediated by thioredoxin- (Trx), tryparedoxin- (TXN) and glutaredoxin-type oxidoreductases (Comini et al., 2013).

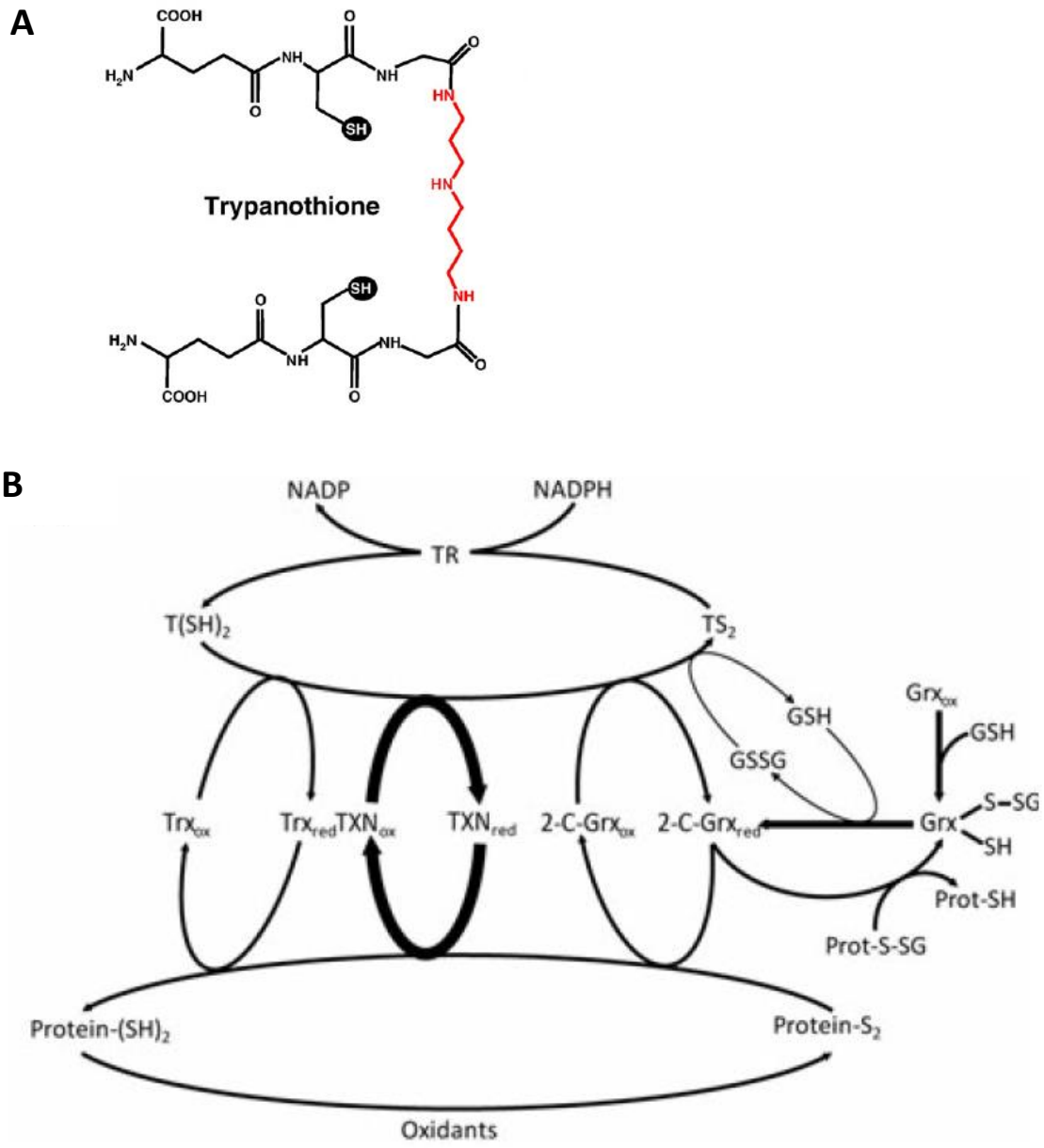


Figure 2. (A) Redox metabolism in trypanosomatids is based on trypanothione which is made up of two glutathione molecules (black) joined by a spermidine bridge (red)(figure taken from Krauth-Siegel and Comini 2008). (B) Trypanothione reductase (TR), an NADPH-dependent flavoenzyme, catalyses the reduction of trypanothione disulfide $(TS)_2$ to trypanothione $(TSH)_2$. Trypanothione is the direct reducing agent for the parasite thioredoxin (Trx), tryparedoxin (TXN) and dithiol glutaredoxin (2-C-Grx) type oxidoreductases, as well as for glutathione disulfide (GSSG). TXN is by far the most efficient multipurpose oxidoreductase of the parasites (Figure and text taken from Comini, Krauth-Siegel and Bellands, 2013).

1.5 Glutaredoxins

Glutaredoxins are small ubiquitous enzymes involved in redox homeostasis. Glutaredoxins are found in every organism and can be localized in different cellular compartments (Stroher and Millar, 2012). There are two main classes for glutaredoxins. Class I consists of glutaredoxins with Cys-Pro-Tyr-Cys sequence in the active site (Lillig et al., 2008). Class II consists of monothiol glutaredoxins with Cys-Gly-Phe-Ser as typical active site sequence and these glutaredoxins have been shown to be involved in iron-sulfur cluster assembly (Lillig et al., 2008). Class III are the CC-type glutaredoxins that have an active site with CC(M/L)(C/S) and is restricted to angiosperms (Stroher and Millar, 2012). ROXY is widely used in *Arabidopsis* as the name for the glutaredoxin of this class III (Stroher and Millar, 2012).

Glutaredoxins are members of the thioredoxin superfamily that share a common structural motif called the Trx fold. Other members of the thioredoxin family include protein disulfide-isomerases (PDI), thioredoxin, glutathione peroxidases and glutathione transferase (Stroher and Millar, 2012). The oxidoreductases of the Trx family share a similar active site motif Cys-X-X-Cys or Cys-X-X-Ser (Lillig et al., 2008). The general structure of the Trx fold is shown in Figure 3.

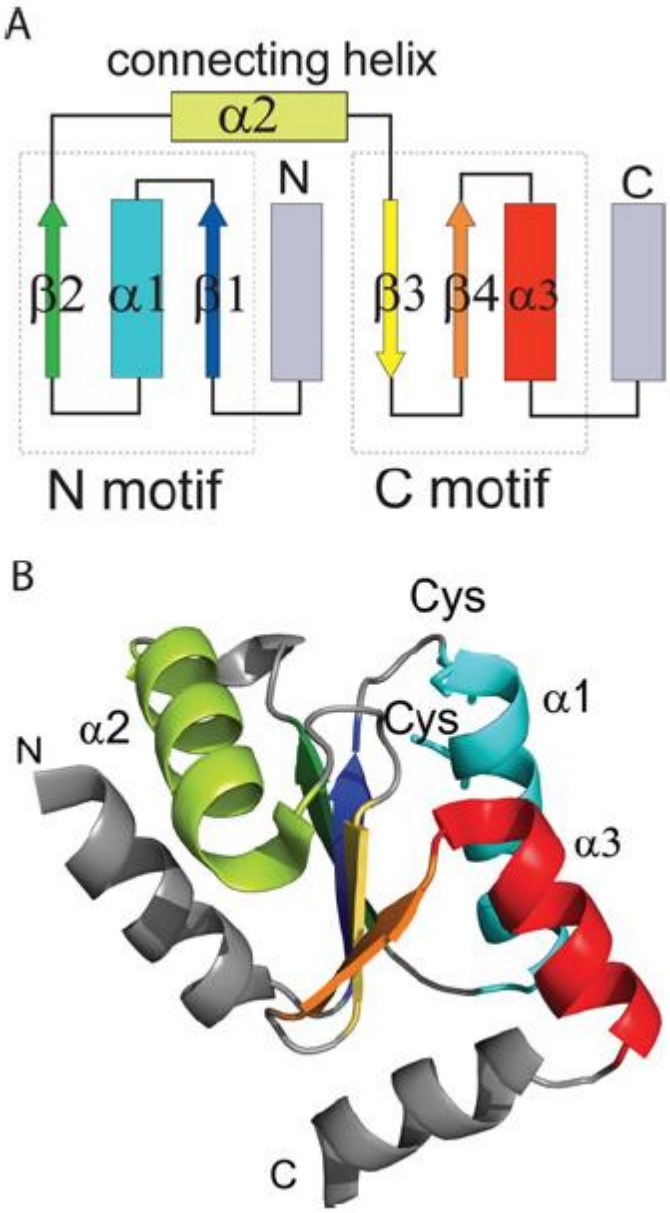


Figure 3. (A) Architecture of Grxs; α-helices are drawn as rectangles, β-sheets as arrows. N- and C- terminal conserved regions of the Trx fold are indicated in colour. (B) Structure of HsGrx2 (PDB code 2FLS). Same colour codes as in (A), the N- and C-terminus are indicated (text and figure from Stroher and Millar, 2012).

Protein glutathionylation is the formation of a mixed disulfide between a protein cysteine and glutathione (Klatt and Lamas, 2000). This modification can have a regulatory role in a cell as well means to store glutathione and to protect proteins under oxidative stress by preventing irreversible oxidation of thiols (Dalle-Donne et al., 2008). Protein deglutathionylation is frequently catalyzed by glutaredoxins (Dalle-Donne et al., 2008). Dithiol glutaredoxins can

catalyze reactions that proceed via two distinct mechanisms - the dithiol and monothiol mechanisms (Lillig et al., 2008). Reduction of protein-mixed disulfides proceed via a monothiol mechanism which requires only the N-terminal active site cysteine to reduce the mixed disulfide between the protein and glutathione (Stroher and Millar, 2012). The reduction of protein disulfides goes via a dithiol mechanism whereby the two active site cysteines are involved (Lillig et al., 2008). In this reaction, the N-terminal cysteine performs a nucleophilic attack on the target disulfide resulting in a mixed disulfide between the cysteine and target. In the next step, the second active site cysteine attacks the mixed disulfide intermediate (Lillig et al., 2008). The intramolecular disulfide formed between the two active site cysteines is then reduced by two glutathione molecules (Stroher and Millar, 2012). The two mechanisms are depicted in Figure 4.

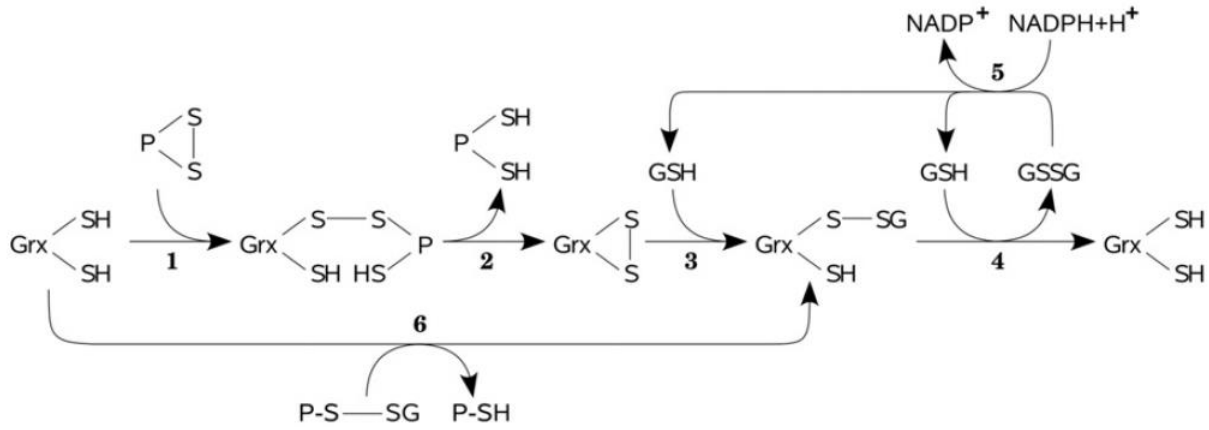


Figure 4. Reaction mechanisms of glutaredoxins. Glutaredoxins catalyze the reversible reduction of protein disulfides utilizing both their active site cysteinyl residues (reactions 1-4). Disulfides between glutathione and proteins or low molecular weight compounds are reduced in the monothiol mechanism that requires only the more N-terminal active site cysteinyl residue (reactions 6 and 4). In either case, glutathione disulfide formed in the reaction is reduced by glutathione reductase at the expense of NADPH (reaction 5)(figure and text taken from Lillig, Berndt and Holmgren 2008).

1.6 Glutaredoxins in *Trypanosoma brucei*

T. brucei contains three monothiol glutaredoxins (1-C-Grx1 to 3) and two dithiol glutaredoxins (Grx1 and Grx2) (Comini et al., 2013). The two dithiol proteins were partially characterized by Ceylan et al. 2010. Grx1 and Grx2 are located in the cytosol and probably in the intermembrane space of the

mitochondrion, respectively. Both proteins are kept reduced by direct reaction with trypanothione. Recombinant *T. brucei* Grx1, but not Grx2, was shown to coordinate iron-sulfur clusters. Grx1 was shown to catalyze the reduction GSH-protein mixed disulfides with 10-fold activity than of Grx2. In addition, Grx1 strongly accelerated the reduction of glutathione disulfide by trypanothione. RNA interference against Grx1 showed no growth phenotype in both bloodstream and procyclic form of *T. brucei*, but RNAi against Grx2 in procyclic cells resulted in a proliferation defect (Ceylan et al., 2010). Recombinant Grx1 could supply electrons for the reduction of ribonucleotide reductase by 20 to 50 % activity of that tryparedoxin (Tpx). Grx2 has a CQFC active site and was shown to catalyze the reduction of protein disulfides and glutathione-protein mixed disulfides although the latter reaction is less efficient when compared to Grx1 (Ceylan et al., 2010).

1.7 Aim of the PhD thesis

This work comprised

(i) To generate bloodstream and procyclic cell lines that lack Grx1, Grx2 or both Grx1 and Grx2 (if possible)

(ii) To characterize the mutant cell lines under various forms of stress

(iii) To determine the *in vivo* redox state of the Grxs under normal and oxidizing conditions

(iv) *In vitro* glutathionylation of Grx1 and determination of its effect on activity

(v) To generate conditional knockouts with either wildtype or mutant ectopic copies of Grx2 in procyclic cells

(v) To study bloodstream knockout cell lines compared to wildtype cells at an elevated temperature

(vi) To determine the role of the dithiol Grxs in the intact parasites

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetone	Applichem, Darmstadt
Agar	Life Technologies,Darmstadt
Agarose (standard) low electroendosmosis	Biomol, Hamburg
AMS	Life Technologies, Darmstadt
Bathocuproinedisulfonic acid disodium salt	Sigma Aldrich, Steinheim
Blasticidin S hydrochloride	Carl Roth, Karlsruhe
Carbenicillin	Serva, Heidelberg
DAPI	Sigma Aldrich, Steinheim
Deferoxamine mesylate	Sigma Aldrich, Steinheim
DTT	Biomol, Hamburg
EDTA	Applichem, Darmstadt
Ethanol	Sigma Aldrich, Steinheim
Fetal calf serum	Biochrom, Berlin
G418 Sulfate, cell culture tested	Merck Millipore, Darmstadt
Hemin	Sigma Aldrich, Steinheim
Hydrogen peroxide	Merck Millipore, Darmstadt
Hygromycin B	Carl Roth, Karlsruhe
Hypoxanthine	Sigma Aldrich, Steinheim
Iron(II) sulfate heptahydrate	Sigma Aldrich, Steinheim
Iron(III) nitrate nonahydrate	Sigma Aldrich, Steinheim
Iscove's modified Dulbecco's medium	Life Technologies, Darmstadt
Kanamycin	Serva, Heidelberg
L-Cysteine, non-animal source	Sigma Aldrich, Steinheim
MEM non-essential amino acids	Sigma Aldrich, Steinheim
MEM vitamins	Sigma Aldrich, Steinheim
Mem-Pros (with L-Glutamine, without $NaHCO_3$)	Merck Millipore, Darmstadt
Milk powder	Carl Roth, Karlsruhe
NaOH	Merck Millipore, Darmstadt
NEM	Sigma Aldrich, Steinheim
Penicillin/Streptomycin	Sigma Aldrich, Steinheim
Phenol red	Merck Millipore, Darmstadt
Phleomycin	Sigma Aldrich, Steinheim

Puromycin dihydrochloride	Sigma Aldrich, Steinheim
SDS	Serva, Heidelberg
Sodium chloride	Sigma Aldrich, Steinheim
Tetracyclin-hydrochloride	Sigma Aldrich, Steinheim
Trypton/Pepton from Casein	Carl Roth, Karlsruhe
Tween 20	Merck Millipore, Darmstadt
Yeast Extract	Carl Roth, Karlsruhe
β-Mercaptoethanol	Sigma Aldrich, Steinheim

2.1.2 Consumables

24-well cell culture plate (662160)	Greiner Bio-One, Frickenhausen
Amicon Ultra-15 concentrator	Merck Millipore, Darmstadt
BL21 (DE3) cells	Merck Millipore, Darmstadt
Cell culture flasks (250 ml, 550 ml)	Greiner Bio-One, Frickenhausen
Digital thermometer (GT11700)	GHM Messtechnik, Regenstauf
DNA *Taq* polymerase	Thermo Fisher Scientific, Lithuania
DNeasy Blood and Tissue Kit (Cat. No. 69504)	Qiagen, Hilden
Filter Tips Universal (10, 100, 1000 µL	Greiner Bio-One, Frickenhausen
Gene Rulers (100 bp and 1 kb)	Thermo Fisher Scientific, Lithuania
Goat anti-guinea pig IgG-HRP	Santa Cruz Biotechnology, USA
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology, USA
Goat anti-rabbit IgG-HRP	Thermo Fisher Scientific, USA
Guinea pig anti-Grx1	Eurogentec, Belgium
Guinea pig anti-Grx2	Eurogentec, Belgium
Mouse anti-myc	Roche, Mannheim
Novablue cells	Merck Millipore, Darmstadt
Nucleobond Xtra Midi	Macherey-Nagel, Düren
Pfu DNA Polymerase	Thermo Fisher Scientific, Lithuania
Stericup-GP Filter unit 0.22 µm, 500 ml	Merck Millipore, Darmstadt
Super Signal West Pico Kit	Thermo Fisher Scientific, USA
Tissue culture flasks (50 ml)	Becton Dickinson, Heidelberg
Whatman paper 3 MM	Whatman, Dassel
Zymoclean Gel DNA Recovery (D4002)	Hiss Diagnostic, Freiburg

2.1.3 Primers

All primers were synthesized by Eurofins MWG Operons, Ebersberg, Germany and are listed in Tables 2A and B.

Table 2A. List of Grx1 primers

Purpose	**Name**	**Sequence (restriction sites in bold)**
Amplification *of grx1* 5‘ UTR for ligation into pHD1747/pHD1748 for the generation of knock-out cell lines	5UTR-5aF-*grx1* -XhoI	AGAT**CTCGAG**GTAACTGCA TCACGCAC
	5UTR-5bR-*grx1*-HindIII	TTA**AAGCTT**GATACCACGTG TTGGGGAC
Amplification *of grx1* 3‘ UTR for ligation into pHD1747/pHD1748 for the generation of knock-out cell lines	3UTR-3aF-*grx1*-EcoRI	AGA**GAATTC** TAGTGCCAAACGAGAGGTG
	3UTR-3bR-*grx1*-NotI	GT**GCGGCCGC**GTGCTAAAA GGCGATCTTC
To confirm insertion of the resistance cassette into the Grx1 locus	Grx1-5utrFW_BM	GTCTATGCTGCGCAATAGG GAT
	PuroR_BM2	CACACCTTGCCGATGTCGAG
	BlaRev	GAGCTGCGCTGGCGACG
To verify the deletion of *grx1* alleles	Grx1 1Fo (P337)	CATG CCC TCT ATC GCT TCG
	Grx1 1Re (P334)	CGT TTC GTG TTT GTA TGC TTG
To verify presence of peroxidase II	Px IIF (Michi's P26)	ATGTCAGCTGCTTCGTCAAT C
	Px IIR (Michi’s P18)	AGACGCGCTCTGCGTGCT

Table 2B. List of Grx2 Primers

Purpose	**Name**	**Sequence (restriction sites in bold)**
Amplification of *grx2* 5‘ UTR for ligation into pHD1747/pHD1748 (*neo*) for the generation of knock-out cell lines	Tb2CGrx2_5’_1f	CTG**CTCGAG**CTCTTTTAC TAAGGTAG
	Tb2CGrx2_5’_2r	CTC**AAGCTT**CCTCACAGC AACGTAC
Amplification *of grx2* 3‘ UTR for ligation into pHD1747/pHD1748(*neo*) for the generation of knock-out cell lines	Tb2CGrx2_3’_7f	CGA**GAATTC**GGGGCTGA AGAGG
	Tb2CGrx2_3’_4r	AAT**GCGGCCG**CCGAGCA GCATACAAC
To confirm insertion of the resistance cassettes into the *grx2* locus	5UTRGrx2F_BM	CTTCACTCTCAAACTTATG TAGG
	PuroR_BM2	CACACCTTGCCGATGTCG AG
	NeoR_BM	CATCAGCCATGATGGATA CTTTC
To verify the deletion of *grx*2 alleles	2CGrx2F_BM	ggatgaataacgcattggatc
	2CGrx2R_BM	cttctgcaaacacttcacgac
	5UTRGrx2-2F_BM	GTACGTTGTTGTGAGGG ACCTA
	3UTRGrx2_2R_BM	CCTATGCTCACAATACAT AAAC
To mutate Cys34 into a serine	2CGrx2_C34S_f 5’	GCCACGTATTGTCAGTTC TCCACAAAACTGAAGATG TTAC
	2CGrx2_C34S_r 5’	GTAACATCTTCAGTTTTG TGGAGAACTGACAATAC GTGGC
To mutate both Cys31 and Cys34 to serine	2CGrx2_Dmut_f 5’	GTCCGCCACGTATTCTCA GTTCTCCACAAAACTGAA GATG
	2CGrx2_Dmut_r 5’	CATCTTCAGTTTTGTGGA GAACTGAGAATACGTGG CGGAC

To introduce a stop codon in the pHD1700-Grx2-c-myc$_2$ construct	2CGrx2_Stop_F 5’	GAAATTGAAAGGGGCTGAGGATCCAACGAGCAAAAG
	2CGrx2_Stop_r 5’	CTTTTGCTCGTTGGATCCTCAGCCCCTTTCAATTTC

2.1.4 Vectors

The vectors (pHD1747, pHD1748 and pHD2171) were kindly provided by Dr. Christine Clayton (ZMBH, Heidelberg).

2.1.5 Antibodies

The antibodies used in this work are listed in Table 3.

Table 3. List of primary and secondary antibodies

Primary antibody		**Secondary antibody**	
	Dilution		Dilution
α-Grx1	1:800	α-guinea pig	1:10000
α-Grx2	1:200	α-guinea pig	1:10000
α-Grx2	1:200	α-rabbit	1:20000
α-TXNPx	1:6000	α-rabbit	1:20000
α-Myc	1:800	α-mouse	1:20000

2.1.6 Media for *E. coli* cultivation

LB Medium (1 l)

Yeast extract		5 g
Sodium chloride		5 g
Trypton/Pepton		10 g
Water (sterile)	ad	1 l

pH 7.5 (NaOH)

2 YT Medium (1 l)

Yeast extract		10 g
Sodium chloride		10 g
Trypton/Pepton		20 g
Water (sterile)	ad	1 l
pH 7.5 (NaOH)		

2.1.7 Media for *T. brucei* cultivation

HMI-9 Medium for bloodstream cells (10 l)

Iscove's modified Dulbecco's Medium	176.6 g
$NaHCO_3$	30.24 g
Hypoxanthine	1.36 g
Sodium pyruvate	1.1 g
Thymidine	0.39 g
Bathocuproine disulfonic acid disodium	0.28 g
Water (sterile) ad	10 l
pH 7-7.5 (NaOH) (adjusted if necessary)	

Complete HMI-9-Medium (1 l)

HMI-9-Medium	900 ml
Fetal calf serum (inactivated at 60 ^{o}C, 1h)	100 ml
Pen/Strep (5000 U/ml; 5 mg/ml)	10 ml
β-Mercaptoethanol (14 M)	14 µl
L-Cysteine (150 mM in sterile water)	10 ml
Phleomycin (2.5 mg/ml in sterile water)	80 µl

Complete MEM-Pros-Medium for procyclic cells (1 l)

*1 x Basic-MEM (pH 7.4 (NaOH))	900 ml
FCS (inactivated)	100 ml
Pen/Strep (5000 U/ml; 5 mg/ml)	10 ml
Hemin (2.5 mg/ml in 0.1 M NaOH)	2.8 ml
100 x MEM-vitamins	10 ml
100 x MEM non-essential amino acids	10 ml
10 mg Phenol red	10 mg
Phleomycin (2.5 mg/ml in sterile water)	200 µl

*1 x Basic MEM with L-glutamine and without $NaHCO_3$ dissolved in water and pH adjusted to pH 7.4 with NaOH. Powder for 5 l dissolved in 4.5 l. Aliquoted into 450 ml or 900 ml.

2.1.8 Parasites

The parasites used in this work were of the *T. brucei brucei* 449 strain which is a descendant of Lister 427 (Cunningham and Vickerman, 1962).

2.1.9 Antibiotics for *T. brucei* cell culture medium

Both bloodstream and procyclic cells were cultured in medium that contained 50 U/ml penicillin and 0.05 mg/ml streptomycin. Additionally, complete HMI-9 and complete MEM-Pros medium contained 0.2 and 0.5 µg/ml phleomycin, respectively. For selecting the clones after transfection (see below), the antibiotics listed in Table 4 were used.

Table 4. Antibiotics used for selection purposes

	Concentration for bloodstream cells (µg/ml)	**Concentration for procyclic cells (µg/ml)**
Hygromycin	10	50
Tetracycline	1	1
Puromycin	0.2	2
Blasticidin	5	10
Neomycin/G418	2.5	30

2.2 Methods

2.2.1 Sequencing

All plasmids were sequenced by EuroFins MWG Operons, Ebersberg, Germany or GATC Biotech AG, Konstanz, Germany.

2.2.2 Cultivation of *T. brucei*

Wildtype bloodstream form parasites were cultivated in HMI-9 medium at 37 ^{o}C in a humidified incubator with 5 % CO_2. Procyclic cells were grown at 27 ^{o}C in MEM-Pros medium. Both media were supplemented with 10 % (v/v) heat-inactivated fetal calf serum. After *grx1* or *grx2* alleles had been knocked out (in the absence of an ectopic copy), the resulting double knockout (KO) clones were grown in the absence of the selecting antibiotics.

2.2.3 Glycerol stocks

Procyclic cells were harvested by centrifugation at 2000 rcf and 4 ^{o}C for 10 min and resuspended in 10 % sterile glycerol (in complete MEM-Pros medium) to a final density of 1 x 10^7 cells/ml. Bloodstream form cells were harvested by centrifugation at 2000 rcf and 4 ^{o}C for 10 min and resuspended in 10 % sterile glycerol (in complete HMI-9 medium) to a final density of 6 x 10^6 cells /ml. The cells were stored as 1 ml aliquots in cryo tubes at -80 ^{o}C.

2.2.4 Isolation of genomic DNA from *T. brucei*

Genomic DNA was isolated from 5 x 10^6 bloodstream form parasites using the Qiagen DNeasy Blood and Tissue kit.

2.2.5 Isolation of plasmids from *E. coli*

Plasmid DNA amplified in *E. coli* was isolated using the NucleoBond Extra Midi kit.

2.2.6 PCR analyses

PCR analyses were performed with the Eppendorf Master Cycler and *Taq* DNA polymerase (for analytical purposes) or *Pfu* polymerase (for cloning purposes). The general program for PCR is given in Table 5.

Table 5. General PCR program

Step	Temperature (^{o}C)	Time
1. Denaturation	95	3 min
2. Addition of polymerase	80 (pause)	
3. Denaturation	95	30 s
4. Annealing	variable	30 s
5. Extension	72	1 min/kb
6. *Repeat		
7. Final elongation	72	10 min

*Steps 3 to 5 were repeated for 30 cycles.

2.2.7 Mutagenesis of plasmids for parasite transfection and expression of recombinant proteins

2.2.7.1 Mutagenesis of pHD1700/*grx2*-c-myc2 vector to express ectopic tag-free Grx2

In order to express a tag-free ectopic of Grx2 protein in the parasites under tet control, the vector pHD1700/*grx2*-c-myc$_2$ (Ceylan et al., 2010) was used as template and a stop codon was introduced after the *grx2* sequence using QuickChange Multi Site-Directed Mutagenesis Kit and the primers 2CGrx2_Stop_F 5' /2CGrx2_Stop_r 5' (see Table 2B) to generate vector pHD1700/*grx2*.

2.2.7.2 Mutagenesis of either one or both active site cysteines to serine in the pHD1700/*grx2* vector

Vector pHD1700/*grx2* generated as described above was used as a template and either cysteine 34 or both cysteines 31 and 34 were replaced by serine using the QuickChange Multi Site-Directed Mutagenesis Kit to generate pHD1700/*grx2c34s* or pHD1700/*grx2c31sc34s*, respectively.

2.2.7.3 Plasmids for expression of wildtype and cysteine mutants of recombinant Grx2

To generate cysteine mutants of recombinant Grx2 (C34S or C31S/C34S), plasmid pETtrx1b/*grx2* (Ceylan et al., 2010) was subjected to site-directed

mutagenesis of either one or both cysteines to serine using the QuickChange Multi Site-Directed Mutagenesis Kit. The pETtrx1b/*grx2c34s* and pETtrx1b/*grx2c31sc34s* constructs obtained were then used to express and purify the recombinant proteins according to published procedure (Ceylan et al., 2010). The concentration of the proteins was determined by the Bradford method.

2.2.8 Agarose gel electrophoresis

Agarose gels were prepared by dissolving 1 - 2 % agarose in 1 x TAE buffer. The DNA samples were mixed with 10 x sample buffer and loaded onto the gel. DNA fragments were separated by electrophoresis at a constant voltage of 100 V in 1 x TAE buffer at room temperature. The gel was put in approximately 0.04 % ethidium bromide solution for about 10 min before the DNA bands were visualized under UV light.

50 x TAE buffer (500 ml)

	MW (g/mol)	Amount
2 M Tris	121.14	121.14 g
0.1 M EDTA	372.24	18.61 g
pH 8.3 (conc. Acetic acid)	28.57 ml	

1 x TAE (2 l)

50 x TAE	40 ml
Water (bidest.) ad	2 l

2.2.9 Western blot analyses

A polyvinylidene fluoride (PVDF) membrane was soaked in methanol and arranged in a ‘sandwich’ composed of a sponge, 4 Whatman papers, the SDS gel, the PVDF membrane, 4 Whatman papers and a sponge. The ‘sandwich’ was put into a Western blotting chamber that contained 1 x Western blotting buffer. The transfer was carried out for 2 h at a current of 150 mA at room temperature. After transfer, the membrane was treated for 1 h with 20 ml of 5 % milk powder in TBS-T buffer, washed 3 x with TBS-T buffer for 5 min and incubated overnight with the primary antibodies, diluted in 5 % milk powder in

TBS-T buffer. Subsequently, the membrane was washed three times with TBS-T buffer for 10 min and then incubated for 1 h with the secondary antibodies diluted in 5 % milk powder in TBS-T buffer. The membrane was washed again 3 x with TBS-T buffer and then developed with SuperSignal West Pico chemiluminescent substrate in the darkroom.

10 x Western blotting buffer (1 l)

	MW (g/mol)	**Amount (g)**
248 mM Tris	121,14	30
1.86 M Glycine	75,07	140

1 x Western blotting buffer (1 l)

10 x Western blotting buffer	100 ml
Methanol	200 ml
Water (bidest.)	700 ml

10 x TBS buffer (1 l)

	MW (g/mol)	**Amount**
1 M Tris	121,14	121 g
1.5 M Sodium chloride	58,44	90 g
pH 7.5 (conc. HCl)		63 ml

1 x TBS-T buffer (1 l)

10 x TBS	100 ml
Tween 20	500 µl
Water (bidest.) ad	1 l

Western blot stripping

To remove bound antibodies, the membrane was incubated with 50 ml of stripping buffer for 1 h at 50 ^{o}C in a water bath. The membrane was then washed 3 x with TBS-T buffer for 10 min each and incubated with new antibodies as described above.

Stock solutions

	MW (g/mol)	Concentration	Volume (ml)	Mass (g)
525 mM Tris (10 x)	121.14	500 mM	50	3.03
pH 6,8 (conc. HCl)				
10 % SDS (5 x)		100 g/l	50	5

Stripping Buffer (50 ml)

	Volume (ml)	Final concentration
525 mM Tris	5	52.5 mM
10 % SDS	10	2 %
14 M β-mercaptoethanol	0.35	98 mM
Water (bidest.)	35	

2.2.10 Silver staining

The gels were stained at room temperature according to the Fast Silver Staining Protocol of the Price Lab (http://www.labspaces.net/protocols/15291206472756_protocol.pdf). The solutions required are listed in Table 6. They were freshly prepared. 100 ml of each solution was used per gel (plus 12.5 ml citric acid). The gel was soaked for 10 min in fixing solution, washed twice with water for 5 min, soaked for 1 min in 0.02 % sodium thiosulfate solution and washed again twice in water for 20 s. The gel was then treated for 10 min with 0.1 % silver nitrate, rinsed with water, followed by a small amount of developing solution and finally soaked in fresh developing solution (formalin was added freshly) until protein bands were visible (approx. 1 - 3 min). 12.5 ml of 2.3 M citric acid was added to stop the reaction and the tray was shaken for 10 min. The gel was washed for 10 min in water and then soaked for at least 30 min in water before drying.

Table 6. Solutions for silver staining

	Final solution	Composition
Fixing solution	40 % (v/v) ethanol 13.5 % (v/v) formalin	40 ml absolute ethanol 13.5 ml formalin water ad 100 ml (bidest.)
Sodium thiosulfate	0.02 % (w/v) $Na_2S_2O_3$	0.02 g of $Na_2S_2O_3$ dissolved in 100 ml water (bidest.)
Silver nitrate	0.1 % (w/v) $AgNO_3$	0.1 g of $AgNO_3$ dissolved in 100 ml water (bidest.)
Developing solution	3 % (w/v) sodium carbonate 0.000016 % (w/v) $Na_2S_2O_3$ 0.05 % (w/v) formalin	3 g of sodium carbonate dissolved in 100 ml water (bidest.). add 80 µl of a 0.02 % $Na_2S_2O_3$ **Before use**: add 50 µl of formalin
Citric acid	2.3 M citric acid	6 g of citric acid dissolved in 12.5 ml water (bidest.)

*Note: Formalin is a commercial solution of 37 % formaldehyde.

2.2.11 Generation of Grx1 knockout cell lines in bloodstream and procyclic cells

2.2.11.1 Cloning of the constructs for deletion of the *grx1* alleles in *T. brucei*

Genomic DNA was isolated from bloodstream form *T. brucei* as described in section 2.2.4. The 5'- untranslated region of *grx1* was amplified from the genomic DNA by PCR with *Pfu* polymerase and 5UTR-5aF-*grx1*-XhoI and 5UTR-5bR-*grx1*-HindIII as primers, generating a fragment of 401 bp. The 3' untranslated region was amplified using the primers 3UTR-3aF-*grx1*-EcoRI and 3 UTR-3bR-*grx1*-NotI, yielding a fragment of 380 bp. Each fragment was purified from a 2 % agarose gel using the Zymoclean Gel DNA Recovery kit and ligated into the pGEM-T vector according to the manufacturer's protocol (pGEM-T and pGEM-T Easy Vector Systems) and amplified in NovaBlue cells. The 5' and 3' untranslated regions were released from the plasmid by digestion, and stepwise cloned into the pHD1747 (confers resistance to

puromycin) and pHD1748 (confers resistance to blasticidin) vectors to generate pHD1747-*grx1*-KO and pHD1748-*grx1*-KO, respectively (Figure 5).

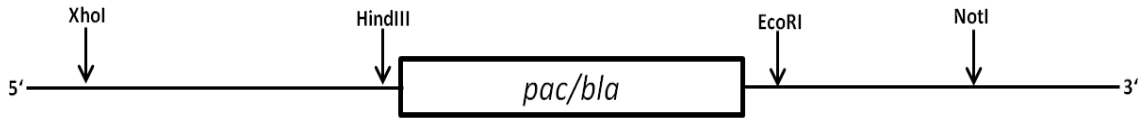


Figure 5. Scheme for the replacement of the *grx1* alleles by resistance genes. The 5'-and 3'-untranslated regions with XhoI/HindIII and EcoRI/NotI restriction sites, respectively, were cloned into the pHD1747 and pHD1748 vectors. The expected sizes of pHD1747-*grx1*-KO and pHD1748-*grx1*-KO cassettes after digestion with XhoI and NotI were 1375 bp and 1171 bp (calculated using Serial Cloner program), respectively. The puromycin and blasticidin resistance genes have sizes of 600 bp and 399 bp, respectively.

2.2.11.2 Preparation of DNA for parasite transfections

12 µg of pHD1747-*grx1*-KO and pHD1748-*grx1*-KO, respectively, were digested with XhoI and NotI. The digest (50 µl) was diluted to 100 µl with sterile water. 100 µl of 4 M ammonium acetate and 500 µl of absolute ethanol (kept at room temperature) were added. The sample was vortexed and incubated on ice for at least 15 min and then centrifuged at 13 000 rpm at 4 ^{o}C for 10 min. The supernatant was discarded and the pellet resuspended in 100 µl of 70 % v/v ethanol (at room temperature) and then centrifuged as before. The supernatant was carefully removed by pipetting and the pellet was allowed to dry under a sterile hood.

2.2.11.3 Transfection of procyclic *T. brucei* and cloning of *grx1*-deficient cell lines

4 x 10^7 wildtype procyclic cells harvested in the logarithmic growth phase were centrifuged at 2000 rcf for 10 min at 4 ^{o}C. The ethanol-precipitated DNA was mixed with 100 µl human T cell nucleofactor solution and added to the cell pellet. Transfection was performed in the Amaxa nucleofactor electroporator with the program X-001. Transfected parasites were transferred into 20 ml of conditional MEM-Pros medium (pre-incubated overnight at 37 ^{o}C). After overnight cultivation at 27 ^{o}C without any antibiotics, the parasites were seeded in 24-well plates by serial dilutions. Stably-transfected clones were selected by the respective antibiotic (2 µg/ml puromycin or 10 µg/ml blasticidin. Clones are obtained after about 7 days. Verified single knockout cells were transfected with the respective other deletion cassette (as depicted

in Figure 6) and clones were selected using both antibiotics. Deletion of both *grx1* alleles was verified by both PCR and Western blot analyses.

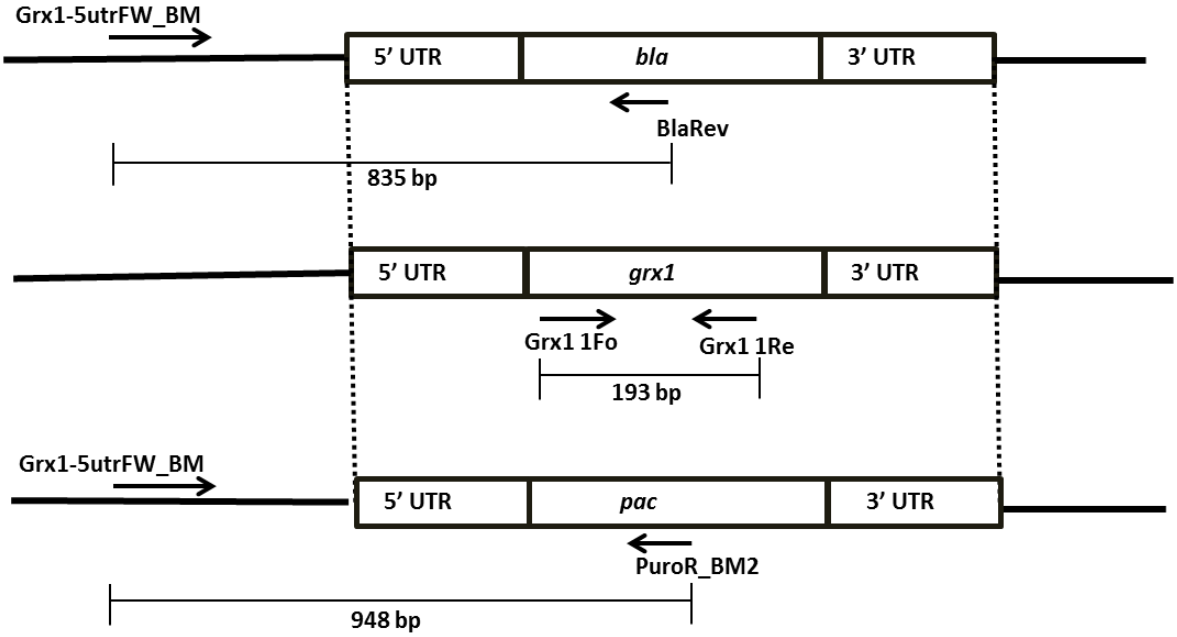


Figure 6. Scheme for the deletion of both *grx1* alleles in procyclic *T. brucei*. Two deletion cassettes conferring resistance to blasticidin (*bla,* blasticidin S-deaminase gene*)* and puromycin (*pac*, puromycin N-acetyltransferase gene) were cloned and used for transfection of the parasites. Various primer combinations (indicated by arrows and expected amplicon sizes below) verified the replacement of both *grx1* alleles by PCR analysis.

2.2.11.4 Transfection of bloodstream *T. brucei* and cloning of *grx1*-deficient cell lines

4 x 10^7 wildtype bloodstream cells were harvested and transfected with 12 µg of digested pHD1747-*grx1*-KO or pHD1748-*grx1*-KO as described before for procyclic cells. Transfected parasites were grown in 50 ml of HMI-9 medium at 37 ^{o}C in a 5 % CO_2 humidified incubator without any selecting antibiotic overnight and then seeded in 24-well plates by serial dilutions. Stably-transfected clones were selected using the appropriate antibiotics (0.2 µg/ml puromycin or 5 µg/ml blasticidin).

2.2.11.5 Generation of bloodstream Grx1 KO cells that express an ectopic copy of Grx1-c-myc2

Grx1 KO harboring an ectopic copy of Grx1-c-myc$_2$ were generated by transfecting 4 x 10^7 Grx1 KO bloodstream cells with 12 µg of digested pHD1700/*grx1-c-myc$_2$* (Ceylan et al., 2010) using the procedure described in section 2.2.11.3. Transfected parasites were grown in 50 ml of HMI-9 medium at 37 ^{o}C in a 5 % CO_2 humidified incubator without any selecting antibiotic overnight and then seeded in 24-well plates by serial dilutions. Stably-transfected clones were selected using 10 µg/ml of Hygromycin. Expression of ectopic Grx1-c-myc$_2$ was achieved by growing the cells overnight in medium containing 100 ng/ml tet.

2.2.12 Generation of Grx2 knockout cell lines in bloodstream and procyclic cells

2.2.12.1 Cloning of the constructs for deletion of the *grx2* alleles in *T. brucei*

Genomic DNA was isolated from bloodstream form *T. brucei* as described in section 2.2.4. The 5' untranslated region of *grx2* was amplified from the genomic DNA by PCR with *Pfu* polymerase and Tb2CGrx2_5'_1f and Tb2CGrx2_5'_2r as primers, generating a fragment of 388 bp. The 3' untranslated region was amplified using the primers Tb2CGrx2_3'_7f and Tb2CGrx2_3'_4r, yielding a fragment of 416 bp. Each fragment was purified from a 2 % agarose gel using the Zymoclean Gel DNA Recovery kit. The 5' and 3' utr of *grx1* in pHD1747-*grx1*-KO were stepwise released by digestion with XhoI /Hind III and EcoRI/NotI, respectively, and replaced with the *grx2* utr fragments to generate the pHD1747-*grx2*-KO deletion cassette. The blasticidin resistance gene in the pHD1748-*grx1*-KO deletion cassette was removed by digestion with HindIII and EcoRI and replaced with the neomycin resistance gene which was obtained from the pHD2171 vector by digestion also with HindIII and EcoRI. Subsequently, the 5' and 3' utr of *grx1* were stepwise replaced by *grx2* utr fragments, generating pHD1748 (*neo*)-*grx2*-KO deletion cassettes.

2.2.12.2 Transfection of bloodstream *T. brucei* and cloning of *grx2*-deficient cell lines

12 µg of pHD1747-*grx2*-KO and pHD1748 (*neo*)-*grx2*-KO, respectively, were digested with XhoI and NotI. The DNA precipitation and drying procedure was described in section 2.2.11.2. Bloodstream parasites were harvested and transfected with the constructs as described in section 2.2.11.4. Stably-transfected clones were selected using the appropriate antibiotics (0.2 µg/ml puromycin or 2.5 µg/ml neomycin). Verified single knockout cells were transfected with the respective other deletion cassette (as depicted in Figure 7) and clones were selected using both antibiotics. Deletion of both *grx2* alleles was verified by both PCR and Western blot analyses.

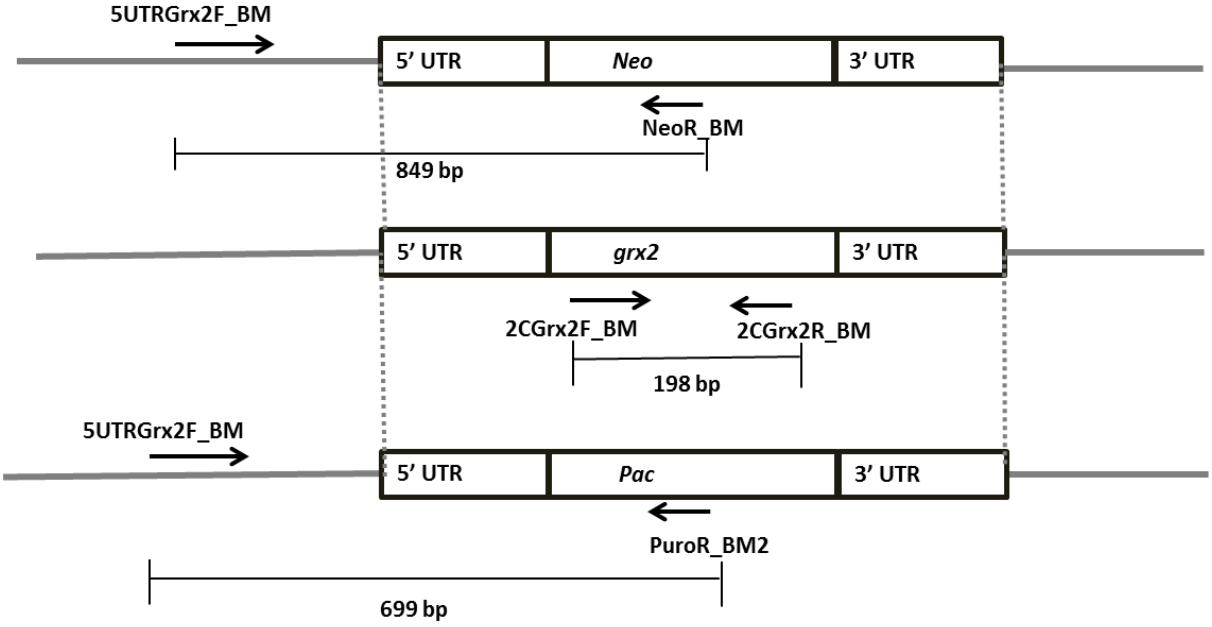


Figure 7. Scheme for the deletion of both *grx2* alleles in bloodstream and procyclic *T. brucei*. Two deletion cassettes conferring resistance to neomycin (*Neo* gene) and puromycin (*pac*, puromycin N-acetyltransferase gene) were cloned and used for transfection of the parasites. Various primer combinations (indicated by arrows and expected amplicon sizes below) verified the replacement of both *grx2* alleles by PCR analysis.

2.2.12.3 Generation of bloodstream Grx2 KO cells that express an inducible ectopic copy of Grx2

4 x 10^7 Grx2 KO bloodstream cells (see section 2.2.12 .2) were transfected with 12 µg of Not I-linearised pHD1700/*grx2* as described before. Transfected parasites were grown overnight in 50 ml of HMI-9 medium at 37 ^{o}C in a 5 %

CO_2 humidified incubator without any selecting antibiotic and then seeded in 24-well plates by serial dilutions. Stably-transfected clones were selected using 10 μg/ml of hygromycin.

2.2.12.4 Transfection of procyclic *T. brucei* and cloning of *grx2*-deficient cell lines

4×10^7 wildtype procyclic cells were harvested and transfected with 12 μg of NotI -digested pHD1747-*grx2*-KO or pHD1748 (*neo*)-*grx2*-KO as in section 2.2.11.3. Stably-transfected clones were selected by the respective antibiotic (2 μg/ml puromycin or 30 μg/ml neomycin). After *grx2* single knockouts (SKO) were verified by PCR, deletion of the second allele was attempted using the other deletion cassette as depicted in Figure 7.

2.2.12.5 Transfection of procyclic *T. brucei* Grx2 SKO cells with vectors to express tet-inducible ectopic copy of wildtype or mutant Grx2

4×10^7 procyclic *grx2* SKO (puro) clones were harvested as described before and transfected with 12 μg of Not I-linearised pHD1700/*grx2,* pHD1700/*grx2c34s* or pHD1700/*grx2c31sc34s*. Stably-transfected clones were selected by both 2 μg/ml puromycin and 50 μg/ml hygromycin.

2.2.12.6 Generation of conditional knockout procyclic cells that express an ectopic copy of Grx2, Grx2C34S or Grx2C31S/C34S

The procyclic *grx2* SKO (puro) clones harboring the tet-inducible Grx2 ectopic copies (pHD1700/*grx2*, pHD1700/*grx2c34s* or pHD1700/*grx2c31sc34s*) were grown overnight in MEM-Pros medium containing 50 μg/ml hygromycin, 100 ng/ml tet and 2 μg/ml puromycin and then transfected with the pHD1748-*grx2*-KO deletion construct as previously described. The cells were cultured overnight at 27 ^{o}C in the presence of hygromycin and tet (without puromycin or neomycin). Stably-transfected clones were selected in medium containing hygromycin, tetracycline, puromycin and neomycin.

2.2.13 Phenotypic analyses of Grx1 KO cell lines

2.2.13.1 Phenotypic analyses of Grx1 KO procyclic cell lines

5 ml cultures of WT and Grx1 KO (Grx1-deficient) procyclic *T. brucei* with an initial density of 5 x 10^5 cells/ml were grown in MEM-Pros medium at 27 ^{o}C in the presence of diamide (0 and 20 µM) for 24 h, hydrogen peroxide (0, 2.5, 5 and 10 µM) for 48 h, Fe (II) (Iron (II) sulfate heptahydrate) or Fe (III) (Iron (III) nitrate nonahydrate) (0 µM, 100 µM and 1 mM) for 48 h and the iron (III) chelator deferoxamine (0, 25, 100 and 500 µM) for 48 h. The stressors were added only once at the beginning of the experiment and viable cells were counted at different time points in a Neubauer chamber. For heat shock stress, a 5 ml culture with 5 x 10^5 cells/ml in medium at 27 ^{o}C in cell culture flask was incubated until the temperature reached 41 ^{o}C (approximately 45 min) in an incubator. Time point zero was defined when the temperature had reached 41 ^{o}C in the reference flask containing only medium, monitored with a digital thermometer. The temperature was maintained at 41 ^{o}C for 60 min and then viable cells were counted in a Neubauer chamber before the cultures were put back to 27 ^{o}C in the incubator for recovery. Cells were counted at various time points and time point zero for the recovery was taken as the time the cultures were put back into the 27 ^{o}C incubator.

2.2.13.2 Phenotypic analyses of Grx1 KO bloodstream form *T. brucei*

5 ml of WT and Grx1 KO bloodstream cells with an initial density of 5 x 10^5 cells/ml were cultured in HMI-9 medium at 37 ^{o}C in the presence of deferoxamine (0, 6.25, and 25 µM) for 30 h. 1 x 10^5 cells/ml of WT and Grx1 KO cells were cultured in medium containing 100 µM Fe (III) (Iron(III) nitrate nonahydrate) for 48 h. In addition to induce a heat shock, 5 ml of 5 x 10^5 cells/ml were transferred into 50 ml falcons and incubated in a water bath until the media reached temperature of 43 ^{o}C. This temperature was maintained for 15 or 30 min depending on the experiment. The temperature was monitored throughout the experiment by a digital thermometer (GT11700). Afterwards, viable cells were counted in a Neubauer chamber before the cells were re-transferred into normal culturing flasks. Recovery of the cells was then analysed at normal culturing conditions at 37 ^{o}C by counting

living cells at various time points. Cells not subjected to heat shock served as controls.

2.2.13.3 Proliferation of bloodstream Grx1 KO cells at 39 °C

As described in section 2.2.2, bloodstream WT and Grx1 KO clones are cultured in HMI-9 medium at 37 ^{o}C in a humidified incubator with 5 % CO_2. Grx1 KO clones that harbor an ectopic copy of Grx1-c-myc$_2$ are normally cultured in the presence of hygromycin. The proliferation of WT, Grx1 KO as well as Grx1 KO cells that express Grx1-c-myc$_2$ (clones 2 and 3) was investigated at an elevated temperature of 39 ^{o}C. To induce overexpression of Grx1-c-myc$_2$, the latter cells were cultured in the presence of 100 ng/ml tet, but without hygromycin. Uninduced clones were grown overnight also without hygromycin. WT cells in medium containing tet were used as controls. On the following day, cells were diluted in 5 ml pre-warmed at 39 ^{o}C to an initial starting density of 2 x 10^5 cells/ml in culture flasks. The flasks cells were put into a 39 ^{o}C humidified incubator with 5 % CO_2. After every 22 h, viable cells were counted and the cultures diluted back to the initial cell density. The induced cells were diluted always in 39 ^{o}C pre-warmed medium containing 100 ng/ml tet. Proliferation was monitored for a total of 110 h (Figure 16). Cells were harvested on the last day, boiled in reducing sample buffer containing 0.05 mg/ml DNase and stored at -20 ^{o}C for subsequent Western blot analyses.

2.2.14 Phenotypic analyses of Grx2 knockout cell lines

2.2.14.1 Phenotypic analyses of procyclic *grx2* SKO clone compared to WT cells

WT and *grx2* SKO cells (in the presence or absence of puromycin) with an initial cell density of 2 x 10^5 cells/ml were grown in MEM-Pros at 27 ^{o}C. Cells were counted and diluted to initial cell density at time points 51, 96 and 144 h.

2.2.14.2 Proliferation of procyclic Grx2 KO clones expressing an ectopic copy of Grx2 or mutant Grx2C34S compared to WT cells

Grx2 KO procyclic cells harboring a tet-inducible ectopic copy of Grx2 or Grx2C34S mutant were overnight cultured in medium containing ±100 ng/ml tet, but with no hygromycin. As control, wildtype cells were also grown in the

presence of tet. On the following day, cells were diluted in 5 ml Mem-Pros medium to an initial density of 5 x 10^5 cells /ml and then cultured under normal conditions, counted after every 22 h and diluted to the initial starting cell density. Induced cells were always diluted in medium containing 100 ng/ml tet. Aliquots were harvested for Western blot analysis.

2.2.14.3 Proliferation of bloodstream WT and Grx2 KO cells at 39 °C

The proliferation of WT, Grx2 KO as well as Grx2 KO cells that express a tet-inducible ectopic copy Grx2 was investigated at 39 ^{o}C as described in section 2.2.13.3.

2.2.15 Immunofluorescence microscopy

WT, Grx1 KO and Grx2 KO cells grown at 39 ^{o}C as described above were harvested at time point 66 h for immunofluorescence analysis using DAPI, following the published procedure (Hiller et al., 2014). Kinetoplast and nuclear DNA analysis was done for at least 200 parasites for each clone in each experiment.

2.2.16 Determination of redox state of glutaredoxins

2.2.16.1 Modification of recombinant Grx1 with thiol reagents

Recombinant *T. brucei* Grx1 was prepared following a published procedure (Ceylan et al., 2010) and stored at 4 ^{o}C. Five 100 µl samples containing 30 µM Grx1 in reaction buffer (50 mM Tris/HCl, 1 mM EDTA, 1% SDS, pH 7.5) were prepared and 25 µl ice-cold TCA solution (100 % TCA, 2 % HCl) was added. The mixtures were incubated on ice for 30 min, centrifuged at 13 000 rpm for 20 min at 4 ^{o}C and the supernatants discarded. The pellets were washed three times with 200 µl ice-cold acetone and centrifuged. Each of the five pellets was then treated differently: (i) As a control, one Grx1 sample was dissolved in 100 µl reaction buffer and stored at 4 ^{o}C. (ii) To alkylate the protein with AMS, the pellet was dissolved in 100 µl of 30 mM AMS dissolved in reaction buffer and incubated at 37 ^{o}C for 1 h. (iii) To alkylate the fully reduced protein with AMS, the pellet was dissolved in 100 µl reaction buffer containing 10 mM DTT and boiled for 5 min. The sample was TCA-treated, washed, dissolved in 100 µl reaction buffer containing 30 mM AMS and incubated for 1 h at 37 ^{o}C as

described above. (iv) To alkylate the fully reduced protein with NEM, the pellet was dissolved in 100 µl reaction buffer containing 10 mM DTT, boiled for 5 min, TCA-precipitated and washed as outlined above. Afterwards, the pellet was dissolved in 100 µl reaction buffer containing 20 mM NEM and incubated for 30 min at room temperature in the dark. (v) To alkylate the fully reduced protein by NEM followed by AMS treatment, Grx1 was incubated with DTT, washed, reacted with NEM and washed again. The final pellet was dissolved in buffer containing 30 mM AMS and incubated at 37 °C for 1 h. 500 ng of each sample was subjected to SDS-PAGE on a 16 % gel under reducing conditions, run at a constant current of 15 mA and room temperature. The protein bands were visualized by silver staining.

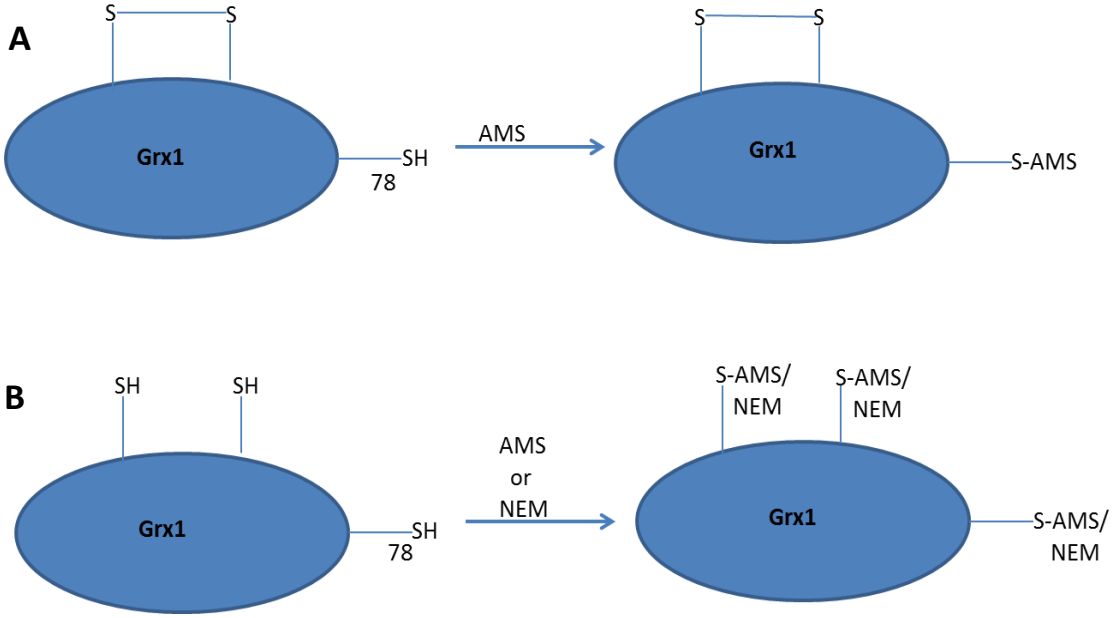


Figure 8. Scheme showing the modification of Grx1 by AMS or NEM. Grx1 has two cysteines in the active site and an additional one at position 78. (A) Reacting the stored recombinant protein with AMS results in the mono-modification of Cys78, resulting in a mass increase of about 500 Da compared to unmodified Grx1. (B) Fully reduced Grx1 reacted with AMS will thus display a mobility shift of about 1500 Da. In contrast, blocking the cysteines with NEM prior to AMS treatment will not result in a mobility shift on the SDS gel as compared to unmodified Grx1.

2.2.16.2 Modification of recombinant Grx2 with AMS

Reaction buffer, TCA precipitation and washing procedures with acetone procedures used here were the same as in section 2.2.16.1.

Three 100 µl samples containing 94 µM of recombinant Grx2 in reaction buffer were prepared and TCA-precipitated on ice for 30 min, centrifuged and the

supernatants discarded. The pellets were washed three times with ice-cold acetone, centrifuged and then treated differently: (i) As a control, one Grx2 sample was dissolved in 100 µl reaction buffer (ii) To alkylate the protein with AMS, the pellet was treated as in procedure (ii) of section 2.2.16.1. (iii) To alkylate the fully reduced protein, the pellet was treated as in procedure (iii) of section 2.2.16.1. 500 ng of each protein was subjected to SDS-PAGE on a 16 % gel under reducing conditions, at a constant current of 15 mA and room temperature. The protein bands were visualized by silver staining.

2.2.16.3 Determination of the *in vivo* redox state of Grx1 in bloodstream cells using AMS and/or NEM

The reaction buffer and TCA solution used here were the same as in section 2.2.16.1. Four samples each with 3 x 10^7 bloodstream cells harvested in logarithmic stage at 2000 rcf for 10 min at 4 ^{o}C ,washed with 15 ml of PBS, resuspended in 1 ml PBS and transferred into Eppendorf cups. After centrifugation at 8000 rpm for 2 min at 4 ^{o}C, the supernatants were discarded. The cells were resuspended in 900 µl PBS and 100 µl ice-cold TCA was added. The mixtures were incubated on ice for at least 30 min and then centrifuged at 13 000 rpm for 20 min at 4 ^{o}C. The protein precipitates were washed three times with 500 µl ice-cold acetone and then treated differently; (i) The control pellet was dissolved in 100 µl reaction buffer. (ii) The pellet was dissolved in 100 µl reaction buffer containing 30 mM AMS and incubated for 1 h at 37 ^{o}C. (iii) The pellet was dissolved in 100 µl reaction buffer containing 20 mM NEM and incubated for 30 min at room temperature in the darkness. (iv)The pellet was dissolved in 100 µl reaction buffer containing 20 mM NEM and incubated for 30 min in the dark. 25 µl of ice-cold TCA was added. The sample was incubated for 30 min on ice and centrifuged at 13000 rpm at 4 ^{o}C for 20 min. The precipitate was washed three times with 500 µl ice-cold acetone and the final pellet was dissolved in 100 µl of 30 mM AMS and incubated at 37 ^{o}C for 1 h. All samples were stored at 4 ^{o}C before analyses. 30 µl of each sample was mixed with 10 µl of reducing sample buffer, boiled for 5 min and 13.5 µl corresponding to approximately 3 x 10^6parasites was loaded per lane of a 16 % gel. The gel was subjected to SDS-PAGE followed by Western blot analyses using anti-Grx1 antibodies (1:800) (Figure 33).

2.2.16.4 Determination of the *in vivo* redox state of Grx1 in bloodstream form parasites under normal and oxidizing conditions

Five samples each with 4 x 10^7 bloodstream cells in logarithmic growth phase were harvested and washed in PBS as in section 2.2.16.3.The cells were resuspended in 900 µl PBS and (i) 100 µl of ice-cold TCA was added, mixture was kept on ice, centrifuged and precipitate was washed with ice-cold acetone and then dissolved in 100 µl reaction buffer (for details, see section 2.2.16.3). (ii) TCA-precipitated and washed with acetone as in i. The pellet was dissolved in 100 µl reaction buffer containing 30 mM AMS and incubated for 1 h at 37 ^{o}C. (iii) 3 mM diamide was added (23 µl of 116.1 mM diamide stock in sterile water). The cells were incubated at room temperature for 5 min and centrifuged at 8000 rpm for 2 min at 4 ^{o}C. The cell pellet was resuspended in 900 µl PBS and 100 µl of ice-cold TCA was added. The mixture was incubated on ice for at least 30 min and then centrifuged at 13000 rpm for 20 min at 4 ^{o}C. The precipitate was washed three times with 500 µl ice-cold acetone and the pellet reacted with AMS as described above. (iv) 3 mM diamide was added. The cells were incubated at room temperature for 5 min, centrifuged, resuspended in 900 µl of 37 ^{o}C pre-warmed HMI-9 medium and incubated for 5 min. After centrifugation the cells were washed three times with 1 ml PBS. The proteins were precipitated and washed as described above and dissolved in 100 µl reaction buffer. (v) treated with diamide and resuspended in medium as in (iv) except that the final pellet was additionally treated with AMS. In all cases, 30 µl of the cell extracts was mixed with 10 µl of non-reducing sample buffer, boiled for 5 min and 17 µl (corresponding to approximately 5 x 10^6 parasites) was loaded per lane of a 16 % gel. The gel was subjected to Western blot analysis using the anti-Grx1 antibodies (1:800) overnight (Figure 34).

2.2.16.*5 In vivo* redox state of Grx2 under normal and oxidizing conditions

Six samples each with 4 x 10^7 procyclic cells in logarithmic growth phase were harvested, washed with PBS as in section 2.2.16.3. The cells were resuspended in 900 µl PBS and (i) the sample was treated the same as in procedure (i) of section 2.2.16.4. (ii) the sample was treated the same as in procedure (ii) of section 2.2.16.4. (iii) 3 mM diamide was added (23 µl of 116.1 mM diamide stock in sterile water). The cells were incubated at room temperature for 5 min and centrifuged at 8000 rpm for 2 min at 4 ^{o}C. The cell pellet was resuspended

in 900 µl PBS and 100 µl of ice-cold TCA was added. The mixture was incubated on ice for at least 30 min and then centrifuged at 13000 rpm for 20 min at 4 ^{o}C. The precipitate was washed three times with 500 µl ice-cold acetone and the pellet dissolved in 100 µl reaction buffer. (iv) treated with 3 mM diamide as in (iii) except that the final pellet was reacted with AMS as described under (ii). (v) 3 mM diamide was added. The cells were incubated at room temperature for 5 min, centrifuged, resuspended in 900 µl of 27 ^{o}C pre-warmed MEM-Pros medium and incubated for 5 min. After centrifugation the cells were washed three times with 1 ml PBS. The proteins were precipitated and treated as described under (i). (vi) treated as in (v) except that the final pellet was treated with AMS. In all cases, 30 µl of the final solution was mixed with 10 µl of non-reducing sample buffer, boiled for 5 min and 34 µl (corresponding to approximately 1 x 10^{7} parasites) was loaded per lane of a 16 % gel. The gel was subjected to Western blot analysis using the anti-Grx2 antibodies (1:200) overnight.

2.2.17 Labelling of recombinant Grx2, Grx2C34S and Grx2C31S/C34S with AMS

In a total volume of 50 µl reaction buffer (see section 2.2.16.1), 120 µg protein (Grx2, Grx2C34S or Grx2C31S/C34S) was incubated with 10 mM DTT for 20 min at 50 ^{o}C. 50 µl of 60 mM AMS in reaction buffer was added and the samples were incubated at 37 ^{o}C for 60 min. As controls, 120 µg of each protein was diluted in 100 µl reaction buffers. Afterwards, 33 µl of reducing sample buffer was added to each sample, boiled and samples stored at 4 ^{o}C. About 5 µg of each protein was loaded on a 16 % gel, subjected to SDS-PAGE and Coomasie-stained afterwards.

2.2.18 *In vitro* glutathionylation of *T. brucei* Grx1

Recombinant Grx1 was prepared according to Ceylan et al., 2010. The protein concentration was determined at 280 nm (A_{280} of 1 corresponds to 1 mg/ml of pure Grx1). The thiol content was measured using Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid),DNTB, (ε_{412} = 13.6 $mM^{-1}cm^{-1}$) (Ellman, 1959). 1 ml of 100 µM Grx1 in 100 mM potassium phosphate, 1 mM EDTA, pH 7, was incubated with 17.5 mM GSH and 13 mM diamide for 2 h at 25 ^{o}C. Excess

diamide and glutathione were removed by three buffer exchanges on a 10 kDa cut-off 15 ml Amicon ultra concentrator. The protein and thiol concentrations were measured again before the treated and untreated Grx1 samples were subjected to ESI-MS analyses.

2.2.19 Determination of reductase activities of glutaredoxins

2.2.19.1 HED assay

The HED (hydroxyethyldisulfide) assay is a commonly used Grx-specific assay. During pre-incubation, HED is spontaneously reduced by glutathione forming mixed β-mercaptoethanol (β-ME) and a mixed disulfide between β-mercaptoethanol and glutathione (β-ME and GSH) (Lillig et al., 2008). The mixed-disulfides is reduced upon addition of Grxs via a monothiol mechanism to produce β-ME and mixed disulfide between GSH and N-terminal active site cysteine of Grx. A second molecule of GSH then reduces the disulfide yielding GSSG. The reaction can be followed by measuring the NADPH consumption at 340 nm by glutathione reductase (GR) during the reduction of GSSG (Lillig et al., 2008).

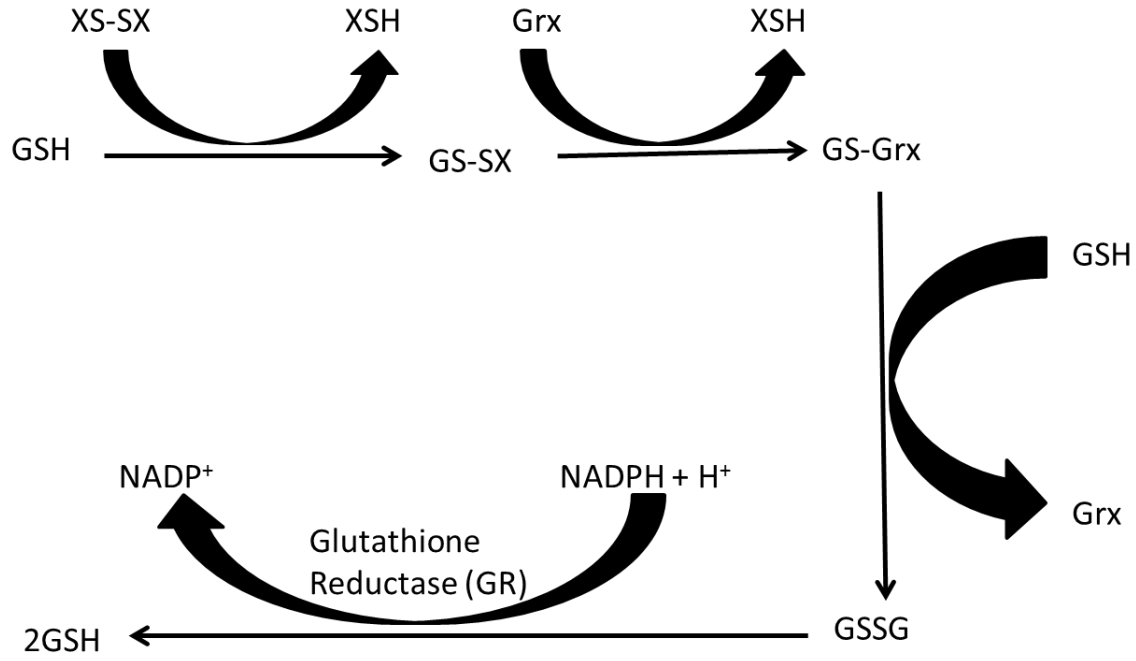


Figure 9. Schematic diagram of the HEDS assay. GSH reacts spontaneously with HEDS (XS-SX) resulting in the release of β-mercaptoethanol (XSH) and formation of a glutathione mixed disulfide (GS-SX). Grx catalyzes the release of a second β-mercaptoethanol molecule and gets glutathionylated (GS-Grx). Attack by a second GSH molecule restores free Grx and

generates glutathione disulfide (GSSG). The overall reaction is monitored spectrophotometrically at 340 nm as GSSG is reduced by glutathione reductase (GR) with the consumption of NADPH (Ceylan et al., 2010).

2.2.19.2 Reduction of glutathione-mixed disulfide using glutaredoxins

HEDS assay was carried out according to Ceylan et al., 2010. 1 ml of 100 mM potassium phosphate, 1 mM EDTA, pH 7, 200 µM NADPH, 1 mM GSH, 1 unit of GR, 0.015 - 1.25 mM HEDS were preincubated for 3 min at 25 ^{o}C to form mixed disulfide between β-mercaptoethanol (HEDS) and glutathione. Reduction of the mixed disulfide was started by addition of 370 nM of either Grx1 or glutathionlyted Grx1 (glut-Grx1). NADPH consumption was measured at 340 nM. The deglutathionylation activities of the Grxs were calculated after subtraction of the background reaction. The experiment was carried out twice and in each experiment, reduction of mixed disulfide was measured twice at each HEDS concentration. The kinetic constants were obtained using the non-linear regression SOLVER add-on in Microsoft Excel (Brown MA., 2000).
In the case of Grx2 species, 2.5 mM HEDS was used and the reduction of the mixed disulfide was started by addition of 400 nM or 1 µM of Grx2, Grx2C34S or Grx2C31S/C34S. The experiment was carried out three times and in each experiment, reduction of mixed disulfide was measured twice.

2.2.19.3 Insulin reduction assay

The assay was performed according to Ceylan et al., 2010. In a total volume of 200 µl reaction buffer (100 mM potassium phosphate, 2 mM EDTA, pH 7.0), 0.9 µM tryparedoxin (Tpx), 9 or 22 µM of Grx2, Grx2C34S or Grx2C31S/C34S were incubated with 2.5 mM DTT (dithioerythritol) for 20 min at room temperature. The reaction was started by adding 600 µl of 1 mg/ml insulin in reaction buffer to resulting in a final concentration of 625 µM DTT and 131 µM insulin. The increase in turbidity was followed at 650 nm at 30 ^{o}C.

3 Results

3.1 Both alleles of *grx1* could be knocked out in procyclic cells

Deletion of both *grx1* alleles in procyclic cells was achieved by replacing one allele at a time. PCR analyses with a forward primer (Grx1-5utrFW_BM) binding upstream of the 5'untranslated region of *grx1* used for recombination and a reverse primer specific for either blasticidin or puromycin (BlaRev and PuroR-_BM2, respectively) verified the replacement of the *grx1* alleles. Internal specific primers (Grx1 1Fo and Grx1 1Re) confirmed the presence of an allele in the SKO clones and the absence of any *grx1* alleles in three selected KO clones (KO clones 1, 2, and 3) (for a scheme see Figure 6). As expected, a single knockout clone (SKO), but none of the KO clones yielded an amplicon with the two internal *grx1* primers (Figure 10A). Primer combinations of Grx1-5utrFW_BM/ BlaRev and Grx1-5utrFW_BM/PuroR_BM2 yielded for all three KO clones PCR products of 853 bp and 948 bp, respectively (Figures 10B and C), in accordance with insertion of the resistance cassettes at the correct locus. Total lysates of wildtype cells, a SKO clone and the three KO clones were subjected to Western blot analyses using anti-Grx1 antibodies (1:800 overnight incubation) and goat anti-guinea pig IgG-HRP (1:10000) as secondary antibodies. Grx1 was detected in both the wildtype cells and the SKO clones, but not in the three double knockout clones (Figure 10D). These results confirmed the successful replacement of both *grx1* alleles. The double knockout (KO) clones were then cultured in MEM-Pros medium without any selecting antibiotics.

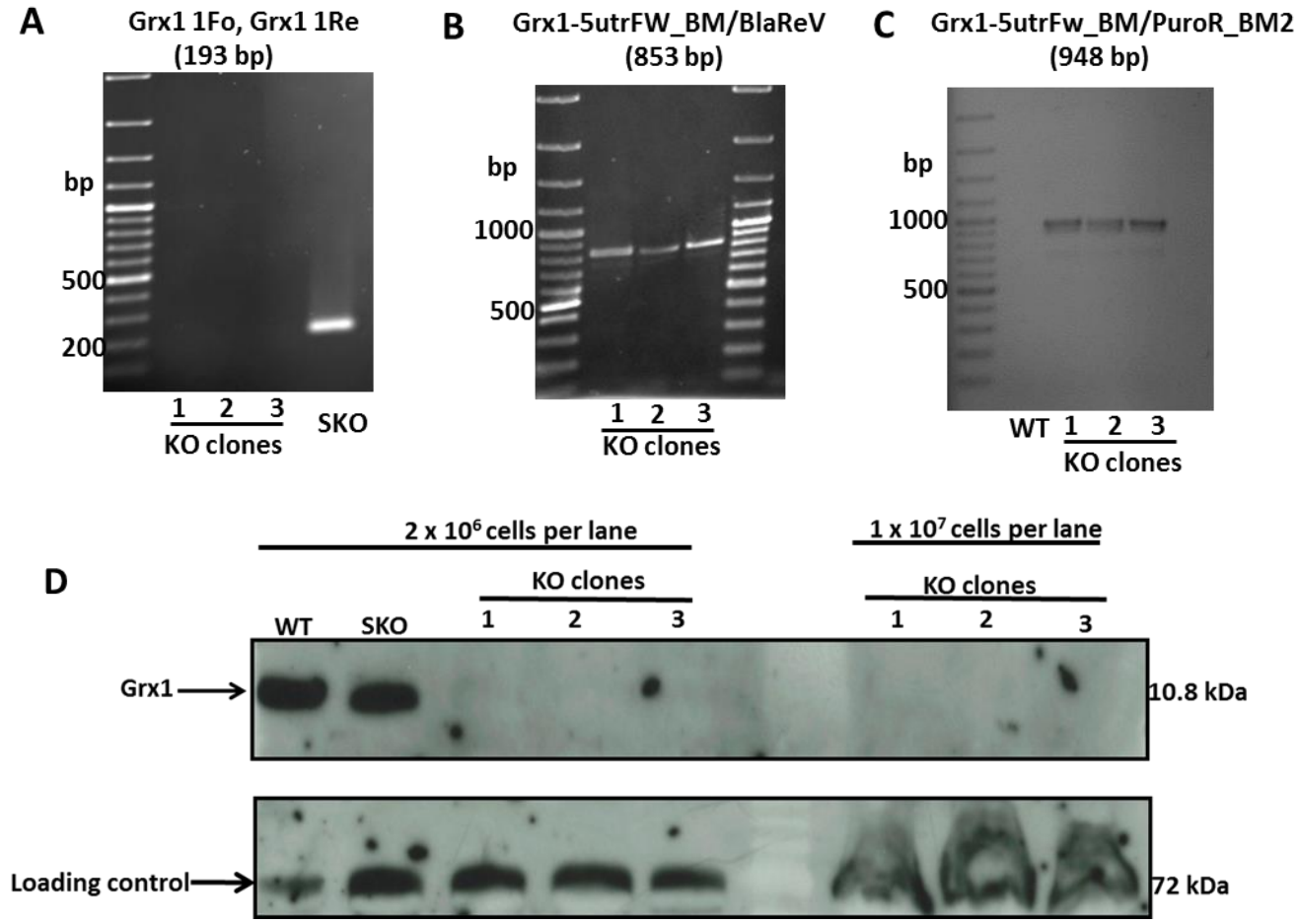


Figure 10. PCR and Western blot analyses of the procyclic WT and Grx1 KO cell lines. (A-C) PCR analyses with the primer combinations indicated above each graph with the expected fragment size in brackets. DNA from a single knockout (SKO) and wildtype (WT) cells served as controls. (D) Western blot analyses using anti-Grx1 antibodies (1:800). Total lysate from 2 x 10^6 WT, a SKO clone and three KO clones was loaded per lane (14 % gel) as well as from 1 x 10^7 cells in the case of the three KO clones. The results showed the absence of Grx1 in the three KO clones. A cross-reacting band at approximately 72 kDa served as loading control. (Clone 1 -C1 (1:4); Clone 2 - C4 (1:2); Clone 3 – C6 (1:2)).

3.2 Bloodstream Grx1 KO cell line was obtained by a loss of heterozygosity

The same strategy used in procyclic cells was applied in an attempt to replace the *grx1* alleles in bloodstream form cells. However, single knock out clones could only be obtained upon selection with blasticidin and not with puromycin. After culturing the SKO cells in the presence of 5 µg/ml blasticidin for several weeks, Western blot analyses were performed to determine the Grx1 level in the mutant compared to that in WT cells. Surprisingly, no Grx1 could be detected in the supposed SKO cells. Thus, I extracted DNA from these cells for

PCR analyses. The primer pair Grx1-5utrFW_BM/BlaRev gave the expected PCR product of 853 bp. However, Grx1 1Fo/Grx1 1Re did not yield a fragment of 193 bp which would have been expected in the case of a remaining *grx1* allele. These cells were then defined as double knock out clones (KO). I then thawed the SKO cells that had been frozen directly after the replacement of the first allele by the blasticidin resistance gene as previously verified by PCR analysis (not shown). Indeed, PCR analyses revealed amplicons for both Grx1-5utrFW_BM/ BlaRev and Grx1 1Fo/Grx1 1Re primer combinations (Figure 11A). These results confirmed the loss of heterozygosity in these bloodstream cells by long-term cultivation in the presence of blasticidin. Western blot analyses using antibodies against Grx1 revealed the protein in wildtype cells and the original single knockout cells but not in the double knockout cells (Figure 11B).

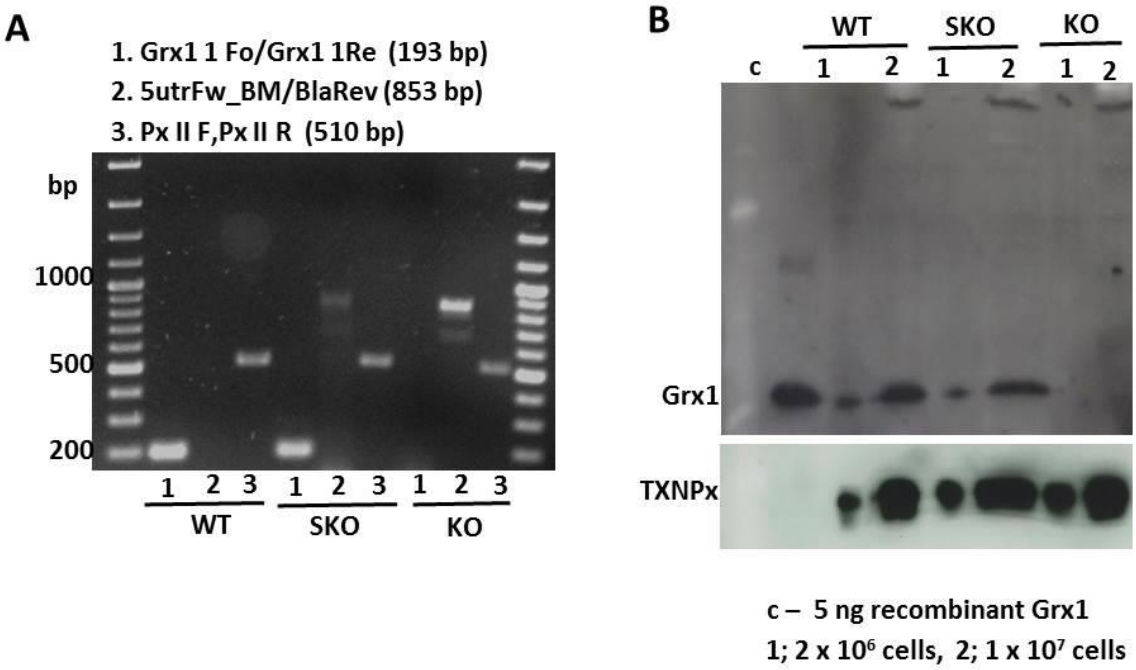
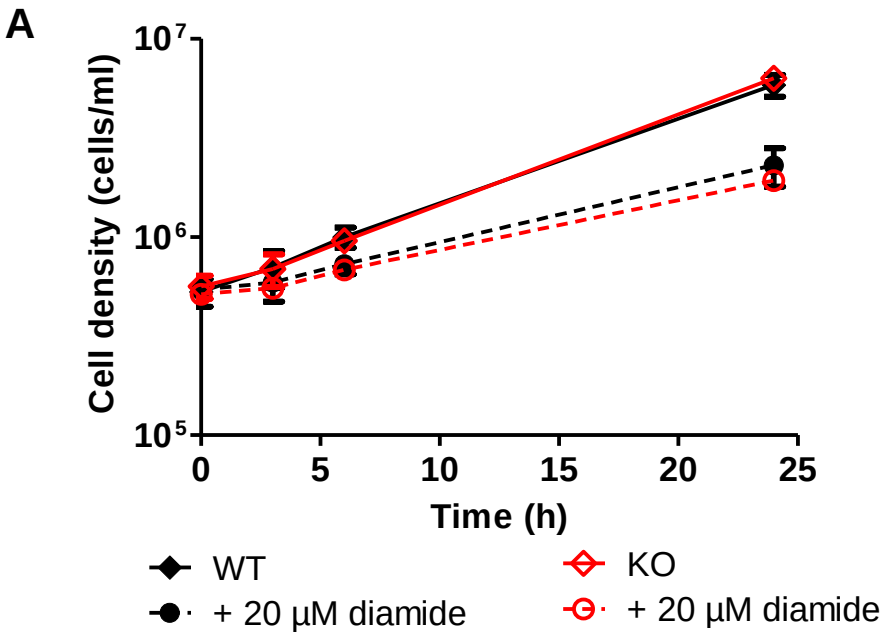
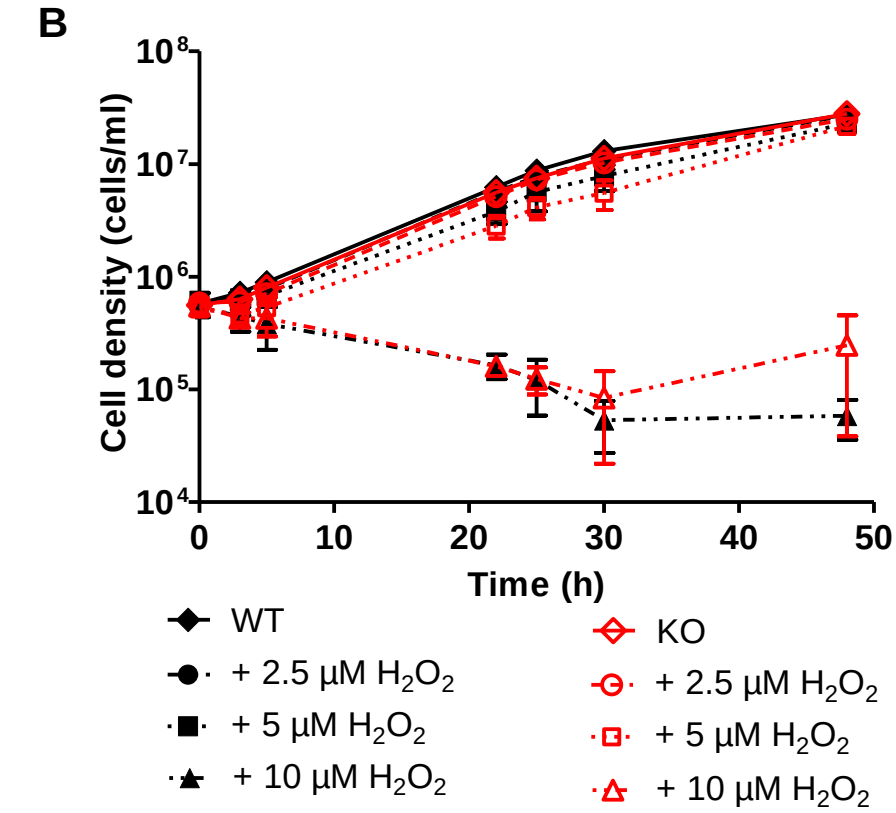


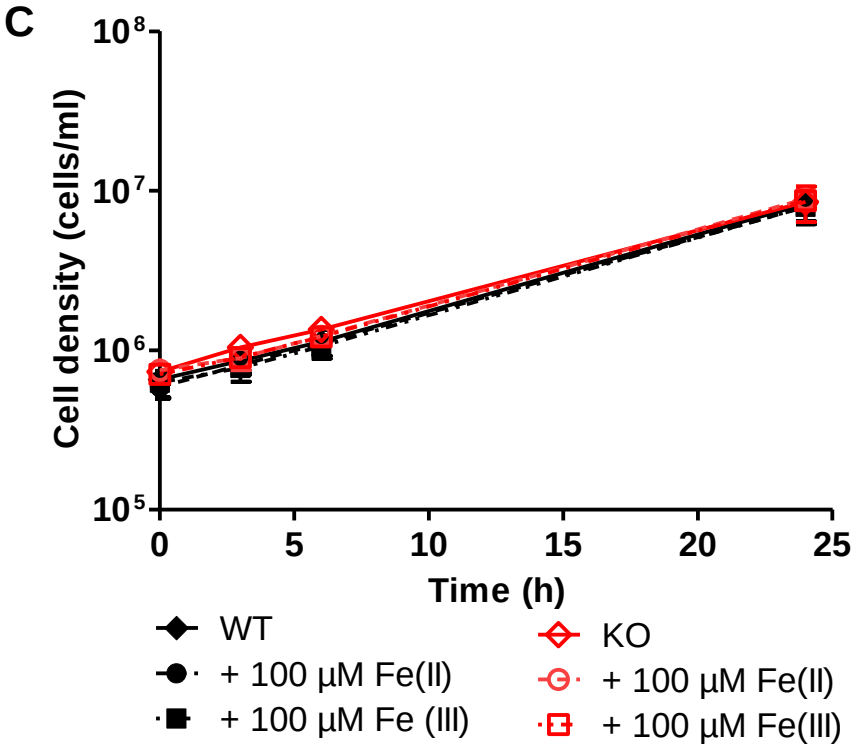
Figure 11. Grx1-deficient bloodstream form *T. brucei* obtained by a loss of heterozygosity. (A) PCR analyses using the indicated primer combinations with the expected sizes in brackets confirmed the replacement of the *grx1* alleles. SKO DNA was extracted from cells that had been frozen directly after successful replacement of one *grx1* allele by the *bla* resistance gene. KO DNA was extracted from those cells after cultivation in the presence of 5 µg/ml blasticidin for several weeks. WT DNA served as control. PCR with PxII (glutathione peroxidase-type tryparedoxin peroxidase) internal primers (specific for PxII) served as positive control. (B) Western blot analyses with the anti-Grx1 (1:800) antibodies revealed the absence of protein in the double-knockout cell line. The membrane was stripped and re-probed for 2-Cys-peroxiredoxin (TXNPx 1:6000) as a loading control.

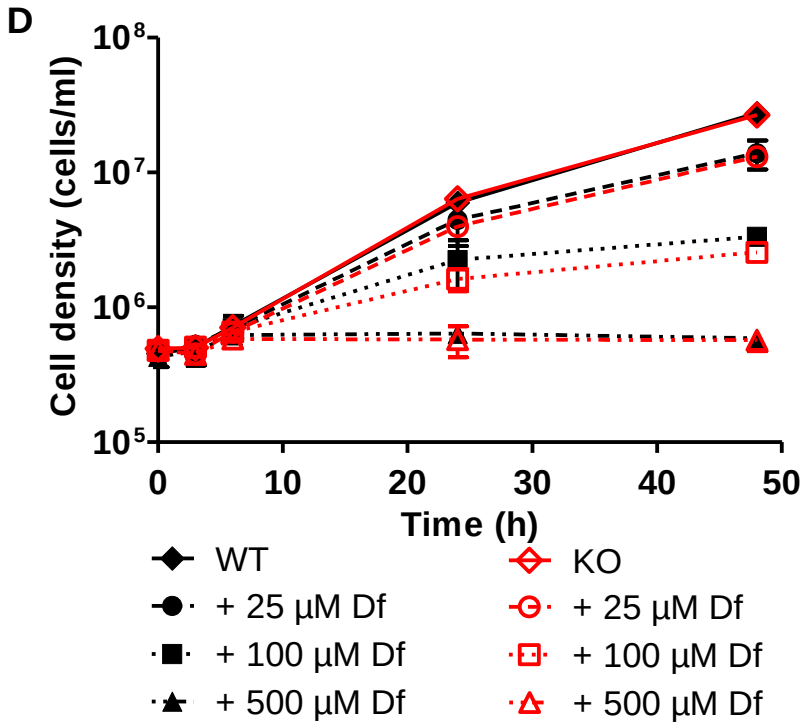
3.3 Phenotypic analyses of procyclic Grx1 KO cells

To investigate a putative role of Grx1 in the overall protection of the parasites against oxidative stress, procyclic WT and Grx1 KO cells (clone C1 (1:4)) (KO clone1 in Figure 10) were cultured in the presence of diamide or hydrogen peroxide as described in section 2.2.13.1. No difference in the sensitivity between the wildtype cells and the Grx1 KO clone was observed (Figures 12 A and B). At a concentration of 100 µM neither iron (II) nor iron (III) had any effect on the proliferation of the WT and Grx1 KO parasites (Figure 12C). There was also no difference in the proliferation when deferoxamine, an iron (III) chelator (Breidbach et al., 2002) was used. WT and KO cells displayed the same sensitivity (Figure 12D). There was also no difference in the recovery of proliferation after a heat shock at 41 °C between the WT and KO cells (Figure 12E). Thus, for none of the stressors studied, a proliferation defect could not be observed for the Grx1 KO procyclic cell line compared to WT cells.




B
Cell density (cells/ml)
Time (h)
WT
+ 2.5 µM H2O2
+ 5 µM H2O2
+ 10 µM H2O2
KO
+ 2.5 µM H2O2
+ 5 µM H2O2
+ 10 µM H2O2


C
Cell density (cells/ml)
Time (h)
WT
+ 100 µM Fe(II)
+ 100 µM Fe (III)
KO
+ 100 µM Fe(II)
+ 100 µM Fe(III)


D
Cell density (cells/ml)
10^8
10^7
10^6
10^5
0
10
20
30
40
50
Time (h)
WT
+ 25 µM Df
+ 100 µM Df
+ 500 µM Df
KO
+ 25 µM Df
+ 100 µM Df
+ 500 µM Df

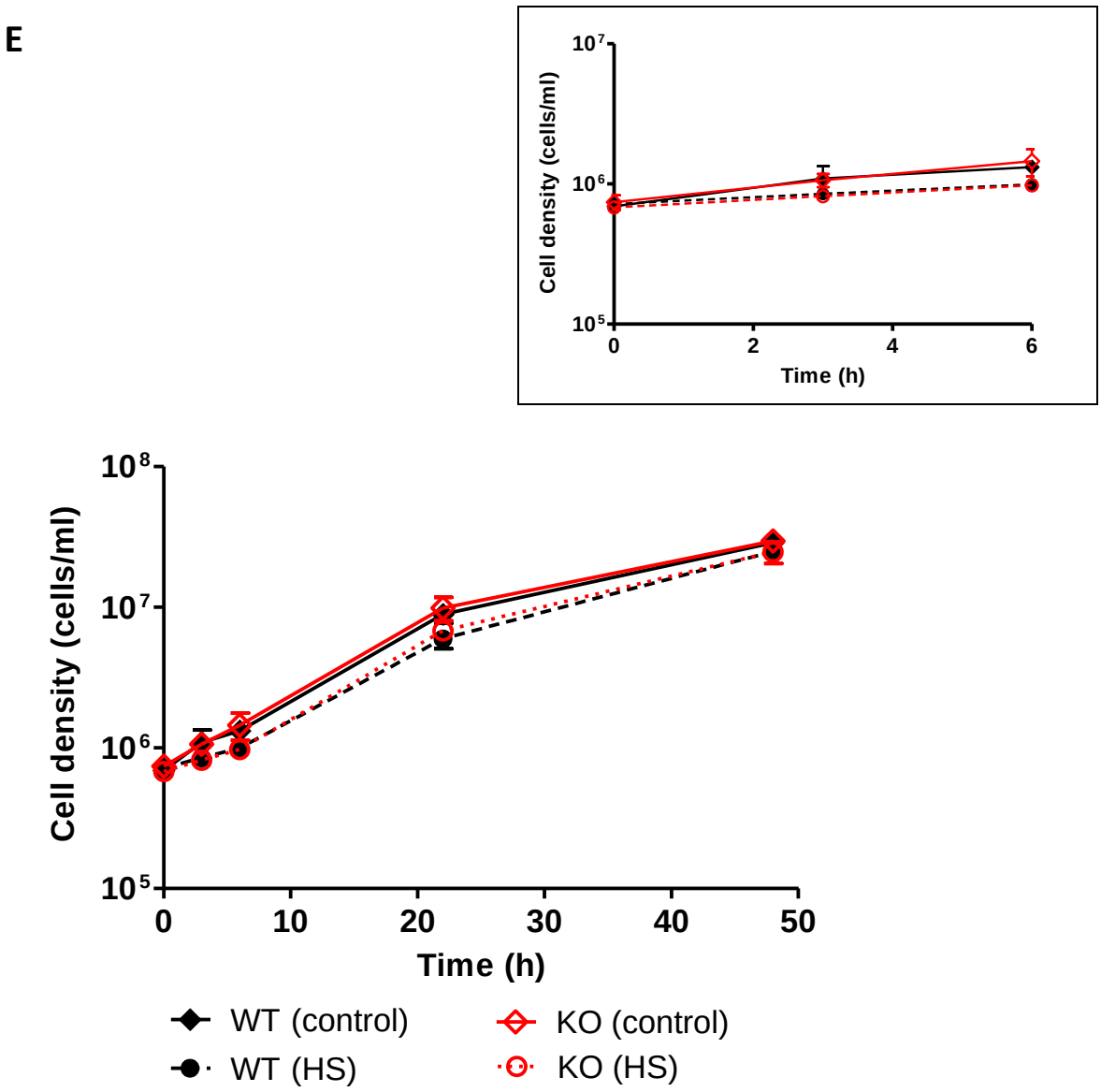
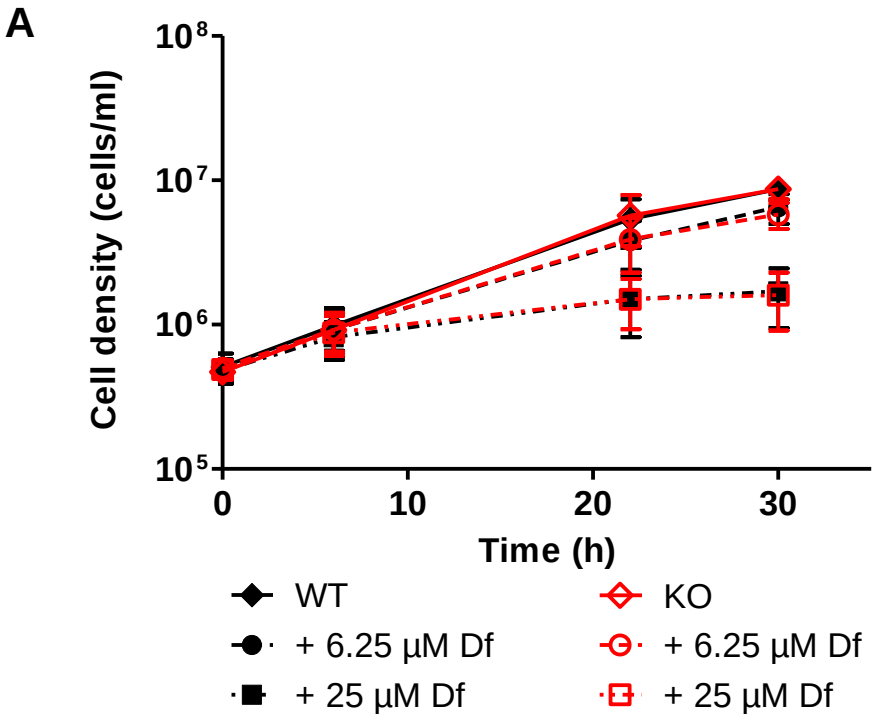


Figure 12. **Proliferation of procyclic Grx1 KO cells compared to WT cells in the presence of exogenous stressors and upon heat shock treatment.** 5 x 10^5 cells/ml of WT and Grx1 KO cells were cultured at 27 ^{o}C in the presence or absence of (A) 20 µM diamide, (B) 2.5, 5 and 10 µM H_2O_2, (C) 100 µM Fe(II) or 100 µM Fe(III), and (D) 25 , 100 and 500 µM deferoxamine (Df). The stressors were added once at time point zero. (E) 5 x 10^5 cells/ml WT andGrx1 KO cells were incubated for 60 min at 41 ^{o}C, viable cells were counted and the culture was put back to 27 ^{o}C. Control cells were not incubated at 41 ^{o}C, but kept at 27 ^{o}C from time point 0 h (inset, enlargement of the 0 to 6 h data). Viable cells were counted at various time points in a Neubauer chamber. The data are the mean ± standard deviation of three independent experiments.

3.4 Phenotypic analyses of Grx1 KO bloodstream *T. brucei*

Deferoxamine, iron (III) and heat shock were also studied as stresses of WT and Grx1 KO bloodstream cells. As in procyclic cells (Figure 12), no differences in the proliferation could be detected between the WT and mutant cells (Figure 13 A-C). Grx1 does not seem to play a role in protecting bloodstream parasite cells from iron depletion by deferoxamine or a 43 ^{o}C heat shock.



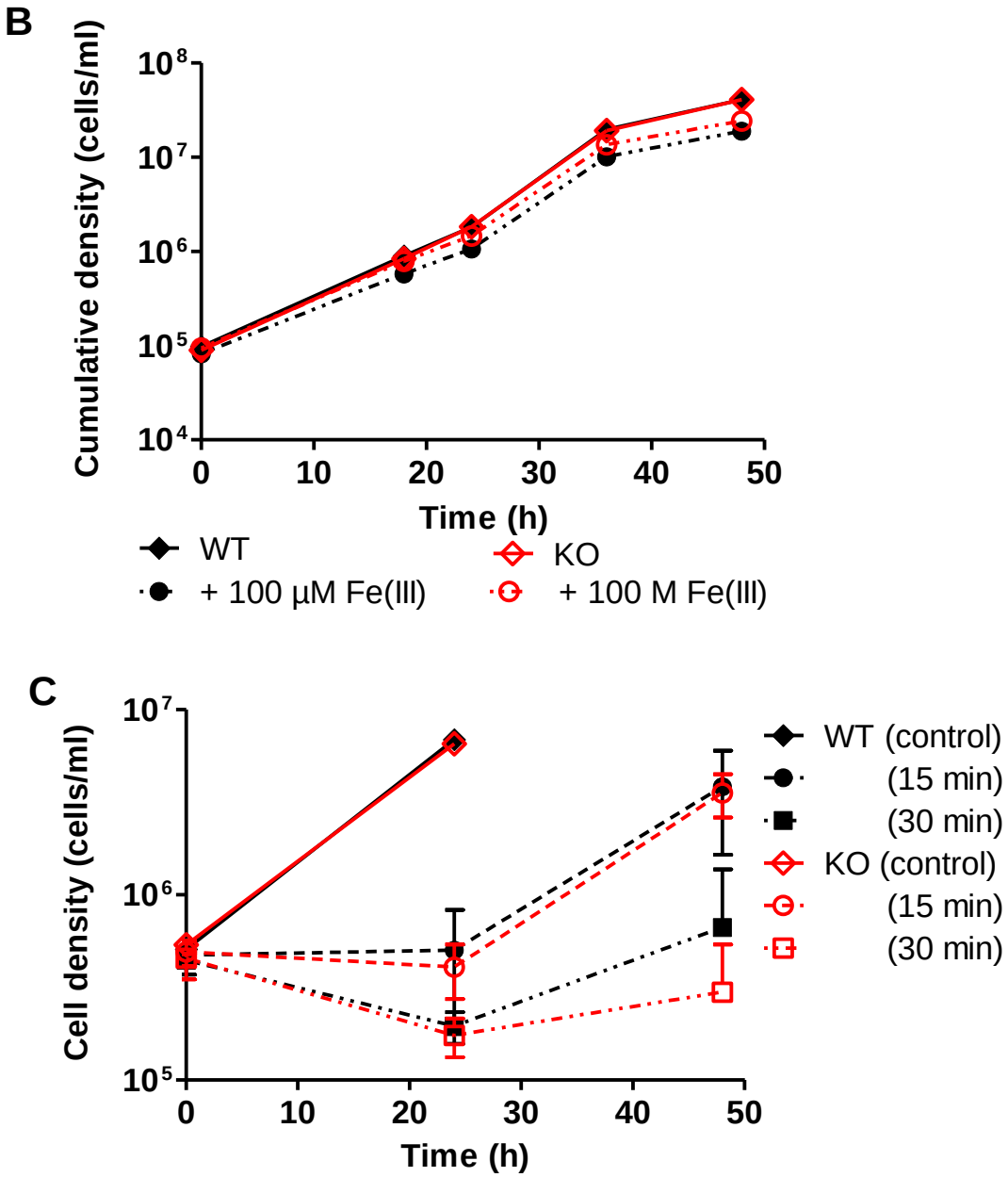


Figure 13. Proliferation of bloodstream WT and Grx1 KO cells subjected to various stresses. (A) 5 x 10^5 cells/ml WT and Grx1 KO parasites were grown in the presence of different concentrations of deferoxamine.(B) Cumulative cell density of WT and Grx1 KO cells with an initial cell density of 1 x 10^5 cells/ml grown in presence of 100 µM iron (III). (C) 5 x 10^5 cells/ml WT and Grx1 KO parasites were incubated at 43 ^{o}C for 15 or 30 min and returned to 37 ^{o}C. The values are the mean ± standard deviation of three independent experiments.

3.5 Grx2 expression levels are not affected by the deletion of Grx1 in bloodstream parasites

Since no proliferation defect could be detected in the Grx1 KO cell lines, the expression of Grx2, the other dithiol glutaredoxin of the parasite, was investigated. Western blot analyses using the anti-Grx2 antibodies (1:200) showed that the Grx2 levels were virtually the same in both cell lines (Figure 14). Thus, the two dithiol glutaredoxins do not appear to exert redundant physiological roles. This is supported by their different subcellular localization. Grx1 and Grx2 were found in the cytosol and mitochondrion, respectively (Ceylan et al., 2010).

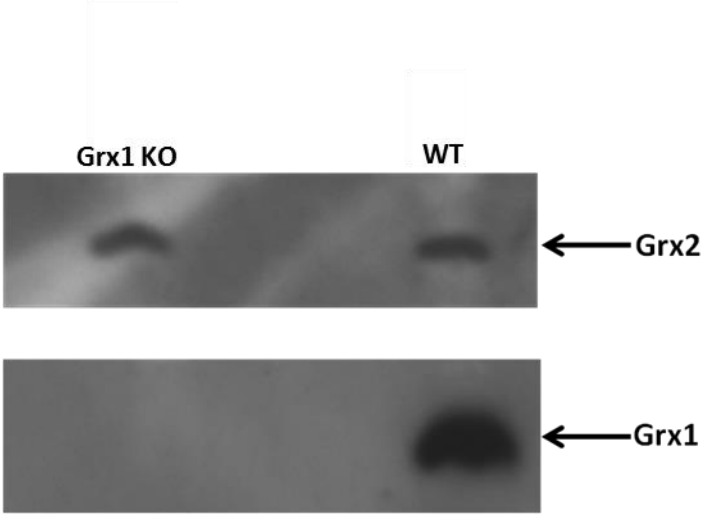


Figure 14. Grx2 expression is not affected in bloodstream Grx1 KO parasites. 2 x 10^7 wildtype and *grx1*-KO bloodstream parasites were subjected to Western blot analyses using the anti-Grx1 (1:800) and anti-Grx2 antibodies (1:400).The results show that there is no upregulation of Grx2 in the Grx1 KO cells.

3.6 Bloodstream Grx1 KO cells had a growth advantage at 39 ^{o}C compared to WT cells

WT and Grx1 KO cells multiply 13 fold within 22 h when grown at 37 ^{o}C (doubling every 6 h). Initial experiments had shown that Grx1 KO clones like WT cells, had growth retardation at 39 ^{o}C as compared to normal temperature of 37 ^{o}C. However, the Grx1 KO clone had a slight growth advantage compared to WT cells at 39 ^{o}C. To confirm that this improved proliferation was indeed due to the absence of Grx1, I transfected Grx1 KO cells with an ectopic copy of

Grx1-c-myc$_2$. Overexpression of Grx1-c-myc$_2$ (clones 2 and 3) was achieved by culturing the cells overnight at 37 ^{o}C in medium containing 100 ng/ml tet. The Western blot analyses with the anti-Grx1 antibodies (1:600) showed for both clones a leaky expression of Grx1-c-myc$_2$ (Figure 15). At 39 ^{o}C, the Grx1 KO clone displayed a slight growth advantage compared to WT cells, whereas the KO cells expressing Grx1-c-myc$_2$ proliferated like WT cells (Figures 16A and B). The results confirmed that the growth advantage observed in the Grx1 KO clone was due to the absence of the protein and suggests that Grx1 may play a role in the proliferation control of bloodstream parasites at elevated temperatures. The average multiplication times during different 22 h time periods of continuous growth (diluting to initial cell density after every 22 h) for WT (- tet) and Grx1 KO cells at 39 ^{o}C (from 3 different experiments) are given in Figure 16 C. Immunoflourescence of DAPI-stained WT and Grx1 KO cells grown until 66h at 39 ^{o}C showed that the Grx1 KO cells had more normal cells with 1 kinetoplast and 1 nucleus compared to WT cells. The number of 'multiple cells', those with at least 2 kinetoplast and 2 nuclei were lower in the Grx1 KO cells compared to WT cell as summarized in the table in Figure 16 D as percentages of total populations counted. The number of cells which had lost kinetoplast or with one kinetoplast and 2 nuclei were similar for both cell lines.

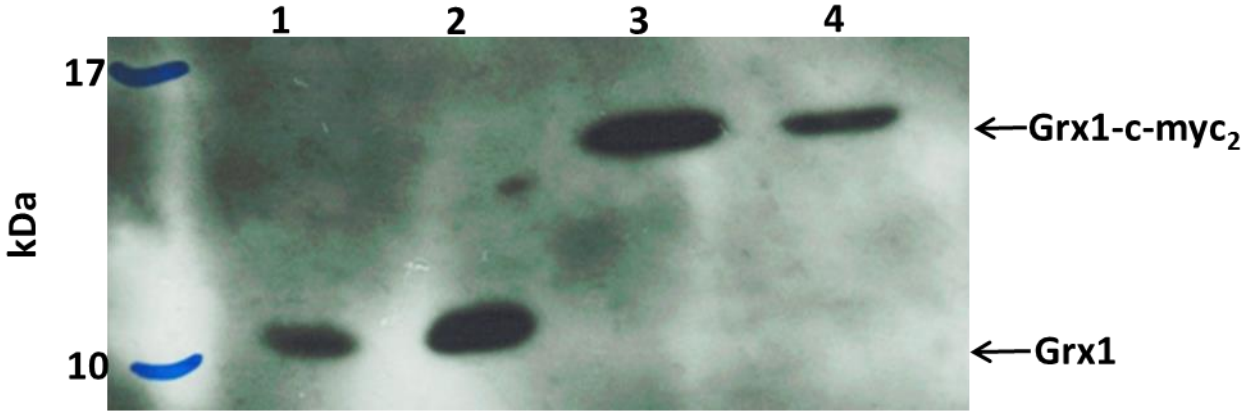
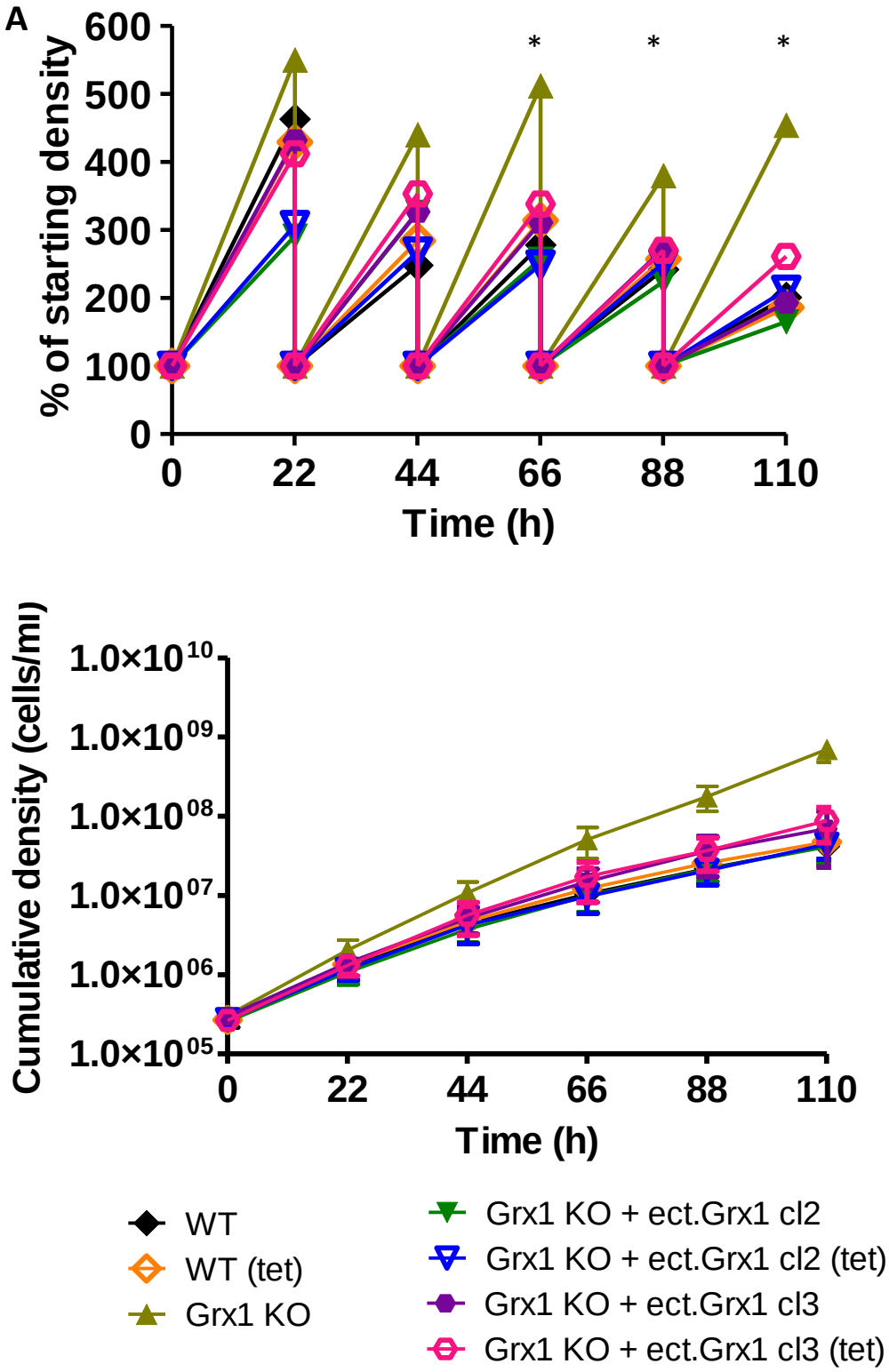
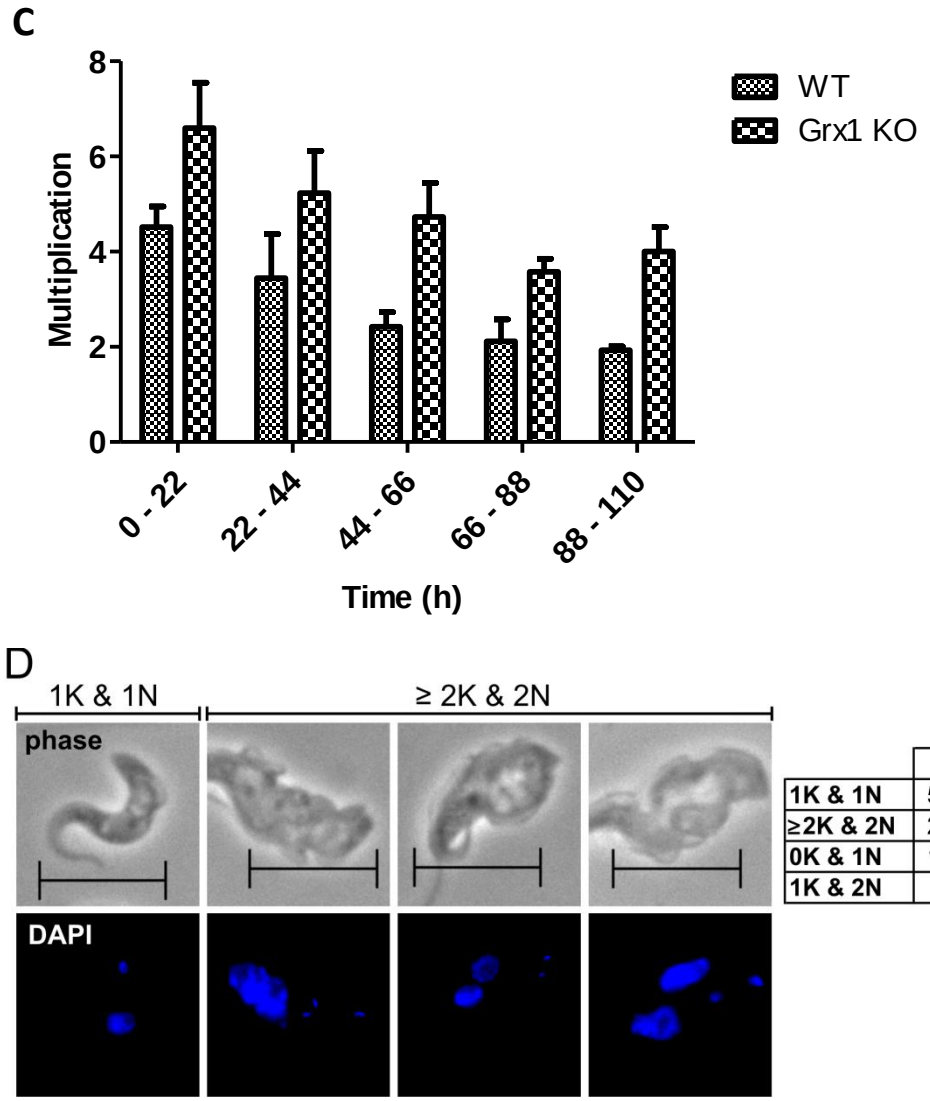


Figure 15. Western blot analyses against Grx1 of bloodstream WT and Grx1 KO cells expressing an ectopic copy of Grx1-c-myc$_2$. Overexpression of Grx1-c-myc$_2$ was achieved by culturing the cells overnight in medium containing 100 ng/ml tet, but without hygromycin. Uninduced cells were grown overnight without any selecting antibiotics. Cell extracts were loaded on a 14 % SDS gel under reducing conditions and subjected to Western blot analyses using the anti-Grx1 (1:600) antibodies overnight. *lane 1*, 4 x 10^6 WT cells. *lane 2*, 1 x 10^7 WT cells. *lane 3,* 4 x 10^6 Grx1 KO cells (clone 2) harboring an ectopic copy of Grx1-c-myc$_2$

(without tet). *lane 4,* 1 x 10^7 Grx1 KO cells (clone 3) harboring an ectopic copy of Grx1-c-myc_2 (without tet).





	WT	Grx1 KO
1K & 1N	50.8 ± 3.8	64.2 ± 3.4
≥2K & 2N	27.2 ± 2.2	18.1 ± 4.4
0K & 1N	14.7 ± 2.3	12.5 ± 3.3
1K & 2N	7.2 ± 1.9	5.1 ± 1.5

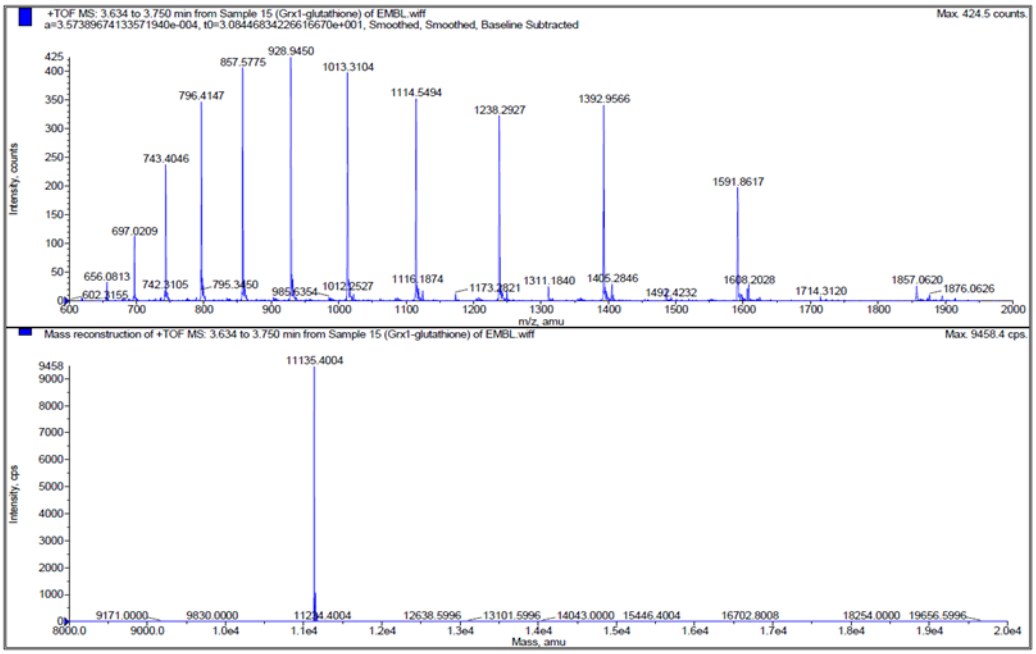
Figure 16. Proliferation of bloodstream cell lines at 39 °C. WT, Grx1 KO and Grx1 KO clones expressing Grx1-c-myc$_2$ (+/- 100 ng/ml tet) were cultured at 39 °C. WT cells were also cultured in presence of tet as control. The cells had an initial density of 2 x 10^5 cells/ml in 5 ml of 39 °C pre-warmed HMI-9 medium. After every 22 h, living cells were counted and the culture was diluted to the initial cell density. (A) A representative graph showing the proliferation at 39 °C for the different cell lines as percentage of starting density. There was a significant difference in the proliferation between WT and Grx1 KO cells from time point 66 to 110 h, indicated by * (p value < 0.05, calculated using Microsoft Excel student's unpaired t-test with equal variance). (B) Cumulative cell density for the different cell lines up to time point 110 h. (C) Average multiplication time of WT and Grx1 KO cells at the 22 h intervals indicated. The results are the mean ± standard deviation of three independent experiments. (D) Immunofluorescence analysis WT and Grx1 KO cells grown at 39 °C. WT and Grx1 KO cells grown at 39 °C were harvested at time point 66 h for immunofluorescence analysis using DAPI stain to visualize kinetoplast (K) (with smaller dots) and nucleus (N) with (large dots), shown on lower panel. The phase contrast pictures are shown on the upper panel (scale bar:10 µm). Analysis of the different phenotypes observed as percentages are

summarized in the table (right). At least 200 parasites were analyzed for each cell line and the results are the mean ± standard deviation of three independent experiments.

3.7 Cys78 of Grx1 is susceptible to *in vitro* glutathionylation

Reacting stored recombinant *T. brucei* Grx1 with Ellman's reagent yielded a protein to thiol ratio of 1:1. Thus only one cysteine reacted per protein molecule most probably because the active site thiols become oxidized upon storage. Incubation of the Grx1 sample with GSH and diamide resulted in a protein species without any free thiol. Treated and untreated Grx1 samples were subjected to ESI-MS analyses. The GSH/diamide treated Grx1 revealed a total mass of 11.135 kDa in comparison to 10.831 kDa for the untreated control (Figures 17 A and B). The mass increase of 304 Da is consistent with the addition of one GSH molecule. Most probably the glutathionylated residue is Cys78. Even if the active site disulfide became transiently reduced by GSH and Cys21 glutathionylated upon GSH/diamide treatment, the adjacent Cys24 should immediately displace the GSH to regenerate the intramolecular disulfide (Beer et al., 2004). This third cysteine is conserved in the Grx1 sequences of *Leishmania major* (Ceylan et al., 2010), human and *T. vivax* (TvY486_1101340) (Figure 18) as well as *E. coli* Grx3 which may suggest a putative regulatory function in the parasite oxidoreductases.

A



B

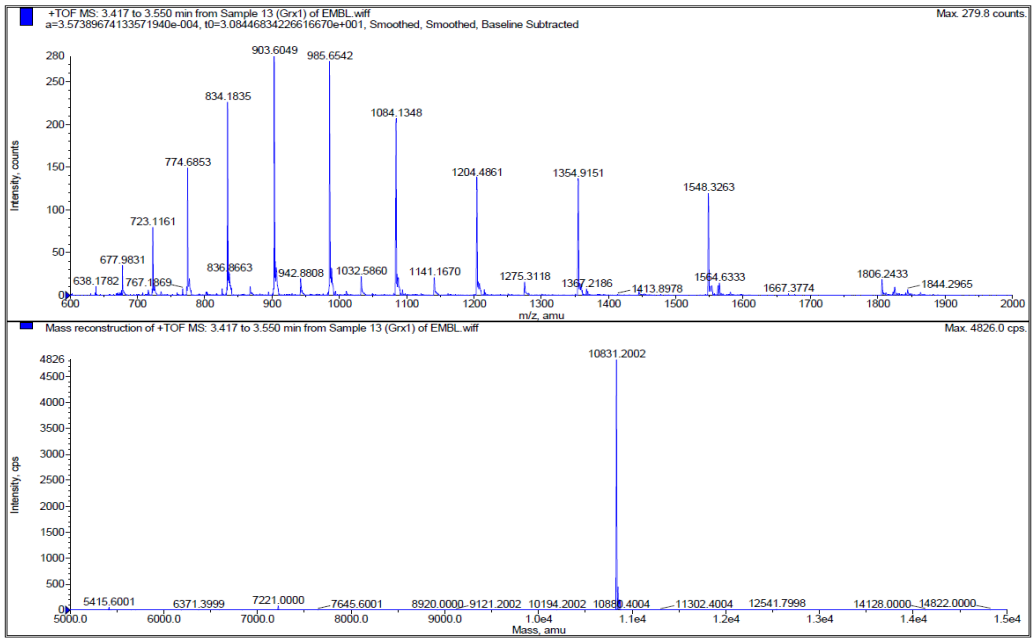


Figure 17. *In vitro* glutathionylation of Grx1. 100 µM Grx1 was incubated with 17.5 mM GSH and 13 mM diamide for 2 h at 25 ^{o}C. After removal of excess diamide and GSH, the Grx1 samples were subjected to ESI-MS analyses (Thomas Ruppert, ZMBH). (A) The GSH/diamide treated protein revealed a total mass of 11.135 kDa in comparison to 10.831 kDa of unmodified Grx1 (B). The mass increase by 304 Da is consistent with the addition of one GSH molecule, most probably at Cys78.

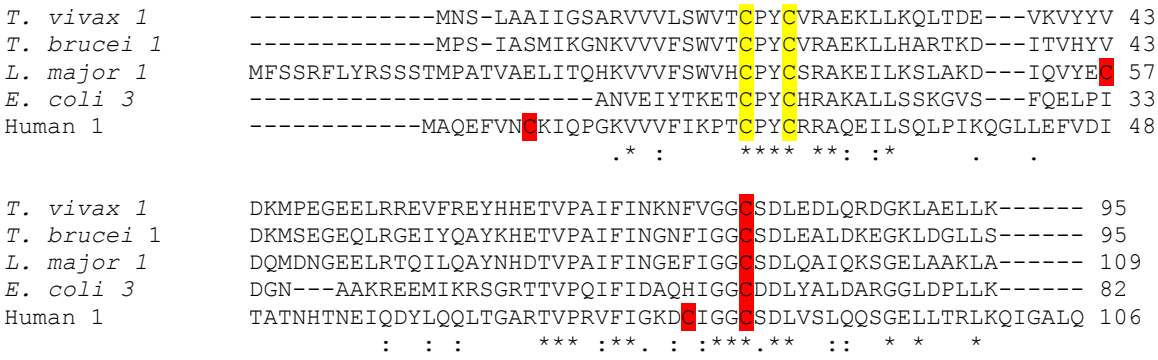


Figure 18. Comparison of *T. brucei* glutaredoxin 1 with Grxs of other organisms. *T. brucei* has two cysteines in the active site (highlighted by a yellow background) and an additional one at position 78 (highlighted in red) which is conserved in glutaredoxins of *T. vivax* (*Trypanosoma vivax,*TvY486_1101340), *L. major* (*Leishmania major, LmjF.27.0810*), *E. coli* (Aslund et al., 1996)and human (sp|P35754|GLRX1_HUMAN Glutaredoxin-1)

3.8 Glutathionylated Grx1 had a diminished reductase activity

The effect of gluathionylation of non-active site of Grx1 on reducing glutathione-protein mixed disulfides compared to unmodified Grx1 was investigated using the HED assay (see section 2.2.19 for method). Results showed that the glut-Grx1 had reductase activity that was three-fold lower compared to unmodified Grx1. However glut-Grx1 had a lower K_m value which resulted in the same k_{cat}/K_m values (Table 7).

Table 7. Reduction of mixed disulfide between β-mercaptoethanol and GSH by Grx1 or glut-Grx1

Protein	V_{max} (U/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Grx1	4.7 ± 0.1	53 ± 5	0.84 ± 0.02	(1.6 ± 0.1) x 10^4
glut-Grx1	1.6 ± 0.1	13 ± 3	0.30 ± 0.01	(2.3 ± 0.2) x 10^4

3.9 Grx2 is essential in procyclic cells and has got a redox function

3.9.1 Only a single *grx2* allele could be deleted in procyclic cells

Using the strategy depicted in Figure 7, *grx2* SKO cells could be obtained in procyclic cells. However, an attempt to replace the second allele in the SKO cells resulted in a double-resistant clone that still kept a *grx2* allele as shown by both PCR and Western blot analysis (Figure 19A and B). Western blot analysis revealed that the amount of Grx2 expressed in the *grx2* SKO clone was lower as compared to WT cells (Figure 19B). To get an insight if the diminished Grx2 have an effect on the proliferation, the proliferation of *grx2* SKO clone was compared to wildtype cells. The *grx2* SKO clone displayed a slight proliferation defect as compared to WT cells (Figure 19C) in accordance to with the previous data obtained by Ceylan et al., 2010 showing that RNAi against Grx2 results in a proliferation defect. However, remarkably, after several months in culture, the proliferation defect was lost (not shown) and Western blot analysis showed that the *grx2* SKO clone had a Grx2 level comparable to that in WT cells (Figure 19D).

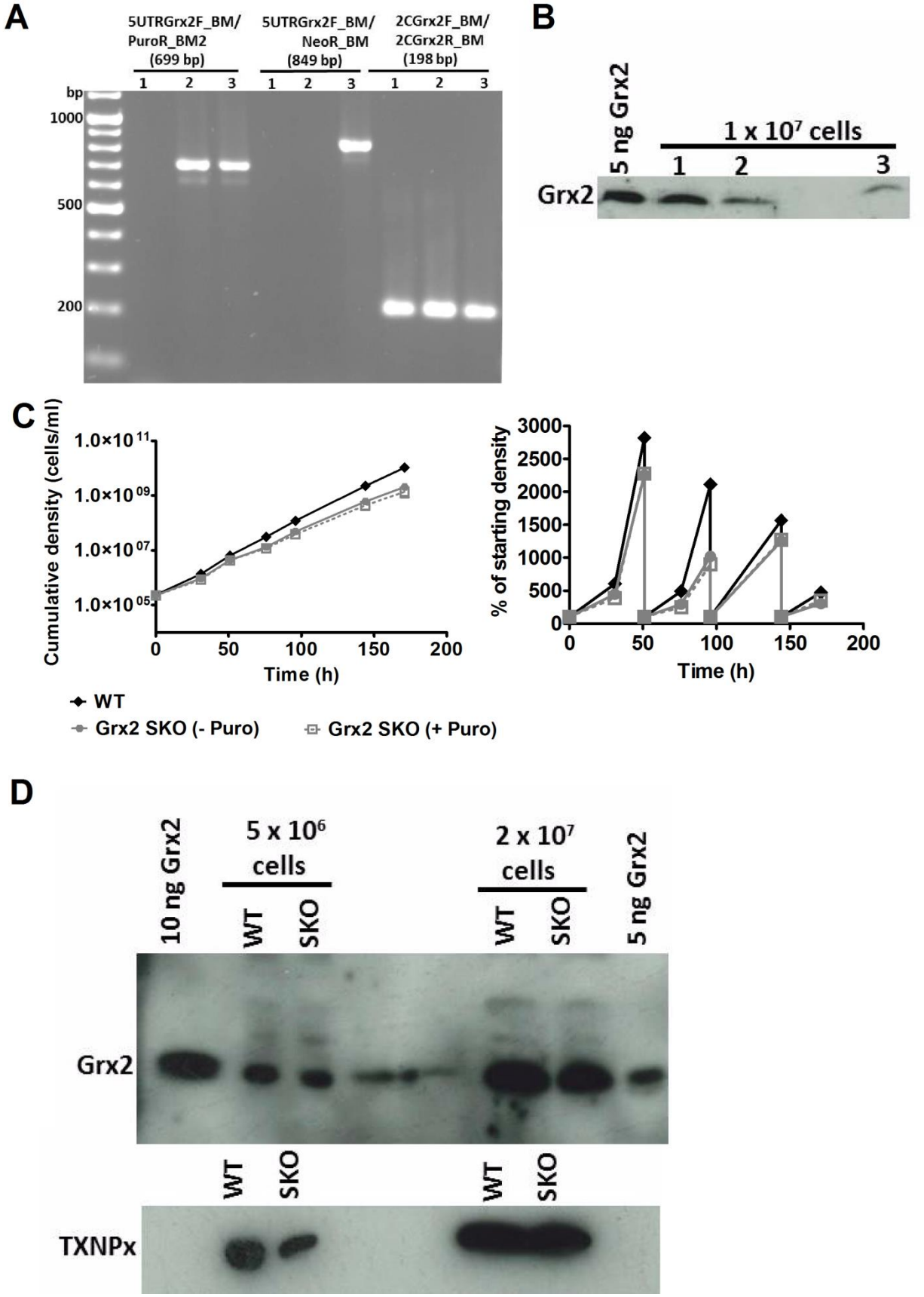


Figure 19. Deletion of both *grx2* alleles was not possible in procyclic cells. (A) PCR analyses using the indicated primer combinations with the expected sizes in brackets confirmed the

insertion of the two resistance genes at the correct positions. However, the double-resistant cells still displayed a *grx2* gene. 1,DNA from WT, 2, a SKO clone, and 3, a double-resistant clone. (B) Total lysates from procyclic cells revealed a decreased protein level in the *grx2* SKO cells (lane 2) as compared to WT cells (lane 1).The double-resistant cell line expressed Grx2 (lane 3). Total lysates from 1 x 10^7 cells were loaded per lane and subjected to SDS-PAGE on a 14 % gel followed by Western blot analyses using anti-Grx2 antibodies (1:200). (C) WT and Grx2 SKO cells (+/- puromycin) with an initial cell density of 2 x 10^5 cells/ml were grown in MEM-Pros at 27 ^{o}C and diluted to initial cell density at time points 51 h, 96 h and 144 h. *left*, The cumulative cell density shows a slight proliferation defect in the SKO cells compared to WT cells. The results are the means ± standard deviation of three different experiments. *right*, A representative graph showing the proliferation of the WT and SKO cell lines as a percentage to starting cell density. (D) After a few months in culture, the procyclic *grx2* SKO cells expressed Grx2 at nearly WT levels. Total lysates from 5 x 10^6 or 2 x 10^7 WT and SKO cells were loaded on a 14 % gel and subjected to Western blot analysis with the anti-Grx2 antibodies (1:200). The membrane was re-probed for TXNPx (1:3000) which served as a loading control.

3.9.2 Both *grx2* alleles could be deleted in procyclic cells expressing an ectopic copy of Grx2 or Grx2C34S

In the presence of tetracycline to express the ectopic copy of Grx2, procyclic clones were obtained that had both the puromycin and neomycin resistance genes in the correct locus as shown by PCR analysis (Figure 21A). Internal primers for *grx2* could not be used to confirm the deletion of the *grx2* alleles, since they would also amplify the *grx2* from the ectopic copy. Thus, primers were designed that in combination with *grx2*-specific primers would amplify the authentic gene, but not the ectopic *grx2* (depicted in Figure 20). The 5UTRGrx2-2F_BM primer binds 5 bp before the *grx2* coding region and the 3UTRGrx2_2R_BM binds 13 bp after the stop codon. PCR analyses with the primer combinations 2CGrx2F_BM /3UTRGrx2_2R_BM and 5UTRGrx2-2F_BM /2CGrx2R_BM showed amplicons for the WT cells, but not for the inducible KO cells (Figure 21B).The obtained clones were then cultured in the presence or absence of tetracycline. Surprisingly, the inducible *grx2* KO clones survived even in medium lacking tetracycline. Western blot analysis revealed expression of protein.This indicates that the inducible expression system became leaky after tet removal in cells lacking the authentic *grx2* alleles (Figure 22).The inducible system was perfectly tight in the SKO background (not shown).

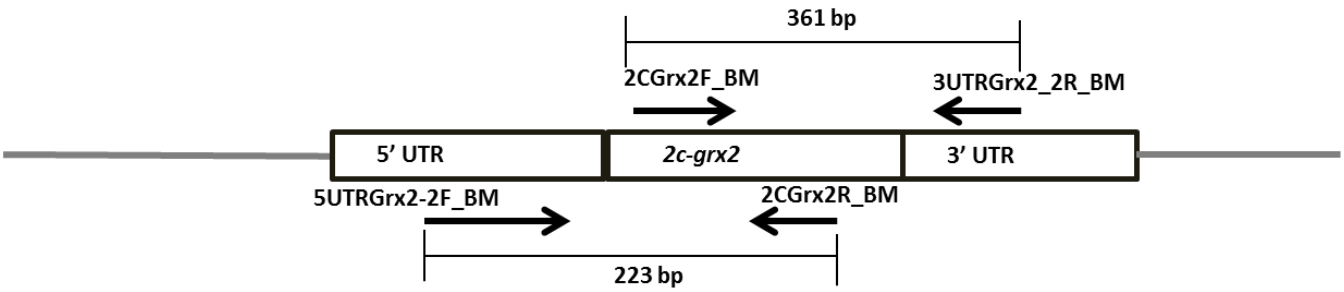


Figure 20. Scheme for the primers specific for authentic *grx2*. To confirm the deletion of the authentic *grx2* alleles in the inducible procyclic Grx2 KO cells, two new sets of primers were used. The primer pairs result in an amplicon for the authentic gene, but not for the ectopic copy which does not contain any parts of either the 5'- or 3'-untranslated regions.

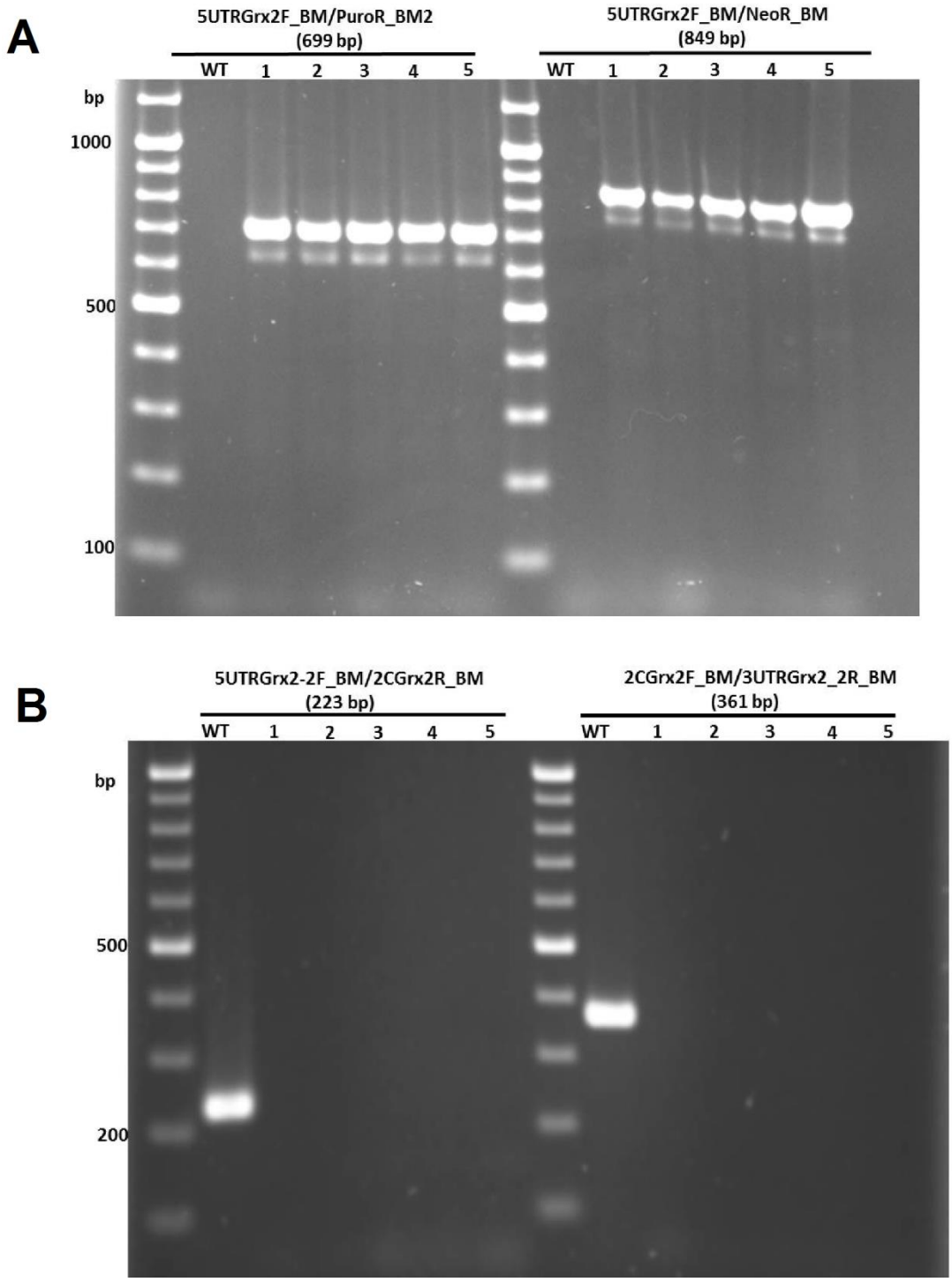


Figure 21. PCR analyses of the inducible *grx2*-deficient procyclic cell lines expressing an ectopic copy of wildtype Grx2. (A) PCR analyses using the indicated primer combinations with the expected sizes in brackets confirmed the insertion of the two resistance genes in the *grx2* locus in the presence of a tet-inducible ectopic copy of wildtype Grx2. The faint lower bands seen are a result of a second binding site of the forward primer. (B) Primer combinations that distinguish the authentic *grx2* gene from ectopic *grx2* gene showed that both *grx2* alleles were replaced in the presence of an ectopic copy of wildtype Grx2. WT DNA served as control. For the primer sequences and binding sites see Table 2B and Figure 7, respectively.

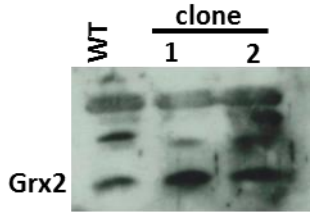


Figure 22. The inducible procyclic Grx2 KO cells express Grx2 independent of tet. Procyclic WT and two Grx2 KO clones harboring an ectopic copy of the *grx2* gene were transferred into MEM-Pros without tetracycline. After one week, cells were harvested and total lysates of 2 x 10^7 cells were loaded on a 14 % gel and subjected to Western blot analysis using the anti-Grx2 antibodies (1:200).Both Grx2 KO clones (1 and 2) expressed the ectopic Grx2 to at least wildtype-like levels and which explains why these clones could survive without induction.

3.9.3 Grx2 has a redox function in procyclic cells

As it was not possible to control the expression of ectopic Grx2 in the inducible Grx2 KO cells, I investigated if in the procyclic cells Grx2 has a redox function requiring one or both of its active site cysteines at positions 31 and 34 (Figure 23).The previously generated *grx2* SKO cells were transfected with constructs to express tet-inducible ectopic copies of either Grx2C34S or Grx2C31S/C34S. Afterwards, I attempted to delete the second *grx2* allele in these cells in the presence of tet. I was successful in deleting both *grx2* alleles in cells expressing an ectopic copy of Grx2C34S as confirmed by PCR (Figures 24 A and B).Western blot analysis showed that the clones also had leaky expression of ectopic Grx2C34S (Figure 25C). The inducible system was perfectly tight in the SKO background (not shown). An attempt to delete both *grx2* alleles in cells expressing an ectopic copy of Grx2C31S/C34S was not successful as the double-resistant clones kept the *grx2* genes. These results showed that Grx2 has a redox function that requires at least the cysteine at position 31.

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T. brucei_Grx2        MNNALDPAKAPQFLDMMLRRNKMVMVSATYCQFCTKLKMLLIELKHRFVSLEIDIIPNGR
T. cruzi_Grx          MNKALDPAKAPQFLDMMLRRNQIVLISATYCEYCTKLKMLLIEMKHRFVSLEINIIPNGR
L. major_Grx2         MNQVLDPARAPQFLDSMLRRNRIVLISATYCQFSTKLKMLLIELKHRFVSLEIDIIPNGR
                      **:.****:****** *****::*::*****::.*********:*********:******

T. brucei_Grx2        EVFAEVVGRTGVHTVPQVFLNGKYFGGYDELVAMYRAGHLSAEIERG
T. cruzi_Grx          EVFAEVVGRTGVHTVPQMFHNGKYLGGYDEIVALYRRGELSATLERR
L. major_Grx2         EVFQEVVARTGVHTVPQVFLNGKYLGGYDDLIALYHKRELSETLEKR
                      *** ***.*********:* ****:****:::*:*:  .**  :*:
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Figure 23. Protein alignments of the Grx2-type proteins from *T. brucei, T. cruzi and Leishmania major.* Grx2 (Tb 927.1.1770) of *T. brucei* has about 80 % similar sequence to that of *T. cruzi* (TcCLB.511431.40) and *L. major* (LmjF.20.1010) (Ceylan et al., 2010). The

active site cysteines are highlighted by a red background. Interestingly, the *L. major* Grx2 has a monothiol active-site unlike those of *T . brucei* and *T. cruzi* which have two active-site cysteines.

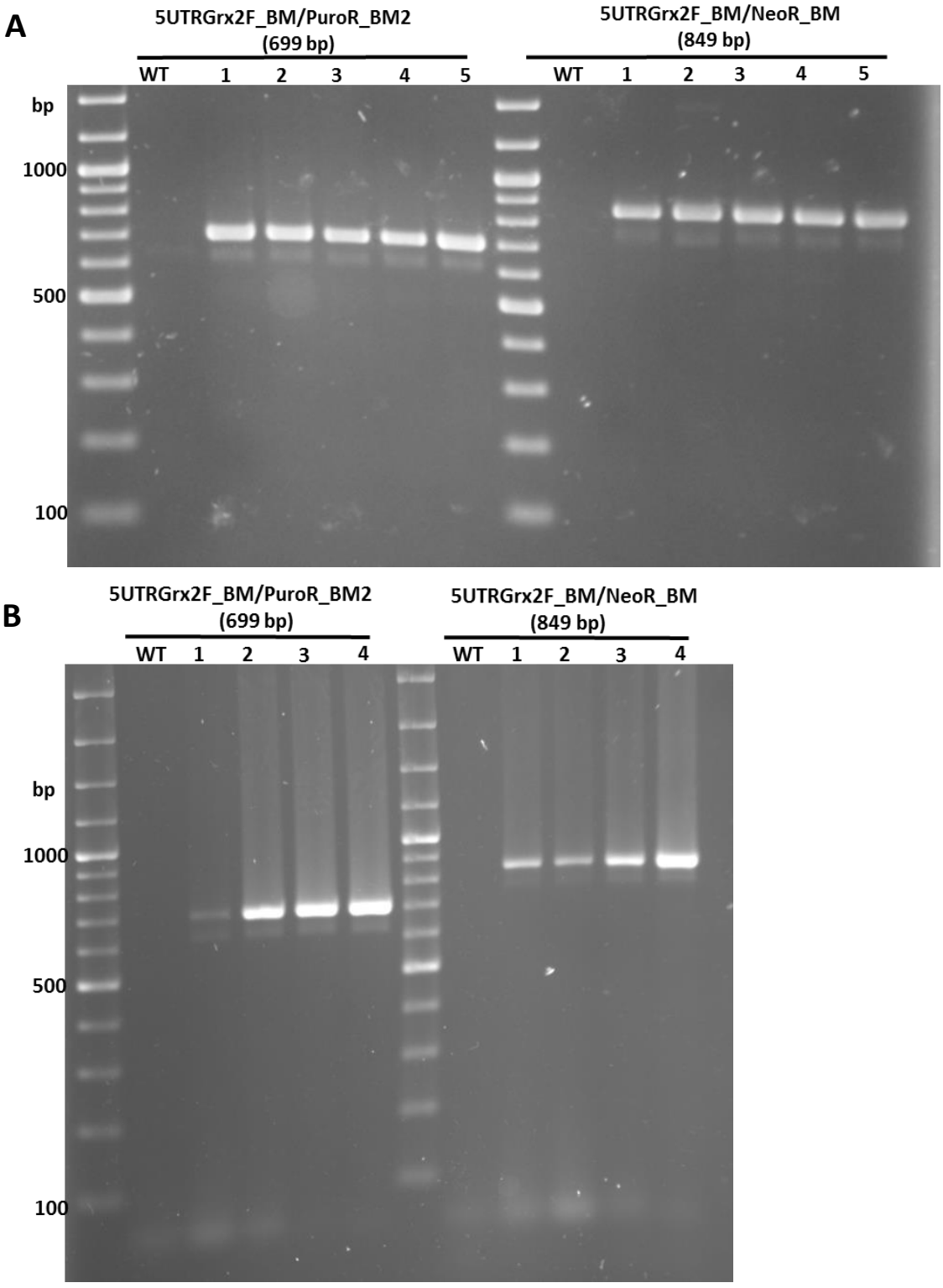


Figure 24. PCR analyses of the inducible *grx2*-deficient procyclic cell lines expressing an ectopic copy of Grx2 C34S mutant. (A) PCR analyses using the indicated primer combinations with the expected sizes in brackets confirmed the insertion of the two

resistance genes in the *grx2* locus in the presence of a tet-inducible ectopic copy of Grx2C34S mutant. (B) Primer combinations that distinguish the authentic *grx2* gene from ectopic *grx2* gene showed that both *grx2* alleles were replaced in the presence of an ectopic copy of Grx2 C34S mutant. WT DNA served as control.

3.9.4 Procyclic Grx2 KO clones that express an ectopic copy of Grx2C34S mutant had a slight proliferation defect

When grown in MEM-Pros medium at 27 ^{o}C, WT cells showed the same proliferation as the Grx2 KO clones expressing a wildtype ectopic copy of Grx2 (clone D1 (1:2)) both in the absence or presence of tet. In comparison, three different Grx2 KO clones that expressed the Grx2C34S mutant (clone 1 - B5(1:2), clone 2 - C6(1:4) and clone 3- D2(1:20)) had a slight proliferation defect as compared to wildtype cells (Figure 25 A). The doubling times for each type of cell lines were very similar in the presence or absence of tet, so I averaged the doubling times obtained +/- tet each obtained from three different experiments (Figure 25B). Western blot analyses revealed that all the inducible cell lines expressing the ectopic copies of Grx2 (either wildtype or mutant) had leaky protein expression that was at least of wildtype-like levels (Figure 25C). Overexpression of the ectopic Grx2 proteins (wildtype or mutants) by tet-induction in the procyclic Grx2 KO clones did not affect the proliferation.

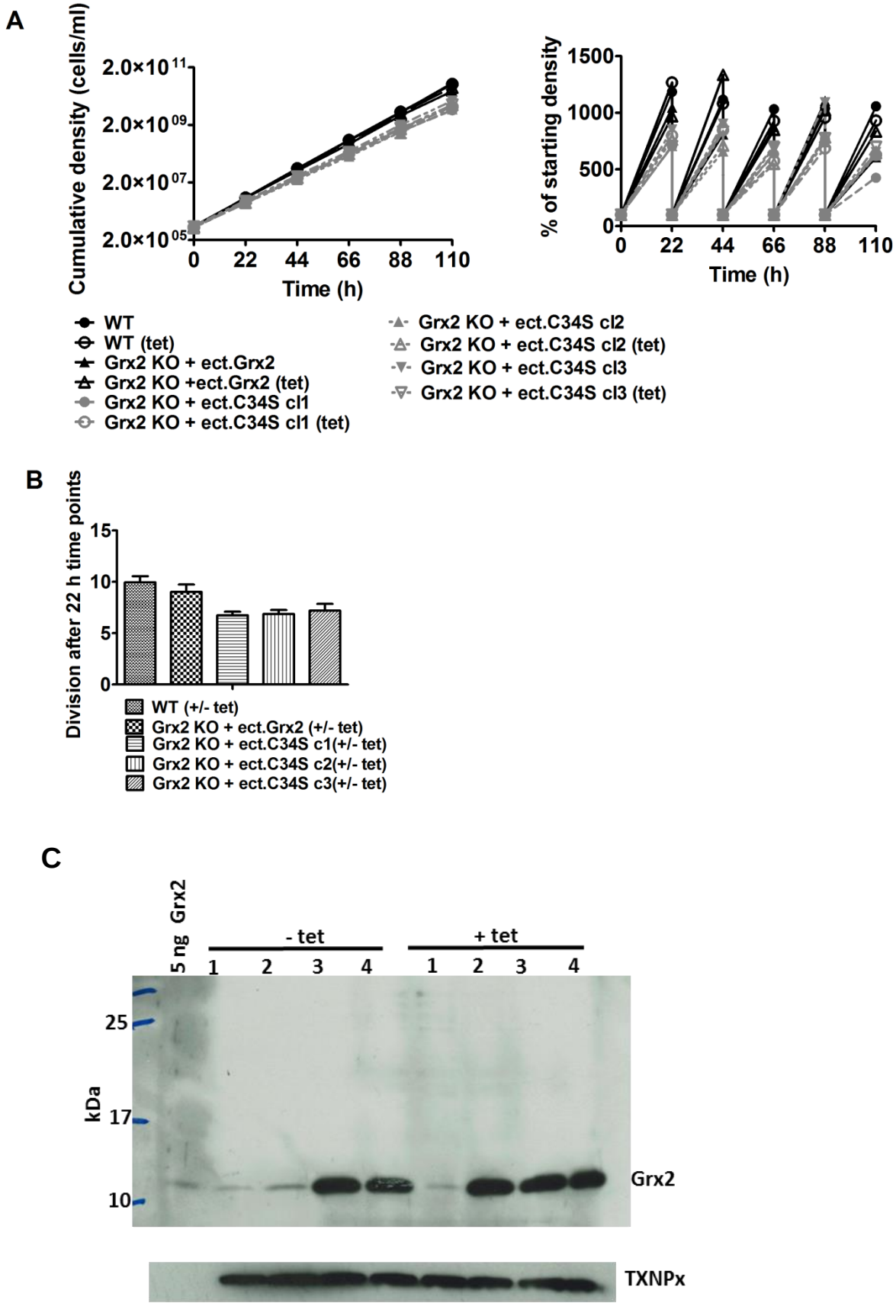


Figure 25. **Procyclic Grx2 KO clones expressing an ectopic copy of Grx2C34S had a slight growth defect as compared to wildtype cells or Grx2 KO cells expressing wildtype Grx2.** (A) Wildtype, Grx2 KO cells expressing ectopic copies of either Grx2 or Grx2C34S mutant ectopic

copies (+/- tet) with an initial density of 5 x 10^5 cells/ml were grown in MEM-Pros medium at 27 ^oC and diluted to the initial cell density after every 22 h. *left*, Cumulative cell density for the different cell lines up to time point 110 h. The values are the mean ± standard deviation of three independent experiments. *right*, A representative graph showing the proliferation of the different cell lines as a percentage to starting cell density. (B) The average doubling times for the different cell lines were calculated after every 22 h in each experiment. The doubling times for each cell line (+/- tet) were very similar and thus summarized together in the analysis. The results are the means ± standard deviation of three different experiments. (C) Aliquots were harvested for Western blot analysis after dilution to initial density at every time. Shown here is a representative blot with extracts from 1.4 x 10^7 cells from experiment 3 (time point 88 h) loaded on a 14 % SDS gel and subjected to Western blot analysis using the anti-Grx2 antibodies (1:200). 1 – WT cells, 2 and 3 - Grx2 KO cells expressing the ectopic Grx2C34S mutant (clone 2 and 3, respectively), 4 - Grx2 KO cells expressing an ectopic copy of wildtype Grx2. All cell lines were grown in the absence or presence of 100 ng/ml tet. The membrane was re-probed for TXNPx which served as a loading control.

3.9.5 Recombinant C34S Grx2 lacks protein disulfide reductase activity

The Grx2 KO cells harboring an ectopic copy of Grx2C34S were able to proliferate. I therefore investigated the possible role of Grx2C34S to reduce protein disulfides. Recombinant Grx2C31S/C34S and Tpx protein served as negative and positive controls, respectively. For method see section 2.2.19.3. 0.9 µM Tpx rapidly reduced insulin as expected (Ceylan et al., 2010). Amongst the Grxs species, Grx2 showed the highest activity. Surprisingly, Grx2C31S/C34S displayed also some activity, but the Grx2C34S mutant did not have any activity (Figure 26 A). The results indicate that both Grx2 and Grx2C34S might not play a role in the reduction of protein disulfides in the parasites as Grx2 KO parasites expressing an ectopic copy of Grx2C34S are fully viable. The activity of the Grx2C31S/C34S might have been caused by a contamination, most probably from some a small amount of thioredoxin which is part of the fusion protein that is then removed by TEV digest. To rule out that the protein samples which were purified at the same time were mixed up, I confirmed their identity by AMS labeling.

Labelling of one cysteine residue of a protein with AMS causes a mobility shift 500 Da compared to the unmodified protein. Silver staining results had previously revealed that both cysteines of Grx2 can be labeled (not shown). Thus, a reduced Grx2 should result in a mobility shift of 1000 Da in contrast to

Grx2C34S which should only result in a mobility shift of 500 Da and Grx2C31S/C34S should not show any shift as it does not contain any cysteine. Results shown in Figure 26B confirmed the identity of the three recombinant proteins used in the insulin assay. Recombinant Grx2C31S/C34S was not stable as it precipitated in the tube. This might also be the reason why some activity was displayed by this recombinant protein in the assay.

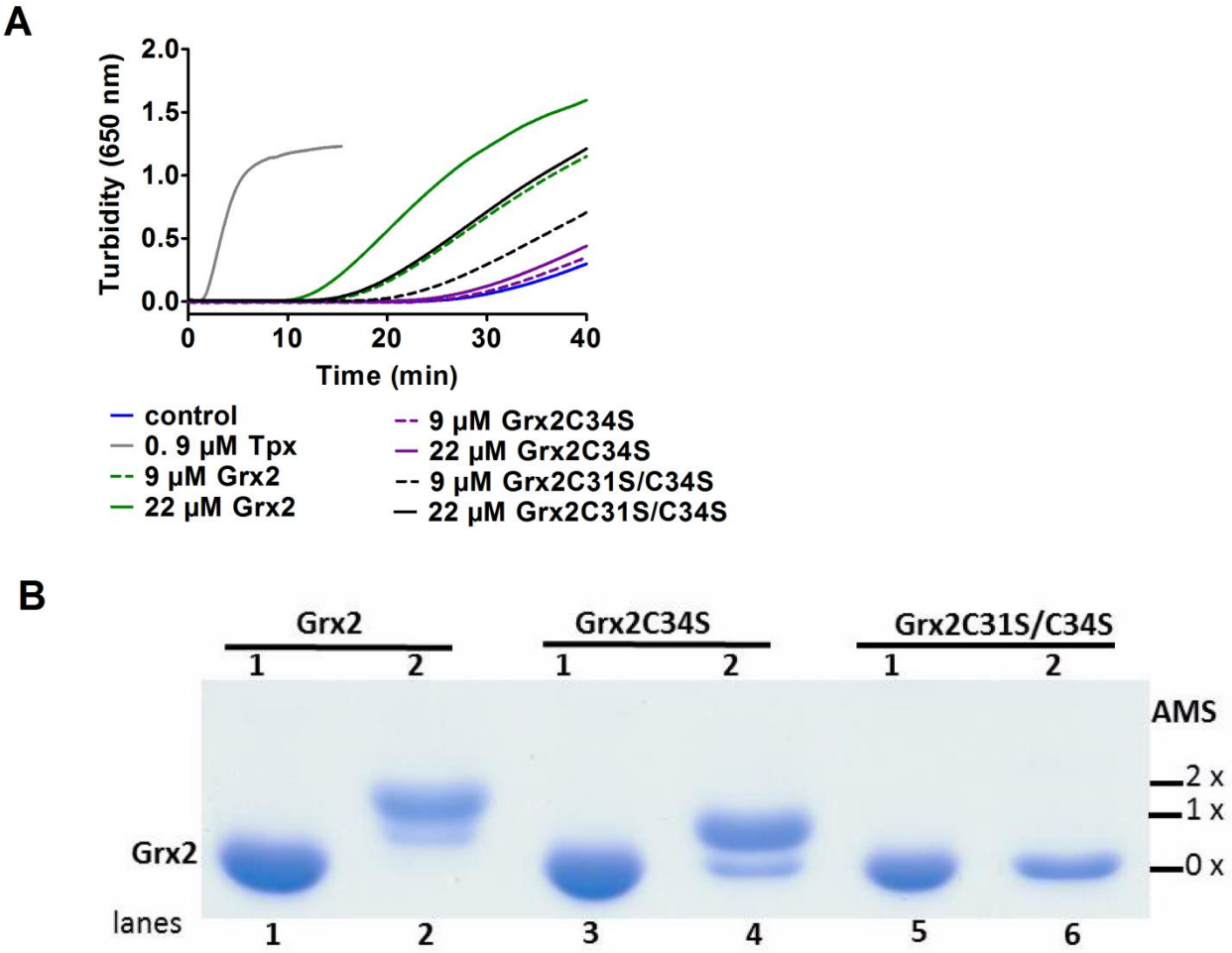


Figure 26. (A) **Reduction of insulin by recombinant Grx2, Grx2C34S and Grx2C31S/C34S.** The reduction of insulin was followed by measuring the turbidity at 650 nm. In a total volume of 800 µl (100 mM potassium phosphate, 2 mM EDTA, pH 7.0), the reaction mixtures contained no putative reductase (control), 0.9 µM Tpx, 9 or 22 µM of Grx2, Grx2C34S or Grx2C31S/C34S, 625 µM DTT and 131 µM insulin. The increase in turbidity was followed at 650 nm at 30 ^{o}C. The graph shown here is a representative of the experiment performed three times.(B) **Reaction of recombinant Grx2 species with AMS.** 120 µg of Grx2, Grx2C34S and Grx2C31S/C34S were treated with 10 mM DTT and then reacted with 30 mM AMS as outlined under Materials and Methods (section 2.2.17). 5 µg of each sample was loaded onto a 16 % gel under reducing conditions and subjected to SDS-PAGE and subsequently visualized by coomasie-staining. 1- untreated controls, 2- samples treated with DTT and AMS. Labelling of maximum of 2 or 1 cysteines by AMS confirmed the identity

of Grx2 and Grx2C34S, respectively. There was no mobility shift in the sample treated with AMS (lane 6) compared to untreated sample (lane 5), confirming the identity of Grx2C31S/C34S. The lower bands in lanes 2 and 4 were due to incomplete labeling of the cysteines by AMS.

3.9.6 Grx2 and Grx2C34S have deglutathionylation activity

Protein deglutathionylation catalyzed by Grxs proceeds via a monothiol mechanism involving the N-terminal cysteine of the active site (Lillig et al., 2008). Since the Grx2 KO procyclic cells proliferated if they expressed an ectopic copy of either Grx2 or the Grx2C34S mutant, I compared the deglutathionylation activity of recombinant Grx2 and Grx2C34S in the HED assay. Recombinant Grx2C34S catalyzed the reaction with 35 % of the wildtype Grx2 activity. The double cysteine mutant, Grx2C31S/C34S, did not have any deglutathionylation activity (Figure 27). Thus, Grx2 and Grx2C34S might be involved in deglutathionylation reactions in the procyclic cells.

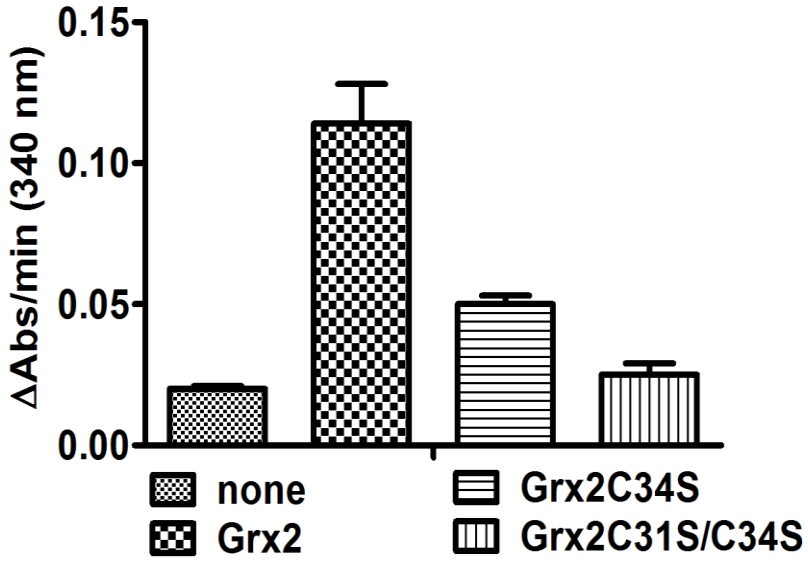


Figure 27.The activities of recombinant Grx2 species in the HEDS assay. In a total volume of 1 ml of 100 mM potassium phosphate, 1 mM EDTA, pH 7.0, 200 µM NADPH, 1 mM GSH, 1 U of human GR and 2.5 mM HEDS (2-hydroxyethyl disulfide) were incubated for 3 min at 25 ^{o}C. The reaction was started by addition of 400 nM of Grx2, Grx2C34S or Grx2C31S/C34S and NADPH consumption was measured at 340 nm. None is the background activity without any Grx. Grx2C34S had about 35 % activity compared to wildtype Grx2. Grx2C31S/C34S displayed an activity similar to the background (none). The data are the mean ± standard deviation of three independent experiments.

3.10 Both *grx2* alleles could be knocked out in bloodstream cells

Using the strategy depicted in Figure 7, *grx2* SKO of bloodstream parasites were stepwise obtained. SKO clones from the first transfection were then used to generate *grx2* KO cell lines by transfecting them with the other deletion cassette. PCR analysis (Figure 28A) confirmed the replacement of both alleles. WT and *grx2* SKO DNA served as controls. The absence of the *grx2* alleles was further confirmed by Western blot analysis (Figure 28B), with WT and Grx2 SKO clone serving as controls.

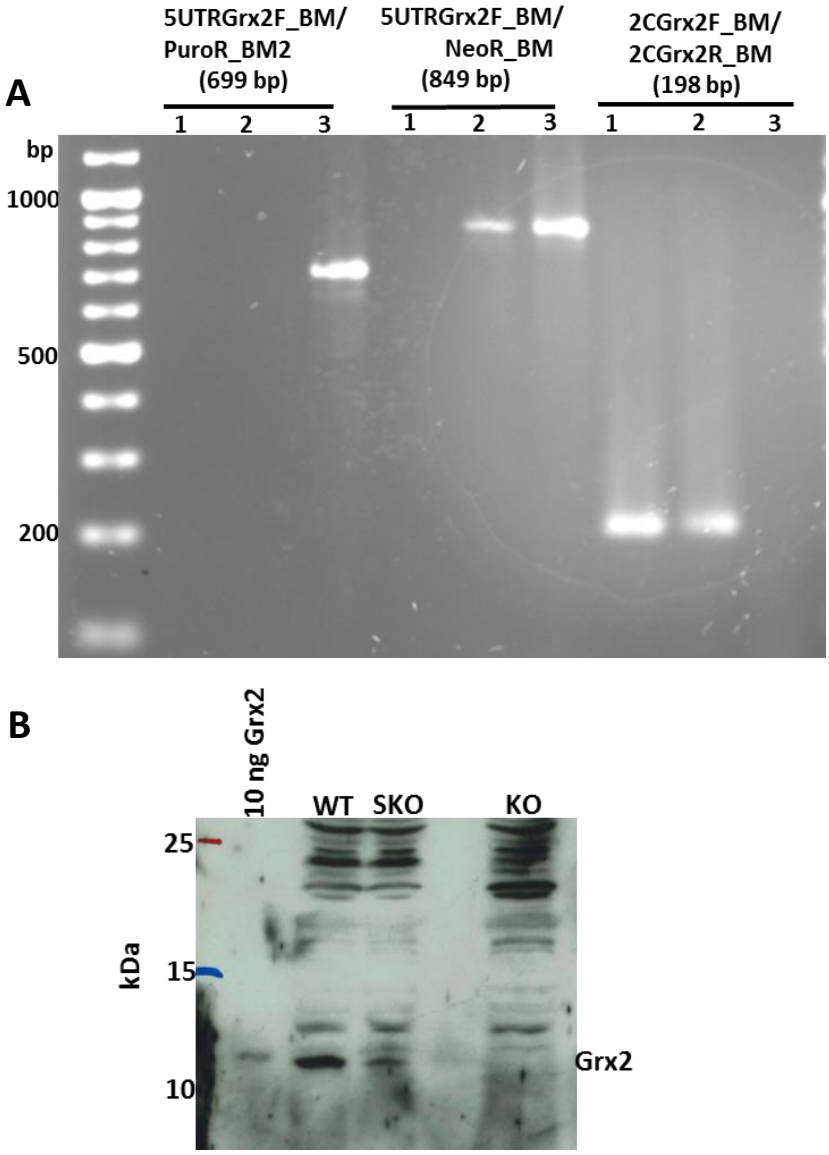


Figure 28. Grx2 deficient bloodstream form *T. brucei*. (A) PCR analyses using the indicated primer combinations with the expected sizes in brackets confirmed the replacement of the *grx2* alleles. 1, 2 and 3 are the PCR products from WT, SKO clone and Grx2 KO clones respectively. (B) Western blot analyses with the anti-Grx2 (1:200) antibodies revealed the presence of the protein in the wildtype and Grx2 SKO cells but not the double-knockout cell line. Total lysates from 2 x 10^7 cells were loaded per lane.

To get an insight if in the *grx2*-deficient cells, Grx1 might be overexpressed, the levels of the cytosolic Grx1 in the Grx2 KO cells was compared with that in wildtype cells. Western blot analysis did not reveal any upregulation of Grx1 in Grx2 KO clone compared to wildtype cells (Figure 29). The membrane was re-probed for TXNPx which served as a loading control.

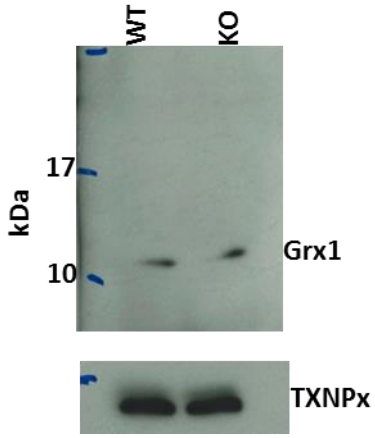


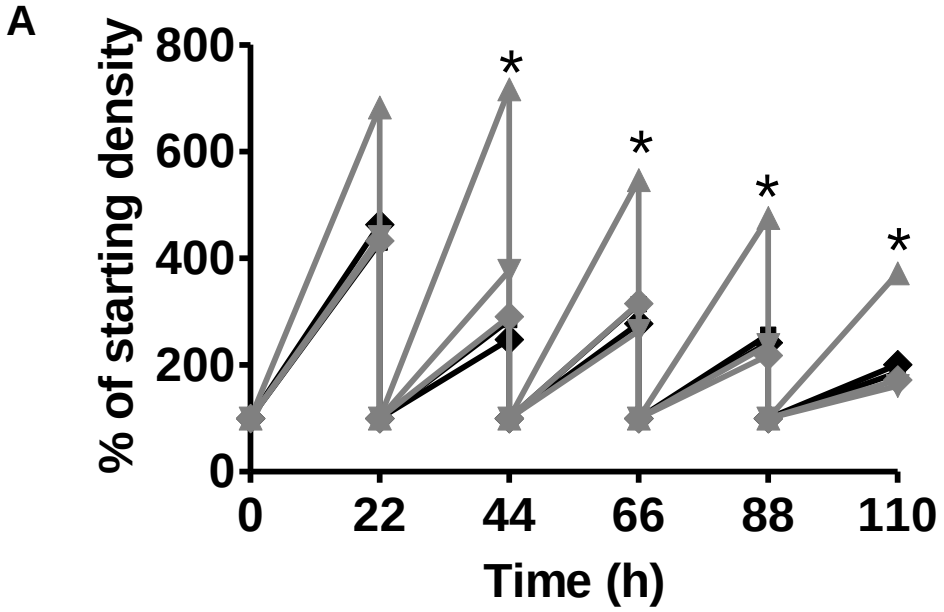
Figure 29. Grx1 expression is not affected in bloodstream Grx2 KO parasites. Total lysates of 1 x 10^7 wildtype and Grx2 KO bloodstream parasites were loaded on a 14 % SDS gel and subjected to Western blot analyses using the anti-Grx1 (1:800) antibodies. The membrane was re-probed for 2-Cys-peroxiredoxin (TXNPx 1:6000) as a loading control. There was no upregulation of Grx1 in Grx2 KO cell line compared to WT bloodstream cells.

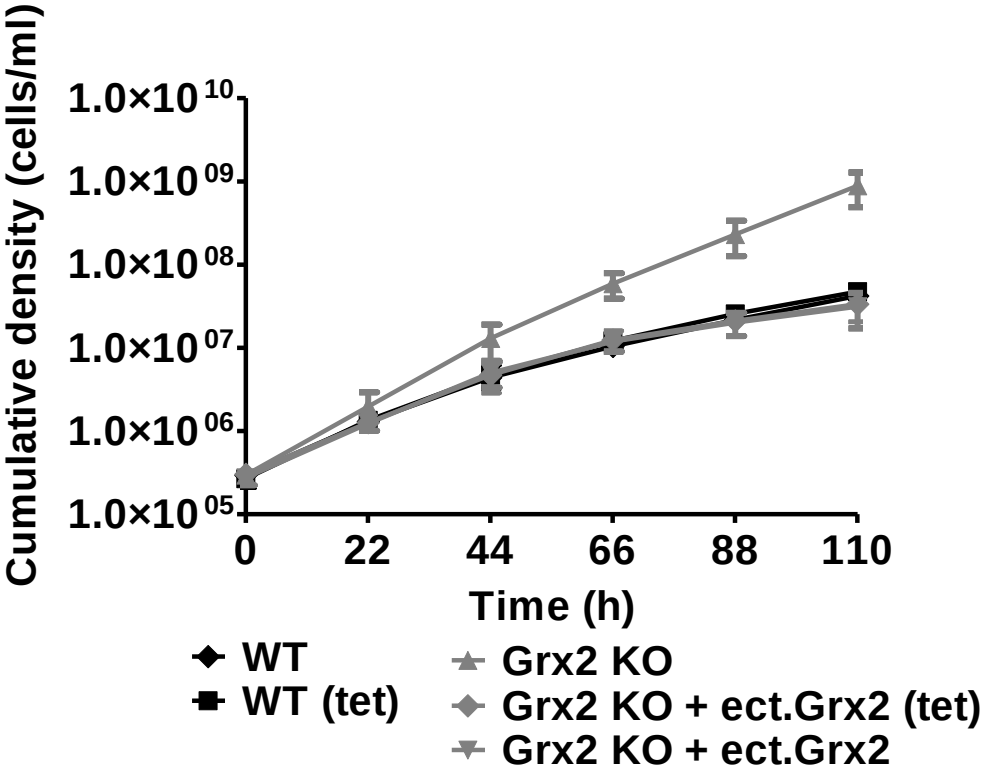
3.11 At 39 ^{o}C, bloodstream Grx2 KO cells proliferate better than the wildtype cells

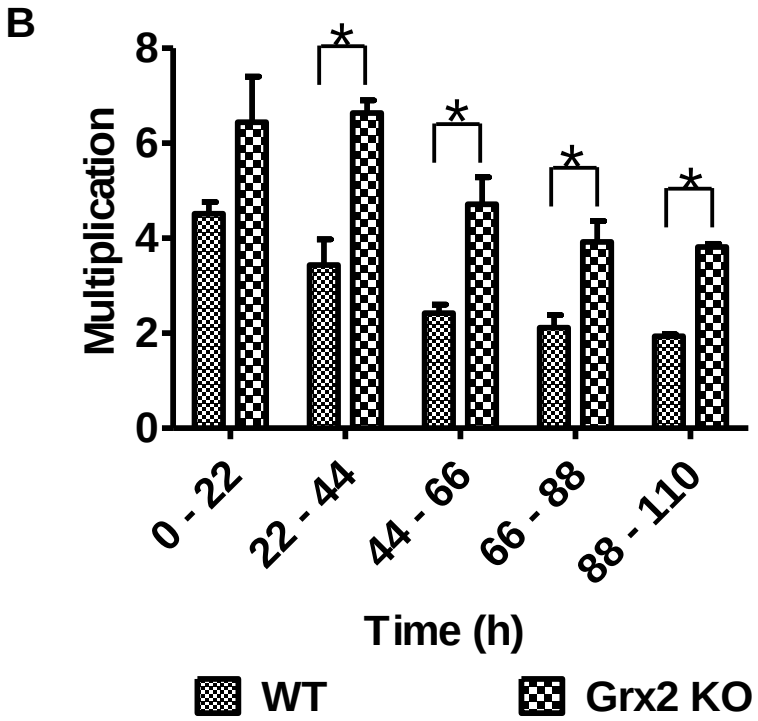
When cultured at 39^{o}C, Grx2 KO had a proliferation advantage compared to WT as was seen in section 3.6 for Grx1 KO cells. However, Grx2 KO cells had the greatest proliferation advantage at 39 ^{o}C (compared to WT and Grx1 KO from time point 44 h onwards).To confirm that the improved proliferation was indeed due to the absence of Grx2, the Grx2 KO cells were transfected with a construct allowing the tet-inducible expression of an ectopic copy of Grx2 and overexpression of Grx2 was achieved using the same procedure as in section 3.6. Grx2 KO cells displayed a significant growth advantage compared to WT cells (between 44 h and 110 h) as mentioned above, whereas the Grx2 KO clone expressing an ectopic copy of Grx2 proliferated like WT cells (Figure 30A). The results confirmed that the growth advantage observed in the Grx2 KO clone was indeed due to the absence of the protein, suggesting that Grx2, like Grx1, may play a role in the proliferation control of bloodstream parasites at elevated temperatures. The average multiplication times during different 22

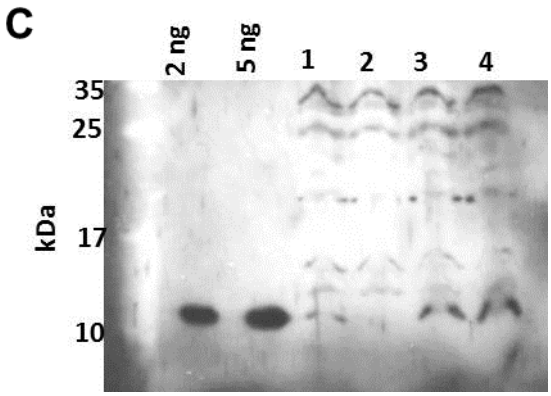
h time periods (as in section 3.6) for WT and Grx2 KO clones (- tet) are shown in Figure 30B. Similar to the phenotypes seen in section 3.6, immunofluorescence results of DAPI-stained WT and Grx2 KO cells grown at 39 ^{o}C till time point 66 h showed that Grx2 KO cells had also more normal cells with one kinetoplast and one nucleus as compared to WT cells. The number of parasites with at least two kinetoplasts and two nuclei were also lower in the Grx2 KO cells compared to WT cells. The number of cells which had lost kinetoplast or with one kinetoplast and two nuclei were similar for both clones. The results as percentages of total population are shown in the table in Figure 30D.The results showed that Grx2 KO clones prevented the formation of cells with least two kinetoplasts and 2 nuclei compared to WT cells when grown at 39 ^{o}C, thereby confirming their better growth at 39 ^{o}C than the WT cells. Compared to results in section 3.6, Grx2 KO cells prevented the formation of the “multiple cells” better than Grx1 KO cells at 39 ^{o}C.

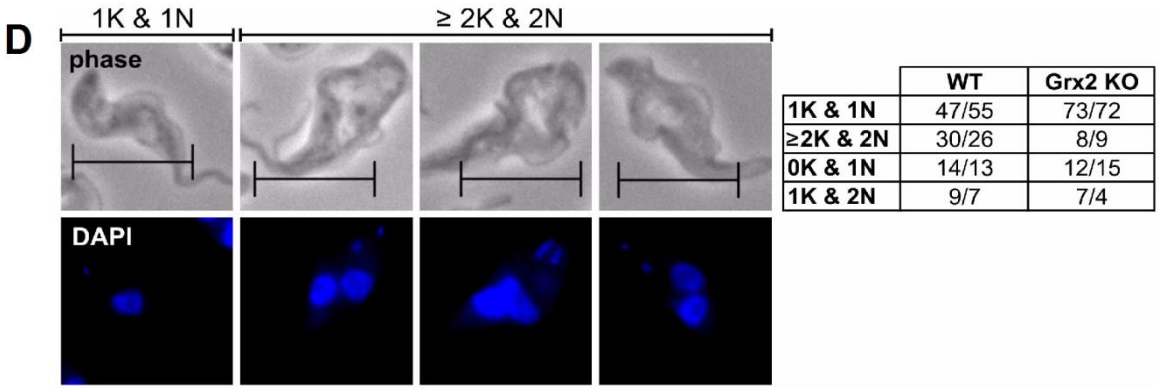
A


% of starting density
800
600
400
200
0
*
*
*
*
0
22
44
66
88
110
Time (h)


Cumulative density (cells/ml)
1.0×10 10
1.0×10 09
1.0×10 08
1.0×10 07
1.0×10 06
1.0×10 05
0
22
44
66
88
110
Time (h)
WT
WT (tet)
Grx2 KO
Grx2 KO + ect.Grx2 (tet)
Grx2 KO + ect.Grx2







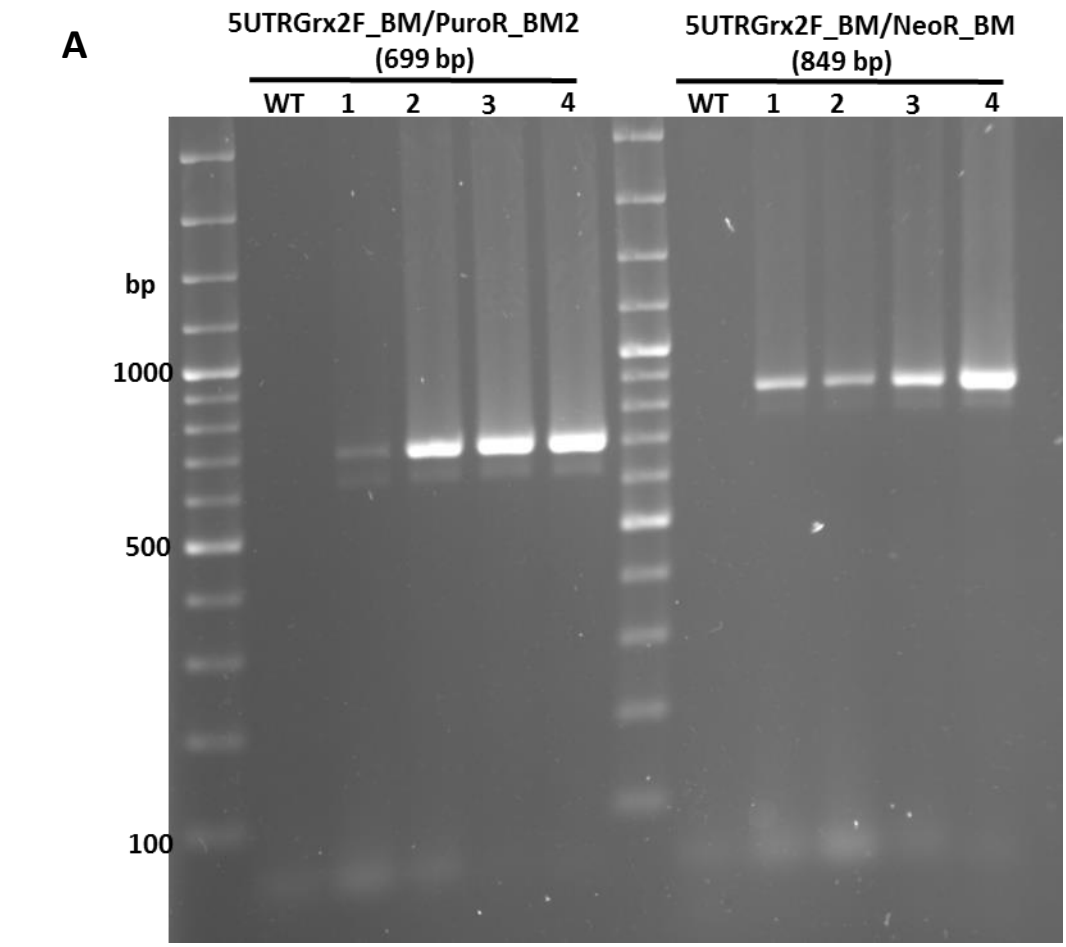
	WT	Grx2 KO
1K & 1N	47/55	73/72
≥2K & 2N	30/26	8/9
0K & 1N	14/13	12/15
1K & 2N	9/7	7/4

Figure 30. Proliferation of bloodstream *T. brucei* cell lines at 39 °C. (A) WT cells as well as a Grx2 KO and Grx2 KO clone expressing Grx2 (+/- 100 ng/ml tet) were cultured at 39 °C. WT

cells were also cultured in presence of tet as control. The cells had an initial density of 2 x 10^5 cells/ml in 5 ml of 39 ^oC pre-warmed HMI-9 medium. After every 22 h, living cells were counted and the culture was diluted to the initial cell density.(A) *top*, representative graph showing the proliferation at 39 ^oC as a percentage to the starting cell density for the different cell lines. There was a significant difference in the proliferation between WT and Grx2 KO cells from time point 44 h onwards, indicated by * (p value < 0.05, calculated using Microsoft Excel student's unpaired t-test with equal variance). *bottom*, Cumulative cell density for the different cell lines. The experiment was done three times. The mean ± standard deviation is depicted. (B) Average multiplication time of WT and Grx2 KO cells at the indicated 22 h intervals.*,time points with a significant difference between the Grx2 KO and WT cells. The results are the mean ± standard deviation of three independent experiments. (C) Western blot analysis against Grx2. Total lysates from 3 x 10^7 cells were loaded on a 14 % SDS gel and subjected to Western blot analysis using anti-Grx2 antibodies (1:100). 1 – WT cells, 2 - Grx2 KO cells, 3 and 4 - Grx2 KO cells expressing an ectopic copy of wildtype Grx2 grown in the absence and presence of 100 ng/ml tet, respectively. 2 and 5 ng recombinant Grx2 were also loaded on the gel.
(D) Immunofluorescence analysis. WT and Grx2 KO cells grown at 39 ^oC (without tet) were harvested after 66 h and subjected to immunofluorescence analysis. DAPI, staining of the kinetoplast (K, smaller dots) and nucleus (N, large dots). Phase, phase contrast pictures (scale bar:10 µm). At least 200 parasites for each cell line displaying one of the few different phenotypes are quantified. The percentages of the cells in two different experiments are given in the table (right).

3.12 Both *grx2* alleles could be deleted in Grx1 KO bloodstream cells

Deletion of both *grx2* alleles was attempted in bloodstream Grx1 KO cells using the same procedure mentioned in section 2.2.12.
PCR analyses using the primer combination above each graph and expected sizes in brackets confirmed the deletion of both *grx2* alleles in Grx1 KO bloodstream cells (Figures 31 A and B) to generate Grx1KO/Grx2 KO.



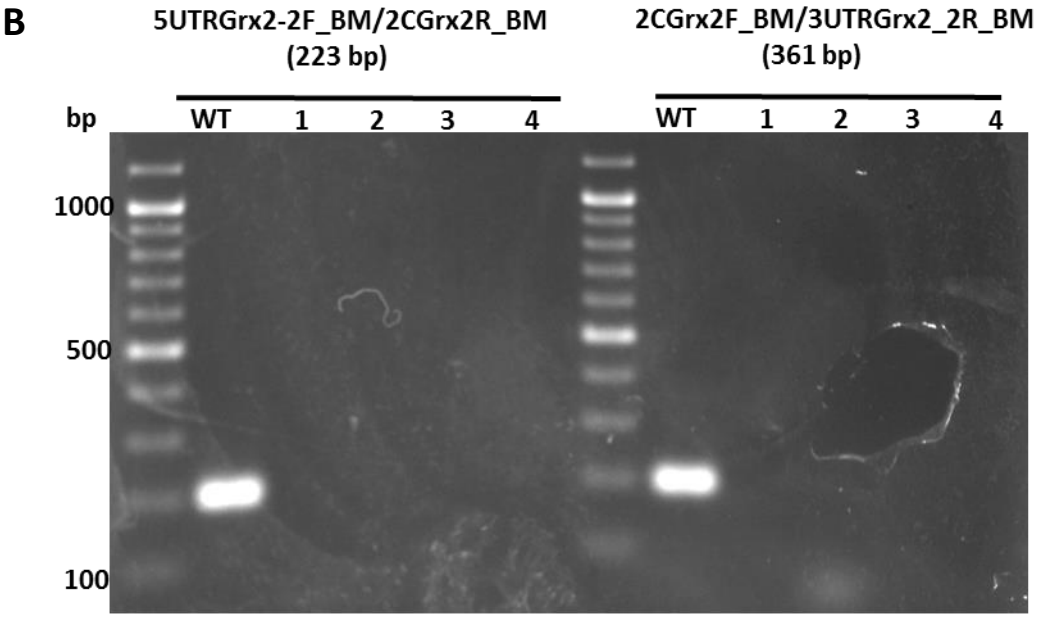


Figure 31. Generation of bloodstream Grx1 KO/Grx2 KO cell lines. (A) PCR analyses using the indicated primer combinations with the expected sizes in brackets confirmed the insertion of the two resistance genes in the *grx2* locus in the Grx1 KO bloodstream cells.

(B)Primer combinations that distinguish the authentic *grx2* gene from ectopic *grx2* gene showed that both *grx2* alleles were replaced. WT DNA served as control. 1-clone B2(1:10), 2-clone B6(1:10), 3-clone A3 (1:20) and 4-clone D2(1:10).

3.13 Redox states of glutaredoxins

3.13.1 All three cysteines of recombinant Grx1 can be modified by AMS and NEM

To determine the redox state of Grx1 in the intact parasite, I first studied the reaction of the cysteine residues in the recombinant protein. Alkylation of one cysteine residue by AMS adds about 500 Da to a protein and thus causes a mobility shift which can be analysed by SDS-PAGE and/or immunoblotting (Ahn and Thiele, 2003). Grx1 possesses three cysteines, namely the redox active Cys21 and Cys24, as well as Cys78. Modification of all three cysteines should thus result in a mass increase of about 1500 Da as compared to the unmodified protein. To facilitate distinction of the protein species, a 16 % SDS gel was used and the protein bands were visualized by silver-staining. Treatment of recombinant Grx1 with AMS resulted in a minor protein fraction that displayed a mobility shift of about 500 Da (Figure 32, *lane 2* in comparison to *lane 1*). This is probably due to modification of Cys78 whereas Cys21 and Cys24 form an intramolecular disulfide bridge in the stored recombinant protein. When first reducing the protein with DTT and then treating it with AMS, all three cysteines were labelled as revealed by a mobility shift of about 1500 Da (Figure 32, *lane 3*). Treatment of pre-reduced Grx1 with NEM did not result in any mobility shift as expected (*lane 4*). NEM efficiently blocked the cysteines since there was no mobility shift when the protein was subsequently treated with AMS (*lane 5*). This experiment showed that all three cysteines could be labeled with NEM and AMS under non-denaturing conditions and the method should be suitable for respective studies in the intact cell.

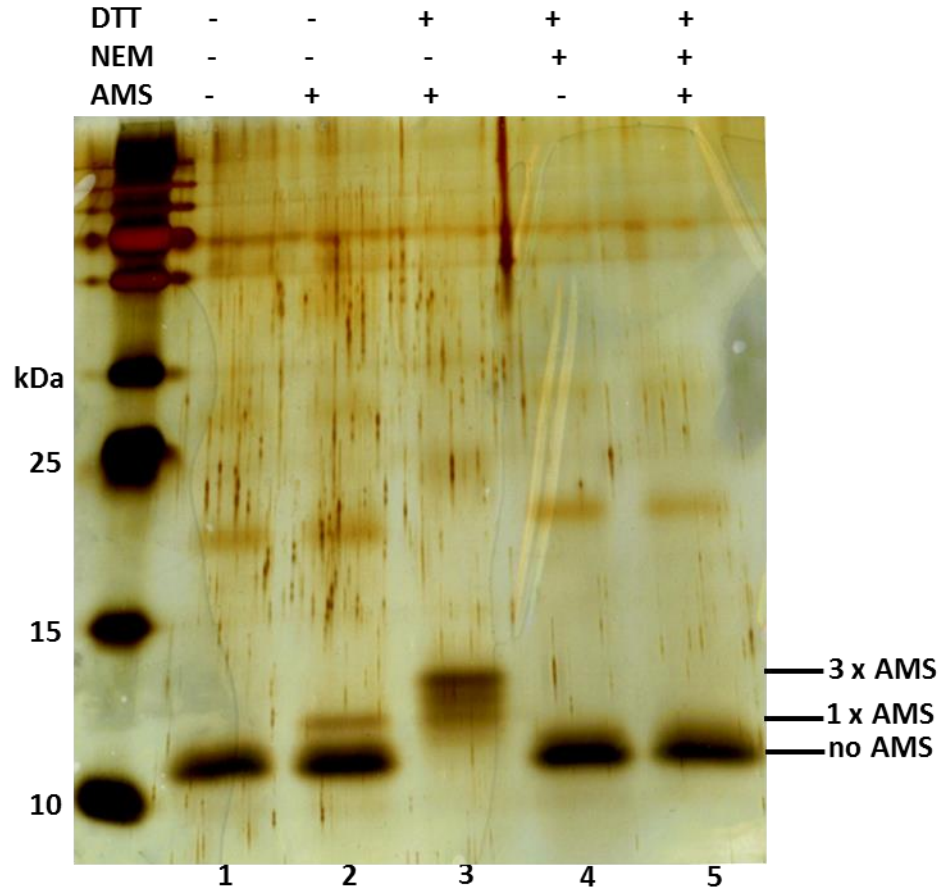


Figure 32. SDS-PAGE and silver-staining of recombinant Grx1 alkylated by NEM or AMS. 500 ng of modified or unmodified protein was loaded per lane of a 16 % gel and subjected to SDS-PAGE under reducing conditions**.** *lane 1,* untreated Grx1 (10.8 kDa). *lane 2*, Grx1 reacted with AMS. *lane 3*, Grx1 treated with DTT and subsequently excess AMS. *lane 4,* Grx1 treated with DTT and then with NEM. *lane 5*, Grx1 treated with DTT, followed by NEM and subsequently AMS. For details see Materials and Methods section 2.2.16.

3.13.2 In bloodstream *T. brucei*, Grx1 is in the fully reduced state

Wildtype bloodstream cells in logarithmic phase were harvested, proteins precipitated and free cysteine residues were blocked with AMS or NEM as described under Materials and Methods. The total protein extracts and recombinant Grx1 samples (as controls, see also Figure 32) were subjected to Western blot analyses using the anti-Grx1 antibodies (Figure 33).The untreated extract displayed a single protein band for Grx1 at the expected size of 10.8 kDa (*lane 1*, Figure 33). In extracts treated with AMS, Grx1 was shifted by about 1500 Da (*lane 2*), revealing that all three cysteines had been labeled. Treatment of the extract with NEM did not result in any mobility shift (*lane 3*). Reacting the protein extract firstly with NEM and then again with AMS also did

not show a mobility shift (*lane 4*) as compared to the untreated control. The upper bands in the samples of recombinant protein are most probably due to covalent dimers. These data clearly showed that under cellular conditions all three cysteines of Grx1 occur in the reduced state.

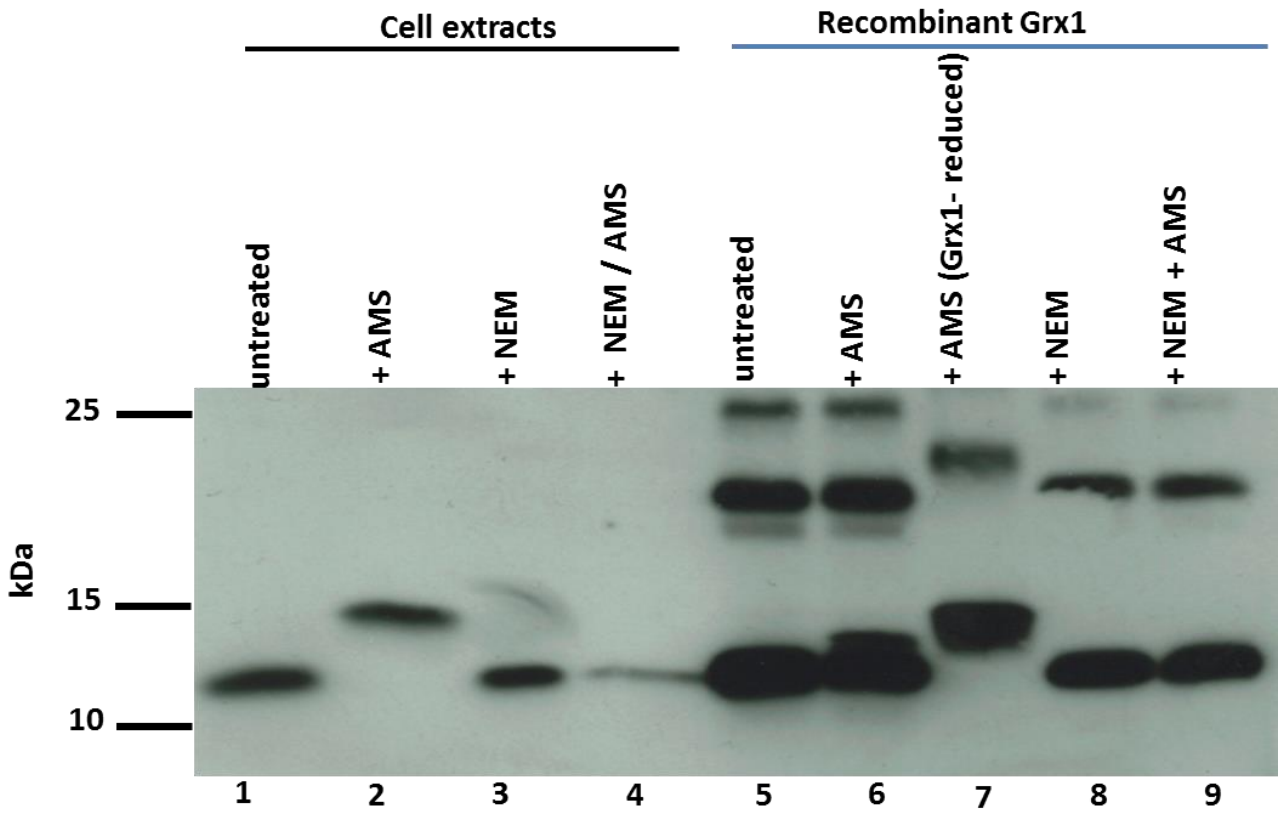


Figure 33. Western blot analyses of bloodstream *T. brucei* and recombinant Grx1 treated with alkylating agents. WT cells were harvested and the proteins were precipitated by TCA. After re-dissolving, free thiols were labeled with AMS and/or NEM. The samples were subjected to SDS-PAGE under reducing conditions followed by Western blotting using anti-Grx1 antibodies (1:800). The extract of 3 x 10^6 cells was loaded per lane. *lane 1*, TCA pellet dissolved in buffer. *lane 2,* TCA pellet dissolved in buffer containing 30 mM AMS. *lane 3*, TCA pellet dissolved in buffer containing 20 mM NEM. *lane 4*, TCA pellet first treated with 20 mM NEM followed by 30 mM AMS. lanes 5-10, recombinant Grx1. *lane 5*, untreated Grx1. *lane 6*, Grx1 treated with 30 mM AMS. *lane 7*, Grx1 first reduced by DTT and then reacted with AMS. *lane 8*, reduced Grx1 reacted with 20 mM NEM. *lane 9*, reduced Grx1 labelled with NEM followed by AMS treatment. For details see section 2.2.16.

3.13.3 Determination of the in vivo redox state of Grx1 in bloodstream form parasites under normal and oxidizing conditions

As described above, under normal culture conditions, Grx1 is in the fully reduced state. Upon diamide treatment of bloodstream cells, Grx1 forms an active site disulfide bridge, but not a covalent dimer as it is observed for the recombinant protein (Figure 33). Upon diamide treatment, Cys78 is still available for reaction with AMS as shown by the 500 Da shift (Figure 34, *lane 3*). Grx1 oxidized by diamide treatment of the parasites is fully reduced within 5 min incubation in normal medium at 37 ^{o}C (*lane 4*). This was confirmed by alkylation of the three cysteines by AMS (*lane 5*).

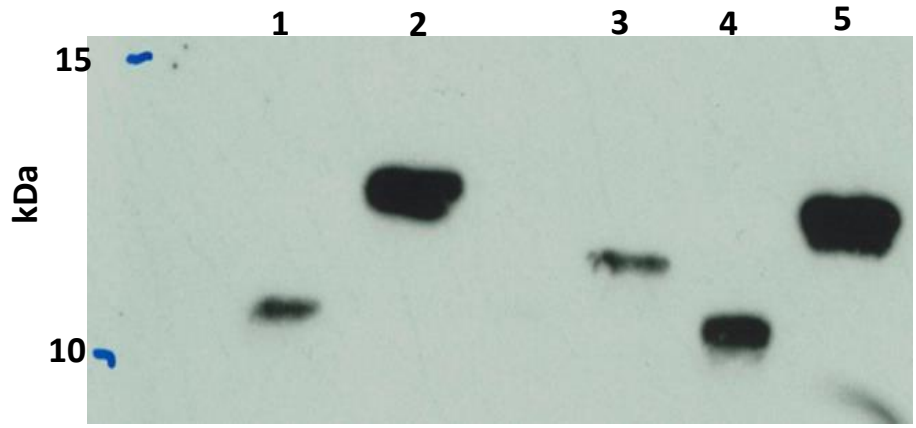


Figure 34. Western blot analyses of bloodstream *T. brucei* Grx1 under normal and oxidizing conditions. Protein extracts from *lane 1*, untreated cells (i). *lane 2,* untreated cells, reacted with 30 mM AMS resulting in a mobility shift of Grx1 of about 1500 Da (ii). *lane 3*, cells treated with 3 mM diamide for 5 min, reacted with AMS (iii). *lane 4*, cells treated with 3 mM diamide and subsequently incubated in HMI-9 medium (iv). *lane 5*, cells treated with 3 mM diamide, incubated in HMI-9 medium and then treated with AMS (v). i – v refers to the conditions outlined in the Materials and Methods section 2.2.16. Cell extracts of 5 x 10^6 cells were loaded per lane on a 16 % gel under non-reducing conditions and subjected to Western blot analysis with the anti-Grx1 antibodies (1:800).

3.13.4 In procyclic *T. brucei*, Grx2 is in the fully reduced state

In procyclic cells grown under standard conditions, Grx2 is in the fully reduced state and rapidly recovers from oxidative stress resulting in a mobility shift upon labelling of cell extracts with AMS (Figure 35, *lane 2*) from cells that were cultured normally. Upon diamide treatment, no cysteines could be labelled with AMS (Figure 35*, lane 4*), as there was no mobility shift compared to

unlabeled Grx2 (lane 3). Most probably the two active-site cysteines make an intradisulfide bridge upon diamide treatment. Upon removal of diamide stress, Grx2 was in fully reduced state as two cysteines could be labelled with AMS (lane 6).

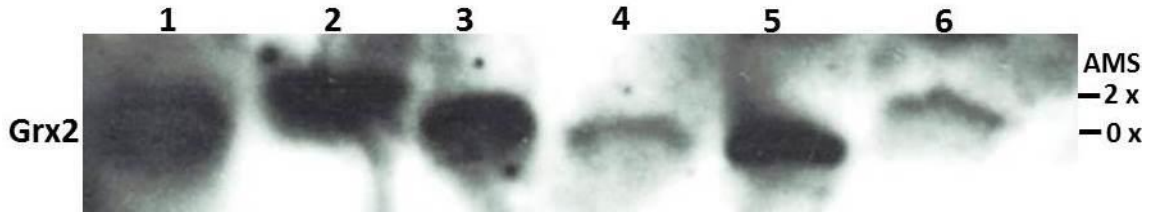


Figure 35. Western blot analyses of the Grx2 in procyclic *T. brucei* under normal and stress conditions. (1 - 6 are lane numbers). *lane 1*, untreated cells (i)., *lane 2,* untreated cells, reacted with 30 mM AMS (ii)., *lane 3*, cells treated with 3 mM diamide for 5 min(iii)., *lane 4*, cells treated with 3 mM diamide for 5 min, reacted with AMS (iv)., *lane 5*, cells treated with 3 mM diamide and subsequently incubated in HMI-9 medium (v). *lane 6*, cells treated with 3 mM diamide, incubated in HMI-9 medium and then treated with AMS (vi). i – vi refers to the conditions outlined in the Materials and Methods section 2.2.16. Cell extracts of 1 x 10^7 cells were loaded per lane on a 16 % gel under non-reducing conditions and subjected to Western blot analysis with the anti-Grx2 antibodies (1:200). Weak bands in lanes 4 and 6 might have been caused by loss of extracts during washing after TCA precipitation.

4 Discussion

4.1 Role of glutaredoxins under various stressors

Bloodstream and procyclic cells lacking Grx1 were viable under culture conditions. This was also reported in yeast where cells lacking *grx1, grx2* or both *grx1* and *grx2* were viable and the glutaredoxins were dispensable under culture conditions (Luikenhuis et al., 1998). Glutaredoxins in various organisms have been reported to protect cells against damage caused by oxidizing agents such as hydrogen peroxide and diamide. For example, human Grx2 (Wu et al., 2010) and *E. coli* Grx2 (Fernandes and Holmgren, 2004) have been shown to protect cells against hydrogen peroxide-induced oxidative stress. Also yeast *Saccharomyces cerevisiae* dithiol *grx2* mutants or *grx1grx2* double mutants were sensitive to hydrogen peroxide compared to WT strains (Luikenhuis et al., 1998). Recombinant *T. brucei* Grx1 was shown to coordinate an iron-sulfur cluster (Ceylan et al., 2010). This led me to study whether the absence of Grx1 affects the sensitivity of parasites towards exogenous iron. There was no difference in the proliferation between WT and Grx1 KO cell lines when iron was used as stress as the cells proliferated like untreated controls. The proliferation defect observed in both WT and Grx1 KO parasites when deferoxamine was used as a stressor, was probably due to an impaired biosynthesis of iron-dependent enzymes like ribonucleotide reductase, superoxide dismutase, and alternative oxidase (Breidbach et al., 2002). In the cyanobacterium *Synechocystis* sp. PCC 6803, the dithiolic GrxA was reported to protect the cell against heat shock (Sanchez-Riego et al., 2013) and also ectopic expression of AtGrxS17 (a monothiol Grx) was reported to increase thermotolerance in tomato plants (Wu et al., 2012). I investigated a respective role of Grx1 in the KO cells compared to WT cells and there was no difference in recovery after heat shock between the two cell lines.

Though Grx1 was not found to play role in protecting both *T. brucei* bloodstream and procyclic cells from the stresses used in our experiments, it will be worthy to investigate the proliferation of the parasites under other forms of stresses such as menadione or osmotic stress. Bloodstream cells lacking Grx2 are viable and the protein is dispensable under culture conditions. *T. cruzi* epimastigotes overexpressing Grx2, a cytosolic protein which has about 80 % identity to *T. brucei* Grx2, were more viable than wildtype cells under

oxidative stress caused by hydrogen peroxide (Marquez et al. 2014). Investigations need to be done on the proliferation of the bloodstream cells lacking Grx2 under various stress conditions compared to wildtype cells. However, it is not possible to investigate the effect of different stresses in procyclic cells compared to WT parasites since cells lacking Grx2 could only be obtained in the presence of a tet-inducible ectopic copy of Grx2 (wildtype or Grx2C34S mutant) and had leaky expression when tet was removed, consistent with an essential role in procyclic cells.

4.2 Glutathionylated Grx1 has decreased activity in HED assay

The third non-active site cysteine of Grx1 is conserved in the Grx1 sequences of *Leishmania major* (Ceylan et al., 2010), human and *T. vivax* (TvY486_1101340) as well as *E. coli* Grx3 which may suggest a putative regulatory function in the parasite oxidoreductases. Protein glutathionylation can result in several effects that include inhibition of a protein function. Examples include transcription factors like Jun and NF-kB which are inhibited by glutathionylation (Ghezzi, 2005). The activity of GAPDH was inhibited upon glutathionlyation during ischemia and reperfusion (Eaton et al., 2002). S-glutathionylation of aldose reductase 2 (ALR2) resulted in decreased activity of the enzyme (Cappiello et al., 2001). It was shown that glutathionlyation of Cys 95 inhibits HIV protease's activity whilst glutathionylation of Cys 67 stabilised the enzyme (Dalle-Donne et al., 2007). Our *in vitro* results showed that the non-active site cysteine (Cys78) of *T. brucei* Grx1 can be glutathionylated. Glutathionylated Grx1 (glut-Grx1) had an activity that was three fold lower compared to the wildtype protein in the HED assay. The K_m value for glut-Grx1 was also about three fold lower as compared to that of Grx1 resulting in an overall same k_{cat}/K_m values between the two protein species of Grx1. These results showed that glutathionylation of the non-active site cysteine might have a regulatory role in *T. brucei.* The decrease in activity after glutathionylation at non-active site cysteine was also mentioned for bacterial Grx3 by Lind et al., 1998.

4.3 Redox state of Grx1 in bloodstream *Trypanosoma brucei* and of Grx2 in procyclic cells

As shown in this thesis, Grx1 in bloodstream parasites is in the fully reduced state under normal culture conditions. Upon diamide treatment, the protein forms an intramolecular disulfide bridge in the active site. In contrast, recombinant Grx1 forms a dimer upon storage which indicates that dimer formation of Grx1 is something artificial that only occurs *in vitro*. The non-active site cysteine of Grx1 could still be labelled with AMS after diamide treatment. Upon removal of the diamide stress, Grx1 was again in the fully reduced state within 5 min, revealing the quick regeneration under normal cellular conditions.

The redox state of Grx2 under normal and oxidizing conditions was investigated in procyclic cells.Grx2 has only two cysteines which are in the active site. As in the case of Grx1 in bloodstream cells, Grx2 is in the fully reduced state in procyclic cells under culture conditions and forms an intradisulfide bridge in the active site upon oxidative stress. It is also fully reduced within 5 min after removal of diamide as there was a mobility shift when cell extracts were labelled with AMS compared to unlabeled controls. The redox state of Grx2 in bloodstream cells was not investigated since Grx2 is very difficult to detect by Western blot analysis in these cells due to very low protein levels (Ceylan et al., 2010).

4.4 Bloodstream parasites lacking Grx1 or Grx2 proliferate better at 39 °C than wildtype cells

My investigations showed that bloodstream parasites lacking either Grx1 or Grx2 have a proliferation advantage at 39 ^{o}C as compared to wildtype cells. It might be possible that at least one of the proteins that function in temperature-sensing in bloodstream trypanosomes might have been inhibited due to the absence of either glutaredoxin, thereby allowing the cells lacking either glutaredoxins to have a proliferation advantage at 39 ^{o}C compared to wildtype cells.Grx1 is located in the cytosol whereas Grx2 occurs in the mitochondrion, probably in the intermembrane space (Ceylan et al., 2010). These two glutaredoxins seem to have a coordinated function in this regard

though they are located in different compartments with Grx2 playing a major role. The two glutaredoxins cannot be said to have an overlapping function since I showed that there was no upregulation of Grx1 in Grx2 KO clone and also that there was no upregulation of Grx2 in a Grx1 KO clone. Furthermore, there is a very low level of Grx2 in bloodstream parasites (Ceylan et al., 2010). A likely modification of any candidate protein in this pathway maybe glutathionylation since it was shown that both Grx1 and Grx2 had deglutathionylation activities *in vitro*, though the former had higher activity (Ceylan et al., 2010).

A cytosolic *Trypanosoma brucei* Hsp70.c. was shown to be upregulated upon heat stress in bloodstream parasite (Burger et al., 2014). ZC3H11, a zinc finger protein that binds and stabilize the 3`-untranslated region of mRNAs of heat-shock proteins in *T. brucei* was shown to stabilise the mRNA of bloodstream Hsp70 (Droll et al., 2013). It is possible that the absence of Grx1 or Grx2 increases the stabilizing effect of ZC3H11 on Hsp70 mRNA, thereby having the parasites lacking either glutaredoxins having a proliferation advantage at 39 ^{o}C than wildtype cells.

Cell division of trypanosomes occurs by binary fission with two distinct phases that must be coordinated whereby the initial process involves the S-phase of the mitochondrial DNA before the nuclear DNA is replicated (Vaughan and Gull, 2008). Immunofluorescence analysis with DAPI of the parasites cultured at 39 ^{o}C till 66 h revealed that the wildtype cells had significantly more difficulties in dividing as compared to either the cells lacking Grx1 or Grx2. The populations of parasites lacking either Grx1 or Grx2 contained more normal cells with 1 nuclei and 1 kinetoplast (1K1N) and less parasites that had at least 2 kinetoplast and 2 nuclei (≥2K2N) as compared to wildtype cells at 39 ^{o}C. These results indicated that bloodstream *T. brucei* have severe problems in starting cytokinesis at 39 °C, a phenotype less pronounced in the Grx1 or Grx2 deficit cells. Several assumptions can be made regarding the observed phenotypes including the following.

Trypanosome receptor for activated C kinase (TRACK) is required for the initiation of cytokinesis in bloodstream parasites (Rothberg et al., 2006). RNAi

against TRACK in bloodstream cells results in growth inhibition. The number of cells with 1N1K (1 nuclei and 1 kinetoplast) decreases whilst those with 2N2K increases with time, revealing problems in cytokinesis (Rothberg et al., 2006). A very similar phenotype was observed for wildtype bloodstream cells when cultured at 39 °C which was attenuated in parasites lacking either Grx1 or Grx2. Thus one may speculate that either the parasite glutaredoxins are negatively involved in the signaling pathway of TRACK or that they directly interact with TRACK. When either or both Grxs are absent, the TRACK is modified in such a way that it retains a comparatively high cytokinesis at 39 ^{o}C, resulting in cells that proliferate better than the wildtype cells. TRACK is localized in the cytosol and in perinuclear region. TRACK has got nine cysteines and five of them are conserved in the receptor for activated C kinases of *Leishmania major* (LACK) and Homo sapiens (RACK1). It might be possible that some of the cysteines from TRACK are prone to glutathionlyation in the absence of either Grxs, resulting in stability and initiating cytokinesis at 39 ^{o}C in bloodstream cells.

The DAPI staining also revealed that in all three cell lines (WT, Grx1 KO and Grx2 KO), there was almost an identical number of parasites that had (i) no kinetoplast and one nuclei (0 K & 1N) and (ii) one kinetoplast and two nuclei (1K & 2N). Failure of kinetoplast segregation can result in trypanosomes that have no kinetoplast or a kinetoplast and two nuclei (Liu et al., 2009). These phenotypes are probably a general consequence caused by the high temperature and cannot be attributed to the lack of Grx1 and Grx2.

Grx2 mutants in yeast were reported to have higher tolerance to cadmium (a toxic heavy metal) exposure as compared to wildtype cells. However this was found to be a temporary putative advantage since the mutants had higher mutagenic rates as compared to wildtype cells (Gomes et al., 2008). The growth advantage at 39 °C of the *T. brucei* Grx1 and Grx2 KO mutants might also be only a temporary benefit. Wildtype cells might not want to keep dividing at the elevated temperature where they will not survive in the long term by decreasing proliferation at the level of cytokinesis at 39 °C. The bloodstream cells lacking Grx1 or Grx2 displayed a significantly higher number

of normal wildtype-like phenotype. However, this might be related with a higher mutagenic rate.

T. cruzi epimastigotes overexpressing Grx2 were found to go under apoptosis more than wildtype when stimulated with human fresh serum (Marquez et al., 2014). This showed that Grx2 was pro-apoptotic in *T.* cruzi. The Grx-type protein might also play a pro-apoptotic role in bloodstream parasites at 39 ^{o}C resulting in fewer number of total parasites for wildtype cells than for those parasites lacking Grx2. Parasites lacking Grx2 retained the highest proliferation amongst the three cell lines studied which might be due to inadequate apoptosis in these cells at 39 °C. This can be investigated in future by checking for apoptosis markers like cytochrome c release (Esseiva et al., 2004), DNA degradation and phosphatidylserine exposure (Tsuda et al., 2005) in wildtype cells compared to the parasites either lacking Grx2 when grown at 39 ^{o}C.

There might be several proteins modified in the same signaling pathway involving Grx1 and/or Grx2 in bloodstream cells. Since it is not yet clear what the targets of the two dithiol Grxs are, the actual roles of the oxidoreductases at 39 °C remain to be elucidated. It will also be highly likely that the most affected protein of this signaling pathway is in the mitochondrion since cells lacking Grx2 retained the highest proliferation rate.
Though the two dithiol glutaredoxins are located in different compartments, it seems that they have a coordinated role at 39 °C in bloodstream parasites. To investigate on this, bloodstream parasite lacking both Grx1 and Grx2 were generated and are still to be characterized upon culturing at 39 °C compared to wildtype cells. At least the phenotype observed for the bloodstream parasites lacking Grx2 should be observed in the parasites lacking both Grx1 and Grx2. Since the wildtype-like proliferation was obtained upon expressing the respective ectopic copies of the Grxs in the KO backgrounds, it should also be investigated if these Grxs have redox function at 39 ^{o}C by expressing ectopic non-redox mutants in KO backgrounds and investigate them upon culturing at 39 ^{o}C compared to wildtype cells.

4.5 Grx2 may be involved in deglutathionylation reactions in procyclic cells

T. brucei procyclic cells lacking Grx2 were fully viable when they express an ectopic copy of either wildtype Grx2 or Grx2C34S mutant. Recombinant Grx2C34S showed 35 % activity of wildtype Grx2 in the HED assay. Similar results were obtained for other recombinant proteins lacking the C-terminal cysteines. The C34S mutant of *Trypanosoma cruzi* Grx2 catalyzes the reduction of GSSG with the same kinetic constants as wildtype Grx2, showing the cysteine at position 34 is not essential for this reaction (Marquez et al., 2014). The respective cysteine mutant of human Grx2, Grx2C40S, catalyses the reduction of GSH-mixed disulfides in the HED assay with 26 % catalytic efficiency of the wildtype protein (Johansson et al., 2004).The yeast Grx2 mutant (C30S) has 30 % of the specific activity in the HED assay compared to wildtype Grx2 (Discola et al., 2009). The corresponding *E. coli Grx1* cysteine mutant (C14S) shows 38 % of the activity of the wildtype protein in the HED assay (Bushweller et al., 1992).

Remarkably, *Leishmania* Grx2 which has about 80 % identity to *T. brucei* Grx2, is a monothiol since it has a serine residue instead of the second cysteine in the active site (Ceylan et al., 2010). Taken together, these data support the results of my *in vitro* assays that the C-terminal cysteine was dispensable in *T. brucei* recombinant Grx2 in a HED assay, though the activity was lower and also why the procyclic cells could survive with an ectopic copy of Grx2C34S. Thus one may speculate that Grx2 in procyclic cells might be involved in essential (de)glutathionylation reactions. Clearly this role is not conserved in bloodstream parasites since Grx2 was dispensable under culture conditions and its absence even conferred a proliferation advantage at elevated temperature.

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6 Supplementary Results

Sequences

pHD1747-*grx1*-KO cassette

CTCGAGGTAACTGCATCACGCACATCAGTGTTCTGTACAACGACGCGAATCCACAAATGCCT
CTGGTACATGCCGTATGCTACCATTTGTATTCTTCCTACGTGTGTACTTTTTTCATTACCTT
CCTTTTATTTATTTTTTTAAATTCTGAAATGTTTTTATGTGATGTGCCCATATAAATGCACC
GGAACCAAAAAAAAAAAAACACAAAGAATCAAGAAGAAACACAAACATTTAGCACTTTCCCA
CCTCGTTTTTATTCACTTACAATACACCTTGAAGTGAATAAAACTAAGGTTACTCTGTAGAG
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TCCTGGCGCCGCACCGGCCCAAGGAGCCCGCGTGGTTCCTGGCCACCGTCGGTGTCTCGCCC
GACCACCAGGGCAAGGGTCTGGGCAGCGCCGTCGTGCTCCCCGGAGTGGAGGCGGCCGAGCG
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CGCAAGCCCGGTGCCTGAATCGAATTCTAGTGCCAAACGAGAGGTGTTTGCATATGTTGAGG
GGGTAAACTACACACCCTTCCTTTATATACATCTCCTTCATCCTTCACTTGATCGCTTTCAT
TTTTTTTATATTTTTGTTCCTGCTTATATTAGGGGTTATAATGCATTATGCATGATGTGTTC
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GAGCGAGTGAACTGACATCAATTCAATCGGGGGAAGTGTTTAAAAACACCTCACCGGTATAA
CTTCCCCTATTGATACGTTACGCACGACGCTATTTCTGTTGTTGAATACTCGCGCTTTGAAG
ATCGCCTTTTAGCACGCGGCCG

CTCGAG – XhoI restriction site

AAGCTT – HindIII restriction site

GAATTC – EcoRI restriction site

GCGGCCG – NotI restriction site

ATG and TGA are the start and stop codon, respectively, of the puromycin resistance gene. The 5'- and 3'- untranslated regions of the *grx1* gene are given in italics.

pHD1748-*grx1*-KO cassette

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CGCACGACGCTATTTCTGTTGTTGAATACTCGCGCTTTGAAGATCGCCTTTAGCACGCGGCC
GC

CTCGAG – XhoI restriction site
AAGCTT – Hind III site
GAATTC – EcoRI site
GCGGCCG – NotI site
ATG and TAA are the start and stop codon, respectively, of the blasticidin resistance gene. The 5'- and 3'- untranslated regions of the *grx1* gene are given in italics.

pHD1747-*grx2*-KO cassette

CTCGAGCTCTTTTACTAAGGTAGAATTTTTTTTTTAATAAAATAGTAAAAGGAACTACTAAT
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CAAACAAACACAAATAAGATTTAGTATAAGGTTACACATATTTAGTTGTTGATTTACATATA
TAGTTGACTTGTTTGCTTTATTTTGTTCTATTTTATTTTATTCTATTTTTTTTTTTGTACGT
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CCGCGACGACGTCCCCAGGGCCGTACGCACCCTCGCCGCCGCGTTCGCCGACTACCCCGCCA
CGCGCCACACCGTCGATCCAGACCGCCACATCGAGCGGGTCACCGAGCTGCAAGAACTCTTC
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GTCACCGCCGACGTCGAGGTGCCCGAAGGACCGCGCACCTGGTGCATGACCCGCAAGCCCGG
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TTTTTCTTATGTTTTTTTTTTGTTTCATCTGTCACCACTTTCTTTCTTTCTTTCTTTCATCG
TTCCATAGAAATTTGAACGATGATGTGGAAAGGGAGGATTGTGTGGAGAAAATGAGGCACAC
GAACATGGAAGTTTGTTAATAGGTATTCATACTCACACATGTATTTGTATGGCGTGCACATC
TCGCTGATTCTCTGTTTTGGTTTTATTTGTTGTTGTATGCTGCTCGGCGGCCGC

CTCGAG – XhoI restriction site

AAGCTT – Hind III site

GAATTC – EcoRI site

GCGGCCG – NotI site

ATG and TGA are the start and stop codon, respectively, of the puromycin resistance gene. The 5'- and 3'- untranslated regions of the *grx2* gene are given in italics.

pHD1748(*neo*)-*grx2*-KO cassette

CTCGAGCTCTTTTACTAAGGTAGAATTTTTTTTTTAATAAAATAGTAAAAGGAACTACTAAT
AACGGAGTTCCAATCCTTTTCGGCCAGCTTTAACGACACTTCTGCATAAGGGGAGGGGGGGA
AGTTATTTTAAAAAAAAAAGTAAAGAAGTTGTGGAGTTCAAGTGCGCTGGTATCAAATACAT
AAGTCACGTGATTGCGTCCAAAGAAAACCAGTGAAGCTGGATTTATTAACGTCAGGCAGTTC
CAAACAAACACAAATAAGATTTAGTATAAGGTTACACATATTTAGTTGTTGATTTACATATA
TAGTTGACTTGTTTGCTTTATTTTGTTCTATTTTATTTTATTCTATTTTTTTTTTTGTACGT
TGCTGTGAGGAAGCTTATGGTGGAACAAgatggattgcacgcaggttctcCGGCCGCTTGGG
TGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTG
TTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCT
GAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCG
CAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCG
GGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGC
AATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATC
GCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAA
GAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGG
CGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCC
GCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCG
TTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCT
TTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCT
TCTGAGAATTCGGGGCTGAAGAGGTAATTATTGTTTATGTATTGTGAGCATAGGCGTAACGA
AATATATTTATGTGTATGTTCACTTCGATGGAGCGTACAGCTGTAATGTTTAATTGTCTCAT
TTTGAGACGGGCAGTGGTCATTTGGTTTTTTTTGTTGTTATTGTTGTTGTTGTTTTTTTTTT
CTTATGTTTTTTTTTTGTTTCATCTGTCACCACTTTCTTTCTTTCTTTCTTTCATCGTTCCA
TAGAAATTTGAACGATGATGTGGAAAGGGAGGATTGTGTGGAGAAAATGAGGCACACGAACA
TGGAAGTTTGTTAATAGGTATTCATACTCACACATGTATTTGTATGGCGTGCACATCTCGCT
GATTCTCTGTTTTGGTTTTATTTGTTGTTGTATGCTGCTCGGCGGCCG

CTCGAG – XhoI restriction site

AAGCTT – Hind III site

GAATTC – EcoRI site

GCGGCCG – NotI site

ATG and TGA are the start and stop codon, respectively, of the neomycin resistance gene. The 5'- and 3'- untranslated regions of the *grx2* gene are given in italics.

Digitonin analysis of bloodstream Grx2 KO cells expressing an ectopic copy of Grx2-c-myc$_2$

Bloodstream Grx2 KO cells expressing and ectopic copy of Grx2-c-myc$_2$ were grown overnight in the presence of 1 µg/ml tet. Cells were harvested and subjected to digitonin lysis basically as described in Ceylan et al., 2010. The supernant and pellet fractions corresponding to 1.9 x 10^6 cells were loaded on a 14 % gel and subjected to Western blot analysis using the anti-Grx2 antibodies (α-guinea pig (1:100).

The results showed that overexpression of an ectopic copy of Grx2-c-myc$_2$ in bloodstream cells resulted in the Grx2-c-myc$_2$ being located mainly in the cytosol (Figure S1).

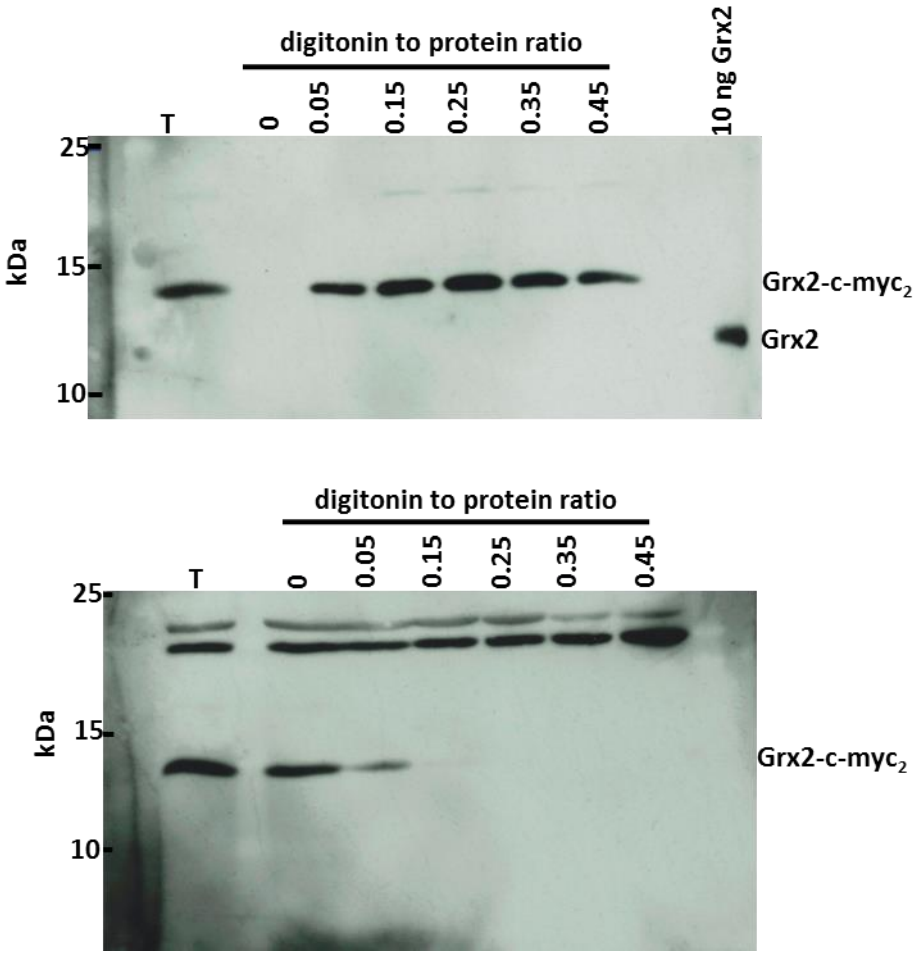

Figure S1. Localisation of Grx2-c-myc$_2$ in bloodstream Grx2 KO cell line. Bloodstream Grx2 KO cells lines expressing an ectopic copy of Grx2 were cultured overnight in presence of 1 µg/ml tet. Fractions corresponding to 1.9 x 10^6 cells after digitonin lysis (mg digitonin to mg protein ratios are indicated above each lane) were loaded on a 14 % gel and subjected to Western blot

analysis using anti-Grx2 antibodies. *Upper blot*, supernatant fractions and *lower blot*, pellet fractions. T is the total lysate (not subjected to digitonin fractionation)