Parasitology Open

cambridge.org/pao

Giardia lamblia: missing evidence for a canonical thioredoxin system

David Leitsch[1,2], Samuel Rout[3,4], Britta Lundström-Stadelmann[1], Vreni Balmer[1], Adrian Hehl[3] and Norbert Müller[1]

[1]Institute of Parasitology, Vetsuisse Faculty, University of Bern, Längassstrasse 122, CH-3012, Bern, Switzerland; [2]Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Kinderspitalgasse 15, A-1090, Vienna, Austria; [3]Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 266a, CH-8057, Zurich, Switzerland and [4]Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012, Bern, Switzerland

Research Article

Cite this article: Leitsch D, Rout S, Lundström-Stadelmann B, Balmer V, Hehl A, Müller N (2017). *Giardia lamblia*: missing evidence for a canonical thioredoxin system. *Parasitology Open* **3**, e13; 1–6. https://doi.org/10.1017/pao.2017.16

Received: 23 May 2017
Revised: 2 August 2017
Accepted: 15 September 2017

Key words:
Giardia lamblia; thioredoxin reductase; redox system

Author for correspondence: David Leitsch, E-mail: david.leitsch@meduniwien.ac.at

Abstract

The microaerophilic protozoan parasite *Giardia lamblia* occurs globally and causes dysentery in humans and animals. Since it is very sensitive to oxygen and reactive oxygen species, *G. lamblia* disposes over several enzymatic pathways to counter oxidative stress. One of the enzymes involved is thioredoxin reductase (TrxR), a central redox regulator that indirectly reduces peroxiredoxins *via* thioredoxin, an electron shuttle protein. Interestingly, the components of the TrxR-mediated redox system, including functional thioredoxins, have so far not been described despite their surmised importance for parasite survival. We aimed at filling this gap and attempted to identify functional thioredoxins and other interaction partners of TrxR in *G. lamblia*. To this end, we conducted database searches and expressed three recombinant candidate thioredoxins in *Escherichia coli* for ensuing enzyme assays. Further, co-immunoprecipitation experiments were conducted in order to identify further components of the thioredoxin redox network. Finally, the cellular localization of TrxR and peroxiredoxin 1 was determined by immunofluorescence microscopy. Surprisingly, our endeavours did not result in the identification of a functional thioredoxin or other credible interaction partners of TrxR. We, therefore, conclude that there is currently no evidence for a canonical thioredoxin redox network in *G. lamblia*.

Introduction

The protist *Giardia lamblia* (syn. *duodenalis*, *intestinalis*) is a worldwide occurring zoonotic parasite that causes dysentery in humans and animals. It has a microaerophilic/anaerobic lifestyle which renders it vulnerable to oxygen and reactive oxygen species (Gillin and Diamond, 1981; Lloyd *et al.* 2000; Paget *et al.* 2004). Like other microaerophilic protist parasites, such as *Trichomonas vaginalis* (Coombs *et al.* 2004) and *Entamoeba histolytica* (Arias *et al.* 2007), *G. lamblia* possesses antioxidant enzyme pathways for the defence against oxidative stress (Mastronicola *et al.* 2014, 2016). It is well established that the thioredoxin-mediated redox system, comprising thioredoxin reductase (TrxR), the electron shuttle protein thioredoxin, and peroxiredoxins (Lu and Holmgren, 2014), plays a central role in the antioxidant defence. Peroxiredoxins reduce harmful hydrogen peroxide to water and molecular oxygen *via* their catalytic cysteines. To this end, their catalytic cysteines need to be reduced by the catalytic cysteines of thioredoxin first. These, in turn, are reduced by TrxR which harnesses reductive power from NADPH and reduces them *via* an FAD cofactor (Lu and Holmgren, 2014). Moreover, in most organisms, thioredoxin has a large number of protein substrates in addition to peroxiredoxins, including transcription factors and ribonucleotide reductase, rendering it a central redox regulator.

In light of its important function as the reducing enzyme of thioredoxin, TrxR has been repeatedly suggested to have considerable potential as a drug target for antigiardial chemotherapy (Tejman-Yarden *et al.* 2013; Watkins and Eckmann, 2016). Comparably little, however, is known about the components of the thioredoxin-mediated redox system in *G. lamblia*. Most importantly, no functional thioredoxin, the primary substrate of TrxR, has been identified so far in this parasite and, in fact, no protein with such designation exists in the GenBank or any other database. In contrast, functional thioredoxins have been identified and described in *T. vaginalis* and *E. histolytica* (Coombs *et al.* 2004; Leitsch *et al.* 2007, 2009).

In order to fill this gap, we identified candidate thioredoxins in the *G. lamblia* genome, expressed them in *Escherichia coli* and tested them for activity with *G. lamblia* TrxR. In the search of a functional thioredoxin and other interaction/binding partners of TrxR, we also performed co-immunoprecipitation (co-IP) experiments with haemagglutinin (HA)-tagged TrxR in *G. lamblia* cell extracts. Immunofluorescence microscopy was conducted in order to determine the intracellular localization of the TrxR and peroxiredoxin 1.

CAMBRIDGE UNIVERSITY PRESS

Materials and methods

Cell culture

Giardia lamblia WB C6 (ATCC 50803) trophozoites were axenically cultivated in Keister's modified Diamond's medium (Keister, 1983) in Nunclon Delta tubes (Nunc, Roskilde, Denmark). The cultures were subcultured every third day. Constituents of the growth medium were purchased from Merck [peptone from casein, yeast extract, sodium chloride, glucose, ammonium iron (III) citrate]. Fetal calf serum was purchased from Biochrom (Bioswisstec AG, Schaffhausen, Switzerland). When large numbers of cells were needed for co-IP, T500 triple layer culture flasks with closed caps (Thermo Fisher Scientific, Waltham, Massachusetts) were inoculated with 60 mL of densely grown *G. lamblia* culture (equalling six culture tubes), filled to the top with growth medium, and incubated for 48 h. *Trichomonas vaginalis* G3 cells were cultivated in TYM medium as described (Leitsch *et al.* 2009).

Recombinant expression of thioredoxin candidate proteins in E. coli

Of the three proteins identified as candidates (Table 1) for a functional thioredoxin (GL50803_104250, GL50803_3910, and GL50803_9355), two (GL50803_104250 and GL50803_3910) are almost identical with only two amino acid substitutions (104250 → 3910: Y59C, V113A) in the polypeptide sequence. In order to amplify each of the alleles, the respective genes were first amplified including parts of the upstream and downstream sequences where more differences in sequence could be found. The PCR products obtained with the respective primer pairs (given in Table S1) were cloned into the TOPO pCR™ 2·1 vector (Invitrogen). Subsequently, the coding sequences within the cloned fragments were amplified using appropriate primers (Table S1) and cloned into expression vector pET-17b (Leitsch *et al.* 2011). The reverse primers encoded a 6× His-tag (*C*-terminal) for isolation of the recombinant proteins in Ni-NTA columns. GL50803_9355 was amplified by PCR (Table S1) and cloned into a pET151 vector, adding a 6× His-tag to the *N*-terminus of the protein. *Giardia lamblia* peroxiredoxin 1 was also cloned into the pET-17b vector (Table S1). All expression plasmids were transformed into *E. coli* BL21 (DE3) cells and protein expression and purification was performed according to established protocols (Leitsch *et al.* 2007; Müller *et al.* 2013). Recombinant *G. lamblia* TrxR, recombinant *T. vaginalis* TrxR and recombinant *T. vaginalis* thioredoxin were expressed as described (Leitsch *et al.* 2009, 2011).

TrxR assays in cell extracts and/or with purified recombinant proteins

TrxR assays were performed as described before (Leitsch *et al.* 2007, 2009, 2012*a*, *b*). Reduction of thioredoxin was measured as the readout of DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] reduction at $\lambda = 412$ nm ($\Delta\varepsilon 412 = 13{\cdot}6$ mM-1 cm-1) in a Lambda 25 UV-Vis spectrometer (Perkin Elmer, Waltham, Massachusetts) at 37 °C. Heating of the sample was achieved using a PTP A Peltier temperature programmer (Perkin Elmer, Waltham, Massachusetts). For reduction of DTNB, thioredoxins have to be reduced previously by TrxR. Thus, reaction mixtures contained 2 μg mL^{-1} *G. lamblia* TrxR, 40 μg mL^{-1} *T. vaginalis* thioredoxin or *G. lamblia* thioredoxin candidate proteins, 0·5 mM NADPH and 1 mM DTNB. Reactions were buffered in 100 mM Tris, pH 7·5. When reduction of candidate thioredoxins was measured with cell extracts, the same assay mixture was used, but recombinant TrxR was replaced with 50 μg protein mL^{-1} *G. lamblia* WB C6 cell extracts. As a positive control, TrxR assays were also performed with *T. vaginalis* TrxR and *T. vaginalis* thioredoxin (Leitsch *et al.* 2009), following the same protocol. Reduction of *T. vaginalis* thioredoxin was also measured with *T. vaginalis* G3 cell extracts (50 μg protein mL^{-1}).

For the measurement of peroxiredoxin 1 reduction by *G. lamblia* TrxR, 5 μg mL^{-1} TrxR and 10 μg mL^{-1} recombinant peroxiredoxin 1 were added to a reaction buffer similar to the one described above, containing, however, a lower concentration of NADPH (0·2 mM) and 4 mM hydrogen peroxide instead of DTNB. Reduction was measured as oxidation of NADPH at $\lambda = 412$ ($\Delta\varepsilon 412 = 6{\cdot}2$ mM-1 cm-1).

Table 1. The putative thioredoxins recombinantly expressed and studied

ID	Size (aa)	Active site
GL50803_104250	123	CKDC
GL50803_3910	123	CKDC
GL50803_9355	134	CPPC

Construction of HA-tagged expression constructs

In order to obtain a *G. lamblia* WB C6 cell line expressing HA tagged TrxR, the *trxR* gene (GL50803_9827) was fused to the sequence for a 3-fold HA-tag at the *C*-terminus and cloned into the pPac-VInteg vector (Štefanić *et al.* 2009). In addition to the gene reading frame, the amplified fragment contained 120 bp of the region 5′ of the start codon in order to express the fused *trxR* gene under its own promoter. By application of the same procedure, peroxiredoxin 1 (GL50803_14521) and β-giardin (GL50803_4812) were fused to a 3-fold HA-tag. Again, each gene was preceded by its own promoter (100 bp upstream sequence in case of peroxiredoxin, 50 bp in case of β-giardin). The sequences of all primers used are given in Table S1. Transfections of plasmids into WB C6 trophozoites were performed in a BTX Electro cell manipulator 600 (Harvard Apparatus, Holliston, Massachusetts) with the settings 500 V, 800 μF and 720 Ω, and transfectants were selected with puromycin (100 mg L^{-1}) as described (Leitsch *et al.* 2016).

Co-IP of proteins

Co-IP of proteins bound to TrxR was performed closely following an established protocol (Rout *et al.* 2016) using immobilized anti-HA antibodies. However, cross-linking of proteins with Lamont's reagent was omitted. For all co-IP experiments approximately 1×10^9 *G. lamblia* cells were used.

Mass spectrometric identification of proteins and data analysis

Sample preparations for mass spectrometry, mass spectrometric identification of proteins and ensuing data analyses were performed as described recently (Rout *et al.* 2016).

Immunofluorescence analysis and microscopy

A polyclonal antiserum was raised in rabbit against the peptide QMFTTTDVENFPS which is part of the *G. lamblia* TrxR polypeptide sequence (GenicBio Biotech, Shanghai, China). Before immunofluorescence microscopy, antibodies were affinity-purified as described (Hemphill and Gottstein, 1996) using recombinant *G. lamblia* TrxR, isolated from *E. coli* BL21 (DE3) (Leitsch *et al.* 2011), as bait on a nitrocellulose membrane. For visualization of peroxiredoxin and β-giardin, anti-HA antibodies from mouse were used for the detection of HA-tagged

peroxiredoxin and β-giardin in the respective transfected cell lines (see above). Samples of *G. lamblia* WB C6 trophozoites were prepared for immunofluorescence microscopy as described (Skarin *et al.* 2011) by the use of a Nikon Eclipse 80i and the software Openlab (version 5.5.2). As secondary antibody either anti-rabbit serum was used for the detection of TrxR, or anti-mouse serum for the detection of anti-HA antibody. Secondary antibodies had FITC tags.

Results

For the identification of potential thioredoxins, we searched the *Giardia* DB database for proteins that have a thioredoxin-like domain with a CXXC catalytic site and are rather small (10–20 kDa). Apart from protein disulphide isomerase 3 which has a strongly hydrophobic N-terminus and resides in the endoplasmic reticulum (Knodler *et al.* 1999), three proteins were found to match these criteria: GL50803_104250, GL50803_3910 and GL50803_9355 (Table 1). The first two proteins have a CKDC catalytic site, whereas the third thioredoxin-like protein (GL50803_9355) has a rather unusual CPPC catalytic site and is not closely related to the first two. The three candidate thioredoxins and *G. lamblia* TrxR were recombinantly expressed in *E. coli* (Leitsch *et al.* 2011) and used for TrxR enzyme assays. Surprisingly, none of the three proteins was found to be reduced by recombinant *G. lamblia* TrxR, although the enzyme was functional as indicated by the reduction of DTNB (Fig. 1A). If GL50803_3910 and GL50803_9355 were used in combination, a minimal enhancement of DTNB reduction could be observed (Fig. 1A). Thioredoxin from *T. vaginalis* (TVAG_125500) was not reduced by *G. lamblia* TrxR. Further, if NADPH was replaced with NADH reduction of DTNB was also observed albeit at a lower rate (Fig. S1). Importantly, also no reduction of the candidate thioredoxins was observed when *G. lamblia* WB C6 cell extracts were used instead of recombinant *G. lamblia* TrxR (Fig. 1B). Addition of FAD, a cofactor of TrxR, did not enhance reduction of DTNB when candidate thioredoxin GL50803_104250 was assayed (Fig. 1B). In contrast, recombinant *T. vaginalis* TrxR (Fig. 1A) efficiently reduced recombinant *T. vaginalis* thioredoxin (TVAG_125500) as described before (Coombs *et al.* 2004; Leitsch *et al.* 2009). Further, strong reduction of *T. vaginalis* thioredoxin was also observed when recombinant *T. vaginalis* TrxR was replaced with *T. vaginalis* G3 cell extracts (205 ± 40 nM min^{-1} mg^{-1}) (Fig. 1B). Since the measured activity was very similar to the one determined in an earlier study (Leitsch *et al.* 2012*a*, *b*), we concluded that our assay was fully functional and, hence, that none of the three candidate *G. lamblia* thioredoxins tested is a substrate for *G. lamblia* TrxR. In addition, it was tested if the three candidate *G. lamblia* thioredoxins could be reduced by *T. vaginalis* TrxR. Interestingly, a very small enhancement of DTNB reduction could be observed when GL50803_104250 and GL50803_3910 were used, possibly due to unspecific reduction of the disulphides in the candidate thioredoxins (Fig. 1A). The effect was quite comparable to the one observed with *G. lamblia* TrxR and GL50803_3910 and GL50803_9355 used in combination (Fig. 1A), suggesting that the latter is also due to unspecific reduction of disulphides. Finally, we also tested whether *G. lamblia* TrxR could directly reduce peroxiredoxin 1 by adding hydrogen peroxide to the assay buffer as substrate but, again, no reduction could be observed.

Since the search for a functional thioredoxin had remained unsuccessful, we performed a co-IP experiment for the isolation and identification of protein factors binding to TrxR following an established protocol (Rout *et al.* 2016). We argued that a protein with thioredoxin function would likely be isolated together with TrxR. To this end, we constructed a *G. lamblia* WB C6

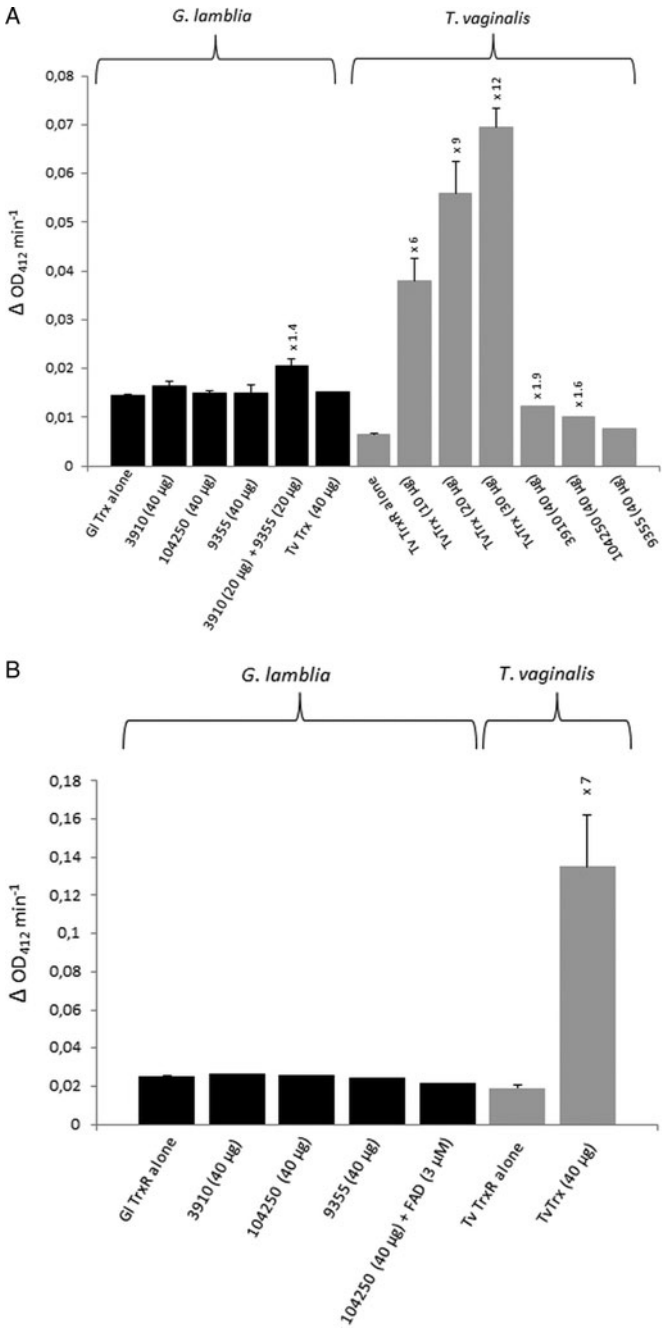

Fig. 1. (A), Reduction of DTNB (at OD_{412}) by thioredoxin reductases (TrxR) of *Giardia lamblia* and *Trichomonas vaginalis* either measured in the presence or in the absence of *G. lamblia* candidate thioredoxins GL50803_3910 (3910), GL50803_104250 (104250) and GL50803_9355 (9355) or *T. vaginalis* thioredoxin (TvTrx). The amounts of candidate thioredoxins and TvTrx used are indicated. In all reactions, 2 µg mL^{-1} TrxR were used. The increases in activity of TrxR after addition of a given candidate thioredoxin or TvTrx are indicated above the columns. Measurements with *G. lamblia* TrxR were performed twice, with the exception of the measurement in the presence of TvTrx, which was only performed once. Measurements with *T. vaginalis* TrxR were all performed three times with the exception of the measurements in the presence of GL50803_104250 (104250) and GL50803_9355 (9355), which were only performed once. Error bars indicate standard error of the mean (SEM). (B) Reduction of DTNB (at OD412) by cell extracts of *G. lamblia* and *T. vaginalis* either measured in the presence or in the absence of *G. lamblia* candidate thioredoxins GL50803_3910 (3910), GL50803_104250 (104250) and GL50803_9355 (9355) or TvTrx. The amounts of candidate thioredoxins and TvTrx used are indicated. In all reactions, 50 µg protein from extract mL^{-1} were used. The increase in activity of *T. vaginalis* TrxR after addition of TvTrx is indicated above the respective column. Measurements with *G. lamblia* extracts were performed once, with the exception of the measurement in the absence of candidate thioredoxins, which was performed twice. Measurements with *T. vaginalis* extract were performed three times. Error bars indicate SEM.

Fig. 2. Schematic presentation of the HA-tagged gene constructs used for co-IP, depicting upstream regulatory sequences (URS), the genes and the 3-fold haemagglutinin tags (3 × HA).

cell line expressing an HA-tagged TrxR (TrxR-HA) from a plasmid (Fig. 2) and isolated TrxR protein complexes from cell extracts with immobilized anti-HA antibodies. Isolated protein complexes were submitted for mass-spectrometric and bioinformatics analyses. The procedure was performed twice on two different days (for all peptides identified see Supplementary Table S2), and only proteins which were found to co-isolate with TrxR-HA in both experiments were considered as positives (Table 2). In total, 28 proteins were confirmed to bind to TrxR-HA, including seven metabolic enzymes, seven ribosomal proteins and four enzymes presumably involved in signalling. However, neither a thioredoxin-like protein nor one of the peroxiredoxins co-isolated twice with TrxR-HA. Peroxiredoxin 1 (GL50803_14521) and thioredoxin-like protein GL50803_104250, however, were each found in one of the experiments. Methionine sulfoxide reductase (GL50803_4946), an enzyme known to depend on reduction by thioredoxin (Lu and Holmgren *et al.* 2014), was also found once. As thioredoxin-like protein GL50803_104250 had been found not to be a substrate of TrxR before (Table 1), we performed another set of co-IP experiments with HA-tagged peroxiredoxin 1 (Prx-HA) (Fig. 2). Since peroxiredoxins depend on reduction by thioredoxins in order to be functional (Mastronicola *et al.* 2016), we hypothesized that a functional thioredoxin might be co-isolated with Prx-HA. As previously, only those proteins were considered as binding partners of peroxiredoxin 1 which were co-isolated twice (Supplementary Table S2). A considerably lower number of proteins, i.e. 10, co-precipitated with Prx-HA as compared with TrxR-HA (Table 3), including three metabolic enzymes, HSP 90-α (GL50803_98054) and a putative ATP-dependent p47 RNA helicase. The only protein which was co-isolated with both baits, i.e. TrxR-HA and Prx-HA, was alcohol dehydrogenase (GL50803_93358), a strongly expressed enzyme in *G. lamblia* (Leitsch *et al.* 2012*b*). Otherwise, the identified protein sets were different. Thus, the co-IP experiments gave no indication of any firm binding of TrxR or peroxiredoxin 1 to a thioredoxin or other factors commonly assigned to the thioredoxin-mediated redox system, e.g. methionine sulfoxide reductase. Given the conspicuous discrepancy with regard to the proteins co-isolated with TrxR-HA and Prx-HA, we performed immunofluorescence microscopy on WB C6 trophozoites with anti-TrxR and anti HA-antibodies in order to check whether TrxR and peroxiredoxin 1 do indeed localize to the same cell compartment. This was found to be the case as TrxR and peroxiredoxin were both found to localize to the cytoplasm (Fig. 3).

Table 2. Proteins isolated with TrxR-HA

Name	ID
Ribosomal protein S3a	GL50803_16265
Ribosomal protein L4	GL50803_17547
Ribosomal protein L7a	GL50803_17244
Ribosomal protein L10a	GL50803_1345
Ribosomal protein SA	GL50803_7766
Translation elongation factor	GL50803_13561
Fibrillarin-like pre-rRNA processing protein	GL50803_97219
Alcohol dehydrogenase	GL50803_93358
Aldose reductase	GL50803_7260
Carbamate kinase	GL50803_16453
Enolase	GL50803_11118
Fructose-bisphosphate aldolase	GL50803_11043
Ornithine carbamoyltransferase	GL50803_10311
Phosphoglycerate kinase	GL50803_90872
Alpha-3 giardin	GL50803_11683
Alpha-7·3 giardin	GL50803_114787
Kinase, NEK	GL50803_14742
Kinase, NEK	GL50803_101534
Kinase, CMGC GSK	GL50803_17625
Phosphatase	GL50803_15215
Cytosolic HSP70	GL50803_88765
Protein 21·1	GL50803_9030
Protein 21·1	GL50803_27925
Protein disulfide isomerase PDI4	GL50803_103713
Ubiquitin	GL50803_7110
WD-40 repeat protein family	GL50803_10822
Hypothetical protein	GL50803_94463
Hypothetical protein	GL50803_113133

Discussion

We aimed at the identification of functional thioredoxins in *G. lamblia*. However, the thioredoxin-like proteins tested were not functional (Fig. 1A and B) and the co-IP experiments conducted with TrxR-HA and Prx-HA did not lead to the identification of any further thioredoxin candidates. Thus, at present our understanding of the thioredoxin system in *G. lamblia* remains incomplete as there is no evidence for any protein that shuttles electrons from TrxR to peroxiredoxin or any other protein. It was clearly demonstrated that *G. lamblia* TrxR exerts disulphide reductase activity (Tejman-Yarden *et al.* 2013; Leitsch *et al.* 2016) but whether it functions as a true TrxR remains unclear. Interestingly, the impact of TrxR activity seems to be rather limited because strong overexpression of the enzyme had only little

Table 3. Proteins isolated with Prx-HA

Name	ID
Ribosomal protein S3	GL50803_7999
ATP-dependent RNA helicase p47, putative	GL50803_16376
Elongation factor 1-γ	GL50803_12102
Alcohol dehydrogenase	GL50803_93358
Pyruvate kinase	GL50803_17143
Pyruvate, phosphate dikinase	GL50803_9909
Xaa-Pro dipeptidase	GL50803_17327
Vacuolar ATP synthase subunit B	GL50803_12216
TCP-1 chaperonin subunit β	GL50803_11397
Heat shock protein HSP 90-α	GL50803_98054

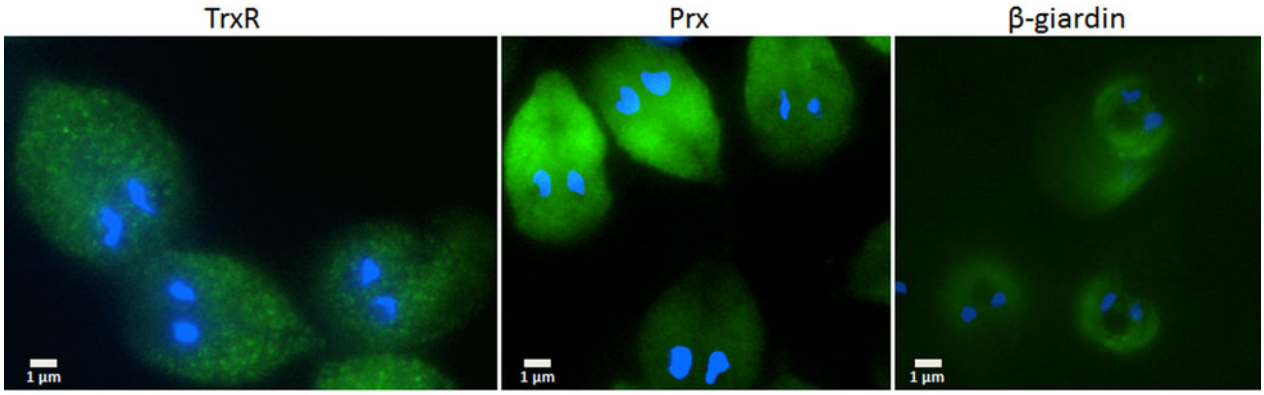

Fig. 3. Immunofluorescent microscopy images of *G. lamblia* WB C6 cells expressing TrxR (left panel), HA-tagged peroxiredoxin 1 (central panel) and HA-tagged *β*-giardin (right panel). Secondary antibodies had FITC tags and nuclei were stained with DAPI. TrxR and peroxiredoxin localize to the cytoplasm. *β*-Giardin, which localizes to the ventral disc, served as a control for the validity of the assay.

effect on the viability or physiology of *G. lamblia* (Leitsch *et al.* 2016; Müller *et al.* in preparation). Moreover, the severalfold overexpression as compared to wild-type TrxR of a mutated TrxR which lacks a functional catalytic site had no negative impact on viability (Leitsch *et al.* 2016). This is surprising, as it would have been expected that the mutated TrxR sequestered factors depending on reduction by TrxR, causing a pleiotropic phenotype. Therefore, it is possible that *G. lamblia* TrxR only functions as a disulphide reductase but is not involved in any essential protein interactions. The list of proteins co-isolated with TrxR rather supports this notion as none of the proteins, with the exception of enolase (Lemaire *et al.* 2004), have been described to be associated with the thioredoxin system. Some of the isolated proteins are known to be very highly expressed, e.g. ornithine carbamoyltransferase (unpublished data) and alcohol dehydrogenase (Leitsch *et al.* 2012*a*, *b*), or can at least be expected to be highly expressed in the proteome, e.g. HSP70 and ribosomal proteins. This implies that they could interact with TrxR in a rather unspecific manner. Further, the numbers of proteins co-isolated with TrxR-HA differed very strongly between the two experiments (59 *vs* 541), again suggesting rather unspecific interactions between TrxR and the identified proteins. Arguably, these occur *via* the reactive cysteine residues of TrxR's catalytic site making it impossible to distinguish between true physiological interactions and artefacts obtained through the co-IP procedure. The numbers of proteins co-isolated with Prx-HA varied less strongly (56 *vs* 120), but only one protein, i.e. alcohol dehydrogenase, was confirmed to bind to TrxR-HA, as well as Prx-HA. This rather argues against any close interaction of TrxR and peroxiredoxin 1 in a protein complex, although both proteins localize to the cytoplasm. Possibly, reduction of peroxiredoxin 1 by TrxR can be brought about via transient interactions only, without any other further organization of the two proteins in a complex. In contrast to this notion, however, we could not observe any reduction of Prx 1 by TrxR in an *in vitro* enzyme assay. Finally, it is interesting to note that the spatial organization of TrxR in *E. histolytica* is different where it distinctly localizes to nodules beneath the cytoplasmic membrane (Arias *et al.* 2007), suggesting a different role for the enzyme in *E. histolytica* as compared with *G. lamblia*.

To conclude, there is currently no evidence for a fully developed canonical thioredoxin-mediated redox system in *G. lamblia*, although this does not fully rule out that a hitherto undiscovered or wrongly annotated protein could function as thioredoxin. Still, the results of this study bring back into focus a recently presented hypothesis (Mastronicola *et al.* 2011) which ascribes *G. lamblia*'s confinement to the small intestine to the weakly developed antioxidant defence of this parasite. The high redox buffering capacity of the small intestine (Blau *et al.* 1999), presumably due to high concentrations of cysteine and glutathione (Mastronicola *et al.* 2011), limits the extent of oxidative stress to which *G. lamblia* is exposed, possibly rendering a fully developed antioxidant defence redundant.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/pao.2017.16.

Acknowledgements. We are grateful to our colleagues at Giardia DB for their continuous support.

Financial support. David Leitsch was supported by grant J3492 by the Austrian Science Fund (FWF). Norbert Müller was supported by grant 31003A_163230 of the Swiss National Fund. Samuel Rout was supported by grant 31-140803/1, awarded to Adrian Hehl by the Swiss National Science Fund. Britta Lundström-Stadelmann was supported by grants 31003A_141039/1 and 31003A_160108/1 of the Swiss National Fund.

Conflict of interest. None.

References

Arias DG, Gutierrez CE, Iglesias AA and Guerrero SA (2007) Thioredoxin-linked metabolism in *Entamoeba histolytica*. *Free Radical Biology and Medicine* **24**, 1496–1505.

Blau S., Rubinstein A, Bass P, Singaram C and Kohen R (1999) Differences in the reducing power along the rat GI tract: lower antioxidant capacity of the colon. *Molecular and Cellular Biochemistry* **194**, 185–91.

Coombs GH, Westrop GD, Suchan P, Puzova G, Hirt RP, Embley TM, Mottram JC and Müller S (2004) The amitochondriate eukaryote *Trichomonas vaginalis* contains a divergent thioredoxin-linked peroxiredoxin antioxidant system. *Journal of Biological Chemistry* **279**, 5249–5256.

Gillin FD and Diamond LS (1981) *Entamoeba histolytica* and *Giardia lamblia*: effects of cysteine and oxygen tension on trophozoite attachment to glass and survival in culture media. *Experimental Parasitology* **52**, 9–17.

Hemphill A and Gottstein B (1996) Identification of a major surface protein on Neospora caninum tachyzoites. *Parasitology Research* **82**, 497–504.

Keister DB (1983) Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77**, 487–8.

Knodler LA, Noiva R, Mehta K, McCaffery JM, Aley SB, Svärd SG, Nystul TG, Reiner DS, Silberman JD and Gillin FD (1999) Novel protein-disulfide isomerases from the early-diverging protist *Giardia lamblia*. *Journal of Biological Chemistry* **274**, 29805–11.

Leitsch D, Kolarich D, Wilson IBH, Altmann F and Duchêne M (2007) Nitroimidazole action in *Entamoeba histolytica*: a central role for thioredoxin reductase. *PLoS Biology* **5**, 1820–1834.

Leitsch D, Kolarich D, Binder M, Stadlmann J, Altmann F and Duchêne M (2009) *Trichomonas vaginalis*: metronidazole and other nitroimidazole drugs are reduced by the flavin enzyme thioredoxin reductase and disrupt

the cellular redox system. Implications for nitroimidazole toxicity and resistance. *Molecular Microbiology* **72**, 518–36.

Leitsch D, Burgess AG, Dunn LA, Krauer KG, Tan K, Duchêne M, Upcroft P, Eckmann L and Upcroft JA (2011) Pyruvate:ferredoxin oxidoreductase and thioredoxin reductase are involved in 5-nitroimidazole activation while flavin metabolism is linked to 5-nitroimidazole resistance in *Giardia lamblia*. *Journal of Antimicrobial Chemotherapy* **66**, 1756–1766.

Leitsch D, Drinić M, Kolarich D and Duchêne M (2012*a*) Down-regulation of flavin reductase and alcohol dehydrogenase-1 (ADH1) in metronidazole-resistant isolates of *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **183**, 177–183.

Leitsch D, Schlosser S, Burgess A and Duchêne M (2012*b*) Nitroimidazole drugs vary in their mode of action in the human parasite *Giardia lamblia*. *International Journal for Parasitology: Drugs and Drug Resistance* **2**, 166–70.

Leitsch D, Müller J, Müller N (2016) Evaluation of *Giardia lamblia* thioredoxin reductase as drug activating enzyme and as drug target. *International Journal for Parasitology: Drugs and Drug Resistance* **6**, 148–153.

Lemaire SD, Guillon B, Le Maréchal P, Keryer E, Miginiac-Maslow M and Decottignies P (2004) New thioredoxin targets in the unicellular photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the USA* **101**, 7475–80.

Lloyd D, Harris JC, Maroulis S, Biagini GA, Wadley RB, Turner MP and Edwards MR (2000) The microaerophilic flagellate *Giardia intestinalis*: oxygen and its reaction products collapse membrane potential and cause cytotoxicity. *Microbiology* **146**, 3109–18.

Lu J and Holmgren A (2014) The thioredoxin antioxidant system. *Free Radical Biology and Medicine* **66**, 75–87.

Mastronicola D, Giuffrè A, Testa F, Mura A, Forte E, Bordi E, Pucillo LP, Fiori PL and Sarti P (2011) *Giardia intestinalis* escapes oxidative stress by colonizing the small intestine: a molecular hypothesis. *IUBMB Life* **63**, 21–5.

Mastronicola D, Falabella M, Testa F, Pucillo LP, Teixeira M, Sarti P, Saraiva LM and Giuffrè A (2014) Functional characterization of peroxiredoxins from the human protozoan parasite *Giardia intestinalis*. *PLoS Neglected Tropical Diseases* **8**, e2631.

Mastronicola D, Falabella M, Forte E, Testa F, Sarti P and Giuffrè A (2016) Antioxidant defence systems in the protozoan pathogen *Giardia intestinalis*. *Molecular and Biochemical Parasitology* **206**, 56–66.

Müller J, Schildknecht P and Müller N (2013) Metabolism of nitro drugs metronidazole and nitazoxanide in *Giardia lamblia*: characterization of a novel nitroreductase (GlNR2). *Journal of Antimicrobial Chemotherapy* **68**, 1781–9.

Paget T, Maroulis S, Mitchell A, Edwards MR, Jarroll EL and Lloyd D (2004) Menadione kills trophozoites and cysts of *Giardia intestinalis*. *Microbiology* **150**, 1231–6.

Rout S, Zumthor JP, Schraner EM, Faso C and Hehl AB (2016) An interactome-centered protein discovery approach reveals novel components involved in mitosome function and homeostasis in *Giardia lamblia*. *PLoS Pathogens* **12**, e1006036.

Skarin H, Ringqvist E, Hellman U and Svärd SG (2011). Elongation factor 1-alpha is released into the culture medium during growth of *Giardia intestinalis* trophozoites. *Experimental Parasitology* **127**, 804–10.

Štefanić S, Morf L, Kulangara C, Regös A, Sonda S, Schraner E, Spycher C, Wild P and Hehl AB (2009) Neogenesis and maturation of transient Golgi-like cisternae in a simple eukaryote. *Journal of Cell Science* **122**, 2846–56.

Tejman-Yarden N, Miyamoto Y, Leitsch D, Santini J, Debnath A, Gut J, McKerrow JH, Reed SL and Eckmann L (2013) Auranofin, a reprofiled drug, is effective against metronidazole-resistant *Giardia lamblia*. *Antimicrobial Agents and Chemotherapy* **57**, 2029–35.

Watkins RR and Eckmann L (2016) Treatment of giardiasis: current status and further directions. *Current Infectious Disease Reports* **16**, 396.