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Trypanosoma cruzi tryparedoxin II interacts with different peroxiredoxins under physiological and oxidative stress conditions

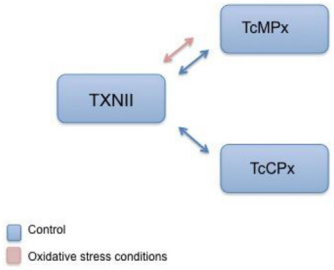
L. Dias [a, 1], E.F. Peloso [a, 1], A.F.P. Leme [b], C.M. Carnielli [b], C.N. Pereira [a], C.C. Werneck [a], S. Guerrero [c], F.R. Gadelha [a, *]

[a] Departamento de Bioquímica e Biologia Tecidual, UNICAMP, Campinas, SP, Brazil
[b] Associação Brasileira de Tecnologia de Luz Sincrotron, Laboratório Nacional de Biociências, Campinas, SP, Brazil
[c] Instituto de Agrobiotecnología del Litoral, Facultad de Bioquímica y Ciencias Biológicas (CONICET, Universidad Nacional del Litoral), Santa Fe, Argentina

HIGHLIGHTS

- TXNII interacts with TcMPx and TcCPx under physiological conditions.
- TXNII has TcMPx as an interacting partner under oxidative stress.
- TXNII interaction partners change following oxidative stress exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

Trypanosoma cruzi, the etiologic agent of Chagas disease, has to cope with reactive oxygen and nitrogen species during its life cycle in order to ensure its survival and infection. The parasite detoxifies these species through a series of pathways centered on trypanothione that depend on glutathione or low molecular mass dithiol proteins such as tryparedoxins. These proteins transfer reducing equivalents to peroxidases, including mitochondrial and cytosolic peroxiredoxins, TcMPx and TcCPx, respectively. In *T. cruzi* two tryparedoxins have been identified, TXNI and TXNII with different intracellular locations. TXNI is a cytosolic protein while TXNII due to a C-terminal hydrophobic tail is anchored in the outer membrane of the mitochondrion, endoplasmic reticulum and glycosomes. TXNs have been suggested to be involved in a majority of biological processes ranging from redox mechanisms to protein translation. Herein, a comparison of the TXNII interactomes under physiological and oxidative stress conditions was examined. Under physiological conditions, apart from the proteins with unknown biological process annotation, the majority of the identified proteins are related to cell redox homeostasis and biosynthetic processes, while under oxidative stress conditions, are involved in stress response, cell redox homeostasis, arginine biosynthesis and microtubule based process. Interestingly, although TXNII interacts with both peroxiredoxins under physiological conditions, upon oxidative stress, TcMPx interaction prevails. The relevance of the interactions is discussed opening a new perspective of TXNII functions.

* Corresponding author. Fernanda Ramos Gadelha, Universidade Estadual de Campinas, Instituto de Biologia, Departamento de Bioquímica e Biologia Tecidual, CEP: 13083-862, Campinas, São Paulo, Brazil.
E-mail address: frgad@unicamp.br (F.R. Gadelha).
[1] These authors contributed equally to this work.

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1. Introduction

Trypanosoma cruzi is the etiologic agent of Chagas disease, a neglected tropical disease, and a public health problem in 21 countries in the Americas (WHO, 2015). In non-endemic countries some cases were reported due to immigration from endemic areas (reviewed in (Gascon et al., 2010)). Chagas disease affects approximately 6–8 million people while 65 millions of people in the Americas are exposed to this disease (WHO, 2015). Current treatment, developed empirically, is not successful especially during the chronic phase of the disease, and can lead to several side effects. In the search for a more suitable target for the development of a specific therapy, rational drug design has pointed out the proteins involved in the parasite antioxidant system as good targets for this purpose.

T. cruzi is able to cope with the oxidative environments found in the hosts and ensure infection due to an efficient antioxidant system. Besides dealing with the reactive oxygen or nitrogen species (ROS or RNS, respectively) generated by the vertebrate host immune response (Tanaka et al., 1983) or the ROS coming from the degradation of heme in the invertebrate host (Nogueira et al., 2015), the parasite has also to deal with the ROS generated by its own metabolism, mainly by the mitochondrial respiratory chain. The antioxidant system centered on trypanothione ($T(SH)_2$), a molecule restricted to this family of parasites (Fairlamb et al., 1985), involves proteins and enzymes that act sequentially in distinct intracellular compartments in order to detoxify hydroperoxides (Wilkinson et al., 2000, 2002a). In the cytosolic pathway, trypanothione reductase (TR), transfers the reduced equivalents from NADPH to $T(S)_2$ that in its reduced form ($T(SH)_2$) is able to reduce glutathione disulfide to glutathione, dehydroascorbate to ascorbate and tryparedoxin (TXN) (reviewed in (Irigoín et al., 2008)). Glutathione acts as a source of reducing equivalents to glutathione peroxidases (TcGPXI and II) (Wilkinson et al., 2002a, 2002b) and ascorbate to ascorbate peroxidase (Wilkinson et al., 2002c). TXN is a source of reducing equivalents for TcGPxI, which detoxify fatty acid and phospholipid hydroperoxides (Wilkinson et al., 2002a, 2002b) or to cytosolic peroxiredoxin (TcCPx) that can metabolize H_2O_2, alkyl hydroperoxides (reviewed in (Wilkinson and Kelly, 2003)) and peroxynitrite (Piacenza et al., 2008). Another peroxiredoxin, with a mitochondrial localization (TcMPx) (Wilkinson et al., 2000; Peloso et al., 2016) metabolizes the same TcCPx's substrates (reviewed in (Wilkinson and Kelly, 2003)) and is also able to interact in vitro with TXN (Piñeyro et al., 2011a).

TXNs are low molecular weight dithiol proteins belonging to the thioredoxin oxidoreductase superfamily and use a dithiol exchange mechanism to reduce protein disulfides. Although part of the thioredoxin family, trypanosomatids TXNs are different from the other members of the family since they display a WCPPC motif, instead of WCG(A)PK, as in most thioredoxins, at their catalytic center (Hofmann et al., 2001). TXNs have been identified in various trypanosomatids with different intracellular locations and distinct roles in parasite viability. Besides their role in antioxidant mechanisms, TXNs can also aid in DNA synthesis, where the trypanothione/tryparedoxin system appears to be the main donor of reducing equivalents for the synthesis of deoxyribonucleotides (Dormeyer et al., 2001), in kinetoplast replication due to TXN interaction with the universal mini circle binding protein (Onn et al., 2004) or in the transfer of reduced equivalents to methionine sulfoxide reductase (Arias et al., 2011). Most importantly, TXN is a suitable drug target for drug development since its interaction with trypanothione is the rate-limiting step of the trypanothione-dependent pathway (Wilkinson et al., 2002b); it has a low catalytic efficiency and its interaction with tryparedoxin peroxidase and glutathione peroxidase exerts the major control of the pathway flux (González-Chávez et al., 2015).

In *Trypanosoma brucei* two TXN were identified, one in the cytosol, essential for parasite survival (Wilkinson et al., 2003; Comini et al., 2007), and another one, which appears to be nonessential to bloodstream forms (Wilkinson et al., 2003), in the mitochondrion with no obvious targeting signal and a putative transmembrane domain in its C-terminus moiety (Wilkinson et al., 2003; Comini et al., 2007). In *Leishmania infantum* the cytosolic TXN (*Li*TXN1) shares the same role in parasite viability described for the cytosolic one present in *T. brucei* (Romao et al., 2009). Besides *Li*TXN1, this parasite has two additional TXNs with a mitochondrial location; one inside the mitochondrion (*Li*TXN2), not essential throughout the parasite life cycle and another one located in the outer mitochondrial membrane (*Li*TXN3) (Castro et al., 2010). *Li*TXN3 although being unable to reduce TXN-dependent peroxidases, retains its oxidoreductase activity making it reasonable to suggest that it is involved in redox-dependent biological processes such as, protein folding assistance (Castro et al., 2010).

T. cruzi possess two TXNs constitutively expressed along its life cycle: one cytosolic (TXNI), representing 3% of the total soluble protein and sharing an identity of 51–63% to others trypanosomatids TXNs and 33% to the other *T. cruzi* TXN (TXNII) (Wilkinson et al., 2002b; Piñeyro et al., 2011b). TXNII is anchored through a C-terminal hydrofobic tail, in the membrane of the glycosome, mitochondrion outer membrane and endoplasmic reticulum (Arias et al., 2013).

Using a mutated TXNI containing a substitution in the resolving cysteine (Cys^{43}) its potential binding patterns were determined (Piñeyro et al., 2011b). The majority of the proteins found interacting with TXNI are localized (or predicted to be) in the cytosol and are involved in oxidative metabolism, cysteine and methionine related pathways and protein translation and degradation (Piñeyro et al., 2011b). In relation to the trypanothione-dependent cytosolic hydroperoxide detoxification cascade, TcCPx was found interacting with this tryparedoxin (Piñeyro et al., 2011b).

TXNII has also a trypanothione-dependent oxidoreductase activity being able to transfer reducing equivalents to TcCPx, TcMPx and also to TcGPxI (Arias et al., 2013). In relation to the kinetic parameters, TXNI and TXNII have a relatively similar $K_{m\ app}$ to TcCPx, whilst TXNII has a higher and a lower affinity to TcMPx and TcGPxI, respectively, than TXNI (Arias et al., 2013). Besides acting as a reductant to peroxidases, TXNII is also capable of transferring reducing equivalents to low-molecular mass disulfides supporting the involvement of this tryparedoxin in the antioxidant hydroperoxide detoxification pathway and in the regeneration of key metabolites (Arias et al., 2013).

Through the same approach used to determine TXNI interactome (Piñeyro et al., 2011b), 16 proteins were found interacting with TXNII. These proteins are involved in antioxidant mechanisms, energy metabolism, cytoskeleton and protein translation and located to (or predicted to be) in the cytosol. Interestingly, both TcCPx and TcMPx were found interacting with TXNII (Arias et al., 2015).

To widen our knowledge regarding TXNII interaction partners, herein without any modification in its structure, its interactome under physiological and oxidative stress conditions was determined. Interestingly, the profile of TXNII-protein interaction changes under physiological and oxidative stress conditions. A new perspective regarding the tools employed by *T. cruzi*, involving

disulfide oxidoreductases, in order to deal with the different levels of ROS encountered during its life cycle is discussed.

2. Material and methods

2.1. Culture procedure

T. cruzi transfected epimastigotes (Y strain) were grown in liver infusion tryptose (LIT) medium supplemented with 20 mg/l hemin, 10% fetal calf serum at 28 °C (Castellani et al., 1967) and 200 μg/l G418. In the *early stationary* phase, cells were collected by centrifugation (1000 × *g* at 4 °C, 5 min) washed and resuspended in phosphate-buffered saline (PBS pH 7.3). The number of cells was determined in a Neubauer chamber.

2.2. Generation of transfected parasites

The TXNII gene (GenBank AAF04973) plus a sequence encoding an N-terminal six-His tag was amplified from *T. cruzi* genomic DNA. The amplification product was cloned into the pGEM-T vector (Promega) and subcloned as an EcoRI/XhoI fragment into the trypanosomal expression vector, pTEx (Kelly et al., 1992) generating

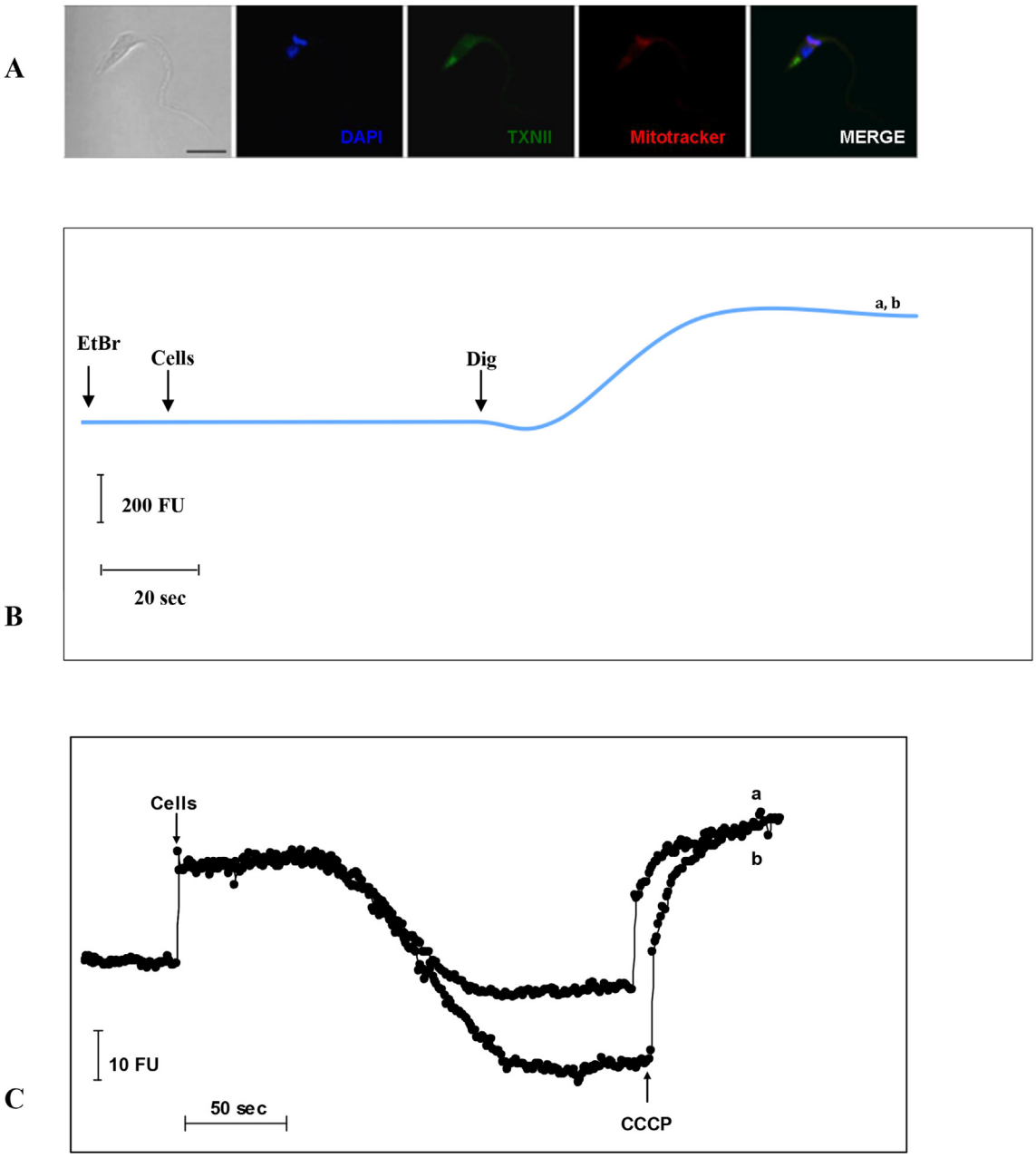

Figure 1. – Expression of His-TXNII does not alter protein distribution and upon H_2O_2-treatment plasma and mitochondrial membrane integrity is preserved. (A) Epimastigotes mitochondria were stained in vivo with the Mitotracker dye; red. After fixation and permeabilization, parasites were incubated with the anti-His antibody followed by incubation with Alexa Fluor 488-conjugated second antibody TXNII; green. Cells were mounted with ProLong Gold Antifade reagent containing DAPI; blue. Scale bar = 5 μm. **(B)** Parasites (10^7/ml) submitted to H_2O_2-treatment (a) or under control conditions (b) were added to PBS containing ethidium bromide (EtBr, 100 μM) and fluorescence was determined in a time-course reaction. Digitonin (Dig, 1.6 mM) was added as indicated by the arrow. **(C)** Parasites (10^7/ml) incubated (a) or not (b) in the presence of 200 μM H_2O_2 were added to PBS medium containing succinate (5 mM), EGTA (0.3 mM), BSA (0.003%) and Safranine O (5 μM). Digitonin (50 μM) and CCCP (1 μM) were added where indicated by the arrows. Curves in **B** and **C** are the best representative of three independent experiments performed in duplicate.

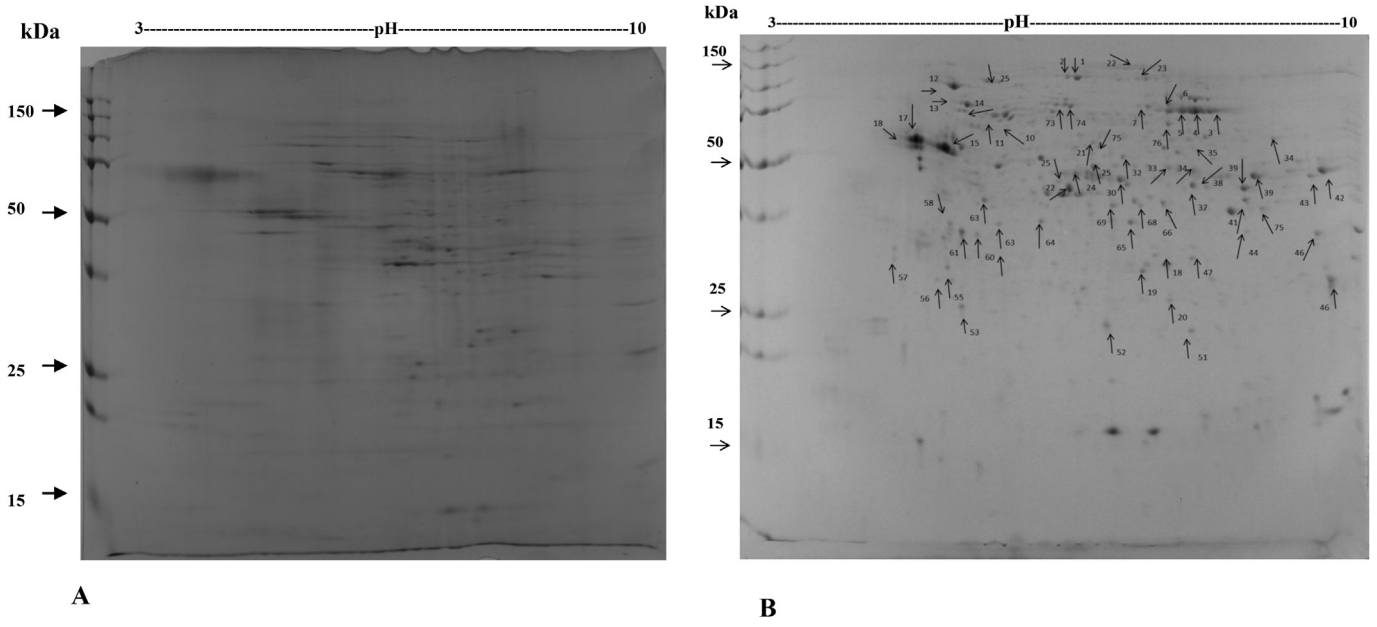

Figure 2. - Identified proteins on a representative pI 3–10 2-D gel under control conditions. Two-dimensional gel of the proteins derived from pull-down assays in cells bearing pTEx (A) or His-TXNII pTEx vector (B). The identified spots are marked. The numbered spots correspond to the identified proteins listed in Table 1.

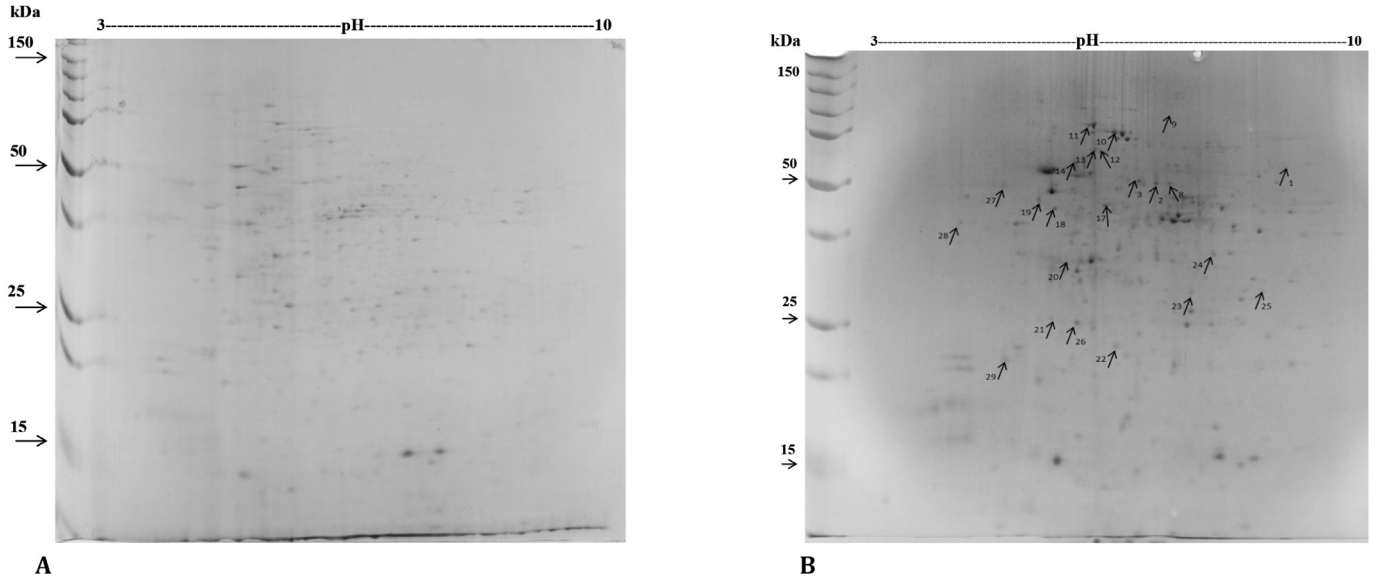

Figure 3. - Identified proteins on a representative pI 3–10 2-D gel under oxidative stress conditions. Two-dimensional gel of the proteins derived from pull-down assays in cells bearing a pTEx (A) or HisTXNII pTEx (B) vector under oxidative stress conditions. The identified spots are marked. The numbered spots correspond to the identified proteins listed in Table 2.

the His-TXNII pTEx vector. Epimastigotes were transfected with the His-TXNII pTEx or the pTEx vector and selected as previously described (Finzi et al., 2004).

2.3. H_2O_2 treatment and pull down

Epimastigotes at the early stationary phase (5.2×10^6/ml) were incubated in PBS (pH 7.3) in the presence or absence (control) of 200 μM H_2O_2 at 28 °C for 2 h. The parasites were then washed, resuspended in PBS (pH 7.3) and subjected to the pull-down procedure as described (Piñeyro et al., 2011b). Briefly, after H_2O_2-treatment or not (control), parasites were washed in PBS and resuspended in 6.5 ml of 300 mM NaCl, 1% Triton X-100, 1 mM PMSF, phosphate buffer (pH 8.0), and protease inhibitor cocktail (Sigma). After 3 freeze-thaw cycles and incubation with DNase I for 30 min at 4 °C, a centrifugation for 1 h at 6000 × *g*, was performed and the resulting supernatant applied to Dynabeads® His-Tag Isolation and Pulldown (Invitrogen). His-tagged proteins were purified according to the manufacturer's recommendations. Two sequential elutions were conducted using the same buffer described above with a second elution including 300 mM imidazole.

Table 1
Identification of TXNII interacting proteins by shotgun LC-MS/MS under control conditions

Spot #	Protein	Accession No. (GI)	Theoretical MW (Da)	Theoretical pI	Sequence coverage (%)	Matches	Protein score
54	14-3-3 protein	50512994	29371	5,03	37	17	382
37	2-amino-3-ketobutyrate coenzyme A ligase, putative	70878944	43987	6,72	38	33	789
58	60S Acidic ribosomal protein P0	70886487	35194	5,02	46	15	144
73	Aconitase, putative	70887285	99328	6,45	17	13	232
47	Aldo-keto reductase	557867556	32666	6,81	21	7	199
70	Aminopeptidase, putative	70885129	56769	6,06	48	23	502
67	Arginine kinase, putative	407844351	40443	6,29	41	24	416
41	Aspartate aminotransferase, mitochondrial, putative	70872491	46413	8,57	39	26	531
16	ATPase beta subunit, putative	407847222	56019	5,39	50	44	966
19	beta tubulin 1.9	18568139	50352	4,74	55	39	499
20	beta tubulin 1.9	18568139	50352	4,74	61	49	855
17	beta tubulin 1.9	18568139	50352	4,74	65	52	941
18	beta tubulin 1.9	18568139	50352	4,74	71	50	734
59	beta tubulin, putative	70881835	50352	4,7	53	35	499
60	beta tubulin, putative	70881835	50352	4,7	42	27	766
21	Calreticulin, putative	70877367	46354	4,77	24	28	347
44	Chaperone DNAJ protein, putative	407849598	36683	8,69	34	9	356
40	Cystathione gamma lyase, putative, partial	70882176	23959	8,36	40	16	197
30	Cystathionine beta-synthase	13241614	42651	6,37	55	40	727
68	D-isomer specific 2-hydroxyacid dehydrogenase-protein, putative	70876009	38771	6,04	32	15	367
69	D-isomer specific 2-hydroxyacid dehydrogenase-protein, putative	70876009	38771	6,04	54	30	337
46	Deoxyribose-phosphate aldolase, putative	407859367	29892	7,49	18	9	168
33	Dihydrolipoyl dehydrogenase, putative	70877058	51240	7,14	33	21	564
1	Elongation factor 2, putative	407835084	95044	5,73	44	51	486
2	Elongation factor 2, putative	70874194	95099	5,67	24	25	541
25	Enolase, putative	70885013	46871	5,92	34	21	533
14	Glucose-regulated protein 78	557866643	52998	5,09	26	14	344
11	Glucose-regulated protein 78, putative	407419687	72222	5,42	34	32	918
13	Glucose-regulated protein 78, putative	407853728	71051	5,21	19	13	265
24	Glutamamyl carboxypeptidase, putative	70867597	44191	5,98	37	17	605
26	Glutamamyl carboxypeptidase, putative	70866993	44094	6,09	30	11	372
27	Glutamamyl carboxypeptidase, putative	70866994	44212	6,24	30	15	514
28	Glutamamyl carboxypeptidase, putative	70866993	44094	6,09	34	15	332
29	Glutamamyl carboxypeptidase, putative	70866993	44094	6,09	34	17	500
43	Glutamamyl carboxypeptidase, putative	70883132	44362	8,15	43	28	209
39	Glutamate dehydrogenase, putative	70884129	45592	8,5	35	17	374
42	Glutamate dehydrogenase, putative	407411773	49247	8,26	38	27	573
50	GTP-binding nuclear protein rtb2, putative	70879067	24724	6,95	39	11	305
10	Heat shock 70 kDa protein, mitochondrial precursor, putative	407416761	71393	5,87	33	35	927
76	Heat shock protein, putative	70879011	94038	5,28	9	8	80
74	Histidine ammonia-lyase, putative	407849669	58529	8,6	23	23	415
75	Hypothetical protein TCDM_08325	557860395	30227	8,05	34	9	368
5	Hypothetical protein, conserved	70881954	73113	7,08	37	46	1243
6	Hypothetical protein, conserved	70881954	73113	7,08	37	38	1090
31	Hypothetical protein, conserved	70883016	43565	6	48	23	331
55	Hypothetical protein, conserved	70885675	28666	5,13	37	12	322
62	Hypothetical protein, conserved	70869980	41120	5,28	25	13	191
3	Hypothetical protein, conserved	70881954	73113	7,08	49	42	777
4	Hypothetical protein, conserved	70881954	73113	7,08	52	56	1632
71	Hypothetical protein, conserved	70883145	55118	6,17	24	15	292
8	Major paraflagellar rod protein	162179	69961	5,85	30	22	540
45	Malate dehydrogenase, putative	70884391	33673	8,44	49	27	782
36	Malic enzyme, putative	70883499	63048	6,55	35	26	251
49	Methylthioadenosine phosphorylase, putative	70881672	33502	6,34	62	28	658
38	N-acetylglucosamine-6-phosphate deacetylase-like protein, putative, partial	70871289	30823	9,26	30	13	222
57	NAC alpha	21435929	19564	4,66	37	6	227
65	Nucleoside phosphorylase, putative, partial	70875997	29250	8,16	59	26	552
9	Paraflagellar rod protein 3, putative	407410649	69050	5,81	8	4	133
53	Peroxiredoxin	4388655	25734	7,62	39	22	438
48	Prostaglandin F synthase	408407927	32779	6,57	30	8	250
23	Prostaglandin F2a synthase	25006239	42463	5,83	65	40	1031
66	Protein disulfide isomerase, putative	70877917	42004	6,69	24	15	469
64	Protein kinase C receptor activated, putative	70882943	35522	5,73	55	23	492
35	Pyruvate kinase 2, putative	70885978	55380	7,11	32	15	454
15	Serine carboxypeptidase	307635437	52512	5,19	46	23	259
34	Succinyl-coA:3-ketoacid-coenzyme A transferase, mitochondrial precursor, putative	70886291	53334	7,94	14	12	239
51	Superoxide dismutase, iron, putative	70873953	23511	7,08	28	8	196
7	Transketolase, putative, partial	70870034	58233	6,57	10	6	140
32	Trypanothione reductase, putative	70868650	54234	6,4	35	22	298
52	Tryparedoxin Peroxidase (txnpx)chain A In The Red State	550545525	22618	6,32	37	13	340
22	Tyrosine aminotransferase	408894	46708	5,82	40	18	321
63	Unknown	2984700	39055	5,72	35	20	475

2.4. SDS-PAGE and two-dimensional electrophoresis (2-DE)

For 2-DE, the protocol described by Peloso et al. (2016) was followed. For each experimental condition, treated or not treated with H_2O_2, two independent samples derived from the pull down from His-TXNII expressing or pTEx bearing parasites were analyzed through 2D. The data was quite reproductible were a similarity of around 94% was found between the independent gel duplicates. Afterwards, gels images were overlapped compared and only the proteins present on the His-TcTXNII pTEx gel were analyzed through mass spectrometry. Sample preparation for shotgun LC-MS/MS analysis was performed essentially as described (Peloso et al., 2016).

2.5. Indirect immunofluorescence assays

Immunofluorescence assays were performed essentially as described (Castro et al., 2002). Briefly, epimastigotes in the early stationary phase expressing the His-TXNII (10^7/mL) were stained with the mitochondrion-specific dye MitoTracker orange (Molecular Probes), fixed with 4% paraformaldehyde (w/v) in PBS, permeabilized with 0.1% (v/v) Triton X-100 in PBS and spotted onto polylysine-coated microscope slides. Parasites were then incubated with anti-His antibody (Molecular Probes). Secondary antibody was Alexa Fluor 488 anti-rabbit IgG (Molecular Probes). Cells were mounted with ProLong Gold Antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) and examined in the National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC) at UNICAMP, using a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) using an objective plan-apochromat 63x/1.4 Oil DIC M27. Images were collected using 405, 488 and 561 nm laser lines for excitation and 415–492, 499–551 and 577–691 emission filters for Dapi, Alexa Fluor 488 and Mitotracker orange fluorophores, respectively, with pinholes set to 1 airy unit for each channel, 2048 × 2048 image format, and 3.5× optical zoom.

2.6. Plasma and mitochondrial membrane integrity

In order to determine plasma membrane integrity, parasites H_2O_2-treated or not (control) (10^7/ml) were added to PBS medium containing ethidium bromide (EtBr, 100 μM) and fluorescence was measured in a Hitachi Fluorimeter 2500 at 365 and 580 nm, Ex and Em respectively. Digitonin (1.6 mM) was added where indicated by the arrow (Cohen et al., 1990).

Mitochondrial membrane integrity was determined in digitonin-permeabilized parasites (Vercesi et al., 1991). Cells (10^7/ml) submitted to H_2O_2-treatment or under control conditions were added to PBS medium containing succinate (5 mM), EGTA (0.3 mM), BSA (0.003%) and Safranine O (5 μM). Digitonin (50 μM) and CCCP (1 μM) were added as indicated. Fluorescence was measured in a Hitachi Fluorimeter 2500 at 495, 586 nm, Ex and Em, respectively.

3. Results and discussion

In order to ensure its survival and virulence, *T. cruzi* relies on its antioxidant mechanisms to deal with the ROS and RNS encountered during its life cycle. TXNII is part of this system and a range of possible interactions for this protein in vitro has been proposed (Arias et al., 2013). The aim of this work was to establish through an in vivo approach TXNII interacting partners under physiological and oxidative stress conditions, which could reflect in distinct functions performed by this tryparedoxin.

In this way, parasites expressing His-TXNII were obtained and the subcellular localization of TXNII was determined to ensure that all the protein was membrane-bound localized and was not present in the cytosol (Arias et al., 2015). In Fig. 1A, it is shown that no protein is found in the cytosol corroborating previous data, which located this protein in the outer membrane of mitochondria, glycosome and endoplasmic reticulum (Arias et al., 2015).

The next step was to ensure that not only the plasma membrane but also the mitochondrial membrane permeability were not altered by H_2O_2-treatment, which in this case could release proteins restricted to the mitochondrion in the cytoplasm. Plasma membrane integrity was checked by incubating parasites previously treated, or not, with H_2O_2, in the presence of ethidium bromide (EtBr), a nucleic acid-binding compound (Cohen et al., 1990). EtBr fluorescence depends on binding of the dye to double-stranded DNA (Cohen et al., 1990). As shown in Fig. 1B, epimastigotes submitted to H_2O_2-treatment or not (control) did not show any alteration in plasma membrane permeability. Influx of EtBr was only achieved when high concentrations of digitonin were added to the incubation medium. Interestingly, at low concentrations, digitonin has been used to selectively permeabilize the

Table 2
Identification of TXNII interacting proteins by shotgun LC-MS/MS in cells submitted to oxidative stress

Spot #	Protein	Accession No. (GI)	Theoretical MW (Da)	Theoretical pI	Sequence coverage (%)	# Peptide	Score
14	alpha Tubulin	1220545	50324	4,90	9	6	193
15	alpha Tubulin	1220545	50324	4,90	5	3	59
20	alpha Tubulin	1314208	47498	5,49	15	7	163
24	Aromatic L-alpha-hydroxyacid dehydrogenase, partial	7109725	34284	6,82	11	5	230
4	Glutamamyl carboxypeptidase, putative	70871330	44090	6,03	29	13	175
5	Glutamamyl carboxypeptidase, putative	70866993	44094	6,09	27	20	407
7	Glutamamyl carboxypeptidase, putative	70867597	44191	5,98	22	13	405
6	Glutamamyl carboxypeptidase, putative	70867597	44191	5,98	24	12	282
9	Heat shock 70 kDa protein, mitochondrial precursor, putative	70869901	71459	5,75	20	15	406
16	Heat shock protein 60 kDa	1495230	59602	5,38	5	3	47
17	Heat shock protein 60 kDa	1495230	59602	5,38	10	5	153
10	Heat shock protein 70	205278868	71478	5,32	28	33	892
11	Heat shock protein 70	50659756	71444	5,06	27	17	338
22	hslvu complex proteolytic subunit-like, putative	70874668	23038	6,77	10	5	117
26	Peroxiredoxin	4388655	25734	7,62	30	20	242
21	Proteasome alpha 5 subunit, putative	70872393	27206	4,96	39	10	396
23	Pyridoxal kinase, putative	70873094	33595	6,00	21	7	96
25	RNA-binding protein, putative	70884753	31489	6,63	16	4	106
2	Thiol transferase Tc52 (TcAc2)	537611	51049	5,60	27	16	392
3	Thiol transferase Tc52 (TcAc2)	537611	51049	5,60	17	8	78
8	Thiol transferase Tc52, partial	32395936	49056	5,42	31	14	275

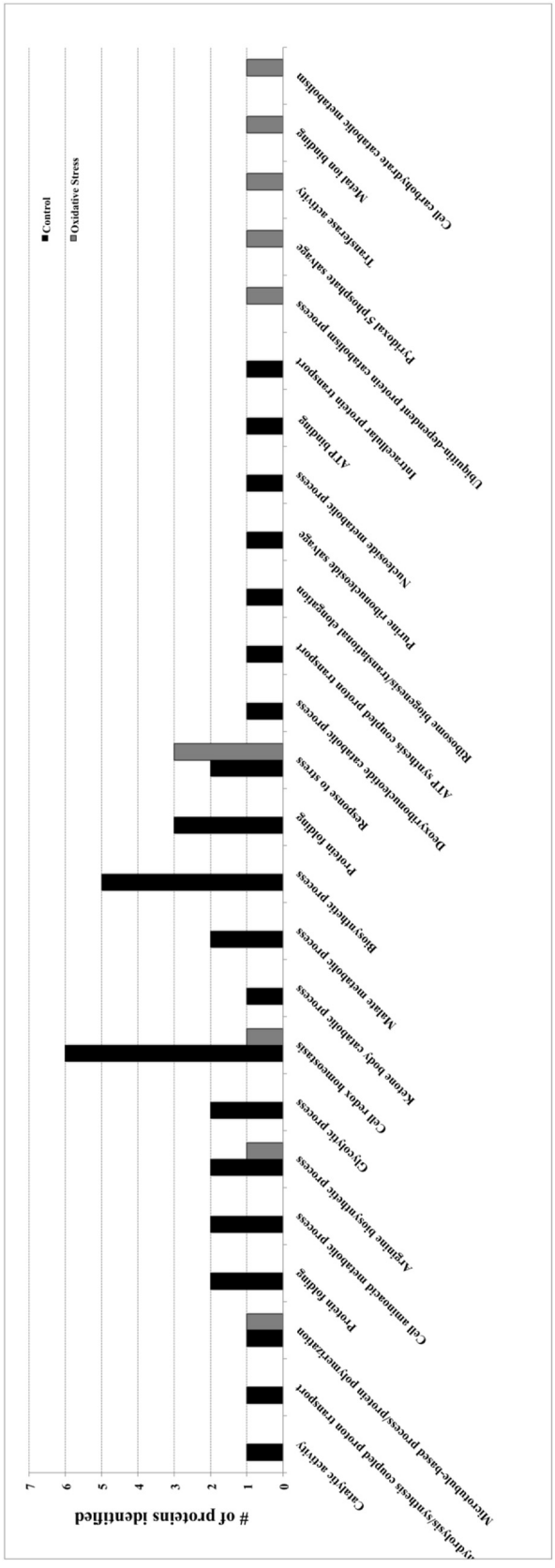

Figure 4. - Biological process annotation of the proteins that interact with TXNII under control and oxidative stress conditions. To a better view of the results, the 19 proteins with unknown biological process annotation were removed from the figure.

plasma membrane of *T. cruzi* without disturbing the functional integrity of the mitochondrion (Vercesi et al., 1991). In Fig. 1C it can be seen that mitochondrial integrity was not altered by H_2O_2-treatment since the same rate of decrease in fluorescence in H_2O_2-treated cells and control cells, due to the penetration of safranine O into the cells, was achieved. The mitochondrial membrane potential in treated cells is lower than in control cells, probably due to the oxidative stress that the parasite has to cope with. Safranine O is retained in mitochondria under both conditions and it is only released upon addition of CCCP, an uncoupler of oxidative phosphorylation leading to the conclusion that no alteration in the mitochondrial membrane permeability occurred.

Bearing in mind that there was no change in protein localization and no alteration in the plasma and mitochondrion membrane permeability, the pull down assay was performed in parasites transfected with pTEx (i.e., negative control) or His-TXNII pTEx under control or oxidative stress conditions. In both situations, the proteins detected in the pTEx were excluded when they were also found in the His-TXNII pTEx gels (Figs. 2 and 3). In this sense, only the proteins pulled down by His-TXNII were submitted to MS analyses.

Under physiological conditions, 53 proteins were found, where 6 are hypothetical and 1 unknown (Table 1, Fig. 2). Five proteins, i.e., TcCPx (spot #52), TcMPx (spot #53), D-isomer specific 2 hydroxyacid dehydrogenase protein, elongation factor 2 and β tubulin, were also identified by Arias et al. (2015) when a mutated TXNII containing a substitution in its resolving cysteine was used as bait.

TXNII is capable of interacting with TcMPx and TcCPx in vitro in a relatively similar way ($K_{m\ app} \approx$) (Arias et al., 2013), and under both experimental conditions, i.e., ours or using a mutated TXNII (Arias et al., 2015), revealed that this is also true in vivo. Besides that, since the redox active domain of TXNII is facing the cytosol, and since it was recently suggested the presence of TcMPx in the cytosol (Peloso et al., 2016), the interaction between these proteins is feasible. Other proteins identified were D-isomer specific 2 hydroxyacid dehydrogenase, i.e., lactate dehydrogenase suggested to be under redox regulation (Arias et al., 2015), and reinforced by our results; β tubulin part of the cytoskeleton and elongation factor 2, implicated in protein synthesis, a process that in rats (Ayala et al., 1996) and also in *Escherichia coli* (Zhong et al., 2015) have been shown to be inhibited by oxidative stress at the elongation step.

In trypanosomatids the sub-pellicular microtubules formed by α and β tubulin, are linked to each other and to the plasma membrane. Our results are in agreement with the ones obtained by Arias et al. (2015) using mutated TXNII, but so far the dependence on the redox state of tubulin assembly and polymerization has not been shown in *T. cruzi*. A second microtubule organization, the flagellum, together with the paraflagellar rod, also identified here, are linked to the cell body by other cytoskeleton elements and it has been suggested that it has a role in mediating cell signaling (Mattos et al., 2012).

The βsubunit of ATPase was found, whilst in the interactome of mutated TXNII, the delta subunit was identified (Arias et al., 2015). Redox regulation of ATP synthase has been described in several organisms (Wang et al., 2013). Interestingly, in the universally conserved ATPase YchF, associated with different cellular processes, such as, DNA repair and apoptosis, the redox activation is performed by a thioredoxin (Hannemann et al., 2015). These results suggest that a similar regulation could occur with *T. cruzi* ATP synthase.

Superoxide dismutase (SOD), trypanothione reductase (TR) and dihydrolipoyldehydrogenase (DHLD) identified here, were also detected in the TcMPx interactome (Peloso et al., 2016). In the case of SOD, and as discussed by Peloso and collaborators (Peloso et al., 2016) this could be due to the formation of supramolecular

complexes through a common substrate or product enhancing the efficiency of ROS detoxification. TR would be used by the peroxiredoxins to reduce hydroperoxides and also DHLD that has also been proposed to function as a reductant, although with less affinity, to *L. infantum* peroxiredoxins (Castro et al., 2008). Proteins involved in stress response, such as HSP70 were also found interacting with TXNII. These proteins are induced during *T. cruzi* differentiation, but it is not clear whether this expression is part of the differentiation process or is an epiphenomenon involved in the adaptation to the new environmental challenges (Folgueira and Requena, 2007).

Arginine kinase, an enzyme involved in trypanosomatids resistance to ROS, pH and starvation (Pereira, 2014) was also identified. It is located in the cytosol (Miranda et al., 2009) and since it is absent in the mammalian host has been pointed out as a suitable drug target (Pereira, 2014).

Another protein found as a TXNII-interacting partner was the 14-3-3 protein, which plays a broad regulatory role in cell signaling (Obsilova et al., 2014). The binding of 14-3-3 to a protein can modulate enzyme activity, subcellular location, structure, stability or the molecular interactions of the target protein (Obsilova et al., 2014).

Interestingly, under oxidative stress conditions, TXNII changes its interaction partners (Table 2, Fig. 3). Fewer proteins were identified under this condition suggesting that in an oxidative stressed cell TXNII undergoes fewer interactions compared to control conditions. In relation to the antioxidant pathways, the most striking difference is the identification of only TcMPx as an interaction partner, corroborating previously in vitro data that showed a higher affinity of TXNII to TcMPx (Arias et al., 2013). In *T. cruzi* both peroxiredoxins have their expression increased under oxidative stress conditions (Finzi et al., 2004; Peloso Ede et al., 2011) and although as stated above the $K_{m\ app}$ for TXNII and both peroxiredoxins are relatively similar, under oxidative stress conditions, the interaction with TcMPx appears to prevail. Furthermore, and regarding the change in interaction, α tubulin instead of β tubulin was found interacting with TXNII under oxidative stress conditions.

An interesting possibility to be considered is the interaction between TXNII and TcMPx during mitochondrial protein import. This reaction could envisage reduction of TcMPx in order to allow its transport through the mitochondrial membrane. This assumption is supported by the fact that in yeast cells, the cytosolic thioredoxin system keeps the small Tim proteins (intermembrane mitochondrial proteins) in reduced forms, which in turn facilitate their mitochondrial import (Durigon et al., 2012). A similar procedure could be happening to TcMPx.

Thiol transferase Tc52 (TcAc2) is another TXNII partner. This protein is overexpressed in *T. cruzi* populations resistant to benznidazol selected in vivo and induced in vitro (Murta et al., 2008). Considering the mechanism of action of benznidazole that involves the generation of an imbalance in the antioxidant defenses, it is reasonable to infer that TcAc2 increased expression also occurs under our experimental conditions. This protein shares a sequence homology to an antioxidant molecule, glutathione transferase (Ouaissi and Dubremetz, 1995).

Regarding the biological process annotation of the proteins that interact with TXNII in Fig. 4 it can be observed that under physiological conditions the majority of the proteins could not have a metabolic process associated with followed by cell redox homeostasis, biosynthetic processes, protein folding and response to stress. These results are in agreement with previous data from the literature (Arias et al., 2013, 2015) supporting the relevance of TXNII to *T. cruzi* redox-based processes and as a drug target for the development of a more specific therapy for Chagas disease treatment. When parasites were submitted to oxidative stress conditions, the majority of the proteins found interacting with TXNII are related to the stress response followed by cell redox homeostasis, arginine biosynthetic process and microtubule-based process.

In relation to the localization of the proteins found interacting with TXNII, the same profile, i.e., with an unknown localization followed by the cytoplasm/microtubule was encountered under both experimental conditions (Fig. 5). These results are supported by the fact that TXNII bears its active site towards the cytosol (Arias et al., 2013) allowing these interactions to occur.

4. Conclusions

The results obtained through an in vivo approach besides giving support to the ones obtained under in vitro conditions or using mutated TXNII also suggest novel TXNII interactions which are distinct when cells are submitted or-not to oxidative stress. The putative TXNII interacting proteins are involved in a wide range of cellular processes, highlighting the importance of TXNII for *T. cruzi* redox-based processes. The different interactomes possibly enables cell redox signaling, which could contribute for parasite adaptation to the different environmental conditions imposed by its life cycle.

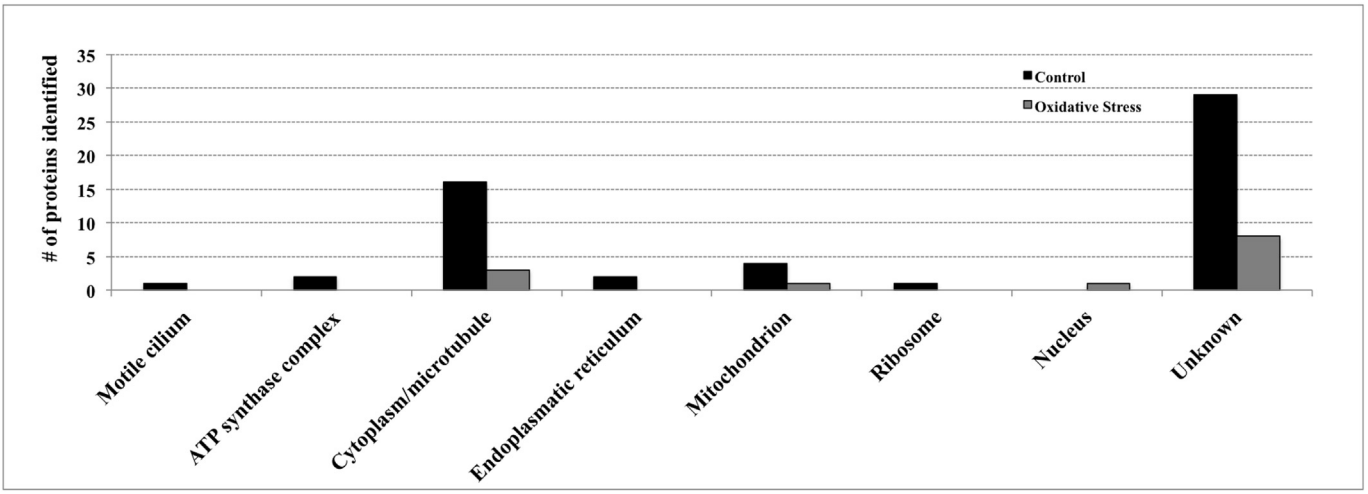

Figure 5. Intracellular localization annotation of TXNII-interacting proteins under control and oxidative stress conditions.

Interestingly, some of the putative TXNII interacting proteins found have homologues known to interact with thioredoxin, also a member of the oxidoreductase superfamily, in other organisms. One of the most interesting points raised by these results was the preferentiality of TcMPx over TcCPx under basal and oxidative stress conditions reinforcing the higher affinity of TXNII to TcMPx. More importantly, as a role the results give support to the notion that this protein could be used as a drug target for the development of a more specific therapy for Chagas disease treatment.

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