

1 **Molecular cloning and characterization of a thioredoxin from *Taiwanofungus***  
2 ***camphorata***

3 **Running title:** Characterization of thioredoxin

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17

18 ABSTRACT

19 Background: Thioredoxin (Trx) is reduced by thioredoxin reductase. Trx is used in  
20 ribonucleoide reduction, assimilatory sulfate reduction, in modulation of protein sulfhydryl  
21 groups, and refolding proteins.

22 Results: A TcTrx (Tc: *Taiwanofungus camphorata*) cDNA (640 bp, GenBank AY838902.1)  
23 encoding a putative thioredoxin (Trx) of 135 amino acid residues with calculated molecular  
24 mass of 16.17 kDa was cloned from *Taiwanofungus camphorata*. The deduced amino acid  
25 sequence containing a motif (Cys-Gly-Pro-Cys) that is highly conserved among the reported  
26 Trxs. A three dimensional structural model of the TcTrx has been created based on the known  
27 structure of *Malassezia sympodialis* Trx (MsTrx, PDB ID: 2j23). To characterize the TcTrx,  
28 the codon optimized coding region was subcloned into an expression vector and transformed  
29 into *Saccharomyces cerevisiae*. The recombinant His8-tagged TcTrx was expressed and  
30 purified by Ni affinity chromatography. The purified enzyme showed a band of approximately  
31 32 kDa (expected dimeric form) on a 12 % SDS-PAGE. The molecular mass determined by  
32 MALDI-TOF is 33.16 kDa which suggests that the purified enzyme is a dimeric enzyme.  
33 Furthermore, the enzyme exhibited TcTrx activity via insulin assay. The Michaelis constant  
34 ( $K_M$ ) value for insulin was  $3.78 \times 10^{-2}$  mM. The enzyme's half-life of deactivation was 13 min  
35 at 45°C. The enzyme was most active at pH 7.

36 Conclusions: A three dimensional structural model of *T. camphorata* Trx based on its TcTrx  
37 cDNA sequence. The active form of the TcTrx has been successfully expressed in yeast. The  
38 enzyme possesses Trx activity and is capable of reduction of disulfide bonds during the  
39 formation of newly synthesized proteins.

40 **Keywords:** *Taiwanofungus camphorata*, Thioredoxin (Trx), Three-dimension structural model,  
41 Insulin

42 **Abbreviations:** Trx, thioredoxin; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; SDS-PAGE,  
43 sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffer saline.

#### 44 **Background**

45 *Taiwanofungus camphorata* (*T. camphorata*) is a valued mushroom found only in the forests  
46 of Taiwan. It has been used for centuries as health food, among others (Ao et al., 2009). *T.*  
47 *camphorata* has been shown to have anti-inflammatory properties (Hsieh et al., 2010). The  
48 active compounds identified from the fruiting bodies of *T. camphorata* in a submerged culture  
49 are benzenoids, diterpenes, maleic and succinic acid derivatives, polysaccharides, steroids, and  
50 triterpenoids (Ao et al., 2009). It can be obtained as health supplements formulated from the  
51 mass of *T. camphorata* from the artificial cultivation by an Asian Nova Biotechnology Inc  
52 (<http://www.asian-bio.com/>) at a high market value. Many studies were aimed to find the exact  
53 bioactive compounds of the mushroom (Ao et al., 2009). In order to search for physiologically  
54 active components including antioxidant enzymes, we have established an expressed sequence  
55 tag (EST) from the fruiting bodies of *T. camphorata*. Based on the established EST, several  
56 antioxidant enzymes including a 2-Cys peroxiredoxin (Huang et al., 2007), a superoxide  
57 dismutase (Liau et al., 2007), a catalase (Ken et al., 2008), a glutathione formaldehyde  
58 dehydrogenase (Huang et al., 2009), a dithiol glutaredoxin (Ken et al., 2009), a 2-Cys  
59 peroxiredoxin isozyme (Liau et al., 2010), a monothiol glutaredoxin (Ken et al., 2011), a  
60 nitroreductase (Chen et al. 2012), a peroxiredoxin (Huang et al., 2014) and an aryl-alcohol  
61 dehydrogenase (Ken et al., 2014) have been cloned and characterized. This encourages us  
62 further to search for active components from the established EST of *T. camphorata* for potential  
63 health food applications.

64 Thioredoxin (Trx) is reduced by thioredoxin reductase with NADPH as the hydrogen

65 donor. Trx is used in refolding proteins ((Li and Churchich, 1997), in modulation of protein  
66 sulfhydryl groups (Buchanan et al., 1994) , and phage assembly (Russel et al., 1986). Here, we  
67 report a three dimensional structural model of *Taiwanofungus camphorata* thioredoxin based  
68 on its sequence. The coding sequence of the TcTrx cDNA was introduced into an *S. cerevisiae*  
69 expression system and the active enzyme purified and characterized its properties.

## 70 **Materials and methods**

### 71 **Isolation of TcTrx cDNA**

72 We have previously established an EST database from fruiting bodies of *T. camphorata* and  
73 sequenced all clones with insert size greater than 0.4 kb (data not shown). The identity of a Trx  
74 cDNA clone was assigned by comparing the inferred amino acid sequence in various databases  
75 using the basic local alignment search tool (BLAST)  
76 (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The Trx cDNA fragment was subcloned into  
77 pCR<sup>®</sup>4-TOPO<sup>®</sup> (invitrogen, CA) vector and transformed into *E. coli* TOPO10. The nucleotide  
78 sequence of the insert was determined in both strands. Sequence analysis revealed that the Trx  
79 cDNA covered an open reading frame of a putative Trx cDNA (640 bp, GenBank AY838902.1).

### 80 **Bioinformatics analysis of TcTrx**

81 The BLAST program was used to search homologous protein sequences in the nonredundant  
82 database (NRDB) at the National Center for Biotechnology Information, National Institutes of  
83 Health (<http://www.ncbi.nlm.nih.gov/>). Multiple alignments were constructed using ClustalW2  
84 program. Protein secondary structure was predicted by SWISS-MODEL program and  
85 represented as  $\alpha$  helices and  $\beta$  strands. A three dimensional structural model of TcTrx was  
86 created by SWISS-MODEL (Arnold et al. 2006) (<http://swissmodel.expasy.org/>) based on the  
87 known crystal structure of *Malassezia sympodialis* Trx (MsTrx, PDB ID: 2j23).

## 88 **Subcloning of TcTrx cDNA into an expression vector**

89 TcTrx cDNA was subcloned into an *E. coli* and yeast expression vector, respectively. The  
90 coding region of the TcTrx cDNA was amplified using gene specific flanking primers. The 5'  
91 upstream primer contains *Eco* RI recognition site (5'GAA TTC GAT GTT ATC TTC GCT  
92 TGC ATC C3') and the 3' downstream primer contains *Eco* RI recognition site (5'GAA TTC  
93 GCG AGG CCC TGG ATG AG3'). Using 0.2 µg of TcTrx cDNA as a template, and 10 pmole  
94 of each 5' upstream and 3' downstream primers, a 405 bp fragment encoding the putative  
95 mature TcTrx gene was amplified by PCR. The fragment was ligated into pCR4.0 and  
96 transformed into *E. coli*. The recombinant plasmid was isolated and digested with *Eco* RI. The  
97 digestion products were separated on a 1.0% agarose gel. The 405 bp insert DNA was gel  
98 purified and subcloned into *Eco* RI site of pET-20b(+) expression vector (Novagen, Darmstadt,  
99 Germany). The recombinant DNA was then transformed into *E. coli* C43(DE3). The  
100 recombinant protein was not expressed in the *E. coli* expression system. Furthermore, we tried  
101 to subclone into the *Eco* RI site of the pYEX-S1 expression vector (Clontech, Mountain View,  
102 CA, USA) and introduced into *Saccharomyces cerevisiae* (*trp<sup>-</sup> ura<sup>-</sup>*). The recombinant protein  
103 was not expressed in the *Saccharomyces cerevisiae* expression system. Thus the TcTrx gene  
104 was optimized based on the yeast codon usage table (The codons were optimized by using the  
105 Codon Optimization Tool provided by the Integrated DNA Technologies  
106 (<http://sg.idtdna.com/CodonOpt>) with codon usage table of *Saccharomyces cerevisiae*. The  
107 GenScript Codon Usage Frequency Table Tool was used as the reference for yeast usage table.  
108 The optimized gene was synthesized by Genomics company, Taiwan as shown in Figure 1 red  
109 color codon) and subcloned into a pET-20b(+) expression vector again for expression. The  
110 recombinant DNA was then transformed into *E. coli* C43(DE3). The recombinant protein was  
111 still not expressed in the *E. coli* expression system. We then re-amplified the codon-optimized  
112 pET-20b(+)-TcTrx DNA using two gene-specific primers: the 5' upstream primer contained

113 *Eco* RI recognition site (5'GAA TTC GAT GTT ATC TTC GCT TGC ATC C3') and the 3'  
114 downstream primer contained a His8-tag and *Eco* RI recognition site (5' GAATTC GAG ACG  
115 TCA GTG GTG GTG GTG GTG GTG GTG GTG3'). Using the 0.2 µg optimized recombinant  
116 DNA of pET-20b(+)-TcTrx as a template, and 10 pmole of each 5' upstream and 3' downstream  
117 primers, a 0.4 kb fragment was amplified by PCR. The fragment was ligated into pCR4.0 and  
118 transformed into *E. coli*. The recombinant plasmid was isolated and digested with *Eco* RI. The  
119 digestion products were separated on 1.0% agarose gel. The 0.4 kb insert DNA was gel purified  
120 and subcloned into the *Eco* RI site of the pYEX-S1 expression vector and introduced into  
121 *Saccharomyces cerevisiae* (*trp<sup>-</sup> ura<sup>-</sup>*). The transformed yeast cells were selected by YNB<sub>5</sub>  
122 (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose) agar plates containing  
123 20 µg Trp/mL. The presence of TcTrx cDNA in the selected transformants was verified by PCR  
124 using gene-specific flanking primers. The recombinant TcTrx protein was expressed in yeast  
125 in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Expression of the functional  
126 recombinant TcTrx was analyzed by enzyme activity assay.

### 127 **Expression and purification of the recombinant TcTrx**

128 The yeast transformant containing the TcTrx gene was grown at 30°C, 170 rpm in 100 mL  
129 of YPD medium for 18 h. The cells were harvested and the soluble proteins extracted in PBS  
130 (phosphate buffer saline) with glass beads as described previously (Ken et al. 2005). The  
131 recombinant TcTrx was purified by Ni-NTA affinity chromatography (elution buffer: 1× PBS/5  
132 % glycerol/1 mM DTT containing 20-250 mM imidazole) according to manufacturer's  
133 instruction (Qiagen). The purified protein was analyzed by a 12% SDS-PAGE followed by  
134 staining with Coomassie Brilliant Blue R-250 and destaining with 10% acetic acid/10%  
135 methanol. The purified protein was pooled, desalted, and buffer exchanged using Amicon ultra  
136 centrifugal filter unit (5000 MWCO). The exchanged buffer exchanged was 0.01×PBS/ 0.01  
137 mM DTT/2.5 mM imidazole/0.1% glycerol. The final recombinant TcTrx protein concentration

138 was determined by a Bio-Rad Protein Assay Kit (Richmond, CA). For storage, one volume of  
139 glycerol was added to the purified protein and stored at -20 °C for further analysis.

#### 140 **Molecular mass analysis via JOEL MALDI-TOF**

141 The purified recombinant TcTrx (0.82 µg/µL) was prepared in 0.01×PBS containing 0.01  
142 mM DTT, 2.5 mM imidazole and 0.1% glycerol. The sample (5 µL) was used for molecular  
143 mass determination using an MALDI-TOF mass spectrometer (JMS-S3000, Japan).

#### 144 **TcTrx activity assay**

145 Trx activity was assayed by the method of Holmgren (Holmgren and Reichard, 1967;  
146 Holmgren, 1979a, b) using the insulin precipitation assay which was monitored by a  
147 spectrophotometric assay of the increase in turbidity at 650 nm. The reaction mixture (200 µL)  
148 at 25°C contained 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.33 mM DTT, 15 mM  
149 NADPH, 0.025 mM insulin and 1.0 µg TcTrxR (thioredoxin reductase from *T. camphorata*,  
150 Huang et al., 2010). The reaction was started by the addition of 1.0 µg TcTrx (0.41 µg/µL).  
151 The reaction was followed by the increase in  $A_{650}$  due to insulin precipitation on reduction.

#### 152 **Kinetic studies**

153 The kinetic properties of the TcTrx (1.0 µg) was determined by varying the concentrations  
154 of insulin (0.025~0.055 mM). The change in absorbance at 650 nm was recorded between 30  
155 and 60 sec. The  $K_M$ ,  $V_{max}$  and  $k_{cat}$  were calculated from Lineweaver-Burk plots.

#### 156 **Enzyme characterization**

157 The TcTrx enzyme was tested for stability in terms of its activity under various conditions.

158 Aliquots of the TcTrx sample (1.0 µg) were treated as follows: (1) *Thermal effect*. Each enzyme  
159 sample (1.0 µg) was heated at 40, 50, or 60°C for 5 min. Then samples were checked for TcTrx  
160 activity: 80% residual activity at 40°C treatment, 40% residual activity at 50°C treatment.  
161 Therefore, we choose at 45 °C heating to this enzyme effect, each enzyme sample (1.0 µg) was  
162 heated at 45 °C for 2, 4, 8, 16 min. (2) *pH effect*. Each enzyme sample (1.0 µg) was adjusted  
163 to desired pH by adding a half volume of buffer with different pHs: 0.2 M citrate buffer (pH  
164 4.0), 0.2 M phosphate buffer (pH 6.0, 7.0 or 8.0) or 0.2 M CAPS buffer (pH 10.0). Each sample  
165 was incubated at 37 °C for 1 h. At the end of each treatment, samples were checked for TcTrx  
166 activity by insulin precipitation assay at pH 7.

## 167 **Results and discussion**

### 168 **A three dimensional structural model of *Taiwanofungus camphorata* thioredoxin based on** 169 **its TcTrx cDNA sequence**

170 A putative TcTrx cDNA (640 bp) clone was identified on the basis of the consensus pattern  
171 and sequence homology to other published Trxs in NCBI database. The entire coding region of  
172 TcTrx cDNA is 405 bp and the deduced protein consists of 135 amino acid residues with a  
173 calculated molecular mass of 16.2 kDa (accession no. GenBank AY838902.1). The TcTrx gene  
174 was optimized based on yeast codon usage table as shown Fig. 1 red color codon. Fig. 2 shows  
175 the optimal alignment of the amino acid sequences of the TcTrx with five selected Trx  
176 sequences from other sources. This TcTrx shared 63% similarity with TvTrx (*Trametes*  
177 *versicolor*, accession no. EIW63845.1), 62% similarity with DsTrx (*Dichomitus squalens*,  
178 accession no. EJJ67406.1), 58% similarity with PsTrx (*Punctularia strigosozonata*, accession  
179 no. EIN14269.1), 57% similarity with MsTrx (*Malassezia sympodialis*, accession no.



180 AJ937746.1, PDB ID: 2j23), and 42% similarity with SmTrx (*Schistosoma mansoni*, accession  
181 no. 2XBI\_A). The deduced amino acid sequence containing highly conserved active site motif  
182 (Cys<sup>62</sup>-Gly-Pro-Cys<sup>65</sup>) (Limacher, et al., 2007). The two Cys residues are the key to the ability  
183 of thioredoxin to reduce other proteins. A three dimensional structural model of the TcTrx  
184 (purple) has been created based on the known structure of *Malassezia sympodialis* Trx (MsTrx,  
185 PDB ID: 2j23). The RMSD is 0.95 Å. The highly conserved active motif is denoted in yellow  
186 underline (Fig. 2A). The secondary structure, predicted by SWISS-MODEL program, showed  
187 4  $\alpha$  helices and 6  $\beta$  strands. Superimposition with SmTrx (PDB ID: 2XBI, white) was shown  
188 using protein solid ribbons. Putative active residues are shown in yellow (Fig. 2B).

### 189 **Expression and purification of the recombinant optimized TcTrx**

190 The optimized coding region for the TcTrx (405 bp) was amplified by PCR and subcloned  
191 into an expression vector, pYEX-S1 as described in the Materials and Methods. Positive clones  
192 were verified by DNA sequence analysis. The recombinant TcTrx was expressed, and the  
193 proteins were analyzed by a 12% SDS-PAGE in the absence of reducing agent and without  
194 boiling (Fig. 3). The recombinant TcTrx was expressed as a His<sub>8</sub>-tagged fusion protein and was  
195 purified by affinity chromatography with nickel chelating Sepharose. A major band with  
196 molecular mass of ~35 kDa (expected size of TcTrx dimer) was detected in Ni-NTA eluted  
197 fractions by SDS-PAGE (Fig. 3, lanes 8-9). Analysis of the TcTrx by MALDI-TOF MS  
198 confirms the presence of a single protein with molecular mass of 33154 Da. This indicates that  
199 the enzyme is predominantly dimeric in nature. As shown in Fig.2, from the point of the  
200 structure that Cys62 and Cys65, we assume that the Trx favors formation of dimers through  
201 intermolecular disulfide linkages. This assumption is supported by our SDS-PAGE analysis of  
202 the purified protein in the absence of  $\beta$ -mercaptoethanol, the dimeric form is predominant (see  
203 Fig. 3, lanes 8-9). The dimers may be linked by two disulfide bonds between Cys62-Cys65 and  
204 Cys65-Cys62, or between Cys62-Cys62 and Cys65-Cys65. The dimers may also be linked by

205 one disulphide bond between Cys62 and Cys65, or Cys62-Cys62, or Cys65-Cys65 (Chae et al,  
206 1994; Liau et al., 2010). The yield of the purified His8-tagged TcTrx was 82 µg from 100 mL  
207 of culture. Functional TcTrx was detected by activity assay as describe below.

### 208 **Kinetic studies of the purified TcTrx**

209 As shown in Fig. 4, the Lineweaver-Burk plot of the velocity (1/V) against 1/insulin gave  
210 the  $K_M$ ,  $k_{cat}$  and  $k_{cat}/K_M$  values were  $3.7 \times 10^{-2}$  mM,  $1.2 \times 10^{-2}$  min<sup>-1</sup>,  $3.1 \times 10^1$  min<sup>-1</sup> mM<sup>-1</sup>.  
211 Comparison of the  $K_M$  with that of Trx from other sources (Table1) reveals that *T. camphorata*'s  
212  $K_M$  is several fold larger. The result indicating that the TcTrx works at higher substrate  
213 concentration. But the  $k_{cat}$  value is similar to that of Trx from other sources.

### 214 **Properties of the purified TcTrx**

215 The TcTrx enzyme was shown to possess Trx activity by its ability to reduction insulin. Heat  
216 stability of the TcTrx was tested to examine the effect of heat on the Trx activity as described  
217 in the Materials and Methods. We found 80% and 40% residual activity when the enzyme was  
218 treated at 40°C and 50°C for 5 min, respectively. The enzyme activity found at 25 °C was  
219 defined as 100 % activity. The enzyme (1.0 µg) was further heated for various time intervals at  
220 45°C. The enzyme activity decreased as the heating time increased (Fig. 5). The enzyme's half-  
221 life of deactivation was 13 min at 45°C. In Fig. 6, the TcTrx is most activity under pH 7.0.

### 222 **Conclusion**

223 A three dimensional structural model of *T. camphorata* Trx based on its TcTrx cDNA sequence.  
224 This study reported the first cloning and expression of an important reduction enzyme, TcTrx,  
225 from *T. camphorata*. The active form of the TcTrx has been successfully expressed in yeast.  
226 The enzyme possesses Trx activity and is capable of reduction of disulfide bonds during the  
227 formation of newly synthesized proteins.

228 The TcTrx has a higher  $K_M$  value (Table 1) therefore can work under higher substrate  
229 concentration. Its  $k_{cat}$  value is compatible to that of Trx from another source. It is likely that the  
230 Trx is one of the enzymes responsible for reducing protein disulfide targets in *T. camphorata*.

### 231 **Competing interest**

232 The authors declare that they have no competing interests.

### 233 **Authors' contributions**

234 P-FH carried out the molecular genetic studies and biochemical studies. C-TL was an adviser  
235 to carry out the molecular genetic and biochemical studies. LW and Y-TC participated in  
236 drafting the manuscript.

237 C-TL and LW drafted the manuscript and revised the manuscript. All authors  
238 read and approved the final manuscript.

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307 .

### 308 **Legends**

309 **Table 1.** Kinetic characterization of TcTrx and that from other sources.

310 **Fig. 1.** Nucleotide sequences of TcTrx cDNA and its codon usage optimization based on the

311 yeast codon usage table.

312 The codon optimization of TcTrx was shown above with red codon.

313 **Fig. 2.** Alignment of the amino acid sequences of TcTrx with other organism's Trx and three

314 dimensional structural model. (A) Sequence alignment: TcTrx (this study), TvTrx (*Trametes*

315 *versicolor*, EIW63845.1), DsTrx (*Dichomitus squalens*, EJF67406.1), PsTrx (*Punctularia*

316 *strigosozonata*, EIN14269.1), MsTrx (*Malassezia sympodialis*, AJ937746.1), SmTrx

317 (*Schistosoma mansoni*, 2XBI\_A). Identical amino acids in all sequences are shaded black,

318 conservative replacements are shaded gray. The highly conserved motif is denoted in yellow

319 underline (Fig. 1A) predicted by SWISS-MODEL program and represented as  $\alpha$  helices and  $\beta$

320 strands. (B) A three dimensional structural model of TcTrx. The structural model of the TcTrx

321 was created based on the known crystal structure of *Malassezia sympodialis* (MsTrx, PDB ID:

322 2j23) via SWISS-MODEL program. Superimposition of TcTrx (purple) and SmTrx (white) was  
323 shown using protein solid ribbons. Yellow structure denotes active site..

324 **Fig. 3.** Expression and purification of recombinant TcTrx in *Saccharomyces cerevisiae*. Fifteen  
325  $\mu$ L (loading buffer without mercaptoethanol and without boiling) of each fraction was loaded  
326 into each lane of the 12% SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining.  
327 Lane 1, crude extract from *Saccharomyces cerevisiae* expressing TcTrx; 2, flow-through  
328 proteins from the Ni-NTA column; 3, wash; 4-9, TcTrx eluted from Ni-NTA column. Molecular  
329 masses (in kDa) of standards are shown at left.

330 **Fig. 4.** Double-reciprocal plot of varying insulin on TcTrx activity. The initial rate of the  
331 enzymatic reaction was measured with change in absorbance at 650 nm between 30 and 60 sec  
332 with the insulin varied from 0.04~0.15 mM. The  $K_M$ ,  $k_{cat}$  and  $k_{cat}/K_M$  were calculated from  
333 Lineweaver-Burk plots.

334 **Fig. 5.** Effect of temperature on the purified TcTrx. The enzyme sample (1.0  $\mu$ g) was heated at  
335 45 °C for 2, 4, 8, 16 min. At the end of each treatment, samples were assayed for TcTrx activity  
336 at pH 7.0.

337 **Fig. 6.** Effect of pH on the purified TcTrx. Aliquots of the enzyme sample were incubated with  
338 different pH buffers at 37 °C for 30 min and then assayed for TcTrx activity. Data are means of  
339 three experiments.

340





342 **Table 1.** Kinetic characterization of TcTrx and that from other sources.

343

Protein	Substrate	$K_m$ (mM)	$k_{cat}$	$k_{cat}/K_m$
TcTrx	insulin	$3.7 \times 10^{-2}$	1.2	$3.1 \times 10^1$
<i>E. coli</i> Trx	insulin	$1.1 \times 10^{-2}$		
Dv Trx	insulin	$6.3 \times 10^{-3}$		
Dr Trx	insulin	$5.7 \times 10^{-3}$	1.1	$1.9 \times 10^2$

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345 Values are from TcTrx (this study), *Escherichia coli* Trx (*E. coli* Trx) ([Holmgren, 1979a](#)),

346 *Desulfovibrio vulgaris* Trx (Dv Trx) ([Pieulle et al., 2011](#)) and *Deinococcus radiodurans* Trx

347 (Dr Trx) ([Obiero et al., 2010](#)).

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