Title

Biochemical and physiological analyses of NADPH-dependent thioredoxin reductase isozymes in *Euglena gracilis*

Author names and affiliations

Shun Tamaki ^a, Takanori Maruta ^{a,c}, Yoshihiro Sawa ^a, Shigeru Shigeoka ^{b,c},
Takahiro Ishikawa ^{a,c,*}

^a Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, 1060 Nishikawatsu, Matsue, Shimane 690-8504, Japan

^b Department of Advanced Bioscience, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan

^c Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Chiyoda-ku, Tokyo 102-0076, Japan

*Corresponding author. Tel/Fax: +81-852-32-6580.

E-mail addresses: ishikawa@life.shimane-u.ac.jp (T. Ishikawa)

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Abbreviations: APX, ascorbate peroxidase; dsRNA, double-stranded RNA; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EF1 α , elongation factor 1 α ; FTR,

ferredoxin-dependent thioredoxin reductase; GC-MS, gas chromatography-mass spectrometry; KD, knockdown; NTR, NADPH-dependent thioredoxin reductase; Prx, peroxiredoxin; RNAi, RNA interference; RNA-Seq, RNA sequencing; ROS, reactive oxygen species; TMHMM, transmembrane helices based on a hidden Markov model; Trx, thioredoxin

Abstract

At least four peroxiredoxins that are coupled with the thioredoxin (Trx) system have been shown to play a key role in redox metabolism in the unicellular phytoflagellate *Euglena gracilis*. In order to clarify Trx-mediated redox regulation in this alga, we herein identified three NADPH-dependent thioredoxin reductases (NTRs) using a homologous search and characterized their enzymatic properties and physiological roles. Each *Euglena* NTR protein belonged to the small, large, and NTRC types, and were named EgNTR1, EgNTR2, and EgNTRC, respectively. EgNTR2 was phylogenetically different from the known NTRs in eukaryotic algae. EgNTR1 was predicted to be localized in mitochondria, EgNTR2 in the cytosol, and EgNTRC in plastids. The catalytic efficiency of EgNTR2 for NADPH was 30~46-fold higher than those of EgNTR1 and truncated form of EgNTRC, suggested that large type EgNTR2 reduced Trx more efficiently. The silencing of *EgNTR2* gene expression resulted in significant growth inhibition and cell hypertrophy in *Euglena* cells. These results suggest that EgNTRs function in each cellular compartment and are physiologically important, particularly in the cytosol.

1. Introduction

Thioredoxins (Trxs) are small proteins (~12 kDa) that act as redox regulators in numerous cellular processes including metabolism, the synthesis of DNA, photosynthesis, respiration, and protein folding and

repair [1]. Since Trxs are able to reduce thiol-containing peroxidases, peroxiredoxins (Prxs), they also play crucial roles in the metabolism of reactive oxygen species (ROS) as well as cellular redox regulation and other metabolic functions including the regulation of gene expression and molecular chaperone [2,3]. The reducing power of Trxs is normally provided by NADPH in a reaction catalyzed by NADPH-dependent Trx reductase (NTR), which together form the so-called Trx system. NTR has been found in all types of organisms from bacteria to plants and animals. Two major classes of NTRs have been identified to date: (i) small type NTRs with a low molecular mass (~35 kDa), which are found in prokaryotes and some eukaryotes, and (ii) large type NTRs with a high molecular mass (~55 kDa) in some eukaryotes such as animals and protists [4]. Photosynthetic organisms such as plants and algae have the enzyme NTRC, which is a bimodular enzyme formed by an NTR and Trx module in the same polypeptide [5].

Among photosynthetic organisms, *Arabidopsis* NTRs have been characterized in detail. *Arabidopsis* NTRs are encoded by three genes and are distributed in three distinct cellular compartments: most NTRA are in the cytosol, most NTRB are in mitochondria, and NTRC exclusively exists in plastids [5,6]. The growth of an *Arabidopsis ntra ntrb* double knockout mutant was previously reported to be slower [7]. The growth of an *Arabidopsis ntrc* knockout mutant was also found to be inhibited and it also exhibited hypersensitivity to different abiotic stresses, such as oxidative, drought, and salt stresses [5]. Genetic and biochemical analyses using an

Arabidopsis ntrc knockout mutant suggested that NTRC regulated the biosynthesis of starch and chlorophyll [8,9]. These findings indicate that NTRs are key regulators in various biological processes in plants. In contrast, although NTR genes have been identified in a few algal species such as *Chlorella vulgaris* and *Emiliania huxleyi* [10,11], their physiological significance in algae has not yet been established.

Euglena gracilis is a motile unicellular flagellate that can grow by photosynthesis and possesses an unusual cell membrane complex, the pellicle complex, but not a cell wall [12]. Metabolism in this organism was previously exhibited to be unique. For example, *Euglena* accumulates a large amount of paramylon (β -1,3 glucan) under aerobic conditions [13]. Under anaerobic conditions, *Euglena* synthesizes medium-chain wax esters from paramylon by a unique metabolic process called wax ester fermentation [14]. We have been studying the molecular mechanisms underlying cellular redox regulation, including ROS metabolism, and their physiological roles in stress responses in *Euglena*. *Euglena* lacks catalase, but contains a single ascorbate peroxidase (APX), which is only localized in the cytosol. In contrast to APX in higher plants, the *Euglena* enzyme can reduce both H_2O_2 and alkyl hydroperoxides [15]. Furthermore, the activities of monodehydroascorbate, dehydroascorbate, and glutathione reductase have only been detected in the cytosol [16]. These findings indicate the physiological significance of an ascorbate-dependent antioxidant system in the cytosol in *Euglena*. We recently identified four genes encoding Prx proteins in *Euglena*. *Euglena* Prxs were predicted to be localized in the cytosol, chloroplasts, and

mitochondria. All enzymes exhibited the reduction activities of H₂O₂ and alkyl hydroperoxides. Knockdown (KD) experiments suggested that cytosolic Prx isoforms were essential for the normal growth of *Euglena* cells [17]. These findings provided important evidence that the Trx-dependent antioxidant system is also a crucial regulator of cellular redox states in this alga. However, the roles of the Trx-dependent redox system(s) in ROS metabolism and other physiological processes remain largely unknown because Trx and NTR have not yet been identified in this organism.

We herein attempted to clarify Trx-dependent redox regulation in *Euglena* by searching for putative *NTR* genes. Three *NTR* genes were identified based on *Euglena* RNA-Seq data (Ishikawa et al., unpublished data), and named *EgNTR1*, *EgNTR2*, and *EgNTRC*. The enzymatic analysis of recombinant EgNTRs indicated that EgNTR2 had the highest catalytic efficiency, likely due to its molecular structure and catalytic mechanism of large type NTR. KD experiments suggested that EgNTR2 plays a key role in the homeostasis of cell growth and size in *Euglena* cells. To the best of our knowledge, this is the first study to have established the physiological importance of NTRs, especially in the cytosol, in eukaryotic microalga.

2. Materials and methods

2.1. Strain and culture

Euglena gracilis strain Z was grown in Koren-Hutner (KH) medium for heterotrophic growth [18] or Cramer-Myers (CM) medium for autotrophic growth [19] under continuous light conditions (50 mol m⁻² s⁻¹) at 26 °C with

rotary shaking (120 rpm). Regarding the anaerobic treatment of the cells, the cultures were completely sealed and stood for 24 h without shaking after the replacement of air with nitrogen gas. Cell number and volume were determined using the electric field multi-channel cell counting system, CASY (Roche Diagnostics).

2.2. Construction of expression plasmids of recombinant *Euglena* NTRs

Total RNA was isolated from *Euglena* cells, as previously described [17]. First strand cDNA was synthesized using PrimeScript RT Master Mix (Takara) according to the manufacturer's instructions. The open reading frames of *Euglena* NTRs were amplified from first strand cDNAs using the following primer sets; EgNTR1-F (5'-GAGCTCATGTCCAAGCTGCTGC-3'), EgNTR1-R (5'-AAGCTTTCAGGGCTCGCCGT-3'), EgNTR2-F (5'-CATATGACGTCATACGACTATGACTACG-3'), EgNTR2-R (5'-AAGCTTTTAGCCGCACTTGC-3'), EgNTRC -F (5'-GAGCTCCAATTGTTTTTCGGG-3'), and EgNTRC -R (5'AAGCTTCTAGTACTCGATGAGCAGC-3'). The amplified DNA fragments were ligated into the pGEM-T easy vector (Promega) to confirm the absence of PCR errors. The resulting constructs were digested with Sac I and Hind III for EgNTR1 and EgNTRC , and Nde I and Hind III for EgNTR2, and were ligated into the expression vector pCold II (Takara) to produce His-tagged proteins. The resulting plasmids were introduced into *Escherichia coli* strain BL21 Star cells (Agilent Technologies).

2.3. Expression and purification of recombinant proteins

E. coli strain BL21 Star cells transformed with each pCold II/EgNTR were grown in 3 mL of LB medium containing 50 g mL⁻¹ of ampicillin. After an overnight culture at 37 °C, the cultures were transferred to 600 mL of LB medium with the ampicillin and grown to an A₆₀₀ of 0.5. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a concentration of 0.5 mM, and the cells were incubated for 20 h at 15 °C. All the buffers used for recombinant protein purification were prepared using metal ion-free water. The harvested cells were resuspended in 100 mM potassium phosphate buffer, pH 7.0, and were sonicated. His-tagged recombinant EgNTR proteins were purified using a TALON Metal Affinity resin (Clontech) according to the manufacturer's instructions. Protein contents were determined following the method of Bradford [20]. The purified enzymes were then desalted and concentrated using an ultrafiltration membrane (Amicon® Ultra-4, Millipore), and stored at -20 °C until later use.

2.4. Enzyme assay

NTR activity was determined by the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) according to the method described by Pascual et al. [21]. The reaction was performed in 100 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 5 mM DTNB, 150 M NADPH, and purified enzymes at the concentrations indicated in the table legends. The reduction of DTNB was monitored by increases in absorbance at 412 nm.

2.5. RNA interference (RNAi) experiments

The KD of EgNTRs by RNAi was performed as described previously [17]. Approximately 500-bp partial cDNA templates of EgNTRs with the T7 RNA polymerase promoter (underlined in the primer sequences below) were amplified using the following primer sets EgNTR1/RNAi-F (TAATACGACTCACTATAGGGGCTTTTCCAAGGGCATC), EgNTR1/RNAi-R (TAATACGACTCACTATAGGGTCACCTTCACACCGGTC), EgNTR2/RNAi-F (TAATACGACTCACTATAGGGAAGAAGCTGATGCACTACGC), EgNTR2/RNAi-R (TAATACGACTCACTATAGGGTTTCTCGGAGCACTGGC), EgNTRC/RNAi-F (TAATACGACTCACTATAGGGTTACAACACAGCGGTGGAAG), and EgNTRC/RNAi-R (TAATACGACTCACTATAGGGGCGACTCGTGGTACAGTTTC).

Double-stranded RNA (dsRNA) was synthesized from PCR products as templates using the MEGAscript RNAi Kit (Life Technologies) according to the manufacturer's instructions. *Euglena* cells from seven-day-old cultures grown in CM medium were harvested and resuspended in CM medium. *Euglena* cells grown in KH medium for two days were used for heterotrophic conditions. Approximately 15 g of dsRNA was introduced into the cell suspension (100 L; approximately 5×10^6 cells) using the NEPA21 electroporator (Nepa Gene). The cell suspension was inoculated into fresh medium and cultured at 26 °C for restoration.

2.6. RT-PCR analysis

Total RNAs were isolated from 2-week-old *Euglena* cells into which dsRNA were introduced. Gene silencing was then confirmed by RT-PCR using RNAi primer sets as described above (see 2.5). EgEF α was chosen as a control using the following primers; EgEF1 α -F (5'-GCCTCTTCGCCTCCCACTG-3'), and EgEF1 α -R (5'-TTCATCAGGACAATCGCAGCA-3'). PCR amplification was performed with 22–34 cycles of 95 °C for 30s, 58 °C for 30s, and 72 °C for 40s. The PCR products were analyzed on 1% agarose gels. Equal loading of each amplified gene sequence was determined with the control EgEF1 α PCR product.

2.7. Microscopy analysis

Cells cultured for 32 days after the introduction of dsRNA were observed. Images were recorded using an Olympus BX51 upright microscope through a 40 $\times$ objective lens (Olympus).

2.8. Paramylon determination

Paramylon was extracted from *Euglena* cells according to the method described by Sugiyama et al. [22]. *Euglena* cells harvested by centrifugation were freeze-dried. After being suspended in acetone, the cells were sonicated and paramylon was harvested. To remove lipids and proteins, this rough paramylon was treated at 100 °C for 30 min with 1% SDS solution. Paramylon was obtained after further centrifugation, and, following

repeated washing with 0.1% SDS solution and H₂O, refined paramylon was obtained. Paramylon was solubilized with 0.5 N NaOH and determined by the phenol-sulfuric acid method.

2.9. Wax ester determination

The extraction of the total wax ester fraction from *Euglena* cells was performed according to the method described by Inui et al. [14]. *Euglena* cells were harvested, freeze-dried, and added to 2.4 mL of a mixture of chloroform, methanol, and water in the ratio 10:20:8 (v/v/v). After thorough agitation, the mixture was centrifuged to remove cells and debris. The extraction was repeated, and the combined supernatants were added to 1 ml each of chloroform and water, followed by vigorous shaking. After centrifugation, the chloroform phase was evaporated and dissolved in hexane. The wax ester extract was filtrated using the PTFE 0.22 μ m filter for the gas chromatography-mass spectrometry (GC-MS) analysis. The wax ester fraction was separated and determined using GCMS-QP2010 (Shimadzu). Separation was performed on an Agilent J&W DB-5ms column (30 m $\times$ 0.25 mm internal diameter, 0.25 μ m film thickness; Agilent Technologies). A 1- μ L portion of the wax ester fraction was injected into GC-MS using a splitless injection; helium was used as the carrier gas (1.16 mL/min). Chromatographic separation was initially set at 100 °C (1 min), then the temperature was increased to 280 °C (10 °C per min), and held for 10 min. The mass transfer line and ion source were at 250 °C. Myristyl myristate was detected with electron ionization (70 eV) in the selected ion monitoring

mode at m/z 229.2 and 57.1 for the quantitative analysis.

3. Results and Discussion

3.1. Identification of *NTR* genes in *Euglena*

Three *NTR* genes of *Euglena* were identified by homology search in *Euglena* RNA-Seq data (Ishikawa et al., unpublished data). The cDNA sequence of each *EgNTR* gene had a unique spliced leader sequence, which was a common short sequence usually found at the 5' end of *Euglena* transcripts [23], indicating that these cDNA sequences were full-length cDNAs.

NTRs are generally classified into three representative subclasses such as small and large types and NTRC as described in [introduction](#). Thus, based on the known classification, the three cDNAs were named *EgNTR1*, *EgNTR2*, and *EgNTRC* (accession numbers AB853316, AB853317, and AB853318, respectively). Sequence comparisons of *EgNTRC* with known NTRCs revealed that *EgNTRC* was a bimodular enzyme formed by an NTR module and Trx module [5]. As shown in Supplementary Table S1, although no Trx module was found in *EgNTR1*, it showed significant identity (54%) with *EgNTRC*. Since the NTR module of NTRC typically belongs to the small type, it was suggested that *EgNTR1* was classified as the small type. This was also supported by *EgNTR1* and *EgNTRC* both being highly identical (53~62%) to known small type NTRs and NTRCs. In contrast, *EgNTR2* had 50~59% identity with large type NTRs, but low identity (15~25%) with small type NTRs and NTRCs. Thus, it was suggested that *EgNTR2* was classified

as the large type.

The sequence alignments of *Euglena* NTRs with the known NTRs were shown in Figure 1. EgNTR1 and EgNTRC included the conserved Cys residues (Cys142 and -145 in EgNTR1, and Cys327 and -330 in EgNTRC) in the active site. These sequences also had the sites for binding NADPH and FAD [24]. Moreover, EgNTRC contained the conserved Cys residues (Cys570 and -573) in the Trx module active site. EgNTR2 had the conserved Cys residues (Cys52 and -57) in the active site, found in known large type NTRs [1]. Among the large type NTRs, NTRs from mammals and eukaryotic algae have been shown to have a Se-Cys at the C-terminus, but not in malaria parasites [25]. The C-terminal sequence of EgNTR2 lacked a Se-Cys, which corresponded to that of the parasites (*Plasmodium falciparum* and *P. vivax*). The phylogenetic tree showed that EgNTR1, EgNTR2, and EgNTRC were close to small, large, and NTRC type clades, respectively (Fig. 2). EgNTR2, in particular, was very close to NTRs from malaria parasites, but not from eukaryotic algae. Thus, the phylogenetic analysis suggested that NTRs from *Euglena* and the apicomplexa phylum including malaria parasites shared a common ancestor.

Predictions of the existence of transit peptides in the deduced amino acid sequences of *Euglena* NTRs were performed using the TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) and TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) programs. The TMHMM program showed that EgNTRC had three transmembrane domains at the N-terminal region (Supplementary Fig. S1C). Since *Euglena* is a secondary symbiont, its

chloroplasts are surrounded by three layer envelopes, unlike chloroplasts from plants. Thus, the plastid-targeting signal is completely different from that in plants, and, as for the signal, two transmembrane helices were shown to exist at the N-terminal region of plastid-targeted proteins [26]. In agreement with the localization of NTRCs from plants, it was suggested that EgNTRC was localized in plastids. The TargetP program is not suitable for predicting plastid-targeting signals in *Euglena*. The third transmembrane domain of EgNTRC, which contains a conserved FAD binding site, is likely irrespective of import into plastids. The TargetP program predicted that EgNTR1 was localized in mitochondria (Supplementary Fig. S1D). In most cases, *Euglena* mitochondria-targeted proteins contain an arginine-rich motif within hydrophobic amino acids at the N-terminus [27]. Since 10 N-terminal amino acids of EgNTR1 are rich in arginine, methionine, and leucine (Supplementary Fig. S1E), EgNTR1 appeared to be localized in mitochondria. The existence of a characteristic organelle-targeting peptide in EgNTR2 was not predicted by either program, suggested the localization of EgNTR2 in the cytosol. The calculated molecular masses of the full-length forms of EgNTR1 and EgNTR2, and predicted mature EgNTRC were 34.6, 53.2, and 51.8 kDa, respectively.

3.2. Enzymatic characterization of recombinant *Euglena* NTRs

To examine the enzymatic properties of EgNTRs, each recombinant EgNTR protein was expressed in *E. coli* and purified to apparent homogeneity, as judged by SDS-PAGE (Supplementary Fig. S2). While

EgNTR1 appeared to have a putative very short mitochondria-targeting signal-like peptide, recombinant EgNTR1 was expressed as a full-length form. Recombinant EgNTRC was expressed as a truncated form without both the plastid-targeting peptide and Trx domain, and was named EgNTRC. NTR activity was assayed by the reduction of DTNB. All recombinant EgNTRs showed significant DTNB reduction activities. To test the electron donor specificity of recombinant EgNTRs, enzyme activities were measured using NADPH and NADH as electron donors. Using NADPH, the specific activities of EgNTR1, EgNTR2, and EgNTRC were 0.92, 7.32, and 0.55 mol min⁻¹ mg⁻¹ protein, respectively (Table 1). By contrast, the specific activities of EgNTR1 and EgNTR2 using NADH were markedly lower, or not detectable, and the specific activity of EgNTRC was 36% using NADPH. These results suggested that electron donor affinities differed between EgNTRC and other EgNTRs.

The kinetic properties of recombinant EgNTRs were examined. As shown in Table 2, the $V_{\max}$, K_m , and k_{cat} values of EgNTR2 for NADPH were determined to be 7.02 mol min⁻¹ mg⁻¹ protein, 5.6 M, and 383 min⁻¹, respectively. The K_m and k_{cat} values of EgNTR2 for DTNB were determined to be 2.5 mM, and 642 min⁻¹, respectively. These kinetic properties were similar to those of *P. falciparum* NTR [28,29] in consistent with their molecular structures. The K_m and k_{cat} values of EgNTR1 for NADPH were determined to be 22.4 M, and 35.5 min⁻¹, while those of EgNTRC were 24.7 M, and 58.0 min⁻¹, respectively, indicating the similar kinetic parameters of both enzymes for NADPH. On the other hand, the K_m value of EgNTRC for

NADH was determined to be 80.6 μ M. Considering the fact that the K_m values of wheat NTR and rice NTRC for NADH were 0.6 mM, and 1.2 mM, respectively [5,30], EgNTRC appeared to have higher affinity for NADH than NTRs from plants. However, the catalytic efficiencies (k_{cat}/K_m) of EgNTRC for NADPH and NADH were determined to be 3.95×10^4 and $2.48 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$, respectively; therefore, the physiologically dominant electron donor of EgNTRC appeared to be NADPH. Although EgNTR1 was predicted to be localized in mitochondria, this preferably used NADPH over NADH at least *in vitro*. This property was similar to that of *Arabidopsis* mitochondrial AtNTRB [31]. The K_m and k_{cat} values of EgNTR1 for DTNB were determined to be 2.4 mM and 52.9 min^{-1} , respectively, while those of EgNTRC were 11.9 mM and 73.5 min^{-1} , respectively. The K_m value of EgNTRC was approximately 5-fold higher than that of EgNTR1, and this might be attributed to deletion of the Trx module in EgNTRC. The catalytic efficiency of EgNTR2 ($1.22 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$) for NADPH was 30~46-fold higher than those of EgNTR1 and EgNTRC ($2.65\sim3.95 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$), and may have been caused by structural and catalytic differences between EgNTR2 and other EgNTRs. Together, our findings demonstrated that all EgNTRs can act as NTRs, and the large type EgNTR2 can reduce DTNB more efficiently than the small type NTRs, EgNTR1 and EgNTRC. Since most species, except for eukaryotic algae, contain either small or large type NTRs, the enzymatic characterization of EgNTRs will provide important insights into the molecular evolution of NTRs.

3.3. Physiological role of *Euglena* NTRs

To examine the physiological role of *Euglena* NTRs, KD cell lines (KD-*ntr1*, -*ntr2*, and -*ntrc*) were generated by electroporational dsRNA transfer into *Euglena* cells grown autotrophically. The suppression of individual *EgNTR* gene expression in each KD-*ntr* cell was confirmed by an RT-PCR analysis. As shown in Figure 3A, corresponding gene expression in all KD-*ntr* cells was specifically suppressed. The growth rate of KD-*ntrc* cells was similar to control cells, while those of KD-*ntr1* and -*ntr2* cells were significantly lower than that of control cells (Fig. 3B). The growth of KD-*ntr2* cells stopped approximately 12 days after the introduction of dsRNA.

Although NTRC is responsible for various chloroplastic processes including defense against oxidative stress [32], growth rates were not affected by the suppression of *EgNTRC* expression in *Euglena* cells. This result suggests a functional compensation by other reductase(s). Ferredoxin-dependent Trx reductase (FTR) is found in photosynthetic organisms, and functions as a chloroplastic Trx reductase during the light period when reduced ferredoxin is available [33]. A putative *FTR* gene existed in our *Euglena* RNA-Seq data (data not shown). Therefore, the FTR gene may mimic NTRC functions in KD-*ntrc* cells. In addition, another reason to consider is that, in contrast to plants, the photosynthesis of *Euglena* is not susceptible to H₂O₂ as a result of the resistance of thiol-enzymes, like fructose-1,6-bisphosphatase, phosphoribulokinase, sedoheptulose-1,7-bisphosphatase, and NADP⁺-glyceraldehyde-3-phosphate dehydrogenase, in the Calvin cycle to H₂O₂ [34]. Therefore, the result

obtained by the suppression of *EgNTRC* expression seems to be reasonable.

The cell volume of KD-*ntr2* cells was unexpectedly 3.4-fold higher than that of control cells for 32 days after the introduction of dsRNA. The cell volume of KD-*ntr1* cells also increased slightly, while that of KD-*ntrc* cells was similar to that of control cells (Fig. 4). These results suggested that EgNTRs, especially EgNTR2, play a key role in the homeostasis of cell growth and size in *Euglena* cells. To understand whether the redox perturbation in the cytosol leads to cell hypertrophy in more detail, we checked the cell volume of *Euglena* cell lines with reduced expression of *APX*, *Prx1*, and *Prx4*, which act as cytosolic peroxidases [15,17]. The results obtained showed that cell volumes in KD-*apx* and -*prx1/4* cells remained unchanged (data not shown). Thus, it is likely that EgNTRs regulate cell size irrespective to ROS levels in the cytosol. In eukaryotic cells, Trx system also regulates a key enzyme of DNA synthesis, ribonucleotide reductase, by dithiol/disulfide exchange reaction [35]. Thus, cell hypertrophy in KD-*ntr2* cells may be due to the inhibition of DNA synthesis. Further analysis will be necessary to explain the molecular mechanism underlying the phenomenon in KD-*ntr2* cells.

Euglena converts paramylon, a storage β -1,3 glucan, into medium-chain wax esters after switching from aerobic to anaerobic conditions. This metabolic process is referred to as wax ester fermentation because of the simultaneous net synthesis of ATP [14]. Since oxygen sensing is one of the key factors for wax ester fermentation, the relationship between wax ester fermentation and redox regulation was also examined. As the

results, the contents of paramylon and myristyl myristate (C14:0-C14:0) in heterotrophically grown KD-*ntr* cells were similar to those in control cells under both aerobic and anaerobic conditions (Supplementary Fig. S3), strongly suggesting that Trx systems did not contribute to the control of wax ester fermentation.

In conclusion, we identified three genes encoding NTR enzymes in *Euglena*, and characterized these enzymes. Unlike the well-characterized NTRs from animals and plants, *Euglena* exhibited the phylogenetic diversity of NTRs. An enzymatic analysis indicated that all EgNTRs were biologically functional enzymes, and EgNTR2, which belongs to large type NTRs, had the highest catalytic efficiency of the three EgNTRs. Silencing experiments showed that the suppression of *EgNTR2* gene expression affected growth rates and cell volumes, suggesting that EgNTR2 plays a key role in the homeostasis of cell growth and size in *Euglena* cells.

ROS metabolism in trypanosomatids, which belong to the protist phylum Euglenozoa as well as *Euglena*, depends on an alternative redox system consisting of tryparedoxin, trypanothione (N¹,N⁸-bis(glutathionyl) spermidine), and trypanothione reductase, but not a Trx system [36]. Trypanothione has been detected in some parasites, except for trypanosomatids, [37, 38]. It is worth mentioning that a previous study demonstrated the existence of trypanothione reductase in *Euglena* [39]. A putative trypanothione reductase gene, the deduced amino acid sequence of which contains the reported peptide sequence (KMGATIRDFYTTIGVH), was identified in our *Euglena* RNA-Seq data (data not shown). *Euglena* is a

fascinating model for research on the evolution of both Trx- and trypanthione-dependent pathways. Furthermore, this study revealed that *Euglena* had the NTRC type enzyme found in only photosynthetic organisms, suggesting the dual aspects of *Euglena* thiol redox systems as photosynthetic organisms and protists. Therefore, further analysis needs to fully understand the significance to earn different photosynthetic organisms and protists-specific thiol redox systems in *E.gracilis*.

Acknowledgments

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Figure legends

Fig. 1 Sequence alignments of *Euglena* NTR proteins with known NTRs

Partial sequence alignments of (A) EgNTR1 and EgNTRC with small type NTRs from *Arabidopsis thaliana* (AtNTRA, Q39242; AtNTRC, O22229), *Saccharomyces cerevisiae* (ScNTR1, P29509), and *Chlamydomonas reinhardtii* (CrNTRC, A8HNQ7) and (B) EgNTR2 with large type NTRs from *Plasmodium falciparum* (PfNTR, Q25861), *P. vivax* (PvNTR, A5K6V2), *Homo sapiens* (HsNTR1, B2R5P6), and *C. reinhardtii* (CrNTR1, A8J448). Letters shown in white on a black background represent conserved Cys and Se-Cys residues in the active site. FAD and NADPH binding sites are highlighted in dark gray and light gray, respectively. Asterisks indicate the amino acids conserved in all sequences, and colons and dots indicate amino acids with similar biochemical characteristics.

Fig. 2 Phylogenetic relationship between NTRs from *Euglena* and other organisms

The phylogenetic tree was constructed with full-length NTR amino acid sequences using the ClustalW program. Large type NTRs were divided into clades with or without Se-Cys at their C-terminal region. Abbreviations and UniProt accession numbers for the NTR orthologues are as follows: At, *A. thaliana* (AtNTRA, Q39242; AtNTRB, Q39243; AtNTRC, O22229); Cr, *C.*

reinhardtii (CrNTR1, A8J448; CrNTR2, A8I208; CrNTR3, A8JFV6; CrNTRC, A8HNQ7); Cv, *Chlorella vulgaris* (CvNTRC, B9ZYY5); Sc, *S. cerevisiae* (ScNTR1, P29509; ScNTR2, P38816); Hs, *H. sapiens* (HsNTR1, B2R5P6; HsNTR2, Q9NNW7); Pf, *P. falciparum* (PfNTR, Q25861); Pv, *P. vivax* (PvNTR, A5K6V2).

Fig. 3 Effects of EgNTR suppression on cell growth

(A) Transcript levels of *EgNTR1*, *EgNTR2*, *EgNTRC*, and *EgEF1 α* (P14963; for constitutive control) in the control and each KD-*ntr* cell. The control represented cells electroporated without dsRNA. A RT-PCR analysis was performed using specific primers for *EgNTR1*, *EgNTR2*, *EgNTRC*, and *EgEF1 α* with cDNA preparations from the control and each KD cell. (B) Growth rates of the control and each KD cell. Cells were grown autotrophically under continuous light (50 mol m⁻² s⁻¹) at 26°C for 32 days. Values are the mean $\pm$ SD (n = 3). Values with asterisks were significantly different, according to the ANOVA ($p < 0.05$).

Fig. 4 Effects of EgNTR suppression on cell morphology

(A) Cell volumes of the control and each KD-*ntr* cell. Values are the mean $\pm$ SD (n = 3). Values with asterisks were significantly different, according to the ANOVA ($p < 0.05$). (B-E) Photographs of (B) control, (C) KD-*ntr1*, (D) KD-*ntr2*, and (E) KD-*ntrc* cells. Photographs showed cells cultured for 32 days. Scale bars are 20 μ m.

Tables

Table 1 Electron donor specificity of recombinant *Euglena* NTRs

Enzyme	NADPH	NADH
	(mol min ⁻¹ mg ⁻¹ protein)	
EgNTR1	0.92 ± 0.03	0.02 ± 0.01
EgNTR2	7.32 ± 0.11	n.d.
EgNTRC	0.55 ± 0.02	0.20 ± 0.02

NTR activities toward DTNB were measured using 150 M NADPH or NADH as the electron donor. Values are the mean ± SD (n=3). n.d.; not detectable.

Table 2 Kinetic parameters of recombinant *Euglena* NTRs

Enzyme	Substrate	$V_{\max}$ ($\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (M)	k_{cat} (min^{-1})	k_{cat} / K_m ($\text{M}^{-1} \text{sec}^{-1}$)
EgNTR1	NADPH	0.98 ± 0.06	22.4 ± 2.1	35.5 ± 2.3	2.65×10^4
EgNTR2	NADPH	7.02 ± 1.35	5.6 ± 2.2	383 ± 74	1.22×10^6
EgNTRC	NADPH	1.50 ± 0.09	24.7 ± 3.4	58.0 ± 3.4	3.95×10^4
	NADH	0.31 ± 0.03	80.6 ± 8.9	11.9 ± 1.0	2.48×10^3
Enzyme	Substrate	$V_{\max}$ ($\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat} / K_m ($\text{M}^{-1} \text{sec}^{-1}$)
EgNTR1	DTNB	1.45 ± 0.22	2.4 ± 0.5	52.9 ± 7.9	3.77×10^2
EgNTR2	DTNB	11.8 ± 0.9	2.5 ± 0.3	642 ± 50	4.33×10^3
EgNTRC	DTNB	1.90 ± 0.20	11.9 ± 1.7	73.5 ± 7.7	1.03×10^2

NAD(P)H and DTNB were used at a concentration in the range of 2.5-150 M and 0.5-20 mM , respectively. Recombinant EgNTR1 and EgNTRC were added at a concentration of 0.4 M , and EgNTR2 at 0.02 M . Values are the mean $\pm$ SD (n=3).

Fig. 1

[illegible]

Fig. 1

Fig. 2

A **B**

Fig. 3

Fig. 4

Fig. 4