Identification of Novel Nuclear Targets of Human Thioredoxin 1*

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The dysregulation of protein oxidative post-translational modifications has been implicated in stress-related diseases. Trx1 is a key reductase that reduces specific disulfide bonds and other cysteine post-translational modifications. Although commonly in the cytoplasm, Trx1 can also modulate transcription in the nucleus. However, few Trx1 nuclear targets have been identified because of the low Trx1 abundance in the nucleus. Here, we report the large-scale proteomics identification of nuclear Trx1 targets in human neuroblastoma cells using an affinity capture strategy wherein a Trx1C35S mutant is expressed. The wild-type Trx1 contains a conserved C32XXC35 motif, and the C32 thiol initiates the reduction of a target disulfide bond by forming an intermolecular disulfide with one of the oxidized target cysteines, resulting in a transient Trx1–target protein complex. The reduction is rapidly consumed by the donation of a C35 proton to the target molecule, forming a Trx1 C32-C35 disulfide, and results in the concurrent release of the target protein containing reduced thiols. By introducing a point mutation (C35 to S35) in Trx1, we ablated the rapid dissociation of Trx1 from its reduction targets, thereby allowing the identification of 45 putative nuclear Trx1 targets. Unexpectedly, we found that PSIP1, also known as LEDGF, was sensitive to both oxidation and Trx1 reduction at Cys 204. LEDGF is a transcription activator that is vital for regulating cell survival during HIV-1 infection. Overall, this study suggests that Trx1 may play a broader role than previously believed that might include regulating transcription, RNA processing, and nuclear pore function in human cells. Molecular & Cellular Proteomics 13: 10.1074/mcp.M114.040931, 3507–3518, 2014.

Oxidative stress and redox signaling imbalance have been implicated in the development of neurodegenerative diseases and tissue injuries (1). One of the most common features observed in the neuronal tissues of patients with Alzheimer or Parkinson disease is the accumulation of misfolded proteins with oxidative post-translational modifications (2). Cells have evolved to utilize diverse defense mechanisms to counter the detrimental impact of oxidative post-translational modifications, including the engagement of the thioredoxin (Trx) family of proteins, which includes cytosolic Trx1 and mitochondrial Trx2 in mammalian cells.

Trxs are evolutionarily conserved antioxidants found in a variety of organisms, including bacteria, yeast, plants, and mammals. Trx is an oxidoreductase enzyme containing a dithiol-disulfide active site. The Trx1 reduction of oxidized protein thiol groups is coupled with the oxidation of Trx1, and the oxidized Trx1 is recycled by Trx reductase. NADPH is required as an electron donor during the reduction by Trx reductase. In mice, the loss of Trx1 results in an early stage embryonic-lethal phenotype, whereas transgenic mice overexpressing Trx1 are more resistant to a variety of oxidative stresses, including infection and inflammation (3) and focal ischemic brain damage (4). Interestingly, the median life span of the Trx transgenic mice was extended by up to 135% relative to that of the controls (5). Among redox regulatory proteins, Trx1 is a unique reducing enzyme because it regulates only specific cysteines within select target proteins. Because Trx1 itself has a limited ability to scavenge reactive oxygen species, the biological functions of Trx1 must be mediated by its target proteins. Trx1 has been shown to regulate DNA synthesis; interact with proteins related to oxi-
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dative stress, cell proliferation, and apoptosis, such as TXNIP (6), ASK1 (7), glucocorticoid receptor (8), and SENP1 (9); and facilitate nerve growth factor-mediated neurite outgrowth (10). Trx1 overexpression in rat cardiomyocytes revealed that Trx1 has a dual role as both an antioxidant and a signaling molecule involved in the development of cardiac hypertrophy (11). In E47 cells, the knockdown of Trx1 partially sensitizes the cells to oxidative stress via the ASK-1 and JNK1 signaling pathways (12). In addition to being a cytosolic disulfide reductase in the cytosol, Trx1 also translocates to the nucleus and regulates the functions of specific nuclear proteins (2), including nuclear factor κ B (NFκB) (13), activator protein 1 (13), and histone deacetylase 4 (14). In contrast to the broad understanding of Trx1 in regulating cytosolic signal transduction pathways (15–17), the significance of Trx1 in the nucleus is well documented but poorly understood (18), perhaps because of the relatively transient association of Trx1 with its targets and the relatively low levels of nuclear targets.

In this study, we identified novel human nuclear Trx1 targets using a substrate-trapping mutant of Trx1. In the past, proteomics approaches have been used to identify Trx1 targets. Fu et al. used the isotope-coded affinity tag approach and identified more than 50 cardiac Trx1 targets from a transgenic mouse model overexpressing Trx1 (15). More recently, Benhar et al. coupled the biotin switch technique with stable isotope labeling by amino acids in cell culture (SILAC) to identify 46 substrates of Trx1-mediated denitrosylation (19). However, only a small number of the Trx1 targets identified in the previous global proteomics studies have been nuclear proteins. Nuclear proteins that are sensitive to oxidative post-translational modifications and Trx1 reduction are likely to play important roles in maintaining neuronal cell functions in the presence of oxidative stress during aging and neurodegenerative diseases. The identification of novel nuclear Trx1 targets could lead to the discovery of key transcription factors, RNA processing enzymes, and nuclear structural components that are important for cell survival and an effective anti-stress response. We previously optimized a method to enrich the nuclear proteins from neuronal cells for proteome-wide studies (20). In this study, in order to obtain Trx1 target protein complexes stable enough for the proteomic identification of Trx1 targets, we expressed a Trx1C35SS mutant engineered to contain an additional nuclear targeting signal in SH-SY5Y neuroblastoma cells. This enabled us to efficiently affinity capture the nuclear proteins linked to Trx1 via stable mixed disulfides. We hypothesized that more Trx1 targets would be released from the protein complexes associated with Trx1C35SS rather than the wild-type Trx1 (Trx1WT) with β-mercaptoethanol. Using a subtractive proteomics approach, we identified 45 putative human nuclear Trx1 targets among the 1500+ affinity-captured proteins. Many of the putative targets are involved in the regulation of RNA transport and processing, nuclear protein import and export, nuclear pore function, and cell signaling. To validate the effectiveness of this approach, we analyzed both known and newly discovered nuclear Trx1 targets via Western blotting. Here, we report the functional validation of PSIP1 as a Trx1-regulated transcriptional regulator; Trx1 directly reduces PSIP1 at Cys 204. PSIP1 is best known as a host protein involved in HIV integration. The interaction between PSIP1 and HIV integrase is under intense study, and PSIP1 is currently being considered as a target for the development of novel anti-HIV therapies. Elevated Trx1 has also been reported in the plasma of HIV-infected patients (21). The discovery of PSIP1 as a Trx1 target in humans might provide novel insights to aid in the design of more effective anti-HIV treatments, perhaps even for neuro-AIDS symptoms. Overall, the identification and investigation of novel nuclear Trx1 targets might provide a foundation for a better understanding of the redox signal pathways involved in neurodegenerative diseases and the development of novel therapeutic approaches.

MATERIALS AND METHODS

Chemicals and Reagents—HPLC-grade acetonitrile (ACN) and water were purchased from Mallinkrodt Baker, Inc. (Phillipsburg, NJ). Triethylammonium bicarbonate, protease inhibitor mixture, and phosphatase inhibitor mixture were purchased from Sigma (St. Louis, MO). Sequencing-grade trypsin was obtained from Promega (Madison, WI). PepClean C18 spin columns were purchased from Pierce (Rockford, IL). Recombinant human PSIP1 (3468-LE, R&D Systems, Minneapolis, MN) and human Trx1 (T8690, Sigma) were used in this study. Anti-PSIP1 (ab110023, Abcam, Cambridge, MA) and anti-GFP (a11122, Invitrogen, Grand Island, NY) antibodies were used in the Western blotting.

Plasmids and Constructs—The human Trx1-containing nuclear localization sequence (PPLKKKMKVEDEP) was subcloned into a pcDNA3-EGFP plasmid encoding Trx1WT (nTrx1WT) (Figs. 1A and 1B). A site-directed mutagenesis was performed on the nTrx1WT construct to introduce the C35S mutation into the Trx1 redox active site to generate nTrx1C35SS using the Gene Tailor site-directed mutagenesis kit (Invitrogen). GFP was used as a tag for the Western blotting, imaging, and immunoprecipitation (IP). The mammalian expression vector for human PSIP1, pCMV-SPORT6-PSIP1, was purchased from Mammalian Gene Collection (Pittsburgh, PA). A minimal promoter luciferase reporter was generated through the insertion of a minimal promoter sequence encoding the stress response element (STRE) from human vascular endothelial growth factor C (VEGF-C) into the XhoI and HindIII sites of pGL3Basic (Promega) to generate pSTRE.

Sequencing-grade trypsin was obtained from Promega (Madison, WI) and a phosphatase inhibitor mixture were purchased from Sigma (St. Louis, MO). Phosphatase inhibitor mixture, and phosphatase inhibitor mixture were purchased from Sigma (St. Louis, MO). Thio-Lysine and pter were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Mammalian Gene Collection (Pittsburgh, PA). A mammalian expression vector for human PSIP1, pCMV-SPORT6-PSIP1, was purchased from Mammalian Gene Collection (Pittsburgh, PA). A minimal promoter luciferase reporter was generated through the insertion of a minimal promoter sequence encoding the stress response element (STRE) from human vascular endothelial growth factor C (VEGF-C) into the XhoI and HindIII sites of pGL3Basic (Promega) to generate pSTRE.

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were conducted to ensure the reproducibility of the identification of the Trx1 targets (Fig. 1C).

**Nuclear Protein Extraction**—Nuclear extracts were prepared from the SHSY-5Y cells using a modified procedure developed in our lab (20). Briefly, PBS-washed cells were gently suspended in a hypotonic lysis buffer consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, protease inhibitors, and phosphatase inhibitors. The mixture was incubated on ice for 15 min, and then we added 0.5% Nonidet P-40. The cellular extract was then centrifuged at 800 × g for 10 min at 4 °C to separate the cytoplasmic components (supernatant) from the nuclei-enriched fraction (pellet). For the purification of the nuclear proteins, we washed the nuclear pellets two additional times with a freshly prepared hypotonic buffer and Nonidet P-40 to thoroughly remove any cytoplasmic proteins that were loosely associated with the nuclear pellets. The nuclear pellets were then suspended in a hypertonic buffer (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 420 mM NaCl, 25% v/v glycerol, 0.5 mM DTT, 0.2 mM EDTA, and protease and phosphatase inhibitor cocktails). The nuclear proteins were extracted via vigorous agitations for 15 min on ice. The solutions were further sonicated three times at 10-s intervals on ice. The resulting solutions were centrifuged at 16,000 × g at 4 °C for 15 min. The supernatants containing the solubilized nuclear proteins were stored at −80 °C until further analysis.

**Immunoprecipitation and Western Blotting**—Protein lysates were prepared from the cells transfected with the nTrxWT and nTrx1C35S plasmids and then subjected to nuclear protein extraction. For the IP of the Trx1 association proteins, 500 µg of each nuclear extract was incubated with an anti-GFP antibody (A11122, Invitrogen) overnight at 4 °C; then 100 µl of protein-A agarose beads (15918–014, Invitrogen) were added and the incubation continued for 1 h with frequent mixing. The beads were then washed three times with a diluted lysis buffer (10 mM Tris, 25 mM NaCl, 0.5% Triton X-100, 25 mM EDTA), with 2 min of centrifugation at 5000 × g between each wash step. The proteins were eluted from the beads with a 2× loading buffer (100 mM Tris (pH 6.8), 4% SDS, 0.2% bromophen blue, 20% glycerol with 5% β-mercaptoethanol (2-ME, Bio-Rad Laboratories, Hercules, CA)) and incubated on a heat block at 100 °C for 5 min. After centrifugation at 10,000 × g for 5 min, the proteins were resolved on 13.5% SDS-polyacrylamide gels and either were stained with Sypro Ruby dye (Invitrogen) or anti-NF-membranes. The membranes were first blocked with 5% milk and then probed with an anti-GFP antibody (A11122, Invitrogen) overnight at 4 °C. The membranes were then washed with 30% ACN and 0.1% formic acid. The identified proteins were then imaged using a Nikon A1R microscope, and the images were processed using Nikon NIS Elements software.

**Fluorescence Microscopy**—SHSY-5Y cells were transfected with the nTrxWT and nTrx1C35S plasmids. Two days after the transfection, the cells were fixed in 4% paraformaldehyde in PBS for 15 min and then permeabilized with 0.1% Triton X-100 for 5 min. The cells were then incubated with either anti-GFP antibody overnight at 4 °C. After being washed three times with phosphate buffer saline tween (PBST), the cells were incubated with a Cy5-conjugated anti-mouse antibody (ab6563, Abcam) for 1 h. The cells were then washed in PBST and mounted with ProLong Gold anti-fade reagent with DAPI (Invitrogen). The cells were then imaged using a Nikon A1R microscope, and the images were processed using Nikon NIS Elements software.

**Redox Assay of PSIP1**—To obtain oxidized PSIP1, we oxidized recombinant human PSIP1 proteins with 100 mM H₂O₂ for 30 min at 37 °C. The reduced Trx1 (rTrx1) was obtained by reduction of Trx1 with 100 mM DTT for 30 min at 37 °C followed by an acetonitrile precipitation to remove the residual DTT, and the protein pellet was further washed with 80% cold acetone three times. The solubilized PSIP1 was mixed with rTrx1 in a 1 to 0, 1, 2, 4, 8, or 16 molar ratio for 30 min at 37 °C. Subsequently, the free PSIP1 thiols were alkylated with N-(6-[bromotinamido]hexyl)-3′-(2-pyridyldithio)-propionamide (bromotinamido HPDP). The biotinylated PSIP1 was detected via Western blotting using an anti-biotin antibody (Vector Lab Inc., Burlingame, CA). For the reduced PSIP1 site identification following the rTrx1 treatment,
the PSIP1 was treated with rTrx1 or buffer for 30 min at 37 °C; and this was followed by an alkylation of the free thiols with 10 mM IAA for 60 min at 37 °C. The excess IAA was quenched by the addition of 50 mM DTT, and the oxidized cysteines in PSIP1 were also reduced during the process. The residual DTT was removed from the proteins by acetone precipitation, and the protein pellets were further washed with 80% cold acetone three times. The newly freed thiols (previously oxidized) were conjugated with 50 mM methyl methanethiosulfonate. The proteins were digested with trypsin, and the resulting peptides were identified via LC-MS/MS. Trx1 reduction results in less oxidized thiols in PSIP1. The relative quantification of the methyl methanethiosulfonate–labeled peptides was performed after normalization with the top three most abundant PSIP1 peptides without cysteine modifications (n = 3 for the statistical analysis).

**RESULTS**

**Identification of the Putative Nuclear Trx1 Targets in Human Neuroblastoma Cells**—The overall strategy for using nTrx1C35S to trap nuclear Trx1 target proteins and to isolate them via IP is depicted in Fig. 1. In the cells expressing nTrx1WT, rTrx1 can be produced by a Trx reductase from oxidized Trx1 with the reducing equivalents derived from NADPH. rTrx1 can then form a mixed disulfide bond with one of the oxidized thiols in a target protein via the Cys32 in its catalytic site (Fig. 1D). This covalent complex can then immediately be converted to a reduced target when Cys35 donates its proton, resulting in oxidized Trx1. This process is very fast; therefore little or no mixed disulfide complex is typically observed in the cells. In order to capture the mixed disulfide complexes and identify the Trx1 targets, we constructed a Trx1 mutant in which the Cys35 was changed to a serine; nTrx1C35S cannot donate its proton to consummate the complete target reduction reaction, but Cys32 can still form mixed disulfides with the target proteins. Because there is no further target reduction by Ser35, the target proteins are trapped within the mixed disulfide complexes, providing an opportunity to isolate these complexes for proteomic analysis. We constructed nTrx1C35S with a nuclear targeting signal (for stable nuclear expression) and a GFP tag on the C terminus (Fig. 1B) for the isolation of stable nuclear Trx1-targeted protein complexes and efficient proteomic identification (Fig. 1C). As a control, we expressed nTrx1WT in separate cells. Following the successful expres-
Fig. 2. The proteins captured through IP of nTrx1C35S and nTrx1WT. The Venn diagram shows a representative dataset. Scaffold was used to filter and compare the unique proteins and peptides identified in the analysis of the nuclear extract from Experiment 2 (supplemental Tables S1 (A–L) and S2). The peptide and protein identification criteria were set with a 1% false discovery rate. As expected, more proteins and peptides were discovered through the IP of nTrx1C35S, with 460 unique proteins (A) and 2031 unique peptides (B) found only with the protein complex associated with nTrx1C35S.

Validation of the Putative Nuclear Trx1 Targets—The IPA molecular network analysis of the 45 putative nuclear Trx1 target proteins found that 23 proteins participated in a physical and functional protein–protein interaction network involved in the regulation of RNA and protein trafficking and nuclear molecular transport (Fig. 3A). Furthermore, among the nuclear Trx1 target proteins discovered in this study (Fig. 3B), six were significantly enriched with RNA trafficking functions (p value of 2.26E-9), six were enriched with protein trafficking functions (p value of 1.46E-8), and three were enriched with assembly of nuclear pore functions (p value of 4.50E-7). Interestingly, two proteins involved in HIV interactions with host cells were also observed to be regulated by Trx1. These proteins, PSIP1 and RANBP2, regulate the integration of HIV into the host genome (p value of 3.66E-04). Ten putative Trx1 target proteins are known to modulate HIV-1 infection (p value of 1.45E-05).

Validation of Our Approach for the Identification of Nuclear Trx1 Targets—To validate the target proteins, we performed Western blotting analyses of select proteins immunoprecipitated with either nTrx1C35S or nTrx1WT. Peroxiredoxin 1 (Prx1) is one of the most studied targets of Trx1. Although Prx1 reduction by Trx1 occurs primarily in the cytosol, we detected it via both Western blotting and LC-MS/MS (supplemental Table S1 (A–L) & Fig. 4). Two known nuclear targets of Trx1 that were not observed in the MS analysis were NF-κB and P53 (18). However, the Western blot analysis indicated that significantly more P53 and NF-κB was associated with nTrx1C35S than with nTrx1WT (Fig. 4). Because the known Trx1 targets are known to associate with nTrx1C35S with greater affinity than with nTrx1WT, these observations support our hypothesis that novel nuclear Trx1 targets are identifiable via the nTrx1C35S trapping strategy.

Validation of PSIP1 as a Direct Trx1 Reduction Target Protein—We conducted an additional biochemical validation of PSIP1, which is commonly known as LEDGF because of its involvement in the stress response and differentiation of lens epithelial cells. PSIP1 interacts with HIV-1 integrase, thereby regulating HIV-1 integrase’s nuclear localization and association with chromatin (24). The Western blot demonstrated that ~20% more PSIP1 associated with nTrx1C35S than with the nTrx1WT complex (Fig. 5A), presumably because of the limited antibody specificity. Protein sequence alignments indicate that PSIP1 is indeed associated with Trx1 in the nucleus, we performed immuno-fluorescence microscopy to validate the co-localization of PSIP1 with Trx1 (Fig. 5B), and we observed a large number of cells with overlapping GFP-tagged Trx1 signals, Cy5-labeled.
PSIP1 signals, and DAPI luminescence in the nucleus. This result suggests that both nTrx1WT and nTrx1C35S largely co-localized with PSIP1 in the nucleus of human cells. Furthermore, we performed in vitro analysis and determined that PSIP1 can be directly reduced by Trx1 (Fig. 6A). A downstream MS analysis identified PSIP1 Cys204 as the site of Trx1 reduction (Figs. 6B and 6C). Finally, using a luciferase reporter assay construct containing a known PSIP1 re-

### Table I

| Protein name | Gene I.D. | Protein accession number | Cys number in protein | Function |
|--------------|-----------|--------------------------|-----------------------|----------|
| A kinase (PRKA) anchor protein 8 | AKA8P1 | O43823 | 10 | Chromatin binding |
| PIN2/TERF1 interacting, telomerase inhibitor 1 | PINX1 | Q86BK5 | 3 | Telomerase inhibitor activity |
| Structural maintenance of chromosomes 1A | SMC1A | A6K78A6 | 5 | ATP binding |
| Structural maintenance of chromosomes 3 | SMC3 | B0AZOQ4 | 7 | Chromosome organization |
| Structural maintenance of chromosomes flexible hinge domain containing 1 | SMCHD1 | A6NHRR9 | 30 | Chromosome organization |
| Survival of motor neuron 1, telomeric | SMN1/SMN2 | E7EQ24 | 8 | Axonogenesis |
| DNA repair | | | | |
| MutS homolog 6 | MSH6 | B4DF41 | 32 | Methylated histone binding |
| Replication factor C (activator 1) 2, 40 kDa | RFC2 | B5BU2 | 8 | DNA repair |
| Replication factor C (activator 1) 5, 36.5 kDa | RFC5 | A6KSS0 | 5 | DNA repair |
| XPA binding protein 2 | XAB2 | Q71SVY | 14 | DNA repair |
| Helicase | | | | |
| DEAD (Asp-Glu-Ala-Asp) box helicase 24 | DDX24 | B4DKV2 | 10 | RNA helicase activity |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 10 | DDX10 | E9IF2 | 6 | ATP-dependent helicase activity |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 50 | DDX50 | B4DW9 | 9 | ATP-dependent helicase activity |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 54 | DDX54 | B7TDD1 | 5 | ATP-dependent helicase activity |
| Small nuclear ribonucleoprotein 200 kDa (U5) | SNRP200 | B8H89 | 7 | ATP-dependent helicase activity |
| RNA processing | | | | |
| Fused in sarcoma | FUS | A8K4H1 | 4 | Nucleic acid binding |
| GLE1 RNA export mediator | GLE1 | A8K3B8 | 6 | mRNA export from nucleus |
| M-phase phosphoprotein 10 (U3 small nuclear ribonucleoprotein) | MPHOSPH10 | B3KPV5 | 2 | rRNA processing |
| Nucleolar complex associated 3 homolog (Saccharomyces cerevisiae) | NO30L | A6NHJ2 | 8 | Poly(A) RNA binding |
| Polypyrimidine tract binding protein 1 | PTBP1 | K7E4K5 | 2 | Alternative nuclear mRNA splicing |
| Ribosomal RNA processing 12 homolog (S. cerevisiae) | RRP12 | B3KMR5 | 25 | RNA processing |
| Ribosomal RNA processing 1B | RRP1B | Q146B4 | 7 | rRNA processing |
| RNA binding motif protein 28 | RBM28 | B8WV13 | 9 | mRNA processing |
| Small nuclear ribonucleoprotein polypeptide A' | SNRPA1 | H0YMAO | 1 | mRNA processing |
| Splicing factor 3b, subunit 3, 130 kDa | SF3B3 | A8K6V3 | 19 | mRNA processing |
| UTP18 small subunit (SSU) processome component homolog (yeast) | UTP18 | B2RXA6 | 8 | mRNA processing |
| Transcription | | | | |
| Apoptosis antagonizing transcription factor | AATF | Q9NY61 | 2 | Protein binding |
| Bromodomain adjacent to zinc finger domain, 1A | BAZ1A | D3S96 | 29 | Chromatin remodeling |
| Centromere protein B, 80 kDa | CENPB | P07199 | 7 | Centromeric DNA binding |
| Deoxynucleotidyltransferase, terminal, interacting protein 2 | DNTTIP2 | Q5QJE6 | 7 | Regulation of transcription |
| KIAA0020 | KIAA0020 | B2R9D4 | 7 | Poly(A) RNA binding |
| PC4 and SFRS1 interacting protein 1 | PSIP1 | Q7SD4 | 2 | Activating transcription factor binding |
| PWP1 homolog (S. cerevisiae) | PWP1 | B4DNL1 | 10 | Transcription |
| Zinc finger CCCH-type containing 18 | ZC3H18 | B4DZ24 | 3 | Metal ion binding |
| Transporter | | | | |
| Exportin 1 | XPO1 | B4DR01 | 13 | Transporter activity |
| Karyopherin (importin) β 1 | KPNB1 | B2RBG9 | 23 | Transporter activity |
| Nucleoporin 107 kDa | NUP107 | P57740 | 13 | Nucleoporin-mediated transporter |
| Nucleoporin 153 kDa | NUP153 | B4DIK2 | 28 | Protein transport |
| Nucleoporin 210 kDa | NUP210 | B8TEM1 | 16 | Protein dimerization activity |
| Nucleoporin 214 kDa | NUP214 | B3P658 | 13 | Nucleoporin-mediated transporter activity |
| RAN binding protein 2 | RANBP2 | B4G4T2 | 77 | RNA binding |
| Transportin 1 | TNP1 | Q8Z9V3 | 26 | Intracellular protein transport |
| Others | | | | |
| DnaJ (Hsp40) homolog, subfamily C, member 9 | DNAJC9 | Q8WXXS | 3 | Chaperone |
| Guanine nucleotide binding protein-like 2 (nucleolar) | GN1L2 | Q1B8R2 | 4 | Ribosome biogenesis |
| Nucleoporin 205 kDa | NUP205 | B4DE72 | 18 | Protein binding |

All of the proteins listed here fulfill the following requirements: (i) the protein was observed only in nTrx1C35S and not in nTrx1WT in at least two experiments; (ii) the spectra were of high quality; (iii) the protein was designated as a nuclear protein by IPA; and (iv) the protein contains at least one cysteine. Functions annotated in the UniProt database and IPA.
sponse element derived from VEGF-C, we confirmed that Trx1 can indeed modulate the transcriptional regulation function of PSIP1 in both HeLa and SHSY-5Y cells (Fig. 7 and supplemental Fig. S2). Interestingly, whether PSIP1 functions as a transcriptional activator or repressor appears to be cell dependent. In SHSY-5Y cells, both PSIP1 and nTrx1WT suppressed pSTRE-luc expression, but in combination, nTrx1WT was able to significantly enhance the ability of PSIP1 to suppress pSTRE-luc expression (supplemental Fig. S2). However, in HeLa cells, PSIP1 behaved as a transcriptional activator, and Trx1 reduction appeared to attenuate PSIP1’s ability to activate target gene expression (Fig. 7).

DISCUSSION

Trx1 is a distinct reductase that restores oxidized cysteine post-translational modifications back to free thiols, thereby modulating the key functions of certain target proteins. Nuclear Trx1 has unique nuclear targets that differ from its cytoplasmic ones. Therefore, the identification of Trx1 targets in the nucleus of human cells might lead to the discovery of novel stress response proteins, including transcription factors and RNA processing enzymes, that might be important in understanding the cellular-nuclear response during the develop-
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Fig. 5. The binding and subcellular co-localization of Trx1 and PSIP1. SHSY-5Y cells were transfected with nTrx1WT and nTrx1C35S and then subjected to nuclear protein extraction. A, the nTrx1-binding proteins were pulled down using an anti-GFP antibody and separated via SDS-PAGE. After the transfer of the proteins onto nitrocellulose membranes, PSIP1 was probed using an anti-PSIP1 antibody. B, SHSY-5Y cells were transfected with nTrx1WT or nTrx1C35S. The fixed cells were stained with anti-PSIP1 and analyzed with a Zeiss confocal laser scanning microscope. The GFP-tagged Trx1 is shown in green, the antibody Cy5-labeled PSIP1 is shown in red, the DAPI-stained nuclei are shown in blue, and the Trx1 and co-localized PSIP1 are shown in yellow.

opment of some neurodegenerative diseases. Until now, most nuclear targets of Trx1 have been identified using traditional molecular biology approaches that have not included modern proteomics technologies. By continuing to adapt and utilize state-of-the-art mass spectrometers, we can identify more nuclear Trx1 target proteins more rapidly and more accurately.

Proteomics approaches have been widely used to identify Trx targets in non-mammalian cells and tissues (supplemental Table S3), including in plants and microbial cells. As early as 1999, Verducq et al. identified a Trx target protein, YLR109, in yeast using His-tagged Trx affinity (25). Yano et al. used monobromobimane to label free thiols in proteins reduced by Trx that were resolved via two-dimensional gel electrophoresis and identified five Trx targets in peanut seeds (26). The Trx affinity technique was subsequently developed to identify Trx targets in diverse cell types, including spinach (27–31), Arabidopsis (32–35), Escherichia coli (36, 37), wheat seeds (38, 39), Synechocystis (40–42), and HeLa cells (16, 43). The most successful of these studies identified up to 80 putative Trx targets (37). In 2008, Hagglund et al. used a quantitative proteomics approach based on the isotope-coded affinity tag method to identify 40 putative Trx targets in the seeds of malting barley (44). More recently, we used the isotope-coded affinity tag approach to identify over 70 targets of Trx1 transnitrosylation and 50 targets of Trx1-mediated denitrosylation (43). Although a Trx mutant has been used to identify Trx targets in non-mammalian cells (25–42, 44), its application in the identification of mammalian targets has been rare, presumably because of the presence of highly abundant cytosolic targets in the total cell lysates, such as Pmr1, that compete for a limited number of binding sites on the Trx1C35S mutant. This is the first report demonstrating that an nTrx1C35S mutant can be used to identify novel Trx targets in the nucleus of mammalian cells. Although we report the identification of a select number of Trx1 targets here, our continued work involves refining this method to increase the speed, specificity, and sensitivity of this process. For example, increasing the salt concentration to eliminate more non-competitively bound proteins prior to the 2-ME elution of the proteins linked to nTrx1C35S via disulfides might reduce the interference of non-specific proteins in LC-MS/MS. Therefore, transcription factors such as P53 and NF-κB might be further identified. Additionally, tandem affinity purification can be used to increase the purity of the protein complexes. Finally, following the protein elution by 2-ME, isotope-coded affinity tag or thiol-based tandem mass tag labels could be used for a more accurate quantification of only the thiol-containing peptides, allowing the identification of specific reduction sites and the quantification of their enrichment using nTrx1C35S.

PSIP1/LEDGF is a stress-regulated protein reported to be important for cell survival (45) and implicated as having oncogenic properties (46). It is also known as Dense Fine Speckles 70-kDa protein (DFS 70) or transcriptional coactivator p75/p52 and is a transcriptional co-activator and a host factor that assists HIV integration (47, 48). It has been found in both the cytosol and the nucleus of epithelial cells (49). Its ability to bind to heat or stress response elements is attenuated in cells with high levels of oxidative stress (50), suggesting that PSIP1/LEDGF itself might be sensitive to high levels of oxidative stress. Our discovery of PSIP1 as a Trx1 reduction target suggests a means for cells to preserve the PSIP1 stress modulatory function. The knockdown of PSIP1/LEDGF can significantly decrease HIV integration into the host genome (51, 52). PSIP1/LEDGF activates HIV-1 integration by interacting with HIV-1 integrase, and genetic variants of PSIP1 were reported to affect the host’s susceptibility to HIV-1 infection and disease progression (53). Given PSIP1’s established role in regulating HIV integrase function, increasing efforts have been made to identify drugs that can block LEDGF binding to HIV integrase (54). HIV-infected patients have elevated levels of plasma Trx1, which might impair the patient’s survival by blocking chemotaxis (21). Whether oxidative stress or Trx1 plays a role in regulating the interaction between PSIP1 and HIV integrase in human cells is currently unknown. According to the National Institute of Neurological Disorders and Stroke, HIV-induced inflammation may damage the brain and spinal cord and lead to confusion, memory loss, and other behavioral and neuro-AIDS conditions. Our finding that PSIP1 is a novel target of Trx1 in the nucleus of...
human neuronal cells might lead to the development of a more effective therapy for neuro-AIDS patients. For example, Trx1 inhibitors are under development as cancer treatments (55); whether the combination of a Trx1 inhibitor and a drug targeting PSIP1/integrase binding would be effective in reducing HIV-associated inflammation remains to be explored.

A select number of putative nuclear Trx1 targets identified in this study have been suggested to play critical roles in diverse nuclear activities. Among the putative targets of Trx1 are several nucleoporins, such as NUP107, NUP153, NUP210, and NUP214 (Table I). Nucleoporins form the nuclear pore complex and are involved in nuclear-cytoplasmic transport. D’Angelo et al. demonstrated that the nuclear pore is leakier in 28-month-old mice than in 3-month-old mice (56). They observed that the age-related increase in nuclear permeability was accelerated by oxidative stress, and as the mice aged, many of the nucleoporins were affected by oxidation. Recent studies have also indicated that the intermolecu-
Proteomics of Trx1 Nuclear Targets

![Graph](image)

**Fig. 7.** The Trx1 modulation of PSIP1-regulated target gene expression. HeLa cells were co-transfected with luciferase alone or in combination with PSIP1, Trx1, or both for 48 h. The cells were washed three times with PBS and collected for a luciferase activity assay (n = 4).

In this study we identified a large number of novel human nuclear Trx1 targets. These target proteins play important and diverse roles in RNA processing, RNA and protein transport, nuclear structure, and gene regulation. Until now, many of these targets were not known to be redox regulated. Our results implicate Trx1 as playing a broader role in the nucleus than previously thought, such as by directly coordinating the concerted cellular responses to oxidative stress. These results might lead to a better understanding of oxidative stress and help further elucidate the role of Trx1 in modulating nuclear processes. For example, the discovery of PSIP1/LEDGF as a direct target of Trx1 establishes a sound rationale for future studies of this interaction in regulating cell survival in models of neurodegenerative disease, as well as other diseases. In cancer and infectious diseases, the interactions between Trx1 and PSIP1/LEDGF will likely be exploited to develop therapies to attenuate the PSIP1/Trx1 interaction, thereby leading to the death of cancer cells or the attenuation of HIV infection in host cells. Our continued optimization of the proteomics technique described in this study will enable the identification of more nuclear Trx1 targets in mammalian cells and thus will allow us to continue to establish a more complete picture of how Trx1 coordinates anti-oxidative stress responses.

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