

Published in final edited form as:

FEBS J. 2014 August ; 281(16): 3545–3558. doi:10.1111/febs.12898.

Reactivation of oxidized PTP1B and PTEN by Thioredoxin 1

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Abstract

The transient inactivation of protein phosphatases contributes to the efficiency and temporal control of kinase-dependent signal transduction. In particular, members of the protein tyrosine phosphatase family are known to undergo reversible oxidation of their active site cysteine. The thiol oxidation step requires activation of co-localized NADPH oxidases and is mediated by locally produced ROS, in particular H₂O₂. How oxidized phosphatases are returned to the reduced active state is less well studied. Both major thiol reductive systems, the thioredoxin and the glutathione systems, have been implicated in the reactivation of phosphatases. Here, we show that the protein tyrosine phosphatase PTP1B and the dual-specificity phosphatase PTEN are preferentially reactivated by the thioredoxin system. We show that inducible depletion of TRX1 slows down PTEN re-activation in intact living cells. Finally, using a mechanism-based trapping approach we demonstrate direct thiol disulfide exchange between the active sites of thioredoxin and either phosphatase. The application of thioredoxin trapping mutants represents a complementary approach to direct assays of PTP oxidation in elucidating the significance of redox regulation of PTP function in the control of cell signaling.

Keywords

insulin signaling; phosphatase; reactive oxygen species; redox regulation; substrate trapping; thioredoxin 1

INTRODUCTION

Historically, reactive oxygen species (ROS) have been perceived predominantly as toxic and unwanted by-products of aerobic metabolism. It did not seem likely that ROS could have positive or even essential functions in cellular physiology. However, this view changed when ROS production was recognized as an immune defense mechanism [1, 2]. The

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Authors' contributions

U.S., A.H., N.K., R.G. and L.W. performed experiments; U.S., A.H., N.K., R.G., L.W., T.D. and N.K.T. designed experiments and analyzed data. U.S and N.K.T. wrote the paper, with input from all the authors. N.K.T. directed the study.

phagocyte NADPH oxidase transfers electrons from NADPH to molecular oxygen, generating superoxide, which is then converted to H_2O_2 . Subsequently, homologs of the phagocytic enzyme were found in all cell types, constituting the NOX and DUOX families of NADPH oxidases [1, 2]. Now it is well accepted that NOX-derived ROS also play crucial roles in signal transduction [3, 4]. Many growth factors and hormones, including epidermal growth factor (EGF) [5] platelet-derived growth factor [6] and insulin [7], induce the generation of H_2O_2 , which acts as a second messenger.

H_2O_2 influences protein function by modifying thiol groups, the oxidation of which initially yields sulphenic acid (R-SOH). Further oxidation, which leads to sulphinic (R-SO₂H) and sulphonic acid (R-SO₃H), results in modifications that are generally irreversible. However, to prevent irreversible oxidation, the cysteinyl sulphenic acid can condense with a nearby thiol group to form intra- or intermolecular protein disulfide bonds (RSSR) or to become S-glutathionylated (RSSG). Alternatively, the cysteinyl sulphenic acid may also form a cyclic sulphenyl amide. In each case, these modifications are reversible by reduction, making them ideally suited for the transient control of protein function.

Although many signaling proteins, including kinases, are now known to be redox regulated, a particular focus of research has been on the regulation of signal transduction by transient oxidation and inactivation of protein phosphatases. All members of the protein tyrosine phosphatase (PTP) family share the same catalytic mechanism, which depends on a cysteine residue that is essential for catalysis and is located at the base of the active site cleft [8, 9]. Due to the architecture of the active site, this cysteine is deprotonated at physiological pH, which favors its function as the nucleophile in catalysis [8, 9]. Deprotonation is also a prerequisite for its susceptibility to oxidation by H_2O_2 [10, 11]. In the classical PTPs, oxidation of the active site thiolate promotes the formation of sulphenic acid, which is rapidly converted to a cyclic sulphenamide that induces a conformational change to expose the sulfur atom on the surface of the phosphatase [12]. Therefore, following this conformational change the oxidized sulphur atom becomes accessible to cellular reductants. Unlike most of the classical PTPs, the dual specificity phosphatases harbor a second cysteine in the active site, which condenses to form an intramolecular disulfide bond with the sulphenic acid. In either case, the catalytic cysteine is protected against irreversible oxidation and can be reconverted to its active form by reduction.

Oxidative inactivation of PTPs promotes tyrosine phosphorylation and thus enhances signaling responses. A range of PTPs, including representatives of both the classical and dual-specificity phosphatases, are oxidized transiently in response to a variety of extracellular stimuli, including growth factors, hormones, antigens and ECM components [8, 13]. For example, PTP1B, the prototypic member of the PTP family, was found to be oxidized in response to EGF [14] and insulin [7, 15, 16]. The dual specificity phosphatase PTEN is oxidized in response to LPS [17], PDGF, EGF [18, 19] and insulin [20]. The reduction and reactivation of such PTPs ensures the transient and reversible nature of the oxidative thiol modification, allowing PTPs to recover their activity as soon as H_2O_2 concentrations decrease and preventing uncontrolled stimulation of signaling pathways. Nevertheless, the exact reductive mechanisms by which PTPs become reactivated have been less well studied than the oxidative mechanisms by which they are inactivated, although

both major thiol-reductive systems, the thioredoxin and the glutathione system, have been implicated in the reactivation of PTP1B and PTEN [14, 21–24]. In this study, we use a combination of biochemical analysis, mechanism-based trapping and RNAi-induced suppression to demonstrate the importance of thioredoxin for the reduction and reactivation of PTP1B and PTEN.

RESULTS

TRX1 reactivated oxidized PTP1B and PTEN *in vitro*

As a first step, we measured inhibition of phosphatase activity by H_2O_2 *in vitro*. For PTEN we obtained an apparent second order rate constant of $14 \pm 0.5 \text{ M}^{-1}\text{s}^{-1}$ (Figure 1A), and a similar rate for PTP1B (Table 1). We then compared different reductants for their ability to reverse the oxidative inactivation: dithiothreitol (DTT), reduced glutathione (GSH), thioredoxin 1 plus thioredoxin reductase (TRX1/TR), and GSH plus glutaredoxin (GRX/GSH). In each case, the rate of reactivation depended on the concentration of the reductant (shown in Figure 1B for the TRX system) and corresponded to a second-order rate constant (Table 1). With all three reductants, more than 90 % of phosphatase activity could be recovered. However, the reactivation of either phosphatase by the TRX1/TR system was 20-fold faster than by GSH and 5-fold faster than by Grx/GSH (Table 1). Thus, the reactivation of H_2O_2 -treated PTEN or PTP1B by the thioredoxin system was kinetically favored over reduction by the glutathione system.

We determined the midpoint redox potential of the active site cysteine of the two phosphatases. To this end, we equilibrated PTEN or PTP1B with redox buffers of varying redox potential and quantified free thiols with a fluorescent probe. The mid-point potential of PTEN was determined as -188 mV and that of PTP1B as -179 mV (Figure 1C). Since the midpoint potential of the dithiol-disulfide pair in the active site of human TRX1 has been reported as -230 mV [25], reduction of either PTEN or PTP1B by TRX1 is thermodynamically favorable.

TRX1 formed disulfide-exchange intermediates with PTP1B and PTEN

Having observed that the thioredoxin system is more efficient than the glutathione system in reactivating oxidized PTEN or PTP1B, we aimed to confirm a direct interaction between TRX1 and the phosphatases that would lead to disulfide exchange. To this end, we made use of a mechanism-based trapping approach. In wild type TRX1, the first cysteine in the CXXC motif forms a mixed disulfide intermediate with the target protein, which is subsequently resolved by the second cysteine [26]. Mutagenesis of the second cysteine to serine thus stabilizes the mixed disulfide intermediate, which then becomes amenable to purification and analysis. TRX1 harbours three additional cysteine residues, which are dispensable for catalytic activity, but can cause inactivation by intra- or intermolecular disulphide bond formation [27, 28]. Therefore, we used constructs in which these additional cysteines had been substituted by alanine, which had no effect on the trapping profile [27].

In a first step, we treated HeLa cells with a bolus of exogenous H_2O_2 to induce oxidation of endogenous proteins. Cells were lysed in the presence of the alkylating reagent *N*-ethyl

maleimide (NEM) to prevent artificial thiol oxidation during or after lysis. Following removal of unreacted NEM, lysates were incubated with recombinant TRX1-C35S, TRX1-WT or TRX1-C32S. The SBP-tagged thioredoxins were collected with streptavidin beads, washed extensively and analyzed by SDS-PAGE and immunoblotting with TRX1 antibodies (Figure 2A). As expected, only the TRX1 trapping mutant (C35S) formed disulfide conjugates with several other proteins (left panel), which were disrupted upon reduction with DTT (right panel).

In the next step, we looked specifically for a disulfide exchange intermediate between TRX1 and PTP1B. HeLa cells were treated with increasing concentrations of H₂O₂ (0.1 – 1 mM) and analyzed by PTP1B-specific immunoblotting. A disulfide-linked PTP1B-TRX1 complex was detected at H₂O₂ concentrations as low as 0.2 mM (Figure 2B; right panel). In addition to the 1:1 complex, we also detected species of higher molecular weight suggesting that additional stoichiometries are possible (Figure 2C). Similarly, treatment of cells with H₂O₂ led to oxidation of PTEN, as visualized by electrophoretic gel mobility shifts; in this case we also detected conjugates of PTEN and the TRX1 (Figure 2D).

TRX1 contributed to PTEN reduction in living cells

As endogenous TRX1 is an abundant protein, with a cytosolic concentration between 1 and 10 μ M [29], it has the potential to be a formidable barrier to oxidation. Therefore, we tested the effects of expressing a tetracycline-inducible shRNA against TRX1. Depletion of TRX1 was induced by the addition of doxycycline for at least five days prior to the experiment. We treated HeLa cells that expressed either TRX1 shRNA or control shRNA with H₂O₂ and prepared lysate samples at different time points after treatment. Initially, we focused on an analysis of PTEN because the oxidized and reduced forms of this phosphatase can be easily differentiated by mobility on SDS PAGE gels. In cells expressing the control shRNA, most PTEN was converted back to its reduced form after 90 min; however, reduction was clearly delayed in cells expressing TRX1 shRNA, with significant amounts of oxidized PTEN still detectable after 120 min (Figure 3). This result suggested that TRX1 substantially contributes to PTEN reduction in intact living cells.

In addition, we considered the possibility that rapid reduction of oxidized phosphatases prior to cell lysis may limit the amount of disulfide-linked TRX1 conjugates detectable by the trapping approach. Therefore, we performed trapping experiments from lysates of HeLa cells in which TRX1 was suppressed by expressing the doxycycline-inducible shRNA. Cells in which TRX1 was efficiently depleted were then stimulated with exogenous H₂O₂. We observed trapping of both PTP1B (Figure 4A) and PTEN (Figure 4B), with the extent of complex formation significantly increased in lysates from cells with diminished levels of endogenous TRX1 relative to controls. Again, these data are consistent with an important role for endogenous TRX1 in reduction of PTEN and PTP1B in intact cells. Nevertheless, complex formation with the trapping mutant form of TRX1 was not detected in lysates of cells stimulated with insulin (Figure 4A & B), or EGF, despite confirmation of insulin-induced phosphorylation of AKT, consistent with activation of cell signaling (Figure 4C). This suggests that a threshold level of oxidation may be required before the TRX trapping mutants can form complexes with the target PTP in cell lysates.

An ectopically expressed TRX1 trapping mutant co-precipitated with PTP1B after insulin stimulation

Since we were not able to detect trapping of PTP1B from lysates of EGF- or insulin-stimulated cells, we tested for interaction by co-expressing the TRX1 trapping mutant and PTP1B. To this end, 293T cells were co-transfected with FLAG-tagged wild type PTP1B and His₆-tagged TRX1 (TRX1-C35S, TRX1-WT, TRX1-C32S). Cells were either left untreated or treated with H₂O₂. Lysates were subjected to immunoprecipitation with either His₆ (TRX1)- or FLAG (PTP1B)-specific antibodies, and interactions were analyzed by TRX1- and PTP1B-specific immunoblotting. As expected, exogenously applied H₂O₂ induced disulfide bond formation between PTP1B and the TRX1 trapping mutant (TRX1-C35S) (Figure 5A). To confirm that the catalytic cysteine of PTP1B is essential for disulfide bond formation with TRX1, we used a PTP1B mutant that lacks the catalytic cysteine, but adopts the conformation of the oxidized protein in a stable manner (PTP1B-CASA) [16]. Indeed, PTP1B-CASA failed to form a conjugate with TRX1-C35S (Figure 5B). Using this approach, we also detected TRX1(C35S)-PTP1B complex formation following stimulation of the cells with a physiological trigger, in this case insulin (Figure 5C). Again, conjugation depended on the catalytic cysteine of PTP1B (Figure 5D).

On the basis of these observations, one would predict that alteration of TRX1 expression would result in a change in tyrosine phosphorylation of PTP1B substrates. To test this, we examined a time course of insulin-induced phosphorylation of the insulin receptor β -subunit (IR- β) in the presence of TRX1 wild type or trapping mutant. The data illustrate that overexpression of wild type TRX1, which would be expected to lead to more reduced, active PTP1B, led to decreased phosphorylation of IR- β . In contrast, the TRX1-C35S trapping mutant led to enhanced phosphorylation of IR- β early in the time course (Figure 6). Together, these results further support the conclusion that TRX1 catalytically reactivates reversibly oxidized PTP1B in intact cells, also in response to physiological stimuli.

DISCUSSION

Like the PTKs, the PTPs display remarkable specificity in their control of cell signaling and disruption of PTP function has been linked to major human diseases [8, 9]. As a barrier to PTK function, and as critical regulators of signaling in their own right, the activity of the PTP family of enzymes must be tightly regulated *in vivo* to permit a normal response to extracellular stimuli. Although this is achieved through multiple mechanisms, the recently described redox regulation of PTP function represents a powerful new tier of control over pTyr-dependent signaling. A unique feature of the PTP family is the presence of an essential reactive cysteine at the active site, which is exquisitely sensitive to oxidation with concomitant abrogation of its function as a nucleophile in catalysis. The reduction of molecular oxygen to produce superoxide is triggered using electrons generated by NOX enzymes or the mitochondrial transport chain in response to physiological stimuli, such as growth factors, hormones and cytokines [1–4]. After spontaneous or enzymatic conversion of superoxide to H₂O₂, which is the major ROS associated with this regulation of signaling, the inactivation of PTPs at defined subcellular locations permits fine-tuning of the signal-induced changes in tyrosine phosphorylation. A critical part of this fine-tuning is the

reversible nature of PTP oxidation, in which reduction of the oxidized PTP permits restoration of activity and a return to the ground state. Therefore, in addition to the mechanisms underlying the control of ROS production, it is important to characterize the control of both the oxidation and reduction of the molecular targets of ROS, so as to understand the full scope of the effects of this covalent modification.

In the study of reversible protein phosphorylation, the initial emphasis of research was placed on the protein kinases and the addition of phosphate to target substrates, with the importance of protein phosphatases and the removal of phosphate not being fully appreciated until later. Interestingly, the study of redox regulation has followed a similar path. Considerable progress has been made in defining the mechanism of ROS production, the controls over the levels of ROS in cells and the scope of potential targets of this modification [10, 11]. Nevertheless, for oxidation and inactivation of the PTPs to represent an effective regulatory mechanism, it is essential that this is a reversible modification. Conversion of the active site Cys residue to higher oxidized, sulphenic or sulphonie acid forms would be essentially irreversible; however, there are protective mechanisms in place to favour production of the singly oxidized, reversible sulphenic acid. As demonstrated for PTP1B [11] and PTP α [30], and potentially applying broadly across the classical pTyr-specific PTPs, a condensation reaction converts the sulphenic acid to a cyclic sulphenamide, which induces a conformational change that exposes the oxidized cysteine on the surface of the protein where it may encounter cellular reducing agents. Several dual specificity phosphatases, including PTEN [23], cdc25 [31, 32] and PRL2 [33], and the classical PTP SHP2 [34], display an alternative protective mechanism in which a vicinal cysteinyl residue promotes formation of an intramolecular S-S bond with the sulphenic acid form of the active site cysteine. In addition, intermolecular S-S bond formation, such as glutathionylation, has been demonstrated for several PTPs [21, 22, 35]. The next step is the reduction and reactivation of the oxidized PTPs, the mechanism and importance of which remains relatively underexplored.

A wide variety of low Mr thiols, particularly those with low pKa, have been shown to reduce and reactivate member of the PTP family [36]. The biologically important thiol glutathione has been shown to act on several oxidized PTPs, with reports of glutathionylated forms of PTP1B [21, 22] and the Low Mr PTP [35]. Indeed, experiments with butathionine sulphoximine, to deplete GSH in cell and animal models, attest to its potential importance in vivo [6, 37]. In addition, thiol-containing enzymes, such as thioredoxin (TRX) have also been implicated in reduction of those oxidized PTPs that employ the S-S bond protective mechanism, including cdc25 [32], PRL2 [33] and PTEN [23]. Nevertheless, although PTEN and TRX have been reported to interact in a redox-dependent manner, at least one mode of this interaction involves S-S bond formation between the active site Cys of TRX1 and Cys-212 of the C2 domain of PTEN, which leads to inhibitory effects on phosphatase function that are indirect [38].

Although TRX is best known as a disulphide reductase [26], there have also been reports that it functions as a reducing agent for proteins that lack the second resolving Cys residue, and so do not form an S-S bond, such as methionine sulphoxide reductase [39]. Interaction of TRX with the classical PTPs SHP1 and CD45 has been implicated in the mechanism by

which SOCS-1 suppresses ROS-mediated apoptosis [40]. In addition, there have been reports that TRX may also reduce PTP1B [14, 24], although a persulphide modification of the PTP active site Cys generated following sulphydration by H₂S was shown to promote reduction by TRX relative to the oxidized form of the phosphatase [41]. These observations suggest that the variety of reducing systems not only represent a barrier to oxidation, but also the reduction and reactivation step may serve as an additional point at which control may be exerted over the redox regulation of PTP function.

In this study, we used a mechanism-based trapping approach, which previously led to identification of TRX target proteins in plants [42], in the secretory pathway of lymphocytes [43, 44], in mitochondria [45] and on the cell surface [27]. This strategy is similar to the approach pioneered by this lab involving substrate trapping mutants to investigate the specificity of PTPs in vivo [46]. We found that the trapping mutant of TRX1 formed mixed disulphide intermediates with both PTP1B and PTEN after H₂O₂ treatment of cells. The amount of phosphatase covalently captured by the trapping mutant depended on the concentration of exogenously applied H₂O₂.

In addition to the expected PTP1B-Trx1(CS) conjugate, we observed another anti-PTP1B-reactive species (~120kD), approximately twice the size of the PTP1B monomer (Figure 2B, right panel). We consider it likely that this band represents a covalent PTP1B dimer which was formed as a secondary product of the trapping reaction. PTP1B has been reported to form non-covalent dimers [47] and several of its 10 cysteines appear to be accessible on its surface [48]. Thus, PTP1B-TRX1(CS) conjugates are likely to coprecipitate non-covalently associate PTP1B molecules. These may engage in thiol-disulfide exchange with the mixed disulphide of the PTP1B-Trx1(CS) conjugates and, thereby, create covalent PTP1B dimers. This notion is supported by the observation that no 120kD band can be detected in whole cell lysates or when wild type TRX1 is used in the control trapping reaction. Moreover, as expected, in two of our experiments the intensity of the 120kD band was increased by H₂O₂ treatment (Fig. 2B, right panel; Fig. 2C, left panel). Although the 120kD band was not further enhanced by H₂O₂ treatment in another experiment (Figure 4A), we consider it unlikely that PTP1B is constitutively oxidized in these cells, because basal ROS levels in HeLa cells are low [49]. Instead, the observed variability regarding the intensity of 120kD band in the absence of H₂O₂ treatment is likely due to incomplete thiol blocking by NEM. Depending on cell type and culture conditions, very high NEM concentrations (100 mM or higher) may be needed for complete blocking [50]. Incomplete NEM blocking can be expected to lead to (variable) oxidation of PTP1B during cell lysis, which would entail increased trapping and thus formation of covalent PTP1B dimers. Thus, in our interpretation the observation of the 120kD band is consequential to and supportive of the PTP1B-TRX1 redox interaction.

Although an interaction was induced by H₂O₂, we were unable to detect trapping of PTP1B or PTEN in lysates from cells stimulated with growth factors. This is consistent with the documented low stoichiometry of oxidation of PTPs produced following acute stimulation with growth factors and hormones, the extent of which may be below the threshold for detection by application of TRX trapping mutants to whole cell lysates [16, 51]. In fact, even in macrophages induced to generate an oxidative burst, only a small fraction of PTEN

(16%) became oxidized [17, 18, 20]. Furthermore, due to the fact that it is an abundant protein, endogenous TRX1 may exert a major influence on reduction of oxidized proteins during cell lysis and, therefore, may limit the amount of oxidized PTPs that can be captured by the trapping mutant. Therefore, to increase the endogenous steady state concentration of oxidized TRX1 substrates, we used cells in which TRX1 was depleted by RNA interference. Although the amount of PTP1B and PTEN trapped from lysates of H₂O₂-stimulated cells was increased after depletion of endogenous TRX1, we still did not detect trapping of PTPs in lysates from growth factor-stimulated cells. Nevertheless, depletion of endogenous TRX1 delayed the recovery of the reduced form of PTEN after H₂O₂ treatment, highlighting the importance of TRX1 as a mediator of PTEN reactivation for regulation of PIP3-dependent signals in intact cells.

In contrast, TRX trapping mutants were effective when expressed in cells so that complex formation occurred *in situ*. Their application in this context allowed us to define the basis for the TRX-PTP1B interaction in more detail (Figure 7). Following co-expression of the TRX1 trapping mutant with PTP1B, we observed that stimulation of the cells with insulin induced co-precipitation of PTP1B with the TRX1 trapping mutant, but not wild type or C32S mutant, in which the active site cysteine of TRX1 was changed to serine. In turn, a mutant form of PTP1B that adopts the structural features of the oxidized form of the enzyme, but lacks its catalytic cysteine, PTP1B-CASA [16], was not targeted by TRX1 after insulin stimulation. Thus, the interaction was based upon the catalytic cysteines of both proteins. Overall, in addition to highlighting the important role of thioredoxin in reduction and reactivation of PTPs, the TRX trapping mutants illustrate another approach to defining the importance of reversible oxidation in the regulation of PTP function in general and tyrosine phosphorylation-dependent signaling in a broad array of signaling contexts.

EXPERIMENTAL PROCEDURES

Antibodies

The following antibodies were used for immunoblotting and immunoprecipitation: anti-His (Genescript), anti-actin and anti-Flag M2 (Sigma), anti-phospho-Akt (Ser473), anti-Akt, anti-PTEN and anti-TRX1 (Cell Signaling), anti-PTP1B (FG6; N.K. Tonks), rabbit polyclonal anti-insulin receptor [pYpY^{1162/1163}] phospho-specific antibody (Invitrogen) and light chain-specific Anti-Mouse IgG (Jackson Immuno Research Laboratories).

Protein expression, purification and quantitation

Bacterial expression and purification of TRX1 (WT and mutant) and PTP1B were described previously [41, 52]. For mammalian expression TRX1 was subcloned into PTT3 vector using Xba1 (5') and BamH1 (3') restriction sites [53]. C32S-TRX1 and C35S-TRX1 mutants were generated using a Mutagenesis Kit (Stratagene) and the sequence was verified. The enzymes Thioredoxin Reductase and Glutaredoxin were obtained from Sigma-Aldrich. Throughout the study protein concentration was determined using Bradford reagent.

TRX1 substrate trapping

HeLa or HeLa T-REx-252 cells at 80–90 % confluency were serum-starved overnight in DMEM Low Glucose, w/o phenol red (Sigma). Cells were stimulated with human EGF (50 ng/ml; PeproTech), insulin (50 or 100 nM; Calbiochem) or H₂O₂ (1 mM; Sigma) for 5 min at room temperature. Cells were lysed using PBS with 1 % (v/v) Triton X-100, 100 mM *N*-ethyl maleimide (NEM) and Complete Mini Protease Inhibitors EDTA-free (Roche) for 20 min on ice. Soluble proteins were harvested by centrifugation at 13,000 × g for 10 min at 4°C and quantitated. Total protein (~25 µg) was used as lysate control. Total protein (0.5–1 mg) was applied to Zeba Spin Desalting Columns (Thermo Scientific) and samples were collected by centrifugation at 1,000 × g for 5 min at 4°C. Streptavidin sepharose beads (GE Healthcare) were loaded with 100 µg of recombinant TRX1/sample in PBS with 20 mM DTT for 2 hrs at 4°C and subsequently washed to remove the reductant. The loaded beads were added to the lysate samples and incubated for 1 h at 4°C. The reaction was stopped with 20 mM NEM and beads were washed with buffer 1 (PBS with 1 % (v/v) Triton X-100, 10 mM NEM and 500 mM NaCl), buffer 2 (PBS with 1 % (v/v) Triton X-100, 1 mM NEM and 0.1 % (w/v) SDS) and PBS. Protein complexes were eluted in PBS with 2 mM biotin for 30 min at 4°C and equal parts were boiled in non-reducing or reducing (20 mM DTT) Laemmli sample buffer and subsequently treated with 40 mM NEM.

Time course of transient PTEN oxidation in HeLa cells

HeLa T-REx-281 (shRNA control) or –252 (shRNA TRX1) cells at 70–90 % confluency were stimulated with 500 µM H₂O₂. At the indicated time points, the supernatant was removed and cells were treated with 10 mM NEM in PBS for 5 min at 4°C. Cells were harvested in lysis buffer (TBS with 1 % (v/v) Triton X-100, 10 mM *N*-ethyl maleimide (NEM) and Complete Mini Protease Inhibitors EDTA-free (Roche)) and samples were incubated for 30 min at 4°C. Lysates were cleared by centrifugation at 13,000 × g for 15 min at 4°C. Equal parts of the lysates were boiled in non-reducing or reducing (20 mM DTT) Laemmli sample buffer.

Rate of inactivation and reactivation of PTP1B/ PTEN

PTEN or PTP1B [10 µM] was incubated with varying concentrations of H₂O₂ (0–5 mM) in 50 mM Tris pH 6.5, 100 mM NaCl and 0.1 % BSA at 25°C and the rate of inactivation was measured using 6,8-Difluoro-4-Methylumbelliferyl Phosphate (DiFMUP) as substrate. To measure the rate of reactivation, PTP1B or PTEN [10 µM] was oxidized and aliquots were diluted 2-fold into 50 mM Tris pH 6.5, 100 mM NaCl, 0.1 % BSA, and 1 mM EDTA containing varying amounts of DTT [10–200 mM], GSH [50–200 mM], TRX1/TR (0.2–5 equivalents of TRX1 to PTP1B or PTEN) or GSH and GR/GRX (10 mM GSH, together with glutathione reductase (GR) and glutaredoxin, both at 5 µM GRX) for varying time periods (1–60 min) and the phosphatase activity was monitored. For assays using TRX1/TR and GSH/Grx as reductants, NADPH (25–300 µM) was included at a ratio of 200:1.

Measurement of redox potentials

To determine the redox potential of the active site cysteine of PTP1B or PTEN, recombinant enzymes (20 µg) were incubated in 100 mM HEPES, pH 7.0 and 1 mM EDTA in the

presence of varying concentrations of GSH/GSSG for 2 hrs at 25°C. Protein samples were precipitated using TCA (20 %) for 30 min at 4°C and the precipitants were centrifuged at $10,000 \times g$ for 10 min and washed with acetone. Pellets were resuspended in 100 mM Hepes, pH 7.0, 1 mM EDTA and 1 % (w/v) SDS and alkylated using 0.1 mM 5-FAM (fluorescein-5-maleimide, Molecular Probes) for 2 hrs at 25°C. The unreacted 5-FAM was removed using PD-10 size exclusion columns (GE Healthcare) and the degree of labeling was calculated using the following equation (molar extinction coefficient (ϵ) of 5-FAM = $68000 \text{ M}^{-1} \text{ cm}^{-1}$):

$$\text{moles fluor/moles protein} = \text{Emission}_{\text{max}} / \epsilon \times \text{protein concentration}$$

Fluorescence spectra were recorded and the fluorescence emission at 520 nm was used to quantitate the reduced and oxidized cysteine. The redox potential was calculated using the Nernst equation $E_h = E_0 - RT/nf \ln 2[\text{GSH}]/\text{GSSG}$ where E_0 is the standard reduction potential (-240 mV).

Acknowledgments

We thank Darryl Pappin (Cold Spring Harbor Laboratory) for his advice on the proposed mechanism for reduction of PTP1B by TRX1. This research was supported by NIH grant GM55989 to N.K.T. and the CSHL Cancer Centre Support Grant CA45508. U.S. was the recipient of a postdoctoral fellowship from the Cold Spring Harbor Laboratory (CSHL). R.G. was supported by a scholarship from the Studienstiftung des deutschen Volkes. N.K.T. is also grateful for support from the following foundations; The Gladowsky Breast Cancer Foundation, The Don Monti Memorial Research Foundation, Hansen Memorial Foundation, West Islip Breast Cancer Coalition for Long Island, Glen Cove CARES, Find a Cure Today (FACT), Constance Silveri, Robertson Research Fund and the Masthead Cove Yacht Club Carol Marcincuk Fund.

Abbreviations used

DTT	dithiothreitol
EGF	epidermal growth factor
Grx	glutaredoxin
GSH	reduced glutathione
GSSG	oxidized glutathione
H₂O₂	hydrogen peroxide
PBS	phosphate-buffered saline
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
ROS	reactive oxygen species
TBS	Tris-buffered saline
TR	thioredoxin reductase
Trx1	thioredoxin 1
WCL	whole cell lysate

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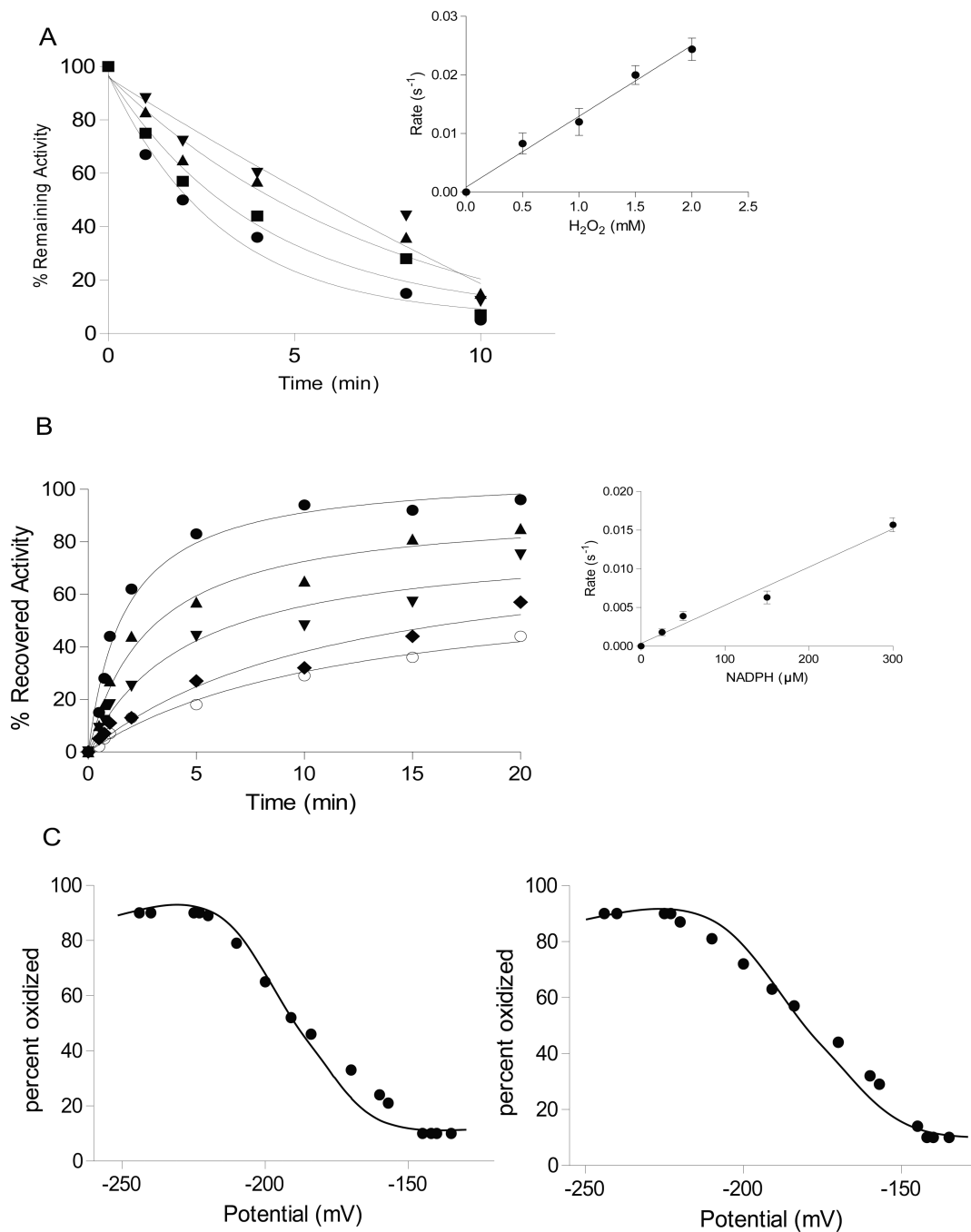


Figure 1. TRX1 reactivated oxidized PTP1B and PTEN in vitro

(A) Time dependent inactivation of PTEN by H_2O_2 , 0.5 mM (\blacktriangledown), 1 mM (\blacktriangle), 1.5 mM (\blacksquare), 2 mM (\bullet). Inset: concentration dependence of the rate of inactivation.

(B) Time dependent reactivation of PTEN by TRX1/TR. NADPH was used at 10 μM (\circ), 25 μM (\blacklozenge), 50 μM (\blacktriangledown), 150 μM (\blacktriangle) or 300 μM (\bullet). Inset: concentration dependence of the rate of reactivation. Data are representative of three independent experiments.

(C) Analysis of the redox potential of PTEN and PTP1B. Recombinant PTEN (left) and PTP1B (right) were incubated in redox buffers with varying ratios of GSH/GSSG and the protein thiol status was determined from the emission spectrum of 5-flourescein maleimide.

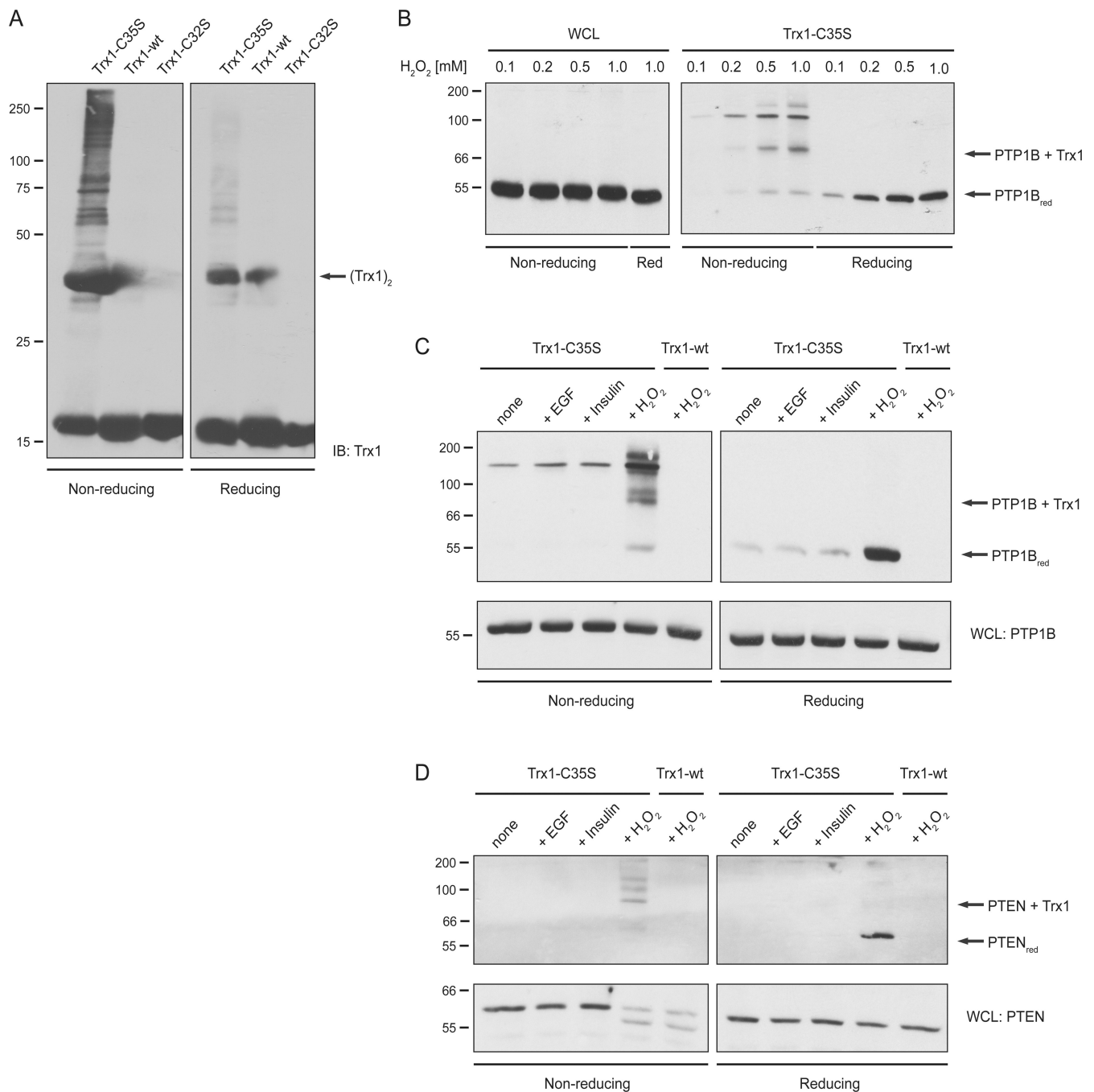


Figure 2. TRX1 formed disulfide-exchange intermediates with PTP1B and PTEN

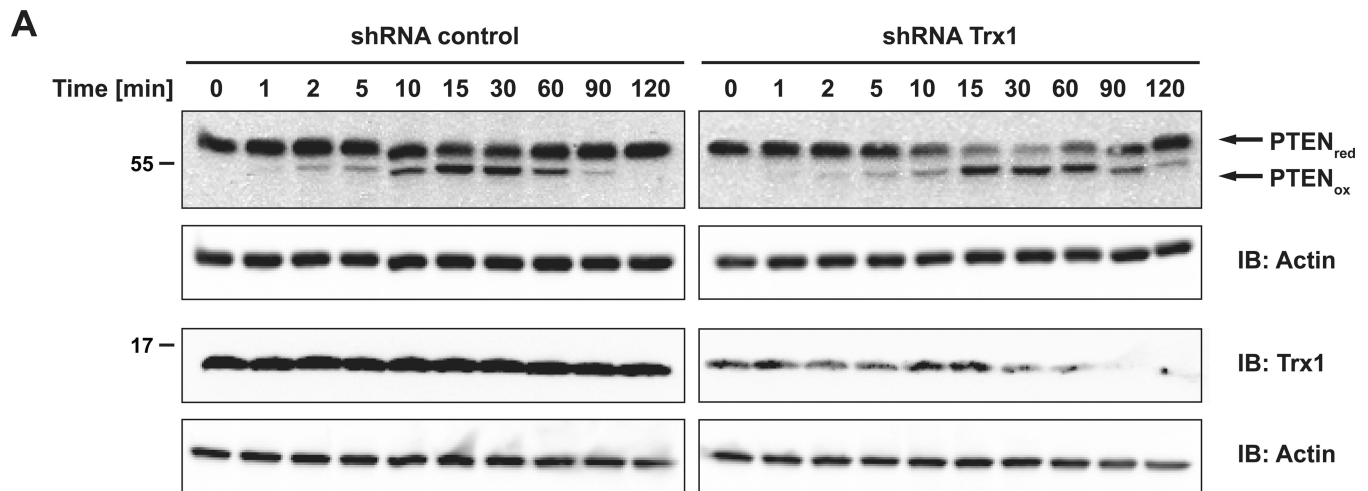
(A) HeLa cells were stimulated with 1 mM H₂O₂ and lysate samples were incubated with different recombinant TRX1-constructs. TRX1-substrate complexes were resolved by non-reducing and reducing SDS-PAGE and analyzed by anti-TRX1 immunoblotting. Dimerization of exogenously added TRX1 is indicated. The molecular marker is indicated in kDa.

(B) HeLa cells were stimulated with different concentrations of H₂O₂ and lysate samples were incubated with the TRX1 trapping mutant (TRX1-C35S). Samples of WCL (left panel)

and TRX1-substrate complexes (right panel) were resolved by non-reducing and reducing SDS-PAGE and analyzed by anti-PTP1B immunoblotting. The disulfide-linked TRX1-PTP1B complex and reduced PTP1B are indicated. The molecular marker is indicated in kDa.

(C) HeLa cells were stimulated with 50 ng/ml EGF, 50 nM insulin or 1 mM H₂O₂ and lysate samples were incubated with the TRX1 trapping mutant (TRX1-C35S) or TRX1-wt as control. TRX1-substrate complexes and samples of WCL were analyzed as described in (A). The disulfide-linked TRX1-PTP1B complex and reduced PTP1B are indicated. The molecular marker is indicated in kDa.

(D) Samples from (B) were analyzed by PTEN-specific immunoblotting. The disulfide-linked TRX1-PTEN complex and reduced PTEN are indicated. The markers indicate kDa.



B

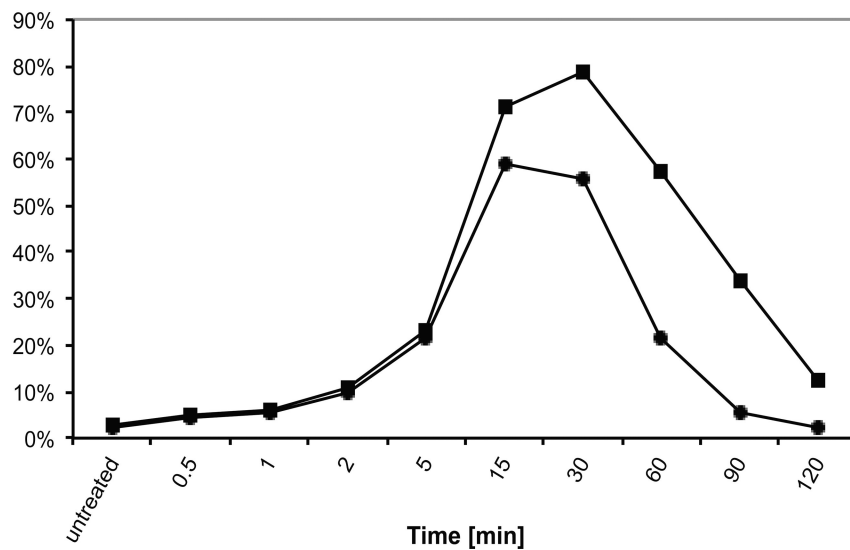


Figure 3. Effect of suppressing TRX1 on oxidation-reduction of PTEN in cells

(A) HeLa T-REx-281 (shRNA control) or HeLa T-REx-252 (TRX1 shRNA) cells were treated with 1 μ g/ml doxycycline for 7 days. Cells were treated with 500 μ M H₂O₂ and lysate samples were prepared at the time points indicated. Samples were resolved by non-reducing (upper panel) or reducing SDS-PAGE and analyzed by anti-PTEN, anti TRX1 and anti-actin immunoblotting.

(B) Quantitation of a time course of H₂O₂-induced oxidation of PTEN in the presence (closed circles) and absence (closed squares) of RNAi-mediated suppression of TRX1. The y-axis represents the % of PTEN migrating as the oxidized form on SDS-PAGE compared to the total population of PTEN.

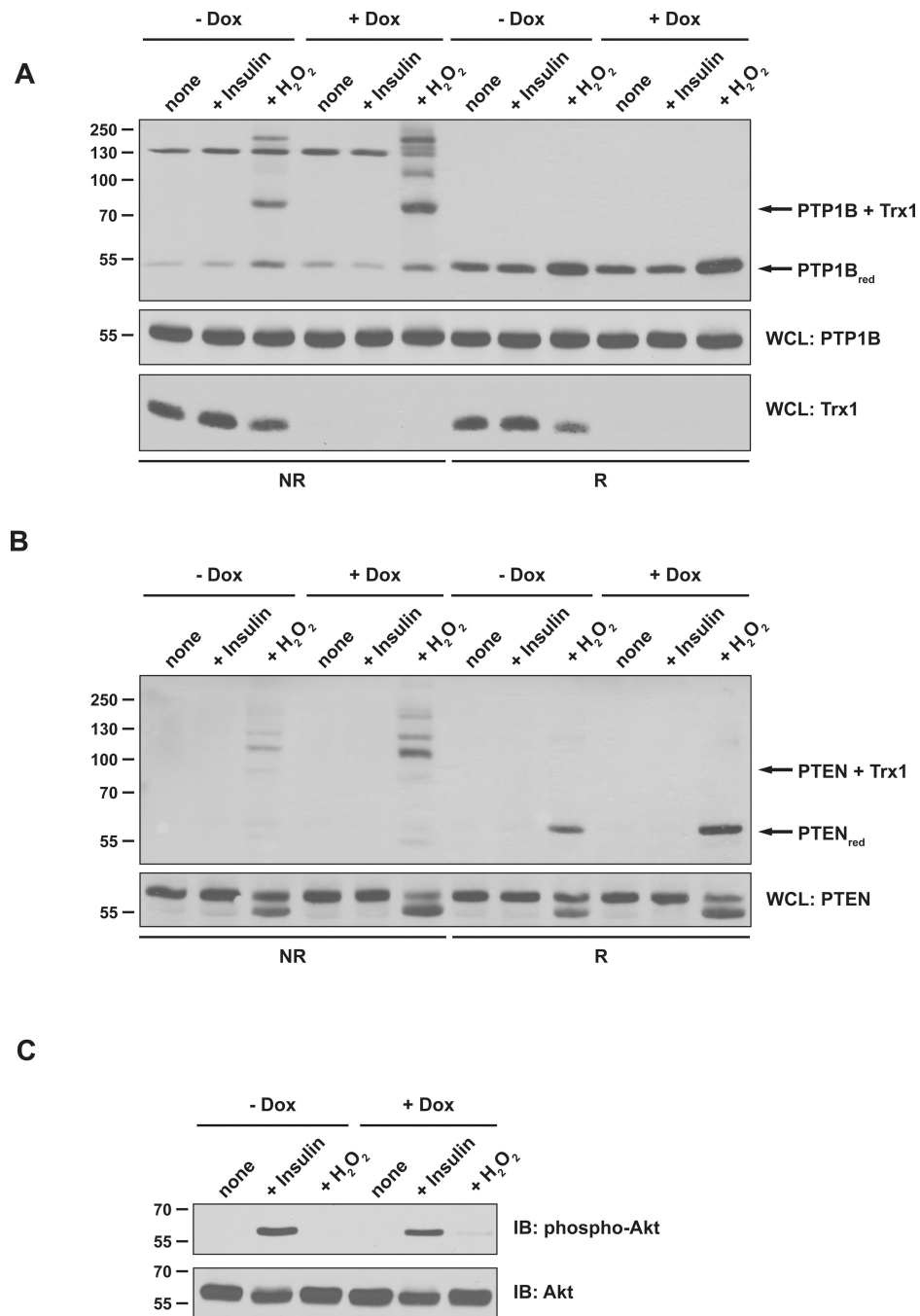


Figure 4. TRX1 contributed to reduction of PTEN and PTP1B in cells

(A) HeLa T-REx-252 cells were either left untreated or treated with 1 μ g/ml doxycycline (Dox) for 5 days. Cells were stimulated with 100 nM insulin or 1 mM H₂O₂ and lysate samples were incubated with the TRX1 trapping mutant (TRX1-C35S). TRX1-substrate complexes (upper panel) and samples of WCL (middle panel) were resolved by non-reducing and reducing SDS-PAGE and analyzed by anti-PTP1B immunoblotting. The disulfide-linked TRX1-PTP1B complex and reduced PTP1B are indicated. WCL samples were analyzed by TRX1-specific immunoblotting to verify TRX1 depletion (lower panel).

(B) Samples as in (A) were analyzed by PTEN-specific immunoblotting. The disulfide-linked TRX1-PTEN complex and reduced PTEN are indicated. The markers indicate kDa.
(C) Samples of WCL were analyzed with anti-phospho-Akt (Ser473) antibody to confirm responsiveness of the cells to insulin.

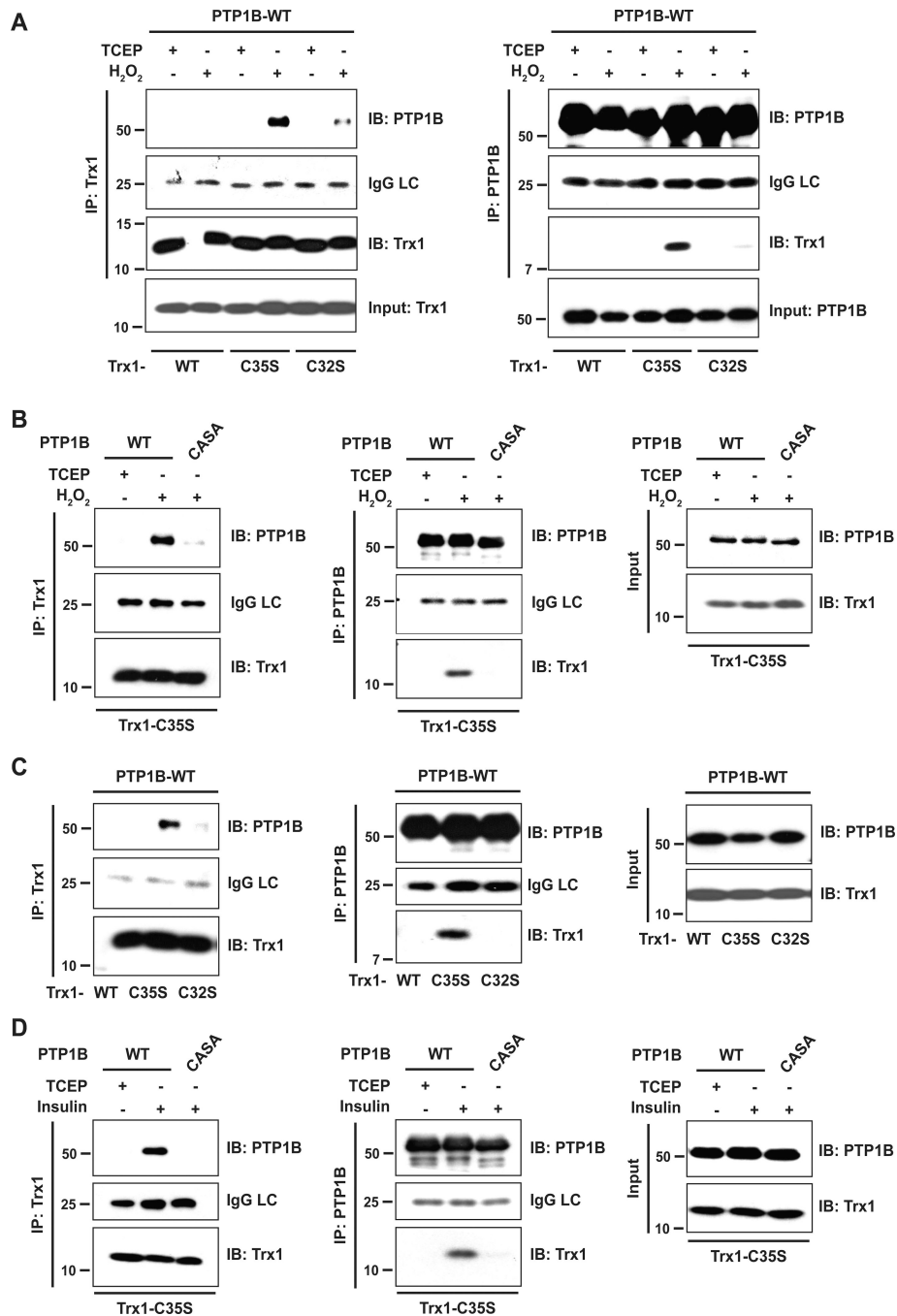


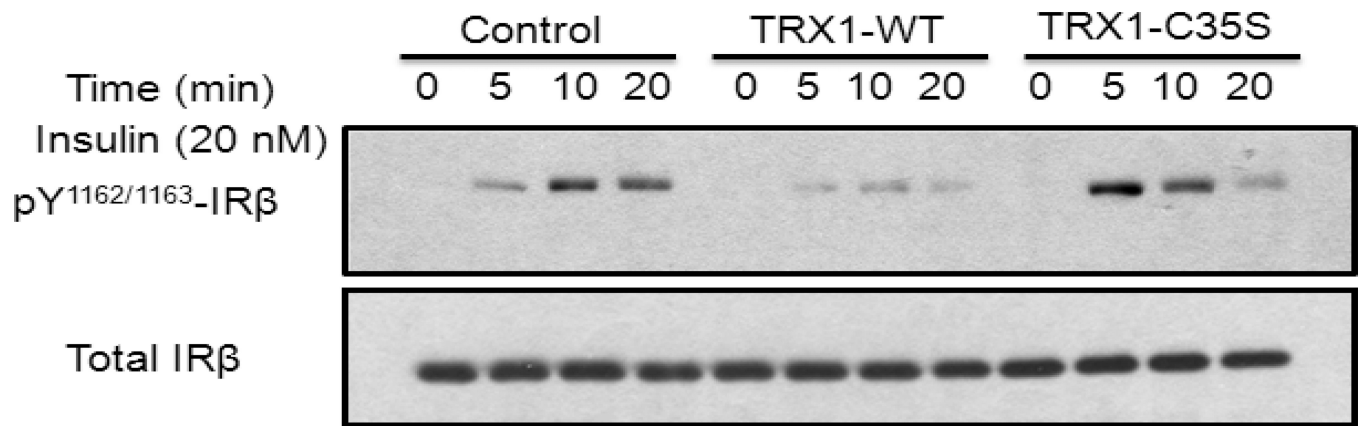
Figure 5. An ectopically expressed TRX1 trapping mutant co-precipitated with PTP1B after insulin stimulation

(A) Different His₆-tagged TRX1 constructs were co-expressed with Flag-tagged PTP1B in 293T cells. Cells were treated with 1 mM H₂O₂ to oxidize intracellular proteins. Untreated cells were harvested in the presence of TCEP. TRX1 (left panel) or PTP1B (right panel) was immunoprecipitated from whole cell lysates and analyzed by immunoblotting. TRX1 and PTP1B expression was confirmed in input lysates. LC: IgG light chain

(B) TRX1-C35S and PTP1B-WT or the CASA mutant were co-expressed in 293T cells. TRX1 (left panel) or PTP1B (middle panel) was immunoprecipitated from lysates of reduced (TCEP) or H₂O₂-treated cells and immunoprecipitates were analyzed as described in (A). TRX1 and PTP1B expression was confirmed in input lysates (right panel).

(C) 293T cells co-expressing different His₆-tagged TRX1 constructs and Flag-tagged PTP1B were treated with 100 nM insulin for 10 minutes, the time point at which insulin receptor β subunit (IR β) and IRS-1 displayed maximum tyrosine phosphorylation [16]. TRX1 (left panel) or PTP1B (middle panel) was immunoprecipitated from whole cell lysates and immunoprecipitates were analyzed as described in (A). TRX1 and PTP1B expression was confirmed in input lysates (right panel).

(D) 293T cells co-expressing TRX1-C35S and PTP1B-WT or -CASA were treated with 100 nM insulin. PTP1B-WT or -CASA were immunoprecipitated and analyzed as described in (A). TRX1 and PTP1B expression was confirmed in input lysates (right panel).

**Figure 6. Effects of TRX1 on insulin signaling**

293T cells expressing vector only, His₆-tagged TRX1-WT or His₆-tagged TRX1-C35S constructs were treated with 20 nM insulin for 0, 5, 10, 20 minutes. Insulin-stimulated phosphorylation of the insulin receptor β-subunit was monitored by immunoblotting with anti-insulin receptor [pYpY^{1162/1163}] phospho-specific antibody. Sample loading on the gel was standardized by immunoblotting with antibody to insulin receptor β-subunit.

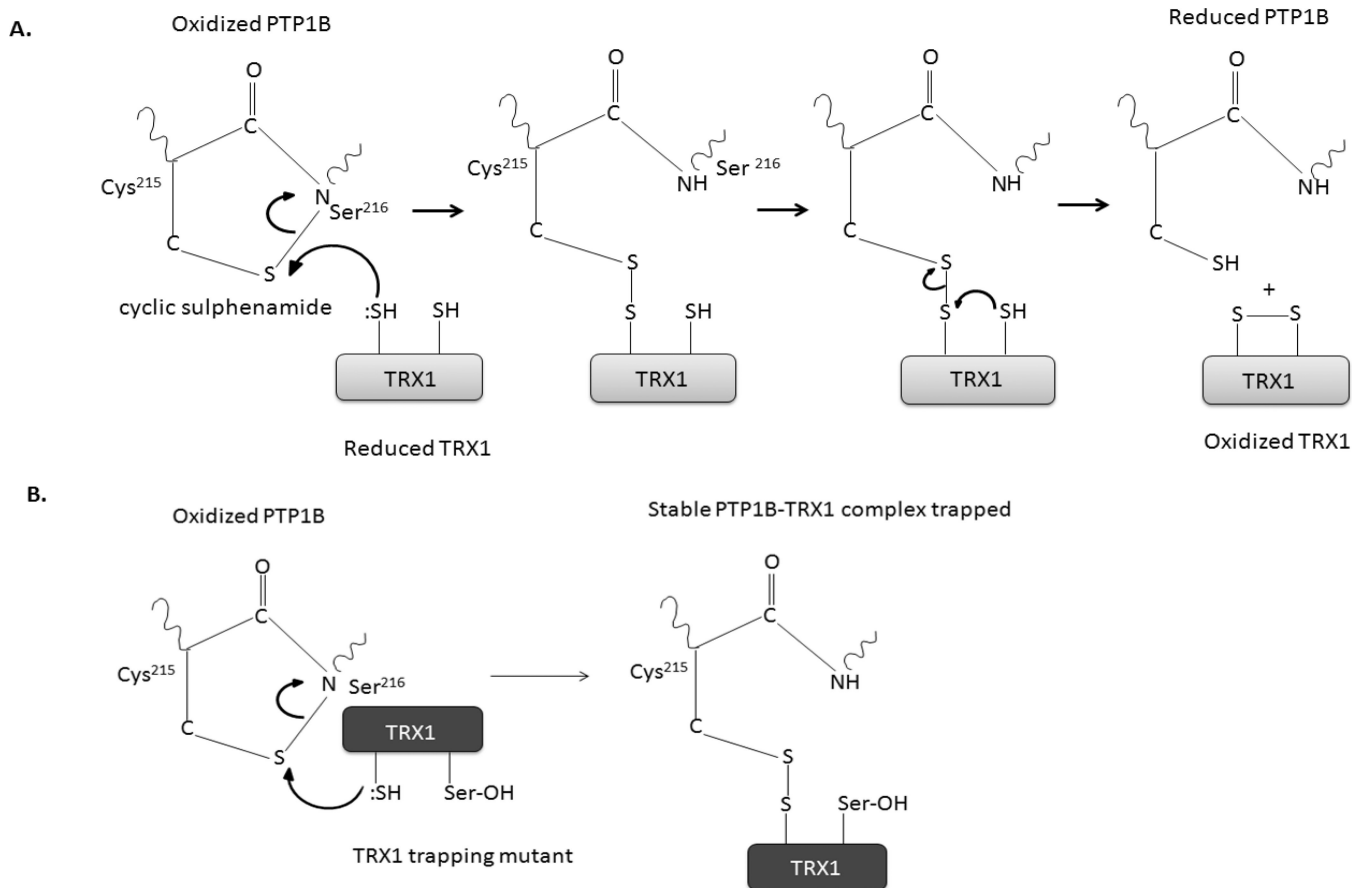


Figure 7. TRX1-mediated reduction of PTP1B

(A) The proposed mechanism for reduction of the cyclic sulphenamide form of oxidized PTP1B by TRX1.

(B) The absence of the resolving cysteine (Cys-35) generates a trapping mutant form of TRX1 that forms a stable complex with oxidized PTP1B (lower panel).

Table 1

Inactivation and reactivation rates of PTEEN and PTP1B upon oxidation with H₂O₂ and reduction with either DTT, GSH, TRX1/TR or GRX/GSH

Enzyme	H ₂ O ₂ (M ⁻¹ s ⁻¹)	DTT (M ⁻¹ s ⁻¹)	GSH (M ⁻¹ s ⁻¹)	TRX1/TR (M ⁻¹ s ⁻¹)	GRX/GSH (M ⁻¹ s ⁻¹)
PTEEN	14± 0.5	0.83± 0.12	0.036± 0.01	0.95± 0.15	0.14± 0.04
PTP1B	10± 0.8	0.55± 0.1	0.042± 0.01	0.75± 0.2	0.12± 0.03