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Delayed cell cycle progression from SEPW1 depletion is p53- and p21-dependent in MCF-7 breast cancer cells

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ABSTRACT

Selenium (Se) is an essential redox-active trace element with close connections to cancer. Most of Se's biological functions have been attributed to the antioxidant properties of Se-containing proteins. However, the relative contribution of selenoproteins and small Se compounds in cancer protection is still a matter of debate. The tumor suppressor p53 is the most frequently mutated gene in human cancer and is often referred to as the "guardian of the genome". In response to genomic stresses, p53 causes cell cycle arrest to allow time for genomic damage to be repaired before cell division or induces apoptosis to eliminate irreparably damaged cells. Selenoprotein W (SEPW1) is a highly conserved small thioredoxin-like protein required for cell cycle progression. The present work shows that SEPW1 facilitates the G1 to S-phase transition by down-regulating expression of the cyclin-dependent kinase inhibitor p21. SEPW1 controls p21 by modulating levels of the p53 transcription factor, and this is associated with changes in phosphorylation of Ser-33 in p53. More work is needed to identify the mechanism by which SEPW1 regulates phosphorylation of Ser-33 and the kinase or phosphatase enzymes involved.

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1. Introduction

The essential trace element, selenium (Se) has been associated with cancer for nearly a century [1]. Dietary Se prevents chemically induced cancers in experimental animals [2] and higher dietary Se intakes are associated with decreased risk of cancer in humans [3]. The main biologically active form of Se in mammals is selenocysteine (Sec)-containing proteins, known collectively as selenoproteins. The mammalian selenoproteome consists of 25 conserved selenoproteins [4], the most well-known of which are the glutathione peroxidases that reduce hydrogen peroxide and/or organic hydroperoxides at the expense of glutathione (GSH). Supplemental Se and over-expression of selenoproteins protects against oxidant challenges, whereas Se-depletion and genetic deletion of selenoproteins makes animals and cells more sensitive to oxidative stress. Thus, anti-oxidation is assumed to be the main biological function of selenoproteins and the anti-cancer activity of Se has been hypothesized to arise from Se's antioxidant properties [5].

Se is the only element specified in the universal genetic code and Sec has become recognized as the 21st protein amino acid [6]. Sec is synthesized from serine after aminoacylation to a unique transfer RNA, $tRNA^{Sec}$, in all three superkingdoms of life [7]. Sec is subsequently incorporated into growing polypeptide chains under control of the UGA codon (TGA in DNA), which usually codes for termination. Incorporation of Sec is directed by a specific stem-loop structure in the mRNA 3′ to the UGA codon known as a "selenocysteine insertion sequence". All of the selenoproteins for which an enzymatic activity has been identified catalyze redox reactions involving oxidation of sulfhydryl groups and/or reduction of disulfides.

The microbial selenoproteome contains over 3600 members in approximately 58 families of homologous selenoproteins [8]. The most abundant and widespread selenoprotein family is "selenoprotein W-like" [9]. Selenoprotein W (SEPW1), the mammalian archetype of the selenoprotein W-like family, is a ubiquitous 9 kDa Sec-containing protein with glutathione-dependent antioxidant activity [10]. SEPW1 occurs in humans, mice, rats, sheep, monkeys, rabbits, guinea pigs, and cattle [11]. SEPW1 is expressed in all 22 human tissues examined, with highest levels in brain, testes, and muscle. SEPW1 is one of the most highly expressed selenoproteins in humans and it is regulated at the level of mRNA stability by Se intake [12]. SEPW1 expression is proportional to dietary Se intake, increasing markedly with Se supplements and decreasing rapidly on Se-restricted diets [13]. SEPW1 mRNA expression is cell cycle-dependent in human epithelial cells and silencing of SEPW1 expression causes cells to accumulate in G0/G1 phase of the cell cycle [14]. Homozygous SEPW1-knockout mouse embryos die at the pre-implantation blastocyst stage [15].

Abbreviations: Se, selenium; SEPW1, selenoprotein W1; Cys, cysteine; Sec, selenocysteine; Ser, serine; Thr, threonine; ROS, reactive oxygen species.

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The evolutionary conservation of SEPW1 and the embryonic lethality of its deletion suggest it has an important role in cell cycle regulation.

The p53 protein is a homotetrameric transcription factor that regulates expression of a wide variety of genes through direct binding to response elements in DNA. The best understood function of p53 is to respond to an array of cellular stresses, such as DNA damage, oncogene expression, nucleotide depletion, and aberrant growth signals by inducing cell cycle arrest, DNA repair, differentiation, senescence, or apoptosis. The p53 protein senses and integrates these various stresses via a panoply of post-translational modifications, including phosphorylation, acetylation, and ubiquitination. p53 protein is expressed constitutively, but levels are normally kept low by its rapid ubiquitination by the HDM2 protein and rapid proteasomal degradation. Following DNA damage and other stresses, the human p53 N-terminal region is phosphorylated on serines 6, 9, 15, 20, 33, and 37 and threonine 18 by ATM, ATR, DNA-PK, p38 MAPK, Chk1, and Chk2. Phosphorylation of p53 disrupts its binding with HDM2, blocks ubiquitination and proteolysis, and results in a rapid increase in p53 protein levels, allowing p53 to enter the nucleus, bind to DNA and induce expression of DNA repair and cell cycle inhibitor genes. Thus, p53 facilitates the repair and survival of damaged cells or eliminates severely damaged cells from the replicative pool to protect the organism, earning it the moniker "guardian of the genome".

We investigated the role of p53 in cell cycle arrest induced by SEPW1 silencing in MCF-7 breast tumor cells. We found that total p53 and p53 phosphorylated on Ser-33 were increased in SEPW1-silenced cells. Stable knockdown of p53 with short hairpin RNA abrogated cell cycle arrest from SEPW1 silencing. p21 (Cip1/WAF1), the cyclin-dependent kinase inhibitor targeted by p53 that mediates cell cycle arrest, was increased in SEPW1-silenced cells, and silencing of p21 expression abrogated cell cycle arrest from SEPW1 depletion. Thus, cell cycle arrest from SEPW1 silencing in MCF-7 cells is mediated by p53 and p21.

2. Materials and methods

2.1. Cell culture

MCF-7 human breast adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in 1:1 DMEM/F12 (HyClone, SouthLogan, UT) supplemented with 10% FBS and 2 mM L-glutamine in the presence of 5% CO_2 in air at 37 °C. The p53-deficient MCF-7 cell line stably-transfected with shRNA targeting the p53 gene was a kind gift from Dr. Xinbin Chen at University of California at Davis and was cultured under the same conditions as the wild type MCF-7.

2.2. siRNA transfections

10^5 cells per well were reverse-transfected in six well dishes with 0.2% Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA) and 5 nM Silencer Select Validated siRNAs (ABI, Foster City, CA) targeting either SEPW1 (#s361), p21 (#s415), or Silencer Select negative control siRNA #1.

2.3. Cell cycle analysis

Propidium iodide staining of cellular DNA and flow cytometry analysis were performed as described before [14].

2.4. Western blots

Total cellular protein was extracted with RIPA buffer containing 1 × HALT Protease and Phosphatase Inhibitor Cocktails (Pierce, Rockford, IL) and 5 mM EDTA. Protein concentrations were determined using a standard BCA assay, and the extracts were stored at −80 °C until use. Twenty or 30 μg protein per well resolved by SDS–PAGE was transferred to Immobilon P PVDF membranes (Millipore, Billerica, MA). The membranes were blocked for 1 h in 5% milk/TBST and then probed with 1 μg/ml anti-p53, anti-beta actin (Sigma, St. Louis, MO), anti-p21, anti-phospho-Ser20-p53, or anti-phospho-Ser33-p53 (Cell Signaling Technologies Beverly, MA) antibodies overnight at 4 °C. Following 1 h incubation with the appropriate secondary antibodies, the blots were covered with Immun-Star Western C reagent (BioRad, Hercules, CA) and chemiluminescence signals were detected with a ChemiDoc XRS Imaging System (BioRad). Membranes were stripped using Restore Plus Stripping Buffer (Pierce) and re-probed when necessary. Densitometry on blot images was performed using ImageLab software (BioRad).

2.5. Immunoprecipitation and Western blotting of SEPW1

Custom rabbit polyclonal antibody (Antibodies Inc., Davis, CA) raised against full-length mutant recombinant human SEPW1 (Sec to Cys mutation introduced to allow expression in *Escherichia coli*) was purified by absorption to Protein G-Agarose and stored in PBS at −70 °C. MCF-7 cells were seeded (6×10^5 cells) in 100 mm culture plates and transfected as described above. After 72 h, cells were lysed in M-Per lysis buffer containing 1 × HALT Protease and Phosphatase Inhibitor Cocktails and 5 mM EDTA (Pierce, Rockford, IL) and 1.75 mg total protein was incubated overnight with 10 μg SEPW1 antibody and 100 μL Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) in 1:1 M-Per lysate:PBS at 4 °C with gentle shaking. The resin was collected by centrifugation, washed four times with PBS and the proteins were eluted into reducing Laemmli buffer by boiling for 5 min. Samples were analyzed by SDS–PAGE and Western blotting as described above, using the polyclonal rabbit SEPW1 antibody as the primary antibody.

2.6. Statistical analyses

Cell cycle distribution data were analyzed with Student's *t*-test with SigmaStat Software (Systat, San Jose, CA). Changes in quantitative densitometry data from Western blots were expressed as fold-change and compared to a reference value of "1" with Student's *t*-test. Only the reported contrasts were tested statistically to minimize errors from multiple hypothesis testing. A probability of $p < 0.05$ was considered significant.

3. Results

SEPW1 protein was immunoprecipitated from MCF-7 lysates, subjected to SDS–PAGE, and analyzed with Western blots to reveal a prominent immunoreactive band at approximately 9 kDa in control siRNA-treated MCF-7 (Fig. 1). In contrast, immunoprecipitates from lysates of MCF-7 cells transfected with SEPW1 siRNA yielded only a faint band of SEPW1 protein, demonstrating that the siRNA efficiently silenced expression of SEPW1 protein.

Previous work showed that depletion of SEPW1 in non-tumorigenic prostate and mammary epithelial cells causes a delay in cell cycle progression at the G1 to S-phase transition [14]. To study the mechanism of this G1 arrest, we examined the effects of SEPW1 depletion in MCF-7 breast cancer cells, which have wild-type p53 and have been used extensively in studies of p53 function and regulation. First, we confirmed that SEPW1 depletion induced the same phenotype in MCF-7 cells as was previously observed in non-tumorigenic MCF-10A cells [14]. Table 1 shows that treatment with SEPW1 siRNA caused a greater fraction of cells to be in

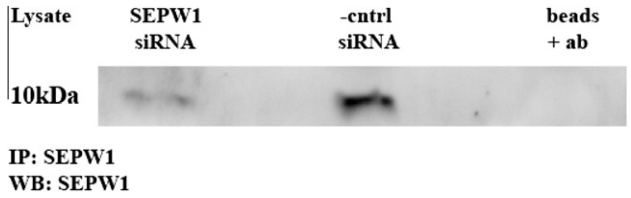

Fig. 1. SEPW1 silencing blocks expression of SEPW1 protein. Lysates of MCF-7 cells transfected with SEPW1 siRNA or a non-targeting control siRNA (-cntrl) were immunoprecipitated with rabbit polyclonal SEPW1 antibody and Protein A/G Agarose as described in Section 2. The proteins pulled down were separated by SDS–PAGE and probed with the polyclonal SEPW1 primary antibody.

G0/G1-phase and a smaller fraction to be in S-phase, indicating that SEPW1 depletion induced a G1 arrest in wild-type MCF-7 cells. This shows that SEPW1 facilitates the G1 to S-phase transition in MCF-7 breast cancer cells. Next, we tested whether SEPW1 depletion affected p53 and p21 protein levels as we had observed in non-tumorigenic prostate cells [16]. Quantitative densitometry of Western blots of p53 and p21 (examples shown in Figs. 2 and 3) indicated that SEPW1 depletion increased p53 by 95 ± 40% (n = 3, p = 0.038) and increased p21 by 206 ± 104% (n = 5, p = 0.024), consistent with the known functions of p53 and p21.

To find out whether delayed cell cycle progression from SEPW1 depletion required p53, we treated p53-deficient MCF-7 cells with SEPW1 siRNA. There was no increase in the fraction of cells in G0/G1-phase from SEPW1 silencing in p53-deficient MCF-7 cells (Table 2), showing that G1 arrest from SEPW1 depletion is p53-dependent. Curiously, depletion of SEPW1 seemed to facilitate entry into mitosis in p53-deficient MCF-7 cells, as the fraction of cells in S-phase was decreased and the fraction of cells in G2/M-phase was increased (Table 2). Next, we asked if delayed cell cycle progression from SEPW1 depletion required p21 expression by testing the effect of SEPW1 siRNA in wild-type MCF-7 cells with or without co-transfection with p21 siRNA. Whereas there was a noticeable increase in G0/G1 cells with SEPW1 siRNA alone (Table 3, "C" vs. "B"), there was no effect of SEPW1 siRNA on cell cycle when it was co-transfected with p21 siRNA (Table 3, "E" vs. "D"), even though p53 protein was increased compared to the negative control (Fig. 3, lanes 3 and 2 vs. lane 4). This result shows that G1 arrest from SEPW1 silencing is mediated by p21 cyclin-dependent kinase inhibitor.

Silencing SEPW1 increased p53 levels, which is typically a result of phosphorylation events on serine and threonine residues in the N-terminal transactivation domain of p53 (residues 1–42). DNA damage induces phosphorylation of Ser-20, but treatment of wild-type MCF-7 cells with SEPW1 siRNA did not change the amount of p53 phosphorylated on Ser-20 (Fig. 4). On the other hand, the form of p53 phosphorylated on Ser-33 was increased by SEPW1 depletion.

Table 1
Effect of SEPW1 silencing on cell cycle distribution in wild-type MCF-7 cells.

siRNA treatment	Percentage of cells (±SEM)		
	G0 and G1	S-phase	G2 and M
No transfection	63.9 ± 1.1	27.4 ± 1.4	8.7 ± 0.3
Non-targeting control siRNA	67.8 ± 0.8	23.6 ± 0.7	8.5 ± 1.1
SEPW1 siRNA	77.0 ± 2.0[a]	14.7 ± 1.4[a]	8.3 ± 0.8

Seventy-two hours after transient transfection, cells were fixed, stained with propidium iodide, and their DNA contents determined by flow cytometry. Data were analyzed with ModFit LT 3.0. The experiment was repeated three times and the data shown are the means ± SEM.

[a] Significantly different from non-targeting control siRNA (p < 0.01, 2-tailed t-test).

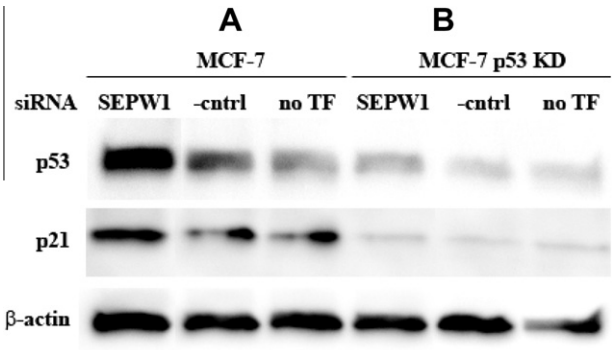

Fig. 2. Effect of SEPW1 silencing on p53 and p21 (Cip1/WAF1) in wild-type and p53-deficient MCF-7 cells. (A) Western blots of p53 and p21 protein from wild-type MCF-7 cells transfected with SEPW1 siRNA s361 or a non-targeting control siRNA (-cntrl). (B) Western blots of p53 and p21 protein from p53-deficient MCF-7 cells transfected with SEPW1 siRNA s361 or a non-targeting control siRNA (-cntrl). Beta-actin was included as a loading control.

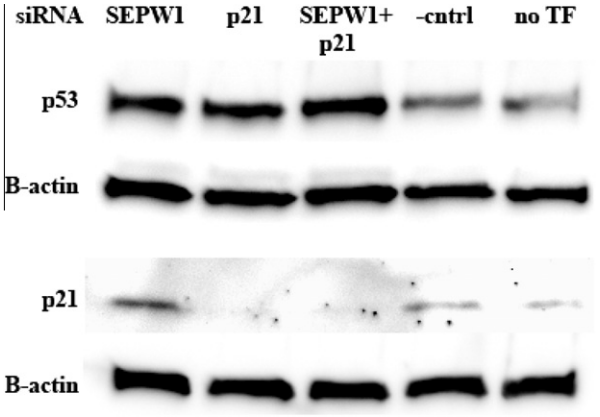

Fig. 3. Effect of SEPW1 and *p21* silencing on p53 and p21 (Cip1/WAF1) in wild-type MCF-7 cells. Western blots of p53 and p21 proteins from wild-type MCF-7 cells transfected with SEPW1 siRNA, *p21* siRNA, both SEPW1 and *p21* siRNAs or a non-targeting control siRNA (-cntrl). Beta-actin was included as a loading control. The experiment was repeated two times and the data shown are representative of both experiments.

Table 2
Effect of SEPW1 silencing on cell cycle distribution in p53-deficient MCF-7 cells.

siRNA treatment	Percentage of cells (±SEM)		
	G0 and G1	S-phase	G2 and M
No transfection	58.1 ± 1.0	30.9 ± 0.5	11.1 ± 0.5
Non-targeting control siRNA	53.3 ± 0.8	37.2 ± 0.3	9.5 ± 0.5
SEPW1 siRNA	54.2 ± 0.6	32.0 ± 0.4[a]	13.8 ± 0.2[a]

Seventy-two hours after transient transfection, cells were fixed, stained with propidium iodide, and their DNA contents determined by flow cytometry. Data were analyzed with ModFit LT 3.0. The data shown are the means ± SEM.

[a] Significantly different from non-targeting control siRNA (p < 0.01, 2-tailed t-test).

4. Discussion

The G1 to S-phase transition in several mammalian cell types requires a transient increase in ROS to activate G1-regulatory proteins in preparation for entry of cells into S-phase [17,18]. Reducing agents such as glutathione and thioredoxin activate p53 and oxidizing agents such as H_2O_2 and diamide inhibit p53. The highly conserved Cys-275 and Cys-277 residues in p53 form an intramolecular disulfide bridge under oxidative stress that negatively

Table 3
Effect of SEPW1 and p21 silencing on cell cycle distribution in wild-type MCF-7.

siRNA treatment	Percentage of cells (±SEM)		
	G0 and G1	S-phase	G2 and M
(A) No transfection	63.8 ± 0.1	27.7 ± 0.8	8.5 ± 0.7
(B) Non-targeting control siRNA	66.2 ± 0.1	23.9 ± 0.1	9.9 ± 0.1
(C) SEPW1 siRNA	75.9 ± 1.1[a]	14.7 ± 0.5[b]	9.4 ± 0.6
(D) *p21* siRNA	54.6 ± 0.7	34.4 ± 1.0	11.0 ± 0.3
(E) *p21* siRNA and SEPW1 siRNA	53.9 ± 0.3	34.0 ± 0.6	12.0 ± 0.3

Seventy-two hours after transient transfection, duplicate samples of cells treated as indicated were fixed, stained with propidium iodide, and their DNA contents determined by flow cytometry. Data were analyzed with ModFit LT 3.0. The data shown are the means ± SEM.

[a] Significantly different from non-targeting control siRNA ($p = 0.0128$, 2-tailed *t*-test).

[b] Significantly different from non-targeting control siRNA ($p = 0.0032$, 2-tailed *t*-test).

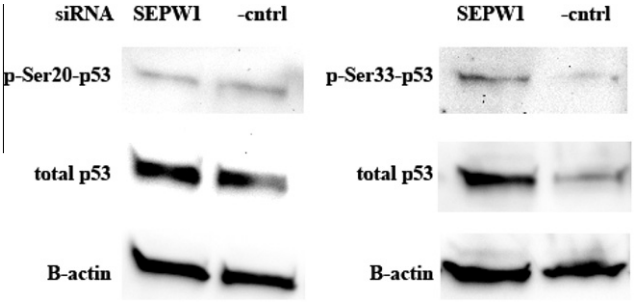

Fig. 4. Western blots of p53 phosphorylated on Ser-20 and Ser-33 in wild-type MCF-7 cells transfected with SEPW1 siRNA s361 or non-targeting control siRNA (-cntrl). Beta-actin was included as a loading control.

regulates p53 DNA binding activity [19]. Exogenous H_2O_2 cause S-glutathionylation of Cys-124 and Cys-141 in p53, leading to decreased DNA binding and loss of tetramer formation [20], which exposes a nuclear export signal that causes p53 to be exported to the cytoplasm [21], where it is subject to rapid ubiquitination and proteasomal degradation. Thus, suppression of p53 activity by oxidation of Cys residues may be part of the mechanism by which H_2O_2 stimulates growth of mammalian cells. SEPW1 has glutathione-dependent antioxidant activity [22] and overexpression of mouse SelW made CHO cells and H1299 human lung cancer cells more resistant to H_2O_2 cytotoxicity [10]. However, oxidation decreases p53, whereas SEPW1 silencing increases p53, so oxidation of p53 cannot explain the increase in p53 from SEPW1 silencing.

The present study shows that SEPW1 affects the phosphorylation of Ser-33. Ser-33 can be phosphorylated in response to ionizing and UV radiation, nitric oxide, topoisomerase II inhibition, microtubule disruption, premature senescence, and osmotic shock [23]. Phosphorylation of p53 on Ser-33 creates a binding site for the peptidyl-prolyl-cis–trans isomerase PIN1 [24], interaction with which is required for optimal transactivation of p21 and cell cycle arrest [25]. Phosphorylation of Ser-33 can be accomplished by p38 stress-activated MAP kinase [26], glycogen synthase kinase 3-beta (GSK3B) [27], cyclin-dependent kinase 7 [28], and cyclin-dependent kinase 9 [29]. Some of these kinases respond to oxidative stress and may be influenced by SEPW1 depletion. For example, p38 alpha MAP kinase is activated by ROS during Ras oncogene-induced malignant transformation [30], and activation of GSK3B by ROS is a critical step in autophagy induction by cadmium in MES-13 mesangial cells [31]. Phosphorylation of Ser-33 by GSK3B activates transcription of a p53 dependent luciferase construct, whereas mutation of Ser-33 to alanine prevents activation of p53 by GSK3B [27], showing that phosphorylation of Ser-33 is sufficient for activation of p53. Thus, the increased phosphorylation of Ser-33 may also mediate the cell cycle arrest from SEPW1 silencing.

Dual specificity phosphatases and Ser/Thr phosphatases are inhibited by oxidative stress [32,33]. It is not known which phosphatases control de-phosphorylation of Ser-33 in p53. However, some phosphatases are known to affect Ser-33 phosphorylation indirectly. For instance, the Wip1 and PPM1D Ser/Thr-protein phosphatases de-phosphorylate and inhibit activated p38, and thus reduced expression of Wip1 or PPM1D leads to increased phosphorylation of Ser-33 in p53 [34,35]. Ser/Thr-protein phosphatase 5 has been shown to de-phosphorylate p53 *in vitro*, but the amino acid residues affected were not characterized [36].

Thus, oxidative stress due to SEPW1 depletion might conceivably activate one or more of the kinases that phosphorylate Ser-33, or inhibit phosphatases that de-phosphorylate Ser-33, or form a disulfide bridge in p53 that influences the rate of phosphorylation/de-phosphorylation of Ser-33. However, these ideas are challenged by a report that depletion of SEPW1 does not cause an increase in reactive oxygen species (ROS), leading the authors to conclude its main role is not as an antioxidant [37]. Furthermore, increased p53 from silencing SEPW1 was not associated with increased phosphorylation of Ser-20 in MCF-7 cells, suggesting that silencing SEPW1 does not increase p53 by activating the DNA damage pathway.

This raises the intriguing possibility that SEPW1 may catalyze oxidative modification of p53 or proteins that modulate p53, as opposed to protecting them from oxidation. The selenoproteins GPX4 and thioredoxin-glutathione reductase use H_2O_2 to form protein disulfides critical to the process of sperm chromatin condensation [38,39]. Glutathione peroxidase 3 serves as part of signal transduction system in plants by converting H_2O_2 signals to intramolecular disulfide bonds in transcription factors [40,41]. Because of the unique reactivity of Sec, H_2O_2 reacts completely with mammalian selenoproteins before reacting with other potential targets [42]. The resulting Sec selenenic acid can then form disulfide bonds in substrate proteins, via a sequence of thiol-disulfide exchange reactions [43]. It is tempting to speculate that SEPW1 may destabilize p53 by catalyzing formation of disulfides, thus facilitating cell cycle progression through the G1 to S-phase transition. More work will be required to identify the molecular mechanism(s) by which SEPW1 affects the phosphorylation state and stability of p53.

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