ARTICLE
Journal of Cellular Biochemistry 113:61–69 (2012)

Journal of Cellular Biochemistry

Selenoprotein W Depletion Induces a p53- and p21-Dependent Delay in Cell Cycle Progression in RWPE-1 Prostate Epithelial Cells

Wayne Chris Hawkes,* Ignat Printsev, and Zeynep Alkan

USDA Agricultural Research Service, Western Human Nutrition Research Center, University of California at Davis, 430 West Health Science Drive, Davis, California 95616

ABSTRACT

The anticancer activity of selenium (Se) has been demonstrated in myriad animal and in vitro studies, yet the mechanisms remain obscure. The main form of Se in animal tissues is selenocysteine in selenoproteins, but the relative importance of selenoproteins versus smaller Se compounds in cancer protection is unresolved. Selenoprotein W (SEPW1) is a highly conserved protein ubiquitously expressed in animals, bacteria, and archaea. SEPW1 depletion causes a delay in cell cycle progression at the G1/S transition of the cell cycle in breast and prostate epithelial cells. Tumor suppressor protein p53 is a master regulator of cell cycle progression and is the most frequently mutated gene in human cancers. p53 was increased in SEPW1 silenced cells and was inversely correlated with SEPW1 mRNA in cell lines with altered SEPW1 expression. Silencing SEPW1 decreased ubiquitination of p53 and increased p53 half-life. SEPW1 silencing increased p21(Cip1/WAF1/*CDKN1A*), while p27 (Kip1/*CDKN1B*) levels were unaffected. G1-phase arrest from SEPW1 knockdown was abolished by silencing p53 or p21. Cell cycle arrest from SEPW1 silencing was not associated with activation of ATM or phosphorylation of Ser-15 in p53, suggesting the DNA damage response pathway was not involved. Silencing GPX1 had no effect on cell cycle, suggesting that G1-phase arrest from SEPW1 silencing was not due to loss of antioxidant protection. More research is required to identify the function of SEPW1 and how it affects stability of p53. J. Cell. Biochem. 113: 61–69, 2012. Published 2011. This article is a U.S. Government work and is in the public domain in the USA.

KEY WORDS: SELENIUM; TUMOR SUPPRESSOR PROTEIN; G1/S TRANSITION; UBIQUITIN; CHEMOPREVENTION

Selenium (Se) is a trace element that is an essential nutrient for organisms in all major phyla, with the apparent exception of fungi and higher plants. The main biochemically active form of Se is selenocysteine (Sec), which functions at the active sites of Se-containing enzymes. Sec is synthesized from serine while attached to a special transfer RNA ($tRNASer^{Sec}$) by replacement of the serine hydroxyl group with a selenohydryl group (–SeH) [Yuan et al., 2006]. The resulting Sec-aminoacyl-tRNA is used during protein synthesis to insert Sec at internal UGA codons that occur in a special nucleotide sequence context. Recoding UGA from a stop codon to Sec requires several unique factors in eukaryotes, including specific secondary structure in the mRNA, a unique tRNA, an RNA-binding protein (SBP2), and a specialized elongation factor (EFsec) [Small-Howard and Berry, 2005]. Se is the only element specified in the genetic code (“TGA”) and Sec has become recognized as the 21st protein amino acid [Commans and Bock, 1999].

Selenoproteins have biological functions in oxidation–reduction (redox) processes, redox signaling, antioxidant defense, thyroid hormone metabolism, and immune responses. They are therefore strongly associated with cancer, Keshan disease, virus infections, male infertility, and abnormalities in immune responses and thyroid hormone function [Lu and Holmgren, 2009]. The mammalian selenoproteome consists of 25 conserved selenoprotein genes [Kryukov et al., 2003]. The mammalian selenoproteins with known enzymatic activities include 4 glutathione peroxidases, 3 thioredoxin (Trx) reductases, 3 iodothyronine deiodinases, and a single form of methionine sulfoxide reductase B. The microbial selenoproteome contains over 3,600 members in approximately 58 families of homologous selenoproteins [Zhang and Gladyshev,

The authors have no financial or other conflicting interest in any product or service mentioned in this article.
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: U.S. Department of Agriculture CRIS Project; Grant number: 5306-51530-018-00D.
*Correspondence to: Wayne Chris Hawkes, PhD, USDA Agricultural Research Service, Western Human Nutrition Research Center, University of California at Davis, 430 West Health Science Drive, Davis, CA 95616.
E-mail: wayne.hawkes@ars.usda.gov
Received 9 August 2011; Accepted 12 August 2011 • DOI 10.1002/jcb.23328 • Published 2011 Wiley Periodicals, Inc.
Published online 22 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

2008]. The most abundant and widespread selenoprotein family is "selenoprotein W-like" [Zhang et al., 2005].

Selenoprotein W (SEPW1), the mammalian archetype of the SEPW1-like family, is a ubiquitous 9.4 kDa Sec-containing protein with a putative antioxidant function [Jeong et al., 2002]. SEPW1 occurs in humans, mice, rats, sheep, monkeys, rabbits, guinea pigs, and cattle, and is expressed in all 22 human tissues examined, with highest levels in brain, testes, and muscles [Bellingham et al., 2003]. SEPW1 is one of the most highly expressed selenoproteins in humans and SEPW1 protein is regulated at the level of mRNA by Se intake [Aachmann et al., 2007]. Silencing of SEPW1 expression causes a delay in cell cycle progression in breast and prostate epithelial cells with cells accumulating in G0/G1-phase of the cell cycle. Moreover, SEPW1 mRNA expression is maximal during G1-phase and is down-regulated after the G1/S transition [Hawkes et al., 2009]. Homozygous *SEPW1*-knockout mouse embryos die at the preimplantation blastocyst stage [Albright, 2004].

The tumor suppressor protein p53 responds to cellular stresses, such as DNA damage, oxidative stress, chemotherapeutic drugs, nucleotide depletion, and aberrant growth signals by regulating the expression of specific sets of genes [Staib et al., 2005]. p53 is involved in several critical pathways including cell cycle, apoptosis, DNA repair, and cellular senescence [Chumakov, 2007]. p53 protein is expressed constitutively, but levels are normally kept low by its rapid ubiquitination by the HDM2 protein, an E3 ubiquitin ligase, and subsequent proteasomal degradation. Phosphorylation of p53 in response to stress (e.g., DNA damage) disrupts its binding with HDM2, blocks ubiquitination and proteolysis, and results in a rapid accumulation of p53 in the nucleus and expression of DNA repair and cell cycle inhibitor genes. Deregulation of p53 activities is involved in cancer, cardiovascular, neurodegenerative, infectious and metabolic diseases, as well as the process of aging [Chumakov, 2007].

We investigated the role of p53 in delayed cell cycle progression induced by SEPW1 silencing in prostate epithelial cells. We found that steady-state p53 protein levels were increased in SEPW1-silenced cells due to stabilization of p53, and that p21—the transcriptional target of p53 that mediates G1-phase arrest—was induced. Both p53 and p21 were required for cell cycle arrest induced by SEPW1 silencing. Knockdown of GPX1 did not mimic SEPW1 silencing and phosphorylations of ATM and Ser-15 in p53 were absent, suggesting that oxidative DNA damage was not the cause of the p53-dependent cell cycle arrest.

MATERIALS AND METHODS

CELL CULTURE

RWPE-1 human prostate epithelial cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in keratinocyte serum-free medium with supplements provided by the manufacturer (Invitrogen, Carlsbad, CA) in the presence of 5% CO_2 in air at 37°C.

siRNA TRANSFECTIONS

10^5 cells/well were reverse-transfected in six-well dishes with 0.2% Lipofectamine RNAiMax reagent (Invitrogen) and 5 nM Silencer Select Validated siRNAs (ABI, Foster City, CA) targeting SEPW1 (#s361 and #s363), p21 (#s415), GPx1 (#s804), non-targeting negative control siRNA #1, or 100 nM custom siRNA with the sequence gacuccagugguaaucuacuu targeting p53 (Dharmacon, Lafayette, CO) [Llanos et al., 2009]. RNA isolation, cDNA synthesis, and quantitative RT-PCR to assess silencing efficiencies were performed utilizing previously published methods [Hawkes et al., 2009]. To measure p53 mRNA levels, the following forward and reverse primers were used, respectively: taagcgagcactgcccaac and tgggcatccttgagttcca.

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 *E. coli*) was purified by absorption to Protein G-Agarose and stored in PBS at −70°C. RWPE-1 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 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 antigen eluted into reducing Laemmli buffer by boiling for 5 min. Samples were analyzed by SDS–PAGE and Western blotting as described below, using the polyclonal rabbit SEPW1 antibody as the primary antibody.

SEPW1 UNDER- AND OVEREXPRESSING CELL LINES

HuSH29 short-hairpin RNA (shRNA) vectors targeting SEPW1 and a non-targeting negative control construct (Origene, Rockville, MD) were amplified in and purified from *E. coli* using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). RWPE-1 cells were reverse-transfected with 1 μg construct per well using 0.2% siPORT NeoFX (Ambion, Austin, TX) reagent in six-well plates. The cells were maintained under 0.5 μg/ml puromycin selection starting 72 h after the transfection. Lines established from surviving cells were assessed for the degree of SEPW1 knockdown with reverse transcriptase-polymerase chain reaction (RT-PCR) utilizing previously published methods [Hawkes et al., 2009]. A cell line overexpressing *SEPW1* mRNA by 2.5-fold and a matching negative control line were created using *SEPW1* TrueClone cDNA in a pCMV6-Neo vector and empty vector (Origene), respectively. The overexpressing cells were maintained continuously under 200 μg/ml G418 selection starting 72 h after the transfection.

CELL CYCLE ANALYSIS

Propidium iodide staining of cellular DNA and flow cytometry analysis were performed as described before [Hawkes et al., 2009].

WESTERN BLOTS

Total cellular protein was extracted with RIPA buffer containing 1× HALT Protease and Phosphatase Inhibitor Cocktails (Pierce) and 5 mM EDTA. Protein concentrations were determined using a standard BCA assay [Smith et al., 1985], and the extracts were stored

at −70°C until use. Twenty to 40 μg protein/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 in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, and then probed with 1 μg/ml anti-p53 (BP53-12), anti-p27 (4B4-E6), anti-beta actin (AC-74; Sigma, St. Louis, MO), anti-p21 (DCS60), anti-ATM (D2E2), anti-phospho-Ser1981-ATM (10H11.E12), anti-GPX1 (C8C4; Cell Signaling Technology, Beverly, MA), or anti-phospho-Ser15-p53 (ab1431) (Abcam, Cambridge, 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 detected either by ChemiDoc XRS Imaging System (BioRad) or exposure to film. Membranes were stripped using Restore Plus Stripping Buffer (Pierce) and re-probed when necessary. Densitometry on blot images was performed using ImageLab software (BioRad).

DETERMINATION OF p53 HALF-LIFE

Seventy-two hours after transient transfection with SEPW1 siRNA or a negative control siRNA, cell growth medium was removed and replaced with medium containing 60 μg/ml cycloheximide. Total cellular protein was harvested at indicated time points after the switch to cycloheximide medium.

IMMUNOPRECIPITATION AND WESTERN BLOTTING OF UBIQUITINATED p53

RWPE-1 cells were seeded (6×10^6 cells) in 100 mm culture plates and transfected with 75 pmol SEPW1 or negative control siRNA plus 24 μg 2X-FLAG-ubiquitin-pcDNA3 construct (A kind gift from Dr. Xinbin Chen, UC Davis Veterinary Oncology) using 60 μl Lipofectamine 2000 Reagent (Invitrogen). Three days after transfection, cells were treated with 10 μM MG-132 for 4 h and lysed in M-Per lysis buffer containing 1× HALT Protease and Phosphatase Inhibitor Cocktails and 5 mM EDTA (Pierce, Rockford, IL). Total protein of 1.5 mg was incubated overnight with 2 μg p53 antibody and 50 μl Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C with gentle shaking. The resin was collected by centrifugation, washed four times with PBS, and the antigen eluted into reducing Laemmli buffer by boiling for 5 min. Western blots were probed with a FLAG-HRP antibody (Sigma).

STATISTICAL ANALYSES

Cell cycle data were analyzed using two-way analysis of variance followed by Tukey's multiple comparisons test with SigmaStat Software (Systat, San Jose, CA) [Lowry, 2010]. Changes in densitometry values obtained from Western blots were analyzed a by comparing signal ratios to the hypothetical mean value of one in one-sample *t*-test. A probability of $P < 0.05$ was considered significant.

RESULTS

SEPW1 siRNA EFFICIENTLY KNOCKS DOWN SEPW1 PROTEIN

SEPW1 protein was immunoprecipitated from RWPE-1 lysates, subjected to SDS–PAGE, and analyzed with Western blots to reveal a prominent immunoreactive band at approximately 9 kDa in lysates from control RWPE-1 cells (Fig. 1). In contrast, immunoprecipitates from lysates of RWPE-1 cells transfected with SEPW1 siRNA (#s361) contained no detectable SEPW1 protein. This demonstrates that the siRNA efficiently silenced expression of SEPW1 protein.

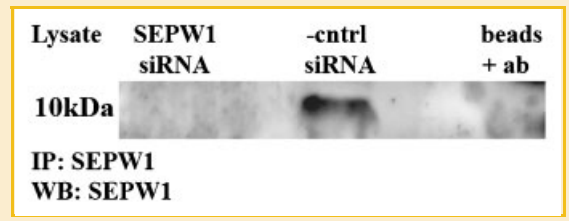

Fig. 1. *SEPW1* silencing blocks expression of SEPW1 protein. Lysates of RWPE-1 cells transfected with *SEPW1* siRNA or a non-targeting control siRNA (-cntrl) were immunoprecipitated with rabbit polyclonal SEPW1 antibody and Protein A/G Agarose as described in the Materials and Methods Section. The proteins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

ROLE OF p53 IN SEPW1-MEDIATED CELL CYCLE PROGRESSION

Transfection of wild-type RWPE-1 cells with siRNA targeting SEPW1 inhibited cell cycle progression, with cells arrested at the G1-phase of the cell cycle (Tables I and II). Silencing SEPW1 increased the fraction of cells in G0/G1-phase from around 63–65% to approximately 69–75%. The D-type cyclins drive cell cycle progression through G1-phase by forming active holoenzymes with cyclin-dependent kinases 4 and 6, which phosphorylate the retinoblastoma protein to allow release of the E2F transcription factor that promotes transcription of S-phase genes. The magnitude of the G1 arrest from silencing SEPW1 was similar to the G1 arrest induced by silencing expression of cyclin D1 (from 52% G1-phase to 65% G1-phase) [Leslie et al., 2006] and cyclin D3 (from 48% G1-phase to 59% G1-phase) [Lu et al., 2005]. Thus, depletion of SEPW1 has a biologically significant effect on cell cycle progression.

p53 was increased an average of 60 ± 42% ($P = 0.016$, $n = 5$) in SEPW1-silenced RWPE-1 cells (Figs. 2 and 3). p53 was also

TABLE I. Expression of p53 Is Required for Cell Cycle Arrest From Silencing SEPW1

	Percentage of cells (±SEM)		
siRNA treatment	G0- and G1-phase	S-phase	G2- and M-phase
No transfection	59.4 ± 0.9^{a}	34.6 ± 1.4^{a}	6.0 ± 0.4^{a}
Non-targeting control siRNA	64.8 ± 1.8^{b}	28.9 ± 1.0^{b}	$6.3 \pm 0.8^{a,b}$
SEPW1 siRNA	73.5 ± 0.9^{c}	20.1 ± 1.3^{c}	6.5 ± 0.4^{b}
p53 siRNA	62.6 ± 3.4^{d}	27.8 ± 2.5^{d}	9.6 ± 0.9^{c}
p53 siRNA and SEPW1 siRNA	62.6 ± 3.3^{d}	$28.0 \pm 2.3^{b,d}$	9.3 ± 1.0^{c}

Seventy-two hours after transient transfection, duplicate cultures of 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 twice and the data shown are the means ± SEM. Means within a column not sharing a common superscript are significantly different ($P < 0.05$, two-way ANOVA with Tukey's multiple comparisons test).

TABLE II. Expression of p21 Is Required for Cell Cycle Arrest From Silencing SEPW1

siRNA treatment	Percentage of cells (±SEM) G0- and G1-phase	S-phase	G2- and M-phase
No transfection	62.6 ± 1.6^{a}	$28.9 \pm 1.7^{a,c}$	$8.5 \pm 0.1^{a,b}$
Non-targeting control siRNA	62.6 ± 0.9^{a}	28.6 ± 0.2^{a}	8.8 ± 1.2^{a}
SEPW1 siRNA	69.4 ± 2.6^{b}	23.3 ± 1.7^{b}	7.3 ± 0.9^{b}
p21 siRNA	59.0 ± 0.1^{c}	30.4 ± 0.0^{c}	10.6 ± 0.1^{c}
p21 siRNA and SEPW1 siRNA	61.9 ± 0.2^{a}	$29.0 \pm 0.0^{a,c}$	9.1 ± 0.2^{a}

Seventy-two hours after transient transfection, duplicate cultures of 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 twice and the data shown are the means ± SEM. Means within a column not sharing a common superscript are significantly different ($P < 0.05$, two-way ANOVA with Tukey's multiple comparisons test).

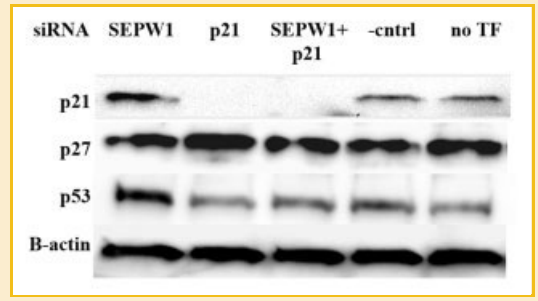

Fig. 3. SEPW1 silencing increases p21 expression, but does not increase expression of p27 (Kip1). Western blots of p21 (Cip1/WAF1), p27 (Kip1), and p53 in RWPE-1 cells transfected with *SEPW1* siRNA, p21 siRNA, or a non-targeting control siRNA (-cntrl). Beta-actin blots were used as loading controls. Data are representative of three independent experiments.

increased in RWPE-1 cells stably under-expressing SEPW1 (Fig. 4). Conversely, p53 was decreased in RWPE-1 cells that overexpressed SEPW1 (Fig. 4), indicating that altered SEPW1 expression can modulate p53 levels both positively and negatively.

Known as the "guardian of the genome," p53 is a central point of regulation controlling the G1/S transition. Therefore, we decided to test if silencing SEPW1 would cause a delay in G1- to S-phase progression when p53 expression was blocked. G1-phase arrest from SEPW1 silencing was completely blocked by silencing p53 (Table I), indicating p53 expression is required for the cell cycle effect of SEPW1. Taken together, these results strongly suggest that delayed cell cycle progression from SEPW1 silencing is mediated by p53.

p53 HALF-LIFE AND STABILITY

p53 is expressed constitutively and regulated by post-translational modifications that control its turnover rate [Jiang et al., 2010]. Consistent with this paradigm, p53 mRNA measured by quantitative real-time PCR was not increased by silencing SEPW1, but was actually decreased by 5% (data not shown); indicating SEPW1 does not regulate p53 at the level of transcription or mRNA stability. This led us to test the effect of SEPW1 silencing on the rate of disappearance of p53 protein. In cells transfected with control siRNA, p53 protein became undetectable on Western blots after protein synthesis had been inhibited for 30 min. On the other hand, in cells transfected with SEPW1 siRNA, p53 protein was still

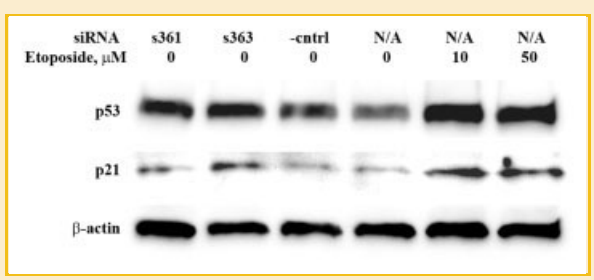

Fig. 2. SEPW1 silencing increases p53 and p21 (Cip1/WAF1). Western blots of p53 and p21 (Cip1/WAF1) proteins from RWPE-1 cells transfected with two different *SEPW1* siRNAs (s361 and s363) or a non-targeting control siRNA (-cntrl). Etoposide-treated lysate was included as a positive control for the classical DNA damage response pathway. Beta-actin was included as a loading control. Data are representative of four independent experiments.

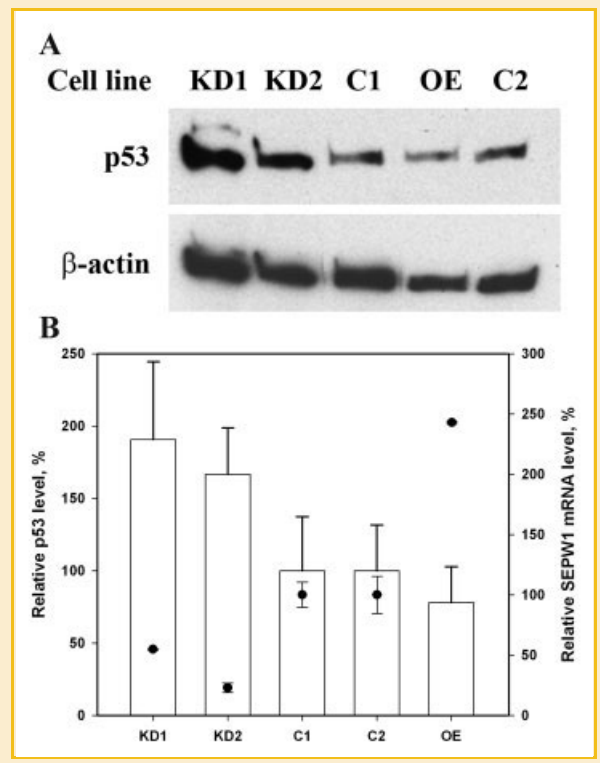

Fig. 4. SEPW1 under-expression and overexpression have opposite effects on p53. A: Western blots of p53 in RWPE-1 cells stably under-expressing or overexpressing SEPW1. "KD1" = RWPE-1 cells expressing a SEPW1 short-hairpin RNA (Origene #TI372740) in a pRS vector. "KD2" = RWPE-1 cells expressing a SEPW1 short-hairpin RNA (Origene #TI372743) in a pRS vector. "C1" = RWPE-1 cells expressing a non-targeting shRNA (Origene #TR30012). "OE" = RWPE-1 cells transfected with SEPW1 cDNA in a pCMV6-Neo vector. "C2" = RWPE-1 cells transfected with an empty pCMV6-Neo vector. B: p53 protein (bars) from densitometric analysis of Western blots and SEPW1 mRNA (circles) from real-time RT-qPCR. Data are from three experiments.

detectable even after protein synthesis had been inhibited for 60 min (Fig. 5A). Thus, silencing of SEPW1 doubled the half-life of p53 from 6.8 ± 2.2 to 14.1 ± 2.1 min (Fig. 5B); indicating that SEPW1 is associated with increased turnover of p53.

The E3 ubiquitin ligase HDM2 rapidly ubiquitinates p53, marking it for proteasomal degradation to maintain a low level of p53 protein in unstressed cells. To test whether the ubiquitination status of p53 was affected by SEPW1 silencing, we treated cells overexpressing FLAG-tagged ubiquitin with the proteasomal inhibitor MG-132 and measured FLAG-specific immunoreactivity in p53 immunoprecipitates. Control cells accumulated high levels of ubiquitinated p53 when proteasomal proteases were inhibited, whereas cells in which SEPW1 was silenced accumulated much less ubiquitinated p53 (Fig. 6). This suggests that the SEPW1 increases turnover of p53 by increasing ubiquitination of p53, thus increasing proteasomal degradation of p53.

ROLE OF p21 IN SEPW1-MEDIATED CELL CYCLE REGULATION

The main mechanism by which p53 induces G1-phase arrest is by up-regulating expression of p21 (Cip1/WAF1), a cyclin-dependent kinase inhibitor that binds to and inhibits the activity of cyclin–CDK2 and cyclin–CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. Consistent with involvement of p53, p21 protein was increased by $274 \pm 184\%$ ($P = 0.01$, $n = 4$) in SEPW1-silenced RWPE-1 cells (Figs. 2 and 3). Besides p21, the other main cyclin-dependent kinase inhibitor controlling cell cycle entry is p27 (Kip1), which is not regulated by p53. Consistent with cell cycle arrest from SEPW1 silencing being mediated by p53, levels of p27 were not increased in SEPW1 siRNA-treated RWPE-1 cells (Fig. 3). To assess if cell cycle arrest from SEPW1 silencing requires p21 expression, we tested the effect of silencing p21. Table II shows that delayed cell cycle progression from SEPW1 silencing was abrogated by silencing p21 expression. This shows that p21 mediates the cell cycle arrest from silencing SEPW1 and confirms the involvement of p53.

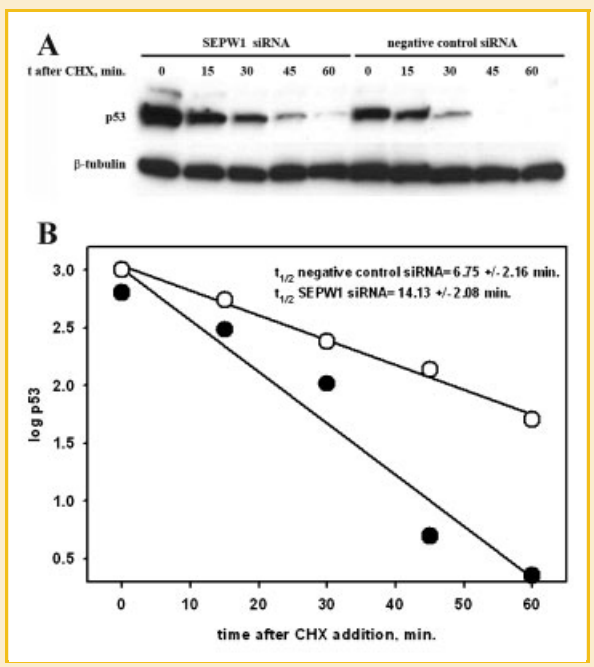

Fig. 5. SEPW1 silencing increases p53 half-life. A: Western blots of p53 from RWPE-1 cells transfected with *SEPW1* siRNA or non-targeting control siRNA. Cell lysates were prepared at the indicated times after inhibition of protein synthesis with 60 μg/ml cycloheximide (CHX). B: Log of p53 protein levels was calculated from quantitative densitometry of Western blots to estimate p53 half-life. Open dots = SEPW1 siRNA, black dots = negative control siRNA. Data are the average of two experiments.

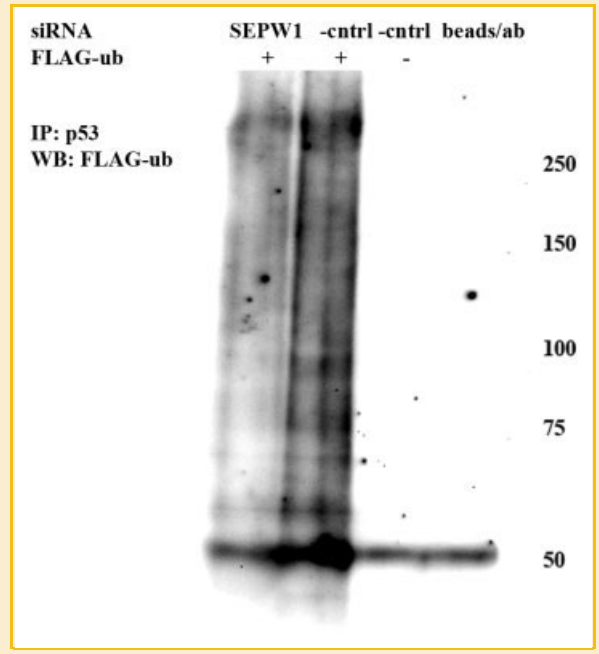

Fig. 6. SEPW1 silencing decreases ubiquitination of p53. RWPE-1 cells were co-transfected with a FLAG-tagged ubiquitin overexpression vector and either a non-targeting control siRNA or SEPW1 siRNA. After 4 h treatment with the proteasomal inhibitor MG132, cells were lysed and immunoprecipitated with p53 antibody. The immunoprecipitates were subjected to SDS–PAGE and probed with anti-FLAG antibodies to detect ubiquitinated p53.

ROLE OF DNA DAMAGE IN CELL CYCLE ARREST FROM SEPW1 SILENCING

G1-phase arrest is induced by p53 and p21 in response to a variety of cellular stresses, such as DNA damage [Chumakov, 2007]. Because SEPW1 has been shown to possess glutathione-dependent antioxidant activity, silencing SEPW1 expression might conceivably cause oxidative stress that could lead to DNA damage and induce a p53 response. To address this possibility, we compared the effect of SEPW1 silencing on cell cycle to the effect of silencing GPX1, the major antioxidant selenoprotein. As shown in Figure 7, GPX siRNA decreased GPX1 protein to undetectable levels. Nevertheless, silencing GPX1 had no effect on cell cycle distribution (Table III). Thus, we conclude that delayed cell cycle progression from silencing SEPW1 is not due to loss of antioxidant protection.

DNA damage activates several signaling pathways that converge on ATM, a Ser/Thr protein kinase that propagates the DNA damage signal by phosphorylating diverse targets, including p53 on Ser-15 [Kruse and Gu, 2009]. We used a phosphospecific antibody to detect

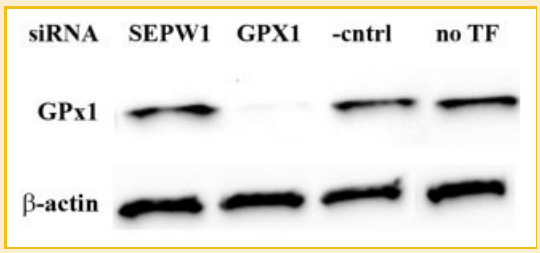

Fig. 7. GPX1 silencing blocks the expression of GPX1 protein. Western blots of GPX1 in lysates of RWPE-1 cells transfected with SEPW1 siRNA, GPX1 siRNA, or a non-targeting control siRNA (-cntrl). Beta-actin blots were used as loading controls.

p53 phosphorylated on Ser-15. Etoposide, a topoisomerase inhibitor that causes DNA single- and double-strand breaks, caused a large increase in phospho-Ser-15-p53, whereas SEPW1 silencing had no effect (Fig. 8A). ATM itself is activated by phosphorylation on Ser-1981. A phosphospecific antibody for ATM detected phospho-Ser-1981-ATM in RWPE-1 cells treated with doxorubicin, another DNA strand breaking drug, but not in cells treated with SEPW1 siRNA (Fig. 8B). Taken together, these results strongly suggest that cell cycle arrest from SEPW1 silencing is not secondary to oxidative DNA damage.

DISCUSSION

Our previous work showed SEPW1 depletion in breast and prostate epithelial cells delays cell cycle progression at the G1/S transition and that SEPW1 mRNA is down-regulated after the start of S-phase [Hawkes et al., 2009], consistent with a role of SEPW1 in the G1/S transition. However, the mechanism by which SEPW1 affects cell cycle progression was not clear. The G1/S transition is a critical juncture in the cell cycle. Once a cell has entered S-phase and begun DNA synthesis it is irrevocably committed to completing the entire cell cycle. Failure to complete cell division or errors in copying the DNA can create tetraploid cells or cells with damaged DNA—either of which may lead to cancer. Thus, the decision to enter the cell cycle must be precisely regulated to protect against genetic instability, a gatekeeper function controlled largely by p53. Turnover of p53 is controlled primarily by E3 ubiquitin ligases, such as HDM2, that target p53 for degradation by 26S proteasomes. Disruption of the p53–HDM2 interaction, for example, by competition or post-translational modifications of p53 and/or HDM2, leads to a rapid accumulation of p53 after stress [Jiang et al., 2010]. The results presented here indicate that SEPW1 promotes cell cycle progression because it increases ubiquitination and proteasomal degradation of p53.

TABLE III. Silencing GPX1 Has No Effect on Cell Cycle

	Percentage of cells (±SEM)		
siRNA treatment	G0- and G1-phase	S-phase	G2- and M-phase
No transfection	61.9 ± 0.4^{a}	31.3 ± 0.2^{a}	$6.9 \pm 0.1^{a,b}$
Non-targeting control siRNA	64.1 ± 1.2^{b}	29.1 ± 1.2^{b}	$6.8 \pm 0.1^{a,b}$
SEPW1 siRNA	74.6 ± 2.4^{c}	19.5 ± 1.7^{c}	5.9 ± 0.6^{a}
GPX1 siRNA	63.9 ± 1.1^{b}	28.9 ± 0.9^{b}	7.2 ± 0.3^{b}

Seventy-two hours after transient transfection, duplicate cultures of 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 twice and the data shown are the means ± SEM. Means within a column not sharing a common superscript are significantly different ($P < 0.01$, one-way ANOVA with Tukey's multiple comparisons test).

The main effector of p53-dependent cell cycle arrest is p21 (Cip1/WAF1). SEPW1 silencing increased protein levels of p53 and p21, implicating both in cell cycle arrest from SEPW1 silencing. Silencing SEPW1 caused a delay in cell cycle progression in wild-type RWPE-1 cells, but had no effect when p53 or p21 was also silenced, showing that p53 and p21 mediate the cell cycle effect of SEPW1. Silencing SEPW1 decreases ubiquitination of p53 and increases p53 half-life and p53 protein, which induces p21 expression and inhibits cell cycle progression at the G1/S transition.

Phosphorylation of p53 at Ser-15 and other sites in the C-terminal transactivation domain in response to DNA damage and other kinds of stress disrupts its binding with HDM2, blocks ubiquitination and proteolysis, and results in a rapid increase in nuclear p53 protein levels [Kruse and Gu, 2009]. SEPW1 silencing did not affect Ser-15 phosphorylation, suggesting SEPW1 regulates p53 stability by a different pathway. SEPW1 silencing also did not increase activation of ATM. Thus, it seems unlikely that the DNA damage response played a role in stabilizing p53. More than 60 post-translational modifications of p53 have been reported [Boehme and Blattner, 2009], and HDM2 is subject to many post-translational modifications that affect its stability, activity, and/or association with p53 [Kruse and Gu, 2009], leading to a large number of possible routes by which p53 stability might conceivably be regulated by SEPW1. More research is needed to reveal the mechanism by which SEPW1 affects p53 ubiquitination and stability.

Silencing gene expression with siRNA is not necessarily completely specific for the targeted gene. For example, it has been shown that p53 and p21 protein and mRNA levels can be differentially affected by off-target effects of siRNAs designed to target the MEN1 gene, due to a combination of mRNA degradation and translational inhibition [Scacheri et al., 2004]. Off-target effects of the SEPW1 siRNAs we used on p53 mRNA, if they exist at all, could only explain a decrease in p53, not an increase as we observed here. Furthermore, we observed delayed cell cycle progression and increased levels of p53 and p21 protein with two siRNAs targeting different sequences in the SEPW1 mRNA (s361 and s363). Finally, siRNA treatment decreased SEPW1 protein to undetectable levels. Thus, it is unlikely our observations were due to off-target effects of the siRNA. In addition, shRNA-mediated knockdown of SEPW1 as well as SEPW1 overexpression increased and decreased p53 protein levels, respectively [Hawkes et al., 2009], independently demonstrating that SEPW1 negatively regulates p53.

There have been many prior reports of Se affecting p53, albeit in the opposite direction to what we observed. Pharmacological doses of several Se compounds have been shown to induce apoptosis in cancer cells via p53-dependent pathways [Lanfear et al., 1994; Jiang et al., 2004; Goel et al., 2006; Guan et al., 2008; Rudolf et al., 2008; Chen and Wong, 2009; Das et al., 2009; Zhao et al., 2009]. However, the doses of Se used are cytotoxic to tumor cells and activate the

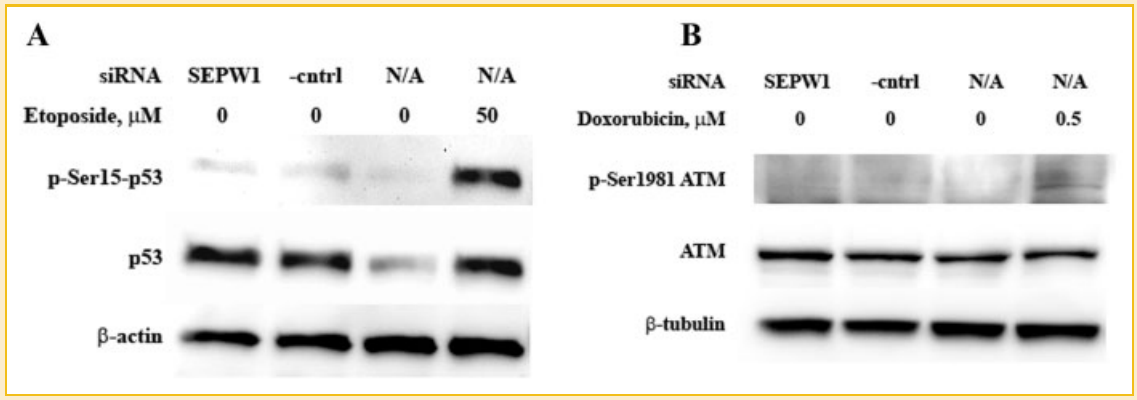

Fig. 8. SEPW1 silencing does not induce a DNA damage response. A: Western blots of p53 phosphorylated on Ser-15 in RWPE-1 cells transfected with *SEPW1* siRNA or non-targeting control siRNA (-cntrl). B: Western blots of Ser-1981-Phospho-ATM in lysates from RWPE-1 cells transfected with *SEPW1* siRNA or non-targeting control siRNA (-cntrl). Lysates from cells treated with DNA-damaging drugs doxorubicin or etoposide were added as positive controls. Beta-actin or beta-tubulin blots were included as a loading control.

p53 pathway as a result of severe oxidative stress, similarly to chemotherapeutic drugs. Sodium selenite at concentrations of 1–20 μM causes oxidative DNA damage that induces phosphorylation, stabilization, and transcriptional activity of p53, thus causing apoptosis [Jiang et al., 2004; Guan et al., 2008; Rudolf et al., 2008; Zhao et al., 2009; Sarveswaran et al., 2010]. Sec at concentrations of 5–20 μM forms superoxide that activates the p53-dependent DNA damage pathway to induce apoptosis in cancer cells [Chen and Wong, 2008]. On the other hand, selenomethionine at 10–20 μM increases p53, enhances DNA repair, and improves survival of UV-treated cells via a ref1/p53/Brca1 protein complex without causing DNA damage [Seo et al., 2002; Fischer et al., 2006]. A recent study found that several small molecular weight Se compounds activate the ATM-dependent DNA damage response via reactive oxygen species [Qi et al., 2010]. In stark contrast, we observed p53-dependent cell cycle arrest from silencing of a selenoprotein, not from addition of a Se compound, and without evidence of a DNA damage response.

Altered selenoprotein expression has previously been reported to affect p53 metabolism. Overexpression in mouse neuronal cells of selenoprotein H, a 13 kDa homolog of SEPW1 with a similar Trx-like Sec active site, suppressed the increase in p53 induced by UV radiation and protected cells from apoptosis [Mendelev et al., 2009]. This is similar to our results insofar as selenoprotein H expression and p53 levels were inversely related. Many effects of selenoproteins are due to their antioxidant properties and SEPW1 is reported to have glutathione-dependent antioxidant activity [Sun et al., 1998]. To see if the loss of antioxidant protection per se would affect cell cycle progression, we silenced GPX1, the main antioxidant enzyme. Silencing GPX1 had no effect on cell cycle, suggesting that loss of antioxidant protection cannot explain the delayed cell cycle progression from SEPW1 silencing. This is consistent with a recent report that that depletion of SEPW1 does not cause an increase in reactive oxygen species, leading the authors to conclude its main role is not as an antioxidant [Xiao-Long et al., 2010].

Recent evidence indicates that p53 can be regulated by redox mechanisms [Liu et al., 2008]. Reducing agents such as glutathione and Trx activate p53 whereas oxidation with hydrogen peroxide 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 regulates p53 DNA-binding activity [Buzek et al., 2002]. Exogenous hydrogen peroxide causes *S*-glutathionylation of Cys-124 and Cys-141 in p53 that exposes a nuclear export signal that causes p53 to be exported to the cytoplasm, where it is rapidly degraded [Foo et al., 2007]. Micromolar concentrations of exogenous hydrogen peroxide cause growth responses in a wide variety of mammalian cells [Day and Suzuki, 2005] and mouse embryonic fibroblasts require a transient increase in reactive oxygen species for entry of cells into S-phase [Menon et al., 2003] that may be at least partly related to oxidative inactivation of p53. This raises the intriguing possibility that SEPW1 might destabilize p53 by mediating an oxidative modification of p53. In support of this possibility, silencing of the selenoprotein thioredoxin reductase in MCF-7 breast cancer cells increased p53 half-life, p53 protein, and p21 protein, and did so without affecting phosphorylation of Ser-15 [Seemann and Hainaut, 2005]. Absence of thioredoxin reductase activity causes an increase in the level of oxidized thioredoxin, which can catalyze formation of protein disulfides. Thus, it is tempting to speculate that SEPW1 may likewise influence p53 stability via formation of regulatory disulfides in p53 or one of the proteins with which it interacts.

ACKNOWLEDGMENTS

This study was supported by U.S. Department of Agriculture CRIS Project 5306-51530-018-00D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. The opinions expressed herein represent those of the authors and do not necessarily represent those of the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

REFERENCES

Aachmann FL, Fomenko DE, Soragni A, Gladyshev VN, Dikiy A. 2007. Solution structure of selenoprotein W and NMR analysis of its interaction with14-3-3 proteins. J Biol Chem 282:37036–37044.

Albright MA. 2004. OSU researcher banks on mutant mice. Corvallis, OR: Corvallis Gazette-Times Online.

Bellingham J, Gregory-Evans K, Fox MF, Gregory-Evans CY. 2003. Gene structure and tissue expression of human selenoprotein W, SEPW1, and identification of a retroprocessed pseudogene, SEPW1P. Biochim Biophys Acta 1627:140–146.

Boehme KA, Blattner C. 2009. Regulation of p53—Insights into a complex process. Crit Rev Biochem Mol Biol 44:367–392.

Buzek J, Latonen L, Kurki S, Peltonen K, Laiho M. 2002. Redox state of tumor suppressor p53 regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. Nucl Acids Res 30:2340–2348.

Chen T, Wong YS. 2008. Selenocystine induces apoptosis of A375 human melanoma cells by activating ROS-mediated mitochondrial pathway and p53 phosphorylation. Cell Mol Life Sci 65:2763–2775.

Chen T, Wong YS. 2009. Selenocystine induces caspase-independent apoptosis in MCF-7 human breast carcinoma cells with involvement of p53 phosphorylation and reactive oxygen species generation. Int J Biochem Cell Biol 41:666–676.

Chumakov PM. 2007. Versatile functions of p53 protein in multicellular organisms. Biochemistry (Mosc) 72:1399–1421.

Commans S, Bock A. 1999. Selenocysteine inserting tRNAs: An overview. FEMS Microbiol Rev 23:335–351.

Das A, Bortner J, Desai D, Amin S, El-Bayoumy K. 2009. The selenium analog of the chemopreventive compound *S*,*S*'-(1,4-phenylenebis[1,2-ethanediyl])-bisisothiourea is a remarkable inducer of apoptosis and inhibitor of cell growth in human non-small cell lung cancer. Chem Biol Interact 180:158–164.

Day RM, Suzuki YJ. 2005. Cell proliferation, reactive oxygen and cellular glutathione. Dose Response 3:425–442.

Fischer JL, Lancia JK, Mathur A, Smith ML. 2006. Selenium protection from DNA damage involves a Ref1/p53/Brca1 protein complex. Anticancer Res 26:899–904.

Foo RS, Nam YJ, Ostreicher MJ, Metzl MD, Whelan RS, Peng CF, Ashton AW, Fu W, Mani K, Chin SF, Provenzano E, Ellis I, Figg N, Pinder S, Bennett MR, Caldas C, Kitsis RN. 2007. Regulation of p53 tetramerization and nuclear export by ARC. Proc Natl Acad Sci USA 104:20826–20831.

Goel A, Fuerst F, Hotchkiss E, Boland CR. 2006. Selenomethionine induces p53 mediated cell cycle arrest and apoptosis in human colon cancer cells. Cancer Biol Ther 5:529–535.

Guan L, Huang F, Li Z, Han B, Jiang Q, Ren Y, Yang Y, Xu C. 2008. p53 Transcription-independent activity mediates selenite-induced acute promyelocytic leukemia NB4 cell apoptosis. BMB Rep 41:745–750.

Hawkes WC, Wang TTY, Alkan Z, Richter BD, Dawson K. 2009. Selenoprotein W modulates control of cell cycle entry. Biol Trace Elem Res 131:229–244.

Jeong D, Kim TS, Chung YW, Lee BJ, Kim IY. 2002. Selenoprotein W is a glutathione-dependent antioxidant in vivo. FEBS Lett 517:225–228.

Jiang C, Hu H, Malewicz B, Wang Z, Lu J. 2004. Selenite-induced p53 Ser-15 phosphorylation and caspase-mediated apoptosis in LNCaP human prostate cancer cells. Mol Cancer Ther 3:877–884.

Jiang L, Sheikh MS, Huang Y. 2010. Decision making by p53: Life versus death. Mol Cell Pharmacol 2:69–77.

Kruse JP, Gu W. 2009. Modes of p53 regulation. Cell 137:609–622.

Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R, Gladyshev VN. 2003. Characterization of mammalian selenoproteomes. Science 300:1439–1443.

Lanfear J, Fleming J, Wu L, Webster G, Harrison PR. 1994. The selenium metabolite selenodiglutathione induces p53 and apoptosis: Relevance to the chemopreventive effects of selenium? Carcinogenesis 15:1387–1392.

Leslie K, Lang C, Devgan G, Azare J, Berishaj M, Gerald W, Kim YB, Paz K, Darnell JE, Albanese C, Sakamaki T, Pestell R, Bromberg J. 2006. Cyclin D1 is transcriptionally regulated by and required for transformation by activated signal transducer and activator of transcription 3. Cancer Res 66:2544–2552.

Liu B, Chen Y, St Clair DK. 2008. ROS and p53: A versatile partnership. Free Radic Biol Med 44:1529–1535.

Llanos S, Cuadrado A, Serrano M. 2009. MSK2 inhibits p53 activity in the absence of stress. Sci Signal 2:ra57.

Lowry R. 2010. VassarStats: Website for statistical computation. Poughkeepsie, NY: Vassar College. Available at: http://faculty.vassar.edu/lowry/VassarStats.html.

Lu J, Holmgren A. 2009. Selenoproteins. J Biol Chem 284:723–727.

Lu M, Kwan T, Yu C, Chen F, Freedman B, Schafer JM, Lee EJ, Jameson JL, Jordan VC, Cryns VL. 2005. Peroxisome proliferator-activated receptor gamma agonists promote TRAIL-induced apoptosis by reducing survivin levels via cyclin D3 repression and cell cycle arrest. J Biol Chem 280:6742–6751.

Mendelev N, Witherspoon S, Li PA. 2009. Overexpression of human selenoprotein H in neuronal cells ameliorates ultraviolet irradiation-induced damage by modulating cell signaling pathways. Exp Neurol 220:328–334.

Menon SG, Sarsour EH, Spitz DR, Higashikubo R, Sturm M, Zhang H, Goswami PC. 2003. Redox regulation of the G1 to S phase transition in the mouse embryo fibroblast cell cycle. Cancer Res 63:2109–2117.

Qi Y, Schoene NW, Lartey FM, Cheng WH. 2010. Selenium compounds activate ATM-dependent DNA damage response via the mismatch repair protein hMLH1 in colorectal cancer cells. J Biol Chem 285:33010–33017.

Rudolf E, Rudolf K, Cervinka M. 2008. Selenium activates p53 and p38 pathways and induces caspase-independent cell death in cervical cancer cells. Cell Biol Toxicol 24:123–141.

Sarveswaran S, Liroff J, Zhou Z, Nikitin AY, Ghosh J. 2010. Selenite triggers rapid transcriptional activation of p53, and p53-mediated apoptosis in prostate cancer cells: Implication for the treatment of early-stage prostate cancer. Int J Oncol 36:1419–1428.

Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M, Collins FS. 2004. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. Proc Natl Acad Sci USA 101:1892–1897.

Seemann S, Hainaut P. 2005. Roles of thioredoxin reductase 1 and APE/Ref-1 in the control of basal p53 stability and activity. Oncogene 24:3853–3863.

Seo YR, Kelley MR, Smith ML. 2002. Selenomethionine regulation of p53 by a ref1-dependent redox mechanism. Proc Natl Acad Sci USA 99:14548–14553.

Small-Howard AL, Berry MJ. 2005. Unique features of selenocysteine incorporation function within the context of general eukaryotic translational processes. Biochem Soc Trans 33:1493–1497.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic-acid. Anal Biochem 150:76–85.

Staib F, Robles AI, Varticovski L, Wang XW, Zeeberg BR, Sirotin M, Zhurkin VB, Hofseth LJ, Hussain SP, Weinstein JN, Galle PR, Harris CC. 2005. The p53 tumor suppressor network is a key responder to microenvironmental

components of chronic inflammatory stress. Cancer Res 65:10255–10264.

Sun Y, Gu OP, Whanger PD. 1998. Antioxidant function of selenoprotein W using overexpressed and underexpressed cultured rat glial cells. FASEB J 12:A824.

Xiao-Long W, Chuan-Ping Y, Kai X, Ou-Jv Q. 2010. Selenoprotein W depletion in vitro might indicate that its main function is not as an anti-oxidative enzyme. Biochemistry (Mosc) 75:201–207.

Yuan J, Palioura S, Salazar JC, Su D, O'Donoghue P, Hohn MJ, Cardoso AM, Whitman WB, Soll D. 2006. RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. Proc Natl Acad Sci USA 103:18923–18927.

Zhang Y, Gladyshev VN. 2008. Trends in selenium utilization in marine microbial world revealed through the analysis of the global ocean sampling (GOS) project. PLoS Genet 4:e1000095.

Zhang Y, Fomenko DE, Gladyshev VN. 2005. The microbial selenoproteome of the Sargasso Sea. Genome Biol 6:R37. [Epub 2005 Mar 29].

Zhao R, Xiang N, Domann FE, Zhong W. 2009. Effects of selenite and genistein on G2/M cell cycle arrest and apoptosis in human prostate cancer cells. Nutr Cancer 61:397–407.