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# Expression of Common Chromosomal Fragile Site Genes, WWOX/FRA16D and FHIT/FRA3B is Downregulated by Exposure to Environmental Carcinogens, UV, and BPDE but not by IR

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## Abstract

Common chromosomal fragile sites are unstable genomic loci susceptible to breakage, rearrangement, and are highly recombinogenic. Frequent alterations at these loci in tumor cells led to the hypothesis that they may contribute to cancer development. The two most common chromosomal fragile sites FRA16D and FRA3B which harbor *WWOX* and *FHIT* genes, respectively, are frequently altered in human cancers. Here we report that environmental carcinogens, ultraviolet (UV) light, and Benzo[a]pyrene diol epoxide (BPDE), significantly downregulate expression of both genes. On the other hand, we observe that ionizing radiation (IR) does not affect expression of these genes, suggesting that the effect of repression exerted by UV and BPDE is not just a consequence of DNA damage but may be a result of different signaling pathways triggered by specific DNA lesions. Such downregulation correlates with an induction of an S-phase delay in the cell cycle. Treatment of UV-irradiated cells with caffeine abrogates the S-phase delay while concomitantly overcoming the repression phenomenon. This suggests the involvement of unique cell cycle checkpoint mechanisms in the observed repression. Therefore, it is hypothesized that protracted downregulation of the putative tumor suppressor genes *WWOX* and *FHIT* by environmental carcinogens may constitute an additional mechanism of relevance in the initiation of tumorigenesis.

### Keywords

fragile sites; UV; BPDE; DNA damage; WWOX; FHIT

## INTRODUCTION

Common chromosomal fragile sites are loci that exhibit gaps and breaks during metaphase in cells that have been cultured under conditions of replicative stress such as folate deficiency or treatment with aphidicolin [1]. These sites are normally stable in cultured human cells. However, following induction with replication inhibitors, these sites become hot spots for increased sister chromatid exchanges, translocations, and deletions [1,2]. The



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instability at such loci in tumor cells and their frequent alteration [3] led to the hypothesis that they may contribute to cancer development. Although the exact number of common fragile sites is a matter of interpretation, more than 70 aphidicolin-induced common fragile sites have been reported. Gaps and breaks at just 20 chromosomal fragile sites, however, represent over 80% of all fragile lesions observed in lymphocytes following treatment with low doses of aphidicolin [1]. *FRA3B* at 3p 14.2 stands out as the most active fragile site in the human genome, followed by 16q23 (*FRA16D*), 6q26 (*FRA6E*), 7q31.2 (*FRA7G*), and Xp22.3 (*FRAXB*) [4]. A recent study suggested that *FRAXB, FRA3B, FRA7G, FRA7H, FRA16D*, and their associated genes are unstable in cancer cells [5]. The frequency of gaps and breaks that occur after aphidicolin treatment varies in the population and appears to be determined by the combination of genetics and exposure to environmental carcinogens (reviewed in References [6,7]). This is exemplified by the fact that smokers show a higher level of expression of common chromosomal fragile sites than nonsmokers and by the association of allele losses at specific fragile sites (e.g., FRA3B) and smoking related cancer [8–11].

The fragile histidine triad (*FHIT*) gene was isolated from the *FRA3B* locus [12]. FHIT protein expression is reduced or absent overall in 60% of tumors, including lung, breast, esophagus, stomach, and bladder cancers (reviewed in Reference [13]) because of genomic deletions [10] or epigenetic changes, including 5’ CpG island methylation [14,15]. Recently, we have cloned a WW domain containing oxidoreductase (*WWOX*) gene from chromosome region 16q23.3–24.1 spanning the region of common fragile site *FRA16D* [16]. *WWOX* expression has been examined in a variety of tumors and cancer-derived cell lines and loss or reduced expression, aberrant transcripts as well as genomic deletions were observed in breast [16], ovary [17], esophageal [18], lung [19], liver cancers [20], and leukemias [21] among other tumor types. WWOX has been demonstrated to behave as a putative tumor suppressor [22,23]. In general, common chromosomal fragile sites do not have high sequence similarities and, therefore, specific sequences do not appear to be responsible for their fragile nature [6]. However, the two fragile genes *FHIT* and *WWOX* have common features; both span huge genomic loci of more than 1 Mb in size including the fragile regions, show frequent altered expression in cancers, and are suspected tumor suppressor genes [6,23]. A recent examination of the coordinate expression of the *WWOX* and *FHIT* genes in hematopoietic disorders provided evidence that loss or alteration of expression of the two genes can occur concordantly in hematopoietic tumors by epigenetic mechanisms such as histone modifications (methylation and deacetylation) at these fragile loci [21]. Additionally, a strong correlation was observed in loss of expression between WWOX and FHIT in invasive breast tumors [24].

Analyses of the mechanism of fragile site induction revealed that fragile sites are late replicating in S-phase and fragile site-inducers delay replication even more [25–27]. If replication is delayed at several different segments of a fragile region, cells would enter the $G_2$/M phase of the cell cycle with such chromosomal segments still unreplicated leading to the formation of gaps and breaks, a phenomenon that would be enhanced by aphidicolin [6]. Of note is a recent demonstration that fragile site induction by aphidicolin does not inhibit expression of fragile site gene *FHIT* [28]. Loss of fragile site gene expression, in cancer, has

been mostly attributed to allelic deletion, chromosomal translocation or gene inactivation as a result of exposure to environmental carcinogens [29]. However, no information is available on the regulation of expression of fragile site genes in cells following DNA damage by environmental carcinogens, some of which do not induce chromosomal fragility. Therefore, here we report on the expression pattern of *WWOX* and *FHIT* following DNA damage by ultraviolet (UV) light and benzo[a]pyrene diol epoxide (BPDE), two ubiquitous environmental carcinogens that induce bulky DNA adducts.

## MATERIALS AND METHODS

### Cell Lines and Treatments

MCF-7 and Saos-2 cells were maintained in complete IMEM (IMEM (Biosource, Rockville, MD) with 5% FBS and Gentamycin (50 μg/mL) and complete DMEM (Cambrex, Walkersville, MD) with 10% FBS Gentamycin (50 μg/mL), respectively. For UV irradiation, monolayers of 60%–70% confluent cells were washed once with phosphate buffered saline (PBS) and irradiated with UV-C emitted by five narrow band germicidal lamps emitting predominantly 254 nm. For protracted (three doses) UV-C exposure (10 $J/m^2$ each time), cells were allowed to recover for 24 h between exposures. Cell survival was monitored by counting viable cells (Trypan blue-excluding) 24 h posttreatment. For ionizing (X-ray) irradiation, monolayers of cells at 60%–70% confluency were irradiated in RS2000 X-ray Irradiator (RadSource Technologies, Inc., Boca Raton, FL). Control cells were sham-irradiated in both the UV-C and ionizing radiation (IR) experiments. For BPDE treatment, (±)-7r,8t-dihydroxy-9,10t-oxy-7,8,9,10-tetrahydrobenzo[a] pyrene (BPDE) was obtained from ChemSyn Laboratories (Lenexa, KS), and stock solutions were prepared in tetrahydrofuran as previously described [30]. The integrity of BPDE stock solutions was checked immediately prior to use by a spectrophotometric assay [30]. Because BPDE is extremely labile in aqueous solution, the carcinogen was added to an aliquot of serum-free IMEM (at a dilution of 1/300) within 5 s of addition of the IMEM to the 60%–70% confluent monolayers of MCF-7 cells, which were previously rinsed once with PBS; final concentration of BPDE was 0.5 μM. Control cells were treated in 1/300 dilution of solvent in serum-free IMEM. Thirty minutes later, the serum-free medium was removed, the cells were rinsed once with PBS, and complete IMEM was added. For caffeine treatment, MCF-7 cells were pretreated for 1 h with complete IMEM containing 5 or 10 mM caffeine (Sigma, St. Louis, MO) prior to UV-C irradiation. The cells were maintained in the caffeine-containing complete IMEM after UV-C irradiation until harvested for RNA or for flow cytometry. Control cells were sham-treated in complete IMEM without caffeine. For cell cycle progression analysis, MCF-7 cells were grown in serum free IMEM for 96 h to arrest cells in $G_1/G_0$ phase. Cell growth stimulated by the addition IMEM containing 5% FBS for the indicated time points until harvested for RNA.

### RNA Isolation and Northern Blot Analysis

Total RNA was isolated 24 h after treatments from the cells with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Northern blots of total RNA were prepared with standard procedures. A 1200 bp *BamHI-EcoRI* restriction fragment of *WWOX* cDNA clone, spanning the whole amino acid-coding region, was used as probe. For *FHIT*, a

300 bp cDNA was used as probe. For *p21*, a 500 bp cDNA was used and for human *GAPDH*, 1200 bp cDNA was used as probe. The cDNA probes were labeled with [$^{32}$P]dCTP with random priming (Prime It II; Stratagene, La Jolla, CA). The membranes were hybridized at 42°C overnight in hybridization buffer (10% Dextran Sulfate, 50% Formamide, 5× SSC, 1× Denhardts, 20 mM Tris (pH 7.5) 20 μg/mL herring sperm DNA) followed by washing once with 2x SSC/0.5%SDS and twice with 0.5 × SSC/0.5%SDS at 60°C. Washed membranes were exposed to X-ray film to develop the signals.

### Western Blot Analysis

At the end of treatment protocols, cell monolayers were washed twice with PBS and lysed with RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl 0.5% Na-deoxycholate, 1% NP40, 0.1% SDS, and protease inhibitors (Roche, Indianapolis, IN). Protein samples were resolved in SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA) with standard protocols. Antibody treatment and signal detection were performed with Protein Detector™ Western blotting Kits (KPL, Inc., Gaithersburg, MD), as per manufacturer's protocol. The affinity purified primary rabbit polyclonal antibody used against WWOX (pure concentration at 280 ng/mL) was developed at our laboratory [23] and was used at a 1:500 dilution and that against actin (ICN, Aurora, OH) was at 1:10 000 dilution. Appropriate Horseradish peroxidase-labeled secondary antibodies were used. The FHIT antibody used for Western analysis was a kind gift of Dr. Kay Huebner, Thomas Jefferson University, Kimmel Cancer Center.

### [$^{3}$H]-Thymidine Uptake

Control, caffeine-, and UV-treated MCF-7 cells were analyzed for DNA synthesis by adding 1 μCi/mL of [$^{3}$H]-Thymidine (6.7 Ci/mmol; ICN) for a period of 4 h prior to the end of the time point. Cells were then washed twice with cold PBS and fixed and washed twice in ice-cold 10% Trichloroacetic acid for 5 min each. The fixed cells were lysed and solubilized in 0.5NNaOH. Aliquots were counted in scintillation counter.

### Flow Cytometry

Cells were harvested with trypsin, pelleted, and washed with PBS. The cells were fixed with 70% ethanol at room temperature for 20 min and resuspended in PBS containing propidium iodide (25 μg/mL), RNAse-A (500 μg/mL), and Triton X-100 (0.5%). Cell cycle analysis was performed with Coulter® EPICS® Elite flow cytometer.

## RESULTS

### UV and BPDE but not Ionizing Radiation (IR) Downregulate Fragile Site Gene Expression

MCF-7 breast cancer cells were chosen as a model system for these studies because *WWOX* is abundantly expressed in this cell line. To determine the effect of UV-C irradiation on *WWOX* as well as *FHIT* expression, the cells were irradiated with UV-C at doses of 10 and 15 J/m$^2$, total RNA was isolated and Northern blot hybridization carried out. Interestingly, as compared to control, *WWOX* and *FHIT* mRNA levels dramatically decreased by 24 h in the UV-irradiated samples (Figure 1A). At the moderate UV-C doses tested [31–34], cell number decreased about twofold at 24 h postirradiation (Figure 1B) without a significant

decrease in cell viability (Figure 1C). As expected [31–33], the *p21* mRNA level increased as a consequence of DNA damage by UV-C (Figure 1A). Thus, the decrease in cell number seen at 24 h is because of growth arrest rather than cell death (see below), which generally requires higher doses of UV-C [31,35]. Next we asked if IR, which causes DNA double strand breaks, also caused a similar effect on *WWOX* and *FHIT* expression. To test this, MCF-7 cells were irradiated with IR at a dose (10 Gy) approximately equitoxic to 10 J/m$^2$ of UV-C radiation (Figure 1A). Northern analysis showed that IR-induced a DNA damage response as expected [32,36] as shown by the robust increase in *p21* mRNA levels (Figure 1A), but did not cause a significant reduction of WWOX or FHIT transcripts. FHIT expression was observed to slightly increase in IR treated cells, however, the significance of this is unknown. Higher doses of IR (20, 30, and 40 Gy) were also ineffective in decreasing *WWOX* and *FHIT* mRNA levels, whereas increasing doses of UV (20 and 30 J/m$^2$) caused downregulation of *WWOX* and *FHIT* transcripts similar to that obtained with 10 and 15 J/m$^2$ (data not shown). Similar to UV, treatment of MCF-7 cells with BPDE, another environmental carcinogen which induces DNA bulky adduct formation, also caused a dramatic decrease of *WWOX* and *FHIT* transcripts at 24 h post-BPDE treatment (Figure 1A).

## Downregulation of Expression of Fragile Site Genes Is p53-Independent

MCF-7 cells have endogenous wild-type p53 protein. In order to ascertain the role, if any, of p53 in the decreased expression of fragile site genes following UV treatment, we tested the p53-null Saos-2 cell line. Similarly to MCF-7 cells, UV irradiation downregulated the levels of *FHIT* and *WWOX* transcripts in Saos-2 cells, indicating that the repressive effect of UV on the fragile site genes was a p53-independent phenomenon (Figure 2). The increase in *p21* mRNA level in Saos-2 can be attributed to a p53-independent mechanism as suggested by others [37].

## Repeated UV Irradiation Reduces the Level of WWOX Protein

In UV-irradiated MCF-7 cells, in spite of a dramatic reduction in the mRNA levels of *WWOX*, the protein expression level did not decrease considerably at 24 h. A considerable decrease in the protein expression was observed following multiple UV-C irradiations (24 h apart) at later time points (48 and 72 h), as shown by Western blot analysis (Figure 3). This suggests that the *WWOX* protein is relatively stable, and repeated genotoxic insults are required to cause a considerable decrease in protein levels. With the same Western blot membranes, we also tested the *FHIT* protein levels. However, we did not observe any significant reduction in levels of this protein (data not shown). The reason for a lack of reduction of FHIT levels, again could be because of a protein stability differences at the limited number of time points tested.

## S-Phase Checkpoint Mechanisms May be Responsible for the Downregulation of Expression of Fragile Site Genes

Different DNA damaging agents are known to alter the cell cycle profile of MCF-7 cells in different ways [38,39]. Flow cytometric analyses of UV-irradiated MCF-7 cells revealed that the fraction of cells in S-phase increased considerably in the total cell population (Figure 4A) indicating that UV-induced an S-phase checkpoint. On the other hand, IR-

induced a $G_2$ arrest in MCF-7 cells (Figure 4B). Similar to UV, BPDE treatment of MCF-7 cells also increased the S-phase fraction of the total cell population (data not shown). In order to confirm that the UV-induced increase in S-phase fraction was because of accumulation of cells in S-phase as a result of a delay and not because of increased cell proliferation, DNA synthetic rates in UV-C treated MCF-7 cells were monitored by uptake of [$^3$H]-Thymidine. As shown in Figure 4C, a significant UV-C-induced reduction in the incorporation of [$^3$H]-Thymidine was observed at 24 h posttreatment. This indicates that the increase in the fraction of S-phase cells shown in Figure 4A is, indeed, because of accumulation of cells at an S-phase checkpoint rather than to an increased rate of entry of cells into S-phase.

Caffeine is known to block the S-phase checkpoint response [40]. Hence, we investigated the effect of caffeine on the UV-induced S-phase delay and on the aforementioned decrease in transcript levels *WWOX* and *FHIT*. Interestingly, treatment of MCF-7 cells with caffeine abrogated the S-phase delay (Figure 4A) and significantly overcame the repression by UV of *WWOX* and *FHIT* expression (Figure 5A). Figure 4A also shows abrogation by caffeine of a small increase of $G_2$/M checkpoint caused by UV that can be attributed to the known pleiotropic effects of caffeine. It is unlikely that the $G_2$/M checkpoint response is involved in WWOX repression because IR causes a significant $G_2$/M checkpoint response without affecting WWOX transcription. Therefore, the signaling pathways triggered at the S-phase checkpoint may play a role in the observed down-regulation.

It was a possibility that the observed repression of WWOX and FHIT expression was a consequence of DNA damage induced alterations in the cell cycle. It has been previously shown that FHIT expression does not change during cell cycle progression [41]. To determine whether WWOX expression was cell cycle regulated, MCF-7 cells were arrested in $G_1$-phase by serum starvation and WWOX expression levels were determined following serum stimulation. We observed normal levels of *WWOX* mRNA after serum starvation ($G_1$ arrest) that remained unchanged as the cells progressed through the S- and $G_2$/M-phases (Figure 5B). This was in sharp contrast to cyclin E mRNA that was undetectable in the $G_1$-phase and dramatically increased as the cells went through S- and $G_2$/M-phases. We conclude that WWOX expression is not dependent on cell cycle-specific signaling in normal growing cells but can be controlled by DNA damage induced S-phase checkpoint mechanisms.

## DISCUSSION

Humans are daily exposed to an enormous variety of DNA-damaging agents. Therefore, it is not surprising that elaborate molecular regulatory systems exist to maintain cellular genomic integrity. UV causes DNA lesions at sites not occupied by histones by producing cyclobutane pyrimidine dimer, which accounts for nearly three-quarters of the damage to DNA induced by this carcinogen. Other types of UV-induced lesions include the pyrimidine-(6-4)pyrimidine dimers and a small fraction of pyrimidine hydrates and glycols [42]. The polycyclic aromatic hydrocarbon (PAH) benzo [*a*] pyrene (BP) is another ubiquitous environmental carcinogen. BP is product from combustion of fuel and tobacco and is one of the most potent carcinogens to which humans are frequently exposed [43,44].

A metabolic pathway mediated by cytochrome P450 and epoxide hydrolase converts BP to BPDE (benzo [*a*] pyrene diol epoxide) [45], the ultimate carcinogenic metabolite responsible for virtually all stable DNA adducts derived from BP. *Cis* or *trans* opening of the epoxide ring of BPDE by the exocyclic amino group $N^2$ of guanine or $N^6$ of adenine results in covalent DNA adducts [46]. IR, on the other hand, produces primarily double strand breaks and thymine glycols. Cells respond to DNA damage by undergoing cell cycle arrest or apoptosis mostly mediated by the tumor suppressor, p53. The first of these two pathways, involves blocking cell cycle progression to allow the DNA repair machinery of the affected cell to remove or repair the damage site prior to replication [47–49]. Nucleotide excision repair is the main pathway involved in the repair of bulky DNA lesions in DNA such as those induced by UV and BPDE [50,51]. The alternative pathway, apoptosis, is considered as a way to eliminate cells that have DNA so extensively damaged that mutation-free recovery is unlikely. During such cell cycle “checkpoints” for DNA repair or apoptosis, certain set of genes are activated whose protein products participate in these pathways whereas certain other genes, whose products may not be required are repressed [52,53]. The kind of response viz., arrest at different phases of cell cycle, or activation of apoptotic pathway is dependent on the nature of the damaging agents (carcinogens) and the type of cells [38,54–56].

The difference in the expression response at the *FHIT* and *WWOX* loci to damage induced by IR or U V is of interest. Both damaging agents produce a response that includes the phosphorylation and stabilization of p53, the activation of MAPK pathways, dramatic changes in transcription of damage response genes, and the establishment of one or more cell cycle checkpoints. However, these damage responses are mediated by different pathways. The ATM kinase pathway is primarily responsible for the IR-dependent response, whereas the related ATR kinase pathway is activated by UV. There is some evidence that BPDE-induced S-phase arrest is also mediated by the ATR kinase pathway [57]. Although similar in many ways, these damage response pathways are qualitatively distinct; note, for example, the differential cell cycle response to IR and UV shown in Figure 4. Thus, the downregulation of *FHIT* and *WWOX* expression by UV and BPDE but not by IR could therefore be a consequence of qualitative differences between the ATR- and ATM-mediated response pathways. Additionally, it has been demonstrated that the in vivo kinase activities of ATR and ATM remain unaffected upon caffeine treatment [40,58]. Within this context, our observation that caffeine is able to significantly block the repressive effect of UV on WWOX and FHIT expression, suggests that the ATR-Chk1 signaling pathway is unlikely to be the major mechanism behind the observed repressive phenomenon.

Alternatively, it is possible to speculate that the observed differences may be a consequence of the type of DNA damage produced by the different agents. As stated above, IR produces primarily double strand breaks and thymine glycols, the latter being a nondistortive DNA lesion. UV, on the other hand, produces cyclobutane pyrimidine dimers and 6-4 photoproducts both of which are classified as “bulky” lesions. The 6-4 photoproducts produce especially large distortions in DNA structure. The major BPDE-DNA adduct, formed by addition of this PAH to the exocyclic amino group of deoxyguanine, is also quite bulky, and has recently been shown to induce large kinks in adducted DNA [59]. These

bulky adducts represent strong blocks to both transcription and replication, and could directly affect the expression of genes such as *FHIT* and *WWOX*, which produce extremely long transcripts. Additionally, BPDE-DNA adducts have been shown to directly affect the function of several transcription factors, including Sp1 and E2F1, by a hijacking mechanism in treated cells [60,61]. This effect is thought to be specific for DNA adducts that distort the DNA, mimicking the binding sites for transcription factors that bend DNA when bound. It is therefore possible that alterations in transcription factor availability at the promoters for the *FHIT* and *WWOX* genes, produced by the distortive DNA adducts in UV- or BPDE-treated cells but not in IR-treated cells are involved in the differential response observed.

Common chromosomal fragile sites contribute to genomic instability by their propensity as prime sites for the occurrence of chromosomal translocations, deletions, gene amplifications, and integration of oncogenic viruses, thereby contributing to cancer development and progression [4,6,7]. In this study, we have clearly demonstrated that the two most commonly altered fragile site genes *WWOX/FRA16D* and *FHIT/FRA3B* are downregulated in a coordinated manner by the common environmental carcinogens, UV and BPDE. Such a decrease in expression level occurred following induction of S-phase checkpoint in an asynchronously cycling cell population. Caffeine is known to inhibit, among others, the S-phase checkpoint and the DNA repair process [40,62]. In this study, caffeine treatment of UV-irradiated MCF-7 cells abrogated the S-phase delay and significantly prevented the decrease of the *WWOX* and *FHIT* transcripts, indicating that the S-phase checkpoint mechanisms contribute to the observed downregulation. It can be hypothesized that DNA damage repair mechanism contributes to the repression of *WWOX* and *FHIT* transcription following UV and BPDE exposure. In the event of DNA damage, elongating RNA Polymerase II is blocked by many DNA lesions in the transcribed strand [63], resulting in transcriptional downregulation of the genes. In stark contrast to the fragile site genes, the observed *p21* upregulation, the unaffected *GAPDH* expression as well as the failure of IR to reduce the expression of *WWOX* and *FHIT* indicate that decrease in mRNA abundance following DNA damage is not a global phenomenon. Additionally, such a decrease in *WWOX* transcripts caused by a single moderate (nonlethal) UV dose (10 $J/m^2$) is transient. In time-course experiments, such reduction was noticeable as early as 3 h postirradiation and dramatically reduced by 18 and 24 h, however, at 48 h the *WWOX* transcripts re-appeared (data not shown). This also implies that the observed repression is unlikely because of gene inactivation by fragile site induction and also speaks for the viability of the cells (Figure 1B). In addition, UV has not been reported to induce chromosomal fragility. It is worth noting that no deleterious effects were noted by the UV treatment (10 $J/m^2$) because examination by phase contrast microscopy and trypan-blue exclusion proved that the cells remained viable and healthy.

At present, no information is available on specific regulatory transcription factor(s) of the two genes that could be affected by these carcinogens. However, their chromosomal localization at fragile regions and their being easy targets for environmental carcinogens could be reasoned for their concordant downregulation. Moreover, the structural nature of both fragile sites spanning huge genomic regions (up to 2 Mb) combined with the presence of large introns may pose considerable delay in the repair/recovery processes through

specific pathways following DNA damage leading to the downregulation of expression of these genes.

Therefore, the observed decrease in expression also appears to be a mechanism for loss of expression of fragile site genes, in addition to the previously described gross genomic abnormalities. It has been recently suggested that chromosomal breaks on the DNA at fragile sites represent regions that have escaped the *ATR-CHK1* DNA damage checkpoint mechanisms [2]. Thus, one could imagine a scenario in which one allele of a fragile site gene could be inactivated by deletion or fragile site induction while the other allele could be subject to downregulation (probably, in transcription) by environmental carcinogens as observed in this study, leading to complete silencing of the gene. Interestingly, supporting this concept and our findings, recently Iida et al. [64] reported that the genotoxic carcinogen methyleugenol induces a marked reduction of Wwox and Fhit expression in mouse liver while the noncarcinogenic congener eugenol has no effect. Importantly, methyleugenol forms bulky DNA adducts making it probable that the observed “in vivo” transcriptional repression could be mediated by the same mechanisms induced by UV and BPDE damaged DNA reported here. We hypothesize that such a continuous (transcriptional) silencing of fragile site associated putative tumor suppressor genes such as *WWOX* and *FHIT* by protracted exposure to environmental carcinogens may play a significant role in the initiation and development of cancer.

Since the submission of our manuscript, Ishii et al. [65] reported that UV irradiation resulted in the reduction of *WWOX* and *FHIT* gene expression and suggested a role for the G1-S checkpoint.

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## Abbreviations

| | |
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| **UV** | ultraviolet |
| **BPDE** | benzo[a]pyrene diol epoxide |
| **IR** | ionizing radiation |
| **FHIT** | fragile histidine triad |
| **WWOX** | WW domain containing oxidoreductase |
| **PBS** | phosphate buffered saline |
| **BP** | benzo [*a*] pyrene |

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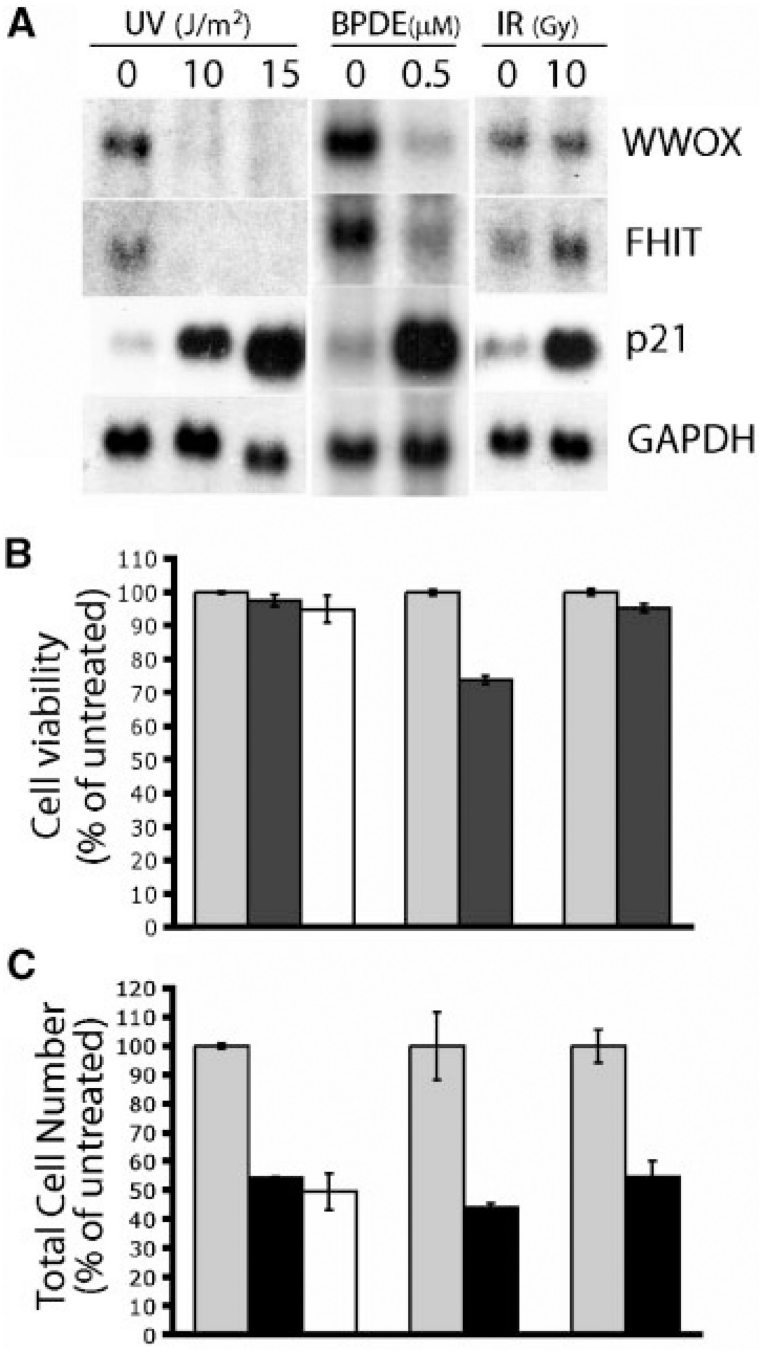


**Figure 1.**

(A) UV and BPDE treatment but not IR downregulate expression of fragile site genes, *WWOX* and *FHIT*. MCF-7 cells were exposed to UV, BPDE, and IR, as described in Materials and Methods. Northern blots of total RNA (20 μg per lane) were hybridized with probes for *WWOX*, *FHIT*, *p21*, and *GAPDH* genes, as indicated. (B) Cell viability of treated cells. Cell viability was determined by trypan-blue exclusion and is expressed as a percentage of untreated controls. (C) UV, BPDE, and IR treatment resulted in cell growth arrest. Total viable cell number was determined by trypan-blue exclusion 24 h

posttreatment. Each treatment was performed in triplicate and is represented as the average ±SD.

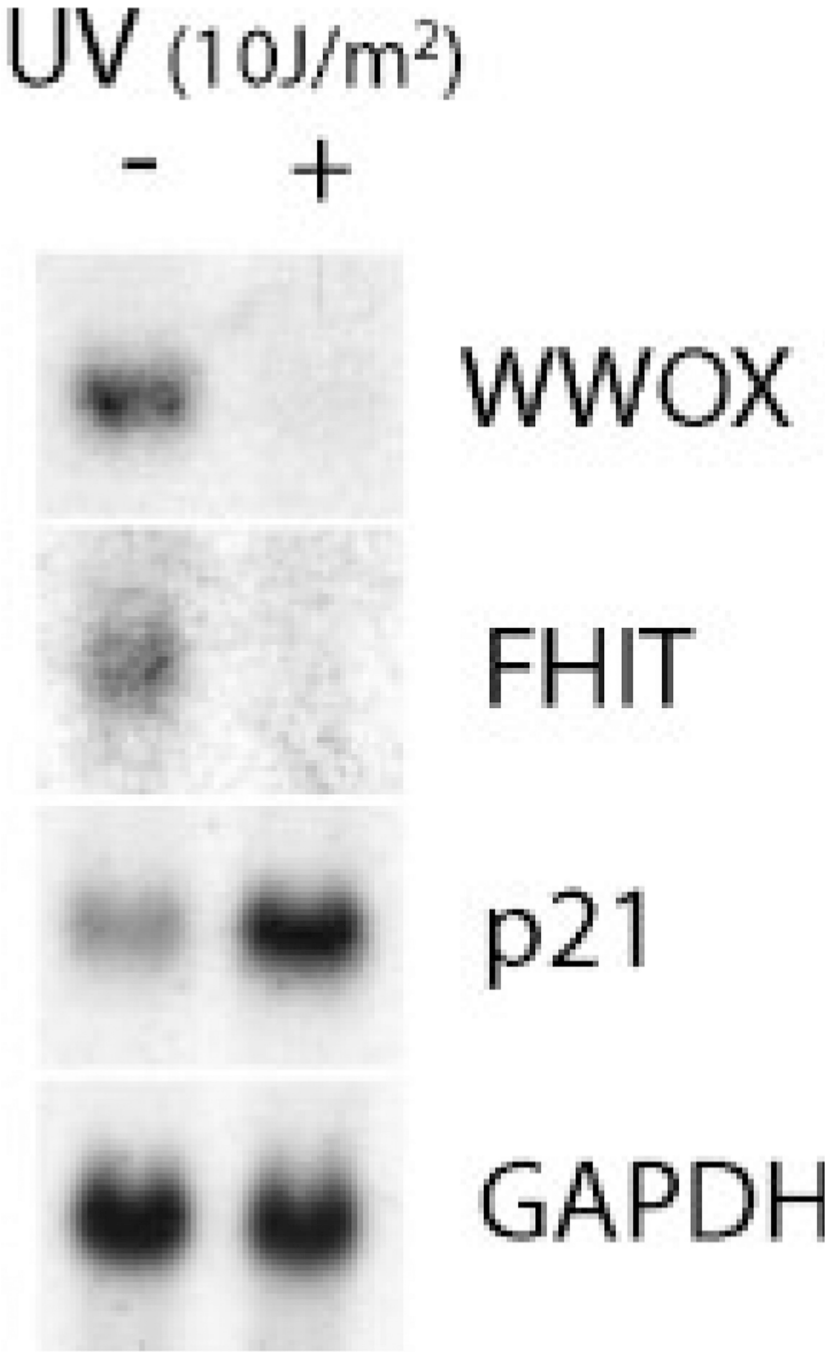


**Figure 2.**

Downregulation of fragile site gene expression by UV is p53-independent. UV treatment of Saos-2 cells and Northern blot hybridization was carried out as mentioned for Figure 1. Note that *WWOX* and *FHIT* transcripts were decreased upon UV irradiation.

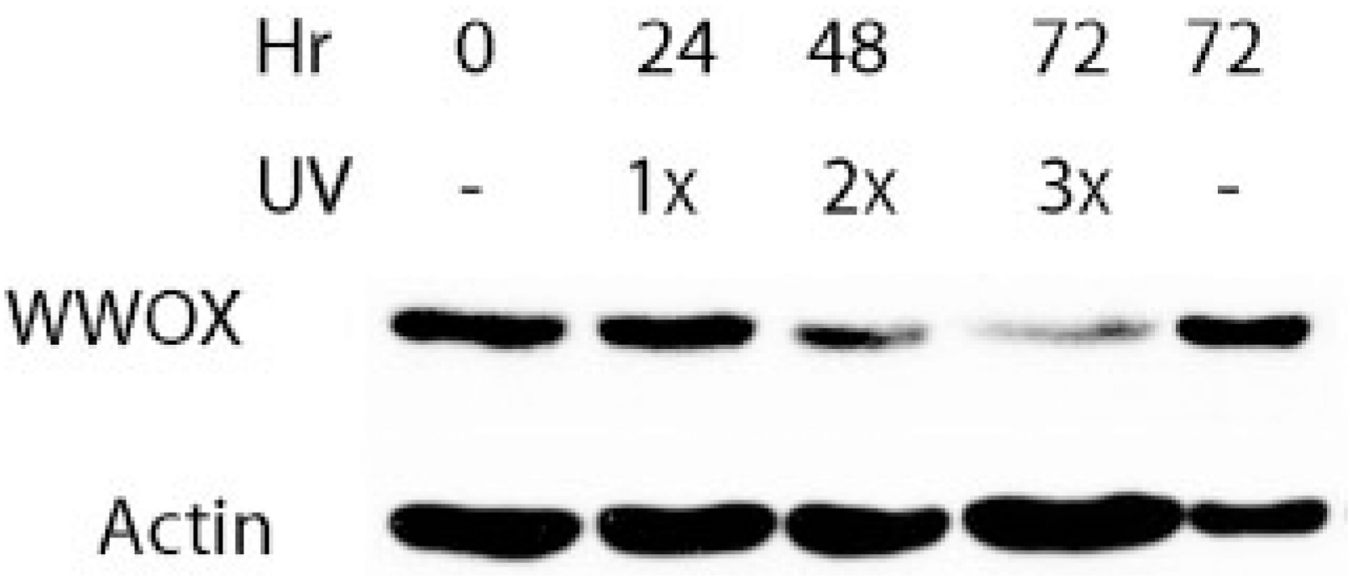


**Figure 3.**

Repeated UV irradiation decreases WWOX protein level. Total protein extracts were made from MCF-7 cells irradiated once (1×), twice (2×) each at 24 h intervals or thrice (3×) each at 24 h intervals or left untreated (−), at indicated time points, 50 μg of protein was resolved in 10% SDS-PAGE, transferred to PVDF membrane and probed with antibodies. Note a progressive decrease in WWOX protein levels upon repeated UV irradiations.

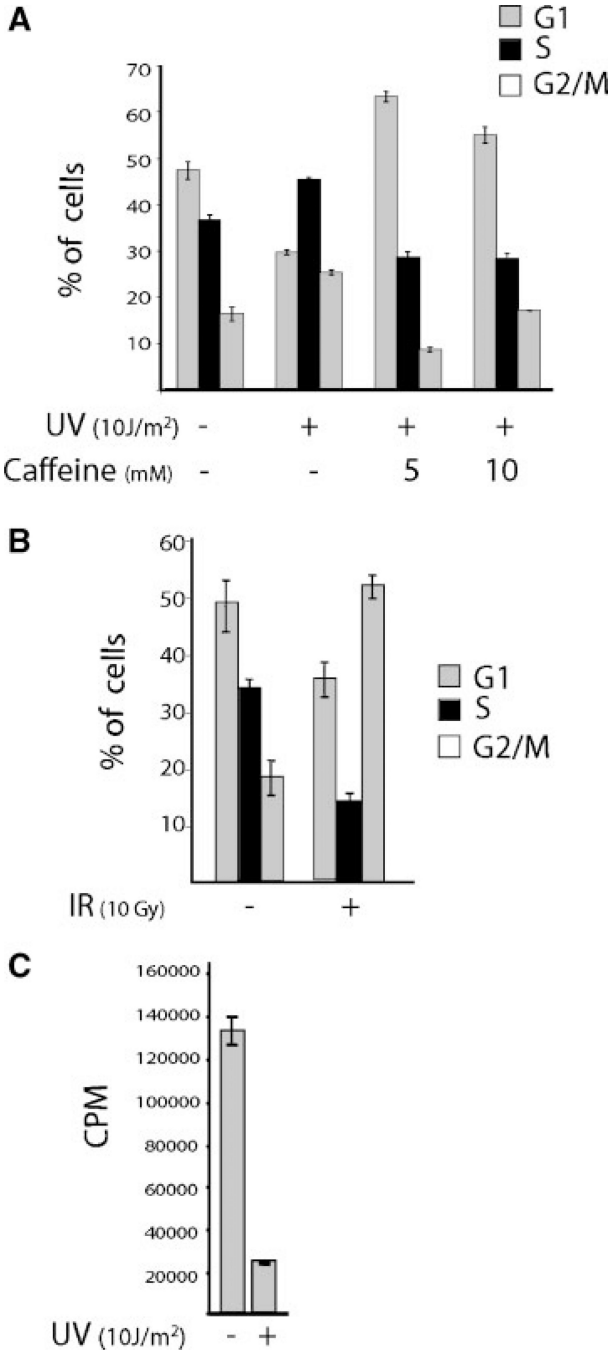


**Figure 4.**

(A) Caffeine abrogates S-phase delay. MCF-7 cells at 60%–70% confluency were pretreated or left untreated for 1 h with the indicated concentrations of caffeine and irradiated with UV. Caffeine treatment was continued for 24 h. The cells were harvested and processed for flow cytometry as mentioned in Materials and Methods. (B) IR causes primarily a $G_2$/M arrest. MCF-7 cells at 60%–70% confluency were subjected to IR, and 24 h later harvested and processed for flow cytometry. (C) UV blocks DNA synthesis. MCF-7 cells were seeded in 12-well plate at $4 \times 10^4$ cells per well. Thirty-six hours later, the cells were irradiated with

UV or left untreated for control. At 20-h post-UV irradiation, [$^3$H]-Thymidine was added. Four hours later the cells were harvested and processed for counting radioactivity, counts per minute (CPM). Triplicate values for each treatment were plotted in the graphs.

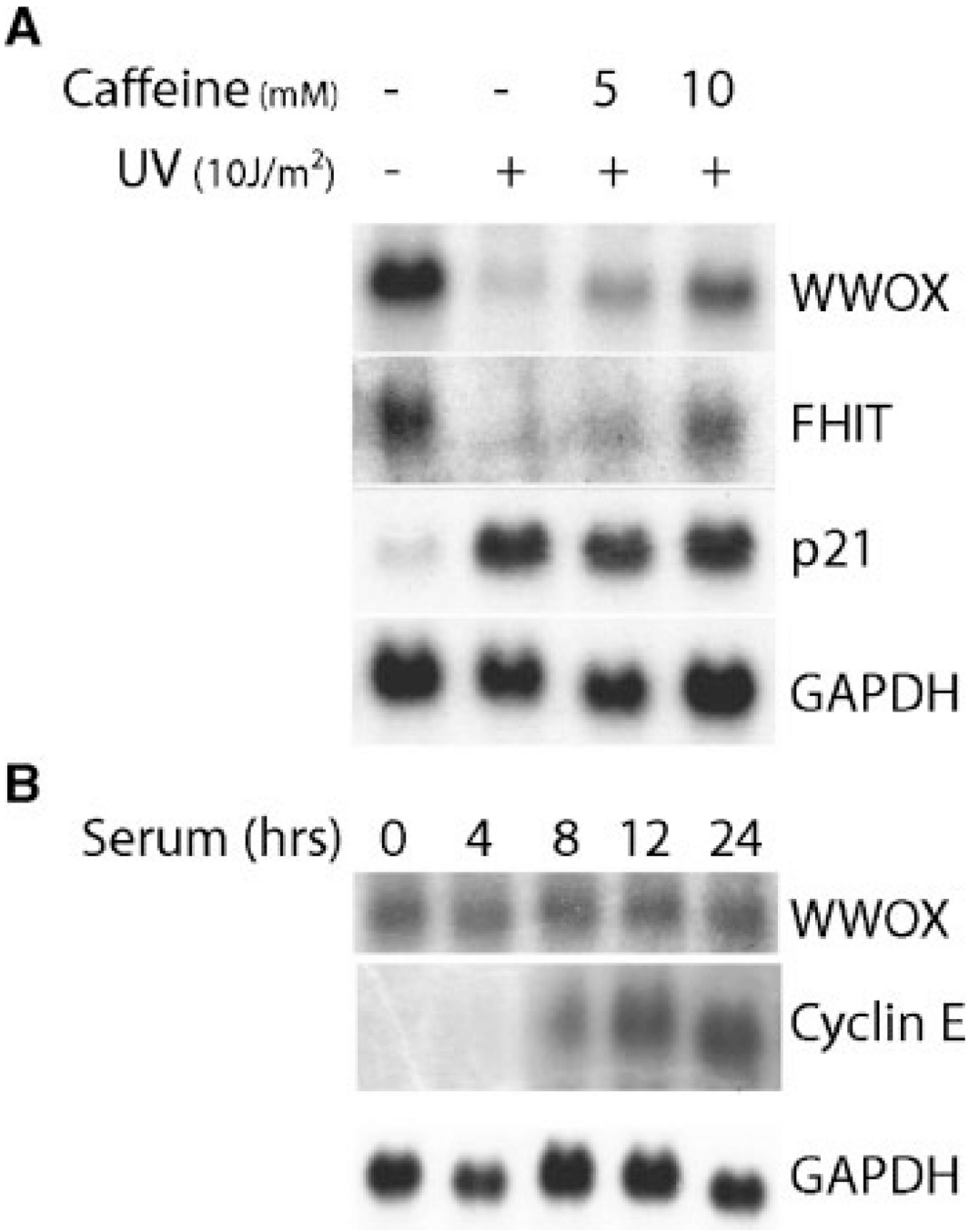


**Figure 5.**

(A) Caffeine significantly overcomes downregulation of expression of fragile site genes. MCF-7 cells were subjected to UV-irradiation and caffeine treatment as indicated in Figure 4A. Northern blots of total RNA (30 μg per lane) from these treated cells were hybridized as indicated in Figure 1. Note the increase in the intensity of *WWOX* and *FHIT* signals upon caffeine treatments. (B) WWOX expression is not cell cycle regulated. MCF-7 cells were grown in serum-free medium for 4 d to synchronize the cells in $G_1/G_0$ phase. Total RNA

was isolated from cells incubated in 5% serum containing medium for the indicated times and analyzed by Northern blotting with WWOX, Cyclin E, and GAPDH probes.