WOX1 Is Essential for Tumor Necrosis Factor-, UV Light-, Staurosporine-, and p53-mediated Cell Death, and Its Tyrosine 33-phosphorylated Form Binds and Stabilizes Serine 46-phosphorylated p53

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WW domain-containing oxidoreductase WOX1, also named WWOX or FOR, undergoes Tyr33 phosphorylation at its first N-terminal WW domain and subsequent nuclear translocation in response to sex steroid hormones and stress stimuli. The activated WOX1 binds tumor suppressor p53, and both proteins may induce apoptosis synergistically. Functional suppression of WOX1 by antisense mRNA or a dominant negative abolishes p53-mediated apoptosis. Here, we determined that UV light, anisomycin, etoposide, and hypoxic stress rapidly induced phosphorylation of p53 at Ser46 and WOX1 at Tyr33 (phospho-WOX1) and their binding interactions in several tested cancer cells. Mapping by yeast two-hybrid analysis and co-immunoprecipitation showed that phospho-WOX1 physically interacted with Ser46-phosphorylated p53. Knockdown of WOX1 protein expression by small interfering RNA resulted in L929 fibroblast resistance to apoptosis by tumor necrosis factor, staurosporine, UV light, and ectopic p53, indicating an essential role of WOX1 in stress stimuli-induced apoptosis. Notably, UV light could not induce p53 protein expression in these WOX1 knockdown cells, although p53 mRNA levels were not reduced. Suppression of WOX1 by dominant negative WOX1 (to block Tyr33 phosphorylation) also abolished UV light-induced p53 protein expression. Time course analysis showed that the stability of ectopic wild type p53, tagged with DsRed, was decreased in WOX1 knockdown cells. Inhibition of MDM2 by nutlin-3 increased the binding of p53 and WOX1 and stability of p53. Together, our data show that WOX1 plays a critical role in conferring cellular sensitivity to apoptotic stress and that Tyr33 phosphorylation in WOX1 is essential for binding and stabilizing Ser46-phosphorylated p53.

Hyaluronidases and hyaluronan are important mediators of tissue remodeling and cancer cell metastasis. Metastatic and malignant breast and prostate cancers frequently overexpress hyaluronidases and hyaluronan (1, 2). Hyaluronidases PH-20, Hyal-1, and Hyal-2 are known to induce the expression of a candidate tumor suppressor WW-domain-containing oxidoreductase WOX1 (also known as WWOX, FOR, or WWOXv1) (3–5).

The human WWOX gene, which comprises nine exons encoding the WWOX/WWOX family proteins, is located on a fragile site on the chromosome 16q23.3–24.1 (6–9). Eight mRNA transcripts of the WWOX gene have been found so far (7). However, it is still not known whether all of the mRNA transcripts are translated successfully into proteins. Isoforms WWOXv1 (46 kDa), WWOXv2 (42 kDa), and WWOXv8 (60 kDa) and several other small proteins can be found in normal and several types of cancer cells (3, 8–11).3 Genetic alterations of the WWOX gene and disappearance of WWOX/WWOX family proteins have been shown in multiple types of cancers, especially at an invasive or a metastatic stage (8, 9, 11–20). Hypermethylation of the WWOX gene may inactivate its expression (21). In contrast, significant up-regulation of WWOX/WWOX family proteins has been shown during progression of breast, prostate and other cancers to a premetastatic state (10, 22, 23). Also, absent expression of these family proteins in metastatic cancer cells is not necessarily due to disruption of the WWOX gene. We have recently determined that post-transcriptional blockade of the full-length mRNA translation to protein may account for the disappearance of the WWOX/WWOX family proteins in cutaneous squamous cell carcinoma cells in patients and in UVB-treated mice (24).

Wild type WOX1 possesses two N-terminal WW domains (containing conserved tryptophan residues), a nuclear localization sequence and a C-terminal short-chain alcohol dehydrogenase/reductase domain (which contains a mitochondria-targeting sequence) (3). Sex steroid hormones such as estrogen and androgen activate WOX1 via Tyr33 phosphorylation (p-WOX1) and nuclear translocation (11). Importantly, p-WOX1 is located in the mitochondria during benign prostatic hypertrophy (11). Nuclear translocation of p-WOX1 occurs when prostate cells progress toward cancerous and metastatic states (11), suggesting a critical role of WOX1 phosphorylation during prostate cancer development.

We determined that WOX1 enhances the cytotoxic function of tumor necrosis factor (TNF)4 and induces apoptosis when overexpressed (3). We also showed that in response to stress or apoptotic stimuli, WOX1 becomes phosphorylated at Tyr33, which allows its complex formation with activated p53 and JNK1 (25). The p53–WWOX complex translocates to the mitochondria and nuclei to mediate apoptosis (25, 26). WOX1 induces apoptosis synergistically with p53 (3, 25). In contrast, JNK1 may block WOX1-induced cell death (25). Src is known to phosphorylate WOX1 at Tyr33 (27). We also determined that Tyr33-phosphorylated WOX1 translocates to the mitochondria and nuclei during light-induced degeneration of photoreceptors in rat eyes, as determined by light and electron microscopy (28).

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4 The abbreviations used are: TNF, tumor necrosis factor; siRNA, small interfering RNA; WOX1si, siRNA-targeting WOX1; dn-WOX1, dominant negative WOX1; CHX, cycloheximide; RT, reverse transcriptase; p-WOX1, phospho-WOX1; EGFP, enhanced green fluorescent protein; zLLL or MG132, benzoyloxycarbonyl-Leu-Leu-Leu-aldehyde.
In this study, we further characterized the binding interaction between p53 and WOX1. We determined that WOX1 plays an essential role in TNF-, UV light-, staurosporine-, and p53-mediated cell death and that Tyr33-phosphorylated WOX1 binds and regulates the stability of Ser46-phosphorylated or activated p53.

EXPERIMENTAL PROCEDURES

Cell Lines and Chemicals—In this study, we have grown the following cell lines for the indicated experiments: 1) murine L929 fibroblasts, 2) human monocytic U937 cells, 3) human neuroblastoma SK-N-SH cells, 4) human p53-deficient lung H1299 cells, 5) monkey kidney COS7 fibroblasts, 6) human colorectal HCT116 cells, and 7) human Molt-4 T lymphocytes. These cells were from the American Type Culture Collection (ATCC) (Manassas, VA). Bovine testicular hyaluronidase PH-20, iron chelator deferoxamine, and DNA-damaging etoposide were from Sigma, TNF-α was from PeproTech and R&D Systems, and MDM2 antagonist nutlin-3 (racemic) and staurosporine were from Calbiochem. Yeast lysis buffer Y-PER was from Pierce.

Antibodies and Antibody Production—We have generated specific antibodies against a Tyr3-phosphorylated peptide of WOX1 (25) and a recombinant full-length WOX1 protein, tagged with glutathione S-transferase (11). The quality of these antibodies has been documented (10, 11, 24, 25, 28). In addition, we constructed the N-terminal WW domain regions of murine WOX1 in pGEX-2T (Amersham Biosciences) and expressed the fusion protein tagged with glutathione S-transferase in bacteria. Antibody against the purified glutathione S-transferase-WOX1ww was performed in rabbits as described (3, 10, 11, 24, 25, 28). In addition, we constructed the N-terminal WW domain regions of murine WOX1 in pGEX-2T (Amersham Biosciences) and expressed the fusion protein tagged with glutathione S-transferase in bacteria. Antibody against the purified glutathione S-transferase-WOX1ww was performed in rabbits as described (3, 10, 11). This antibody recognized a 46-kDa WOX1 as determined in Western blotting using L929 and other cells and was shown to be effective for co-immunoprecipitation (see “Results”).

Additional specific antibodies used in Western blotting were against the following proteins: polyclonal IgG against WWOX (N-19), MDM2 (C-18), and p53 (FL-393) and monoclonal IgG against p53 (Pab240) from Santa Cruz Biotechnology; p53 and 1×BS from BD Biosciences; phospho-p53 at Ser15 from Calbiochem; phospho-p53 at Ser46 from R&D Systems; α-tubulin from Accurate Chemicals; and histones and their acetylated forms from Cell Signaling.

cDNA Expression Constructs, Transfection and Expression in Cell Lines, and Stable Transfectants—The following expression constructs were made as previously described: 1) murine EGFP-WOX1 (3), 2) murine dominant negative WOX1 (dn-WOX1) tagged with EGFP (25), 3) human wild type p53 tagged with DsRed (p53-pDsRedN1) (29), and 4) human p53-pDsRedN1 with Ser46 deletion (29). Control vectors pEGFP-C1 and pDsRedN1 were from Clontech.

Where indicated, L929 and other cell lines were electroporated with the above constructs (200 V, 50 ms; Square Wave BTX ECM830; Genetronics), cultured overnight, and then treated with bovine testicular hyaluronidase PH-20 (Sigma) for the indicated times. Alternatively, the cells were exposed to UV irradiation (240 mJ/cm2; using a UV cross-linker from Fisher), followed by culturing for 1 h. The cells were then subjected to extraction and separation of cytosolic and nuclear fractions using an NE-PER nuclear and cytoplasmic extraction reagent (Pierce). The extent of protein expression was determined using the indicated specific antibodies.

In addition, we generated stable transfectants using the above transduced cells by exposure to G418 (up to 500 μg/ml) for 2 weeks (3, 10, 25). The extent of protein expression was examined by fluorescence microscopy and Western blotting.

siRNA Targeting WOX1 and Stable WOX1 Knockdown L929 Cells—We have previously constructed a siRNA-expressing plasmid (in pSuppressorNeo vector; Imgenex), targeting the expression of human and mouse WOX1 (10). A scrambled sequence construct in the pSuppressorNeo plasmid was used as a control (10). Stable transfectants of L929 cells were also established using G418 selection (10). The extent of WOX1 protein knockdown was examined by Western blotting using our specific antibodies. We have also established stable WOX1 knockdown of neuroblastoma SK-N-SH cells (10). These cells were also used for experiments.

Where indicated, we used retroviral expression of WOX1si in human and murine cell lines to knock down the expression of WOX1 (10). In controls, an empty retrovirus was used (10).

Reverse Transcription (RT)-PCR—RT-PCR was performed as described (3). One μg of the first stranded cDNA from the established cell lines was used as templates. Primer pairs for RT-PCR were as follows: 1) murine WOX1 coding region, forward (5’-ATGCCCTTGGACCTGGCC) and reverse (5’-TCACTCTGAGCCTCCTCG) (3), 2) human WWOX/WOX1 coding region, forward (5’-ATGAGCAGCCTGCGCTAC) and reverse (5’-TAGGCCGACTGGCTGCC), 3) human p53 coding region, forward (5’-ATGAGGACGGCCAGTCA) and reverse (5’-CATCACTGGATCTGACGGC), and 4) murine p53 coding region, forward (5’-ATGACTGCCATGGAGGATC) and reverse (5’-TACGACTGGTTTGGCTTTC). Primer sets for mouse and human glyceraldehyde-3-phosphate dehydrogenase were from Clontech/BD Biosciences.

Co-immunoprecipitation—Co-immunoprecipitation was performed as previously described (3, 10, 11, 25, 29). Briefly, Molt-4 T cells or other indicated cell lines were grown overnight (in 150-mm Petri dishes) and exposed to UV light (or other indicated reagents), followed by culturing for 1 h. Both cytosolic and nuclear fractions were prepared using the NE-PER nuclear and cytoplasmic extraction reagent (Pierce) and quantified (Bio-Rad protein assay). 3–4 μg of anti-p53 or WOX1 IgG were added to these protein preparations (~0.5–0.7 μg in 500 μl of NE-PER extraction buffer), followed by rotating end-over-end at 4 °C for 5–16 h. The mixtures were then added to 20 μl of protein A-agarose beads (Pierce), rotated for 2 h at 4 °C, washed with Tris-buffered saline containing 0.5% Nonidet P-40, and processed for SDS-PAGE (3). The presence of WOX1, p53 and other indicated proteins in the precipitates was determined by Western blotting, using specific antibodies. Where indicated, nonimmune serum or IgG was used in negative controls.

Immunofluorescence Microscopy—The indicated cells were exposed to UV light (240 μl/cm2), cultured for 10–40 min, and subjected to immunofluorescence staining with anti-p53 (FL-393) and anti-WWOX (N19) IgG (Santa Cruz Biotechnology) (3, 4). Cy2-tagged anti-rabbit IgG and Texas Red-tagged anti-goat IgG were used for secondary staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole DAPI (Sigma). In negative controls, secondary antibodies were used for cell staining only.

p53 Stability—p53-deficient H1299 cells, suspended in Dulbecco’s modified Eagle’s medium containing 0.5% albumin, were electroporated with DNA constructs of p53-pDsRedN1 or p53-pDsRedN1 and human p53 coding region, forward (5’-ATGACCCTGAGCCTCCTCG), 2) human WWOX/WOX1 coding region, forward (5’-ATGCCCTTGGACCTGGCC) and reverse (5’-TCACTCTGAGCCTCCTCG), 3) human p53 coding region, forward (5’-ATGAGGACGGCCAGTCA) and reverse (5’-CATCACTGGATCTGACGGC), and 4) murine p53 coding region, forward (5’-ATGACTGCCATGGAGGATC) and reverse (5’-TACGACTGGTTTGGCTTTC). Primer sets for mouse and human glyceraldehyde-3-phosphate dehydrogenase were from Clontech/BD Biosciences.

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WOX1 and p53 Interactions

FIGURE 1. p53 and WOX1 binding interactions. A, Molt-4 T cells were exposed to UV light (240 mJ/cm²) and stained with WOX1 and p53 antibodies and anti-rabbit IgG (conjugated with Cy2). Nuclei were stained with 4',6-diamidino-2-phenylindole. B, Molt-4 T cells were exposed to UV light (240 mJ/cm²) and stained with p53, WOX1, and anti-rabbit IgG (conjugated with Texas Red). Secondary staining was performed using anti-goat IgG (conjugated with Cy2). Nuclei were stained with 4',6-diamidino-2-phenylindole. C, monocytic U937 cells were exposed to UV light (240 mJ/cm²) and stained with WOX1, p53, and anti-rabbit IgG (conjugated with Cy2). Nuclei were stained with 4',6-diamidino-2-phenylindole. D, similarly, neuroblastoma SK-N-SH cells were exposed to deferroxamine (500 μM) for 1 h to induce hypoxic stress and shown to have an increased binding of p53 with WOX1.

RESULTS

Binding of Ser46-phosphorylated p53 with Tyr33-phosphorylated WOX1—We determined the characteristics of p53 and WOX1 binding interactions. Exposure of Molt-4 T cells to UV light resulted in a time-dependent phosphorylation of p53 at Ser46 and WOX1 at Tyr33 and their nuclear translocation (Fig. 1A). Ser46 phosphorylation plays a critical role in p53-induced apoptosis and gene transcription (30–32). Thompson et al. (33) showed that under the inhibition of MDM2 by nutlin-3, phosphorylation of p53 on key serines is not essential for transcriptional activation and apoptosis. UV light also increased the levels of nuclear histone H3 and its acetylation at lysine 9 (H3K9) (Fig. 1A). Immunoprecipitation using anti-p53 antibody showed that there was an increased binding of p53 with WOX1 (Fig. 1A). Precipitating with anti-WOX1 IgG also showed an increased binding of WOX1 with p53 (data not shown; see Fig. 8 from using HCT116 cells). Phosphorylation of p53 at other sites such as Thr18 was also observed (data not shown). UV light-induced nuclear translocation of p53 and WOX1 was further confirmed by immunofluorescence microscopy (Fig. 1B). Similarly, exposure of L929 fibroblasts to UV light resulted in Tyr33 phosphorylation and nuclear translocation of p53 and WOX1, along with p53. (see supplemental Fig. 1).

Under similar conditions, UV light increased phosphorylation of p53 at Ser46 and WOX1 at Tyr33 in monocytic U937 cells (Fig. 1C). An increased binding of p53 with p-WOX1 was observed in UV-irradiated U937 cells (Fig. 1C).

IkBα binds a portion of cytosolic p53, and this interaction appears to stabilize p53 from degradation (29, 34). Exposure of neuroblastoma SK-N-SH cells to genotoxic stress, such as UV light, anisomycin, and etoposide, also caused phosphorylation and nuclear translocation of p53 and WOX1 and their binding interaction (data not shown). IkBα was also present in the cytoplasmic p53-WOX1 complex.

Similarly, hypoxic stress increased the binding of p53 with WOX1. SK-N-SH cells were exposed to deferroxamine for 1 h to induce hypoxic stress (29) and were shown to have an increased binding of WOX1 with p53, as determined by immunoprecipitation with anti-WOX1 IgG (Fig. 1D).

We mapped the domain regions and critical amino acid residues responsible for binding of p53 with WOX1, using a CytoTrap yeast two-hybrid analysis for protein/protein interaction in the cytoplasm (3, 10, 25, 29). In agreement with our previous observations (3), human p53 bound to the N-terminal WW domains of WOX1 (Fig. 2A) and p53 binding with -terminal WW domains of WOX1 (Fig. 2B). Similarly, deletion of other critical phosphorylation residues such as Thr18 and Ser20 in p53 could not abolish its binding with WOX1 (Fig. 2A). In positive controls, self-binding of MafB was shown (Fig. 2A). In negative controls, no binding interactions were observed for empty vectors and collagenase versus lamin C (Fig. 2A).
Tyr\(^{33}\) is a conserved phosphorylation site in WOX1, and stress stimuli and sex steroid hormones induce Tyr\(^{33}\) phosphorylation (11, 25). We have shown that alteration of Tyr\(^{33}\) to Arg abolishes binding of WOX1 with JNK1 (25). Similarly, alteration of Tyr\(^{33}\) to Arg abolished the binding of WOX1 with p53 (Fig. 2A), indicating an essential role of Tyr\(^{33}\) phosphorylation in WOX1 for interacting with p53. Human WWOX/WOX1 binding with p53 is essential for binding with Tyr\(^{33}\)-phosphorylated WOX1.

We examined WOX1 phosphorylation at Tyr\(^{33}\) in the above mentioned yeast cells. These cells were grown at room temperature for 2 days in a galactose-containing broth (to reach an exponential phase). To induce the activation of Ras pathway, some of these cells were then cultured at 35°C for 1 h to induce the activation of Ras pathway. Heat induces the expression of p53, WOX1, and its phosphorylation at Tyr33 (p-WOX1) in yeast cells transfected with wild type p53 and WOX1. In contrast, no WOX1 phosphorylation was observed in cells transfected with p53S46 mutant and wild type WOX1.

To confirm the above observations, p53-deficient H1299 cells were transfected with wild type p53 or p53S46, tagged with DsRed. By co-immunoprecipitation using antibodies against p-WOX1 (25), we determined that UV light induced physical association of p-WOX1 with the wild type p53-DsRed but not p53S46-DsRed (Fig. 3). These results were reproducible using L929 and SK-N-SH cells (data not shown). These observations clearly indicate that Ser\(^{46}\) phosphorylation in p53 is essential for binding with Tyr\(^{33}\)-phosphorylated WOX1.

WOX1 Is Essential for TNF-, UV Light-, Staurosporine-, and p53-mediated Cell Death—We have recently generated plasmid and retroviral vectors for expressing small interfering RNA (siRNA)-targeting WOX1 (WOX1si) (10). Murine L929 cells were transfected with a WOX1si or a scrambled siRNA plasmid construct by electroporation. Stable transfecants were selected using G418. WOX1si-expressing cells had a significantly reduced expression of WOX1 protein (>50%), compared with control cells (Fig. 4A). We have determined that WOX1 enhances TNF cytotoxicity in L929 cells (3). Expectedly, suppression of WOX1 expression by siRNA resulted in cellular resistance to TNF (Fig. 4A).

Similarly, these WOX1si-expressing cells resisted staurosporine and UV light-induced cell death (Fig. 4, B and C). Similar results were observed when challenging WOX1si-expressing SK-N-SH cells to UV light and staurosporine (data not shown). In parallel, we have recently determined that suppression of WOX1 by siRNA in cutaneous squa-
WOX1 and p53 Interactions

![Graph A](image)

**FIGURE 4.** Suppression of WOX1 expression by siRNA induces resistance to death by TNF, staurosporine, and UV light in L929 cells. A, we established stable L929 cell lines expressing scrambled siRNA or WOX1 siRNA (WOX1si) (constructed in pSuppressorNeo) (10). Compared with the “scrambled” control cells, the WOX1si-expressing cells resisted TNF-mediated cell death during exposure for 16–24 h (n = 8; mean ± S.D.; p < 0.001, Student’s t tests). Inhibition of WOX1 protein expression was more than 50% in the WOX1si-expressing cells. The extent of cell death was determined by crystal violet staining. Similar results were observed using non-radioactive cell proliferation assay (Promega; data not shown). B, these WOX1si-expressing cells also resisted staurosporine-induced cell death during exposure to staurosporine for 16 h (n = 8; mean ± S.D.; p < 0.01, Student’s t tests). Compared with “scrambled” control cells, WOX1si-expressing cells resisted UV light-induced DNA fragmentation or apoptosis. Both established cells were exposed to UV light (720 mJ/cm²), followed by culturing for 16 h and then a processing DNA fragmentation assay (3).

![Graph B](image)

**FIGURE 5.** p53-Induced cell death is blocked in WOX1-knockdown cells. p53-deficient H1299 cells were electroporated with p53-DsRed or DsRed vector (empty) only. The cells were then cultured in the presence of retrovirus expressing siRNA-targeting WOX1 (WOX1si) or “empty” retrovirus only for 24 h. For determining the extent of death, cells were stained with crystal violet (representative data on the right (2)). Ectopic p53-DsRed-mediated death of H1299 cells was inhibited in the presence of WOX1si (n = 8; mean ± S.D.). Similar results were observed from a 48-h culture (data not shown).

![Graph C](image)

**FIGURE 6.** Absence of p53 expression in hyaluronidase PH-20- or UV-treated WOX1 knockdown cells. A, stable L929 cells, expressing a WOX1si or a scrambled siRNA, were established. WOX1 protein level was reduced by ~50% in these cells. Compared with scrambled control cells, no induction of p53 expression in WOX1si-expressing cells was observed when these cells were exposed to PH-20 (200 units/ml) for 30 min. PH-20 induces p53 expression in L929 cells (3, 4, 37). B, these cells were exposed to UV light, followed by culturing for 30 min at 37 °C and examination of p53 expression. Compared with control cells, UV light could not restore p53 and WOX1 expression in the WOX1si-expressing cells (data not shown for WOX1 expression). C, stable L929 transfectants, expressing dn-WOX1 (tagged with EGFP) or a control EGFP vector, were established. UV light could not induce p53 protein expression in the dn-WOX1-expressing cells (data not shown for the PH-20 induction). D, the reduced p53 expression was not due to down-regulation of p53 mRNA in WOX1 knockdown cells, as determined by RT-PCR.

We have previously designed a dominant negative WOX1 (dn-WOX1; tagged with EGFP), which blocks stress stimuli-induced WOX1 phosphorylation at Tyr^{33} as well as p53-mediated apoptosis (25). Failure of induction of p53 protein expression in WOX1 knockdown cells is probably not due to interference of p53 mRNA by WOX1si. We established stable transfectants expressing dn-WOX1 (and EGFP as controls). Again, these dn-WOX1-expressing cells resisted UV-induced p53 expression, compared with control cells (Fig. 6G; data not shown for the PH-20 induction). Similarly, COS7 cells express a high level of inducible p53 expression in the WOX1si-expressing cells (Fig. 6A). L929 cells express wild type p53 and are sensitive to TNF cytotoxicity (3, 11).

UV light activates p53 and WOX1 in various cultured cell lines (25, 29, 38). Similarly, UV light rapidly induced p53 expression in the control cells but not in the WOX1si-expressing cells (Fig. 6B). In addition to UV light and PH-20, we also determined that apoptosis-inducing chemicals, such as anisomycin and etoposide, could not induce p53 expression in these WOX1 knockdown cells (data not shown).

UV Light Does Not Induce p53 Expression in WOX1 Knockdown Cells—We examined p53 expression in the WOX1 knockdown L929 cells. Hyaluronidase PH-20 induces the expression of WOX1 and p53 in L929 cells (3, 4, 37). Stimulation of the WOX1si-expressing cells with PH-20 failed to restore WOX1 expression (Fig. 6A). PH-20 could not induce Tyr^{33} phosphorylation in L929 cells (data not shown). PH-20 up-regulated the expression of cytosolic p53 in control cells but suppressed p53 nuclear translocation (Fig. 6A). Notably, PH-20 could not induce p53 expression in the WOX1si-expressing cells (Fig. 6A). L929 cells express wild type p53 and are sensitive to TNF cytotoxicity (3, 11).

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endogenous p53. dn-WOX1 suppressed p53 expression by 70–80%, and UV light could not restore p53 expression (data not shown).

By RT-PCR, we showed that failure of UV light-induced p53 protein expression was not due to down-regulation of p53 mRNA in the WOX1si-expressing cells (Fig. 6D). Also, UV light induced WOX1 gene expression in control cells but not in the WOX1si-expressing cells (Fig. 6D). Similar results were observed by testing SK-N-SH cells expressing scrambled or WOX1 siRNA (data not shown).

p53 Is Destabilized in WOX1 Knockdown Cells—p53-deficient lung H1299 cells were electroporated with p53-DsRed or p53ΔS46-DsRed, in the presence of a scrambled or WOX1 siRNA (WOX1si) construct. After culturing for 48 h, these cells were treated with CHX (300 μM) to block protein synthesis for the indicated times. A, p53-DsRed was relatively stable in control cells expressing scrambled siRNA (less than 15% reduction). In contrast, the stability of p53-DsRed was significantly reduced in the WOX1si-expressing cells (~30–40% reduction). B, the stability of p53ΔS46-DsRed was relatively unchanged in the presence or absence of WOX1si. C, breast MDA-MB-231 cells were exposed to empty or WOX1si retrovirus for 48 h and then treated with zLLL (MG132) for 1 h. Suppression of WOX1 expression by siRNA induced spontaneous ubiquitination of p53, and zLLL further stimulated the accumulation of ubiquitinated p53 in these cells. In the right panel, an average of two experiments is shown.

Inhibition of MDM2 by Nutlin-3 Increases p53 Conformational Changes and Binding with WOX1—MDM2 is an inhibitor of p53 and promotes p53 degradation (for reviews, see Refs. 40–43). We determined whether WOX1 affects the binding of p53 with MDM2. WOX1 physically interacted with both p53 and MDM2 in HCT116 cells, as determined by co-immunoprecipitation (Fig. 8A). Full-length MDM2 (90 kDa) and its small size isoforms (60 and 42 kDa) were found in the precipitates (Fig. 8A). HCT116 cells express wild type p53 (44). The presence of MDM2 isoforms has been shown in different types of cells (45–47). When HCT116 cells were stimulated with nutlin-3 (racemic) for 16–24 h to inhibit MDM2 (41, 48), MDM2 was released from the p53–WOX1 complex (Fig. 8A). Nutlin-3 is known to stabilize p53 without inducing phosphorylation at key serine residues (33, 48). In contrast, UV light enhanced the binding of WOX1 with p53 and MDM2 in HCT116 cells (Fig. 8B). Similar results were observed when precipitating the MDM2-p53-WOX1 complex using IgG antibody against full-length p53 (FL-393), in UV- or nutlin-3-treated HCT116 cells (data not shown).

Immune precipitation of p53 by a monoclonal antibody (Pab240) showed that p53 bound MDM2 of 60 and 42 kDa, but not the full-length 90 kDa, in HCT116 cells (Fig. 8C). Also, p53 could not bind WOX1 effectively. Pab240 binds p53 mutant forms (49). Nutlin-3 appeared to further induce p53 conformational changes, thus leading to its interaction with phosphorylated WOX1, but not MDM2, in HCT116 cells (Fig. 8C). Similar results were observed in L929 cells (data not shown). Nutlin-3-activated p53 was resistant to CHX-mediated degradation in HCT116 cells (Fig. 8D).

We also show the presence of p53–MDM2–WOX1 complex in COS7 fibroblasts, as determined by co-immunoprecipitation using anti-WOX1 IgG (see supplemental Fig. 2). Several MDM2 isoforms were present in COS7 cells. However, only a 42-kDa MDM2 interacted with p53–WOX1 (see supplemental Fig. 2). Under similar conditions, UV light induced complex formation of p53, MDM2, and WOX1 in Molt-4

**FIGURE 7. Reduced p53 stability in the absence of WOX1.** p53-deficient lung H1299 cells were electroporated with p53-DsRed or p53ΔS46-DsRed, in the presence of a scrambled or WOX1 siRNA (WOX1si) construct. After culturing for 48 h, these cells were treated with CHX (300 μM) to block protein synthesis for the indicated times. A, p53-DsRed was relatively stable in control cells expressing scrambled siRNA (less than 15% reduction). In contrast, the stability of p53-DsRed was significantly reduced in the WOX1si-expressing cells (~30–40% reduction). B, the stability of p53ΔS46-DsRed was relatively unchanged in the presence or absence of WOX1si. C, breast MDA-MB-231 cells were exposed to empty or WOX1si retrovirus for 48 h and then treated with zLLL (MG132) for 1 h. Suppression of WOX1 expression by siRNA induced spontaneous ubiquitination of p53, and zLLL further stimulated the accumulation of ubiquitinated p53 in these cells. In the right panel, an average of two experiments is shown.
WOX1 and p53 Interactions

A schematic model for p53 interactions with WOX1 and MDM2. A, UV light induces phosphorylation of p53 at Ser46 and WOX1 at Tyr33 and their complex formation in the presence of MDM2. Nutlin-3 inhibits MDM2 and appears to alter p53 conformation (p53*), thereby stabilizing p53 and its interaction with WOX1. This p53 is not phosphorylated at key serines. UV light restores the complex formation of p-p53-p-WOX1-MDM2. B, WOX1 alone binds MDM2 probably via its C-terminal short-chain alcohol dehydrogenase/reductase domain, and nutlin-3 dissociates the binding. UV light restores the binding.

**DISCUSSION**

In this study, we have demonstrated for the first time that WOX1 is involved in binding and stabilizing p53. During stress response, both p53 and WOX1 undergo phosphorylation at Ser46 and Tyr33, respectively, and the phosphorylation is essential for their binding. Ser46 phosphorylation is involved in p53-mediated apoptosis and gene transcription (30–32). WOX1 and p53 induce apoptosis in a synergistic manner (3). p53-induced apoptosis is blocked when WOX1 phosphorylation or protein expression is blocked by dn-WOX1 or antisense mRNA (3, 25). We further demonstrate here that knockdown of WOX1 protein expression by siRNA enables L929 cells to resist to apoptosis by TNF,
WOX1 and p53 Interactions

MDM2 is involved in the regulatory control of nucleocytoplasmic trafficking of p53. As determined by immunofluorescence microscopy, we show that nutlin-3 increases nuclear localization of WOX1 and p53 in the nuclei but restricts MDM2 to the cytoplasm. It is likely that WOX1 increases p53 stability by enhancing the duration of p53 nuclear localization. UV light increases nuclear localization of WOX1, MDM2, and p53, along with their complex formation. We believe that WOX1 is likely to counteract with MDM2 in promoting nuclear export of p53.

Numerous proteins have been shown to stabilize p53. For example, hypoxia-inducible factor 1a has been shown to stabilize p53 when cells are under hypoxic stress (54). NAD(P)H:quinone oxidoreductase 1 also stabilizes p53 during oxidative stress (55). Human vaccinia-related kinase 1 stabilizes p53 by phosphorylating Thr18 and disrupting p53-MDM2 interaction (56). Interestingly, promyelocytic leukemia p53 stability is sequestered MDM2 to the nucleolus (57). Similarly, merlin (neurofibromatosis 2) increases the p53 stability by inhibiting the MDM2-mediated degradation of p53 (58). PTEN physically associates with endogenous p53 and regulates the transcriptional activity of p53 by modulating its DNA binding (59).

Prolyl isomerase Pin1 possesses an N-terminal WW domain and binds to phosphorylated p53 on the Ser/Thr-Pro motifs, particularly at Ser20, Ser15, and Thr28, and the polyproline region (60, 61). Ser46 phosphorylation in p53 has no effect on Pin1 binding (60, 61). The isomerase activity of Pin1 stabilizes and induces conformational changes of p53, thereby stimulating the DNA binding activity and transactivation function of p53 (60, 61). Whether WOX1 stimulates p53-mediated DNA binding and transactivation function remains to be established.

We have shown that the non-ankyrin C terminus of IκBα physically interacts with the proline-rich region and Ser46 in p53. The binding could be abolished by deletion of Ser46 or alteration of Ser46 to Gly in p53 (29). However, alteration of Ser46 to a conserved phosphorylation residue Thr still retains the binding interaction, suggesting that Ser46 phosphorylation in p53 is involved in binding with IκBα. The p53-IκBα complex dissociates in response to apoptotic stress, hypoxia, DNA damage, and transforming growth factor-β1-mediated growth suppression (29). Zhou et al. (34) confirmed our findings regarding p53 and IκBα interactions.

WW domain-containing proteins are known to bind proline-rich motifs, such as PPXY, PPLP, and others (62). However, phosphorylation of the conserved Tyr35 in the first WW domain of WOX1 enables its interaction with a large context of proteins, independently of polyproline binding. For example, WOX1 binds Smad4 in a Tyr35 phosphorylation-dependent manner, whereas there is no apparent proline-rich motif in JNK1 (25). Similarly, Tyr35-phosphorylated WOX1 binds Smad4 in a Tyr35 phosphorylation-dependent manner. No proline-rich motifs are shown in Smad4. How Tyr35-phosphorylated WOX1 interacts with its binding partners remains to be established.

In summary, we have shown that in resting cells, a portion of cytosolic p53 complexes with MDM2 and WOX1. This binding is critical in controlling p53 activation, conformational changes, nuclear translocation, and ubiquitination/proteosomal degradation.

REFERENCES
1. Bertrand, P., Girard, N., Duval, C., d’Anjou, J., Chauzy, C., Menard, J. F., and Delpech, B. (1997) Int. J. Cancer 73, 327–331
2. Lokeswar, V. B., Robinowicz, D., Schroeder, G. L., Forgacs, E., Minna, J. D., Block, N. L., Nadji, M., and Lokeswar, B. L. (2001) J. Biol. Chem. 276, 11922–11932
3. Chang, N.-S., Pratt, N., Heath, J., Schulz, L., Slate, D., Carey, G. B., and Zevotek, N. (2001) J. Biol. Chem. 276, 3361–3370
4. Chang, N.-S. (2002) BMC Cell Biol. 3, 8
5 L.-J. Hsu and N.-S. Chang, unpublished data.
