

UV-mediated Regulation of the Anti-senescence Factor Tbx2*

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Several lines of evidence have implicated members of the developmentally important T-box gene family in cell cycle regulation and in cancer. Importantly, the highly related T-box factors Tbx2 and Tbx3 can suppress senescence through repressing the cyclin-dependent kinase inhibitors *p19^{ARF}* and *p21^{WAF1/CIP1/SDII}*. Furthermore, Tbx2 is up-regulated in several cancers, including melanomas where it was shown to function as an anti-senescence factor, suggesting that this may be one of the mechanisms by which T-box proteins contribute to the oncogenic process. However, very little is known about whether Tbx2 is regulated by p21-mediated stress-induced senescence signaling pathways. In this study, using the MCF-7 breast cancer cell line known to overexpress Tbx2, we show that in response to stress induced by ultraviolet irradiation the Tbx2 protein is specifically phosphorylated by the p38 mitogen-activated protein kinase. Using site-directed mutagenesis and *in vitro* kinase assays, we have identified serine residues 336, 623, and 675 in the Tbx2 protein as the p38 target sites and show that these sites are phosphorylated *in vivo*. Importantly, we show by Western blotting, immunofluorescence, and reporter assays that this phosphorylation leads to increased Tbx2 protein levels, predominant nuclear localization of the protein, and an increase in the ability of Tbx2 to repress the *p21^{WAF1/CIP1/SDII}* promoter. These results show for the first time that the ability of Tbx2 to repress the *p21* gene is enhanced in response to a stress-induced senescence pathway, which leads to a better understanding of the regulation of the anti-senescence function of Tbx2.

The T-box gene family encodes transcription factors that are evolutionarily defined by the conservation of the DNA-binding domain known as the T-box (1–4). This family has achieved great prominence in the field of developmental biology because its members have been shown to play important roles in embryonic development (5) and mutations within several T-box genes are associated with a number of human congenital diseases (6). Furthermore, members of the T-box family have also

been implicated in cell cycle regulation and cancer. Tbx2 and the highly related factor Tbx3 function as transcriptional repressors (7–10) and can prevent senescence in mouse embryonic fibroblasts and ST.Hdh^{Q111} striatal cells through a mechanism involving their ability to repress the cyclin-dependent kinase inhibitors *p19^{ARF}* (11–13) and *p21^{WAF1/CIP1/SDII}* (referred to as p21) (14, 15). Both Tbx2 and Tbx3 have also been linked to several cancers, primarily because their expression levels have been found to be deregulated in these cancers. Tbx2 and Tbx3 are required for normal breast development, and their expression was found to be up-regulated in a subset of human breast tumors and breast cancer cell lines (16, 17). Both these factors are also overexpressed in a subset of pancreatic cancers (18, 19) and melanomas (15, 20). In addition, Tbx3 has been implicated in bladder (21), liver, (22) and ovarian cancers (23). One mechanism by which Tbx3 may be involved in oncogenesis has been suggested by a study that showed that Tbx3 can cooperate with oncogenic Myc and Ras to induce cellular transformation and suppress apoptosis through repression of *p19^{ARF}* expression and p53 protein levels (24). It is also possible that Tbx2 and Tbx3 may contribute to the oncogenic process through their anti-senescence function, especially since a dominant negative form of Tbx2 has been shown to induce senescence in melanomas overexpressing Tbx2 (15). Taken together, the above reports would suggest that the levels of Tbx2 and Tbx3 need to be tightly regulated during the cell cycle. Indeed a recent study by Bilican and Goding (25) showed that Tbx2 protein levels are regulated during the various phases of the cell cycle, peaking at G₂. In the same study the investigators show that changes in Tbx2 protein levels are not matched by changes in *Tbx2* mRNA levels, suggesting that the protein may be regulated by post-translational modifications such as phosphorylation. Identifying signaling pathways, especially those involved in inducing senescence, that regulate Tbx2 levels and activity is therefore potentially important for the development of novel therapeutic targets to treat cancers by inhibiting Tbx2 function.

Cellular senescence can be triggered by alterations of telomeres as well as in response to a variety of stresses, such as DNA damage caused by irradiation and cytotoxic drugs or chemically induced oxidative stress (26). The p38 pathway plays an important role in diverse cellular responses such as cell cycle progression, differentiation, and apoptosis (27) and has also been identified as a senescence signaling pathway that is activated in response to both telomere-dependent and -independent senescence (28–30). In response to DNA damaging agents, such as ultraviolet (UV) radiation, the p38 mitogen-

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activated protein (MAP)³ kinase signal transduction cascade is initiated to inhibit cell cycle progression and to activate DNA repair processes, thereby maintaining the integrity of the genome. It has previously been shown that in response to stress, p38 phosphorylates and stabilizes the p21 protein (31), which is thought to be the major mediator of a senescence-like growth arrest after UV-induced DNA damage. The subsequent role and regulation of p21 in the DNA repair pathway is, however, unresolved. One line of evidence suggests that p21 levels are induced and that high levels of the protein are maintained in order to facilitate the effect of proliferating cell nuclear antigen in DNA repair (32, 33). However, another line of evidence suggests that the activation of p21 is only transient and that p21 expression needs to decrease after the establishment of growth arrest in order to promote DNA repair by facilitating proliferating cell nuclear antigen recruitment to DNA repair sites (34–36). Although there is compelling evidence to suggest that the decrease in p21 levels seen in the latter scenario results from ubiquitin-dependent protein degradation (36), there is also evidence to suggest that a decrease in *p21* mRNA levels may be involved (37).

Here we show that in response to ultraviolet C (UVC) irradiation, Tbx2 is specifically phosphorylated by the p38 MAP kinase, which leads to an increase in Tbx2 protein levels, predominant nuclear localization of Tbx2, and an increase in the ability of Tbx2 to repress the *p21* promoter. Furthermore, in response to UVC irradiation the increase in Tbx2 protein levels correlates inversely with p21 protein and mRNA levels. Taken together, the data suggest a possible mechanism by which *p21* mRNA levels are repressed in response to UV-induced DNA damage.

EXPERIMENTAL PROCEDURES

Plasmids—The wild-type (WT) p21-LUC reporter construct contains a 2.2-kb SacI-Hind III fragment of the human *p21* promoter inserted upstream of a LUC reporter gene (38). As has previously been described, the pCMV19.Tbx2 and pGEX.Tbx2 were constructed by cloning a full-length BamHI fragment of the mouse *Tbx2* cDNA into the BamHI site of the pCMV19a and pGEX.2TK vectors (7). The pGL3-Basic-p14^{ARF} (642) luciferase construct was kindly provided by Dr. D. Holzschu (Ohio University, Athens, Ohio). Point mutations were introduced into full-length WT *Tbx2* cDNA by site-directed mutagenesis using the Stratagene QuikChange system and appropriate primer pairs.

Cell Cultures and Transfection Assay—COS-7 monkey kidney, NIH 3T3 mouse fibroblast, and HT1080 human fibrosarcoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 units/ml penicillin, and 100 μg/ml streptomycin. MCF-7, human breast epithelial adenocarcinoma cells, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM minimal essential medium non-essential amino acids, and 0.01 mg/ml insulin. Cells were maintained in a 37 °C incubator (95.0% air/5% CO₂, 65% humidity).

Transfections were performed using FuGENE 6 (Roche Applied Science) and/or FuGENE HD (Roche Applied Science) for COS-7 cells and Transfectin (Bio-Rad) for NIH 3T3 cells, according to the manufacturers' instructions. Cells were plated at 0.5×10^5 /ml in 35-mm dishes 1 day before transfection. For luciferase assays, COS-7 cells were transfected with 700 ng of the p21 or p14^{ARF} luciferase reporter plasmid plus 200 ng of the Tbx2 expression plasmid or 200 ng of an empty vector plasmid. The vector pRL-TK containing the thymidine kinase promoter driving the expression of a *Renilla* reporter was used as an internal control for transfection efficiency (50 ng/transfection). Cells were cultured for 30 h, and extracts were assayed for firefly and *Renilla* luciferase activity using the dual luciferase assay system (Promega, Madison, WI). Luciferase activities were measured using the Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA). Firefly luciferase values were normalized to the *Renilla* luciferase activity and expressed relative to empty vector control. All transfections were performed in duplicate, and at least three independent experiments were done to confirm reproducibility. For transfected cells, UVC irradiation (254 nm, 40 J/m²) was performed using a UV Stratalinker 1800 apparatus (Stratagene) 30 h post-transfection. For cells expressing endogenous Tbx2, UVC irradiation was performed at 100 J/m². For UVC treatment, the medium was completely removed and replaced immediately after irradiation. Pretreatment with the specific p38 inhibitor SB203580 (Calbiochem) was at 10 μM for 30 min prior to UVC irradiation.

Quantitative Real-time PCR—Total RNA was extracted from MCF-7 cells using the RNeasy Plus Mini kit (Qiagen). Reverse transcription of RNA (1 μg) was performed according to the manufacturer's instructions using the QuantiTect Reverse Transcription kit (Qiagen). Using 2 μl of cDNA, PCR was conducted with the QuantiTect SYBR Green PCR master mix (Qiagen) according to the manufacturers' protocol. Real-time PCR was performed on a LightCycler Version 3 (Roche Applied Science) using the following parameters: denaturation (15 min at 95 °C), annealing and amplification at 35 cycles (15 s at 94 °C; 20 s at 55 °C; 20 s at 72 °C), melting temperature (15 s at 65 °C), and a cooling step (30 s at 40 °C). Each DNA sample was quantified in duplicate, and a negative control without cDNA template was run with every assay to assess the overall specificity. Melting curve analyses were carried out to ensure product specificity, and data were analyzed using the 2^{−ΔΔC_t} method (39). Relative mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase for each reaction with PCR efficiency correction calculated using the formula Ratio = (E_{target})^{CP_{target}(control−sample)}/(E_{ref})^{CP_{ref}(control−sample)}. E, real-time PCR efficiency, CP, crossing-point. Primers used to amplify the human Tbx2 (QT00091266) and p21 (QT00062090) were purchased from Qiagen. Glyceraldehyde-3-phosphate dehydrogenase, forward 5'-GAAGGCTGGGGCTCATTT-3'; reverse 5'-CAGGAGGCATTGCTGATGAT-3').

Western Blot Analyses—Whole cell extracts were prepared from cells using a lysis buffer containing 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl, pH 7.5, 1% deoxycholate, supplemented with 1 mg/ml aprotinin, 1 mg/ml pepstatin A, and 2 mM phenylmethanesulfonyl fluoride protease inhibitors (Sigma). Proteins were resolved on 7.5–15% SDS-

³ The abbreviations used are: MAP, mitogen-activated protein; UVC, ultraviolet C; WT, wild-type; FBS, fetal bovine serum; GST, glutathione S-transferase; NLS, nuclear localization signal; MOPS, 4-morpholinepropanesulfonic acid; JNK, c-Jun N-terminal kinase.

polyacrylamide gels as required and transferred to Hybond ECL (Amersham Biosciences). The membranes were probed with appropriate primary antibodies and detected using peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:5000) and visualized by ECL (Pierce). The primary antibodies used were mouse monoclonal anti-Tbx2 62-2 antibody (1:2000), rabbit polyclonal anti-phospho-p38 (1:1000) and anti-p38 (1:5000) (Cell Signaling Technology Inc., Beverly, MA), anti-Pk-Tag (SV5) antibody (1:3000) (Serotec, Oxford, UK), and rabbit polyclonal p21 (1:200) and mouse monoclonal α -tubulin (1:500) from Santa Cruz Biotechnology, Santa Cruz, CA.

Protein Expression and Purification—All glutathione S-transferase fusion proteins were expressed in *Escherichia coli* strain pLysS. Isopropyl-1-thio- β -galactopyranoside induction was performed for 4 h at 37 °C. Fusion proteins were purified using glutathione-Sepharose 4B beads (Amersham Biosciences).

p38 Protein Kinase Assays—Recombinant active p38 α was obtained from Upstate Biotechnology. Kinase assays were performed in 40 μ l of reaction volume (20 mM MOPS, pH 7.2, 25 mM β -glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) at 30 °C for 30 min under agitation. 25 ng of active p38 protein kinase were added to Tbx2 recombinant protein in the presence of [γ - 32 P]ATP (10 μ M Ci diluted with 9 μ l of 400 μ M unlabeled ATP, 75 mM MgCl₂) and incubated for 30 min at 30 °C. Following the kinase reaction, beads were washed five times with 1 ml of reaction buffer, and 20 μ l of protein denaturing buffer were added. Phosphorylation of Tbx2 by the p38 MAP kinase was detected by autoradiography after SDS-PAGE gel electrophoresis.

Immunofluorescence—MCF-7 cells grown on glass coverslip slides 1 h after UVC irradiation were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature before permeabilization with 0.2% Triton X-100 for 10 min at room temperature. Slides were incubated overnight at 4 °C with mouse Tbx2 monoclonal antibody (62-2) at a dilution of 1:750 and then incubated with Alexa 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) at a dilution of 1:1000 for 2 h at room temperature. Cells were incubated in the dark with 1 μ g/ml 4',6-diamidino-2-phenylindole in phosphate-buffered saline for 10 min, mounted on a slide with Mowial mounting medium (Hoescht, Germany) containing anti-fade (Sigma), and examined by fluorescent microscopy.

RESULTS

Tbx2 Is Phosphorylated *in Vivo*—To determine whether the Tbx2 protein is regulated post-translationally by phosphorylation, protein extracts from MCF-7 cells were treated with shrimp alkaline phosphatase and subjected to Western blot analyses. Fig. 1A shows that when probed with an antibody specific to the Tbx2 protein, the higher molecular weight band seen in the untreated sample disappears in the phosphatase-treated sample. These results suggest that the top band seen in the untreated sample represents phosphorylated Tbx2 and that the protein is phosphorylated *in vivo*.

UVC-induced Phosphorylation of Tbx2—Having established that Tbx2 is phosphorylated *in vivo*, we next examined the Tbx2 protein sequence for potential phosphorylation sites

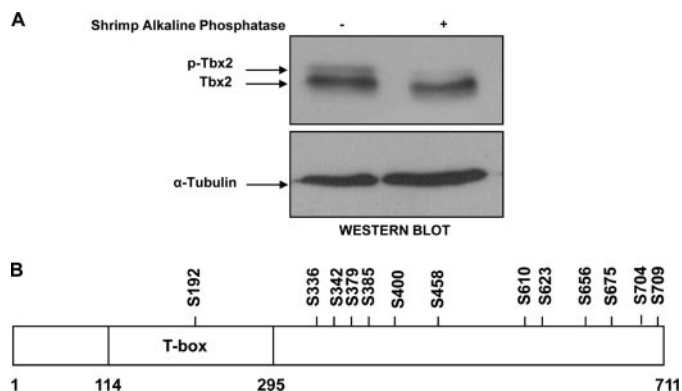


FIGURE 1. Tbx2 is phosphorylated *in vivo*. A, dephosphorylation of Tbx2 by shrimp alkaline phosphatase. Protein extracts (30 μ g) from MCF-7 cells were dephosphorylated by incubating the samples with 1 unit of shrimp alkaline phosphatase at 37 °C for 30 min prior to Western blot analyses. For optimal separation of phosphorylated Tbx2, protein extracts were separated by 7.5% SDS-PAGE. Tubulin is included as a loading control. p-Tbx2, phosphorylated Tbx2. B, schematic representation of Tbx2 showing the thirteen SP motifs that are potential phosphorylation sites.

using an on-line motif data base (au.expasy.org/tools/scan-prosite/). Thirteen serine-proline (SP) motifs (Fig. 1B), which are minimum potential target sites for phosphorylation by several kinases, including members of the MAP kinase family, were identified (40). To investigate whether Tbx2 is a target for stress-responsive kinase(s), COS-7 cells were transfected with a Tbx2 expression vector and exposed to increasing doses of UVC irradiation and protein extracts were analyzed by Western blotting. Fig. 2A shows that with increasing doses of UVC irradiation there is an increase in phosphorylated Tbx2 from 10 J/m². This observed increase in phosphorylated Tbx2 is consistent with the appearance of activated p38, as detected using an antibody specific for phosphorylated p38. To investigate whether there was a similar effect of UVC irradiation on the phosphorylation status of endogenous Tbx2, MCF-7 cells were exposed to a fixed dose of 100 J/m² UVC and protein harvested at the indicated time points post-treatment (Fig. 2B). An increase in the phosphorylation status of Tbx2 was observed immediately after UVC radiation, with high levels persisting up to 180 min post-treatment. It is interesting to note that this effect is also seen for the lower Tbx2 band which, at the early time points, is probably due to post-translational modification(s) rather than post-transcriptional event(s). The dose of UVC irradiation selected in this experiment was based on previous results that showed that it consistently yielded high levels of phosphorylated Tbx2 in MCF-7 cells (data not shown). Both the JNK and p38 MAP kinase pathways are strongly activated by a variety of cellular stresses, including UV irradiation (41). To identify which of the two pathways was responsible for Tbx2 phosphorylation, we exposed cells to 100 J/m² UVC irradiation in the presence or absence of SB203580, which specifically inhibits p38 while being ineffective against JNK. Based on earlier results, protein was harvested 60 min post-UVC irradiation and analyzed by Western blotting. The results show that the UVC-induced increase in Tbx2 phosphorylation is specifically via the p38 MAP kinase, as treatment with the SB203580 inhibitor significantly reduces this effect (Fig. 2C). We next investigated the effect of UVC irradiation on Tbx2 mRNA levels by

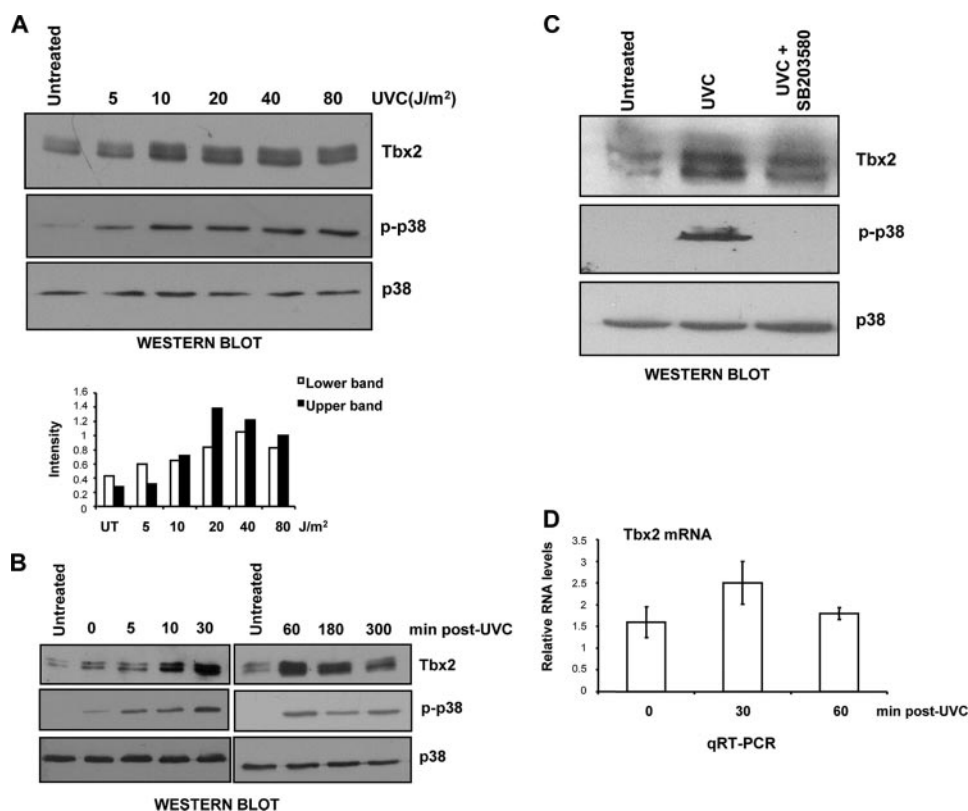


FIGURE 2. UVC-induced phosphorylation of Tbx2. A, dose-dependent increase in the phosphorylation status of transfected Tbx2 in response to UVC irradiation. COS-7 cells were transfected with a vector expressing SV5 epitope-tagged Tbx2 and UVC irradiated at the indicated dosages. Protein extracts were prepared 1 h post-treatment. To detect phosphorylated Tbx2, protein samples were analyzed on a 7.5% SDS-PAGE and by Western blotting using an antibody to SV5. Levels of phospho-p38 and total p38 in these samples were assessed on a 12% SDS-PAGE and by Western blotting with appropriate antibodies. The bar graph compares the intensity of the upper and lower bands in each sample normalized to the p38 loading control. B, time-dependent increase in the phosphorylation of endogenous Tbx2 in response to UVC irradiation. Protein extracts from MCF-7 cells at the indicated time points post-UVC irradiation (100 J/m²) were assayed as described in panel A. The proteins extracted from 0 to 30 min and 60 to 300 min post-UVC treatment were from different experiments and were therefore analyzed on separate gels. C, endogenous Tbx2 is phosphorylated *in vivo* by the p38 kinase. MCF-7 cells irradiated with UVC (100 J/m²) in the presence or absence of SB203580 (10 μ M, 30 min prior to UVC irradiation), a highly specific inhibitor of the p38 family kinases. Protein extracts were assayed as described in panel A. D, Tbx2 mRNA levels increase in response to UVC irradiation. Quantitative real-time PCR of endogenous Tbx2 mRNA extracted from MCF-7 cells was performed at the indicated times post-UVC irradiation (100 J/m²). The levels of Tbx2 mRNA expression were normalized against glyceraldehyde-3-phosphate dehydrogenase. Error bars represent standard deviations.

quantitative real-time PCR. RNA extracted from MCF-7 cells at 0, 30, and 60 min post-UVC irradiation was amplified using primers specific for Tbx2, and Fig. 2D shows that Tbx2 mRNA levels increased immediately post-UVC irradiation with levels peaking at 30 min. Taken together, these results suggest that Tbx2 protein levels increase in response to UVC irradiation due to both a phosphorylation-dependent increase in the stability of the protein and an increase in mRNA levels.

Tbx2 Is Phosphorylated by the p38 MAP Kinase at Three Serine Residues—To map the p38 phosphorylation sites, each of the thirteen candidate SP sites previously identified in the Tbx2 protein was individually mutated to alanine by site-directed mutagenesis. These proteins were expressed with a glutathione S-transferase tag and were used as substrates for *in vitro* p38 α kinase assays. In these kinase assays the GST-Tbx2 fusion proteins were expressed as either N-terminal (1–371) or C-terminal (371–711) proteins (Fig. 3A). Glutathione S-transferase alone was used as a negative control. The results (Fig. 3B) indicate that the WT GST-Tbx2 fusion proteins (1–371) and (371–

711) were both phosphorylated by the p38 MAP kinase. Although S192A and S342A had no effect on phosphorylation of the N-terminal portion of Tbx2, the S336A mutation abolished phosphorylation by the p38 kinase. The single mutations S623A and S675A reduced phosphorylation of the C terminus of Tbx2, and when both these sites were mutated, phosphorylation was severely reduced. Mutating the remaining eight potential p38 sites in the C terminus of Tbx2 had no effect on the phosphorylation of Tbx2 in these experiments (data not shown). These results show that Tbx2 is phosphorylated specifically by the p38 MAP kinase at Ser-336, Ser-623, and Ser-675 *in vitro*. To test whether these sites were phosphorylated *in vivo*, COS-7 cells were transfected with vectors expressing either SV5-tagged WT Tbx2 or a SV5-tagged Tbx2 S336A,S623A,S675A mutant, and the Tbx2 phosphorylation status was compared by Western blot analyses (Fig. 3C). Compared with WT Tbx2, which is expressed as two bands, the Tbx2 mutant is expressed as a single band that migrates with the lower WT Tbx2 band. These results suggest that serine residues 336, 623, and 675 are indeed targets for *in vivo* phosphorylation. Given that mutating these three serine residues results in the complete loss of the upper phosphorylated band, it

would imply that they are critical for phosphorylation by an additional kinase(s). We next investigated whether these residues were phosphorylated in response to UVC irradiation. Cells were transfected with vectors expressing either SV5-tagged WT Tbx2 or the SV5-tagged Tbx2 S336A,S623A,S675A mutant in the presence or absence of UVC, and the Tbx2 phosphorylation status was compared by Western blot analyses (Fig. 3D). As expected, the intensity of both WT Tbx2 bands increased in response to UVC irradiation. Mutating the serine residues 336, 623, and 675, however, abolished this effect because only a single band was observed in both treated and untreated samples. These results confirmed that the identified sites were the only p38 targets for phosphorylation of Tbx2 in response to UVC irradiation.

UVC-induced Phosphorylation by p38 Affects Tbx2 Protein Stability and Subcellular Localization—To explore the functional significance of Tbx2 phosphorylation by p38, we first investigated its effect on Tbx2 protein stability. This was achieved by mutating the p38 target sites to either alanine (Ala),

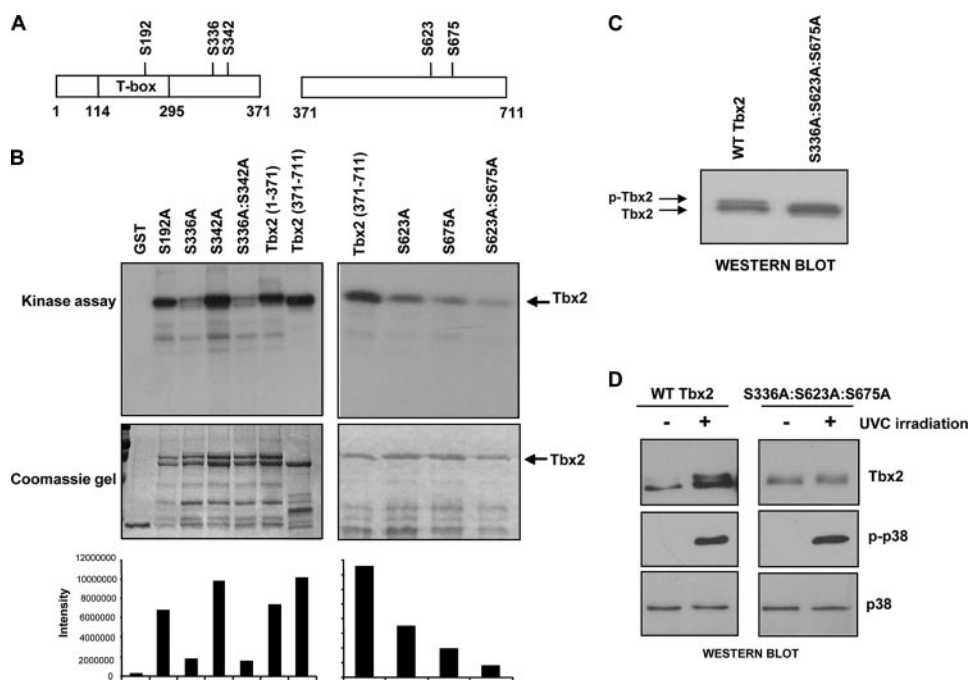


FIGURE 3. Tbx2 is phosphorylated by the p38 MAP kinase at three serine residues. A, schematic representation of the N-terminal (1–371) and C-terminal (371–711) Tbx2 proteins used as substrates in p38 kinase assays. B, mapping the p38 MAP kinase target sites within Tbx2. *In vitro* p38 kinase assays were performed using purified GST-Tbx2 fusion proteins as substrates in the presence of the recombinant activated p38 α kinase and [γ - 32 P]ATP. Kinase assays using the indicated Tbx2 proteins are shown in the upper panels after SDS-PAGE and autoradiography. The lower panels show the same gels stained with Coomassie Blue, indicating that equivalent amounts of protein were used in each assay. The bar graph shows densitometric values of radioactive levels measured for each construct in the kinase assay. C, mutating the p38 MAP kinase sites affects the phosphorylation of Tbx2 *in vivo*. SV5 epitope-tagged Tbx2 or the S336A,S623A,S675A mutant were expressed in COS-7 cells, and the phosphorylation status of Tbx2 was analyzed by 7.5% SDS-PAGE and by Western blotting using an anti-SV5 antibody. D, the identified p38 target serine residues 336, 623, and 675 in the Tbx2 protein are the only sites phosphorylated in response to UVC irradiation. Cells were transfected with SV5 epitope-tagged WT Tbx2 or the Tbx2 S336A,S623A,S675A mutant and exposed to UVC irradiation (40 J/m 2) 29 h post-transfection. Protein extracts were prepared 1 h post-UVC treatment and analyzed by Western blotting as described in Fig. 2A.

to abolish phosphorylation, or glutamic acid (Glu), to mimic phosphorylation. NIH 3T3 cells, transiently transfected with either WT Tbx2 or the Tbx2 mutants, were incubated with cycloheximide, a protein synthesis inhibitor, over a period of 6 h (Fig. 4A). To accurately quantify total levels of Tbx2 protein, cell lysates were separated on a 10% SDS-PAGE in this experiment, and hence Tbx2 is detected as a single band. The Tbx2 S336A,S623A,S675A mutant protein was unstable with an approximate 30% decrease in protein levels at 6 h. On the other hand, levels of the pseudo-phosphorylated Tbx2 S336E,S623E,S675E mutant and WT Tbx2 proteins remain unchanged. This finding implicates p38 kinase as a modulator of Tbx2 protein stability in response to cellular stress.

We next wished to establish by means of immunofluorescence whether phosphorylation by the p38 MAP kinase in response to UVC irradiation affected the subcellular localization of Tbx2 (Fig. 4B). Endogenous Tbx2 was found to exhibit diffuse staining in both the cytoplasm and nucleus of the mock-irradiated cells. In MCF-7 cells exposed to UVC irradiation, however, Tbx2 was predominantly localized in the nuclei of cells. Furthermore, in cells pretreated with the SB203580 p38 kinase inhibitor this effect was reversed as staining was obtained in both the cytoplasm and nucleus. These results show that phosphorylation of Tbx2 by the p38 kinase in response to

UVC irradiation results in the translocation of the protein to the nucleus.

UVC-induced Phosphorylation by p38 Enhances the Ability of Tbx2 to Repress p21—Taken together, the above results suggest that Tbx2 may be playing a role in the UV stress pathway through repressing specific target genes. Interestingly, p21, a known Tbx2 target (14), has been shown to inhibit DNA repair (34–36) and to be down-regulated in response to UV irradiation (36, 37, 42). This UV-mediated down-regulation of p21 was previously observed at both the protein and mRNA levels in MCF-7 cells (37), suggesting that UV-induced phosphorylation of Tbx2 may be required to inhibit p21 in these cells. We therefore tested this hypothesis by investigating whether there was an inverse correlation between Tbx2 and p21 protein levels in UVC-irradiated MCF-7 cells (Fig. 5A). Immediately post-UVC irradiation (0 min post-UVC) there was a significant increase in p21 protein levels followed by a decrease that correlated inversely with the increase in Tbx2 protein levels at all subsequent time points. It is important to point out that the protein

extracted from 0 to 30 min and 60 to 300 min post UVC-treatment were analyzed on different gels and this may account for the difference in p21 protein levels seen in the untreated lanes of the two blots. This does not, however, detract from the results obtained. Quantitative real-time PCR results (Fig. 5B) revealed that relative to the non-irradiated cells p21 mRNA levels were down-regulated immediately after UVC treatment and that this down-regulation persisted over the period post-UVC treatment when Tbx2 protein levels went up. These results showed an inverse correlation between Tbx2 protein and p21 mRNA levels in UVC-irradiated MCF-7 cells and suggested that Tbx2 may be preventing activation of the p21 promoter under these conditions.

To investigate the possibility that stress-induced phosphorylation by the p38 kinase potentiates the ability of Tbx2 to repress transcription of the p21 gene, we co-transfected COS-7 cells with WT Tbx2 together with a p21 promoter-luciferase reporter in the presence and absence of UVC irradiation (Fig. 5C). Consistent with our previously published data (14), co-transfection with a WT Tbx2 expression vector in the absence of UVC irradiation resulted in an ~2-fold repression of the p21 promoter. In the presence of UVC irradiation, however, repression of the p21 promoter by Tbx2 was significantly enhanced by ~5-fold. Tbx2 has previously been shown to repress the p14^{ARF}

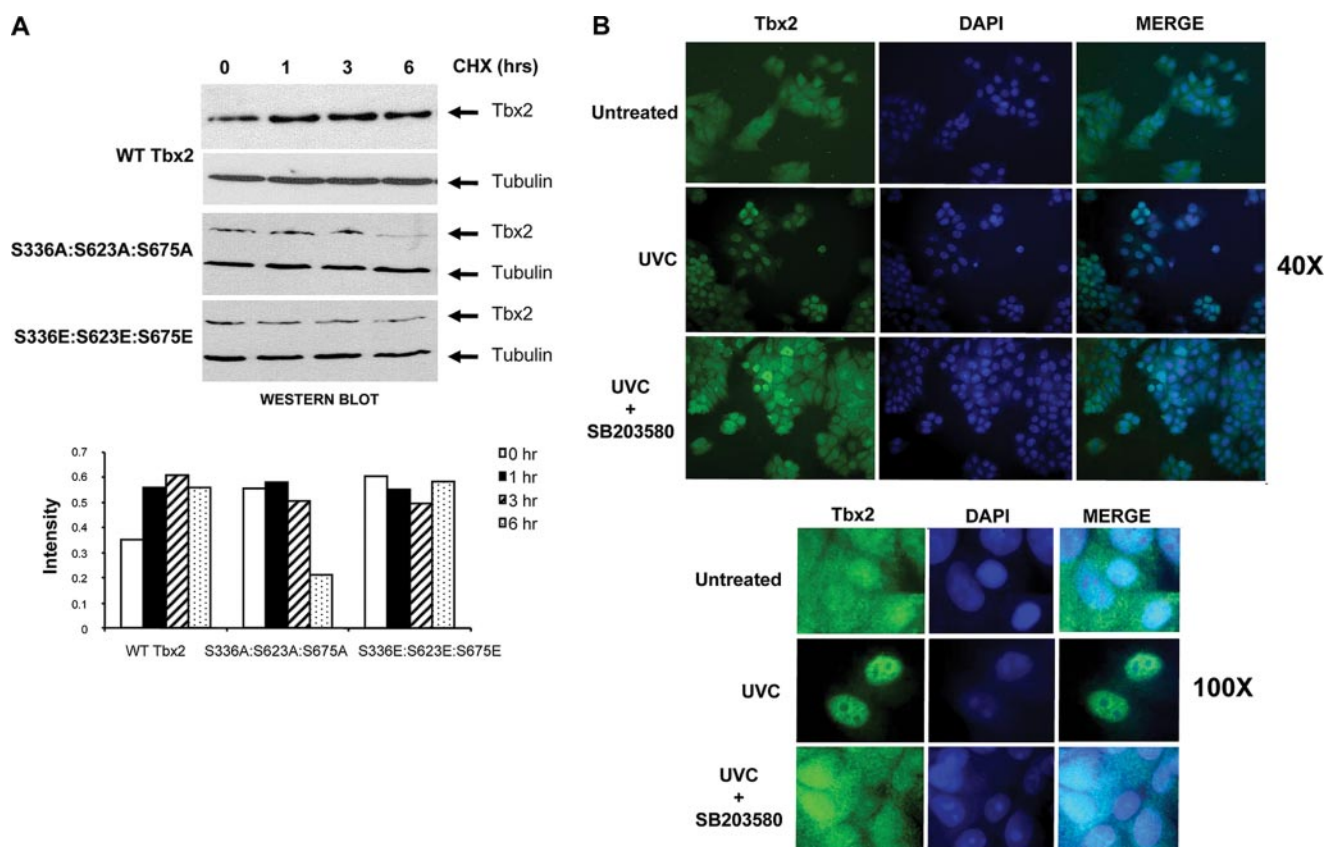


FIGURE 4. UVC-induced phosphorylation by p38 affects Tbx2 protein stability and subcellular localization. A, the Tbx2 S336A,S623A,S675A mutant displays a reduced half-life compared with wild-type Tbx2 and the Tbx2 S336E,S623E,S675E mutant. NIH 3T3 cells, transiently transfected with vectors expressing SV5 epitope-tagged Tbx2 proteins as indicated, were incubated 48 h post-transfection with 30 μ g/ml cycloheximide for the indicated times to block *de novo* protein synthesis. To accurately detect total levels of the Tbx2 protein as a single band, cell lysates were analyzed on a 10% SDS-PAGE and by Western blotting with anti-SV5 antibodies. The bar graph compares the intensity of the Tbx2 protein normalized to the loading control. B, p38 phosphorylation induces nuclear translocation of Tbx2. MCF-7 cells were treated with the p38 inhibitor SB203580 prior to UVC irradiation (100 J/m²) and then analyzed 1 h post-treatment by immunofluorescence using an anti-Tbx2 antibody. The upper and lower panels represent images captured at $\times 40$ and $\times 100$ magnifications, respectively.

gene (11, 13), and we therefore next tested the effect of UVC irradiation on this repression. Fig. 5C shows that whereas Tbx2 repressed the *p14^{ARF}* promoter by ~ 6 -fold, this repression was only marginally enhanced by UVC treatment. These results suggest that the enhancement of Tbx2 transcriptional activity by UVC irradiation is specific for selective targets. We next compared the transcriptional repression of the *p21* promoter by WT Tbx2 and the Tbx2 S336A,S623A,S675A mutant. The results in Fig. 5D indicate that whereas WT Tbx2 repressed the *p21* promoter, abolishing the p38 phosphorylation target sites led to an abrogation of this ability. Taken together, these results suggest that the identified p38 target sites do indeed play a role in regulating the transcriptional activity of Tbx2 on the *p21* promoter in response to UVC irradiation.

DISCUSSION

Despite the pivotal role that members of the T-box family play in a wide variety of developmental processes, very little is known about the biochemical pathways that regulate their levels and transcriptional activity. In view of the detrimental consequences resulting from altered levels of T-box proteins, as seen in both developmental disorders and in certain cancers, the need to identify such pathways is important. Here we provide several lines of evidence that Tbx2 is phosphorylated by

the p38 MAP kinase in response to stress induced by UVC irradiation. First, we show that phosphorylation of Tbx2 increases with increasing doses of UVC irradiation and that this is reversed in the presence of the p38 inhibitor SB203580. Second, we show that Tbx2 is phosphorylated both *in vitro* and *in vivo* at serine residues 336, 623, and 675. Importantly, we show that phosphorylation by the p38 MAP kinase has significant consequences for Tbx2 function. For example, the Tbx2 protein appeared less stable when the three p38 target serine residues were mutated to block phosphorylation. This effect of p38 phosphorylation on the stability of its substrates has been reported for the G₁ proteins p53 and cyclin D1. Whereas p38 phosphorylation of p53 led to increased protein stability, phosphorylation of cyclin D1 by this kinase resulted in ubiquitin-dependent degradation and consequently a G₁ arrest (43–45). Moreover, *Tbx2* mRNA levels also increased in response to UVC irradiation, but the mechanism(s) for this still need to be elucidated. It is tempting to speculate that the ubiquitous bHLH-LZ transcription factor USF-1 may be involved. Both Tbx2 and USF-1 are expressed in melanocytes, and USF-1 has been shown to play an important role in mediating the tanning response induced by UV irradiation. This mechanism involves phosphorylation of USF-1 by the p38 MAP kinase (46), result-

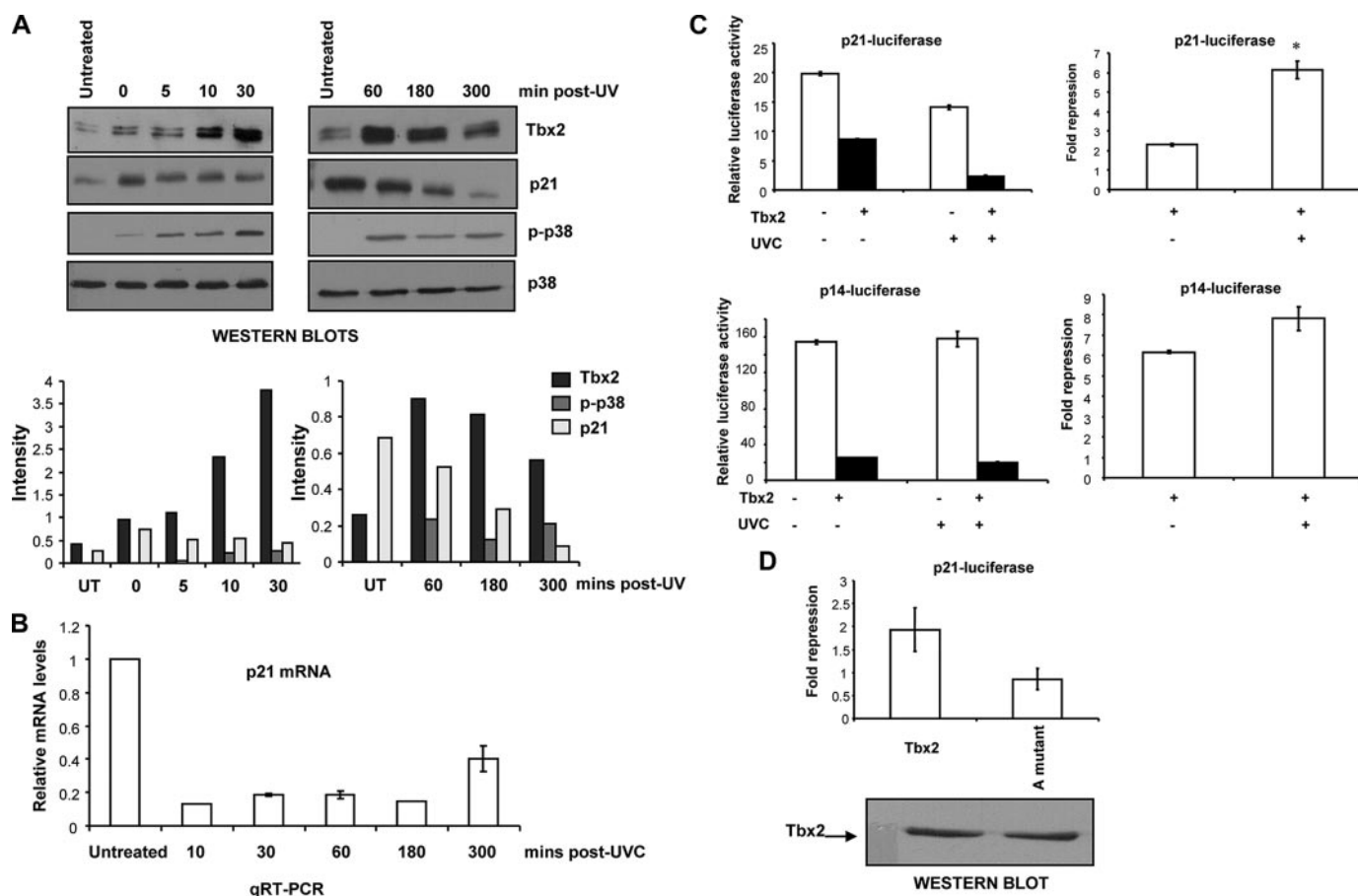


FIGURE 5. UVC-induced phosphorylation by p38 enhances the ability of Tbx2 to repress p21. A, inverse correlation between Tbx2 and p21 protein levels in response to UVC irradiation. Protein extracts (the same used in Fig. 2B) from MCF-7 cells at the indicated time points post-UVC irradiation (100 J/m²) were analyzed by 15% SDS-PAGE and Western blotting using an antibody to p21. The proteins extracted from 0 to 30 min and 60 to 300 min post-UVC treatment were analyzed on different gels. The results for Tbx2, p-p38, and total p38 are the same as shown in Fig. 2B. The bar graph compares the intensity of the Tbx2, p-p38, and p21 bands in each sample normalized to the p38 loading control. B, p21 mRNA levels are repressed in response to UVC irradiation. Total RNA was extracted from MCF-7 cells at the times indicated post-UVC irradiation. Quantitative real-time PCR was then performed on reverse-transcribed RNA using primers specific to p21, and mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase. Error bars represent standard deviations. C, UVC irradiation enhances the transcriptional repression of p21 by Tbx2. The p21 promoter-luciferase reporter (700 ng) was co-transfected into COS-7 cells either with the empty pCMV (200 ng) vector or a Tbx2 (200 ng) expression vector in the presence or absence of UVC (40 J/m²) and luciferase activity determined. Promoter activity is indicated as -fold repression, which represents the ratio of the luciferase activity generated by the pCMV empty vector (without Tbx2) to that obtained in the presence of pCMV-Tbx2. The above experiment was repeated under the same conditions using 700 ng of the p14^{luciferase} promoter-luciferase reporter. *, significant difference from the control (no UVC treatment) at *p* < 0.05. D, COS-7 cells were transfected as described in panel C with the Tbx2 expression vectors as indicated and luciferase activity determined. Western blotting shows equal expression of WT Tbx2 and the Tbx2 mutant in the luciferase assays. Error bars represent standard deviations.

ing in an increase of USF-1 binding to conserved E- and M-box motifs and activation of melanogenic target genes. The *Tbx2* promoter has been shown to possess an E-box (47), and it would be interesting to see if it is capable of responding to USF-1, particularly in response to UV irradiation.

Importantly, we show that Tbx2 is localized to both the cytoplasm and nucleus, but upon UVC irradiation phosphorylation of Tbx2 by the p38 MAP kinase led to the predominant nuclear localization of the protein. This result would suggest that the molecular activity of Tbx2 may be controlled, in part, by regulation of its subcellular localization. Interestingly, in *Caenorhabditis elegans*, *tbx-2* is involved in olfactory adaptation and is expressed in AWB, AWC, ASJ, and many pharyngeal neurons where the protein is mostly localized to the cytoplasm, suggesting that nuclear translocation may regulate its activity (48). There are several other lines of evidence to suggest that the transcriptional activity of T-box factors is regulated, in part, by the regulation of their subcellular localization. For example,

certain T-box family members have been found to possess nuclear localization signals (NLS), which may explain the shuttling of these proteins into the nucleus. In patients suffering from DiGeorge syndrome, the deletion of the NLS in Tbx1 has been shown to prevent the mutant protein from localizing to the nucleus, resulting in haploinsufficiency of the protein (49). Furthermore, deletion of the Tbx3 NLS, RREKRR, at amino acids 292–297 results in mislocalization of the protein to either perinuclear or cytoplasmic sites (9). Given the high degree of homology between Tbx2 and Tbx3, we compared the mouse and human Tbx2 protein sequences and found that the Tbx3 NLS was conserved at exactly the same position in the Tbx2 protein. It will be interesting to investigate whether this NLS plays a role in shuttling Tbx2 into the nucleus and whether phosphorylation of the protein by the p38 MAP kinase can regulate this process or whether the latter regulates Tbx2 nuclear localization through a mechanism independent of the NLS.

The effect of UV-induced p38 phosphorylation on Tbx2 protein levels and nuclear localization would suggest a role for this transcription factor in the stress signaling pathway. There has been a lot of controversy in the literature regarding the role and regulation of the Tbx2 target *p21* in response to UV-induced DNA damage. The present study shows that in response to UVC irradiation, p21 protein levels are rapidly increased but decrease equally rapidly post-UVC treatment. This is consistent with reports that in response to DNA damage, p21 levels need to increase in order to establish a senescence-like growth arrest and subsequently have to be down-regulated in order for DNA repair to occur (34–36). One mechanism by which p21 is down-regulated in response to UV-induced DNA damage has been shown to involve ubiquitin-dependent proteolysis (36). Here we show that *p21* mRNA levels also decrease in response to UV irradiation, which is consistent with a study by Wang *et al.* (37) that demonstrates that both p21 protein and mRNA levels are down-regulated post-UV irradiation of MCF-7 cells.

Interestingly, we show that UVC irradiation leads to the enhanced transcriptional repression of Tbx2 on the *p21* promoter, which raises the possibility that Tbx2 may be required to inhibit *p21* in the UV-induced DNA-damaged pathway. This is a particularly attractive possibility because to our knowledge no other mechanism has yet been identified to explain how *p21* mRNA levels are down-regulated following UV irradiation.

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