JNK1 Physically Interacts with WW Domain-containing Oxidoreductase (WOX1) and Inhibits WOX1-mediated Apoptosis*

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Nan-Shan Chang‡, Joan Doherty§, and Amy Ensign¶
From the Guthrie Research Institute, Laboratory of Molecular Immunology, Guthrie Medical Center, Sayre, Pennsylvania 18840

Transient activation of c-Jun N-terminal kinase (JNK) promotes cell survival, whereas persistent JNK activation induces apoptosis. Bovine testicular hyaluronidase PH-20 activates JNK1 and protects L929 fibroblasts from staurosporine-mediated cell death. PH-20 also induces the expression of a p53-interacting WW domain-containing oxidoreductase (WOX1, also known as WWOX or FOR) in these cells. WOX1 enhances the cytotonic function of tumor necrosis factor and mediates apoptosis synergistically with p53. Thus, the activated JNK1 is likely to counteract WOX1 in mediating apoptosis. Here it is demonstrated that ectopic JNK1 inhibited WOX1-mediated apoptosis of L929 fibroblasts, monocytic U937 cells, and other cell types. Also, JNK1 blocked WOX1 prevention of cell cycle progression. By stimulating cells with anisomycin or UV light, JNK1 became activated, and WOX1 was phosphorylated at Tyr33. The activated JNK1 physically interacted with the phosphorylated WOX1, as determined by co-immunoprecipitation. Alteration of Tyr33 to Arg33 in WOX1 abrogated its binding interaction with JNK1 and its activity in mediating cell death, indicating that Tyr33 phosphorylation is needed to activate WOX1. A dominant negative WOX1 was developed and shown to block p53-mediated apoptosis and anisomycin-mediated WOX1 phosphorylation but could not inhibit JNK1 activation. This mutant protein bound p53 but could not interact with JNK1, as determined in yeast two-hybrid analysis. Taken together, phosphorylation of JNK1 and WOX1 is necessary for their physical interaction and functional antagonism.

WW domain-containing oxidoreductase (WOX1, also known as WWOX or FOR) is a proapoptotic protein and is known to enhance the cytotonic function of tumor necrosis factor (TNF) as well as overexpressed TNF receptor-associated death domain protein (1–3). Enhancement of TNF cytotoxicity by WOX1 is due, in part, to its significant down-regulation of the apoptosis inhibitors Bcl-2 and Bcl-xL but...
nisms of JNK1 and WOX1 binding interaction and the functional significance of this interaction were investigated.

EXPERIMENTAL PROCEDURES

Cell Lines—Cell lines used in these studies were murine L929 fibroblasts, human monocyte U937 cells, human prostate DU145 cells, and human neuroblastoma SK-N-SH cells. These cells were grown according to the instructions of the ATCC (Manassas, VA).

cDNA Expression Constructs—Expression constructs for the murine wild type Wox1 cDNA (sense and antisense directions) and a nuclear localization mutant Wox1mtNLS were established as previously described (1). The constructs were made in pEGFP-C1 vector (Clontech, Palo Alto, CA). The expressed proteins were tagged with an N-terminal enhanced green fluorescent protein.

The FLAG-tagged wild type JNK1 (pDNA3-Flag-JNK1) and dominant negative JNK1 (DN-JNK1 or pDNA3-Flag-JNK1apf) expression constructs were kind gifts of Dr. Roger Davis (University of Massachusetts Medical Center, Worcester, MA). The HA-tagged wild type JNK2 and JNK3 (peVRFO-HA-JNK2 and peVRFO-HA-SAPKβ) and dominant negative JNK2 and JNK3 (peVRFO-HA-JNK2-K55R and peVRFO-HA-SAPKβ-K55R or DN-JNK2 and DN-JNK3) were kind gifts of Dr. Michael Kracht (Medical School Hannover, Hannover, Germany).

Cell Cycle Analysis and Cell Death Assays—Where indicated, L929 cells were co-transfected with the wild type or DN-JNK constructs and Bovine testicular hyaluronidase PH-20 (100 units/ml; Sigma) for 24 h. The cell layers, reaching 80–90% confluency in 48 h, were gently washed once with phosphate-buffered saline, then harvested by repeat pipetting, precipitated by centrifugation (500 g), and then stained with propidium iodide. The cellular DNA content was analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

In some experiments, L929 and other cells were electroporated with WOX1 and/or JNK1 constructs and cultured for 24 h (in eight replicates). The extent of cell death was examined by crystal violet staining (1) or by Promega’s MTS proliferation assay (Promega, Madison, WI).

Antibodies, Western Blotting, and Co-immunoprecipitation—Anti- body against the N-terminal amino acid sequence of WOX1 was generated as previously described (1). Commercial antibodies used in the Western blotting were against JNK1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Co-immunoprecipitation was performed as previously described (1, 20). Briefly, U937, L929, or SK-N-SH cells were treated with anisomycin (100 μM) for various indicated times to activate JNK1. The cytosolic and nuclear fractions of these cells were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Endogenous JNK1 was precipitated by the specific anti-JNK1 antibody (Santa Cruz Biotechnology) and protein A-agarose beads (Pierce), followed by separation in SDS-PAGE and detection by immunoblotting. Co-expression of WOX1 in the precipitates was examined similarly by immunoblotting. Alternatively, immunoprecipitation was performed using anti-WOX1 or p53 (Santa Cruz Biotechnology) antibody.

Generation of Antibody against a Phospho-Tyr33 WOX1 Peptide—Generation of antibody against synthetic peptides in rabbits was performed as described (1). A synthetic peptide NH2-CKDGWVpYY-ANHTEEK-COOH, with tyrosine 33 phosphorylation (pY), was made (Genemed Synthesis, South San Francisco, CA). A control peptide without phosphorylation was made also. The N-terminal cysteine was added to allow the peptide to covalently conjugate to keyhole limpet hemocyanin for immunization or to SulfoLink beads for antibody purification (Pierce). The peptide sequence is located at the first WW domain of murine WOX1 (acidic amino acids 28–38). Antibody generation was performed using the EZ antibody production and purification kit (Pierce). The antisera was preadsorbed with a column covalently conjugated with the control peptide, and then the antibody was purified by affinity chromatography using a column immobilized with the synthetic phosphopeptide.

Yeast Two-hybrid Interactions and Constructs—The Ras rescue-based CytoTrap yeast two-hybrid system (Stratagene, La Jolla, CA) for protein binding interaction was used (1, 20). Briefly, binding of a cytosolic Sos-targeted bait protein to a cell membrane-anchored target protein (tagged with a myristoylation signal) results in activation of the

FIG. 1. Hyaluronidase PH-20 promoted L929 fibroblast progression toward S and G2/M phases in the cell cycle. Freshly harvested L929 cells were grown overnight and treated with bovine testicular hyaluronidase PH-20 for 24 h, followed by determining cellular DNA content using flow cytometry (see “Experimental Procedures”). PH-20 increased cellular distribution in the S and G2/M phases and a reduced distribution in the G0/G1 phase. No PH-20-mediated cell death or apoptosis was observed (sub-G1/A1 phase). A representative set of data from two experiments is shown.

Ras signaling pathway in yeast. Activation of the Ras signaling pathway allows mutant yeast Cdc25H to grow in 37 °C using a selective agarose plate containing galactose. Without binding, yeast cells fail to grow at 37 °C. The human JNK1 cDNA was constructed in a pMyr vector and utilized as target. The bait constructs in a pSos vector for binding interactions with JNK1 were murine Wox1 (1), human WOX3 (1), and the first WW domain of WOX1 (1). Additional constructs for control experiments were human p53 in pMyr, MafB in both pMyr and pSos, collagenase in pSos, and lamin C in pMyr (1, 20).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Tyr33 in WOX3 was mutated to Arg33 (designated Y33R) using the following primers by PCR: forward, 5′-AAAGCCGCCTGGTTCTG-ATACGCCAACACTACACCC; reverse, 5′-GGTTGGATGGCAGTGAAACCCGTTCT; Lys36 and Asp39 in Wox1 were mutated to Thr36 and Val39 (designated K36T/D39V); forward, 5′-AGAGAACACACGCGTGGCGTGGTGAATCT; reverse, 5′-AGTACACACCGCGTGGTTGTGCCT.

Statistical Analysis and Data Presentation—Where indicated, Student’s t tests were performed to analyze the experimental data (using the Microsoft Excel program). All experiments indicated above were performed 2–5 times. In most cases, a representative set of data is shown.

RESULTS

Dominant Negative JNK1 Induces Apoptosis in Murine L929 Fibroblasts—Bovine testicular hyaluronidase PH-20 activates JNK1 in L929 fibroblasts (19). PH-20 promotes the proliferation of L929 cells (4). By measuring cellular DNA content using flow cytometry, PH-20 was shown to increase cell cycle progression toward the S and G2/M phases during 24-h exposure (Fig. 1). No PH-20-mediated apoptosis or cell death was observed (Sub G0/G1) (Fig. 1).

To examine the possible role of JNK1 in the PH-20-mediated growth regulation, L929 cells were electroporated with a wild type human JNK1 or a dominant negative JNK1 construct. The cells were cultured overnight, exposed to PH-20 for 24 h, and then subjected to cell cycle analysis by flow cytometry. In controls, the cells were electroporated with nothing (medium only) and treated similarly. The results showed that ectopic wild type JNK1 did not induce apoptosis or death of L929 cells (Fig. 2). Expression of these JNK1-expressing cells to PH-20 could not induce apoptosis (Fig. 2). In contrast, dominant-negative JNK1 significantly induced L929 cell death (56.4%), and PH-20 did not significantly increase the extent of cell death (60.9%) (Fig. 2).
In comparison, ectopic human wild-type JNK2 and JNK3 mediated apoptosis or death of L929 cells by \( \sim 30\% \), and PH-20 limitedly increased the cell death (\( \sim 40\% \)) (Fig. 2). In contrast, dominant negative JNK2 and JNK3 significantly mediated cell death up to \( \sim 60\% - 70\% \), and PH-20 slightly increased the cell death (Fig. 2).

**JNK1 Inhibition of WOX1-mediated Cell Death—PH-20 induces the expression of proapoptotic p53 and WOX1 in L929 cells, thereby increasing cellular sensitivity to the cytotoxic effect of TNF (1). Nonetheless, PH-20 also activates JNK1 in these cells (19). Here, we examined whether JNK1 inhibited WOX1-mediated apoptosis of L929 cells. In agreement with our previous observations (1), electroporation of L929 cells with a wild-type WOX1 expression construct (tagged with enhanced green fluorescent protein) resulted in cell death (Fig. 3). The extent of cell death was determined by the MTS assay. Similar results were observed by staining the cells with crystal violet (data not shown). The extent of WOX1 protein expression was \( \sim 60\% - 75\% \) in L929 cells, as determined by counting cells under fluorescence microscopy. When L929 cells were cotransfected with both wild type JNK1 and WOX1 constructs, and JNK1 blocked WOX1-mediated cell death (Fig. 3). In contrast, WOX1mtNLS, which possesses a mutated sequence at the NLS, had a significantly reduced activity in mediating cell death (Fig. 3). When in combination, JNK1 and WOX1mtNLS could not induce cell death (Fig. 3). The mutation in WOX1mtNLS prevents nuclear translocation of this protein (1). In agreement with our previous observations (1), expression of antisense WOX1 mRNA in L929 cells failed to result in cell death, and JNK1 had no effect in mediating cell death during cotransfection (Fig. 3). Together, WOX1 mediates apoptosis in the nucleus, and JNK1 blocks the WOX1-mediated cell death at the nuclear level.

**Fig. 2.** Dominant-negative JNK1 mediated apoptosis of L929 cells. L929 cells were electroporated with a wild type or a dominant-negative (dn) JNK1 (B), JNK2 (C), or JNK3 (D) construct and cultured overnight. The cells were treated with or without hyaluronidase PH-20 (100 units/ml) for 24 h and then subjected to cell cycle analysis by flow cytometry. In controls, the cells were electroporated with nothing or medium (mock) and treated similarly (A). The wild type JNK1 did not induce apoptosis of L929 cells. In contrast, dominant-negative JNK1 significantly induced L929 cell death, and hyaluronidase (HAase) slightly increased the extent of cell death. In comparison, wild type JNK2 and JNK3 mediated L929 cell death by \( \sim 30\% \), and the dominant negatives of these proteins significantly caused cell death (\( \sim 60\% - 70\% \)). A representative set of data from two experiments is shown.

**Fig. 3.** JNK1 inhibited WOX1-mediated death of L929 cells. L929 cells were transfected with JNK1 and/or various WOX1 constructs by electroporation and cultured for 24 h. The extent of cell death was measured by the MTS assay. Wild type WOX1 mediated L929 cell death (\( \sim 40\% \)), which was blocked by JNK1 (\( \sim 20\% \) death) (\( p < 0.0001, n = 8 \), Student’s t test). JNK1 alone slightly increased cell proliferation (\( 0.001 < p < 0.05, n = 8 \), Student’s t test). JNK1 and the antisense WOX1, in combination, could not significantly mediate cell death (\( p > 0.1, n = 8 \), Student’s t test). A representative set of data from two experiments is shown.
mediated reduction of cellular distribution in the G0/G1, S, and G2/M phases. JNK1 alone had litter or no effect on the cell cycle progression, whereas it prevented WOX1-mediated apoptosis and inhibition of the cell cycle progression. A representative set of data from two experiments is shown.

**JNK1 Blocks WOX1-mediated Inhibition of Cell Cycle Progression in U937 Cells**—To further verify the above observations, we examined the ability of wild type JNK1 in blocking apoptosis of monocytic U937 cells by WOX1. U937 cells were electroporated with various amounts of WOX1 in the presence or absence of wild type JNK1. The cells were cultured 48 h and subjected to cell cycle analysis by flow cytometry. The results showed that WOX1-mediated apoptosis was suppressed by JNK1 (see the sub-G0/G1 phase) (Fig. 4). Additionally, WOX1-mediated reduction of cellular distribution in the G0/G1, S, and G2/M phases was reversed by JNK1 (Fig. 4). Thus, our data strongly support the protective role of JNK1 in blocking WOX1-mediated cell death.

**WOX1 Physically Interacts with JNK1**—We investigated whether JNK1 physically interacts with WOX1, thereby blocking WOX1-mediated apoptosis. As determined by yeast two-hybrid analysis (1, 20), the wild type human JNK1 interacted with murine WOX1 and human WOX3 in vivo, as evidenced by the growth of mutant Cdc25H yeast at 37 °C in the presence of galactose. In negative controls, no binding interactions were observed when testing empty pSos vector versus empty pMyr vector or collagenase versus lamin C. The wild type human JNK1 physically interacted with murine WOX1 and human WOX3. Human WOX3 has a deletion of its ADH domain at the C-terminus, whereas its N-terminal WW domain region is highly homologous to that of murine WOX1 (1). The first WW domain interacted with JNK1. Alteration of a conserved phosphorylation site at Tyr33 to Arg33 at the first WW domain in WOX3 was performed. This mutant, designated WOX3(Y33R), could not bind JNK1. A representative set of data from three experiments is shown.

**Anisomycin Activates JNK1 and Mediates Tyr33 Phosphorylation in WOX1 and Their Binding Interactions**—To further verify the above observations, U937 cells were treated with anisomycin to activate JNK1. Anisomycin is a potent activator of mutant Cdc25H yeast at 37 °C (Fig. 5). In negative controls, no binding interactions were observed when testing empty pSos vector versus empty pMyr vector, or collagenase versus lamin C (Fig. 5).

Similarly, exposure of SK-N-SH cells to anisomycin for 30 min also resulted in an increased binding interaction between JNK1 and WOX1, as determined using anti-WOX1 antibody in co-immunoprecipitation (Fig. 6B). Similar results were observed when L929 cells were treated with anisomycin, or the above cells were exposed to UV light (data not shown).

To further verify Tyr33 phosphorylation in WOX1, we synthesized a peptide containing phospho-Tyr33 for immunization in rabbits. Anisomycin rapidly increased Tyr33 phosphorylation in WOX1, as well as JNK1 phosphorylation, in SK-N-SH cells (Fig. 7A). Similar results were observed using L929 and U937 cells (data not shown).

Exposure of L929 cells to UV light, followed by growing for 1 h, resulted in increased p53 expression (Fig. 7B). p53 is known to interact with WOX1 (1) and JNK1 (21). Co-immunoprecipitation of p53 with a specific antibody resulted in the
presence of p53, JNK1, and phosphorylated WOX1 in the precipitates (Fig. 7B).

**Dominant Negative WOX1 Abolishes WOX1 Phosphorylation and p53 Apoptosis but Not JNK1 Activation**—We developed a dominant-negative WOX1. Lys28 and Asp29 in Wox1 were mutated to Thr28 and Val29, respectively. These residues are located at the first WW domain. In agreement with our previous observations (1), p53 and WOX1 mediated U937 cell death in a synergistic manner, and antisense WOX1 abolished p53-mediated cell death (Fig. 8A). The mutated full-length WOX1 (WOX1mt) abolished p53 apoptosis (Fig. 8A). Similarly, the mutated WW domain region (WOX1wwmt) also abolished p53 apoptosis (Fig. 8A). These data support the dominant negative effect of the generated WOX1 mutant.

Anisomycin-mediated WOX1 phosphorylation in Tyr33 was abolished by the dominant negative WOX1 (Fig. 8B). SK-N-SH cells were transfected with a GFP-tagged WOX1wwmt or a control GFP construct by electroporation. The cells were cultured for 48 h and then treated with anisomycin for 30 min. The dominant negative WOX1wwmt abolished anisomycin-mediated WOX1 phosphorylation in Tyr33 (Fig. 8B). However, WOX1wwmt failed to inhibit anisomycin-induced JNK1 activation (Fig. 8B).

By yeast two-hybrid analysis, both WOX1mt and WOX1wwmt physically interacted with p53, whereas WOX1wwmt could not bind JNK1 (Fig. 8C). Apparently, these dominant negative WOX1 proteins have an altered conformation, and inhibition of p53 apoptosis by the dominant negatives is due to a direct binding interaction. In contrast, these dominant negatives failed to bind and prevent JNK1 activation.

**Tyr33 Phosphorylation Is Necessary for WOX1 Apoptotic Function—Overexpression of the WW domain region of WOX1, designated WOX1ww, induces cell death (1). Alteration of Tyr33 to Arg33 was performed in WOX1ww, and the resulting mutant, WOX1ww(Y33R), had a significantly reduced activity (∼50% decrease) in mediating death of L929 and Du145 cells (Fig. 9). The findings were also observed using U937, SK-N-SH, and other types of cells (data not shown). JNK1 significantly blocked WOX1ww-mediated death of L929 and Du145 cells by 30–50% (Fig. 9). Interestingly, JNK1 increased the apoptotic function of WOX1ww(Y33R) mutant in L929 but not in Du145 cells (Fig. 9).

**DISCUSSION**

In this study, we demonstrated that stress stimuli such as anisomycin and UV light mediated WOX1 phosphorylation at Tyr33. The phosphorylation is essential for functional activation of WOX1 in mediating apoptosis. Alteration of Tyr33 to Arg33 reduced the WOX1 activity. In agreement with our previous observations (1), WOX1-mediated apoptosis occurs at the nuclear level. Mutation of the nuclear localization signal reduced the WOX1 apoptotic function.

We determined that ectopic JNK1 counteracted WOX1-mediated apoptosis, as well as cell cycle progression. By stimulating cells with anisomycin, JNK1 became activated and physically interacted with the Tyr33-phosphorylated WOX1, as determined by co-immunoprecipitation. A dominant negative WOX1 blocked p53-mediated apoptosis and anisomycin-mediated WOX1 phosphorylation but failed to inhibit JNK1 activation. This dominant negative WOX1 bound p53 but could not interact with JNK1, as determined in yeast two-hybrid analy-
An additional conserved phosphorylation site in WOX1 is Tyr\(^{61}\). Alteration of this residue to Arg\(^{61}\) results in abrogation of the binding interaction between WOX1 and p53 in yeast.\(^2\) Thus, it is likely that both Tyr\(^{53}\) and Tyr\(^{61}\) in WOX1 are phosphorylated when cells are exposed to stress stimuli. Functional significance of Tyr\(^{61}\) phosphorylation in WOX1 is being determined in this laboratory.

The functional property of WOX3 in vivo and in vitro is unknown. Whether WOX3 expression is increased during stress response is unknown. By RT-PCR and Northern blot, Driouch demonstrated that about 50% of breast tumors have overexpressed WOX1 and WOX3.\(^2,22\) WOX3 appears to have a tumor suppressor function.\(^10,11\) Presumably, the overexpressed WOX3 enhances cancer growth by neutralizing the WOX1 function.

In addition to activate JNK1, anisomycin inhibits protein synthesis and mediates apoptosis.\(^{23,24}\) Anisomycin-mediated apoptosis of leukemia HL-60 cells depends upon JNK1.\(^24\) Most interestingly, anisomycin-activated JNK1 phosphorylates Bcl-2, thereby increasing cell survival.\(^{25}\) Previously, we found that when L929 cells were exposed to anisomycin for less than 1 h, these cells became resistant to TNF cytotoxicity\(^\text{(17)}\). We determined that IxB\(\alpha\), the inhibitor of NF-\(\kappa\)B, maintains the basal level of JNK1 activation and regulates JNK1-mediated TNF resistance.\(^\text{(17)}\) However, whether this protective event is related to JNK1-mediated Bcl-2 phosphorylation remains to be determined.

Hyaluronidase PH-20 activates JNK1, and the activation is necessary to prevent L929 cell death by various anticancer drugs such as staurosporine, daunorubicin, and doxorubicin.\(^\text{(19)}\) In contrast, PH-20 increases L929 cell sensitivity to TNF cytotoxicity by increasing the expression of WOX1.\(^\text{(1)}\) Apparently, different mechanisms are involved in the regulation of cellular sensitivity to TNF and anticancer drugs. Indeed, we determined that ectopic JNK1 and exogenous PH-20 act synergistically in blocking L929 cell death by WOX1 in the presence of staurosporine, daunorubicin, or doxorubicin.\(^2\) We believe that PH-20 activates both endogenous and ectopic JNK1 that block the cell death. Whether JNK1 mediates Bcl-2 phosphorylation in these tested cells is unknown. The hyaluronidase \(HYAL1\) gene, also known as \(LUCA1\), is a candidate tumor suppressor (for a review, see Ref. \(\text{26}\)). Inactivation of the \(HYAL1\) gene by aberrant splicing of pre-mRNA has been shown in head and neck squamous cell carcinomas (27). Nonetheless, overexpressed hyaluronidases are frequently associated with metastatic cancers (28–30). Also, plasma \(HYAL1\) is capable of promoting tumor cell cycling (31). PH-20 enhances cell proliferation \(\text{(4)}\). Here, we demonstrated that PH-20 increased cell cycle progression in L929 cells. PH-20-activated JNK1 is essential for cell survival, since dominant negative JNK1 mediated apoptosis in L929 cells.

UV light up-regulates and activates p53 (32) and also activates JNK1.\(^\text{(12)}\) Co-immunoprecipitation of the anisomycin-treated cells with anti-p53 resulted in the presence of p53, JNK1, and WOX1 in the precipitate. The observation suggests the presence of a p53-WOX1-JNK1 complex during stress response. Most recently, we determined that a portion of cytosolic IxB\(\alpha\) binds p53 in resting cells and the complex dissociates in response to apoptotic stress, hypoxia, DNA damage, and tumor growth factor-\(\beta\)-mediated growth suppression.\(^\text{(20)}\) The dissociation allows p53 nuclear translocation. Alternatively, when dissociated from IxB\(\alpha\), p53 may complex with WOX1 and JNK1.

Although transient JNK1 activation provides a signal for cell

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\(\text{a}\) N.-S. Chang, J. Doherty, and A. Ensign, unpublished results.
Fig. 9. Tyr33 phosphorylation is necessary for WOX1 apoptotic function. Alteration of Tyr33 to Arg33 reduced WOX1ww-mediated death of L929 and Du145 death by ~50% (p < 0.0001, Student’s t test). JNK1 significantly blocked WOX1ww-mediated death of L929 cells (p < 0.01, Student’s t test; see the representative crystal violet stain). Interestingly, JNK1 increased the apoptotic function of WOX1ww(Y33R) mutant in L929 but not in Du145 cells. Experiments were done in eight replicates for L929 cells and four replicates for Du145 cells (mean ± S.D.). A representative set of data from two experiments is shown. The extent of cell death was determined by crystal violet stain.

JNK1 and WOX1 Interactions

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