

WISP-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase

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WISP-1 (Wnt-1-induced secreted protein) was identified as an oncogene regulated by the Wnt-1- β -catenin pathway. *WISP-1* belongs to the CCN family of growth factors, which are cysteine-rich, heparin-binding, secreted proteins associated with the extracellular matrix, and can interact with cellular integrins. Expression of *WISP-1* in some cells results in transformation and tumorigenesis. Here it is shown that *WISP-1* can activate the antiapoptotic Akt/PKB signaling pathway. It also is demonstrated that *WISP-1* can prevent cells from undergoing apoptosis following DNA damage through inhibition of the mitochondrial release of cytochrome *c* and up-regulation of antiapoptotic Bcl-X_L. Furthermore, the results show that *WISP-1* protects cells from p53-dependent cell death, but not Fas-ligand activated cell death, suggesting that there may be cross talk between the tumor suppressor protein p53 and *WISP-1* signaling pathways. *WISP-1* acts to block cell death at a late stage in the p53-mediated apoptosis pathway.

[Key Words: *WISP-1*; Akt; apoptosis; p53; DNA damage]

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WISP-1 (Wnt-1-induced secreted protein) was first identified as a downstream target gene of the Wnt-1- β -catenin signaling pathway using subtractive hybridization between the mouse mammary epithelial cell line C57MG and *Wnt-1*-expressing C57MG cells (Pennica et al. 1998). The *WISP-1* gene is located on human chromosome 8q24.1-8q24.3 and encodes a protein of 367 amino acids with four potential N-linked glycosylation sites. *WISP-1* genomic DNA is amplified in some human colon cancer cell lines, and its mRNA is overexpressed (2- to >30-fold) in 84% of the human colon tumors examined as compared with patient-matched adjacent mucosa (Pennica et al. 1998). Recent studies have demonstrated that *WISP-1* can accelerate cell proliferation and can morphologically transform cells. *WISP-1* overexpressing cells, when injected into nude mice, form tumors (Xu et al. 2000).

WISP-1 is a member of the CCN family of growth factors, which are secreted, heparin-binding, cysteine-rich, and extracellular matrix-associated proteins, including CTGF (connective tissue growth factor), Cyr-61 (cysteine-rich 61), nov (neuroblastoma overexpressed), Cop-1/*WISP-2*, and *WISP-3* (Brigstock 1999). These highly conserved proteins share four cysteine-rich modular domains with sequence similarities to insulin-like growth

factor-binding proteins (IGFBP), von Willebrand factor (VWC), thrombospondin (TSP), and growth factor cysteine knots (CT) (Lau and Lam 1999).

Two members of the CCN family, CTGF and Cyr61, signal through the integrin receptors $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, and $\alpha_6\beta_1$ (Kireeva et al. 1998; Babic et al. 1999; Jedsaday-anmata et al. 1999; Chen et al. 2001; Grzeszkiewicz et al. 2001). Integrin receptors have been shown to play a role in cell migration and proliferation, and can affect cancer cell invasion and growth (Giancotti and Ruoslahti 1999; Ruoslahti 1999). It has been reported that integrins can signal through integrin-linked kinase (ILK) (Dedhar et al. 1999) or focal adhesion kinase (FAK) (Ruoslahti 1999) to activate downstream targets, such as Akt (Delcommenne et al. 1998; Sonoda et al. 1999; Persad et al. 2001). Akt, also known as protein kinase B (PKB), is a serine/threonine kinase, which is phosphorylated and activated by a variety of growth and survival signals including insulin, epidermal growth-factor receptor (EGFR), platelet-derived growth factor (PDGF), Ras, and Her2/neu. The Akt signaling pathway is negatively regulated by the PTEN tumor suppressor lipid phosphatase (Verdu et al. 1999; Di Cristofano and Pandolfi 2000). Activation of Akt promotes cell survival by inhibiting apoptosis through several downstream effectors. Bad, for example, which is normally a proapoptotic bcl-2 family member, releases the antiapoptotic Bcl-x following phosphorylation by Akt (Zha et al. 1996; Datta et al. 1997). Caspase-9, an effector protein of the intrinsic cell-death machinery, can also be inactivated following Akt phosphoryla-

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tion (Cardone et al. 1998). The forkhead family of transcription factors, which normally function to up-regulate the expression of proapoptotic proteins such as Fas ligand, is translocated from the nucleus to the cytoplasm following its phosphorylation by Akt (Brunet et al. 1999; Kops et al. 1999). Finally, the universal cell-cycle inhibitor p21^{Cip1/WAF1}, has also been shown to relocalize into the cytoplasm following Akt phosphorylation to promote cell growth in *Her2/neu*-overexpressing cells and breast cancers (Zhou et al. 2001).

Most, if not all, apoptotic cell death signaling pathways ultimately lead to the activation of members of the caspase family of proteases. In general, activation of the caspase cascade requires both initiator caspases, such as caspase-8 and caspase-9, and effector caspases such as caspase-3 (Cryns and Yuan 1998; Nunez et al. 1998). There are two well-described pathways by which initiator caspases become activated. One of these pathways initiates at the plasma membrane where death receptor (such as Fas) and ligand binding results in activation of caspase-8 (Ashkenazi and Dixit 1998). In the second apoptotic pathway, cellular stress such as DNA damage causes the release of cytochrome *c* from mitochondria. Cytochrome *c*, in association with a cytoplasmic protein known as Apaf-1 (apoptotic protease-activating factor 1), recruits caspase-9 and leads to caspase-9 activation (Hu et al. 1999). The active caspase-9, in turn, can process procaspase-3, and the activated caspase-3 then cleaves several key cellular substrates such as PARP and DFF45, leading to apoptosis (Zou et al. 1997). Recently it was shown that, in irradiation- or oncogene-induced p53-dependent apoptosis, Apaf-1 and caspase-9 were essential downstream targets of p53 (Soengas et al. 1999).

The p53 tumor-suppressor protein has at least two roles in preventing cancers: cell cycle arrest in G₁, which allows time for the repair of damaged DNA, or apoptosis, which eliminates cells with damaged genomes (Ko and Prives 1996; Levine 1997). p53 can be activated by many types of stress, including DNA damage, oncogene activation, hypoxia, and loss of normal cell-cell contacts (Bates and Vousden 1999; Prives and Hall 1999), and the activation of p53-dependent apoptosis can contribute to the inhibition of cancer development at several stages during tumorigenesis (Lowe and Lin 2000). It has been well documented that the transcriptional activity of p53 is important in initiating apoptosis through such genes as *Bax* (Miyashita and Reed 1995), *Noxa* (E. Oda et al. 2000), *p53AIP1* (K. Oda et al. 2000), *KILLER/DR5* (Wu et al. 2000), *PERP* (Attardi et al. 2000), *PIDD* (Lin et al. 2000), and *PUMA* (Nakano and Vousden 2001; Yu et al. 2001). So far, it seems that there is no single gene that is the principal mediator of the p53-dependent apoptotic signal, and it has been suggested that different sets of p53 downstream target genes may be regulated coordinately dependent on the nature of the apoptotic stimulus (Zhao et al. 2000).

Recent results showed that overexpression of *WISP-1* induced accelerated growth, morphological transformation of NRK-49F fibroblastic cells, and conferred tumorigenic potential on these cells (Xu et al. 2000). However,

the mechanism by which *WISP-1* promotes tumorigenicity is still unknown. In this study, the hypothesis that *WISP-1* promotes tumorigenicity by suppression of p53-regulated apoptotic pathway was tested. Evidence is provided that *WISP-1* signaling inhibits cell death following DNA damage by activation of the prosurvival Akt pathway and the antiapoptotic Bcl-X_L, and by inhibition of the mitochondrial release of cytochrome *c*. Moreover, we find that *WISP-1* signaling acts only against p53-dependent apoptosis because *WISP-1* does not inhibit the cell death induced by DNA damage in p53-null cells. These studies suggest that *WISP-1* promotes tumorigenicity not only by accelerating cell proliferation but also by the inhibition of programmed cell death.

Results

WISP-1 induces phosphorylation and activation of Akt

Akt promotes cell survival by inhibiting apoptosis via several possible downstream effectors, including phosphorylation and inactivation of Bad (Zha et al. 1996; Datta et al. 1997), inactivation of caspase-9 (Cardone et al. 1998) and repression of the forkhead transcription factor (Brunet et al. 1999). Because *WISP-1* might bind to integrins like the other members of the CCN family, and because it has been shown that integrin engagement stimulates ILK/FAK activity leading to the activation of Akt (Delcommenne et al. 1998), we sought to determine whether *WISP-1* is upstream of Akt, and may regulate its phosphorylation and activation. Because phosphorylation of Akt on Ser 473 is required for its activation, an antibody specific to phosphorylated Ser 473 was used to detect Akt activation by *WISP-1*. Serum starved normal rat kidney fibroblasts (NRK49F) were treated with either mock- or *WISP-1*-conditioned medium collected from NRK49F cells that express either no exogenous proteins (an empty vector) or human *WISP-1*, respectively, or with serum, which is known to activate Akt. Phosphorylation of Akt was detected by Western blotting of whole-cell extracts with a phosphorylated Ser 473-specific antibody. As shown in Figure 1A, Akt was activated by *WISP-1*-conditioned medium 10 min after treatment, and this activation peaked at 30 min after adding the conditioned medium, whereas, in the cells that were treated with mock-conditioned medium, Akt activation was not observed (Fig. 1A).

It is possible that Akt activation by *WISP-1*-conditioned medium might be caused by other proteins or components in the conditioned media and not be a direct effect of *WISP-1* signaling. To examine this possibility, *WISP-1*-conditioned medium was incubated with anti-*WISP-1*-neutralizing antibody before addition to the serum-starved cells. As shown in Figure 1B, anti-*WISP-1*-neutralizing antibody did abrogate the phosphorylation of Akt at Ser 473 in cells treated with *WISP-1*-conditioned medium, but had no effect on mock-conditioned medium or serum-treated cells, indicating that Akt activation by *WISP-1*-conditioned medium is inhibited by a neutralizing antibody specific against *WISP-1* (Fig. 1B).

It has been well documented that Wnt signaling in-

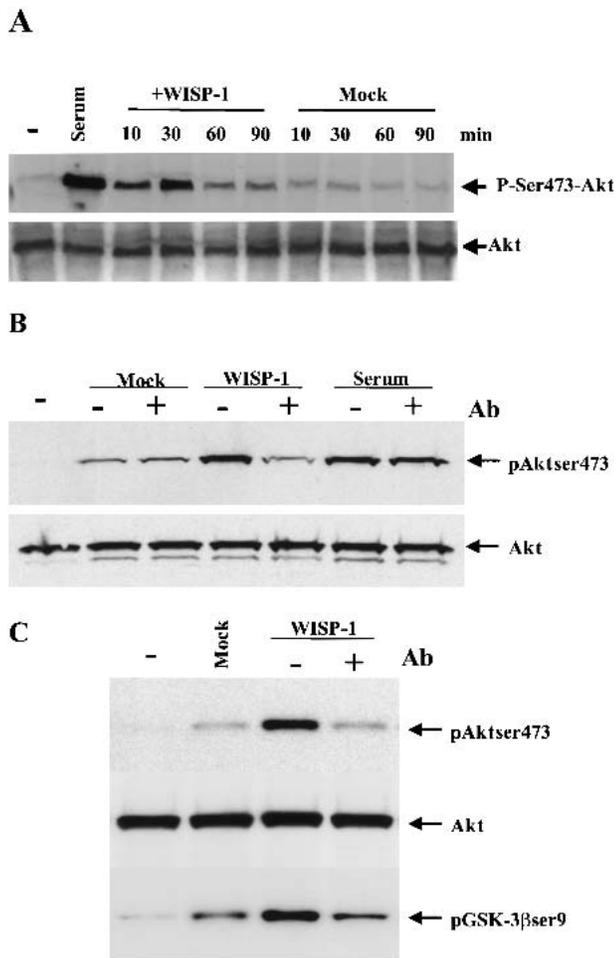


Figure 1. WISP-1 induces Akt activation. (A) Serum starved NRK-49F cells were incubated without (-) or with conditioned media collected from either mock infected (Mock) or WISP-1 (WISP-1) infected NRK-49F cells for the indicated time. Cells were also treated with 10% FBS (Serum). Whole-cell extracts were prepared and 50 μ g of lysate were subjected to Western analysis using antibodies against phosphorylated Ser 473 of Akt (p-Ser 473-Akt). The Western blot was stripped and probed with antibody against total Akt (Akt) to confirm that similar amounts of whole-cell extracts were analyzed. (B,C) Before they were used to treat the cells, serum or conditioned media collected from either mock or WISP-1 infected NRK-49F cells were incubated with anti-WISP-1 antibody (1:100) at room temperature for 2 h. Cells were harvested 30 min after the indicated treatments.

duces the phosphorylation and inactivation of the constitutively active glycogen synthase kinase 3 β (GSK3 β). This, in turn, no longer can phosphorylate β -catenin. Stabilized cytosolic β -catenin can translocate into the nucleus and form a complex with T-cell factor/lymphocyte enhancing factor (TCF/LEF) family of transcription factors to activate the expression of target genes, such as *cyclin D1*, *c-Myc*, and *WISP-1*, which are oncogenes and promote cell survival (Polakis 1999). Using conditioned medium, we also sought to test whether GSK3 β , a known specific Akt target (Cross et al. 1995), can be

phosphorylated and inactivated by WISP-1. Again, serum-starved NRK49F cells were treated with mock- or WISP-1-conditioned media for 30 min. Phosphorylation of GSK3 β was detected by a specific antibody against Ser 9 of GSK3 β in a Western analysis. As shown in Figure 1C, WISP-1-conditioned medium, but not the mock-conditioned medium, induced a strong phosphorylation of both Akt at Ser 473 and GSK3 β at Ser 9, and this induction was abrogated by preincubation of the WISP-1 conditioned medium with an anti-WISP-1 neutralizing antibody. Taken together, these data demonstrate that WISP-1 indeed can induce phosphorylation and activation of the Akt kinase, and subsequent phosphorylation of GSK3 β .

WISP-1 inhibits apoptosis induced by DNA damage

It has been shown that overexpression of *WISP-1* can accelerate cell growth and induce transformation, and that *WISP-1*-overexpressing cells form tumors in nude mice (Xu et al. 2000). The experiments described here showed that *WISP-1* activates Akt (Fig. 1). As mentioned previously, Akt is a proto-oncogene that stimulates cell survival by inhibiting apoptosis. We therefore investigated whether *WISP-1* would inhibit apoptosis, and more specifically, p53-dependent apoptosis. Several H460 (human lung carcinoma cells containing wild-type *p53*) stable cell lines transfected with Flag-tagged *WISP-1* were established. We induced apoptosis in these cells by means of DNA damage, such as UV irradiation, etoposide treatment, and γ -irradiation. Vector alone and Flag-*WISP-1*-expressing H460 cells were treated with either UV or etoposide at the indicated dosages. As shown in Figure 2, UV and etoposide treatment caused high amounts of cell death in the mock-transfected cells; however, there is much reduced cell death in *WISP-1*-expressing cells, indicating that *WISP-1* can protect cells from apoptosis caused by DNA damage (Fig. 2A).

The percentage of apoptotic cells within a given population can be quantitated by labeling cells with propidium iodide, subjecting them to flow cytometric analysis, and calculating the percentage of cells with a sub-G₁ DNA content. As indicated in Figure 2B, UV irradiation induced a significant amount of cell death (35% sub-G₁), and etoposide treatment induced ~30% apoptosis in mock-treated cells. However, the level of apoptosis was reduced in the *WISP-1*-expressing cells treated by UV irradiation (11% sub-G₁), and the level of cell death was decreased to an even greater extent (~sixfold reduction) in the *WISP-1*-expressing cells treated with etoposide (Fig. 2B). To determine whether *WISP-1* inhibition of apoptosis was a general observation and not cell type-specific, we used MCF-7 cells that are human breast adenocarcinoma cells that also express wild-type *p53*. As with H460 cells, ~two- to threefold inhibition of cell death by *WISP-1* was observed in MCF-7 cells treated with γ -irradiation (Fig. 2C). These results confirm that *WISP-1* indeed can attenuate cell death following DNA damage.

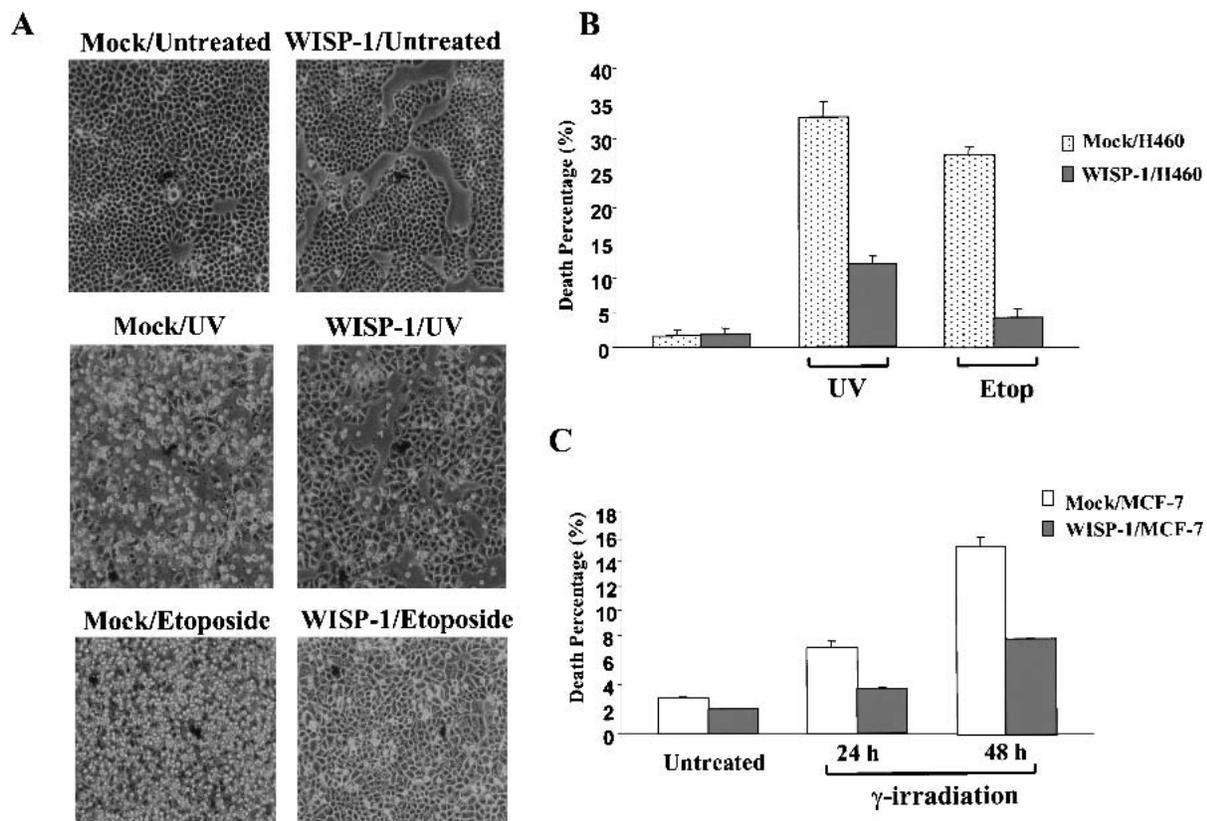


Figure 2. WISP-1 protects cells from apoptosis following DNA damage. (A) Phase contrast microscopy of mock or *WISP-1*-expressing H460 cells, 24 h after UV (20 J/m²) or etoposide (50 μ M) (Etop) treatment. (B) Quantitative analysis of cell death. Vector (mock) or *WISP-1*-expressing H460 cells were treated as in A. Apoptosis percentage (sub-G₁ population) was measured by flow cytometry with propidium iodide staining. Data are means from four independent experiments. (C) The percentile ratio of apoptotic MCF-7 cells following γ -irradiation (40 Gy) measured by flow cytometric analysis for sub-G₁ population.

WISP-1 blocks the activation of caspase-3 and inhibits the cytochrome c release induced by DNA damage

Caspase activation is known to be a critical event in the induction of the cell death process. Among the members of the caspase family, caspase-3 is a common and important effector of the apoptotic process. It is well documented that both caspase-8 and caspase-9 can activate caspase-3 in response to different death signals. Caspase-3 cleaves several important cellular substrates such as PARP and DFF45, leading to apoptosis (Nunez et al. 1998). We therefore examined whether the activation of caspase-3 was inhibited by WISP-1. Caspase-3 exists in normal cells as an inactive procaspase-3, p32. Upon death signals triggering the caspase cascade, procaspase-3 is cleaved by caspase-9 into an active form, which is p20/p17. Control and *WISP-1*-overexpressing cells were treated with etoposide or UV irradiation, and whole-cell lysates were subjected to Western blot analysis using an antibody against both forms of caspase-3. The cleaved, active form of caspase-3 (p20/p17) was observed in both etoposide and UV irradiation treated control cells (Fig. 3, lanes 3,5). However, the processing of procaspase-3 was attenuated in the *WISP-1*-expressing cells treated with UV irradiation (Fig. 3, lane 4) and com-

pletely abolished in the *WISP-1*-expressing cells treated with etoposide (Fig. 3, lane 6), indicating that WISP-1 signaling can inhibit the processing of procaspase-3. The observed cleavages of procaspase-3 in this experiment correlate with the apoptosis data shown above (Fig. 2) where WISP-1 protected cells better from etoposide-induced apoptosis than UV-induced apoptosis.

The major mechanism of processing and activation of procaspases is mediated by the Apaf-1 protein. Apaf-1 is activated by binding to cytochrome *c*, which is released from mitochondria in response to various death stimuli (Zou et al. 1997). We therefore examined whether WISP-1 is capable of regulating the release of cytochrome *c* from mitochondria. Cytosolic extracts were prepared 24 h after etoposide treatment, and cytochrome *c* released from mitochondria to the S-100 cytosolic fraction was detected by immunoblotting. As shown in Figure 3B, etoposide treatment induced a dramatic release of cytochrome *c* in the vector-expressing cells (Fig. 3B, lane 3), however, WISP-1 was able to block the release of cytochrome *c* from mitochondria in response to DNA damage (Fig. 3B, lane 4). Taken collectively, these results indicate that WISP-1 signaling can inhibit the mitochondrial release of cytochrome *c* and the subsequent activation of caspase-3.

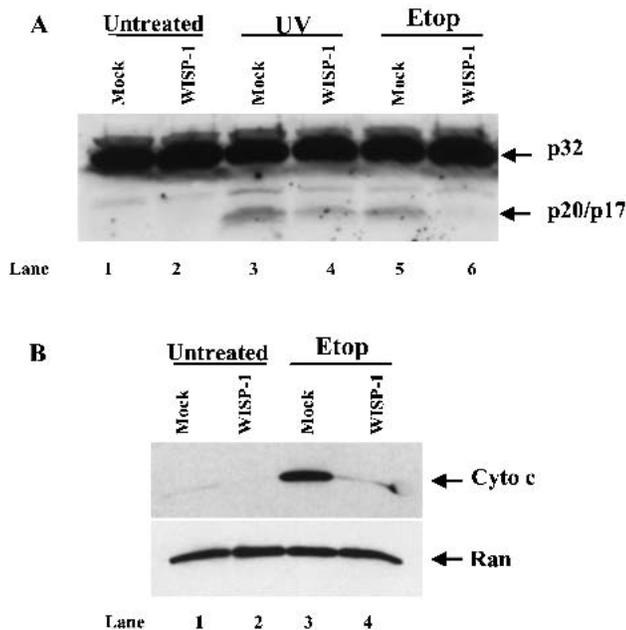


Figure 3. (A) WISP-1 blocks the activation of caspase-3 induced by DNA damage. Vector or *WISP-1* expressing H460 cells were either UV (20 J/m²) irradiated or treated with 50 μ M of etoposide (Etop) for 24 h. Expression and processing of caspase-3 were examined by Western blot analysis. (B) WISP-1 prevents the cytochrome *c* release from mitochondria. Cytosolic extracts were prepared 24 h after the cells were treated with etoposide (Etop), the release of cytochrome *c* from mitochondria was detected by Western blotting using a monoclonal antibody against cytochrome *c*. Anti-Ran blotting serves as an indication that the samples were loaded in similar amounts.

WISP-1 cannot inhibit p53-independent apoptosis

Because both H460 and MCF-7 cells express wild-type *p53*, and the level of *p53* protein was elevated in both vector- and *WISP-1*-expressing cells following DNA damage, we attempted to determine whether *WISP-1* can protect cells from apoptosis in the absence of *p53*. We overexpressed *WISP-1* in H1299, a human lung carcinoma cell line that is null for *p53*. *WISP-1* expressing H1299 cells showed accelerated cell growth compared with vector expressing cells (data not shown). Vector- and *WISP-1*-expressing H1299 cells were treated with 50 μ M of etoposide for 24 h; floating and attached cells were collected and subjected to FACS analysis with propidium iodide staining. The cells mock-transfected with vector were susceptible to etoposide induced cell death, with ~9% of cells becoming apoptotic, and there was a similar level of cell death in *WISP-1*-expressing cells (Fig. 4). These data suggest that *WISP-1* cannot protect cells against DNA damage-induced apoptosis in the absence of *p53*.

WISP-1 does not protect cells from Fas-induced apoptosis

As mentioned previously, there are two major apoptotic signaling pathways: One is signaling via stress, including

DNA damage, inducing *p53* and cytochrome *c*/Apaf-1/caspase-9 pathway, and the other is signaling through death receptors such as Fas, and the caspase-8 pathway. Having demonstrated that *WISP-1* can inhibit apoptosis following DNA damage, we next tested whether *WISP-1* could prevent cells from death receptor-induced apoptosis. It has been well established that coupling of the cell surface receptor Fas with its natural ligand, FasL, triggers apoptosis (Nagata and Golstein 1995). This death pathway can also be triggered upon Fas cross-linking with the cytolytic monoclonal antibody CH-11 (Nagata and Golstein 1995; Tanaka et al. 1996). Vector alone and *WISP-1*-expressing H460 cells were treated with anti-Fas monoclonal antibody CH-11 at indicated concentrations. Thirty hours after treatment, cells were stained with Hoechst 33258 and the apoptotic percentage was determined by scoring condensed nuclei as apoptotic and diffuse staining as normal nuclei. As shown in Figure 5, there were similar amounts of cell death in both control and *WISP-1*-expressing cells treated with different concentrations of anti-Fas antibody, indicating that *WISP-1* can not inhibit Fas-induced cell death (Fig. 5A). Similar results were obtained by calculating sub-G₁ population using FACS analysis (data not shown). We also examined the cleavage product of an endogenous substrate of caspase-3, PARP, which is a marker of apoptosis. Vector alone or *WISP-1*-expressing cells were treated with 500 ng/mL of anti-Fas antibody for 30 h, floating and attached cells were collected, and whole-cell lysates were prepared. A Western blot analysis was then carried out with antibody against PARP. As shown in Figure 5B, PARP was not cleaved in untreated vector or *WISP-1*-expressing H460 cells (Fig. 5B, lanes 1,2), but it was cleaved in both vector and *WISP-1*-expressing cells treated with anti-Fas antibody (Fig. 5B, lanes 3,4). Taken together, these results suggest that *WISP-1* signaling can only protect against *p53*-mediated apoptosis, and not Fas-mediated apoptosis.

Does WISP-1 act by blocking p53 activation?

It has been well documented that *p53* triggers apoptosis or causes cell-cycle arrest in response to DNA damage

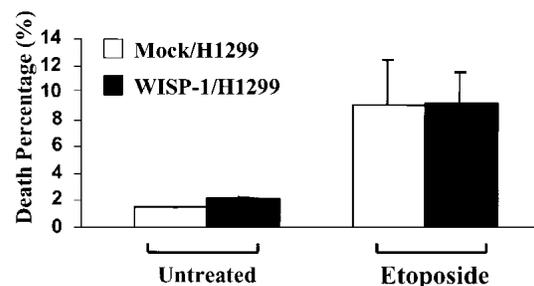


Figure 4. *WISP-1* does not inhibit *p53*-independent apoptosis. Mock or *WISP-1* transfected H1299 cells were seeded 24 h before treatment with 50 μ M of etoposide. Apoptotic percentage (sub-G₁ population) was measured by flow cytometry with propidium iodide staining.

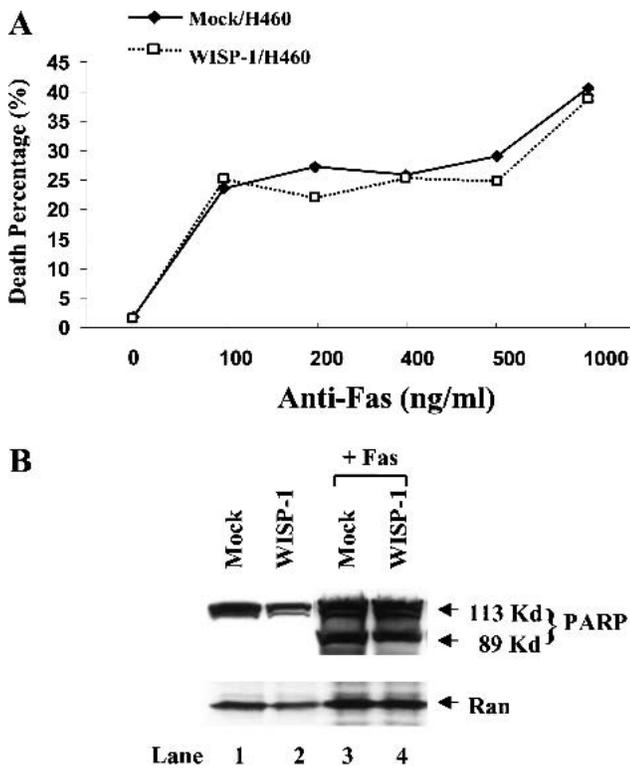


Figure 5. WISP-1 does not protect cells from Fas-induced apoptosis. (A) Vector or *WISP-1*-expressing H460 cells were split onto coverslips 20 h before being treated with anti-Fas monoclonal antibody CH-11 at indicated concentrations for 30 h. Cells were then fixed and stained with Hoechst stain for nuclei visualization. The percentages of apoptotic cells were calculated by counting ≥ 500 cells for each sample and scoring apoptotic cells with condensed staining compared with normal diffuse staining. (B) Vector or *WISP-1*-expressing cells were treated with anti-Fas antibody for 30 h. Whole cell extracts were then prepared and Western blot analysis was performed using antibody against PARP. Anti-Ran antibody is used for loading control.

(Levine 1997; Prives and Hall 1999). Therefore, to determine the mechanisms by which WISP-1 protects cells against apoptosis induced by DNA damage, we first examined whether WISP-1 inhibited cell death by inhibiting p53 activation following DNA damage. Vector alone or *WISP-1*-expressing cells were treated with 20 J/m² of UV irradiation or with 50 μ M of etoposide. Whole-cell lysates were prepared 24 h after treatment, and proteins were analyzed by an SDS-PAGE. p53 levels were measured by Western blotting with the DO-1 anti-p53 monoclonal antibody. The p53 levels in *WISP-1*-expressing cells were equal to or even higher than levels in vector-expressing cells following DNA damage (Fig. 6A), suggesting that WISP-1 does not block the activation of p53 following damage to DNA. It is well established that DNA damage leads to phosphorylation of p53 at several N-terminal residues, including Ser 15, Ser 20, and Ser 37, which appears to be important for stabilization the p53 protein (Shieh et al. 1997, 1999; Unger et al. 1999). We therefore tested whether p53 could be phosphorylated

properly in the *WISP-1*-expressing cells. Phosphorylated p53 at Ser 15 was detected by an antibody that is specific for phospho-Ser 15 of p53. We found that Ser 15 of p53 was phosphorylated in both vector- and *WISP-1*-expressing cells following UV or etoposide treatment (Fig. 6A). Therefore, WISP-1 does not inhibit the activation or Ser 15 phosphorylation of p53.

To determine whether the activated p53 is transcriptionally functional, we examined the protein levels of three downstream target genes of p53, namely *mdm2*, *p21*, which is known to be involved in cell cycle arrest, and *Bax*, which is believed to play a role in inducing apoptosis. Again, both control and *WISP-1*-expressing cells were treated with UV irradiation and etoposide. Twenty-four hours after treatment, whole-cell extracts were prepared and proteins were analyzed by Western blotting using antibodies against p21, *Bax*, or *mdm2*. We found that all three genes, *p21*, *mdm2*, and *Bax*, were activated in both control and *WISP-1*-expressing cells upon DNA damage (Fig. 6A,B; *mdm2* results not shown). Similar results were obtained with the breast cancer cell line, MCF-7 cells. Taken collectively, these results dem-

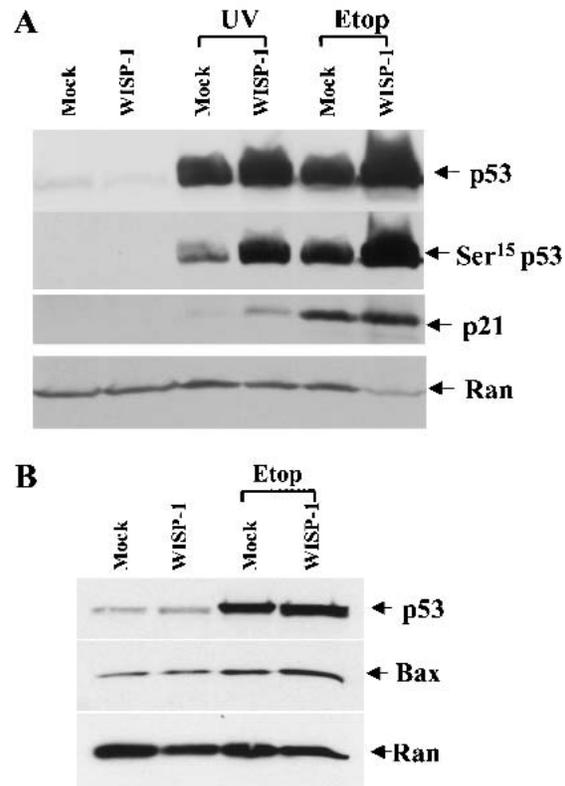


Figure 6. (A) Induction of p53 and p21 in *WISP-1*-expressing cells following DNA damage. Whole-cell extracts were prepared 24 h after exposure to 20 J/m² of UV-C light or 50 μ M of etoposide (Etop), and the samples were processed for Western blot analysis. Anti-Ran antibody is used to confirm that similar amounts of whole-cell extracts were analyzed. (B) *Bax* was induced by p53 in response to DNA damage. Western analysis was performed as indicated in A except using a polyclonal anti-*Bax* antibody.

onstrate that WISP-1 inhibits cell death but not the induction of p53 following DNA damage, and interestingly the induced p53 protein is transcriptionally functional.

WISP-1 inhibition of apoptosis requires Akt activity

Akt promotes cell survival by regulating several key factors in the apoptotic machinery, including Bad, caspase-9, forkhead transcription factor, GSK3 β and NF- κ B (for review, see Datta et al. 1999). This study demonstrated that WISP-1 can inhibit apoptosis in response to DNA damage and can induce phosphorylation and activation of Akt (Figs. 1,2). We therefore sought to determine whether Akt is required for WISP-1 inhibition of DNA damage-induced apoptosis. An *Akt* mutant that has a K179A substitution (*Akt-KD*) is a kinase dead mutant because it cannot bind ATP or phosphorylate substrates. In addition, this mutant has a dominant negative or inhibitory activity over the activation of endogenous Akt by insulin or other growth factors (Dudek et al. 1997). *Akt-KD* was delivered into vector alone or *WISP-1*-expressing H460 cells by retroviral infection, and several positive clones expressing *Akt-KD* were identified and used for this experiment. Both floating and attached cells were collected after 30 h of etoposide treatment, and cells were subsequently stained with propidium iodide for FACS analysis. Again, treatment with etoposide induced a significant level of cell death in vector alone-expressing cells (~24% sub-G₁) and *Akt-KD*-expressing cells (~27% sub-G₁). WISP-1 was able to protect cells from etoposide-induced apoptosis in the *WISP-1*-expressing cells (~4% sub-G₁), however, this protection was attenuated by *Akt-KD* in the cells expressing both *WISP-1* and *Akt-KD* (~16% sub-G₁) (Fig. 7). There may be two explanations for the fact that *Akt-KD* exerted an attenuation but not a complete abrogation of WISP-1 protection. First, the dominant negative effect of this *Akt-KD* mutant may not be potent (Wang et al. 1999) enough to inhibit all the Akt activation induced by WISP-1; second, WISP-1 may also act on some other signaling pathways in addition to the Akt signaling pathway to inhibit p53-mediated cell death. These results demonstrate that Akt

activation was required at least in part for WISP-1 to prevent cells from undergoing apoptosis in response to DNA damage.

WISP-1 up-regulates antiapoptotic protein Bcl-X_L

The results above suggest that WISP-1 may act on some other signaling pathways in addition to the Akt signaling pathway to inhibit p53-mediated apoptosis. It is well established that members of the Bcl-2 family are major regulators of mitochondrial apoptotic events (Kroemer and Reed 2000). Expression of antiapoptotic members such as Bcl-2 and Bcl-X_L prevents the redistribution of cytochrome *c* in response to multiple death-inducing stimuli, whereas proapoptotic members such as Bax and Bak promote cytochrome *c* release (Gross et al. 1999). It is possible that WISP-1 functions by regulating the Bcl-2 family of proteins. We have shown that *Bax* was moderately activated by p53 in response to DNA damage in both vector- and *WISP-1*-expressing cells (Fig. 6B). There was no change of the steady-state expression level of the Bak protein (data not shown). We therefore examined the ability of WISP-1 to activate antiapoptotic members of the Bcl-2 family. Whole-cell extracts were prepared 20 h after the cells expressing either vector alone or *WISP-1* were treated with etoposide. The expression patterns of Bcl-2 and Bcl-X_L were detected by Western blot analysis. There was no difference in Bcl-2 protein levels in both vector- and *WISP-1*-expressing H460 cells, suggesting that WISP-1 does not affect *Bcl-2* expression (data not shown). However, endogenous levels of Bcl-X_L were dramatically increased in the *WISP-1*-expressing cells, and the activation was independent of etoposide treatment (Fig. 8), indicating that WISP-1 up-regulates antiapoptotic *Bcl-X_L*, which can protect the integrity of mitochondria, and thus inhibit apoptosis.

Discussion

WISP-1 belongs to the CCN family of growth factors that have been getting increasing attention lately because some of the family members have been reported to be

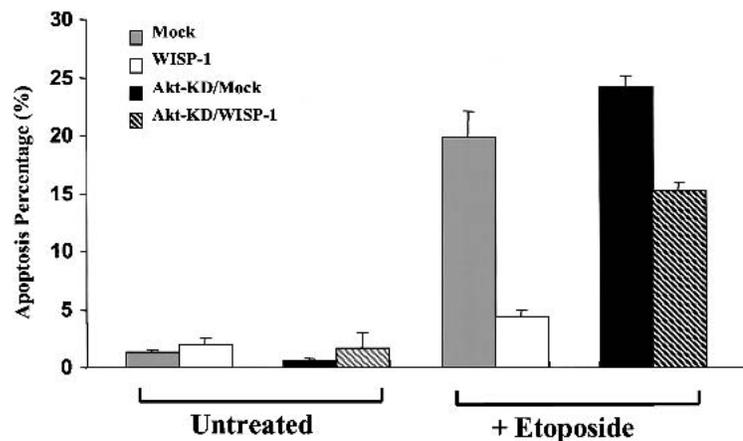


Figure 7. Akt activity is required for WISP-1 protection of cells from apoptosis. H460 cells expressing empty vector (mock), expressing *WISP-1* (*WISP-1*), expressing both vector and *Akt-KD* (*Akt-KD/mock*) and expressing both *WISP-1* and *Akt-KD* (*Akt-KD/WISP-1*) were treated with 50 μ M of etoposide for 30 h. Cells were then stained with propidium iodide for FACS analysis. Apoptotic percentage was calculated by scoring sub-G₁ populations. Experiments were done with four different clones, three independent experiments, and duplicate samples.

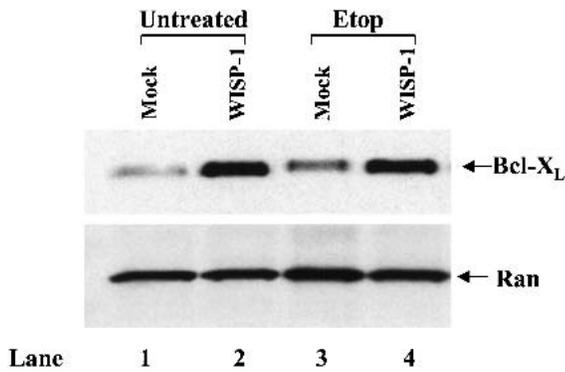


Figure 8. WISP-1 up-regulates antiapoptotic protein Bcl-X_L. H460 cells expressing empty vector (mock) and expressing *WISP-1* (*WISP-1*) were treated with 50 mM of etoposide for 20 h. Whole-cell extracts were then prepared and subjected to Western blot analysis using antibody against Bcl-X_L.

involved in angiogenesis and tumorigenesis (Ayer-Lelievre et al. 2001). After ascertaining that WISP-1 can accelerate cell growth and promote cellular tumorigenicity, this study demonstrated that WISP-1 activates the antiapoptotic Akt signaling pathway, inhibits the mitochondrial release of cytochrome *c*, up-regulates antiapoptotic protein Bcl-X_L, and therefore prevents cells from undergoing apoptosis induced by DNA damage. Furthermore, the results showed that WISP-1 protects cells only from p53-dependent cell death. This is the first report that suggests that there is cross talk between the tumor suppressor protein p53 and WISP-1 signaling pathways.

The activation of Akt by WISP-1, the known activities of Akt, and the impact of the dominant negative Akt on apoptosis make it very likely that WISP-1 inhibits apoptosis by activating the antiapoptotic Akt kinase. Akt has been reported to block the release of cytochrome *c* from mitochondria and thereby prevent initiation of the apoptotic cascade leading to activation of caspases (Kennedy et al. 1999; Gottlob et al. 2001). The results presented here show that WISP-1 can induce Akt activation and inhibit the mitochondrial release of cytochrome *c* (Figs. 1,3B), thus indicating that WISP-1 activation of Akt may directly inhibit the cytochrome *c* release from mitochondria (Fig. 9). It has also been demonstrated that Akt can promote cell survival of T lymphocyte and pre-mast cells through activation of antiapoptotic Bcl-X_L (Jones et al. 2000; Tang et al. 2000). Bcl-X_L has been reported to promote the survival of stress-damaged cells by maintaining mitochondrial integrity and function, and this antiapoptotic ability correlates with its ability to form heterodimers with proapoptotic members of the Bcl-2 family such as Bax (Sedlak et al. 1995; Kelekar et al. 1997). Proapoptotic *Bax* was moderately activated by p53 in response to DNA damage in both vector- and *WISP-1*-expressing cells (Fig. 6B), yet only WISP-1 can protect cells from undergoing apoptosis induced by DNA damage. This study has demonstrated that WISP-1 up-regulates Bcl-X_L (Fig. 8). Thus, WISP-1 may inhibit p53-me-

diated cell death by activating Bcl-X_L, which antagonizes Bax. In other studies, Bcl-X_L has been shown to interact with caspase-9 and Apaf-1, resulting in the inhibition of caspase activation (Hu et al. 1998; Pan et al. 1998). Increased levels of Bcl-X_L induced by WISP-1 may form inhibitory complexes with caspase-9, Apaf-1, and cytochrome *c* after DNA damage, thus blocking apoptosis. Bcl-X_L has also been shown to block DNA damage induced cytochrome *c* release from mitochondria by binding to the cytochrome *c* (Kharbanda et al. 1997), therefore it is possible that WISP-1 prevents cell death by inhibiting the release of mitochondrial cytochrome *c* (Fig. 3B) through up-regulating Bcl-X_L (Fig. 9). This is most consistent with the findings in this study.

Two lines of evidence demonstrate that WISP-1 only inhibits p53-dependent apoptosis. First, WISP-1 cannot protect H1299 cells, which do not have endogenous *p53*, from cell death following etoposide treatment (Fig. 4); Second, WISP-1 showed no protection against apoptosis in anti-Fas antibody treated cells (Fig. 5). Yet this study clearly demonstrated that cell death, but not p53 induction, was blocked in the *WISP-1*-expressing cells. It has been reported that p53-dependent apoptosis requires Apaf-1, which in turn requires release of cytochrome *c* from mitochondria (Yoshida et al. 1998; Soengas et al. 1999). It appears then that WISP-1 acts to attenuate p53-mediated apoptosis by blocking activities downstream of the p53 pathway, such as release of cytochrome *c* from

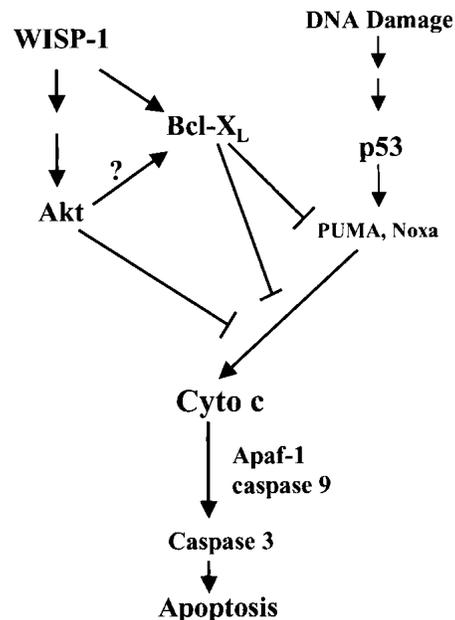


Figure 9. A schematic illustration of WISP-1 inhibition of p53-mediated apoptosis in response to DNA damage. WISP-1 promotes cell survival by activating Akt, which may inhibit the release of cytochrome *c* from mitochondria. WISP-1 also activates the antiapoptotic protein Bcl-X_L, which in turn, through sequestering p53-mediated BH3 domain-only molecules such as PUMA or Noxa, prevents the mitochondrial release of cytochrome *c*, thus blocking the subsequent caspase cascade and eventual cell death.

mitochondria. There are multiple pathways downstream of p53 that are potentially involved in the induction of apoptosis (Prives and Hall 1999). The p53 protein is a transcription factor; many experiments have demonstrated that transcriptional activation by p53 is critical for the induction of apoptosis (Gottlieb and Oren 1998). Moreover, several p53 target genes, such as *Bax*, *Noxa*, *PERP*, *KILLER/DR5*, *p53IAP1*, *PIDD*, or *PUMA* (Ryan et al. 2001), have been identified and are known to play a role in inducing apoptosis, although some of them such as *Bax* may have a redundant function because p53-induced death is not affected by its absence (Brady et al. 1996). Two of these p53 target genes, *Noxa* and *PUMA*, are both Bcl-2 homology (BH) 3 domain-only proteins, localize to the mitochondria, interact with antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L, and function to induce cytochrome *c* release, thereby activating caspase-9 and 3 (E. Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001). The p53 protein is a major upstream determinant for stress-induced apoptosis via the mitochondrial cytochrome *c*/caspase-9 pathway (Soengas et al. 1999), thus *PUMA* and *Noxa* provide a link between p53 and cytochrome *c* release. It was reported very recently that antiapoptotic Bcl-2 and Bcl-X_L act to sequester BH3 domain-only molecules such as Bad, *Noxa* and *PUMA*, thereby preventing the release of cytochrome *c* from mitochondria and subsequent apoptosis (Cheng et al. 2001). This study has demonstrated that *WISP-1* up-regulates the antiapoptotic Bcl-X_L (Fig. 8). Therefore it seems likely that *WISP-1* increases the level of Bcl-X_L, which in turn combines with and suppresses the p53-inducible genes *PUMA* and *Noxa*. This in turn blocks cytochrome *c* release, and apoptosis is halted even though p53 is activated (see Fig. 9). Whether Akt activation plays an important role in the increase in Bcl-X_L levels or adds to the antiapoptotic phenotype of Bcl-X_L remains to be elucidated (Fig. 9).

Materials and methods

Cell culture, transfection, and retroviral infections

NRK-49F, H460, H1299, and MCF-7 cell lines were maintained in DMEM supplemented with 10% FBS. *WISP-1*-expressing H460 and H1299 cell lines were established by transfection of HA- and Flag-epitope-tagged *WISP-1* cDNA, whereas *WISP-1*-expressing MCF7 cell lines were established by retroviral infections. Retroviral expression vector pBabe-puro was used to construct and express tagged human *WISP-1* cDNA. Recombinant retroviruses were generated by transfecting the retroviral constructs into amphotropic PhoenixA packaging cells with lipofectamine (GIBCO BRL). Forty-eight hours posttransfection, retrovirus-containing supernatant was collected. Target cells were infected at about 40% confluence by adding the retrovirus-containing medium supplemented with 8 µg/mL polybrene. Infection medium was replaced with DMEM and 10% FBS 10–16 h later. Cells were split into DMEM that contains 2.5 µg/mL puromycin at 48 h postinfection. Drug-resistant clones were picked and expanded. Total protein was extracted for IP-Western analysis to assess exogenous *WISP-1* expression. A number of stable clones that express human *WISP-1* at high levels were obtained for H460, H1299, and MCF7 cell lines.

WISP-1-conditioned medium

The *hWISP-1*-expressing NRK-49F cell line was established by retroviral infections as described previously (Xu et al. 2000) and was maintained in DMEM supplemented with 10% FBS and 2.5 µg/mL puromycin. *WISP-1*-expressing and control cell lines were split into DMEM supplemented with 3% FBS, then *WISP-1*- and control-conditioned medium were collected ≥5 d later.

DNA damage treatments

Cells were seeded 24 h prior to treatment and were 50%–60% confluent at the time of treatment. *WISP-1*-expressing and control cells were treated with etoposide (Sigma) at a concentration of 50 µM for 24 h, or exposed for 10 sec to a UVC lamp at 20 J/m² or irradiated by 30 Gy using a ⁶⁰Co source, then incubated for 24 h after the irradiation.

Detection of apoptosis

For FACS analysis 24 h or the indicated times after DNA damage, adherent and detached cells were combined and fixed overnight or longer with 80% ethanol in PBS at –20°C. After washing twice with PBS, cells were incubated for 1 h or longer with 1 mL of PBS containing 1 mg of boiled RNaseA and 10 µg of propidium iodide. A total of 5 × 10⁵ cells were then analyzed in a flow cytometer (FACScalibur, BD).

For Hoechst staining, 125,000 cells were plated onto six-well plates where each well contained a microscope coverslip the day before the treatment with anti-Fas antibody. About 24 h after treatment, cells were gently washed with PBS, and fixed/permeabilized with freshly made 70% acetone/30% methanol at –20°C for 7 min. Cells were stained with Hoechst 33258 for nuclei. The condensed DNA was determined by intense local staining of DNA in the nucleus compared with the diffuse staining of DNA in normal cells. The percentage of apoptotic cells was determined from cells containing normal DNA staining compared with cells with condensed DNA. A minimum of 500 cells was scored for each sample, and each experiment was performed in duplicate.

Preparation of cytosolic extracts

Cells were collected by centrifugation at 600g for 5 min at 4°C. The cell pellets were resuspended in five volumes of extraction buffer, containing 250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. After 30 min incubation on ice, cells were homogenized with a Teflon homogenizer (20 strokes). Cell homogenates were centrifuged twice at 750g for 10 min at 4°C. The supernatants were centrifuged at 10,000g for 15 min at 4°C, and the resulting mitochondria pellets were saved at –80°C. The supernatants of the 10,000g spin were further centrifuged at 100,000g for 90 min at 4°C, and the resulting supernatants were designated as S-100 fractions.

Western blot and antibodies

All cells were harvested and lysed in extraction buffer (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA, 1 mM Na₃VO₄, 10 mM NaF, 10 mM Na₄P₂O₇, 1 mM PMSF, 1 µg/mL leupeptin, and 0.1 U/mL of aprotinin) and cleared by centrifugation for 10 min at 4°C to obtain whole-cell extracts. Each 20–50 µg sample of total protein was loaded onto 4%–15% gradient SDS-PAGE gels and

blotted onto Hybond membranes (Amersham Pharmacia Biotech). Protein bands were visualized by chemiluminescent detection (ECL) (Amersham).

Anti-p53 monoclonal antibody (DO-1), anti-caspase-3, anti-PARP and anti-Ran antibody were purchased from Santa Cruz. Anti-p21 antibody was purchased from Oncogene Science. Anti-GSK3 β , anti-phospho-GSK3 β (Ser 9), anti-Akt, anti-phospho-Akt (Ser 473) 4E2 monoclonal antibodies and anti-Bcl-X_L were purchased from New England BioLabs. Anti-Fas monoclonal antibody CH-11 was purchased from Kamiya Biomedical Company. Anti-Bax polyclonal antibody was purchased from Upstate Biotechnology. Anti-cytochrome *c* monoclonal antibody (7H8) was purchased from BD Pharmingen. Anti-hWISP-1 polyclonal antibody was generated at Covance by using the variable domain of hWISP-1 (amino acids 173–213) linked to GST as an antigen. The polyclonal antibody was then purified by affinity chromatography using GST as the negative and the antigen as the positive immunoabsorbent.

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References

- Ashkenazi, A. and Dixit, V.M. 1998. Death receptors: Signaling and modulation. *Science* **281**: 1305–1308.
- Attardi, L.D., Reczek, E.E., Cosmas, C., Demicco, E.G., McCurrach, M.E., Lowe, S.W., and Jacks, T. 2000. PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. *Genes & Dev.* **14**: 704–718.
- Ayer-Lelievre, C., Brigstock, D., Lau, L., Pennica, D., Perbal, B., and Yeager, H. 2001. Report and abstracts on the first international workshop on the CCN family of genes. *Mol. Pathol.* **54**: 105–120.
- Babic, A.M., Chen, C.C., and Lau, L.F. 1999. Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin $\alpha\beta 3$, promotes endothelial cell survival, and induces angiogenesis in vivo. *Mol. Cell. Biol.* **19**: 2958–2966.
- Bates, S. and Vousden, K.H. 1999. Mechanisms of p53-mediated apoptosis. *Cell. Mol. Life Sci.* **55**: 28–37.
- Brady, H.J., Salomons, G.S., Bobeldijk, R.C., and Berns, A.J. 1996. T cells from bax α transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. *EMBO J.* **15**: 1221–1230.
- Brigstock, D.R. 1999. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr. Rev.* **20**: 189–206.
- Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**: 857–868.
- Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., and Reed, J.C. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282**: 1318–1321.
- Chen, C.C., Chen, N., and Lau, L.F. 2001. The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. *J. Biol. Chem.* **276**: 10443–10452.
- Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. 2001. Bcl-2, bcl-x(l) sequester bh3 domain-only molecules preventing bax- and bak-mediated mitochondrial apoptosis. *Mol. Cell* **8**: 705–711.
- Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**: 785–789.
- Cryns, V. and Yuan, J. 1998. Proteases to die for. *Genes & Dev.* **12**: 1551–1570.
- Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**: 231–241.
- Datta, S.R., Brunet, A., and Greenberg, M.E. 1999. Cellular survival: A play in three Akts. *Genes & Dev.* **13**: 2905–2927.
- Dedhar, S., Williams, B., and Hannigan, G. 1999. Integrin-linked kinase (ILK): A regulator of integrin and growth-factor signalling. *Trends Cell. Biol.* **9**: 319–323.
- Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J., and Dedhar, S. 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc. Natl. Acad. Sci.* **95**: 11211–11216.
- Di Cristofano, A. and Pandolfi, P.P. 2000. The multiple roles of PTEN in tumor suppression. *Cell* **100**: 387–390.
- Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., and Greenberg, M.E. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275**: 661–665.
- Giancotti, F.G. and Ruoslahti, E. 1999. Integrin signaling. *Science* **285**: 1028–1032.
- Gottlieb, T.M. and Oren, M. 1998. p53 and apoptosis. *Semin. Cancer Biol.* **8**: 359–368.
- Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R.B., and Hay, N. 2001. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes & Dev.* **15**: 1406–1418.
- Gross, A., McDonnell, J.M., and Korsmeyer, S.J. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes & Dev.* **13**: 1899–1911.
- Grzeszkiewicz, T.M., Kirschling, D.J., Chen, N., and Lau, L.F. 2001. CYR61 Stimulates human skin fibroblast migration through integrin $\alpha\beta 5$ and enhances mitogenesis through integrin $\alpha\beta 3$, independent of its carboxy-terminal domain. *J. Biol. Chem.* **276**: 21943–21950.
- Hu, Y., Benedict, M.A., Wu, D., Inohara, N., and Nunez, G. 1998. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc. Natl. Acad. Sci.* **95**: 4386–4391.
- Hu, Y., Benedict, M.A., Ding, L., and Nunez, G. 1999. Role of cytochrome *c* and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. *EMBO J.* **18**: 3586–3595.
- Jedsadayanmata, A., Chen, C.C., Kireeva, M.L., Lau, L.F., and Lam, S.C. 1999. Activation-dependent adhesion of human platelets to Cyr61 and Fisp12/mouse connective tissue

- growth factor is mediated through integrin $\alpha(\text{IIb})\beta(3)$. *J. Biol. Chem.* **274**: 24321–24327.
- Jones, R.G., Parsons, M., Bonnard, M., Chan, V.S., Yeh, W.C., Woodgett, J.R., and Ohashi, P.S. 2000. Protein kinase B regulates T lymphocyte survival, nuclear factor κB activation, and Bcl-X(L) levels in vivo. *J. Exp. Med.* **191**: 1721–1734.
- Kelekar, A., Chang, B.S., Harlan, J.E., Fesik, S.W., and Thompson, C.B. 1997. Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol. Cell. Biol.* **17**: 7040–7046.
- Kennedy, S.G., Kandel, E.S., Cross, T.K., and Hay, N. 1999. Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. *Mol. Cell. Biol.* **19**: 5800–5810.
- Kharbanda, S., Pandey, P., Schofield, L., Israels, S., Roncinske, R., Yoshida, K., Bharti, A., Yuan, Z.M., Saxena, S., Weichselbaum, R., et al. 1997. Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. *Proc. Natl. Acad. Sci.* **94**: 6939–6942.
- Kireeva, M.L., Lam, S.C., and Lau, L.F. 1998. Adhesion of human umbilical vein endothelial cells to the immediate-early gene product Cyr61 is mediated through integrin $\alpha\text{v}\beta 3$. *J. Biol. Chem.* **273**: 3090–3096.
- Ko, L.J. and Prives, C. 1996. p53: Puzzle and paradigm. *Genes & Dev.* **10**: 1054–1072.
- Kops, G.J., de Ruiter, N.D., De Vries-Smits, A.M., Powell, D.R., Bos, J.L., and Burgering, B.M. 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**: 630–634.
- Kroemer, G. and Reed, J.C. 2000. Mitochondrial control of cell death. *Nat. Med.* **6**: 513–519.
- Lau, L.F. and Lam, S.C. 1999. The CCN family of angiogenic regulators: The integrin connection. *Exp. Cell Res.* **248**: 44–57.
- Levine, A.J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88**: 323–331.
- Lin, Y., Ma, W., and Benchimol, S. 2000. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. *Nat. Genet.* **26**: 122–127.
- Lowe, S.W. and Lin, A.W. 2000. Apoptosis in cancer. *Carcinogenesis* **21**: 485–495.
- Miyashita, T. and Reed, J.C. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**: 293–299.
- Nagata, S. and Golstein, P. 1995. The Fas death factor. *Science* **267**: 1449–1456.
- Nakano, K. and Vousden, K.H. 2001. PUMA, a novel proapoptotic gene, is induced by p53. *Mol. Cell* **7**: 683–694.
- Nunez, G., Benedict, M.A., Hu, Y., and Inohara, N. 1998. Caspases: The proteases of the apoptotic pathway. *Oncogene* **17**: 3237–3245.
- Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**: 1053–1058.
- Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y., et al. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* **102**: 849–862.
- Pan, G., O'Rourke, K., and Dixit, V.M. 1998. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J. Biol. Chem.* **273**: 5841–5845.
- Pennica, D., Swanson, T.A., Welsh, J.W., Roy, M.A., Lawrence, D.A., Lee, J., Brush, J., Taneyhill, L.A., Deuel, B., Lew, M., et al. 1998. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc. Natl. Acad. Sci.* **95**: 14717–14722.
- Persad, S., Attwell, S., Gray, V., Mawji, N., Deng, J.T., Leung, D., Yan, J., Sanghera, J., Walsh, M.P., and Dedhar, S. 2001. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: Critical roles for kinase activity and amino acids arginine 211 and serine 343. *J. Biol. Chem.* **276**: 27462–27469.
- Polakis, P. 1999. The oncogenic activation of beta-catenin. *Curr. Opin. Genet. Dev.* **9**: 15–21.
- Prives, C. and Hall, P.A. 1999. The p53 pathway. *J. Pathol.* **187**: 112–126.
- Ruoslahti, E. 1999. Fibronectin and its integrin receptors in cancer. *Adv. Cancer Res.* **76**: 1–20.
- Ryan, K.M., Phillips, A.C., and Vousden, K.H. 2001. Regulation and function of the p53 tumor suppressor protein. *Curr. Opin. Cell. Biol.* **13**: 332–337.
- Sedlak, T.W., Oltvai, Z.N., Yang, E., Wang, K., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. 1995. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc. Natl. Acad. Sci.* **92**: 7834–7838.
- Shieh, S.Y., Ikeda, M., Taya, Y., and Prives, C. 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**: 325–334.
- Shieh, S.Y., Taya, Y., and Prives, C. 1999. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. *EMBO J.* **18**: 1815–1823.
- Soengas, M.S., Alarcon, R.M., Yoshida, H., Giaccia, A.J., Hakem, R., Mak, T.W., and Lowe, S.W. 1999. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* **284**: 156–159.
- Sonoda, Y., Watanabe, S., Matsumoto, Y., Aizu-Yokota, E., and Kasahara, T. 1999. FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line. *J. Biol. Chem.* **274**: 10566–10570.
- Tanaka, M., Suda, T., Haze, K., Nakamura, N., Sato, K., Kimura, F., Motoyoshi, K., Mizuki, M., Tagawa, S., Ohga, S., et al. 1996. Fas ligand in human serum. *Nat. Med.* **2**: 317–322.
- Tang, X., Downes, C.P., Whetton, A.D., and Owen-Lynch, P.J. 2000. Role of phosphatidylinositol 3-kinase and specific protein kinase B isoforms in the suppression of apoptosis mediated by the Abelson protein-tyrosine kinase. *J. Biol. Chem.* **275**: 13142–13148.
- Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M., and Haupt, Y. 1999. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *EMBO J.* **18**: 1805–1814.
- Verdu, J., Buratovich, M.A., Wilder, E.L., and Birnbaum, M.J. 1999. Cell-autonomous regulation of cell and organ growth in *Drosophila* by Akt/PKB. *Nat. Cell. Biol.* **1**: 500–506.
- Wang, Q., Somwar, R., Bilan, P.J., Liu, Z., Jin, J., Woodgett, J.R., and Klip, A. 1999. Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* **19**: 4008–4018.
- Wu, G.S., Kim, K., and el-Deiry, W.S. 2000. KILLER/DR5, a novel DNA-damage inducible death receptor gene, links the p53-tumor suppressor to caspase activation and apoptotic death. *Adv. Exp. Med. Biol.* **465**: 143–151.
- Xu, L., Corcoran, R.B., Welsh, J.W., Pennica, D., and Levine, A.J. 2000. WISP-1 is a Wnt-1- and β -catenin-responsive oncogene. *Genes & Dev.* **14**: 585–595.
- Yoshida, H., Kong, Y.Y., Yoshida, R., Elia, A.J., Hakem, A., Hakem, R., Penninger, J.M., and Mak, T.W. 1998. Apaf1 is

- required for mitochondrial pathways of apoptosis and brain development. *Cell* **94**: 739–750.
- Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W., and Vogelstein, B. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol. Cell* **7**: 673–682.
- Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**: 619–628.
- Zhao, R., Gish, K., Murphy, M., Yin, Y., Notterman, D., Hoffman, W.H., Tom, E., Mack, D.H., and Levine, A.J. 2000. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. *Genes & Dev.* **14**: 981–993.
- Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H., and Hung, M.C. 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat. Cell. Biol.* **3**: 245–252.
- Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X. 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* **90**: 405–413.