Functional association between Wwox tumor suppressor protein and p73, a p53 homolog

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The **Wwox** gene is a recently cloned tumor suppressor gene that spans the **FRA16D** fragile region. Wwox protein contains two WW domains that are generally known to mediate protein–protein interaction. Here we show that Wwox physically interacts via its first WW domain with the p53 homolog, p73. The tyrosine kinase, Src, phosphorylates Wwox at tyrosine 33 in the first WW domain and enhances its binding to p73. Our results further demonstrate that Wwox expression triggers redistribution of nuclear p73 to the cytoplasm and, hence, suppresses its transcriptional activity. In addition, we show that ectopic expression of Wwox in breast cancer cells inhibits tumor growth in vivo (9). Altogether, these findings suggest that Wwox is a candidate tumor suppressor gene.

The WWOX gene encodes a 46-kDa protein that contains two WW domains and a short-chain dehydrogenase/reductase domain (SDR) (1). The former domain is usually a globular domain consisting of ~40 amino acids, of which two tryptophans and an invariant proline are highly conserved (10). Like the SH3 domain, the WW domain is characterized by interaction with proline-containing ligands and mediates protein–protein interaction (10, 11). Generally, WW domains can be grouped into four classes according to their ligand binding preferences, and, recently, it was suggested that they can be regulated by tyrosine phosphorylation (11).

The p73 protein is a structural and functional homolog of the p53 tumor suppressor protein (12, 13). p73 not only recognizes and binds to the p53-responsive elements found in the promoter regions of diverse p53-target genes but can also transactivate the transcription of these target genes to various degrees (14). Unlike **TP53**, **TP73** encodes seven distinct isoforms (α–n) that are generated as a result of alternative splicing at the 3′ end. Another p73 variant, which lacks the amino-terminal domain, was also reported, known as deltaNp73 (ΔNp73). ΔNp73 lacks the transactivation domain and possesses a dominant negative activity against p73, as well as p53 (15). Physical interaction of p73 with different proteins may modulate its transcriptional as well as its biological activity (16, 17). Here we report a functional interaction between p73 and Wwox and its role in apoptosis.

Materials and Methods

**Cell Culture.** Human embryonic kidney 293 cells, human osteosarcoma SOAS-2 cells, and NIH3T3 cells were grown in DMEM supplemented with 10% FBS and gentamicin (GIBCO/BRL). MCF-7 breast carcinoma cells were grown in RPMI media.

**Plasmid Constructs and Transient Transfections.** The mammalian expression plasmids encoding hemagglutinin (HA) epitope-tagged TAp73α, HA-TAp73β, HA-TAp73γ and HA-ΔNp73 in pCDNA3 vectors have been described (18). Full-length **Wwox** cDNA was cloned into a Myc-tagged pCMV vector (BD Clontech) by using standard protocols. pCMV-Myc-WWY33R, pCMV-Myc-WWY34F, pCMV-Myc-WWY36R, and pCDNA3-HA-p73βY487A were obtained by site-directed PCR mutagenesis (Stratagene), according to the manufacturer’s instruction. Activated Src and dominant negative (DN) Src mutant plasmids were purchased from Upstate Biotechnology (Lake Placid, NY). Overexpression of the proteins was achieved by transient transfection with FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Applied Science).

**Immunoprecipitation and Immunoblot Analysis.** Cells were lysed by using Nonidet P-40 lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors. Lysates were precleared with mouse IgG, immunoprecipitations were carried out in the same buffer, and lysates were washed four times with the same buffer containing 0.1% Nonidet P-40 and 0.1% SDS. Antibodies used were mouse monoclonal anti-β-actin (Covance, Princeton), mouse monoclonal anti-Myc (Zymed), mouse monoclonal anti-p73 (NeoMarkers, Fremont, CA), mouse monoclonal anti-p53 (Santa Cruz Biotechnology). Western blotting was performed under standard conditions. Antibodies used for immunoblot were anti-HA-HRP (Roche Applied Science), anti-Myc-HRP (Santa Cruz Biotechnology), anti-p73 Ab-5 (NeoMarkers), anti-phosphotyrosine-HRP (Transduction Laboratories, San Diego), mouse monoclonal anti-p21 (Transduction Laboratories), and mouse monoclonal anti-Tubulin (Oncogene, Cambridge, MA). Both monoclonal and polyclonal antibodies against the human Wwox protein were raised by using a GST-Wwox fusion protein according to standard protocols.

**GST Pull-Down Assay.** Using standard protocols, we used pGEX6P-2 plasmid (Amersham Pharmacia) to construct and express full-length WT-Wwox or mutated WWY33R fusions. In addition, we designed and constructed GST fusions of the first 50 amino acids of Wwox that include the first WW (WI) domain. GST-W1-WT, -WI-Y33F, -WI-Y334F, and -WI-Y333,34F were generated by using PCR and internal primers. Pull-down assays were performed by using immobilized purified GST fusion proteins or WT GST that was incubated with total cellular proteins prepared from 293 cells transiently transfected with the indicated plasmids. The lysates were precleared with glutathione-

Abbreviations: HA, hemagglutinin; siRNA, small interfering RNA; WI, first WW. 

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Association of Wwox and p73. (A) 293 cells were transiently transfected with the expression plasmids encoding HA-p73α, HA-p73β, or HA-ΔNp73 (as indicated at the top) and Myc-Wwox. Thirty-six hours after transfection, whole-cell lysates were immunoprecipitated (IP) with anti-HA, anti-igG, or anti-Myc antibodies. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA (Upper) or anti-Myc (Lower) antibodies. (B) 293 cells were cotransfected with 50 μM Metoposide for 16 h, and lysates were immunoprecipitated with anti-p73, anti-igG, or monoclonal anti-wwox antibodies, followed by immunoblotting with anti-p73 (Upper) or anti-wwox (Lower). (C) p53 and Myc-Wwox were coexpressed in 293 cells and immunoprecipitated by anti-p53, anti-igG, or anti-Myc antibodies, followed by immunoblotting with anti-p53 (Upper) or anti-Myc (Lower). (D) 293 cells were transiently transfected with the HA-p73β, HA-p73βY487A (D Lower), or HA-p73β (E) and Myc-Wwox expression plasmids. Immunoprecipitation and immunoblotting were as in A. (F) MCF-7 cells were cotransfected with HA-p73β (Upper) or HA-p73β (Lower). Lysates were immunoprecipitated with anti-HA, anti-igG, or monoclonal anti-wwox antibodies, followed by immunoblotting with anti-HA. (G) 293 cells were transiently transfected with the different Wwox expression constructs (as indicated at the top) and HA-p73β. Immunoprecipitation and immunoblotting were as in A. (H) 293 cells were transfected with HA-p73β or HA-p73β, and lysates were mixed with GST-WI-WT or GST alone. Complexes were captured with glutathione-Sepharose, and bound protein was detected by HA immunoblot. (I) 293 cells were transfected with HA-p73β or HA-p73β, and lysates were mixed with GST-WI-WT or GST alone and then treated as in H.

Apoptotic Assays. SAOS-2 cells were transfected as indicated and harvested 48 h later. DNA content was assessed by propidium iodide staining of methanol-fixed cells (19) and monitoring by FACSscan (Becton Dickinson).

In Vitro Kinase Assay. Purified active c-Src kinase (Biomol, Plymouth Meeting, PA) was incubated with GST-W1 fusions and ATP in kinase buffer (20 mM Hepes, pH 7.5/1 mM DTT/10 mM MgCl2) for 0 or 30 min at 30°C. Samples were analyzed by Western Blot with anti-phosphotyrosine (Transduction Laboratories, Lexington, KY).

Immunofluorescence. Cells were seeded on fibronectin-covered cell culture slides (Becton Dickinson), fixed for 10 min in 3.7% PBS-buffered formaldehyde, permeabilized with 0.05% Triton X-100 in PBS for 5 min, blocked with goat serum (GIBCO/BRL), and incubated with a primary antibody for 1 h in 10% goat serum in PBS and with secondary antibody under the same conditions. Antibodies used were mouse monoclonal anti-HA, anti-Myc rabbit polyclonal antibody (Upstate Biotechnology), anti-gst-Wwox serum, anti-mouse Fluoro-conjugated antibody, and anti-Rabbit Texas red-conjugated antibody (Molecular Probes). Cells were examined by confocal microscopy (Bio-Rad) under ×63 magnification.

Small Interfering RNA (siRNA) Treatment. A pool of siRNA duplexes directed against Wwox or GFP were designed and constructed by Dharmacon (Lafayette, CO). MCF-7 cells were transfected with 50 or 100 nM siRNA by using Oligofectamine reagent (Invitrogen). Forty-eight hours after siRNA transfection, cells were transfected with HA-p73α and lysates were mixed with the indicated GST fusions or GST alone. Cells were transfected with HA-p73β or HA-p73β, and lysates were mixed with GST-WI WT or GST alone and then treated as in H.
complexes were readily detectable from lysates by immunoprecipitating with monoclonal anti-p73 antibodies or monoclonal anti-Wwox and blotting with anti-p73 (Fig. 1B Upper, lane 3) or blotting with anti-Wwox antibodies (Fig. 1B Lower, lane 1). In a recent report, Chang et al. (21) reported that the murine Wox1 protein binds p53. To determine whether p53 also coimmunoprecipitates with human Wwox, we cotransfected 293 cells with Myc-Wwox and pCMV-p53 and immunoprecipitated them with anti-HA or anti-p53 antibodies. Under the same conditions where p73 interacts with Wwox, we were unable to demonstrate p53 binding to Wwox (Fig. 1C).

We next proceeded to identify regions responsible for Wwox–p73 association. Regarding p73, we had a strong indication from the affinity assays that the PPPPY motif of p73 was responsible for this interaction. We generated point mutations in this motif and determined the ability of these mutants to bind Wwox. Indeed, once we replaced Y487 with alanine (A), generating HA-p73(Y487A), the protein can no longer bind Wwox (Fig. 1D Lower, lane 3). This association was further investigated by cotransfecting 293 cells with HA-p73 Y (18), which naturally lacks the PPPPY motif, and Myc-Wwox. Coimmunoprecipitation followed by immunoblotting demonstrated the absence of interaction between p73 Y and Wwox (Fig. 1E, lane 6). Furthermore, we transfected MCF-7 breast carcinoma cells, which express endogenous Wwox at a high level (1, 9), with HA-p73β or HA-p73γ. Our results revealed that p73β, but not p73γ, interacts with endogenous Wwox in MCF-7 cells (Fig. 1F, lane 3). Taken together, these data suggest that binding to the PPPPY motif is responsible for the association between Wwox and p73. Because p53 lacks the PPPPY motif, this may provide an explanation for our inability to detect p53–Wwox complexes.

As mentioned above, the Wwox protein contains two WW domains, and the interaction of WW domains with other proteins can be regulated by tyrosine phosphorylation (11). To map the region responsible for binding with p73, we focused our attention on two tyrosines. Tyrosine 33 (Y33) located in the first WW domain was previously reported to affect the murine Wox1 interaction with JNK (22). We used tyrosine 61 (Y61) located in the second WW domain as a control. To determine the role of Y33 in p73 binding, we generated two point mutations, Y33 and Y61 were mutated to arginine (R), producing Myc-WWY33R and Myc-WWY61R. We then tested the ability of the mutated Wwox constructs to interact with p73 by coimmunoprecipitation. 293 cells were cotransfected with plasmids encoding HA-p73β and Myc-WWY33R or Myc-WWY61R, and extracts were precipitated with anti-HA or anti-Myc and blotted with anti-HA (Fig. 1G Upper) or anti-Myc (Fig. 1G Lower). We found that a point mutation, Y33R, in the first WW domain of Wwox abolishes Wwox association with p73 (Fig. 1G Upper, lane 6, and Fig. 1G Lower, lane 4). However, WWY61R can still bind p73 at a level comparable with WT-Wwox (Fig. 1G Upper, lane 9, and Fig. 1G Lower, lane 7). These results imply that Y33 is in the first WW domain of Wwox is critical for the association with p73.

To demonstrate direct binding between Wwox and p73, we carried out in vitro GST pull-down experiments. 293 cells were transfected with HA-p73β. Protein lysates were incubated with GST fusions that contain either a full-length Wwox proteins (Wwox or mutated WWY33R) or GST fusions that contain the first 50 amino acids of Wwox sequence that code for the WI domain and includes Y33. The pulled down proteins were immunoblotted with anti-HA antibody. A specific complex was pulled down when WT-Wwox or WT-WI GST fusions, but not GST alone, were incubated with p73β-expressing lysate (Fig. 1H). Moreover, both mutated constructs that harbor a point mutation in Y33, GST-WWY33R and GST-WWY33F, were unable to pull down p73 (Fig. 1H). In addition, we found that whereas p73β can be pulled down by GST-WL-WT fusion protein, p73γ could not (Fig. 1I), confirming that the PPPPY motif is responsible for this interaction.

Wwox Sequesters p73 in Cytoplasm. p73 was reported to localize in the nucleus, where it binds DNA and acts as a transcriptional factor to transactivate target genes such as p21 and Bax (14). In contrast, Wwox is a cytoplasmic protein. The question thus arises: in which subcellular compartment do Wwox and p73 interact? To answer this, we studied the localization of both proteins with the aid of confocal microscopy. HA-p73 alone or coexpressed with WT or mutated Myc-Wwox was transiently expressed in NIH 3T3 cells. Localization of the HA- or Myc-tagged proteins was then determined by immunofluorescent staining with the appropriate antibodies, as described in Materials and Methods. As shown in Fig. 2A, p73 alone localizes in the nucleus (a), whereas Wwox is localized in the cytoplasm (b). Interestingly, coexpression of p73 and WT-Wwox or WWY61R

Fig. 2. Subcellular localization of Wwox and p73. (A) NIH 3T3 cells were transfected as follows. (a) HA-p73β alone; (b) Myc-Wwox alone; (c1) Myc-Wwox; (c2) and HA-p73β; (d1) Myc-WWY33R; (d2) HA-p73β; (e1) Myc-WWY61R; (e2) HA-p73β. After fixation and permeabilization, the cells were stained with anti-HA and anti-Myc, followed by FITC-conjugated anti-mouse IgG (green; p73) and Texas red-conjugated anti-rabbit IgG (red; Wwox). (B) MCF-7 cells were transfected as follows. (f) empty vector; (g) HA-p73α; (h) HA-Δp73β; (i) HA-p73γ; (j) HA-p73ßY487A; (k) p53. Cells were prepared as in A. Antibodies used were polyclonal anti-wwox (f, g1, h1, i, j, and k), anti-HA (g2, h2, i, and j), and anti-p53 (k). (C and D) Effect of RNA interference directed against endogenous Wwox on p73 subcellular localization. MCF-7 cells were transfected with 100 nM siRNA as indicated. Forty-eight hours after treatment with siRNA, cells were transfected with HA-p73α plasmid for another 24 h. (C) Western blot analysis of siRNA effect on endogenous Wwox expression. (D) Immunofluorescence images showing Wwox-specific RNA interference effects in MCF-7 cells. Wwox is in red and p73 is in green. (Top) Images of mock-treated cells (no siRNA added). (Middle) Images of siRNA-treated cells. (Bottom) Images of irrelevant siRNA-treated cells. (Middle) Images of Wwox siRNA-treated cells.
causes the sequestration of p73 and colocalization with Wwox in the cytoplasm (Fig. 2 Ac and Ae). By contrast, cotransfection of WWY33R with p73 does not affect the nuclear localization p73 (Fig. 2Ad). To further show that relocalization of p73 to the cytosol is due to its interaction with Wwox, we transfected NIH 3T3 with equal amounts of HA-p73β and different quantities of Myc-Wwox. We found that a reduced amount of Wwox in transfection causes a decrease in expression of Wwox and retention of p73 in the nucleus of some cells (Fig. 5, which is published as supporting information on the PNAS web site, b vs. c).

To verify that these localization results also apply to endogenous Wwox, we transfected MCF-7 cells, which express high levels of endogenous Wwox (Fig. 2Bf), with HA-p73α or HA-ΔNp73 and examined intracellular localization. More than 50% of MCF-7 cells exhibited cytoplasmic p73α where it colocalized with endogenous Wwox (Fig. 2Bg). Moreover, we were able to detect colocalization of endogenous Wwox and ΔNp73 in MCF7 cells (Fig. 2Bh). Once again, expression of p73γ, p73βY487A, or p53 in MCF-7 cells did not lead to cytoplasmic localization (Fig. 2 Bi–Bk).

We further confirmed nuclear sequestration of p73 by using RNA interference (RNAi) technology. We constructed a pool of siRNA directed against Wwox. MCF-7 cells were transfected with Wwox-specific siRNAs for 48 h, followed by transfection with HA-p73α for another 24 h. Western blot analysis of cell extracts transfected with siRNA directed against Wwox showed specific knock down of the endogenous Wwox expression (Fig. 2C). To examine whether reduced expression of Wwox affects p73 localization, we assayed siRNA-treated cells by using confocal microscopy. Immunofluorescence images showed that cells that expressed much less Wwox (indicated by arrows) and coexpressed p73 retained nuclear p73 (Fig. 2D). By contrast, p73 colocalized with endogenous Wwox in the cytoplasm when we used an irrelevant siRNA directed against GFP (Fig. 2D). These results suggest that Wwox specifically sequesters p73, causing its retention in the cytoplasm.

**Wwox Suppresses p73-Mediated Transactivation.** To determine whether Wwox affects p73 transactivation function, we transfected SAOS-2 osteosarcoma cells, which express very low levels of Wwox and are p73 and p53 deficient, with a construct containing the luciferase gene driven by a p53 cis-element from the p53 promoter (p53-Luc) that can be used in a p73 transactivation assay (12, 13, 17). As expected, p73β had significant transactivation of luciferase activity (Fig. 3A, bars 2 and 9). Expression of Wwox significantly suppressed the transactivation function of p73 in a dose-dependent manner (Fig. 3A, bars 6, 7, 10, and 11). However, WWY33R enhanced p73 transactivation activity (Fig. 3A, bars 8 and 12).

To assess the role of Wwox in p73 transactivation, we assayed SAOS-2 cells for expression of p21, because it is known that transactivation by p73β is associated with increased expression of this protein (14). Lysates of the above-tested samples were immunoblotted with monoclonal antibodies against p21. Cotransfection of p73β and Wwox reduced the expression levels of p21 (Fig. 3B, lanes 3, 4, 7, and 8) compared with cells transfected with p73β alone (Fig. 3B, lanes 2 and 6). In contrast to WT-Wwox, cotransfection of p73β with WWY33R restores levels of p21 protein expression similar to those in cells transfected with p73 alone (Fig. 3B, lanes 5 and 9). Expression of Wwox and WWY33R were comparable in all experiments (Fig. 6, which is published as supporting information on the PNAS web site). These findings show that Wwox represses the transactivation function of p73 in a mode related to their interaction. Cotransfection of SAOS-2 cells with p73 results in the induction of apoptosis (13), we were prompted to test whether interaction between Wwox and p73 affects this process. Similarly, Wwox was also shown to induce apoptosis in transfected cells (21). Transfection of Wwox or p73β alone increases the number of cells with sub-G1 DNA content compared to cells expressing empty vector (Fig. 3C and Fig. 7, which is published as supporting information on the PNAS web site). Cotransfection of p73β and Wwox markedly increased the number of apoptotic cells. To confirm the importance of the interaction between Wwox and p73 in the induction of apoptosis, we cotransfected ΔNp73, which had no apoptotic effect when published as supporting information on the PNAS web site, b vs. c).
expressed alone, together with Wwox and found that the apoptotic population still increased under these circumstances (Fig. 3C). Taken together with our intracellular localization results, this finding suggests that Wwox sequestered p73 in the cytoplasm, where p73 increases Wwox proapoptotic activity independently of its transcriptional activity. Because WWY33R increases the transcriptional activity of p73 (Fig. 3A and B), we determined whether this increase affects the p73 proapoptotic effect. Because alteration of Y33 in Wwox was previously shown to reduce the proapoptotic activity of Wwox (22), we transfected SAOS-2 cells with WWY33R alone and examined its effect on the number of apoptotic cells. Because we observed that disturbing Y33 in the first WW domain of Wwox that contain the WI domain and include Y33, Y34, or both tyrosines were mutated to phenylalanine (F) producing WI-Y33F, WI-Y34F, and WI-Y33,34F. The three GST-WI fusions together with WT-WI domain were incubated with recombinant Src in the presence of ATP. Analysis of the products by Western Blot with anti-phosphotyrosine antibody showed that both WI-WT and WI-Y34F GST fusions, but not WI-Y33F and WI-Y33,34F, could be directly phosphorylated by Src (Fig. 4E).

**Discussion**

In the present study, we have demonstrated the physical interaction between the recently discovered Wwox and the p53 homolog, p73. This interaction involves the first WW domain of Wwox and a p73 region containing the PPPPY408 motif that immediately precedes the SAM domain (Fig. 1). WW domain association with proline-rich ligands depends mainly on the structure of these motifs (10, 11). Our results suggest that the first WW domain of Wwox binds to ligands containing a PPPY motif and belongs to the first class (class I) of the current classification of the diverse WW domains. Unlike p73α, p73β, and ΔNp73, Wwox does not coimmunoprecipitate with p73γ or p53 (Fig. 1). Both p73γ and p53 lack the proline-rich motif PPPPY, perhaps explaining the absence of complex formation with Wwox. Interestingly, a point mutation in the terminal tyrosine of the PPPPY motif also abrogates Wwox–p73 interaction, emphasizing the principal of sequence-specific protein–protein interaction as a determinant of precise biological output(s).
On the Wwox side, tyrosine (Y) 33 in the first WW domain has an important role in binding to p73. Alteration of Y33 disturbed the Wwox–p73 association, whereas Y34 in the first WW domain and Y61 in the second WW domain are dispensable for this interaction (Fig. 1G). We have also demonstrated specific phosphorylation of Y33 (Fig. 4), which regulate Wwox–p73 interaction. The first WW domain of Wwox harbors a sequence that can be phosphorylated by Src-family tyrosine kinases, perhaps indicating that Src directly phosphorylates Wwox. p73 can also be phosphorylated by tyrosinekinases such as c-Abl (24). Our data showed that Wwox bound phosphorylated p73 (Fig. 4B); however, levels of phosphorylated p73 were independent of Src kinase.

Interestingly, the Wwox–p73 interaction takes place in the cytoplasm, where Wwox usually resides (Fig. 2). Overexpression of Wwox caused the redistribution of p73 from the nuclear compartment to the cytosol. p73 has a nuclear localization sequence as well as a nuclear exporting sequence, located at its C terminus (25). Inoue et al. had shown that the nuclear export is necessary for efficient degradation of p73. We have now showed that nuclear export of p73 may also be important for interaction with and modulation of cytosolic proteins, such as Wwox (Figs. 2 and 3). p73 interacts with Wwox in the cytoplasm and enhances its proapoptotic activity; direct evidence for this conclusion comes from the expression of a p73 variant that lacks the amino-terminal transactivation domain (ΔNp73). ΔNp73 interacts with Wwox in the cytoplasm (Fig. 2B) and enhances its apoptotic activity (Fig. 3D). ΔNp73 by itself is incapable of inducing cell death (15), and thus, the increased apoptosis resulting from the coexpression of Wwox and ΔNp73 may be due to increased Wwox-related apoptosis. Although Wwox suppresses p73 transcriptional activity, as shown by luciferase assay (Fig. 3A), we detected increased apoptotic activity (Fig. 3D). We believe that this effect is due to p73 function in the cytoplasm through interaction with Wwox, and therefore, loss of Wwox in tumor cells could result in reduced apoptotic activity of p73 in the cytoplasm. By contrast, WWY33R that does not bind p73 increases its transcriptional activity. Interestingly, this increase in p73 transactivation was reflected in the apoptotic activity of p73. One possible explanation of this effect of WWY33R is that this mutation has a dominant negative effect on endogenous Wwox.

The WWOX gene is located within a very active common fragile site, FRA16D. Similar to FHit (26), its expression is altered in several cancer types (1, 2, 4–7) and it behaves as a tumor suppressor protein (9). Although mutations of p73 in cancer are not a frequent event (14) and p73-deficient mice show no tumor phenotype (27), several reports established the role of p73 in apoptosis, and its modulation of p53 function can be important for malignancy development (14). Thus, it is important to understand how these gene products function and how their loss affects malignant transformation. We provide evidence that p73 and Wwox interact both in vitro and in vivo, and that this interaction results in an increased rate of cell death. Additional genetic and biochemical approaches will elucidate the biological consequences of this association in normal and cancer cells.

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