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Essential Role for Caspase-8 in Transcription-independent Apoptosis Triggered by p53*

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Han-Fei Ding‡§, Yi-Ling Lin‡, Gaël McGill‡¶, Peter Juo‖, Hong Zhu‖, John Blenis‖, Junying Yuan‖, and David E. Fisher‡**

From the ‡Department of Pediatric Hematology/Oncology, Dana-Farber Cancer Institute and Children's Hospital, Boston, Massachusetts 02115 and ‖Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

p53's dual regulation of arrest *versus* apoptosis may underlie tumor-selective effects of anti-cancer therapy. p53's apoptotic effect has been suggested to involve both transcription-dependent and -independent mechanisms. It is shown here that caspase-8 is activated early in cells undergoing p53-mediated apoptosis and in S100 cell-free extracts that recapitulate transcription-independent apoptosis. Depletion or inactivation of caspase-8 either in cells or cell-free extracts completely prevents this transcription-independent apoptosis and significantly attenuates overall death induced by wild-type p53. Importantly, caspase-8 activation appears to be independent of FADD, and caspase-8 is found in a novel 600-kDa complex following p53 activation. These findings highlight the roles of both transcription-dependent and -independent apoptosis by p53 and identify an essential role for caspase-8 in the transcription-independent pathway.

Dysregulation of apoptosis plays a major role in the development of cancer (1). p53 modulates the apoptotic response in tumor cells following signals such as DNA damage, growth factor deprivation, and hypoxia (2–4). p53-mediated apoptosis has been implicated as an important mechanism by which many antitumor treatments kill cancer cells (5, 6). Its unique ability to trigger cell cycle arrest (7) or apoptosis (8–10) suggests that p53 may confer a favorable therapeutic index to cancer cells during antineoplastic treatment (11, 12). The mechanism(s) by which p53 modulates activation of caspases during apoptosis remain incompletely understood. p53 is a sequence-specific transcription factor (2–4). A number of studies provide evidence that transcriptional activation by p53 is required for apoptosis in some experimental systems. For example, baby rat kidney cells stably expressing E1A and a temperature-sensitive p53 mutant underwent apoptosis when shifted to the permissive temperature, whereas baby rat kidney cells transformed by E1A and a transcriptionally defective temperature-sensitive p53 mutant failed to do so (13). In another study, microinjection of wild-type p53, but not transcription-defective mutants, into p53-null mouse embryo fibroblasts (MEFs)[1] expressing E1A induced apoptosis. Moreover, a chimeric transcription activator protein in which the p53 transactivation domain located at the amino-terminal region was replaced with that of herpes simplex virus VP16 protein remained competent in mediating apoptosis, arguing that transcription activation, rather than the amino-terminal region of p53 *per se*, is important for the p53 apoptotic function (14). Consistent with these findings, several transcriptional target genes of p53 have been identified whose up-regulation has been shown to promote apoptosis (15–18).

By contrast, there is also strong evidence that p53 can induce apoptosis independent of its transcription function. Using three somatotropic cell lines that express a temperature-sensitive p53 mutant, Caelles *et al.* (19) found that a shift to the permissive temperature (32.5 °C) triggers apoptosis following UV-C irradiation even in the presence of transcriptional and translational inhibitors. Similarly, activation of Myc in MEFs expressing a temperature-sensitive p53 mutant induced apoptosis at the permissive temperature in the presence of the translational inhibitor cycloheximide (20). p53 mutations have also been identified that uncouple transcriptional and apoptotic activities for p53 (21–24). These findings suggest the existence of a transcription-independent pathway for mediating apoptosis by p53. This apoptotic pathway has been further identified through use of an apoptosis assay in which S100 cellular extracts display a requirement for p53 protein in mediating caspase activation during *in vitro* incubation (25).

Apoptosis is executed by the caspase family of cysteine proteases (26–29). Caspase-9 appears to play a major role in p53-dependent apoptosis, based on loss of thymocyte radiosensitivity (30, 31) and oncogene-dependent apoptosis in embryonic fibroblasts (32) from caspase-9 null mice. Caspase-9 was also identified as an intermediate in oncogene-induced apoptosis of human fibroblasts (33). Since cytochrome *c* is an essential coactivator of caspase-9 (34), these studies suggest that apoptotic effectors of p53 may target mitochondria to induce cell death, a possibility further corroborated by the observation that p53 overexpression up-regulates Bax, which can directly mediate mitochondrial cytochrome *c* release (15, 35, 36). Other caspases, such as caspase-2, -7, and -8, have been found to be activated in cells undergoing p53-dependent apoptosis (37–39), but it has been unclear whether their activation is required for p53-dependent apoptosis.

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** Nirenberg Fellow at the Dana-Farber Cancer Institute. To whom correspondence should be addressed: D630, Division of Pediatric Hematology/Oncology, Dana-Farber Cancer Institute and Children's Hospital, 44 Binney St., Boston, MA 02115. Tel.: 617-632-4916; Fax: 617-632-2085; E-mail: david_fisher@dfci.harvard.edu.

[1] The abbreviations used are: MEF, mouse embryo fibroblast; FADD, Fas-associated protein with a novel death domain; DN, dominant negative; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; CMV, cytomegalovirus.

Here, using both cell-free (S100) and cell-based systems, we demonstrate that activation of caspase-8 significantly precedes caspase-3 during p53-mediated apoptosis. Caspase-8 activation is found to be essential for transcription-independent apoptosis triggered by p53 and is accompanied by formation of a newly identified 600-kDa complex containing a processed form of caspase-8 but no measurable FADD. Within cells, inactivation of FADD by a dominant negative mutant fails to interfere with the transcription-independent apoptotic process, suggesting a novel pathway for caspase-8 activation during p53-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Preparation of Cell-free Extracts—p53$^{+/+}$ or p53$^{-/-}$ E1A/Ras-transformed MEFs (gift of Scott Lowe, Tyler Jacks, and David Housman (5, 6)) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% newborn calf serum. Exponentially growing cells at ~80% confluence were either untreated or treated with 10 grays of ionizing radiation using a Gammacell 40 irradiator equipped with a ^{137}Cs source. At various times after irradiation (1–8 h, thereby designated as 1–8-h extracts), the cells were harvested, collected by centrifugation, and washed once with ice-cold phosphate-buffered saline. To make S100 cell-free extracts, the cell pellet was resuspended at 10^8 cells/0.5 ml of ice-cold buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 3 mM dithiothreitol, 4 mM Pefabloc, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 15 min, the cells were disrupted by Dounce homogenizing 15 times in a Wheaton Dounce homogenizer with a tight pestle. The lysates were directly centrifuged at 100,000 $\times$ *g* for 1 h in a Beckman Optima TLX-100 ultracentrifuge. The resulting supernatants (S100 cell-free extracts) were collected.

Immunoblot Analysis and Colorimetric Assay of Caspase Activation—For examination of *in vivo* caspase activation, extracts prepared at various time points postirradiation were subjected to 12.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then probed sequentially with antibodies against mouse caspase-8 and -3 and visualized by ECL (Amersham Pharmacia Biotech). For examination of *in vitro* caspase activation, samples of 10 μl of extracts prepared at 1 h following irradiation were incubated at 32 °C for various times, followed by immunoblot analyses. The antiserum against caspase-8 was generated by injecting rats with a peptide encompassing the active site of caspase-8. The antibody to caspase-3 (p20, L-18) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For colorimetric assays of caspase-3 activity, 10 μl of extract was incubated at 32 °C either for 12 h in the absence of cytochrome *c* or for 1 h in the presence of cytochrome *c* (200 ng; Sigma). At the end of the incubation, 10 μl of 2$\times$ reaction buffer (BioVision) and 2 μl of DEVD-pNA (4 mM, BioVision) were added, and the mixtures were incubated for 30 min at 32 °C, followed by reading in a spectrophotometer at 405 nm, which measures pNA cleaved from the labeled substrate DEVD-pNA by activated caspase-3.

Immunodepletion—15 μl of rat anti-caspase-8 serum or 20 μg of rabbit IgG against caspase-9 (H170, Santa Cruz Biotechnology) was incubated with 250 μl of S100 extract for 1 h on ice. The immune complexes were then cleared by incubation with a bovine serum albumin-precoated and washed protein A/G-agarose bead pellet (from 50 μl of 50% suspension; Life Technologies) for 4 h in a rotator at 4 °C. The beads were subsequently pelleted by centrifugation for 2 min in a microcentrifuge at 4 °C, and the resulting supernatants were collected and analyzed by immunoblotting.

Plasmid Constructs—The following plasmids have been used in transient transfection studies. pCMV-p53wt encodes wild-type human p53, and pCMV-p53(Gln^{22}/Ser^{23}) codes for human p53 carrying two tandem point mutations at residues 22 and 23, which is defective in transactivation function (40). pcDNA3-FLICE(C360S) encodes a dominant negative mutant of human caspase-8 with cysteine to serine mutation at the residue 360 (41). pcDNA3-Mch6(C287A) encodes a dominant negative mutant of human caspase-9 in which the residue 287 is altered from cysteine to alanine (42). pcDNA3-FADD DN codes for a dominant negative mutant of human FADD in which part of the N-terminal death effector domain is deleted (43). Full-length cDNAs coding for CrmA, a potent inhibitor of caspase-8 (44), and Bcl-2 were cloned into the expression vector pBabe. pRSV-LacZ and pEGFP (CLONTECH) were used as markers in transient transfection studies.

Transient Transfection, Apoptosis, and Survival Assays—Saos2 cells (p53-null) were seeded at a density of 1.5 $\times$ 10^5 per well in six-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Jurkat cells were seeded at a density of 10^6 cells/well in six-well plates in RPMI 1640 supplemented with 10% fetal bovine serum. 24 h later, the cells were transfected with a total of 1.2 μg of plasmid DNA, 0.2 μg of pRSV-LacZ (Saos2) or pEGFP (Jurkat) plus 1 μg of effector plasmids (as listed in the Figs. 3–5), using the FuGene 6 protocol (Roche Molecular Biochemicals). All effectors were controlled using empty vector transfections. For Saos2 cells, plasmids encoding caspase-8 DN (C360S), FADD DN, CrmA, caspase-9 DN (C287A), or Bcl-2 were used at a ratio of 2:1 with the wild-type p53 or the p53(Gln^{22}/Ser^{23}) expression construct. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells were discriminated from apoptotic cells using criteria previously described for this cell system (45). Apoptotic cells are smaller and round with membrane blebbing. Cells were counted from 20 randomly selected fields (200X) (46), with numbers normalized to the vector control. For Jurkat cells, 24 h after transfection, cells were harvested and washed once with ice-cold phosphate-buffered saline. The cells were then incubated sequentially with annexin V-biotin (Trevigen) and R-phycoerythrin-streptavidin (Jackson ImmunoResearch), followed by flow cytometry analysis. The transfected cells undergoing apoptosis were detected based on both green fluorescent protein expression and annexin V staining.

Size Exclusion Chromatography Analysis of a Caspase-8-containing Complex—0.6 ml (2.5 mg of protein) of extract from irradiated, transformed MEFs was applied to a Superose-6 HR (10/30) column (Amersham Pharmacia Biotech). The column was eluted with buffer A plus 50 mM NaCl at 0.3 ml/min, and 1.0-ml fractions were collected. Aliquots (100 μl) of the Superose-6 column fractions were analyzed by immunoblotting with antibodies against caspase-8 and FADD (StressGen). The column was calibrated with protein standards (Bio-Rad), including bovine thyroglobulin (670 kDa), bovine IgG (158 kDa), chicken ovalbumin (44 kDa), and horse myoglobin (17 kDa).

RESULTS

Caspase-8 Activation in p53-mediated Apoptosis—E1A/Ras-transformed p53$^{+/+}$ MEFs, but not p53$^{-/-}$ MEFs, undergo rapid and extensive apoptosis following γ-irradiation (5). To examine caspase activation during p53-dependent apoptosis, E1A/Ras-transformed p53$^{+/+}$ or p53$^{-/-}$ MEFs were studied at various time points following γ-irradiation. For p53$^{+/+}$ cells, immunoblots reveal processing of caspase-8 within 4 h (p44, Fig. 1*A*, *lanes 1–7*). Activation of caspase-3 is observed about 2 h later (Fig. 1*A*, *lanes 1–7*). No caspase activation is observed in extracts from irradiated p53$^{-/-}$ cells (Fig. 1*A*, *lanes 8–11*). The temporal pattern of caspase-8 cleavage is consistent with the possibility that it may function upstream of caspase-3 during p53-dependent apoptosis.

We next examined the kinetics of caspase-8 and -3 activation in a S100 cell-free system that recapitulates key features of p53-dependent apoptosis in the absence of transcriptional activities (25). In this system, p53-dependent apoptosis is triggered by ionizing radiation, and whole cell extracts (S100) are prepared early enough that removal of endogenous p53 protein prevents subsequent activation of caspase-3 after *in vitro* incubation at 32 °C. As with *in vivo* processing, caspase-8 cleavage precedes caspase-3 by several hours (Fig. 1*B*, *lanes 1–12*). S100 extracts from irradiated, transformed p53$^{-/-}$ MEFs reveal no caspase-8 or -3 activation, even after 16-hours (Fig. 1*B*, *lanes 13–16*). Lack of caspase activation in p53$^{-/-}$ extracts is not due to intrinsic resistance to activation *in vitro*, because the addition of cytochrome *c* leads to efficient activation of caspase-3 *in vitro* without regard to the presence or absence of p53 (Fig. 1*C*, *lanes 1–4*). The cell-free system is thus also consistent with the possibility of an upstream role for caspase-8 in transcription-independent apoptosis by p53.

Requirement of Caspase-8 Activity for Transcription-independent Apoptosis Triggered by p53—To test the functional requirement of caspase-8, *in vitro* immunodepletion was carried out at 4 °C prior to incubation at 32 °C. Immunodepletion of caspase-8 or -9 had no significant effect on levels of caspase-3 protein (Fig. 2*A*). Extracts containing or lacking caspase-8 or -9

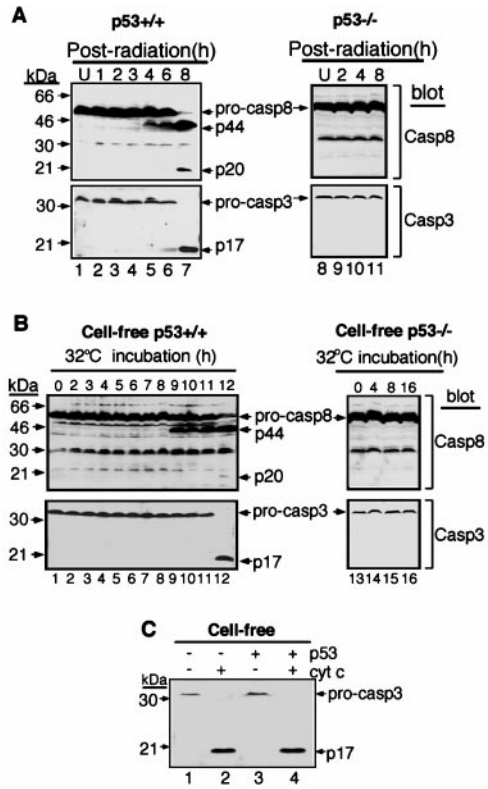

FIG. 1. **Caspase-8 is activated early in p53-mediated apoptosis.** *A*, caspase activation in transformed MEFs ($p53^{+/+}$ or $p53^{-/-}$) following 10-gray irradiation. Extracts were prepared from untreated (*U*) or irradiated cells at the indicated time points (hours) after γ-irradiation. Activation of caspases was examined by immunoblotting with antibodies against individual caspases. The characteristic cleavage products of caspase-8 (p44 and p20) and caspase-3 (p17) are indicated. *B*, caspase activation in S100 cell-free extracts. Extracts were prepared from E1A/Ras-transformed MEFs ($p53^{+/+}$ or $p53^{-/-}$) at 1 h after 10-gray irradiation and incubated at 32 °C for the indicated times, followed by immunoblot analyses for caspase activation. *C*, activation of caspase-3 in cell-free extracts by cytochrome *c* (*cyt c*). Extracts were prepared from transformed MEFs ($p53^{+/+}$ or $p53^{-/-}$) and incubated at 32 °C for 1 h in the absence or presence of cytochrome *c* (200 ng), followed by immunoblot analyses for caspase-3 activation.

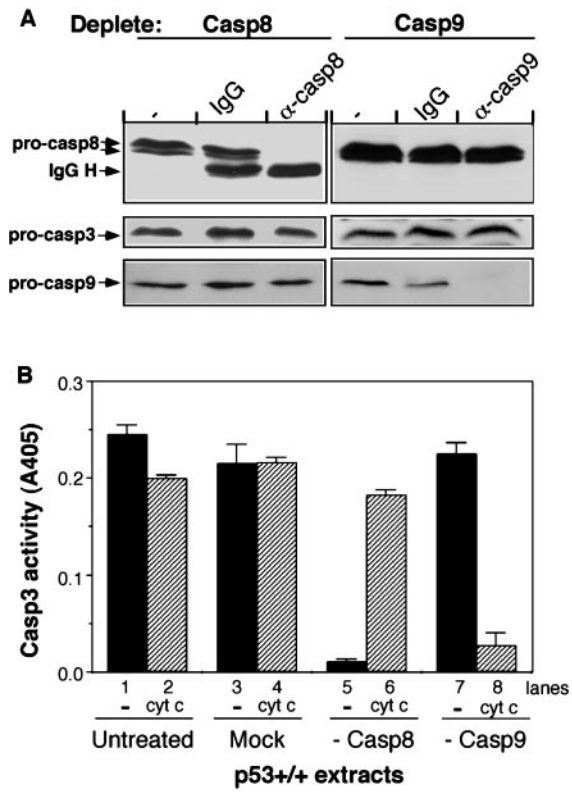

FIG. 2. **Caspase-8 is required for caspase-3 activation in p53-dependent cell-free extracts.** *A*, immunodepletion of caspase-8 or -9. Extracts were prepared from transformed $p53^{+/+}$ MEFs at 1 h after 10-gray irradiation. Caspase-8 or -9 was depleted from the extracts with a rat antiserum against mouse caspase-8 or rabbit polyclonal IgG against caspase-9. Control immunodepletion was performed with normal rat serum or rabbit IgG. The immunoblots were sequentially probed with antibodies against caspase-8, -3, and-9. The position of rat IgG heavy chain (IgG H) is indicated. *B*, colorimetric assays of caspase-3 activity. Extracts containing or lacking caspase-8 or -9 were incubated at 32 °C either for 12 h in the absence of cytochrome *c* or for 1 h in the presence of cytochrome *c* (200 ng). The caspase-3 substrate DEVD-pNA was then added for 30 min at 32 °C. Caspase-3 activity was determined by measuring the cleavage of DEVD-pNA. Data are mean ± S.D. from three independent experiments. p53-dependent apoptosis (*black bars*) was ablated by depletion of caspase-8 but was intact following depletion of caspase-9. In contrast, cytochrome *c*-mediated activation was ablated by depletion of caspase-9 but was intact following depletion of caspase-8.

were then incubated at 32 °C, and subsequent caspase-3 activation was measured. Depletion of caspase-8 blocked subsequent induction of caspase-3 activity (Fig. 2*B*, compare *lanes 1*, *3*, *5*, and *7*). This effect was specific for caspase-8, because cytochrome *c* was still able to trigger caspase-3 activation. In contrast, depletion of caspase-9 did not affect p53-mediated caspase-3 activation, whereas it ablated cytochrome *c* responsiveness (Fig. 2*B*, *lanes 7* and *8*). In all cases, antibody control immunodepletions did not affect caspase-3 activation. These data suggest that caspase-8 is essential for the p53-dependent caspase-3 activation observed in these S100 cell-free extracts.

To study the role of caspase-8 in transcription-independent apoptosis in cells, a transactivation-deficient mutant, $p53(Gln^{22}/Ser^{23})$ (40), was employed, which triggers apoptosis in multiple cell lines, including p53-deficient Saos2 cells and HeLa cells (21, 47). We first asked whether inhibition of caspase-8 activity by expression of a caspase-8 DN mutant (C360S) (41) or CrmA, a cowpox virus serpin that is a potent inhibitor of caspase-8 (44), would protect cells from p53-mediated apoptosis. As shown in Fig. 3*A*, overexpression of either wild type p53 or $p53(Gln^{22}/Ser^{23})$ in Saos2 cells resulted in significant cell death 30 h after transfection together with LacZ to mark transfected cells (*lanes 1*, *4*, and *7*). Expression of either caspase-8 DN (C360S) or CrmA completely blocked cell death induced by $p53(Gln^{22}/Ser^{23})$ (Fig. 3*A*, *lanes 7–9*). Caspase-8 DN (C360S) or CrmA also partially protected Saos2 cells from cell death induced by wild-type p53, as indicated by a 2-fold increase in cell survival (Fig. 3*A*, *lanes 4–6*). The partial effect of caspase-8 DN on wild-type p53 is consistent with the existence of a second mechanism through which p53 can activate caspases, probably a transcription-dependent one not measured by the Gln^{22}/Ser^{23} mutant.

We next asked whether $p53(Gln^{22}/Ser^{23})$ could induce apoptosis in a mutant Jurkat cell line specifically lacking caspase-8 (48). This cell line lacks the ability to undergo Fas-dependent apoptosis but has been shown to retain apoptotic effectors including other caspases (48). Parental and caspase-8-deficient Jurkat cells were transfected with either wild-type p53 or $p53(Gln^{22}/Ser^{23})$. Both wild-type and mutant p53 induced significant levels of apoptosis in the parental Jurkat cells (Fig. 3*B*, *lanes 1* and *3*). Caspase-8-deficient Jurkat cells showed complete resistance to $p53(Gln^{22}/Ser^{23})$ and intermediate sensitivity to wild-type p53 at 24 h (Fig. 3*B*). This effect was maintained through 72 h, and expression of either caspase-8 DN (C360S) or CrmA in the parental Jurkat cells also inhibited mutant p53-induced cell death (data not shown). These distinct cell-based systems and cell-free manipulations demonstrate

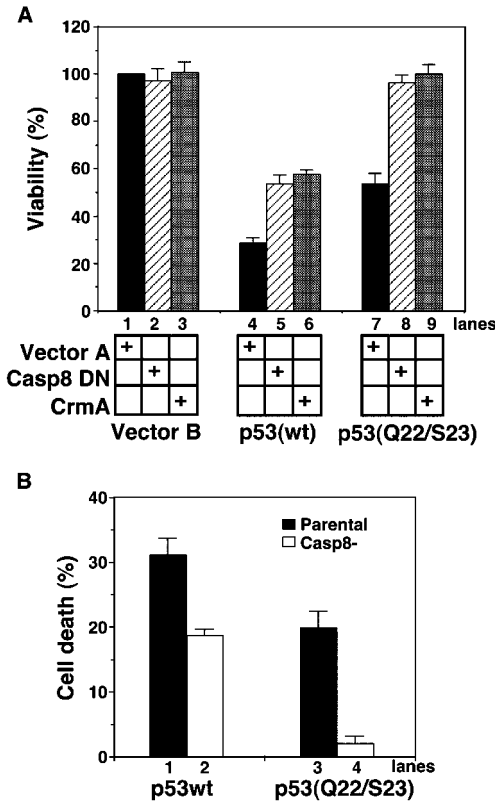

FIG. 3. **Caspase-8 is required for transcription-independent apoptosis induced by p53.** *A*, Saos2 cells were cotransfected with pRSV-LacZ and expression vectors coding for either wild-type p53, p53(Gln^{22}/Ser^{23}), caspase-8 (C360S), or CrmA, as indicated. pcDNA3 (vector A) and pRc/CMV (vector B) were used as controls. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells, determined by morphology, were counted from 20 randomly selected fields (200X). Data were normalized to the vector control (defined as 100% survival) and are displayed as mean ± S.E. from representative experiments done in triplicate. *B*, the parental and caspase-8-deficient mutant Jurkat cells were cotransfected with pEGFP and either pCMV-p53(wt) or pCMV-p53(Gln^{22}/Ser^{23}). 24 h after transfection, apoptotic cells were quantitated by annexin V-based fluorometric assays. Data are mean ± S.D. from four experiments.

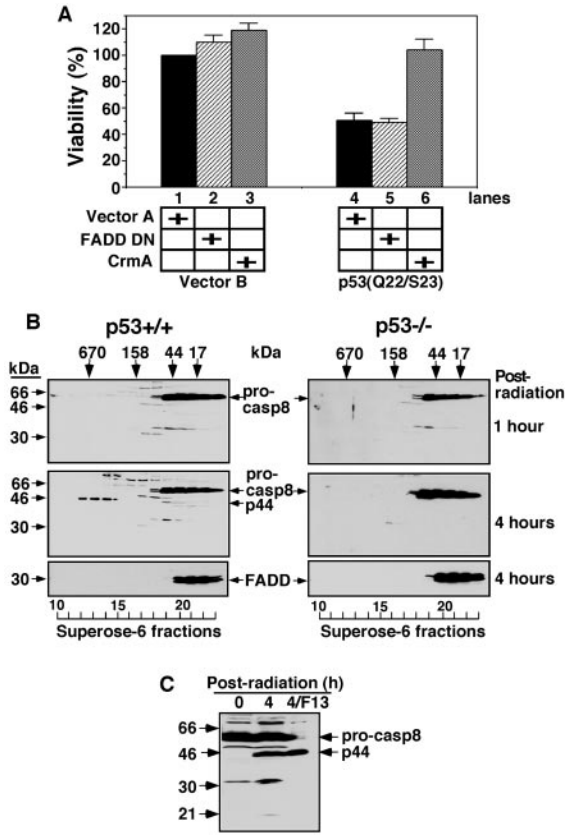

FIG. 4. **FADD is not required p53-dependent apoptosis.** *A*, Saos2 cells were cotransfected with pRSV-LacZ and expression vectors coding for either p53(Gln^{22}/Ser^{23}), FADD DN, or CrmA, as indicated. pcDNA3 (vector A) and pRc/CMV (vector B) were used as controls. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells, determined by morphology, were counted from 20 randomly selected fields (200X). Data were normalized to the vector control (defined as 100% survival) and are displayed as mean ± S.E. from representative experiments done in triplicate. *B*, extracts prepared from transformed $p53^{+/+}$ or $p53^{-/-}$ MEFs at indicated hours following irradiation were fractionated by size exclusion chromatography using a Superose-6 HR column. Aliquots (100 μl) of the indicated fractions were analyzed by immnunoblotting for the presence of procaspase-8, its processed form (p44), and FADD. The elution positions of molecular weight markers are indicated. *C*, unfractionated $p53^{+/+}$ extracts (untreated or 4 h after irradiation) and fraction 13 of the $p53^{+/+}$ 4-h extracts (shown as *U*, *4*, or *4/F13*) were analyzed by immunoblotting with an antibody against caspase-8. Procaspase-8 and its processed p44 form are indicated and demonstrate that the caspase-8 species in fraction 13 co-migrates with p44.

that caspase-8 is an essential component in p53-induced, transcription-independent apoptosis. Moreover, wild type p53's intermediate effects in the setting of caspase-8 deficiency suggest the existence of a second (probably transcription-dependent), caspase-8-independent death mechanism. Consistent with these observations, non-oncogene-transformed embryonic fibroblasts from caspase-8 knockout mice retain sensitivity to UV radiation, etoposide, ceramide, staurosporine, or serum starvation (49).

Caspase-8 Activation in p53-mediated Apoptosis Is Independent of FADD—Caspase-8 is an essential initiator caspase in the Fas-induced apoptotic pathway (48–51). Activation of caspase-8 in Fas-triggered apoptosis requires FADD (52), a death domain-containing protein (53, 54). Since FADD is a candidate scaffold for caspase-8 in this pathway, and in light of several studies suggesting a link between p53 and Fas in signaling apoptosis (55–58), we examined the role of FADD in p53-triggered, transcription-independent apoptosis. Saos2 cells were transfected with p53(Gln^{22}/Ser^{23}) along with an expression vector coding for either FADD DN, a dominant negative inhibitor that blocks Fas-induced apoptosis (43), or CrmA. Inhibition of caspase-8 activity by expression of CrmA protected Saos2 cells from apoptosis induced by p53(Gln^{22}/Ser^{23}); in contrast, no protective effects were observed with expression of FADD DN (Fig. 4*A*, compare *lane 5* with *lane 6*). The same FADD-DN was significantly protective against Fas-induced apoptosis in these same cells and similarly had no effect on wild type p53 (data not shown). These data are consistent with the report that E1A/Ras transformed, FADD-deficient MEFs are as sensitive as wild type transformed MEFs to the induction of apoptosis by the chemotherapeutic drug adriamycin (52), a p53-mediated response (5).

Oligomerization of caspase-8 molecules is a critical step in the process of caspase-8 activation (59, 60), which generally results from their association with a caspase-8-recruiting complex, such as the death-inducing signaling complex in Fas-dependent apoptosis (61). We therefore investigated the possibility that caspase-8 may also associate in a complex during p53-dependent apoptosis. S100 extracts from E1A/Ras-transformed $p53^{+/+}$ or $p53^{-/-}$ MEFs were prepared at 1 or 4 h after irradiation (1- or 4-h extracts) and fractionated by size exclusion chromatography, using Superose-6 high resolution gel filtration. Fractions were collected and analyzed for the presence of caspase-8. In both 1- and 4-h extracts from $p53^{-/-}$ cells,

caspase-8 eluted as the unprocessed monomeric form (Fig. 4*B*, *right panels*, fractions 19–23), correlating with lack of caspase activation in the absence of p53 (see Fig. 1). The same state of caspase-8 was also observed in 1-h extracts from $p53^{+/+}$ cells (Fig. 4*B*, *top left panel*). However, a different elution profile was observed in 4-h extracts from $p53^{+/+}$ cells. Whereas unprocessed procaspase-8 molecules still migrated as monomers (Fig. 4*B*, *middle left panel*, fractions 19–23), the 44-kDa cleaved caspase-8 intermediate eluted in a larger complex of about 600 kDa (fractions 12–14). Side by side analysis of unfractionated $p53^{+/+}$ extracts (untreated or 4 h after irradiation) together with the ~600-kDa fraction (F13, Fig. 4*C*) confirmed that the 44-kDa species displays the same mobility as the prominent 44-kDa processed form of caspase-8 in cells and p53-dependent extracts (Fig. 4*C*, see also Fig. 1, *A* and *B*). These results are reminiscent of the recent finding that p35/37 processed forms of caspase-9 are associated with Apaf-1-containing apoptosome complexes (62, 63). Since FADD is known to recruit caspase-8 to the death-inducing signaling complex in Fas-induced apoptosis, we next asked whether FADD is present in this high molecular weight complex following ionizing radiation. In contrast to caspase-8, FADD eluted exclusively in small (probably monomeric) size fractions regardless of p53 status or time after irradiation (Fig. 4*B*, *bottom panels*, fractions 20–23). Although it is possible that FADD could have been released prior to or upon association of caspase-8 with the complex and thus could not be detected by our analysis, the gel filtration results are consistent with the observations that FADD is not required for caspase-8 activation in p53-dependent apoptosis, as suggested by studies with FADD DN (above) and cells derived from FADD-null mice (52).

Requirement for Caspase-9 in p53-induced Transcription-independent Apoptosis—Studies with caspase-9-null cells show that caspase-9 is essential for p53-dependent apoptosis (30–32). To address caspase-9's functional role in the transcription-independent pathway examined here, we transfected Saos2 cells with p53(Gln^{22}/Ser^{23}) along with an expression vector encoding caspase-9 DN. Inhibition of caspase-9 activity by expression of a caspase-9 dominant negative mutant (C287A) (42) protected Saos2 cells from apoptosis induced by p53(Gln^{22}/Ser^{23}) (Fig. 5*A*), consistent with data from caspase-$9^{-/-}$ cells (30–32). Caspase-9 thus appears to be an essential component in the transcription-independent apoptotic pathway within cells. The dispensability of caspase-9 for caspase-3 activation in S100 extracts may reflect sufficient accumulation of active caspase-8 to mediate direct cleavage of caspase-3, which is well described in other cell-free caspase-8 apoptosis systems (64, 65). In cells, the caspase-8 signal may be significantly amplified through caspase-9 activation.

How might caspase-8 lead to activation of caspase-9 in cells? Caspase-8 could activate caspase-9 through either direct or indirect means. Caspase-9 is activated in transformed $p53^{+/+}$ MEFs at 4 h following γ-irradiation, as indicated by the appearance of p35/37-processed forms of caspase-9 (Fig. 5*B*, *top panel*, *lane 2*). Consistent with recent reports (62, 63), we found that activated caspase-9 proteins p35/37 reside in a complex of ~700 kDa (data not shown). However caspase-9 was not detected in anti-caspase-8 immunoprecipitates from fractions containing the 600-kDa/caspase-8 complex (Fig. 5*B*, *top panel*, *lane 3*), whereas activated caspase-8 was present in the same precipitates (Fig. 5*B*, *bottom panel*, *lane 3*). It is possible that physical interactions between caspase-8 and caspase-9 could be weak or transient, thus escaping detection. However, these observations are also consistent with the possibility that activation of these two caspases occurs through formation of distinct apoptotic complexes.

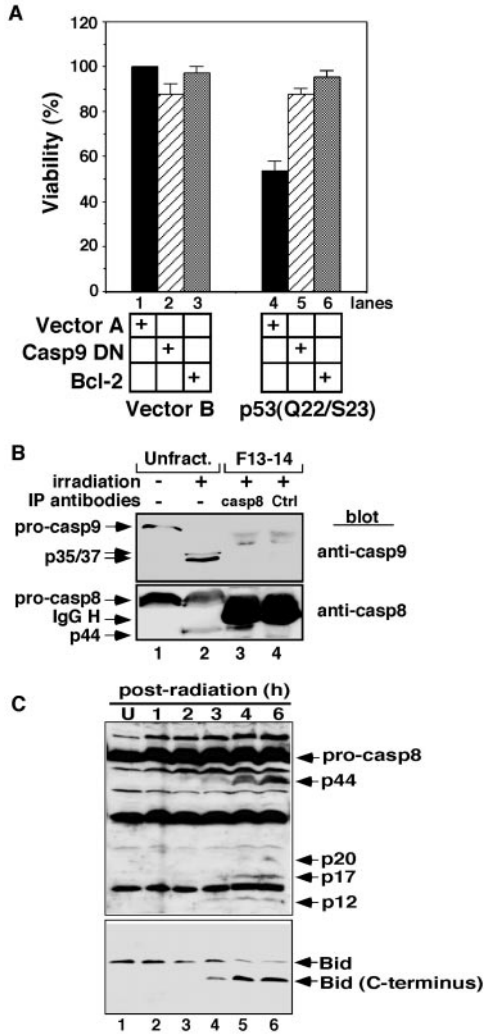

FIG. 5. **Requirement for caspase-9 in transcription-independent apoptosis triggered by p53.** *A*, Saos2 cells were cotransfected with pRSV-LacZ and expression vectors coding for either p53(Gln^{22}/Ser^{23}), caspase-9 DN, or Bcl-2, as indicated. pcDNA3 (vector A) and pRc/CMV (vector B) were used as controls. 30 h after transfection, the cells were fixed and stained with X-gal. Viable blue cells, determined by morphology, were counted from 20 randomly selected fields (200X). Data were normalized to the vector control (defined as 100% survival) and are displayed as mean ± S.E. from three experiments done in triplicate. *B*, extracts prepared from transformed $p53^{+/+}$ MEFs at 4 h following irradiation were fractionated by size exclusion chromatography using a Superose-6 HR column. Fractions 13–14 (*F13–14*), which contain the p44-processed form of caspase-8, were pooled and immunoprecipitated with either a rat antiserum against caspase-8 (*lane 3*) or a control rat serum (*lane 4*). The immunoprecipitates were analyzed by immunoblotting for the presence of caspase-9 (*top panel*) and caspase-8 (*bottom panel*). Unfractionated $p53^{+/+}$ extracts (unirradiated, *lane 1*; 4 h after irradiation, *lane 2*) were used as controls. The positions of procaspases and their processed forms are indicated. *C*, extracts were prepared from untreated (*U*) or irradiated, transformed $p53^{+/+}$ MEFs at the indicated time points (hours) after γ-irradiation. The proteins were separated by a 12.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was then sequentially probed with antibodies against caspase-8 and the C terminus of Bid. Procaspase-8, its cleavage products (p44, p20, p17, and p12), and the C-terminal fragment of Bid are indicated.

One indirect means through which caspase-8 might regulate caspase-9 activation is through a Bcl-2-regulated pathway. Bcl-2 is thought to block mitochondrial permeability of apoptotic factors including cytochrome *c* (66, 67), an essential co-activator for caspase-9 activation (34). As shown in Fig. 5*A*, Bcl-2 protected against transcription-independent apoptosis triggered by the double mutant of p53 (Fig. 5*A*). Moreover,

immunoblot analyses with an antibody against the C terminus of Bid, a proapoptotic member of the Bcl-2 family, showed that Bid was cleaved at 3 h following irradiation of transformed $p53^{+/+}$ MEFs, correlating closely with the appearance of activated caspase-8 (Fig. 5*C*). It has been shown that after cleavage by caspase-8, truncated Bid translocates from the cytosol to the mitochondrial membrane and induces release of cytochrome *c* that in turn binds to Apaf-1 and promotes activation of caspase-9 (68, 69). Together, these data are consistent with a potential role for mitochondria in activation of caspase-9 by caspase-8 in p53-dependent apoptosis, although other mechanisms, such as direct activation by proteolysis, could also be involved.

DISCUSSION

Our studies with both cell-free and cell-based systems demonstrate an essential role for caspase-8 in p53-mediated, transcription-independent apoptosis. Caspase-8 activation during p53-triggered apoptosis appears to be mediated by a FADD-independent mechanism, probably involving a 600-kDa complex associated with processing of caspase-8. These findings suggest a biochemical pathway for p53-triggered, transcription-independent apoptosis. In response to DNA damage and other forms of genotoxic stress, the p53 protein level and its biochemical activity are rapidly increased. Activated p53 protein may recruit or facilitate the assembly of a caspase-8-activating complex through protein-protein interactions. Caspase-8 may then target caspase-9 either through a mitochondrial pathway or by direct proteolysis, resulting in activation of caspase-3 and apoptosis.

Several lines of evidence suggest that the 600-kDa complex participates in caspase-8 activation during p53-dependent apoptosis. Initiator caspases, such as caspase-8 and -9, are in general activated by association within a complex (70). So far, the 600-kDa complex is the only larger form of caspase-8 we have identified in extracts from cells undergoing p53-dependent apoptosis, and it is clearly observed within membrane-free S100 lysates (unlike Fas/FADD-recruitment of caspase-8 (61)). The identification of partially processed caspase-8 (p44) within a complex is analogous to the recent finding that p35/37 processing intermediates of caspase-9 are associated with Apaf-1-containing apoptosome complexes (62, 63). The 600-kDa complex may function in a similar manner for caspase-8 activation. Finally, the timing of complex formation correlates with the start of caspase-8 processing during p53-dependent apoptosis (Fig. 1*A*), suggesting its role in the initiation of caspase-8 activation. Further identification of components within this complex as well as the pathway leading to its formation should shed light on this alternate path to caspase-8 activation.

Caspase-8 requires caspase-9 to mediate transcription-independent apoptosis triggered by p53. This observation is consistent with a number of recent studies showing a role for caspase-9 in caspase-8-induced apoptosis of certain types of cells. Notably, Fas-induced apoptosis is significantly inhibited in Apaf-$1^{-/-}$ MEFs (71). In addition, the dominant negative caspase-9 mutant (C287A) has been found to block apoptosis of MCF-7 cells induced by Fas agonist antibody, TRAIL, and overexpression of TRAIL receptors DR4 and DR5 (42). Finally, hepatocytes deficient in Bid, which mediates cytochrome *c* release from mitochondria in response to caspase-8 cleavage, are resistant to apoptosis triggered by Fas, although caspase-8 has been activated (72). In our study, the cleavage of Bid correlates closely with the appearance of activated caspase-8, suggesting a molecular mechanism by which caspase-8 may activate the mitochondria-caspase-9 death pathway during p53-dependent apoptosis. It is currently unclear whether other intermediates, such as Smac/DIABLO (73–75) and direct proteolysis, are involved in caspase-9 activation by caspase-8 in our systems.

Immunodepletion of caspase-9 from the S100 cell-free extracts has no effect on activation of caspase-3 (Fig. 2*A*), demonstrating that caspase-9 is dispensable in the extracts for caspase-8-mediated activation of caspase-3. This behavior probably reflects sufficient accumulation of active caspase-8, which has been shown to be able to directly activate caspase-3 (64, 65, 76). *In vivo*, the caspase-8 signal may be significantly amplified through caspase-9 activation, a step that appears to be of particular importance for p53-mediated apoptosis. It will be interesting to determine which cell types and death triggers employ such amplification via caspase-8 and to understand the biochemical basis for that need.

It may appear redundant that p53 retains both transcription-dependent and -independent pathways leading to stress-induced cell death. Yet such stress-induced apoptosis may explicitly fail if the stress were severe enough to affect transcription/translation. In such settings, a cascade involving protein-protein interactions is reminiscent of posttranslational stress response mechanisms and may be required to produce apoptotic death. It is unclear to what degree the two pathways (transcription-dependent *versus* -independent) are employed in different physiologic contexts, but the inhibition of caspase-8, as employed here, appears to uncouple the two and may aid in the examination of one pathway in the absence of the other.

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REFERENCES

1. Thompson, C. B. (1995) *Science* **267,** 1456–1462
2. Ko, L. J., and Prives, C. (1996) *Genes Dev.* **10,** 1054–1072
3. Levine, A. J. (1997) *Cell* **88,** 323–331
4. Gottlieb, T. M., and Oren, M. (1998) *Semin. Cancer Biol.* **8,** 359–368
5. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993) *Cell* **74,** 957–967
6. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994) *Science* **266,** 807–810
7. Kuerbitz, S., Plunkett, B., Walsh, W., and Kastan, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7491–7495
8. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991) *Nature* **352,** 345–347
9. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) *Nature* **362,** 847–849
10. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993) *Nature* **362,** 849–852
11. Fisher, D. E. (1994) *Cell* **78,** 539–542
12. Kastan, M. B., Canman, C. E., and Leonard, C. J. (1995) *Cancer Metastasis Rev.* **14,** 3–15
13. Sabbatini, P., Lin, J., Levine, A. J., and White, E. (1995) *Genes Dev.* **9,** 2184–2192
14. Attardi, L. D., Lowe, S. W., Brugarolas, J., and Jacks, T. (1996) *EMBO J.* **15,** 3693–3701
15. Miyashita, T., and Reed, J. C. (1995) *Cell* **80,** 293–299
16. Wu, G., Burns, T., McDonald, E., III, Jiang, W., Meng, R., Krantz, I., Kao, G., Gan, D.-D., Zhou, J.-Y., Muschel, R., Hamilton, S., Spinner, N., Markowitz, S., Wu, G., and El-Deiry, W. (1997) *Nat. Genet.* **17,** 141–143
17. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) *Nature* **389,** 300–305
18. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000) *Science* **288,** 1053–1058
19. Caelles, C., Helmberg, A., and Karin, M. (1994) *Nature* **370,** 220–223
20. Wagner, A. J., Kokontis, J. M., and Hay, N. (1994) *Genes Dev.* **8,** 2817–2830
21. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995) *Genes Dev.* **9,** 2170–2183
22. Rowan, S., Ludwig, R. L., Haupt, Y., Bates, S., Lu, X., Oren, M., and Vousden, K. H. (1996) *EMBO J.* **15,** 827–838
23. Sakamuro, D., Sabbatini, P., White, E., and Prendergast, G. C. (1997) *Oncogene* **15,** 887–898
24. Bissonnette, N., Wasylyk, B., and Hunting, D. J. (1997) *Biochem. Cell Biol.* **75,** 351–358
25. Ding, H.-F., McGill, G., Rowan, S., Schmaltz, C., Shimamura, A., and Fisher, D. E. (1998) *J. Biol. Chem.* **273,** 28378–28383
26. Salvesen, G. S., and Dixit, V. M. (1997) *Cell* **91,** 443–446
27. Cryns, V., and Yuan, J. (1998) *Genes Dev.* **12,** 1551–1570
28. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
29. Wolf, B. B., and Green, D. R. (1999) *J. Biol. Chem.* **274,** 20049–20052
30. Kuida, K., Haydar, T. F., Kuan, C.-Y., Gu, Y., Taya, C., Karasuyama, H., Su,

M. S.-S., Rakic, P., and Flavell, R. A. (1998) *Cell* **94,** 325–337
31. Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penninger, J. M., and Mak, T. W. (1998) *Cell* **94,** 339–352
32. Soengas, M. S., Alarcon, R. M., Yoshida, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. (1999) *Science* **284,** 156–159
33. Fearnhead, H. O., Rodriguez, J., Govek, E., Guo, W., Kobayashi, R., Hannon, G., and Lazebnik, Y. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13664–13669
34. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91,** 479–489
35. Rosse, T., Olivier, R., Monney, L., Rager, M., Conus, S., Fellay, I., Jansen, B., and Borner, C. (1998) *Nature* **391,** 496–499
36. Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., and Green, D. R. (2000) *J. Biol. Chem.* **275,** 7337–7342
37. Chandler, J. M., Alnemri, E. S., Cohen, G. M., and MacFarlane, M. (1997) *Biochem. J.* **322,** 19–23
38. Gao, C., and Tsuchida, N. (1999) *Jpn. J. Cancer Res.* **90,** 180–187
39. Kim, A. L., Raffo, A. J., Brandt-Rauf, P. W., Pincus, M. R., Monaco, R., Abarzua, P., and Fine, R. L. (1999) *J. Biol. Chem.* **274,** 34924–34931
40. Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994) *Genes Dev.* **8,** 1235–1246
41. Vincenz, C., and Dixit, V. M. (1997) *J. Biol. Chem.* **272,** 6578–6583
42. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998) *Mol. Cell* **1,** 949–957
43. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 4961–4965
44. Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V. M., and Salvesen, G. S. (1997) *J. Biol. Chem.* **272,** 7797–7800
45. Hardenbergh, P. H., Hahnfeldt, P., Hlatky, L., Takemoto, C., Shimamura, A., McGill, G., Fung, C. Y., Bodis, S., and Fisher, D. E. (1998) *Int. J. Rad. Oncol. Biol. Phys.* **43,** 601–695
46. Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z. (1999) *Genes Dev.* **13,** 2514–2526
47. Chen, X., Ko, L., Jayaraman, L., and Prives, C. (1996) *Genes Dev.* **10,** 2438–2451
48. Juo, P., Kuo, C. J., Yuan, J., and Blenis, J. (1998) *Curr. Biol.* **8,** 1001–1008
49. Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Bechmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O., Lapidot, T., Soffer, D., Sobe, T., Avraham, K. B., Goncharov, T., Joltmann, H., Lonai, P., and Wallach, D. (1998) *Immunity* **9,** 267–276
50. Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996) *Cell* **85,** 803–815
51. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., Orourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85,** 817–827
52. Yeh, W.-C., de la Pompa, J. L., McCurrach, M. E., Shu, H.-B., Elia, A. J., Shahinian, A., Ng, M., Wakeham, A., Khoo, W., Mitchell, K., El-Deiry, W. S., Lowe, S. W., Goeddel, D. V., and Mak, T. W. (1998) *Science* **279,** 1954–1958
53. Chinnaiyan, A., O'Rourke, K., Tewari, M., and Dixit, V. (1995) *Cell* **81,** 505–512
54. Boldin, M., Varfolomeev, E., Pancer, Z., Mett, I., Camonis, J., and Wallach, D. (1995) *J. Biol. Chem.* **270,** 7795–7798
55. Muller, M., Strand, S., Hug, H., Heinemann, E. M., Walczak, H., Hofmann, W. J., Stremmel, W., Krammer, P. H., and Galle, P. R. (1997) *J. Clin. Invest.* **99,** 403–413
56. Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R., and Weissberg, P. (1998) *Science* **282,** 290–293
57. Fukazawa, T., Fujiwara, T., Morimoto, Y., Shao, J., Nishizaki, M., Kadowaki, Y., Hizuta, A., Owen-Schaub, L. B., Roth, J. A., and Tanaka, N. (1999) *Oncogene* **18,** 2189–2199
58. Rakkar, A. N., Katayose, Y., Kim, M., Craig, C., Ohri, E., Li, Z., Cowan, K. H., and Seth, P. (1999) *Cell Death Differ.* **6,** 326–333
59. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) *J. Biol. Chem.* **273,** 2926–2930
60. Yang, X., Chang, H. Y., and Baltimore, D. (1998) *Mol. Cell* **1,** 319–325
61. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995) *EMBO J.* **14,** 5579–5588
62. Rodriguez, J., and Lazebnik, Y. (1999) *Genes Dev.* **13,** 3179–3184
63. Cain, K., Bratton, S. B., Langlais, C., Walker, G., Brown, D. G., Sun, X.-M., and Cohen, G. M. (2000) *J. Biol. Chem.* **275,** 6067–6070
64. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) *J. Biol. Chem.* **272,** 2952–2956
65. Stennicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) *J. Biol. Chem.* **273,** 27084–27090
66. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136
67. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129–1132
68. Li, H., Zhu, H., Xu, C.-J., and Yuan, J. (1998) *Cell* **94,** 491–501
69. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) *Cell* **94,** 481–490
70. Hengartner, M. (1998) *Science* **281,** 1298–1299
71. Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A., and Gruss, P. (1998) *Cell* **94,** 727–737
72. Yin, X., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) *Nature* **400,** 886–891
73. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) *Cell* **102,** 33–42
74. Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Mortz, R. L., Simpson, R. J., and Vaux, D. L. (2000) *Cell* **102,** 43–53
75. Green, D. R. (2000) *Cell* **102,** 1–4
76. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) *EMBO J.* **17,** 1675–1687

Essential Role for Caspase-8 in Transcription-independent Apoptosis Triggered by p53

Han-Fei Ding, Yi-Ling Lin, Gaël McGill, Peter Juo, Hong Zhu, John Blenis, Junying Yuan and David E. Fisher

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