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Expression by the p53 Tumor Suppressor Protein

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13. ABSTRACT (Maximum 200 Words) This proposal focuses on the tumor suppressor protein p53. This work had three goals: the first goal was to identify the promoter elements in two genes, survivin and c-IAP2, that confer transcriptional repression by p53. The second was to use these p53-repressible elements in order to create a mutant adenovirus, in which a key gene for viral replication (E1A) was controlled by p53-repressible elements. The resultant virus should replicate only in cells where p53 was mutated or deleted, but not in normal cells; we are poised to test this hypothesis in Year 3. The final goal involved the codon 72 polymorphic variants of p53. We previously found that the Arginine 72 form of p53 (R72), a naturally occurring polymorphic variant, has greatly enhanced ability to kill cells (Dumont et al., Nat Genet 2003) due to increased mitochondrial localization. In the last progress report we proposed to elucidate the mechanism underlying enhanced cell death by R72; here-in we report that this involves direct binding of p53 to the pro-apoptotic protein BAK. A manuscript describing this work is included in the Appendix. Efforts for year 3 will follow up on this finding.				
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Progress Report
ARMY grant DAMD-17-02-1-0383
Maureen E. Murphy, Ph.D.

I. Introduction

The goal of the work proposed for this award, which has just completed Year 2, is to understand the function of the p53 tumor suppressor protein. p53 is mutated or inactivated in over 30% of breast carcinomas, making it one of the most frequently mutated genes in breast cancer. It is currently accepted that the strong selection for mutation of p53 during breast tumor development is due to the ability of this protein to detect inappropriate cell proliferation, and to induce the cell to commit cellular suicide, or programmed cell death. The goals of proposed work are two-fold: the first is to use information generated in year 1, on the identification of promoter elements in the p53-repressed *survivin* and *cIAP-2* promoters that confer transcriptional repression by p53, toward the creation of a novel adenovirus for gene therapy. Specifically, these p53-repressible elements would be placed upstream of the adenovirus E1A gene in a recombinant adenovirus (dl1520). The resultant virus would express the E1A gene only in cells with inactive p53, and consequently this virus would be expected to be selectively cytolytic, or toxic, only to tumor cells that had inactive p53, and not to normal cells.

The second goal is more directly related to the ability of p53 to kill a tumor cell. Specifically, our previous work had found that a common polymorphic variation in the p53 gene dramatically alters its ability to induce programmed cell death (apoptosis). This common variant, encoding arginine at codon 72 (R72) has over 15-fold increased ability to induce cell death than the P72 (proline 72) form. Despite this fact, the P72 form of p53 has been the subject of the overwhelming majority of functional studies on this protein, and is the only form of p53 used for gene therapeutic approaches. R72 is homozygous in up to 36% of Caucasians, but only 10% of African Americans. We proposed in Year 2 to generate recombinant adenoviruses encoding the R72 and P72 forms of p53, and compare their efficacy at cell killing. A natural corollary of this goal involved first gaining better insight into the mechanism whereby the R72 form of p53 has enhanced cell killing of breast carcinoma cells.

II. Body

A. Overview of Progress, Year 2:

Year 2 was spent engrossed with two goals; the first was to create a *survivin* promoter-driven version of the E1A gene; this would be used in transfections, along with the dl1520 adenovirus genome, toward the goal of using homologous recombination to generate a mutant form of adenovirus dl1520 in which the *survivin* promoter replaces the E1A promoter. Such a virus should be selectively cytolytic to tumor cells with mutant p53, as cells with normal p53 would repress the expression of E1A, and the virus would fail to replicate. Our homologous recombination approach has proven unsuccessful in generating this recombinant adenovirus, possibly due to the low replication efficiency of the parental dl1520 virus. Therefore, we have chosen to follow the advice from an expert in adenovirus construction (Dr. Yuqiao Shen, ONYX Pharmaceuticals), and use direct cloning techniques to create our mutant virus. Toward that goal, we have successfully created unique restriction sites flanking the *survivin* promoter construct, using site directed mutagenesis. We plan to use these novel sites to directly ligate the *survivin* promoter into the dl1520 genome. This latter approach has been successful generating recombinant adenovirus in a recent publication¹, and is now the preferred mechanism for generating mutant viruses.

The second goal of year 2 was to elucidate the mechanism whereby the R72 form of p53 has enhanced ability to kill tumor cells. We reasoned that such information would be critical before we could effectively compare the cell killing effects of P72 and R72 in adenoviral vectors. Knowing from our previous work that the basis for enhanced cell kill by the R72 form of p53 involved an increased trafficking of that protein to mitochondria, we used a mass spectrometry based approach to identify proteins that interact with p53 in mitochondria purified from a breast cancer cell line. This analysis revealed that following genotoxic stress p53 traffics to mitochondria and binds to the BCL2 family member BAK. BAK is a known pro-apoptotic protein on the mitochondrial outer membrane that, when induced to oligomerize, forms a supra-molecular pore that allows cytochrome c to be released, which then activates the 'apoptosome', leading to caspase activation. We identified the BAK binding domain on p53, and found that p53 can directly oligomerize BAK and cause cytochrome c to be released in cells. This work was recently published in *Nature Cell Biology* (this manuscript acknowledges funding from this award, and is included in Appendix); it sets the stage for our analysis of the differences in cell death potential by adenoviral infection of P72 and R72. Notably, it also has relevance for understanding how the codon 72 polymorphism of p53 may be a risk factor for breast cancer, as has been reported by others^{2,3}.

B. Detailed Progress:

Task 2. Creation of a selectively replicating version of adenovirus dl1520. Test this virus for lytic cell death in breast carcinoma samples with mutant, versus wild type, p53, as well as immortalized breast epithelial cells.

At the end of the last progress report, we had identified the p53-repressible element in the *survivin* promoter (Task 1). This was found to consist of a non-canonical p53 binding site, adjacent to an E2F-inducible element⁴. We cloned approximately 400 nucleotides spanning this element, but not including the start site of transcription, flanked by HindIII/XbaI sites. Before cloning this into the dl1520 genome, we used site-directed mutagenesis to change this XbaI site to BamHI. This was necessary to take advantage of unique HindII/BamHI sites upstream of the E1A coding region in dl1520. This was recently accomplished. We are currently ligating this construct into genomic DNA isolated from the dl1520 virus. This DNA will be used in a transfection approach to generate mutant virus (Figure 1).

Figure 1

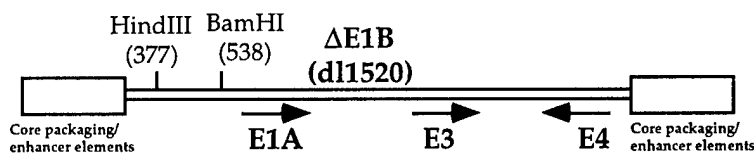


Figure 1. Diagram of adenovirus genome, for use in construction of the survivin-promoter driven E1A mutant adenovirus.

The unique HindIII/BamHI sites will be used to replace the E1A promoter with the p53-repressible *survivin* promoter.

Task 3. Identification of the mechanism whereby R72 kills tumor cells better than P72. Creation of recombinant adenoviruses expressing either the P72, or the R72, forms of p53; compare these for cell kill.

It is important to note that we have only recently found that the R72 form of p53 kills tumor cells 15-fold better than the P72 polymorphic variant⁵. However, the P72 variant was the first form of p53 cloned, and hence **the P72 variant is the only form used to date in gene therapy approaches to tumor kill**. Toward elucidating the pathway whereby the R72 form of p53 kills cells better (and hence would be predicted to be a more effective gene therapy device), we have focused on our finding that the R72 form of p53 has significant localization to mitochondria following genotoxic stress.

Using sucrose gradient centrifugation, we purified mitochondria from the breast carcinoma cell line ZR-75-01, before and after treatment with the chemotherapeutic drug adriamycin. We used an immunoprecipitation approach to identify p53-interacting proteins in these mitochondria, and compared these with proteins that interact with p53 in mitochondria from a cell line with inducible p53 (as an added control for specificity). Mass spectrometry (MALDI/TOF) analysis of these interacting proteins revealed the identity of nine proteins that interact with p53 in mitochondria. Notably, two of these proteins were previously identified, by our group and others, to interact with mitochondrial p53 (Hsp 60 and Grp 75, both heat shock proteins) (see Figure 2).

Figure 2

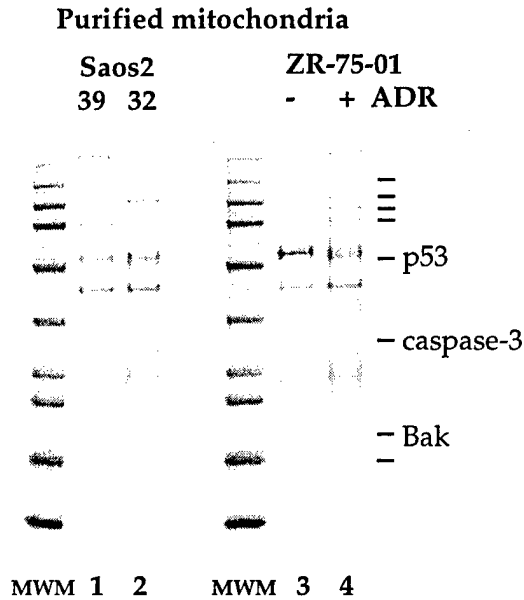


Figure 2. Purification of p53-interacting proteins in mitochondria isolated from the breast carcinoma cell line ZR-75-01.

Sucrose gradient centrifugation was used to purify mitochondria from cells with inducible p53 (Saos2 at 32 degrees = wild type R72 form of p53) or a breast carcinoma cell line where p53 is induced by exposure for 24 hours with 0.5 ug/mL adriamycin (ADR). p53, and interacting proteins, was immunoprecipitated using p53 antisera (p53 is marked, running just under IgG heavy chain). The co-associated proteins BAK, and caspase-3, identified by mass spectrometry, are indicated. MWM= molecular weight markers.

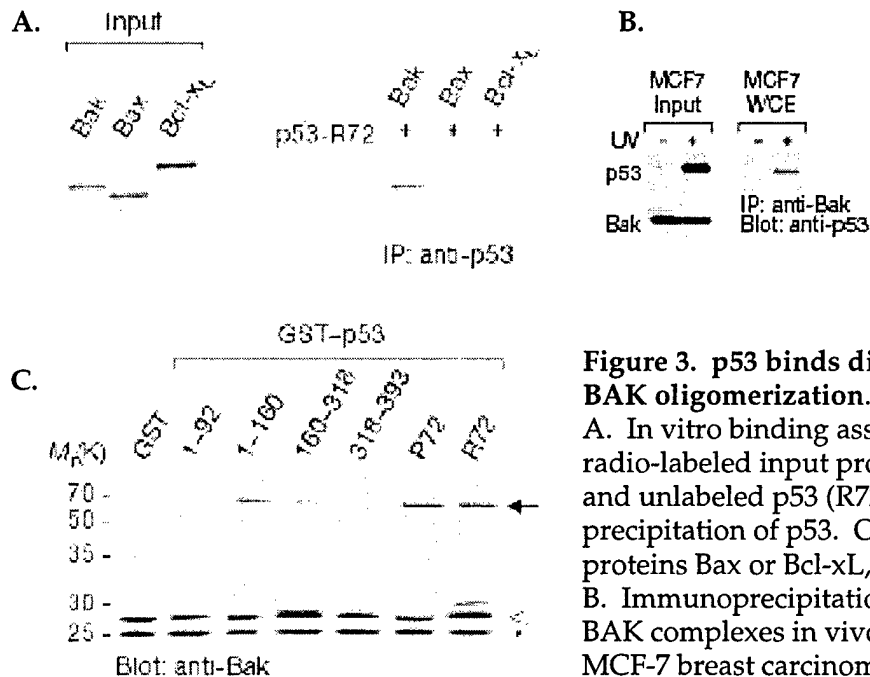


Figure 3. p53 binds directly to BAK, and catalyzes BAK oligomerization.

A. In vitro binding assay using in vitro translated, radio-labeled input proteins (BAK, BAX, or Bcl-xL) and unlabeled p53 (R72) followed by immunoprecipitation of p53. Only BAK, and not the related proteins Bax or Bcl-xL, bind in this assay.

B. Immunoprecipitation-western analysis of p53-BAK complexes in vivo, following DNA damage of MCF-7 breast carcinoma cells (7.5 J/m² UV-C).

C. Addition of p53 GST fusion proteins that can bind to BAK in vitro (GST 1-160, GST 160-318, and R72 and P72) to mitochondria purified from healthy cells is sufficient to induce oligomerization of BAK. Following 20 minute incubation with GST-p53, mitochondria are treated with cross-linking reagent (BMH) and the BAK oligomers (marked by arrow) are induced.

We identified the pro-apoptotic BCL2 family member BAK as a p53-interacting protein in the mitochondria of the breast carcinoma ZR-75-01. We confirmed that p53 interacts with BAK in other cell lines (including MCF-7 breast carcinoma, and prostate carcinoma LnCaP). Significantly, we showed that p53 directly interacts with BAK, and

more importantly, can catalyze the oligomerization of BAK (Figure 3). We found that the oligomerization of BAK by p53 is both necessary and sufficient for cytochrome c release from purified mitochondria. A manuscript describing this work, and acknowledging funding from this source, was recently published in *Nature Cell Biology*⁶, and was accompanied by a New and Views highlighting the significance of this work⁷.

Interestingly, we recently assessed the p53-BAK interaction in four breast carcinoma cell lines; two of these contain wild type p53 (heterozygous P/R), and two contain mutant forms of p53 protein. Both tumor cell lines with wt p53 (MCF-7 and ZR-75-01) has robust p53-BAK interaction after incubation with an agent of DNA damage (camptothecin). Interestingly, however, one tumor cell line with mutant p53, MDA-468, also showed a significant p53-BAK interaction, along with apoptotic cell death (see Figure 4). These data indicate that certain mutant forms of p53 may still respond to DNA damaging agents by interacting with BAK and inducing cell death. These data are consistent with data by Moll and colleagues, indicating that certain tumor-derived mutant forms of p53 are constitutively localized to mitochondria⁸. It will be important to test other breast carcinoma cell lines with mutant p53, to determine if there are particular mutants that consistently are able to bind and oligomerize BAK.

Figure 4

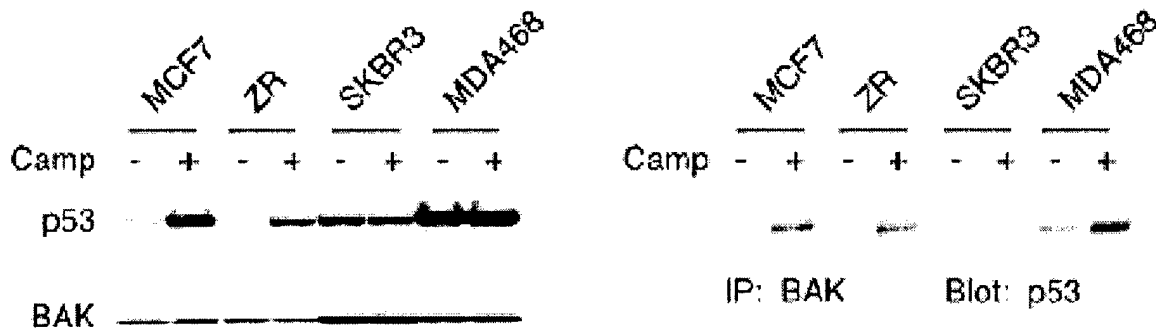


Figure 4. Breast carcinomas with mutant forms of p53 (MDA 468, R273H mutation) do not induce p53 protein in response to camptothecin, but do form p53-BAK complexes after treatment, and do undergo apoptosis (not shown). Data on the left are westerns of whole cell lysates; data on the right are the results of immunoprecipitation of BAK followed by western analysis for associated p53. The data are representative of three independent experiments.

Goals for the next year: With our mutagenized survivin promoter-E1A construct successfully made, we are poised to generate and test our mutant dl1520 adenovirus. With the knowledge that the key mechanism of cell death induction by mitochondrial p53 is binding and oligomerization of BAK, we are poised to compare the ability of P72, and R72, and certain tumor-derived mutant forms of p53, to respond to DNA damage and induce apoptosis. Specific Goals for Year 3 include:

- **Creation of the *survivin*-promoter driven dl1520 mutant adenovirus.**
 - a. We will complete ligation of the *survivin* promoter to the digested dl1520 genome. We will package this genome to create mutant virus.
 - b. We will test the hypothesis that the *survivin*-promoter driven dl1520 virus is selectively toxic to tumor cells, and not normal cells. We will test the ability of

this virus to kill multiple different human breast carcinoma cell lines containing mutant, and wild type, p53.

- **Generation of adenoviral vectors encoding the proline 72 (P72) and arginine 72 (R72) versions of p53.**
 - a. We will create these viruses using the ViraPower Adenoviral Gene expression system (Invitrogen) and compare the ability of these viruses to kill breast carcinoma cell lines, using our Guava Flow Cytometer, which performs 3 different apoptosis assays (Annexin binding, caspase activation, TUNEL assay).
 - b. We will determine the ability of virally-infected p53 to bind and oligomerize endogenous BAK in breast tumor cell lines.

- **Elucidation of the mechanism whereby mutant forms of p53 can be activated by DNA damaging agents, and bind and oligomerize BAK.**
 - a. We will acquire ten different breast carcinoma cell lines with known mutant forms of p53. We will treat these cell lines with DNA damaging agents and test the interaction of p53 with BAK, and test the ability of these DNA damaging agents to induce apoptosis.
 - b. We will use short interfering RNAs (SmartPool RNA, Clontech) for p53, to eliminate the mutant p53 in these breast tumor cell lines, in order to test the hypothesis that tumors with mutant p53 are killed better than tumors with no p53, due to the ability of mutant p53 to bind and oligomerize BAK.

III. Key Accomplishments:

- Created the SpV-E1A construct (*survivin* promoter-driven adenovirus E1A). Used site-directed mutagenesis to create novel restriction sites, for use in cloning the survivin promoter upstream of the E1A/E1B locus in dl1520, a mutant adenovirus with deletion of the E1B locus (inactivates p53).
- Mass spectrometry based analysis of p53-interacting proteins in mitochondria purified from the ZR-75-01 breast carcinoma. p53-interacting proteins included BAK, caspase-3, myosin heavy chain, myosin light chain, and the DNA repair protein RAD 51. We have confirmed the p53-caspase 3 interaction, and are following this up.
- Discovered that certain tumor-derived mutant forms of p53 can still bind and oligomerize BAK. This constitutes a novel avenue for chemotherapeutic approaches toward killing tumor cell with mutant forms of p53.

IV. Reportable Outcomes

Manuscripts:

1. Leu JI, Dumont P, Hafey M, Murphy ME, George DL. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol.* 6:443-50. 2004.
2. Murphy ME, Leu JI, George DL. p53 moves to mitochondria: A turn on the path to apoptosis. *Cell Cycle*, *in press*.

Cell lines: Saos2 ts Lp53. The human, p53-null osteosarcoma stably transfected with an inducible version of p53 that has the total of p53 directed to mitochondria after induction. Useful for identifying p53-interacting proteins in mitochondria.

Plasmids: pSpV-E1A. Has the *survivin* promoter, core p53-repressible element, linked to the viral E1A gene, for use in construction of the mutant dl1520 adenovirus.

CMV-Lp53. Has the p53 tumor suppressor protein linked to the ornithine transcarbamylase leader peptide, which directs the total of p53 to mitochondria. This has proven useful in our studies to identify mitochondrial p53-interacting proteins.

V. Conclusions

- We have identified the mechanism whereby mitochondrial p53 (which would primarily consist of the R72 polymorphic variant of this protein) induces apoptosis. This is via direct binding and oligomerization of BAK.
- We have found that some tumor-derived mutant versions of p53 in breast carcinomas still retain the ability to bind to BAK, and oligomerize it. This indicates that this pathway of tumor suppression may remain intact in some tumor cells, and is therefore an attractive target for chemotherapeutic intervention. We plan in year 3 to further define which mutant forms of p53 retain this ability, and further to begin to delineate why these mutants are still retained in human tumors.
- We have generated all of the DNA constructs necessary for creation of a mutant form of adenovirus that selectively replicates (and lyses) tumor cells with mutant, or inactive, p53.

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7. Perfettini JL, Kroemer RT, Kroemer G. Fatal liaisons of p53 with Bax and Bak. **Nat Cell Biol**. 6:386-8, 2004

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VII. Appendix Contents:

1. Leu JI, Dumont P, Hafey M, Murphy ME, George DL. Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. **Nat Cell Biol.** 6:443-50. 2004.
2. Murphy ME, Leu JI, George DL. p53 moves to mitochondria: A turn on the path to apoptosis. **Cell Cycle**, *in press*.

Mitochondrial p53 activates Bak and causes disruption of a Bak–Mcl1 complex

J. I-Ju Leu¹, Patrick Dumont², Michael Hafey², Maureen E. Murphy² and Donna L. George¹

The tumour suppressor activity of the p53 protein has been explained by its ability to induce apoptosis in response to a variety of cellular stresses^{1,2}. Thus, understanding the mechanism by which p53 functions in the execution of cell death pathways is of considerable importance in cancer biology. Recent studies have indicated that p53 has a direct signalling role in the induction of apoptosis at mitochondria^{3–6}, although the mechanisms involved are not completely understood. Here we show that, after cell stress, p53 interacts with the pro-apoptotic mitochondrial membrane protein Bak. Interaction of p53 with Bak causes oligomerization of Bak and release of cytochrome *c* from mitochondria. Notably, we show that formation of the p53–Bak complex coincides with loss of an interaction between Bak and the anti-apoptotic Bcl2-family member Mcl1. These results are consistent with a model in which p53 and Mcl1 have opposing effects on mitochondrial apoptosis by interacting with, and modulating the activity of, the death effector Bak.

The role of p53 in apoptosis is multi-faceted and most probably involves its transcriptional regulatory functions, as well as less well-characterized transcription-independent activities^{1,2}. Recent studies have demonstrated that a fraction of stress-activated p53 translocates to mitochondria after an apoptotic stimulus, but not during p53-dependent growth arrest^{3,4}. Further support for the significance of p53 mitochondrial localization followed from our analysis of the apoptotic potential of two polymorphic variants of p53 (ref. 5). It has been known for some time that a common coding region polymorphism occurs in human p53, resulting in either an arginine (R72) or proline (P72) at amino acid 72. The frequency of the polymorphic variants of p53 varies in different populations: for example, approximately 35–40% of Caucasians, but only 10–12% of African Americans, are homozygous for the R72 allele⁷. In an earlier study, we found that the R72 variant of p53 exhibits a greater ability to induce apoptosis than does the P72 form, and at least one reason for this difference is enhanced localization of the R72 variant to mitochondria⁵. The greater mitochondrial localization of the R72 variant correlates with enhanced nuclear export, suggesting that differences in nucleo-cytoplasmic shuttling may influence trafficking of p53 to mitochondria.

Reasoning that p53 functions with one or more mitochondrial factors to execute its pro-apoptotic functions, we sought to identify p53-bound mitochondrial proteins. As a source of mitochondrial p53, we used an inducible Saos2 cell line that stably expresses a temperature-sensitive (ts)-R72 variant of p53 (ref 5). The ts-p53 protein exists in a denatured, inactive, form at 39 °C and becomes wild type in conformation and activity at 32 °C. Importantly, a significant fraction of the p53 protein localizes to mitochondria after an apoptosis-inducing temperature shift to 32 °C (see ref. 5 and Fig. 1a). Among several protein bands that were detectable in mitochondrial p53 immunoprecipitates from cells incubated at 32 °C, but not at 39 °C, was a major species with a relative molecular mass (M_r) of approximately 28,000 (Fig. 1a). This band was excised from SDS-PAGE gels, subjected to tryptic digestion, and the resulting peptides were analysed by liquid chromatography and tandem mass spectrometry. Two tryptic fragments of the protein Bak were identified (Fig. 1a). Bak is a multidomain pro-apoptotic member of the Bcl-2 family of proteins, which are key regulators of programmed cell death⁸. We then verified that Bak co-immunoprecipitates with p53 from mitochondrial lysates using immunoprecipitation-western blot analysis (Fig. 1a).

Bak is an integral protein of the mitochondrial membrane, and the R72 form of p53 localizes to mitochondria more effectively than the P72 variant. Therefore, we addressed whether these two p53 isoforms would exhibit differences in binding to Bak *in vivo* that would correlate with apoptotic potential. Analysis of whole-cell and mitochondrial lysates by immunoprecipitation-western blotting confirmed that, compared with P72, much more of the R72 variant is complexed with Bak (Fig. 1b, c). A longer exposure of the autoradiogram detected the less abundant interaction of P72 with Bak (data not shown). In these analyses, little association was detected between p53 and two other Bcl-2 family members, the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-x_L, both of which were reported to function in mitochondrial apoptosis induction by p53 (refs 4, 6). Similarly, *in vitro* binding assays using *in-vitro*-translated proteins identified a robust association of p53 with Bak, but failed to identify a detectable interaction of p53 with either Bax or Bcl-x_L (Fig. 1d). We extended these studies to verify that the interaction between p53 and Bak occurs in different cell lines expressing endogenous wild-type p53. The human prostate carcinoma cell line LNCaP and the human breast carcinoma

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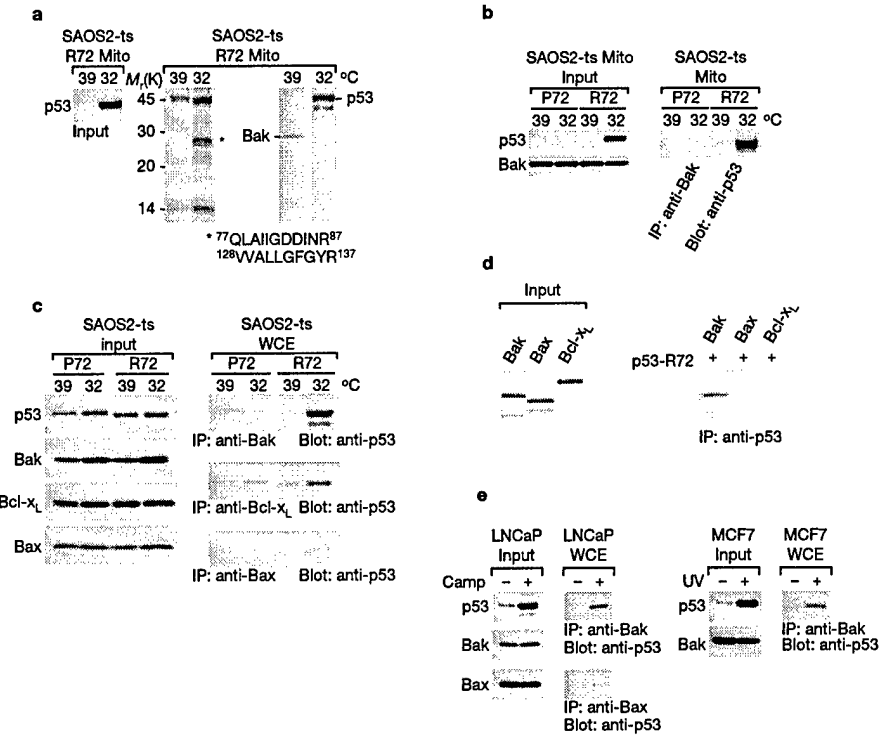


Figure 1 Bak and p53 interact. (a) Immunoblotting of mitochondrial p53 in ts-p53(R72)-Saos2 cells grown at 39 °C or 32 °C (left). Mitochondrial extracts were immunoprecipitated using agarose-conjugated anti-p53 (middle). The silver-stained protein band of approximately 28K was excised and subjected to tryptic digestion and liquid chromatography-tandem mass spectrometry. Asterisk indicates the peptide sequences of Bak obtained from this analysis. Immunoblots of proteins co-precipitating with p53 from cells at 32 °C were probed with antibodies to Bak and p53, as indicated (right). (b) Immunoblots of mitochondrial extracts from ts-P72-Saos2 and ts-R72-Saos2 cells were analysed with an antibody against p53 or Bak (left). The same extracts were immunoprecipitated (IP) with anti-Bak followed by immunoblotting for associated p53 (right). (c) Immunoblots of whole-cell extracts (WCEs) from ts-P72-Saos2 and ts-R72-Saos2 cells analysed for p53, Bak, Bcl-x_L and Bax (left). IP of the same lysates for Bak, Bcl-x_L or Bax, followed by immunoblot analysis for p53 (right). The

autoradiogram exposure times were equivalent for the Bak, Bcl-x_L and Bax IP blots, and the results depicted were consistent with different antibodies for each protein. (d) *In-vitro*-translated full-length p53 (R72) was mixed with *in-vitro*-translated-³⁵S-labelled full-length Bak, Bax or Bcl-x_L. Input analyses confirm similar expression levels for the proteins. Binding to p53 was assayed by IP with an antibody to p53. The products were analysed by SDS-PAGE and autoradiography (right). (e) Immunoblots of whole-cell extracts from LNCaP cells (± 5 μM camptothecin for 24 h) or MCF7 cells (± 7.5 J m⁻² ultraviolet irradiation) were probed with anti-p53, anti-Bak or anti-Bax, as indicated. IP of the same lysates with anti-Bak or anti-Bax, followed by blotting with anti-p53 (right). For these and similar assays, protein interactions were examined after solubilization of cellular fractions in Chaps, which, unlike non-ionic detergents, does not induce an activating, conformational change in Bak or Bax^{12,30}.

cell line MCF7 were treated with camptothecin or ultraviolet irradiation, respectively, to induce upregulation of p53 [AU: OK?]. Immunoprecipitation-western blot analysis confirmed that after its stress-induced activation, p53 interacts with Bak (Fig. 1e). In these cells, as with the p53-Saos2 transfectants, a much stronger association was detected between p53 and Bak, than between p53 and Bax (Fig. 1e) or Bcl-x_L (data not shown).

To map the domain of p53 that interacts with Bak, we used glutathione S-transferase (GST) pull-down assays, in which ³⁵S-labelled *in-vitro*-translated Bak (full-length) bound to immobilized GST-p53 (full-length), but not to GST alone (Fig. 2a). In these *in vitro* assays, the interaction of the R72 and P72 forms of p53 with Bak was indistinguishable (Fig. 2a); this is consistent with our conclusion that the greater association of R72 to Bak observed *in vivo* (Fig. 1b) reflects the enhanced nuclear export and mitochondrial localization of this vari-

ant in cells⁵. Bak does not bind to the amino-terminal domain (amino acids 1–92), or to the carboxy-terminal domain (amino acids 318–393), of p53. Instead, the central region of p53, encompassing amino acids 92–318, contains the Bak-binding domain (Fig. 2b). In fact, these data suggest that there may be two Bak-interacting domains within the central DNA-binding domain of p53. Other proteins, including mSin3a and TBP, also have been found to interact with two distinct regions of the p53 protein^{9,10}. We used similar assays to map the p53-interacting domain of Bak. These analyses demonstrated that the introduction of mutations within critical residues of any of the three Bcl-2-homology regions of Bak (BH1, BH2, BH3) resulted in substantially reduced p53 binding (Fig. 2c). Existing data suggest that these three segments of Bak most probably form a binding pocket that would be disrupted by mutations within any one of the domains^{11,12}. Thus, *in vitro* binding analysis suggests that p53 interacts directly with

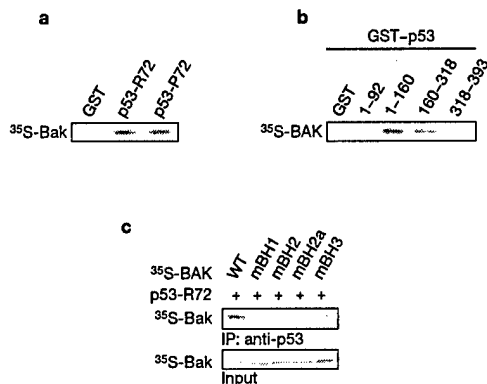


Figure 2 p53 binds to Bak *in vitro*. (a) GST pull-down assays were performed using *in-vitro*-translated-³⁵S-labelled full-length human Bak and GST-tagged recombinant proteins: GST alone, GST-p53-R72 or GST-p53-P72. Protein complexes were analysed by SDS-PAGE and fluorography. (b) *In-vitro*-translated ³⁵S-labelled full-length human Bak was mixed with GST alone, or GST-tagged proteins containing different amino-acid residues of p53, as indicated. (c) *In-vitro*-translated full-length p53-R72 was mixed with *in-vitro*-translated ³⁵S-labelled full-length wild-type Bak, or the different BH-domain Bak mutants indicated (see Methods for the amino-acid changes in the Bak mutants). p53-Bak interactions were assayed by IP with anti-p53. The products were resolved by 10–20% SDS-PAGE and the autoradiogram was developed.

Bak through a pocket structure formed by the BH1, BH2 and BH domains.

In unstressed cells, Bak is located at the mitochondrial outer membrane as an inactive monomer. After diverse apoptotic stimuli, Bak undergoes an activating conformational change that results in the formation of higher-order multimers; this leads to the release of cytochrome *c* and other pro-apoptogenic factors from mitochondria into the cytosol^{13–15}. To assess the ability of p53 to induce oligomerization of Bak, we used a well-established assay using highly purified mitochondria isolated from sucrose gradients^{14,15}. The addition of full-length recombinant p53 (R72 or P72) to mitochondria isolated from unstressed p53-null cells readily shifted Bak from a monomer into higher-order multimers (Fig. 3a). Using deletion mutants of p53, we confirmed that the ability to bind to Bak correlates positively with their ability to induce Bak oligomerization, as well as to stimulate release of cytochrome *c* from purified mitochondria (compare Figs 2b and 3a). Activation of Bak is accompanied by conformational changes, resulting in the exposure of an amino-terminal epitope of Bak that becomes susceptible to trypsin digestion^{14,16,17}. Therefore, we also examined whether p53 could induce such a conformational change in Bak. First, we confirmed that Bak exists in a relatively inactive trypsin-resistant form in untreated mitochondria (Fig. 3b). Significantly, incubation of mitochondria with recombinant p53, but not GST alone, resulted in oligomerization of Bak and an increased susceptibility of Bak to trypsin digestion. This enhanced susceptibility to trypsin proteolysis was consistent with two different antibodies directed against the Bak N terminus (Fig. 3b). Together, these data demonstrate that, similarly to other molecules that activate and oligomerize Bak, p53 induces an amino-terminal conformational change in Bak that is associated with Bak oligomerization. To determine whether p53-induced cytochrome *c* release is dependent on the ability of p53 to bind and oligomerize Bak, we isolated mitochondria from mouse embryo fibroblasts (MEFs) derived from wild type (*Bak*^{+/+}) and Bak-deficient

(*Bak*^{-/-}) mice¹⁸. Consistent with our results obtained from mitochondria purified from cultured human cells, p53 readily induced cytochrome *c* release from mitochondria isolated from wild-type MEFs. In contrast, incubation with p53 failed to release cytochrome *c* from the Bak-deficient mitochondria (Fig. 3c). These data further support a model in which p53, operating at the mitochondria, binds directly to Bak, resulting in Bak oligomerization and cytochrome *c* release.

To better understand the role of mitochondrial p53 in apoptosis, we focused on identifying mitochondrial signalling pathways that might be altered, or mediated, by the p53-Bak interaction. It has been proposed that in unstressed cells, Bak is complexed with other mitochondrial proteins that function to maintain this pro-apoptotic factor in an inactive conformation^{12,16}. Anti-apoptotic members of the Bcl-2 family are attractive candidates to perform such a role. In unstressed cells, however, the majority of the anti-apoptotic Bcl-2 and Bcl-x_L are in different cellular compartments and do not seem to interact appreciably with inactive Bak (refs 12, 19, 20). The anti-apoptotic Mcl1 protein, however, is predominantly mitochondrial in healthy cells (ref. 21 and see Fig. 4a), and has been shown to bind to Bak and Bax in yeast two-hybrid analyses, but not to Bcl-x_L or Bcl-2 (ref. 22). To confirm that mammalian Mcl1 is complexed with Bak in unstressed cells, we performed immunoprecipitation-western blot analyses in unstressed Saos2 cells. These analyses identified an interaction of mitochondrial Mcl1 with Bak; in contrast, very little interaction of Mcl1 with Bax or Bcl-x_L was detected in these assays (Fig. 4b). A recent report has demonstrated that Bak is, in fact, complexed with Mcl1 in healthy cells¹⁶. We performed *in vitro* mapping experiments of this interaction and determined that an intact BH3 domain of Bak is necessary for an efficient interaction with Mcl1 (Fig. 4c).

Elevated Mcl1 expression is associated with increased protection from cell death^{20,23–25}. We found that in isogenic cell lines with different levels of Mcl1, increased Mcl1 attenuates Bak oligomerization (Fig. 4d). These data suggest that Mcl1 and p53 have opposing roles in modulating Bak function. Thus, we sought to determine whether the stress-induced activation of p53 alters the Bak-Mcl1 interaction. For these analyses, we took advantage of two well-established cell lines that are isogenic, except for their p53 status. The ovarian teratocarcinoma cell line PA1 contains wild-type p53. However, its derivative PA1-E6 expresses the human papillomavirus 16 E6 protein, which targets p53 for degradation. It was shown previously^{20,21} that Mcl1 is a short-lived protein, and that agents such as ultraviolet irradiation and etoposide (which can inhibit new protein synthesis) reduced cellular Mcl1 levels^{20,24}. As this would interfere with our analyses, we sought stress-induction protocols that would not significantly decrease RNA or protein synthesis and Mcl1 levels. Treatment of cells with the DNA-damaging agent doxorubicin did not result in decreased steady-state levels of Mcl1, as illustrated for the PA1 and PA1-E6 cells (Fig. 5a). Notably, induction of p53 by doxorubicin resulted in a significant decrease in the Bak-Mcl1 interaction. In PA1-E6 cells lacking p53, however, the amount of Mcl1 co-precipitating with Bak was not altered by doxorubicin treatment (Fig. 5a). Similarly, there was no decrease in cellular Mcl1 protein abundance in p53-inducible Saos2 cells after temperature shift and apoptosis induction at 32 °C; however, p53 induction was accompanied by a substantial decrease in the amount of Mcl1 complexed to Bak (Fig. 5b). Thus, p53 activation results, directly or indirectly, in disruption of the Bak-Mcl1 complex.

The stress-induced localization of p53 to mitochondria is rapid and precedes mitochondrial alterations, such as caspase activation and cytochrome *c* release^{3,4}. Therefore, we assessed whether the interaction of p53 and Bak correlates temporally with disruption of the Bak-Mcl1

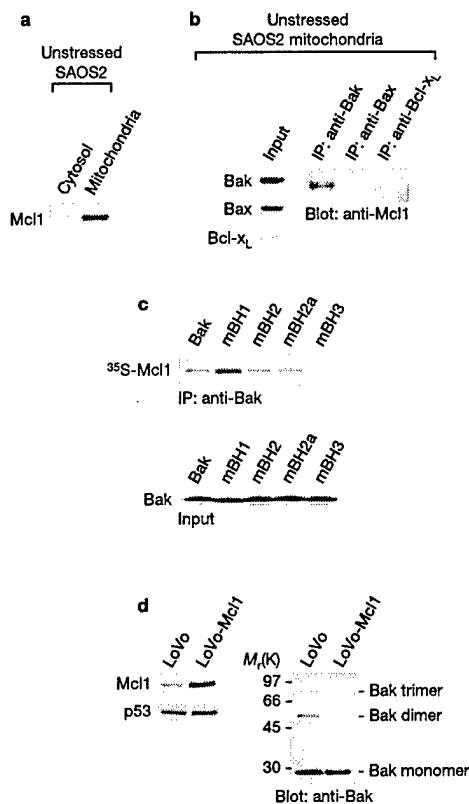


Figure 4 Characterization of the Mcl1-Bak interaction. (a) The presence of Mcl1 in cytosolic and mitochondrial fractions of unstressed Saos2 cells was analysed by immunoblotting with an anti-Mcl1 antibody. (b) To evaluate the interaction of Mcl1 with Bak, Bax and Bcl-x_L, mitochondria (300 µg) from unstressed p53-null Saos2 cells were solubilized in 1% Chaps. Mitochondrial proteins were immunoprecipitated with anti-Bak, anti-Bax or anti-Bcl-x_L antibodies, followed by immunoblotting with an anti-Mcl1 antibody. (c) To determine the region of Bak required for Mcl1 interaction, *in-vitro*-translated ³⁵S-labelled full-length Mcl1 was mixed with *in-vitro*-translated full-length wild-type Bak or the BH-domain Bak mutants indicated (top). Protein interactions were assayed by immunoprecipitation with an anti-Bak antibody before SDS-PAGE (bottom). (d) Overexpression of Mcl1 is associated with attenuated Bak oligomerization *in vivo*, as illustrated for LoVo colon carcinoma cells and for an Mcl1-overexpressing derivative (LoVo-Mcl1). Bak multimer formation was detected in a small percentage of cells undergoing spontaneous apoptosis in an otherwise asynchronous population. Protein expression levels for Mcl1, p53 and Bak were examined by immunoblotting.

target proteins such as Mdm2, caspase-3 and caspase-8, was not detectable at 6 h, but was clearly present by 15 h (see Fig. 5c and ref. 5). Additionally, we found that the p53-Bak complex formed independently of caspase activation, as p53-Bak complexes were detectable even in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (Fig. 5d). These data reinforce the conclusion that the formation of a p53-Bak complex and the disruption of the Bak-Mcl1 interaction represent opposing upstream events in the mitochondrial cell death pathway.

Finally, to determine if p53 can disrupt the Bak-Mcl1 complex, we performed *in vitro* assays with highly purified mitochondria and

recombinant p53 protein. In these studies, incubation of mitochondria with bacterially expressed His-tagged p53 protein resulted in a clear decrease in the amount of Mcl1 interacting with Bak, as tested by immunoprecipitation with an antibody to Bak and western blotting for Mcl1 (Fig. 5e). Notably, disruption of the Mcl1-Bak interaction correlated with formation of a p53-Bak complex (Fig. 5e), Bak oligomerization (data not shown) and the release of cytochrome c from mitochondria (Fig. 5e). In contrast, incubation of mitochondria with a control His-tagged protein, or with recombinant tBid, had no effect on the Mcl1-Bak complex (Fig. 5e). Identical results were obtained using mitochondria purified from other cells, as well as a different form of recombinant p53 (GST-p53; data not shown).

In this study, we provide new insight into the mitochondrial apoptosis pathway by demonstrating that p53 and Mcl1 have opposing actions in regulating the death effector Bak. These studies further define a non-transcriptional role for mitochondrial p53 as a direct upstream activator of Bak-mediated mitochondrial dysfunction. Our data also support a model in which the anti-apoptotic Mcl1 protein functions to maintain Bak in an inactive conformation in normal cells. This could explain why enhanced expression of Mcl1 observed in some tumours is associated with a reduced responsiveness to chemotherapy and a poor prognosis^{20,23-25}. Previous studies have indicated that Mcl1 downregulation alone may not be sufficient to induce apoptosis^{16,20}; our analyses are consistent with the idea that both a disruption of a Bak-Mcl1 complex, as well as activation of Bak, may be required. Accumulating data shows that p53 can respond rapidly to adverse conditions by trafficking to mitochondria³⁻⁵; the ability of p53 to directly bind and activate Bak provides another mechanism (in addition to its transcriptional functions) for p53 to promote an enhanced, multifaceted apoptotic response. Consistently, it has recently been reported that the pro-apoptotic Bcl-2 family member Bax can undergo oligomerization after treatment with cytoplasmic p53 (ref. 26), although no direct interaction between p53 and Bax could be detected^{4,26}. Although these and other studies support a direct mitochondrial role for p53 in apoptosis induction, the relative contribution of this activity to p53-mediated cell death remains to be assessed. Although it has been shown that a well-characterized mutant of p53 lacking transcriptional function is defective at apoptosis induction in mice²⁷, this mutant was shown to be defective at nuclear export, which we have shown is important for mitochondrial localization⁵. Therefore, that study does not shed light on this issue. Our finding that the P72 polymorphic variant of p53, which has greatly reduced ability to localize to mitochondria, also has significantly reduced ability to induce apoptosis⁵, suggests that further assessment of the degree to which mitochondrial p53 contributes to apoptosis induction is warranted. □

METHODS

Cell lines and purification of mitochondria. H1299 lung adenocarcinoma and Saos2 osteosarcoma cells are p53 null. The following human cell lines contain wild-type p53: LNCaP prostate carcinoma (R72); MCF7 breast carcinoma (R72/P72); LoVo colon carcinoma (R72); PA1 ovarian teratocarcinoma (R72). MEF cell lines were established from *Bak*^{+/+} and *Bak*^{-/-} mice (kindly provided by T. Lindsten and C. Thompson, University of Pennsylvania, Philadelphia, PA). Mitochondria were purified using a differential centrifugation method as described^{14,28}. Briefly, cells were harvested, centrifuged at 500g for 5 min at 4 °C, and resuspended in fractionation buffer A (10 mM Hepes-KOH at pH 7.4, 0.1 mM EDTA, 1 mM EGTA and 250 mM sucrose) supplemented with protease inhibitors (6 µg ml⁻¹ aprotinin, 6 µg ml⁻¹ leupeptin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)). Cell disruption was performed by passing the cells through a 23-gauge needle 3-5 times. The homogenates were spun at 700g for 10 min at 4 °C. The supernatants were removed and spun at 7,000g for 10 min at

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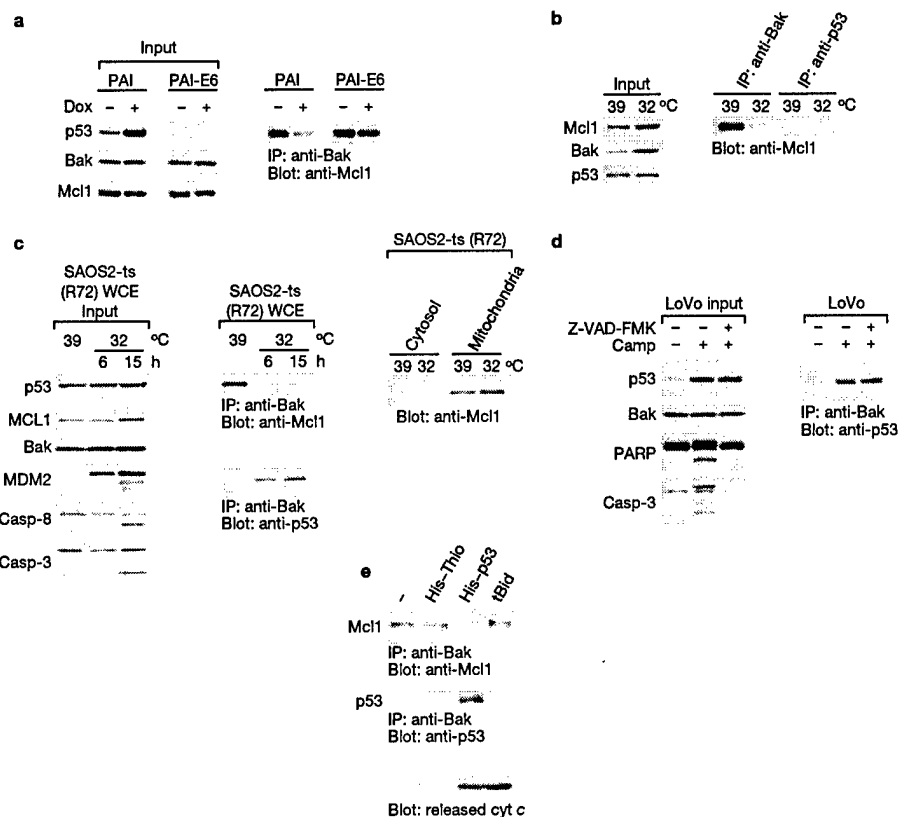


Figure 5 The p53-Bak interaction correlates with disruption of the Mcl1-Bak interaction. (a) Whole-cell extracts (100 μ g) from PA1 and PA1-E6 cells, either left untreated or treated with doxorubicin (Dox; 0.5 μ g ml⁻¹, 5 h), were analysed by immunoblotting with antibodies to Mcl1, p53 or Bak, as indicated (left). Whole-cell extracts (2 mg) from PA1 and PA1-E6 cells were immunoprecipitated with an antibody against Bak before immunoblotting for Mcl1 (right). (b) Whole-cell extracts from ts-R72 Saos2 cells, incubated at 39 °C or 32 °C, were analysed with antibodies raised against p53, Mcl1 or Bak, as indicated (left). The same whole-cell extracts were immunoprecipitated with anti-Bak or anti-p53 antibodies before immunoblotting for Mcl1 (right). (c) Whole-cell extracts from ts-R72 Saos2 cells incubated at 39 °C or 32 °C for 6 h or 15 h were analysed by immunoblotting with antibodies against p53, Mcl1, Bak, Mdm2, caspase-8 or caspase-3, as indicated (left). The same extracts were

immunoprecipitated with an anti-Bak antibody before immunoblotting for Mcl1 or p53 (middle). Cytosolic and mitochondrial fractions from ts-R72 Saos2 cells incubated at 39 °C or 32 °C for 6 h were analysed by immunoblotting for Mcl1 (right). (d) LoVo cells were treated with camptothecin \pm zVAD-fmk for 5 h before immunoblotting for p53, Bak, PARP or caspase-3 (left). The same extracts were subjected to immunoprecipitation with an antibody against Bak before immunoblotting for p53 (right). (e) Purified mitochondria were incubated with the indicated recombinant proteins for 30 min at 30 °C. The mitochondria were pelleted, solubilized in 1% Chaps, immunoprecipitated with an antibody to Bak and analysed by immunoblotting for Mcl1 (top). The same blot was reprobed with an antibody against p53 (middle). The supernatant fraction was analysed for released cytochrome c by immunoblotting (bottom).

4 °C. The mitochondrial pellets were washed with fractionation buffer B (10 mM HEPES-KOH at pH 7.4, 5 mM KH₂PO₄, 5 mM succinate and 250 mM sucrose) and resuspended in fractionation buffer B to a final protein concentration of 2–3 mg ml⁻¹. Protein concentration was determined using the Bradford Reagent (Bio-Rad Laboratories, Inc., Hercules, CA).

Identification of mitochondrial p53-interacting proteins. Mitochondria were purified from the ts-R72 Saos2 cells incubated at 39 °C or 32 °C for 22 h and lysed in 1% Chaps Buffer (5 mM MgCl₂, 137 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Chaps and 20 mM Tris-HCl at pH 7.5) supplemented with protease inhibitors. For immunopurification, an anti-p53 antibody conjugated to agarose resin (EMD Biosciences, Inc., Oncogene Research Products, San Diego, CA) was used. After five washes with 1% Chaps buffer, the associated proteins were resolved by SDS-PAGE in a 10–20% gradient gel (Cambrex Bio Science Rockland, Inc., Rockland, ME) and visualized by silver stain (Bio-Rad). The sil-

ver-stained band corresponding to ~28K was excised from the gel, subjected to trypsin digestion, and the resulting peptides were analysed by liquid chromatography-tandem mass spectrometry at the Genomics Institute and Abramson Cancer Center Proteomics Core Facility (University of Pennsylvania School of Medicine, Philadelphia, PA).

Recombinant protein production. Recombinant GST-tagged proteins were generated using the pGEX-4T-3 vector (Amersham Biosciences, Piscataway, NJ), and recombinant His-tagged proteins were produced using pET-32 EK/LIC vector (EMD Biosciences). The GST-tagged proteins were induced for 6 h with 0.1 mM IPTG in BL21 cells doubly deficient for glutathione reductase and thioredoxin reductase²⁹. His-tagged proteins were induced for 6 h with 0.5 mM IPTG in BL21 (DE3) cells doubly deficient for glutathione reductase and thioredoxin reductase. For purification of GST-p53, the bacterial pellets were resuspended in BugBuster HT Extraction Buffer (EMD Biosciences) and

applied to glutathione-Sepharose 4B (Amersham Biosciences). The GST-p53-glutathione-Sepharose complex was washed five times with 20 column volumes of PBS lacking calcium and magnesium ions (Invitrogen, Carlsbad, CA) and eluted with 10 mM glutathione in 50 mM Tris-HCl at pH 8.0. For purification of His-p53, the bacterial pellets were resuspended in BugBuster HT Extraction Buffer (EMD Biosciences) with 5 mM Imidazole and applied to Ni-NTA resin (Qiagen Inc., Valencia, CA). The His-p53-Ni-NTA complex was washed five times with 20 column volumes of wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl and 10 mM imidazole at pH 8.0) and eluted with wash buffer containing 250 mM Imidazole. The eluted GST-p53 and His-p53 were dialysed against fractionation buffer B.

Bak activation and cytochrome *c* release. For cytochrome *c* release and Bak oligomerization assays^{14,15} involving human H1299 or Saos2 cells, 15 μg of mitochondria were incubated with 30–60 pmol of the indicated recombinant p53 proteins at 30 °C for 30 min. The mitochondria were pelleted at 10,000g for 15 min at 4 °C. Mitochondria were also isolated from *Bak*^{+/+} and *Bak*^{-/-} MEFs. For these studies, 15 μg of mitochondria were incubated with or without 600 pmol of the GST-p53-R72 proteins at 30 °C for 45 min. The mitochondria were pelleted at 10,000g for 15 min at 4 °C. After incubation, the supernatant fractions were separated by SDS-PAGE and subjected to western blotting with an anti-cytochrome *c* antibody (BD Bioscience-Pharmigen, San Diego, CA). For control reactions, mitochondrial pellet fractions were separated by SDS-PAGE in 4–20% gradient gels and subjected to western blotting using antibodies directed against the following proteins: cytochrome *c* (BD Bioscience-Pharmigen), GRP75 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PCNA (Santa Cruz Biotechnology), Mdm2 (EMD Biosciences), Bak (anti-Bak NT, Upstate USA, Inc., Charlottesville, VA), and p53 (BD Bioscience-Pharmigen).

For detection of Bak oligomers using the uncleavable protein crosslinker 1,6-bismaleimidoethane (BMH), 20 μg of mitochondria were incubated with the indicated recombinant p53 proteins (15–30 pmol) for 1 h at 4 °C. After incubation, the proteins were crosslinked with 2.8 mM BMH (Pierce) for 30 min at 25–30 °C. The mitochondria were pelleted, dissolved in SDS sample buffer, and loaded on Nupage Novex 10% Bis-Tris gels (Invitrogen). For Bak-Mcl1 disruption assay, 100 μg of mitochondria were incubated with His-thioredoxin, GST-tBID, His-p53, or GST-p53 at 30 °C for 30 min. The mitochondria were pelleted at 10,000g for 15 min, resuspended in 1% Chaps buffer, and immunoprecipitated with an anti-Bak antibody (Upstate). Protein samples were separated by SDS-PAGE in 10% gels and analysed by western blotting using an anti-Mcl1 antibody (BD Bioscience-Pharmigen) or anti-p53 antibody (EMD Biosciences).

Treatment of Bak with trypsin. Mitochondria from H1299 or Saos2 cells were purified as described above. 15–20 μg of mitochondria were incubated with or without 25 or 125 pmol of recombinant p53 proteins, as indicated, at 30 °C for 30 min (125 pmol) or 60 min at 4 °C (25 pmol). The mitochondria were pelleted at 10,000g for 15 min at 4 °C and washed using fractionation buffer B without protease inhibitors. The pellets were resuspended in 25 μl of fractionation buffer B with or without trypsin (75–125 $\mu\text{g ml}^{-1}$; Invitrogen). Reaction mixtures were incubated on ice for 20 min. Trypsin digestion was stopped by adding 25 μl of 2 \times sample buffer (100 mM Tris-HCl at pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 10% 2-mercaptoethanol) followed by boiling for 5 min. Reaction mixtures (10 μl) were removed from each fraction and resolved by SDS-PAGE in 4–20% gradient gels before western blotting with an anti-GRP75 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The remaining 40- μl reaction mixtures were resolved by SDS-PAGE in 4–20% gradient gels and analysed by western blotting using two different antibodies directed against the N terminus of human Bak (anti-Bak NT, Upstate USA; anti-Bak Ab-1, Oncogene Research Products, San Diego, CA).

Co-immunoprecipitation and western blotting. For *in vivo* co-immunoprecipitation studies, whole-cell extracts (500–2,000 μg) or mitochondrial lysates (50–300 μg) were prepared in 1% Chaps buffer and immunoprecipitated with the anti-Bak (Upstate), anti-Bcl-x_L (Cell Signaling Technology, Inc., Beverly, MA) or anti-Bax antibodies (Cell Signaling Technology). Precipitated proteins were washed three times in 1% Chaps buffer, loaded onto 4–20% or 10–20% Tris-glycine gradient gels, transferred overnight onto immunoblot polyvinylidene difluoride (PVDF) membranes (Bio-Rad) and detected with ECL Western

Blotting Detection Reagents (Amersham Biosciences). Protein interactions were examined after solubilization of mitochondrial fractions using the zwitterionic detergent Chaps, which unlike non-ionic detergents does not induce a conformational change in Bak or Bax (refs 11, 31). For input analysis, 100 μg of whole-cell extracts or 25 μg of mitochondrial fractions lysed in 1% Chaps buffer were resolved by SDS-PAGE in 4–20% Tris-Glycine gels. Western blot analyses were performed as described previously^{5,9}.

Where indicated, colon carcinoma LoVo (Bax deficient) cells were pretreated with the broad-spectrum caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone zVAD-fmk (general caspase inhibitor; BDPharmigen) at 40 μM for 30 min at 37 °C followed by treatment with or without 5 μM camptothecin for 5 h. Whole-cell extracts (5 mg) were immunoprecipitated with the anti-Bak antibody (Upstate) followed by western blotting with anti-p53 antibody (EMD Biosciences). For input analysis, 150 μg of whole-cell extracts were resolved by SDS-PAGE in 4–20% Tris-Glycine gels, followed by western blotting using anti-Bak antibody (anti-Bak NT; Upstate), anti-p53 antibody (EMD Biosciences), anti-Caspase-3 antibody (Cell Signaling Technology) and anti-PARP antibodies (EMD Biosciences).

***In vitro* mapping, *in vitro* mixing and GST-binding assays.** *In vitro* transcription-translation was performed with the TNT T7 Quick Coupled Transcription/Translation System (Promega Corporation, Madison, WI). Redivue L-³⁵S-Methionine was purchased from Amersham Biosciences (Piscataway, NJ). For *in vitro* mapping, 20 μl of ³⁵S-labelled Bak proteins were incubated with 15 μl of *in vitro*-translated p53-R72 in 365 μl of 0.5% Chaps buffer and immunoprecipitated with 1 μg of anti-p53-antibody (AB-6; EMD Biosciences). For *in vitro* mixing experiments, 15 μl of ³⁵S-labelled Bak proteins, 45 μl of ³⁵S-labelled Bax protein or 15 μl of ³⁵S-labelled Bcl-x_L protein was incubated with 15 μl of *in vitro*-translated p53-R72 in 1% Chaps buffer and immunoprecipitated with 1 μg anti-p53-antibody (AB-6; EMD Biosciences). Analysis of the Mcl1-Bak interaction *in vitro* was performed by mixing 15 μl of ³⁵S-labelled Mcl1 with 20 μl of wild-type or mutant Bak proteins, followed by immunoprecipitation using 1 μg of anti-Bak antibody (Upstate). After washing Protein G-agarose with 0.5% Chaps buffer three times, 30 μl of pre-washed Protein G-agarose bead slurry (15 μl of packed beads; Invitrogen) was added to each reaction, followed by gentle rocking for 30 min at 4 °C. The *in vitro* binding reactions were washed three times using 1 ml of 0.5% Chaps buffer. GST pull-down assays were performed as described⁹. Briefly, 1 μg of GST-p53 fusion proteins were incubated with 25 μl of ³⁵S-labelled full-length Bak proteins at 4 °C for 30 min with agitation. After brief centrifugation, the beads were washed five times and resuspended in protein sample buffer. Samples were subjected to SDS-PAGE. To visualize ³⁵S-labelled proteins by fluorography, SDS-PAGE gels were fixed, incubated in Amplify (Amersham Biosciences) and dried before exposure to X-ray film.

Plasmids and site-directed mutagenesis. Plasmids expressing different GST-p53 proteins were described previously⁹. The Mcl1 cDNA was generated from human placenta total RNA using the Titanium One-Step RT-PCR kit according to the manufacturer's instructions (BD Bioscience-Clontech, San Diego, CA). Full-length human Bak and Bax were obtained by PCR amplification using the pSG5-HA-Bak and pSG5-HA-Bax constructs (kindly provided by J. Marie Hardwick, The Johns Hopkins University School of Medicine, Baltimore, MD). Constructs for Bak mtBH1 (W125A, G126E, R127A), Bak mtBH2 (G175E, G176E, W177A), Bak mtBH2a (W170A, I171A, G175A, G176A) and Bak mtBH3 (L78A, D83A) were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit System (Stratagene, La Jolla, CA). Mutations were verified by DNA sequencing.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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EXTRA VIEWS

p53 MOVES TO MITOCHONDRIA
A Turn on the Path to Apoptosis

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ABSTRACT

It has been said that no matter which direction cancer research turns, the p53 tumor suppressor protein comes into view. The widespread role of p53 as a suppressor of tumor development is believed to rely on its ability to induce programmed cell death in response to stress, either the replicative stress associated with uncontrolled cellular proliferation, or the environmental stresses that accompany tumor development, such as hypoxia. For some time it has been believed that the role of p53 in inducing apoptosis in response to such stress was as a master regulator coordinating the expression of other molecules whose ultimate role was the execution of the cell. New data, however, suggest that p53 itself also has a direct role in accomplishing cell death, at the mitochondria.

RUNNING TITLE

p53 moves to mitochondria

Since its discovery a quarter century ago, the p53 tumor suppressor protein has been recognized as a key factor in the induction of programmed cell death and cell cycle checkpoint control in response to genotoxic and environmental stress.^{1,2,3} The p53 pathway is inactivated in the majority of human cancers, most likely because the pro-apoptotic function of p53 is critical to the inhibition of tumor development and progression. Thus, the past twenty-five years have seen intensive and varied investigations to better understand the functions that p53 uses to mediate apoptosis. Clearly established is p53's role as a nuclear transcription factor with the ability to activate, or repress, the expression of many genes.¹⁻⁵ A number of p53 transcriptional targets, such as the p53-induced genes *BAX*, *PUMA*, *NOXA*, and the p53-repressed genes *BCL2* and *SURVIVIN*, represent genes with the potential to promote or inhibit apoptosis, respectively, in stressed cells. More recently, however, a somewhat unexpected turn in the p53-mediated pathway to programmed cell death has emerged with accumulating data that p53 has a direct cytoplasmic role at mitochondria in activating the apoptotic machinery.⁶⁻¹¹

Because others^{2,3,6,7,9,12} have expertly summarized the evidence supporting a transcription-independent function of p53 in apoptosis, that information will not be repeated here. Rather, we will touch briefly on a few points to exemplify the phenomenon. It has been reported, for example, that under some circumstances, p53-dependent apoptosis can occur in the absence of new protein synthesis, thereby excluding p53's function as a transcriptional activator.^{13,14} Also, certain transcriptionally inactive mutants of p53 can still induce apoptosis when overexpressed in tumor cells.^{15,16} Finally, in response to some stresses, such as hypoxia, p53 induces apoptosis but does not function as a transactivator.¹⁷ Intriguingly, Moll and colleagues demonstrated that during p53-dependent apoptosis a fraction of cellular p53 protein localizes to mitochondria and induces cytochrome c release; however, this is not observed during p53-mediated cell-cycle

arrest.⁶ Additional support for the concept that p53 has a cytoplasmic role in apoptosis induction resulted from our functional analysis of polymorphic variants of p53.⁸

Within exon 4 of the p53 gene, a common single-nucleotide polymorphism (SNP) at codon 72 leads to the incorporation of either an arginine (R72) or a proline (P72) at this position of the protein. This SNP was first noted more than twenty years ago as a non-tumor derived amino acid difference that affected the mobility of p53 on SDS-polyacrylamide gels.^{18,19} This observation raised the possibility that this particular amino acid change might affect the structure of the p53 protein and, perhaps, its biological activities as well. The codon 72 polymorphism is maintained at different allele frequencies in different populations, and there appears to be a selection against the R72 allele in populations living near the equator.²⁰ These and other studies indicated that functional differences between the R72 and P72 variants might exist.²⁰⁻²² We further investigated this possibility using cell lines containing inducible versions of alleles encoding the R72 and P72 protein forms, as well as in human cell lines expressing endogenous p53. Our studies revealed that the R72 form of p53 induces apoptosis markedly better than the P72 variant.⁸ A similar conclusion has also been reached in two recent publications.^{23,24} When we explored the potential mechanisms underlying the observed functional difference between the two p53 variants, we made the initially surprising discovery that the greater apoptotic potential of the R72 form correlated with its much better ability to traffic to mitochondria. We showed that this enhanced mitochondrial localization of the R72 protein was associated with increased nuclear export, due to increased binding of R72 to MDM2, which catalyzes nuclear export of p53.⁸ Based on these data, therefore, we concluded that the enhanced apoptosis-inducing activity of the R72 protein related, at least in part, to its greater mitochondrial localization.

A major question arising from that study centered on defining the apoptotic function of mitochondrial p53. We reasoned that mechanistic insight to this process could be obtained from the identification of mitochondrial p53-interacting proteins. Using affinity chromatography protocols and mass spectrometry, we have now uncovered a direct interaction between the p53 tumor suppressor protein and the mitochondrial death-effector protein BAK.¹¹ BAK is a pro-apoptotic member of the BCL2-family of proteins.^{25,26} An analysis of whole cell or mitochondrial extracts by immunoprecipitation-western blot analysis, demonstrated that the R72 form of p53 binds better to BAK than does the P72 variant, correlating with the differences in apoptotic potential of the two p53 variants. Interestingly, however, our *in vitro* analyses revealed that the R72 and P72 proteins bind equally well to BAK. These observations are consistent with the conclusion that the enhanced interaction between BAK and the R72 variant observed in intact cells reflects the enhanced nuclear export and mitochondrial trafficking of this p53 isoform.

In healthy cells, BAK resides at mitochondria as an inactive monomer. In response to various death stimuli, it undergoes an activating allosteric conformational change that promotes homo-oligomerization. This leads to formation of a pore in the outer mitochondrial membrane, and allows the release of cytochrome c and other pro-apoptogenic factors from the mitochondria resulting in the activation of a caspase cascade.²⁷⁻²⁹ Having established that stress-activated p53 physically interacts with BAK, we then sought to test whether p53 has the ability to conformationally alter BAK and mediate the release of cytochrome c from mitochondria. In well-established assays utilizing purified mitochondria, we determined that p53 behaved in a manner similar to that of another BAK-interacting protein, tBID, which also catalyzes the oligomerization of BAK.²⁸ Specifically, incubation of mitochondria with even nanomolar amounts of recombinant p53 rapidly induced a conformational change in BAK, which led to

BAK oligomerization, and cytochrome c release. Importantly, we showed that p53 requires the presence of BAK on purified mitochondria in order to induce cytochrome c release, indicating that p53 is acting through a previously recognized genetic pathway of mitochondrial apoptosis.

Given the discovery that p53 can bind and activate BAK, we sought to elucidate how this interaction might impact on other key factors that regulate the programmed cell death machinery. Reasoning that p53 might alter the interaction of BAK with anti-apoptotic BCL-2 family members, we focused attention on another mitochondrial protein, MCL1. This decision was based, in part, on previous observations that anti-apoptotic proteins such as BCL-2 and BCL-XL are not normally present at the mitochondria and do not appreciably interact with BAK in unstressed cells.³⁰⁻³² Even more interesting, however, were recent data³² indicating that this anti-apoptotic BCL-2 family member, MCL1, functions as a critical upstream regulator of the mitochondrial apoptotic program. The downregulation or loss of MCL1 seems to be necessary, albeit insufficient, to initiate the cell death process, preceding events such as BAX/BCL-XL translocation to mitochondria and BAK oligomerization. In this regard, Cuconati et al. have found that in unstressed cells, BAK is complexed with MCL1, and this interaction is lost following adenovirus E1A-initiated apoptosis.³³ We determined that MCL1 is complexed with BAK in normal cells, but that the stress-induced activation of p53 leads to disruption of the BAK-MCL1 complex.¹¹ Additionally, we demonstrated that formation of a p53-BAK complex and concomitant disruption of the BAK-MCL1 interaction represent early events in apoptosis that occur prior to cytochrome c release and caspase activation. Collectively, then, these observations support a model in which the tumor suppressor p53 and the anti-apoptotic MCL1 protein have opposing upstream roles in mitochondrial apoptosis: by directly binding to

mitochondrial BAK, the MCL1 and p53 proteins function either to inhibit or to promote, respectively, BAK's pro-apoptotic behavior (Figure 1).

The data described above provide new insight into the mechanisms involved in an extra-nuclear direct role of p53 in apoptosis induction, binding and oligomerization of BAK. Recently, like BAK, the BCL2 family members BAX and BCL-XL have also been implicated in mitochondrial apoptosis induction by p53.^{9,10} Employing both *in vivo* and *in vitro* binding assays, we have detected much less, if any, association between these proteins and p53. These discrepant results may stem from variations in experimental conditions used by different groups; indeed, binding conditions must be stringently controlled in such assays, as the conformation and binding activity of BCL-2 family proteins, including BAX and BCL-XL, are known to be strongly influenced by buffer and detergent conditions.^{31,34} The biological importance of a p53-BAK interaction is supported by the finding that p53 can efficiently cause cytochrome c release from isolated mitochondria even if these organelles contain little if any BAX,⁹ but cannot do so if these organelles are BAK-deficient.¹¹ Furthermore, tumor cells deficient in BAX, or BCL-XL, are still competent for p53-dependent apoptosis associated with the formation of a p53-BAK complex at mitochondria (our unpublished observations).

The combined data argue for a direct role of p53 in programmed cell death. However, if one is to accept the relevance and significance of the mitochondrial pathway of p53-dependent apoptosis, at least two findings in the literature must be explained. The first is the finding by Wahl and colleagues that a synthetic p53 mutant that is transcriptionally inactive (p53QS) is also completely unable to function as a tumor suppressor in genetically-engineered mice.³⁵ Additionally, mice that are genetically engineered to lack the BH3-only protein PUMA, a transcriptional target of p53 and BCL-2 family member, have cells that are greatly impaired for

apoptosis induction.³⁶ These two findings have led to the notion that the transcriptional activity of p53 is largely, if not wholly, responsible for apoptosis induction by this protein. Such a conclusion would not be supported by the existent data, however. For example, the transcriptionally-inactive p53QS mutant is also known to be unable to bind to MDM2.^{35,37} It has been shown that MDM2 is essential for efficient nuclear export of p53,^{38,39} and further that forms of p53 that interact less well with MDM2, like the proline 72 polymorphic variant, also exhibit impaired mitochondrial localization.⁸ In line with this, Wahl and colleagues reported³⁵ that the p53QS mutant had greatly impaired nuclear export, and abnormal retention of nuclear p53; therefore, this synthetic mutant would be predicted to have a dysfunctional mitochondrial pathway. Interestingly, the p53 target gene PUMA is also a component of the mitochondrial apoptosis pathway; it has been proposed that PUMA induces cell death by inhibiting the activity of anti-apoptotic BCL-2 family members.^{40,41} Therefore, it will be of interest to determine if the loss of PUMA alters the threshold of active BAK at the mitochondria, thereby impairing oligomerization of BAK by p53. This hypothesis remains to be tested.

While some questions about the actions of mitochondrial p53 have now been answered, many others remain. Certainly a key issue centers on defining the relative contribution of the mitochondrial function of p53 to overall p53-mediated cellular apoptosis. Also, it remains to be determined if this extra-nuclear function of p53 is preferentially activated in tumor cells, relative to normal cells, and if there are other factors (including cell type or nature of the stress) involved in targeting p53 to mitochondria. It is of interest that the stress-induced localization of p53 to mitochondria has been observed in most tumor cells and rapidly proliferating normal cells, but not in cells that tend to growth arrest in response to p53, such as human fibroblasts.^{6,8} These findings raise the possibility that the mitochondrial localization of p53 may be the elusive

deciding factor that dictates whether cells die in response to p53, or whether they growth arrest. Another factor that should be considered in evaluating the literature and in designing future studies in this area concerns the codon 72 polymorphism of p53. As discussed above, this common polymorphism strongly influences the degree of p53 mitochondrial localization and apoptosis.⁸ Recent data also suggest that it is an important determinant of the response to chemotherapeutic agents in tumor cells with wild type p53.²⁴

The loss of p53 function directly contributes to tumor progression and chemoresistance. Thus, restoring key elements of this pathway represents an obvious and attractive target for cancer diagnosis and therapeutics. Approaching this goal, however, requires a clearer understanding of the mechanisms by which p53 acts in the execution of cell death pathways. The studies summarized above strengthen the conclusion that p53 can function directly at the mitochondria to promote apoptosis, and provide the groundwork for a novel therapeutic pathway.

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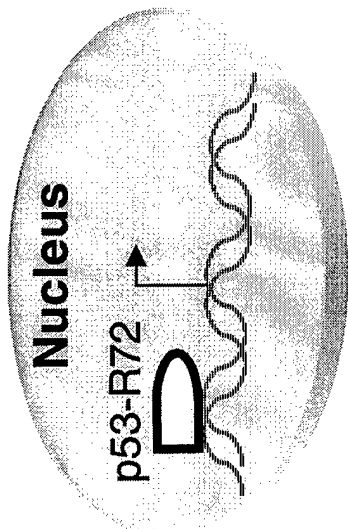
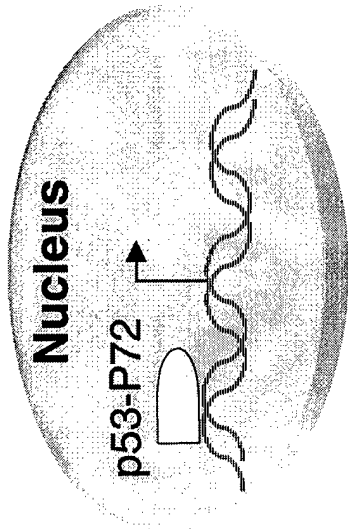
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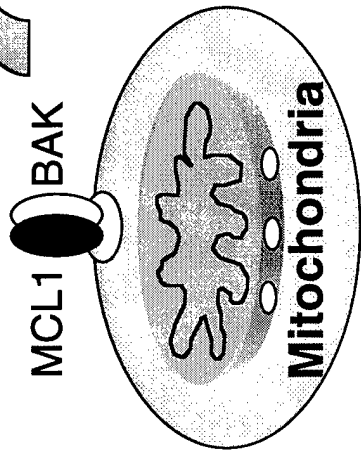
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FIGURE LEGEND

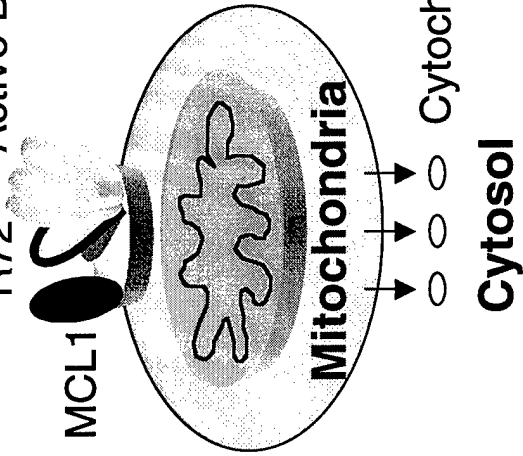
Figure 1. A model for the role of mitochondrial p53. In response to cell stress signals, p53 is activated. Relative to the P72 form of p53, the R72 variant exhibits a greater ability to traffic to mitochondria. Binding of the R72 variant to pro-apoptotic mitochondrial membrane protein BAK leads to disruption of BAK-MCL1 interaction. The formation of a p53-BAK complex induces a conformational change in BAK, BAK oligomerization, and the release of cytochrome c from mitochondria to the cytosol.



Nuclear Export



R72 Active BAK



Cytochrome c
Cytosol

○ ○ ○ ○