A role for caspase 2 and PIDD in the process of p53-mediated apoptosis

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When treated with some DNA-damaging agents, human tumor-derived H1299 cells expressing inducible versions of wild-type or mutant p53 with inactive transactivation domain I (p53$^{Q22/S23}$) undergo apoptosis as evidenced by cytochrome *c* release, nuclear fragmentation, and sub-G_1 DNA content. Apoptosis induced by p53$^{Q22/S23}$ is relatively slow, however, and key downstream effector caspases are not activated. Nevertheless, with either version of p53, caspase 2 activation is required for release of cytochrome *c* and cell death. Remarkably, although p53$^{Q22/S23}$ is known to be defective in transcriptional activation of numerous p53 target genes, it can induce expression of proapoptotic targets including PIDD and AIP1 at least to the same extent as wild-type p53. Furthermore, RNAi silencing of PIDD, previously shown to be required for caspase 2 activation, suppresses apoptosis by both wild-type p53 and p53$^{Q22/S23}$. Thus, the initial stage of DNA damage-facilitated, p53-mediated apoptosis occurs by a PIDD- and caspase 2-dependent mechanism, and p53's full transcriptional regulatory functions may be required only for events that are downstream of cytochrome *c* release.

transactivation | DNA damage | programmed cell death | p53(Q22/S23)

The apoptotic response to p53 is likely to be critical for its role as a tumor suppressor (1, 2). p53 is a sequence-specific transcriptional activator, and several target genes of p53 have been shown to mediate either cell cycle arrest or apoptosis (2, 3).

Among the target genes that are induced by p53 that cause cell cycle arrest in G_1 or G_2 (or both) are p21, GADD45, 14-3-3 sigma, and Reprimo (reviewed in refs. 3 and 4). A substantially greater number of p53 target genes have been identified that play proapoptotic roles. These include, among others, Bax, PIG3, PUMA, NOXA, KILLER/DR5, FAS/APO-1, p53AIP1, Apaf-1, Perp, and PIDD (reviewed in ref. 5). p53 can also repress a number of prosurvival genes such as Bcl2, MAP4, and survivin (reviewed in ref. 6).

Importantly as well, a number of studies have revealed transcription-independent activities of p53 in apoptosis (reviewed in refs. 7 and 8). Whether by transcription-dependent or transcription-independent means, apoptosis induced by p53 has been shown to require the family of cysteine proteases known as caspases (9). p53-dependent caspase activation is a hallmark feature of the intrinsic pathway, and mitochondria are the key integrators of these stress stimuli (9).

Particularly relevant to our study is caspase 2 (Ich1/Nedd2), one of the first and most well conserved mammalian caspases to be identified (10). Caspase 2 has been shown to be involved in DNA damage-mediated apoptosis in a number of human cell lines and to act upstream of mitochondrial outer membrane permeabilization and cytochrome *c* release (reviewed in ref. 11). Caspase 2 is also required for cleavage of Bid, which is necessary for cytochrome *c* release from mitochondria (12–14). Interestingly, whereas processing of caspase 2 is required for cytochrome *c* release, its enzymatic activity is not needed (15, 16). The above findings, implicating a role for caspase 2 early in apoptosis, are consistent with the fact that it possesses a long prodomain characteristic of initiator caspases. Nevertheless, some studies have placed caspase 2 downstream of Bax and even caspase 3 and to serve as a substrate of caspase 3 (17). Thus, caspase 2 may play roles both as an initiator caspase and also as a participant in a caspase 3-dependent apoptotic amplification loop (reviewed in ref. 11).

Activation of caspase 2 has been shown to involve its oligomerization (18, 19). The formation of a large, multimeric protein complex was shown to result in the activation of caspase 2 (20). This complex, termed the "PIDDosome," contains PIDD (p53-induced protein with a death domain), RAIDD, and caspase 2. Genetic inactivation or siRNA silencing of any of the PIDDosome components results in the reduction of apoptosis (20, 21). Most relevantly, p53-mediated apoptosis has been linked to caspase 2 activation in a number of experimental systems (22–26).

We reported that, in H1299 cell lines engineered to express various ectopically expressed inducible forms of p53, the transcriptionally impaired p53 mutant (p53$^{Q22/S23}$) can be enabled by a number of antineoplastic agents to induce an apoptotic response (27). In the present study we have examined several aspects of the process of apoptosis induced by wild-type and p53$^{Q22/S23}$. Our data show both similarities and striking differences between the two versions of p53 and identify activation of caspase 2 as a requirement for p53-mediated cell death.

Results

Characteristics of Apoptosis in Cells Expressing Wild-Type p53 and p53$^{Q22/S23}$. Both wild-type p53 and the transcriptionally impaired mutant p53$^{Q22/S23}$ can induce DNA damage-facilitated apoptosis in H1299 cells as measured by accumulation of cells with sub-G_1 DNA content (27). Here we characterize in detail the features of apoptotic cells treated or untreated with daunorubicin expressing either wild-type p53 or p53$^{Q22/S23}$. Because p53$^{Q22/S23}$ is present at high levels, it is possible that it cannot produce apoptosis when expressed at levels similar to wild-type p53.

Therefore, we regulated p53$^{Q22/S23}$ protein by maintaining different amounts of tetracycline (tet) in the culture medium and observed that levels of p53$^{Q22/S23}$ equivalent to or even lower than wild-type p53 led to significant apoptosis (Fig. 1*B*).

Whereas induction of wild-type p53 resulted in growth arrest, under similar conditions p53$^{Q22/S23}$ had no effect on cell division [supporting information (SI) Fig. 6 *A* and *B*]. By contrast, treatment of cells expressing wild-type p53 or p53$^{Q22/S23}$ with daunorubicin led to decreased numbers of adherent cells in either case, although p53$^{Q22/S23}$-expressing cells displayed a significantly delayed course of cell death (SI Fig. 6 *C* and *D*).

We went on to examine the morphological features of apoptosis in the two cell lines. After treatment with daunorubicin, both wild-type p53- and p53$^{Q22/S23}$-expressing cells were able to

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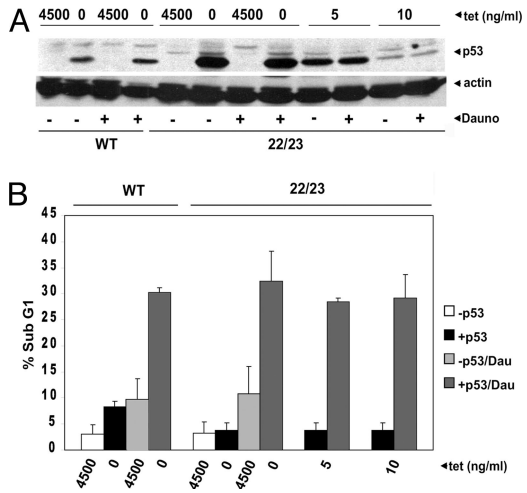

Fig. 1. Comparable protein levels of wild-type p53 and transcriptionally impaired $p53^{Q22/S23}$ produce apoptosis in H1299 cells treated with daunorubicin. (*A*) Cultures expressing inducible wild-type p53 (WT) in the presence of 0 ng/ml tet and inducible $p53^{Q22/S23}$ (22/23) in the presence of 10, 5, and 0 ng/ml tet were left untreated or were treated with daunorubicin for 48 h. Cell extracts from these samples were prepared and resolved by SDS/PAGE followed by transfer to nitrocellulose membranes. Blots were probed with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies. (*B*) Cell lines expressing inducible wild-type p53 in the presence of 4,500 and 0 ng/ml tet and inducible $p53^{Q22/S23}$ in the presence of 4,500, 10, 5, and 0 ng/ml tet were left untreated or were treated with daunorubicin for 48 h. FACS analysis was used to obtain values for the percentage of sub-G_1 cells. The bars show the percentage of sub-G_1 DNA content of cells 48 h after daunorubicin treatment. White bars, untreated, without p53; black bars, untreated, with p53; light gray bars, treated, without p53; dark gray bars, treated, with p53.

induce apoptotic membrane blebbing and undergo nuclear fragmentation. Formation of apoptotic bodies was the predominant end point in wild-type p53-expressing cells. Cell death in $p53^{Q22/S23}$-expressing cells led to appearance of cells with fragmented nuclei but not apoptotic bodies (SI Fig. 7) although at later time points nuclei from cells expressing wild-type p53 and $p53^{Q22/S23}$ nuclei look similar (data not shown). The number of TUNEL-positive $p53^{Q22/S23}$ cells was approximately half of that seen with wild-type p53-expressing cells (SI Fig. 8). Taken together, both nuclear and membrane changes occur in wild-type p53- and $p53^{Q22/S23}$-expressing cells after DNA damage although nuclear changes induced by $p53^{Q22/S23}$ appear to be less advanced than those seen with wild-type p53.

Unexpectedly, when we examined $p53^{Q22/S23}$-expressing cells, there was virtually no phosphatidylserine externalization, even under conditions that gave rise to a marked increase in sub-G_1 DNA (SI Fig. 9). A transiently transfected construct expressing $p53^{Q22/23}$ did give rise to phosphatidylserine externalization in this particular clone of cells, however, and we speculate that the much higher levels of mutant p53 that occur upon transfection are sufficient to activate this pathway. The similarities and differences in apoptotic features of cells expressing wild type and $p53^{Q22/23}$ are summarized in SI Table 1.

Apoptotic Cells Expressing Both Wild-Type p53 and $p53^{Q22/S23}$ Release Mitochondrial Cytochrome *c*, but Only Wild-Type p53 Activates the Canonical Apoptosomal Caspases. Wild-type p53 expression leads to release of cytochrome *c*, a crucial step in triggering the formation of the apoptosome and subsequent effector caspase activation (reviewed in ref. 28). When H1299 cells were treated with daunorubicin, induction of either wild-type p53 or $p53^{Q22/S23}$ led to comparable extents of cytochrome *c* release (Fig. 2*A*). Cytochrome *c* release precedes the mitochondrial death pathway executed by a number of activated caspases. In daunorubicin-treated cells expressing wild-type p53, activation of caspase 9 (Fig. 2*B Upper*), caspase 7 (Fig. 2*C*), and caspase 3 (Fig. 2*D*) was evident. Furthermore, cleavage of PARP, a caspase 3 substrate (Fig. 2*B Lower*), and lamin A, a caspase 6 substrate (data not shown), was readily evident in apoptotic cells. Unexpectedly, $p53^{Q22/S23}$-expressing cells showed no evidence of caspase 3, 7, and 9 activation or caspase substrate cleavage under the same conditions (Fig. 2 *B–D*). Because cleavage and activation of executioner caspases were apparently unique to wild-type p53, it was of interest to determine whether their activation was required to promote apoptosis. Cells expressing either wild-type p53 or $p53^{Q22/S23}$ were treated with caspase 9 and caspase 3 inhibitors before determination of apoptosis by sub-G_1 analysis. In fact, both inhibitors effectively blocked apoptosis by wild-type p53 but not by $p53^{Q22/S23}$ (Fig. 2*E*). This indicates that wild-type p53 and mutant p53 share features of the upstream component of the apoptotic program leading to cytochrome *c* release but diverge in their execution of the downstream pathway to cell death.

A Role for Caspase 2 in Cytochrome *c* Release and Apoptosis Caused by Wild-Type p53 and $p53^{Q22/S23}$. The results shown above suggested that apoptosis triggered by $p53^{Q22/S23}$ occurs through a caspase-independent mechanism. Surprisingly, when both wild-type and mutant p53-expressing cell lines were pretreated with the broad-spectrum caspase inhibitor z-VAD-fmk, apoptotic nuclear fragmentation as assessed by DAPI staining was inhibited in both wild-type p53- and $p53^{Q22/S23}$-expressing cells (SI Fig. 10 *A* and *B*). This result was also corroborated by using sub-G_1 DNA analysis (see Fig. 3*B*). These data indicate that the apoptotic program induced by both the wild-type p53 and mutant $p53^{Q22/S23}$ requires one or more caspases.

Because caspase 2 has been implicated in stress-induced apoptosis (29, 30) we sought to determine whether this caspase is involved in the apoptotic process observed in these cell lines. Indeed, we found that daunorubicin-treated wild-type p53- and $p53^{Q22/S23}$-expressing apoptotic cells displayed a marked increase in caspase 2 activity (Fig. 3*A*).

To assess whether caspase 2 activation is required for the apoptotic pathway induced by wild-type p53 and $p53^{Q22/S23}$, cells were pretreated with the caspase 2 inhibitor z-VDVAD-fmk. As shown in Fig. 3*B*, apoptosis was greatly reduced in the presence of either z-VAD-fmk or z-VDVAD-fmk in either wild-type p53- or $p53^{Q22/S23}$-expressing cells although inhibition by z-VAD-fmk was more complete. Furthermore, wild-type p53- and $p53^{Q22/S23}$-expressing cells containing caspase 2 siRNA underwent significantly less cell death when compared with controls (Figs. 3 *C* and *D* and 4*A*). Taken together, our results implicate caspase 2 in DNA damage-dependent p53-mediated apoptosis.

It was reported that cytochrome *c* release occurs subsequent to caspase 2 activation (29, 30). To determine whether caspase 2 regulates cytochrome *c* release, cells were treated with the caspase 2-inhibitor z-VDVAD-fmk (or with z-VAD-fmk) before addition of daunorubicin. Subsequently, cytochrome *c* release was assessed by immunofluorescence microscopy (SI Fig. 11). In the presence of either inhibitor cytochrome *c* release was blocked in both wild-type p53- and $p53^{Q22/S23}$-expressing cells. Thus, cytochrome *c* release requires caspase 2 when H1299 cells expressing wild-type p53 or $p53^{Q22/S23}$ undergo DNA damage-facilitated apoptosis.

PIDDosome Components Are Involved in Apoptosis in Wild-Type p53- and $p53^{Q22/S23}$-Expressing Cells, and $p53^{Q22/S23}$ Can Induce Transcription of PIDD. Caspase 2 can be activated by a PIDDosome complex containing RAIDD and PIDD. We therefore asked whether PIDD and RAIDD were also required in our system. Indeed siRNA-mediated down-regulation of RAIDD or PIDD (as well as caspase 2 again) resulted in a significant reduction of apoptosis in cells expressing wild-type p53 and $p53^{Q22/S23}$ after

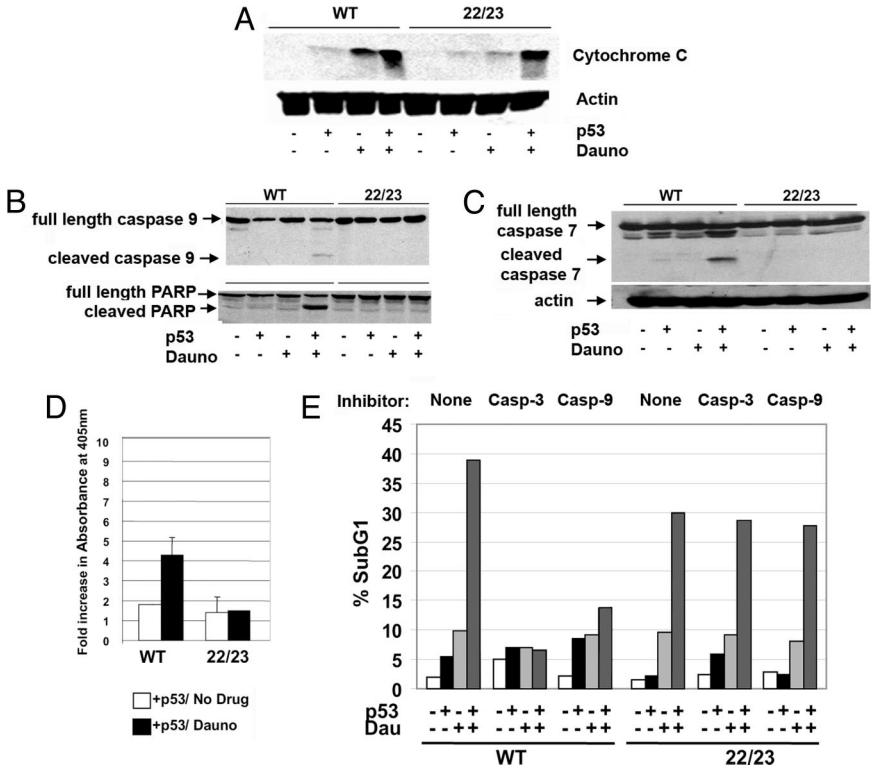

Fig. 2. Cytochrome *c* release is facilitated by both wild-type p53 and $p53^{22/23}$, but apoptosis facilitated by $p53^{22/23}$ is caspase 9/3-independent. (*A*) Expression of wild-type p53 and mutant $p53^{Q22/S23}$ leads to mitochondrial release of cytochrome *c*. Cell lines expressing (+) or not expressing (−) inducible wild-type p53 (WT) and $p53^{Q22/S23}$ (22/23) were left untreated (−) or were treated (+) with daunorubicin for 48 h as in Fig. 1. Cytosolic extracts were obtained from whole-cell extracts and analyzed for the release of cytochrome *c* by Western blotting. Actin was used as a loading control. (*B* and *C*) Cell lines expressing inducible wild-type p53 and $p53^{Q22/S23}$ were left untreated or were treated with daunorubicin for 48 h. Total cell extracts were prepared, and the cleaved products of caspase 9 and PARP (*B*) and caspase 7 (*C*) were detected by Western blot analysis. (*D*) Extracts from *B* were incubated with the caspase 3 substrate DEVD-pNA, and caspase 3 cleavage activity, as measured by increased absorbance at 405 nm, was analyzed in a 96-well microplate reader. (*E*) Cell lines expressing inducible wild-type p53 and inducible $p53^{Q22/S23}$ were left untreated or were treated with daunorubicin for 48 h in the presence or absence of the caspase 3 inhibitor, z-DEVD-fmk, or the caspase 9 inhibitor z-LEHD-fmk. FACS analysis was used to obtain values for the percentage of sub-G_1 cells. The bars show the percentage of sub-G_1 DNA content of cells 48 h after daunorubicin treatment. White bars, untreated, without p53; black bars, untreated, with p53; light gray bars, treated, without p53; dark gray bars, treated, with p53.

DNA damage (Fig. 4*A* and SI Fig. 12). The mutant $p53^{Q22/S23}$ has been shown to be impaired in activation of numerous p53 target genes and has been used frequently to determine transcription-dependent vs. -independent functions of p53. Nevertheless, some reports have indicated that $p53^{Q22/S23}$ has limited transcriptional activity (31–33). The involvement of the p53 target PIDD in apoptosis led us to consider the possibility that $p53^{Q22/S23}$ may not be transcriptionally inert in H1299 cells. Remarkably, RT-PCR analyses showed that $p53^{Q22/S23}$ is capable of efficient induction of PIDD and AIP1 (Fig. 4*B*) as well as KILLER/DR5 and Puma (data not shown) when compared with wild-type p53. In fact, when fully expressed, $p53^{Q22/S23}$ induced the proapoptotic target AIP1 significantly better than wild-type p53 although, as expected, it was very poor in inducing the cell cycle arrest target p21 (Fig. 4*B*).

In light of the fact that reduction of caspase 2, RAIDD, and PIDD each reduces cell death, it is interesting that $p53^{Q22/S23}$ can induce PIDD expression to the same extent as wild-type p53. We conclude that PIDD is a key p53 target gene in DNA damage-facilitated cell death. Because both forms of p53 induce PIDD to similar extents in the presence or absence of DNA damage, additional events are required for apoptosis to occur.

Discussion

Our analysis of the features of apoptosis caused by wild-type p53 and $p53^{Q22/S23}$ has revealed that there are at least two genetically separable functions of p53 capable of inducing cell death through mitochondria (Fig. 5 and SI Table 1). One requires the full repertoire of transactivation/repression functions of p53 and involves the canonical pathway involving activation of multiple initiator and effector caspases, whereas the other entails a very restricted transcription program and a cell death pathway that is distinct from the better-studied pathway. In either case, p53-mediated apoptosis requires activation of caspase 2. Our results provide new information about p53-mediated apoptosis and yet pose some interesting questions.

The p53 transactivation region can be subdivided into two discrete subdomains, AD1 (spanning residues 20–40) and AD2 (spanning residues 40–60) (reviewed in ref. 34). Mutation of AD2 and/or the proline-rich region extending between amino acids 60 and 90 produces a version of p53 that is incapable of inducing apoptosis but still retains the ability to transactivate genes such as p21 that cause cell cycle arrest (reviewed in ref. 34). By contrast, the p53 variant we have studied here, which is simultaneously mutated at two residues L22Q and W23S, is severely impaired for activation of most of the commonly studied p53 target genes (32) and is compromised in its interactions with TAF9 (35), CBP (36), and Mdm2 (37) although it is capable of inducing cell death. In this study we show that $p53^{Q22/S23}$ is not fully dysfunctional as a transcriptional regulator and can induce some proapoptotic targets including AIP1, PIDD, KILLER/DR5, and Puma to the same extent or better (AIP1) than

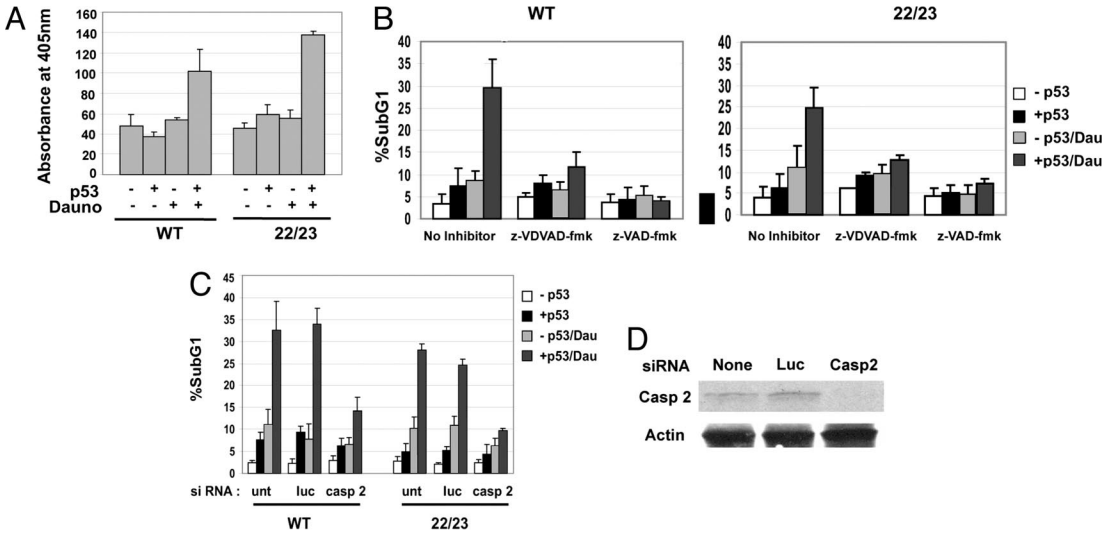

Fig. 3. Caspase 2 is activated by and required for apoptosis induced by wild-type and mutant p53. (*A*) Caspase 2 activation by wild-type and mutant p53. Cell lines expressing inducible wild-type p53 (WT) and $p53^{Q22/S23}$ (22/23) were left untreated or were treated with daunorubicin for 48 h. Whole-cell extracts were incubated with the caspase 2 substrate VDVAD-pNA, and cleavage activity, as measured by increased absorbance at 405 nm, was analyzed in a 96-well microplate reader. (*B*) Caspase 2 inhibition blocks apoptosis induced by wild-type p53 and mutant $p53^{Q22/S23}$. Cell lines expressing inducible wild-type p53 (*Left*) and $p53^{Q22/S23}$ (*Right*) were left untreated or were treated with daunorubicin for 48 h in the presence or absence of z-VDVAD-fmk or z-VAD-fmk. FACS analysis was used to obtain values for the percentage of sub-G_1 cells. The bars show the percentage of sub-G_1 DNA content of cells 48 h after daunorubicin (Dau) treatment. White bars, untreated, without p53; black bars, untreated, with p53; light gray bars, treated, without p53; dark gray bars, treated, with p53. The results show the average of three experiments. (*C*) Caspase 2 siRNA blocks apoptosis by wild-type and mutant p53. Cell lines expressing inducible wild-type p53 and $p53^{Q22/S23}$ were transfected with caspase 2 siRNA or luciferase GL3 siRNA or were left untransfected. After 24 h, cells were left untreated or were treated with daunorubicin for 48 h. FACS analysis was used to obtain values for the percentage of sub-G_1 cells. The bars show the percent of sub-G_1 DNA content of cells 48 h after daunorubicin treatment. White bars, untreated, without p53; black bars, untreated, with p53; light gray bars, treated, without p53; dark gray bars, treated, with p53. The results show the average of three individual experiments. (*D*) Caspase 2 protein expression after siRNA transfection. H1299 cells were untransfected or transfected with caspase 2 siRNA or luciferase GL3 siRNA for 48 h. Caspase 2 levels were determined by immunoblotting using a caspase 2 monoclonal antibody and actin as a loading control.

wild-type p53. This mutant may use a restricted transcription program during apoptosis that is different from wild-type p53.

Recent articles have provided detailed information about transcription-independent roles for p53 in the cytoplasm. Mihara *et al.* (38) reported the ability of p53 to interact with BclXL at the mitochondria in the apparent absence of transcription by p53. Furthermore, when murine mutant $p53^{Q25/S26}$ is forced into cytoplasm of MEFs by wheat germ agglutinin treatment, apoptosis that involves p53 activation of Bax protein ensues (ref. 39; reviewed in ref. 40). Whether caspase 2 activation is involved in the nontranscriptional role of p53 in apoptosis remains to be determined.

Our studies show that expression of the p53-responsive gene PIDD is induced by wild-type p53 or $p53^{Q22/S23}$ in the absence or presence of DNA damage. Moreover, siRNA gene silencing of RAIDD and PIDD suppresses caspase 2-dependent apoptosis induced by wild-type p53 or $p53^{Q22/S23}$. We speculate that wild-type p53 or $p53^{Q22/S23}$ up-regulation of PIDD results in the enhanced recruitment of PIDD and caspase 2 to form the PIDDosome complex with RAIDD resulting in the activation of caspase 2. However, because we have yet to determine whether formation of an active PIDDosome complex occurs in our system, it remains possible that the activation of caspase 2 by wild-type p53 or $p53^{Q22/S23}$ does not require increased levels of PIDD. A recent report indicates an alternative pathway for caspase 2-dependent activation and apoptosis in 5-FU-treated HCT116 cells that does not rely on RAIDD or PIDD (26), suggesting that either cell- or stimulus-specific differences exist in requirement for PIDDosome components in apoptosis.

Finally, it is interesting that cytochrome *c* is released after caspase 2 activation during $p53^{Q22/S23}$-mediated apoptosis but does not result in caspase 3 or caspase 9 activation. Because inhibition of caspase 3 or 9 blocks apoptosis in wild-type p53-expressing cells this implies that the apoptotic pathway initiated by $p53^{Q22/S23}$ is not functional in cells expressing wild-type p53. We cannot formally rule out that $p53^{Q22/S23}$ elicits an alternative form of programmed cell death distinct from the canonical apoptosis process. Nonetheless, we consider the following intriguing questions in thinking about future directions. First, why does cytochrome *c* release not cause the formation of the apoptosome? Perhaps the defect in caspase 9 activation results from its inhibition by IAP expression or activity in the absence of wild-type p53. Alternatively, Apaf1, a transcriptional target of p53 (5) that acts as part of the apoptosome to activate caspase 9, may be differentially regulated by wild-type and mutant p53. Preliminary experiments have not supported these possibilities, but further work needs to be done. Second, how does the mutant p53 cause apoptosis without activation of the apoptosome? We have found that apoptosis-inducing factor (AIF) is released during apoptosis caused by both wild-type and mutant p53 (data not shown). AIF (and/or endoG) thus may play a role in $p53^{Q22/S23}$- but not wild-type p53-mediated apoptosis. Third, does wild-type p53 actively suppress the alternative pathway, or does $p53^{Q22/S23}$ produce effects that are entirely distinct from wild-type p53? Speculative scenarios include the induction of proapoptotic genes that are exclusively regulated by $p53^{Q22/S23}$ but not wild-type p53, or deficits in Apaf1/cytochrome *c* interaction, Smac/DIABLO release, or HtrA2 expression/activity only when the cells are expressing $p53^{Q22/S23}$. It will be of great interest to determine whether any particular cell stresses or cell types preferentially activate upstream signaling networks that act through the second transactivation domain of wild-type p53. Finally, both wild-type and $p53^{Q22/S23}$ activation of (and requirement for) caspase 2 requires both induction of p53 and DNA damage. It will therefore be challenging but interesting to determine how DNA damage facilitates caspase 2-dependent

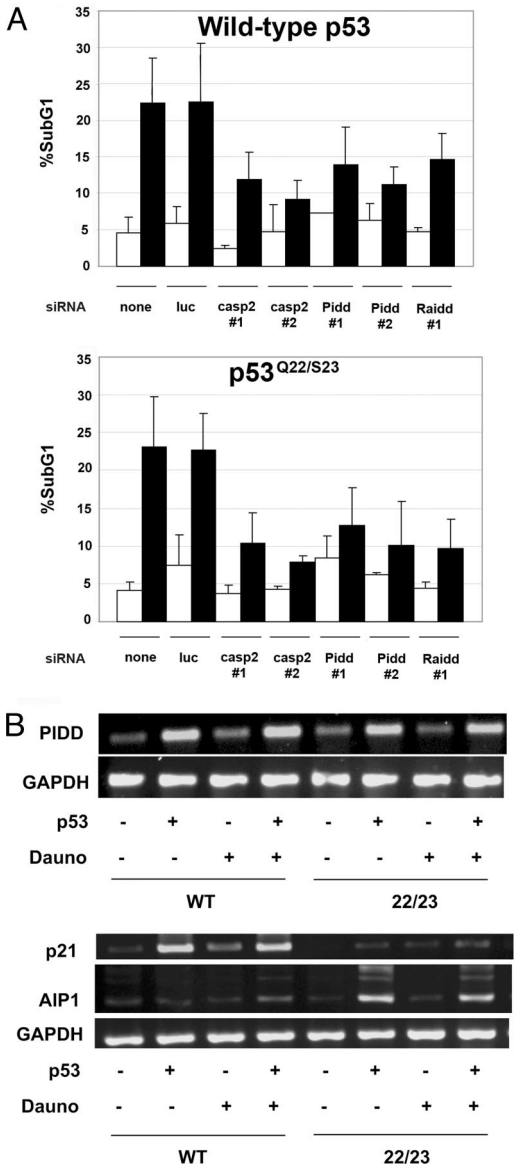

Fig. 4. Caspase 2, RAIDD, or PIDD inhibition reduces apoptosis by wild-type and mutant p53$^{Q22/S23}$, while p53$^{Q22/S23}$ retains the ability to transactivate AIP1 and PIDD. (*A*) Cells expressing inducible wild-type and p53$^{Q22/S23}$ were transfected with luciferase GL3 siRNA (luc), caspase 2 siRNAs (casp2 #1 and casp2 #2), PIDD siRNAs (Pidd #1 and Pidd #2), or RAIDD siRNA (Raidd #1) or were left untransfected. After 24 h, cells were treated with daunorubicin for 48 h. FACS analysis was used to obtain values for the percentage of sub-G_1 DNA content of cells. The bars show the percentage of sub-G_1 DNA content of cells 48 h after daunorubicin treatment. White bars, untreated, with p53; black bars, treated, with p53. The results show the average of at least three separate experiments. (*B*) Cell lines expressing inducible wild-type p53 and p53$^{Q22/S23}$ were left untreated or were treated with daunorubicin for 48 h. Total RNA was isolated, and RT-PCR analysis was performed to examine the expression of the p53 targets p21, AIP1, and PIDD. GAPDH expression was used as a loading control.

apoptosis caused by p53. Future experiments are needed to provide answers to these questions.

Materials and Methods

Cell Culture and Transfection. H1299 cells stably expressing either wild-type p53 or p53$^{Q22/S23}$ as described (27) were maintained in RPMI medium 1640 (GIBCO) containing 10% FBS, 2 μg/ml G418, 2.5 μg/ml puromycin, and 4.5 μg/ml tetracycline (tet). Cultures (6-mm wells) were transfected with GFP (5 μg) as previously described (41). siRNA oligos from Dharmacon (caspase2 no. 1, caspase2 no. 2, and luciferase GL3) and Qiagen (RAIDD no. 1, PIDD no. 1, and PIDD no. 2; 0.27 μg/ ml) were transfected into six-well plates by using Lipofectamine 2000 reagent (Invitrogen). Details about RNA oligos for siRNA analysis are in *SI Materials and Methods*.

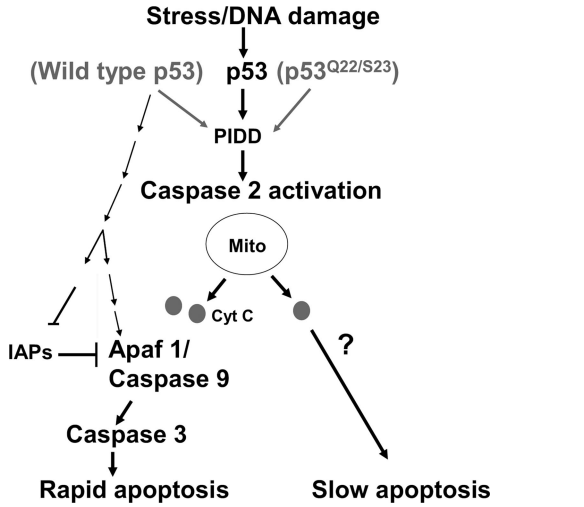

Fig. 5. Stress-induced apoptosis mediated by p53 can involve two pathways and requires caspase 2 for cytochrome *c* release.

Antibodies. The following primary antibodies were used: p53, 1801 hybridoma supernatant; actin, α-actin (Sigma); Bcl2, 100 (Santa Cruz Biotechnology); caspase 2, α-caspase2 (BD Pharmingen); PARP, C2-10 (Santa Cruz Biotechnology); caspase 7, α-caspase 7 (BD Pharmingen); cytochrome *c*, α-cytochrome *c* (BD Pharmingen); caspase 9, no. 9501 (Cell Signaling Technology).

p53 and Apoptosis Induction. Cells were plated with or without medium containing tet. To induce p53, cells were plated in media lacking tet, incubated for 24 h, and washed twice in media lacking tet. After 40 h cells were treated with 0.22 μM daunorubicin (Calbiochem) for an additional 48 h. For caspase inhibition experiments, the following inhibitors (all from Calbiochem) were added to the medium immediately after p53 induction: z-VAD-fmk (100 μM), z-VDVAD-fmk (25 μM), z-LEHD-fmk (10 μM), and z-DEVD-fmk (50 μM).

Immunoblot Analysis. Cells were collected from 10-cm plates in cold PBS followed by centrifugation at 10,000 $\times$ *g*. The pellets were lysed on ice for 15 min in Nonidet P-40 lysis buffer [10 mM Tris, pH 7.5/1 mM EDTA/10% glycerol/0.5% Nonidet P-40/300 mM NaCl/1 mM DTT/0.5 mM PMSF/protease inhibitors (1 μM benzamidine, 3 μg/ml leupeptin, 0.1 μg/ml bacitracin, and 1 μg/ml macroglobulin)]. Lysates were cleared by centrifugation (13,000 rpm for 10 min), and samples were analyzed according to standard immunoblotting procedures by using enhanced chemiluminescence (Amersham). For PARP analysis, cells were collected in cold PBS, resuspended in an extraction buffer containing 62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, and 5% 2-mercaptoethanol, sonicated for 60 s, and then incubated at 65°C for 15 min. Samples were then loaded onto a 7.5% gel and immunoblotted with anti-PARP antibody.

Flow Cytometry Analysis. Cells were seeded on 6-cm plates with or without 4.5 μg/ml tet. After 24 h medium was removed from plates without tet and replaced with fresh tet-free medium for wild-type p53- and p53$^{Q22/S23}$-expressing cells or with 5 and 10 ng/ml tet for only the p53$^{Q22/S23}$-expressing cells. After 40 h cells were treated with 0.22 μM daunorubicin, and 48 h later cells were trypsinized and analyzed by flow cytometry for sub-G_1 DNA content (FACSCalibur; Becton Dickinson) as previously described (27). Cell cycle stages were analyzed by using the ModFit LT Version 3.0 program.

Cytochrome *c* Release Assay. Cells in 10-cm dishes were trypsinized, and floating and adherent cells were pooled and centrifuged at 650 $\times$ *g*. Pellets were washed once with PBS and then resuspended in five volumes of sucrose cell extraction buffer (250 mM sucrose/20 mM Hepes-KOH, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/1 mM sodium EDTA/1 mM sodium EGTA/1 mM DTT/0.1 mM PMSF/20 μg/ml leupeptin/10 μg/ml aprotinin/10 μg/ml pepstatin A). The resuspended cells were Dounce homogenized with 50 strokes followed by

centrifugation twice at 850 × *g* for 15 min at 4°C to remove nuclear contaminants and debris. Supernatants were then centrifuged at 14,000 × *g* for 30 min, and the mitochondrial pellets were resuspended in sucrose cell extraction buffer. The final supernatants represented the soluble cytosolic fractions.

Caspase Cleavage Colorimetric Assay. Caspase 2 and caspase 3 activities were detected by using assay kits from Calbiochem and Clontech, respectively. The assays were performed according to the manufacturer's instructions.

Semiquantitative RT-PCR. H1299 cells were treated as described and then were washed two times with cold PBS. Total RNA was isolated by using the RNeasy Total RNA Isolation Kit (Qiagen). Approximately 4 μg of total RNA was used to prepare cDNA using the SuperScript First Strand cDNA Synthesis Kit (Invitrogen) for oligo(dT) priming. cDNA reaction mixtures (2 μl) were used as template for RT-PCR in which samples were initially heated at 94°C for 5 min, and then 20 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min were carried out. For details about primer constructions, see *SI Materials and Methods*.

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1. Fridman JS, Lowe SW (2003) Control of apoptosis by p53. *Oncogene* 22:9030–9040.
2. Bargonetti J, Manfredi JJ (2002) Multiple roles of the tumor suppressor p53. *Curr Opin Oncol* 14:86–91.
3. Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. *Nature* 408:307–310.
4. Stark GR, Taylor WR (2006) Control of the G_2/M transition. *Mol Biotechnol* 32:227–248.
5. Yu J, Zhang L (2005) The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun* 331:851–858.
6. Ho J, Benchimol S (2003) Transcriptional repression mediated by the p53 tumour suppressor. *Cell Death Differ* 10:404–408.
7. Chipuk JE, Green DR (2003) p53's believe it or not: Lessons on transcription-independent death. *J Clin Immunol* 23:355–361.
8. Murphy ME, Leu JI, George DL (2004) p53 moves to mitochondria: A turn on the path to apoptosis. *Cell Cycle* 3:836–839.
9. Schuler M, Green DR (2001) Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans* 29:684–688.
10. Wang L, Miura M, Bergeron L, Zhu H, Yuan J (1994) Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78:739–750.
11. Zhivotovsky B, Orrenius S (2005) Caspase-2 function in response to DNA damage. *Biochem Biophys Res Commun* 331:859–867.
12. Gao Z, Shao Y, Jiang X (2005) Essential roles of the Bcl-2 family of proteins in caspase-2-induced apoptosis. *J Biol Chem* 280:38271–38275.
13. Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T, Alnemri ES (2002) Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. *J Biol Chem* 277:13430–13437.
14. Wagner KW, Engels IH, Deveraux QL (2004) Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. *J Biol Chem* 279:35047–35052.
15. Robertson JD, *et al.* (2004) Processed caspase-2 can induce mitochondria-mediated apoptosis independently of its enzymatic activity. *EMBO Rep* 5:643–648.
16. Enoksson M, *et al*. (2004) Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. *J Biol Chem* 279:49575–49578.
17. Paroni G, Henderson C, Schneider C, Brancolini C (2001) Caspase-2-induced apoptosis is dependent on caspase-9, but its processing during UV- or tumor necrosis factor-dependent cell death requires caspase-3. *J Biol Chem* 276:21907–21915.
18. Schweizer A, Briand C, Grutter MG (2003) Crystal structure of caspase-2, apical initiator of the intrinsic apoptotic pathway. *J Biol Chem* 278:42441–42447.
19. Read SH, Baliga BC, Ekert PG, Vaux DL, Kumar S (2002) A novel Apaf-1-independent putative caspase-2 activation complex. *J Cell Biol* 159:739–745.
20. Tinel A, Tschopp J (2004) The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 304:843–846.
21. Berube C, *et al*. (2005) Apoptosis caused by p53-induced protein with death domain (PIDD) depends on the death adapter protein RAIDD. *Proc Natl Acad Sci USA* 102:14314–14320.
22. Jiang M, Milner J (2003) Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. *Genes Dev* 17:832–837.
23. Ren J, *et al*. (2005) The Birc6 (Bruce) gene regulates p53 and the mitochondrial pathway of apoptosis and is essential for mouse embryonic development. *Proc Natl Acad Sci USA* 102:565–570.
24. Seth R, Yang C, Kaushal V, Shah SV, Kaushal GP (2005) p53-dependent caspase-2 activation in mitochondrial release of apoptosis-inducing factor and its role in renal tubular epithelial cell injury. *J Biol Chem* 280:31230–31239.
25. Tyagi A, Singh RP, Agarwal C, Agarwal R (2006) Silibinin activates p53-caspase 2 pathway and causes caspase-mediated cleavage of Cip1/p21 in apoptosis induction in bladder transitional-cell papilloma RT4 cells: Evidence for a regulatory loop between p53 and caspase 2. *Carcinogenesis* 27:2269–2280.
26. Vakifahmetoglu H, Olsson M, Orrenius S, Zhivotovsky B (2006) Functional connection between p53 and caspase-2 is essential for apoptosis induced by DNA damage. *Oncogene* 25:5683–5692.
27. Baptiste N, Friedlander P, Chen X, Prives C (2002) The proline-rich domain of p53 is required for cooperation with anti-neoplastic agents to promote apoptosis of tumor cells. *Oncogene* 21:9–21.
28. Moll UM, Wolff S, Speidel D, Deppert W (2005) Transcription-independent pro-apoptotic functions of p53. *Curr Opin Cell Biol* 17:631–636.
29. Lassus P, Opitz-Araya X, Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297:1352–1354.
30. Robertson JD, Enoksson M, Suomela M, Zhivotovsky B, Orrenius S (2002) Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *J Biol Chem* 277:29803–29809.
31. Lin J, Chen J, Elenbaas B, Levine AJ (1994) Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev* 8:1235–1246.
32. Zhu J, Zhou W, Jiang J, Chen X (1998) Identification of a novel p53 functional domain that is necessary for mediating apoptosis. *J Biol Chem* 273:13030–13036.
33. Johnson TM, Hammond EM, Giaccia A, Attardi LD (2005) The p53QS transactivation-deficient mutant shows stress-specific apoptotic activity and induces embryonic lethality. *Nat Genet* 37:145–152.
34. Scoumanne A, Harms KL, Chen X (2005) Structural basis for gene activation by p53 family members. *Cancer Biol Ther* 4:1178–1185.
35. Lu H, Levine AJ (1995) Human TAFII31 protein is a transcriptional coactivator of the p53 protein. *Proc Natl Acad Sci USA* 92:5154–5158.
36. Gu W, Shi XL, Roeder RG (1997) Synergistic activation of transcription by CBP, p53. *Nature* 387:819–823.
37. Kussie PH, *et al*. (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* 274:948–953.
38. Mihara M, *et al*. (2003) p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 11:577–590.
39. Chipuk JE, *et al.* (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 303:1010–1014.
40. Baptiste N, Prives C (2004) p53 in the cytoplasm: A question of overkill? *Cell* 116:487–489.
41. Boussif O, *et al*. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc Natl Acad Sci USA* 92:7297–72301.