14-3-3σ Is a p53-Regulated Inhibitor of G2/M Progression

Heiko Hermeking,* Christoph Lengauer,*
Kornelia Polyak,*† Tong-Chuan He,*†
Lin Zhang,*† Sam Thiagalingam,*
Kenneth W. Kinzler,* and Bert Vogelstein*†
*The Johns Hopkins Oncology Center
†The Howard Hughes Medical Institute
Johns Hopkins University School of Medicine
Baltimore, Maryland 21231

Summary

Exposure of colorectal cancer (CRC) cells to ionizing radiation results in a cell-cycle arrest in G1 and G2. The G1 arrest is due to p53-mediated induction of the cyclin-dependent kinase inhibitor p21[WAF1/CIP1/SDI1], but the basis for the G2 arrest is unknown. Through a quantitative analysis of gene expression patterns in CRC cell lines, we have discovered that 14-3-3σ is strongly induced by γ irradiation and other DNA-damaging agents. The induction of 14-3-3σ is mediated by a p53-responsive element located 1.8 kb upstream of its transcription start site. Exogenous introduction of 14-3-3σ into cycling cells results in a G2 arrest. As the fission yeast 14-3-3 homologs rad24 and rad25 mediate similar checkpoint effects, these results document a molecular mechanism for G2/M control that is conserved throughout eukaryotic evolution and regulated in human cells by p53.

Introduction

It has long been known that DNA-damaging agents induce a cell-cycle arrest, allowing time for repair and thus protecting the organism from the deleterious consequences of mutation (reviewed in Paulovich et al., 1997). In mammalian cells, these arrests are often dependent on the functionality of the p53 gene product, a transcription factor that is translationally and post-translationally activated following DNA damage (reviewed in Cox and Lane, 1995; Levine, 1997; Morgan and Kastan, 1997). Because p53 is mutated in a large fraction of cancers of diverse types, it is thought that the tumorigenic process may be intimately related to the disruption of p53-mediated control of the cell cycle. Accordingly, there has been much effort to define the molecular links between DNA damage, p53 expression, and cell-cycle regulation.

Cells treated with ionizing radiation or other DNA-damaging agents arrest in both G1 and G2, with a consequent decrease in the fraction of cells in S phase. In colorectal-cancer cells and many other epithelial cell types, 15%–40% of the cells arrest in G1 and the remainder arrest in G2/M. The G1 block is in part mediated by p21[WAF1/CIP1/SDI1] (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1995), a cyclin-dependent kinase inhibitor that is transcriptionally controlled by p53 (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993). Several

studies have suggested that the G2/M block following DNA damage is also p53-dependent (Agarwal et al., 1995; Aloni-Grinstein et al., 1995; Stewart et al., 1995; Goi et al., 1997). However, the basis for this G2/M block, though accounting for the predominant form of arrest induced by radiation in many cell types, is unknown.

In this study, we have analyzed the patterns of gene expression following irradiation of a human CRC cell line expressing wild-type p53. These cells arrested mostly in G2, and this arrest was accompanied by changes in gene expression. The most intriguing of the identified changes involved an increase in the expression of 14-3-3σ, a gene originally discovered through its expression in differentiating epithelial cells and a member of the 14-3-3 protein family (Prasad et al., 1992; Leffers et al., 1993). Numerous members of this family, including seven distinct human genes, have been identified, with diverse properties ascribed to them (for review, see Aitken, 1995; Wang and Shakes, 1996). The function of 14-3-3σ (also called HME1 or stratifin and here referred to simply as "σ") has heretofore been unknown. We now show that σ expression in human cells is regulated by p53 and that exogenous expression of σ results in a G2 block similar to that observed following γ irradiation. As the fission yeast 14-3-3 homologs rad24 and rad25 have similar cell cycle control functions, the results suggest a remarkable conservation of the molecular basis for eukaryotic checkpoints and a model for p53-mediated cell-cycle control involving the coordinate expression of both p21 and σ and their inhibition of cyclin-dependent kinases.

Results

Analysis of Gene Expression Following γ Irradiation

The human colorectal cancer cell line HCT116 expresses wild-type p53 and arrests following γ irradiation in an apparently normal fashion, with ∼25% of cells in G1 and ∼75% of cells in G2 (see Figure 5C). RNA was purified from HCT116 cells 60 hr following irradiation and analyzed by the SAGE (serial analysis of gene expression) technique. With SAGE, each RNA species is represented by a 15-base tag uniquely positioned near its 3′ end. Tags are concatamerized and sequenced, and the abundance of each transcript determined from the relative number of tags. A library of 55,429 tags from γ-irradiated HCT116 cells was generated and found to represent 20,291 different mRNAs (of which 6831 were represented by entries in GenBank, release 94). We initially focused on the 100 tags that appeared to be induced to the highest levels in irradiated HCT116 cells compared to exponentially growing HCT116 cells (see Experimental Procedures). Next, we determined whether any of these 100 tags were represented at significantly higher levels in a tag library from colon-cancer cells expressing an exogenous p53 gene compared to the same cells in the absence of exogenous p53 (Polyak et al., 1997). Only 3 of the 100 tags were expressed at 5-fold or higher levels in the p53-expressing cells. Database
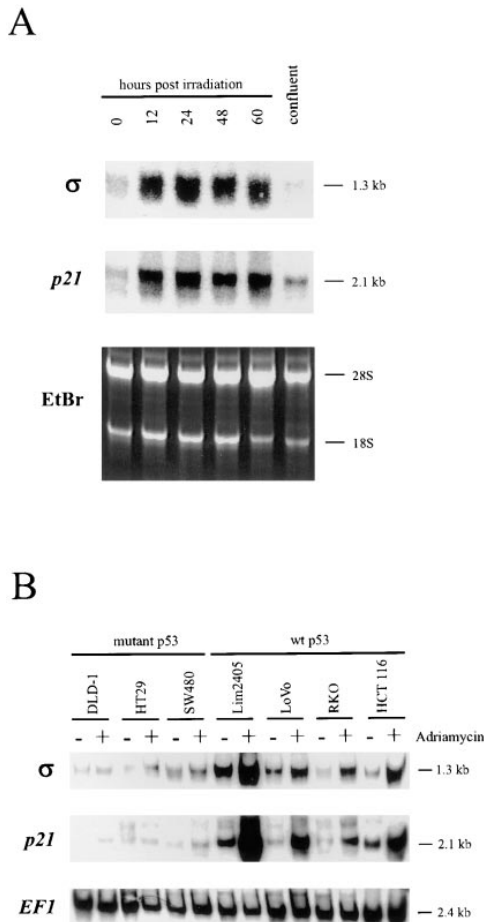
A

B

Figure 1. σ Expression Following DNA Damage

(A) σ expression following irradiation. HCT116 cells were γ-irradiated during exponential growth. RNA was prepared at the indicated times after irradiation or after reaching confluency in the absence of irradiation (confluent). Autoradiographs of Northern blots performed with σ and p21 probes are presented along with a picture of the ethidium bromide (EtBr)–stained gel from which the blots were made. Sizes of the detected transcripts are indicated on the right.

(B) σ expression following adriamycin treatment. Seven different CRC cell lines were treated with 0.2 μg/ml adriamycin (+) or vehicle (−) as indicated. RNA was prepared 24 hr following adriamycin treatment. Autoradiographs of Northern blots performed with σ, p21, and EF1 probes are shown. The p53 status of the respective cell lines is indicated on the top.

searching with the tag sequences tentatively identified one of the genes as that encoding σ and one as *ISG*, encoding a 15 kDa protein induced by interferon (Blomstrom et al., 1986); there were no matches for the third tag. The tag corresponding to σ was of particular interest in light of previous studies demonstrating that *14-3-3* homologs are essential for irradiation-induced G2 arrest in Schizosaccharomyces pombe (Ford et al., 1994; Weinert et al., 1994). A total of six different *14-3-3* family members (σ, ε, τ, β, η, and ζ) were represented in our SAGE libraries, but only σ was found to be expressed at higher levels following both irradiation and p53 expression.
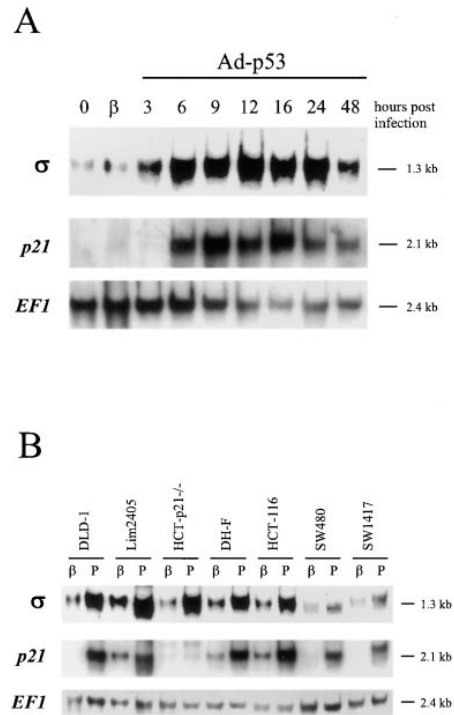
A

B

Figure 2. σ Expression Following p53 Expression

Cells were infected with Ad-*p53* for the indicated time periods in (A) and with Ad-*p53* (lanes marked [P]) or Ad-*β* (lanes marked [β]) in (B). Northern blotting was performed as in Figure 1.

Induction of σ by DNA Damage

Northern blotting with a radiolabeled probe generated from σ cDNA was performed to confirm the SAGE results. As shown in Figure 1A, σ transcripts were induced within 12 hr of irradiation and remained elevated for at least 60 hr. Interestingly, no induction of σ was found when cells (85% of the total) were arrested in G1 by growing them to confluence (Figure 1A). In addition to γ irradiation, other DNA-damaging agents, such as adriamycin, were found to similarly induce σ expression in HCT116 cells (Figure 1B). The induction of σ by DNA damage was not confined to HCT116 cells, as several other colorectal cancer cell lines were shown to express σ at relatively high levels following adriamycin treatment (Figure 1B). Previous reports have shown that σ expression is limited to epithelial cells (Prasad et al., 1992; Leffers et al., 1993). In agreement with these observations, we found that σ was not detectably expressed in either exponentially growing or γ-irradiated human diploid fibroblasts or in human endothelial cells (data not shown).

Of 7 CRC cell lines analyzed, the 4 that expressed higher levels of σ following DNA damage contained endogenous wild-type *p53* alleles, while the 3 in which σ was uninduced contained mutant *p53* genes (Figure 1B). This observation was consistent with the hypothesis that p53 was responsible for the DNA damage-induced expression of σ. To more directly test the ability of p53 to modulate σ expression, we infected CRC cells with a replication defective adenovirus engineered to express
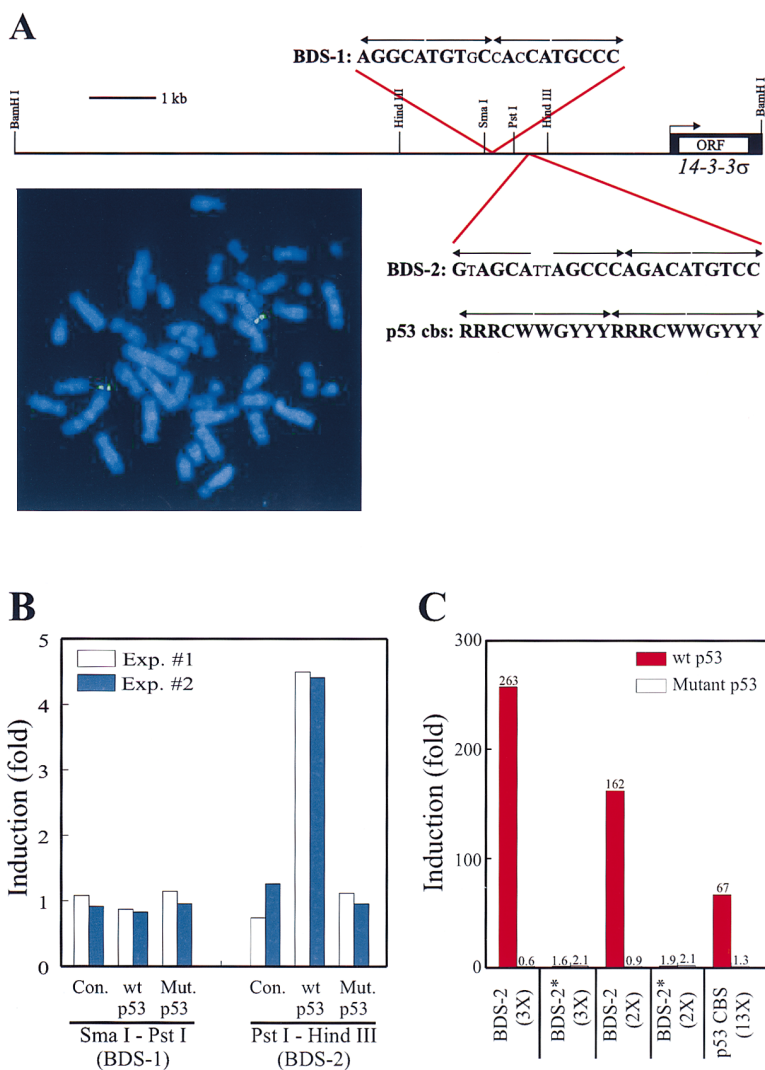
A

BDS-1: AGGCATGTGCCACCATGCCC

1 kb

BamH I   Hind III   Sma I   Pst I   Hind III   BamH I

ORF

14-3-3σ

BDS-2: GTAGCATTAGCCCAGACATGTCC

p53 cbs: RRRCWWGYYYRRRCWWGYYY

B

Induction (fold)

- ☐ Exp. #1
- ■ Exp. #2

Con. / wt p53 / Mut. p53 — Sma I - Pst I (BDS-1)

Con. / wt p53 / Mut. p53 — Pst I - Hind III (BDS-2)

C

Induction (fold)

- ■ wt p53
- ☐ Mutant p53

263 0.6 1.6,2.1 162 0.9 1.9,2.1 67 1.3

BDS-2 (3X) | BDS-2* (3X) | BDS-2 (2X) | BDS-2* (2X) | p53 CBS (13X)

Figure 3. The σ Gene: Localization to *1p35*, Genomic Organization, and Identification of a p53-Binding Site

(A) Genomic Structure and chromosomal localization of σ. A map of a 9.5 kb BamHI fragment of σ, indicating the potential *p53* binding sites *BDS-1* and *BDS-2* identified by sequencing (see text), is presented. The position of the σ open reading frame (ORF), the presumptive transcription start site (arrow), and selected restriction endonuclease sites used for vector constructions are indicated. The previously characterized p53-consensus binding site (cbs; El-Deiry et al., 1992) is shown under the *BDS-2* sequence, with (R) = purine, (Y) = pyrimidine, and (W) = A or T. The insert shows an example of FISH, localizing σ to *1p35*.

(B) p53 responsiveness of σ promoter fragments. The indicated DNA fragments (*BDS-1* and *BDS-2*) were cloned into a luciferase reporter vector containing a minimal promoter. SW480 cells were cotransfected with these reporters, a β-galactosidase encoding plasmid to control for transfection efficiency, and wild-type p53 (wt), mutant p53 (Mut.), or empty (Con.) expression constructs. Luciferase activity is expressed as a fold induction relative to the average obtained with the empty vector control.

(C) p53 responsiveness of the σ p53 binding site *BDS-2*. Reporters containing either two or three copies of the *BDS-2* sequence were constructed as described in Experimental Procedures. Four critical p53-binding residues of *BDS-2* were altered in the reporter marked *BDS-2**. These reporters were transfected and analyzed as in (B). The results are represented as fold induction by either wild-type p53 or mutant p53 compared to that resulting from transfections with an empty vector. Each bar represents the average of two independent experiments; the results in the two experiments varied by less than 20% of the average value indicated in the graph. The plasmid PG$_{13}$-luc, containing 13 copies of a consensus p53-binding site, was used as a positive control (Kern et al., 1992). The numbers in parentheses on the X axis indicate the number of copies of the indicated p53-binding site in each reporter.

wild-type p53. Exogenous p53 expression induced a pronounced rise in σ RNA that was first observed at 3 hr following infection, earlier than *p21* or any other genes known to be induced by p53 in these cells (Figure 2A and data not shown). The maximum induction (18-fold) of σ was achieved at ~12 hr after infection. The induction of σ by exogenous p53 (compared to a control adenovirus, Ad-β, encoding β-galactosidase) was consistently observed in each of seven CRC lines tested (examples in Figure 2B). Additionally, σ was induced by p53 in a CRC line with a deletion of *p21*, demonstrating that the induction of σ was not secondary to an induction of a *p21*-mediated growth arrest stimulated by p53 (Figure 2B).

Basis for the p53 Dependence of σ Induction

To determine the molecular basis for the p53 induction of σ, a genomic clone containing the human σ gene and ~100 kb of surrounding sequences was obtained by screening a bacterial artificial chromosome (BAC) library. Fluorescence in situ hybridization (FISH) analysis of metaphase chromosomes with a probe generated from this BAC clone localized σ to chromosome band *1p35* (Figure 3A). A 9.5 kb BamHI fragment containing the entire σ coding region plus 8.5 kb of upstream sequences was then subcloned and completely sequenced. This region of the BAC revealed a presumptive transcription start site (TSS) when assessed with the TSSG program (Baylor College of Medicine); the TSS coincided with the 5′ end of the most complete σ cDNA clone. To test the ability of sequences within the σ promoter to mediate p53-dependent transcription, subclones from the BAC, containing 1 kb or 4 kb of sequences upstream of TSS, were placed upstream of a promoterless luciferase reporter gene (Figure 3A). The reporter containing 4 kb of promoter sequences was efficiently activated by wild-type (but not mutant) p53, while the reporter with 1 kb had no p53-dependent activity (data not shown). This localized the presumptive p53-regulatory region to the region between 1 and 4 kb from the TSS. The sequence revealed two potential p53-binding
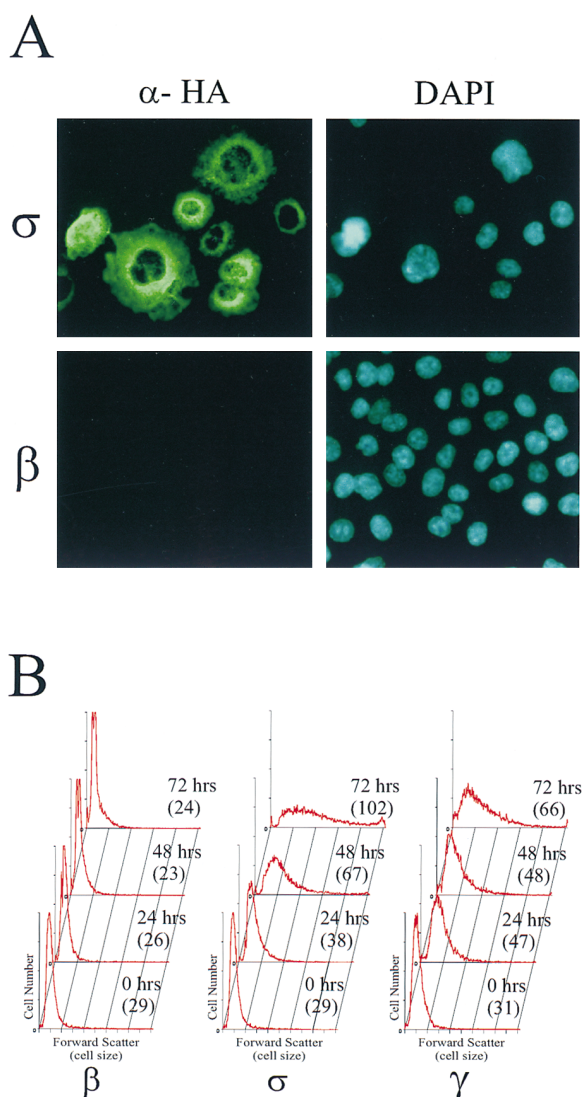
A

| α- HA | DAPI |

σ

β

B

Figure 4. Morphology of σ-Expressing Cells

(A) Immunohistochemical analysis of σ-expressing cells. HCT116 cells were infected with Ad-σ or Ad-β for 30 hr, then stained with an anti-HA antibody, recognizing the HA tag appended to the σ protein. Chromatin staining of the same cells with DAPI revealed the perinuclear localization of σ protein.

(B) Flow cytometric analysis of σ-expressing cells. The same cells and γ-irradiated HCT116 cells were fixed at the indicated times, analyzed by flow cytometry, and assessed for forward light scatter to determine relative size. Each curve represents 10,000 cells.

sites, named BDS-1 and BDS-2, located 2.5 and 1.8 kb upstream of the TSS, respectively, and within the region of the promoter predicted to contain functionally active p53 response elements in the transfection experiments (Figure 3A). Restriction fragments containing each of these two sites were then individually cloned upstream of a luciferase reporter containing a minimal promoter. A PstI–HindIII fragment containing BDS-2, but not a similarly sized fragment containing BDS-1, was found to have substantial p53-dependent activity in this assay (Figure 3B). To prove that the BDS-2 sequence within the PstI–HindIII fragment was indeed responsible for

p53-dependent activation, a 37 bp sequence containing the 20 bp BDS-2 was multimerized and cloned upstream of a minimal promoter and luciferase reporter. The BDS-2 sequences endowed the reporter with exceptional p53-dependent activation properties. Two to three copies of BDS-2 provided substantially more activation than 13 copies of the p53-binding consensus sequences present in the standard p53 reporter PG$_{13}$-luc (Figure 3C; Kern et al., 1992). Mutant p53 had no activating effect, and nucleotide substitutions at residues of BDS-2 predicted to be critical for p53 binding completely abrogated p53-dependent activity (Figure 3C).

σ Expression Disrupts G2/M Progression

cDNA clones of σ were obtained from EST depositories. The sequences of the cDNA clones revealed important corrections to previously published sequences, which were verified by the sequence of genomic clones. To determine whether the expression of σ was causally related to the G2 arrest associated with its induction, we tagged the cDNA with a hemagglutinin (HA) epitope and cloned it into an adenoviral vector to create Ad-σ. HCT116 cells underwent remarkable morphological changes following infection with Ad-σ, and by 30 hr were flattened and enlarged, resembling cells treated with γ irradiation. Staining of Ad-σ-infected cells with an anti-HA antibody showed σ protein to be cytoplasmic, and in some cells a striking perinuclear localization was evident (Figure 4A). DAPI staining demonstrated a nuclear enlargement that accompanied the size increase in cells expressing σ (Figure 4A).

Flow cytometry allowed us to confirm and quantitate the increase in size of HCT116 cells expressing σ (Figure 4B). A similar size increase occurred in γ-irradiated cells (Figure 4B). Flow cytometry additionally revealed a G2/M arrest (DNA content of 4N) in cells infected with Ad-σ compared to those infected with the control Ad-β (24 hr time point in Figures 5A and 5B). There was no evidence of chromosome condensation or nuclear membrane dissolution in these cells, even after treatment with the microtubule disrupting agents nocodazole or colcemid to stabilize any mitoses that may have occurred. There was a complete block of cellular proliferation after Ad-σ infection, with no increase in cell number observed over 7 days (data not shown). Infection of four other CRC cell lines, as well as normal prostate epithelial cells, resulted in a similar arrest of the cell cycle, with a build-up of cells with a DNA content of 4N and no evidence of mitosis (data not shown).

σ Expression Can Induce an Uncoordinated Growth Arrest

At 24 hr following Ad-σ infection of HCT116 cells, the cell-cycle profiles were very similar to those observed in γ-irradiated cells (Figures 5B and 5C). However, a significant difference became apparent at longer times after infection. While irradiated cells remained in a G2-arrested state, with a nuclear DNA content of 4N, many σ-expressing cells entered into a DNA synthetic phase, resulting in a significant population of cells with a DNA content greater than 4N at 48 and 72 hr (Figures
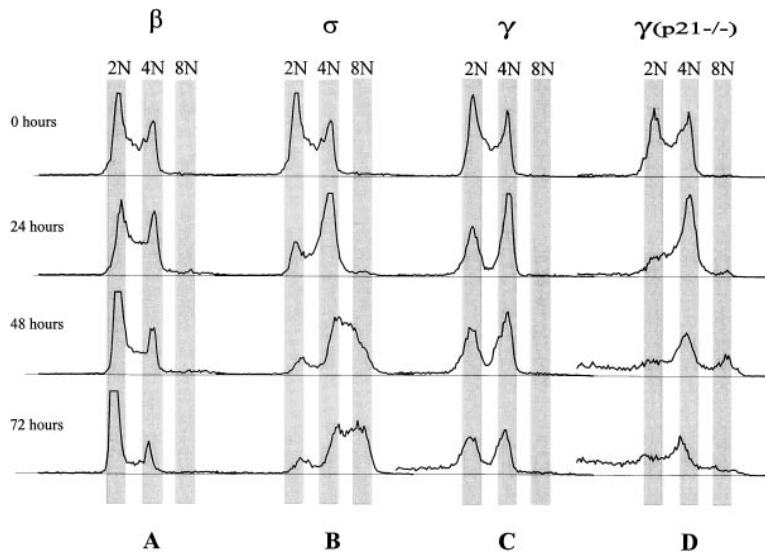
Figure 5. Exogenous Expression of σ Induces a G2 Arrest Followed by Polyploidy

Subconfluent HCT116 cells were infected with Ad-β (A), Ad-σ (B), or treated with γ irradiation (C). HCT116 cells made *p21*-deficient through homologous recombination (D) were similarly treated with γ irradiation. The *p21*-deficient cells began to undergo apoptosis at the late time points. DNA content of at least 10,000 cells was analyzed by flow cytometry at the indicated times.

5B and 5C). Addition of colcemid 30 hr after Ad-σ infection did not prevent this polyploidization (data not shown). This increased ploidy was further demonstrated to result from true DNA reduplication by interphase FISH analysis. Two different chromosomal loci were assessed 72 hr following infection with either Ad-σ or Ad-β. For each analyzed chromosomal locus, nearly half of the σ-expressing cells contained more than the four hybridization signals per locus expected in G2 (one signal from each chromatid; Figure 6A, right panel). Thirty percent of the cells contained 8 signals, 11% contained 16 signals, and 1% contained 32 signals, representing cells with DNA contents of 8N, 16N, and 32N, respectively (Figure 6B). The signals in polyploid cells were nearly always paired, representing bivalent chromosomes (115 of 125 cells examined). However, each pair was separated from other pairs in the same nucleus (Figure 6A, right). This clearly indicated that chromatids had detached from each other prior to a new round of DNA replication and creation of new bivalents. Very few cells infected with Ad-β exhibited more than four hybridization signals/probe, and many exhibited two signals (representing cells in G1; Figure 6A, left panel and Figure 6B).

These results suggested that exogenous σ expression can produce an uncoordinated block in which cycles of DNA synthesis can proceed without intervening mitoses. One potential reason for this uncoordinated arrest, compared to the coordinated arrest observed in γ-irradiated cells, involves *p21*. Irradiated cells express both σ and *p21* in a p53-dependent manner, and p21 can prevent DNA synthesis through its inhibition of cyclin-dependent kinases. However, if σ expression alone is generated by infection with Ad-σ, no concomitant increase in cdk inhibitor expression would be expected and the cells, though unable to undergo a normal mitosis, may still be able to reenter S phase. This explanation predicts that in *p21*-deficient cells, a similar uncoordinated arrest, with chromosome duplication in the absence of mitosis, would occur after γ irradiation. Previous experiments (Waldman et al., 1996) were consistent with this prediction and confirmed by the experiments shown in Figure

5D. Forty-eight hours after γ irradiation of *p21*-deficient HCT116 cells, a marked increase in cells with 8N chromosome complements was observed, resulting in a cell-cycle profile similar to that observed in cells infected with Ad-σ.

To further explore the nature of the block and subsequent polyploidization produced by σ-expressing cells, cells were observed with time-lapse microscopy. Of 24 σ-expressing cells observed continuously for 3 days, none underwent a normal cell division such as that consistently observed in β-galactosidase-expressing cells (Figure 7). Forty-eight percent of the cells rounded up as if they were beginning mitosis and then initiated cytokinesis, yielding two connected daughter cells that never completely separated. These incompletely divided cells subsequently re-fused, forming one large cell with a single nucleus (Figure 7, top row). The remainder of the cells (52%) rounded, never divided, and finally died through an apoptotic event (accompanied by cytoplasmic blebbing and nuclear degradation). *p21*-deficient HCT116 cells (but not parental HCT116 cells) exhibited similar aberrant cytokinetic events following γ irradiation, with 19 of 20 cells examined in detail entering into an abortive cytokinesis morphologically identical to that shown in Figure 7.

Discussion

The results described above demonstrate that γ irradiation and other DNA-damaging agents induce a substantial increase in the expression of σ in CRC cells. This induction is dependent on p53, as cells with an endogenous mutant *p53* gene displayed no increase in σ expression. The molecular basis of the p53 dependence was shown to involve a single p53-binding site, located 1.8 kb upstream of the transcription start site. When exogenously expressed, σ caused a cellular phenotype remarkably similar to that observed following γ irradiation, with an increase in cell size and an arrest in G2. These results strongly suggest that one of the molecular mechanisms underlying the G2 arrest following γ irradiation
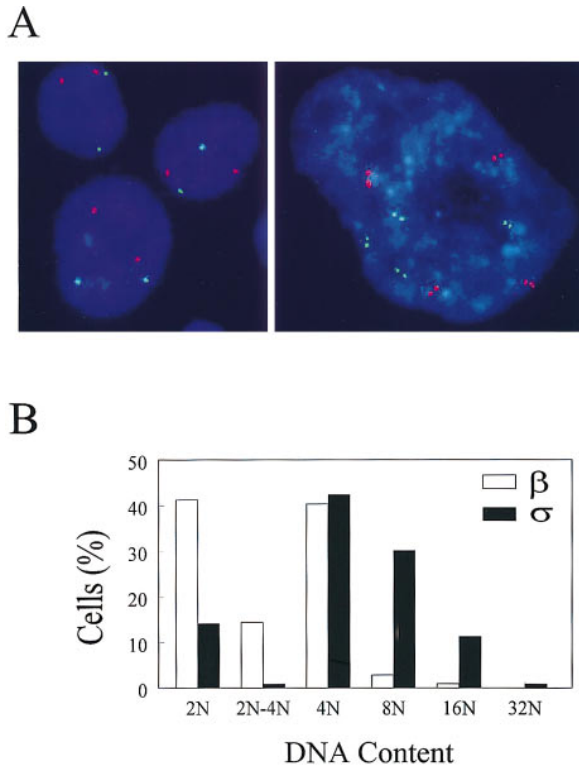
A

B

Figure 6. FISH Analysis of σ-Expressing Cells

(A) Representative examples of HCT116 cells infected with Ad-β (left) or Ad-σ (right) and analyzed by interphase FISH. Hybridizations were performed with a chromosome *2p* probe (red signals) and a chromosome *11q25* probe (green signals) 72 hr after infection. Note the paired signals in the σ-expressing cells. The Ad-β-infected cell nuclei are apparently in G1, containing two unpaired signals per probe in each nucleus.

(B) Distribution of DNA content in σ-expressing cells. The percentage of cells with the indicated DNA content per nucleus, as determined by FISH, is plotted. For Ad-β- and Ad-σ-infected cells, a total of 104 and 106 cells, respectively, were analyzed. "2N-4N" cells represent cells in which one doublet and one single signal were detected.

is based on an activation of p53, which in turn transcriptionally activates σ. The combination of *p21* and σ is likely to mediate a significant part of the cell-cycle-regulatory effects of p53 in epithelial cells following DNA damage (Figure 8).

In S. pombe, the two σ homologs *rad24* and *rad25* function as checkpoints that ensure that DNA damage

is repaired before mitosis is attempted (Ford et al., 1994; Weinert et al., 1994). On the basis of the data presented above, we propose that σ functions similarly in human cells, arresting them in G2 and preventing mitosis after treatment with γ irradiation or other DNA-damaging agents. Interestingly, deletion of *rad24*, and to a lesser extent *rad25*, led to premature entry into mitosis and a small cell size at division (Ford et al., 1994). This is exactly the counterpart of what occurred in human cells after overexpression of σ: there was a delay in mitotic entry associated with an increase in cell size.

The data reveal a conservation of mechanisms underlying cell-cycle regulation following DNA damage. Moreover, results published during the review of this paper suggest a biochemical mechanism by which 14-3-3 homologs control this checkpoint. DNA damage results in the Rad3-dependent activation of Chk1 kinase, which subsequently phosphorylates the Cdc25C phosphatase on Ser-216 (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997). The motif surrounding the phosphorylated Ser-216 represents a previously defined consensus binding site for 14-3-3 proteins (Muslin et al., 1996). The phosphorylated Cdc25C is thereby bound and sequestered by 14-3-3 and therefore unable to dephosphorylate Cdc2, a cyclin-dependent kinase required for entry into mitosis (Peng et al., 1997). Our data demonstrate that DNA damage not only results in phosphorylation of Cdc25C but also strongly induces expression of a 14-3-3 protein in colorectal cancer cells. Moreover, our results suggest that the *14-3-3* gene responsible for sequestering Cdc25C in colorectal cancer cells is likely to be *14-3-3σ*. Interestingly, Cdc25C is also phosphorylated on Ser-216 in cycling cells in the absence of DNA damage. Overexpression of σ, as in the experiments reported here, would presumably bind and sequester the phosphorylated Cdc25 protein and prevent entry into mitosis in a similar manner as that observed following irradiation. The cytoplasmic localization of the exogenously introduced 14-3-3σ protein (Figure 4A) suggests that 14-3-3 may prevent entry of Cdc25C into the nucleus. The p53 dependence of σ induction connects DNA damage and p53 with the cdk required for G2/M progression in much the same way as *p21* connected p53 with the cdks required for G1/S progression (Figure 8).

The new data also provide an excellent example of the independence of the mitotic, cytokinetic, and DNA synthetic phases of the cell cycle (Figure 8). Though the events that characterize these three phases have historically been viewed as sequential, it is becoming
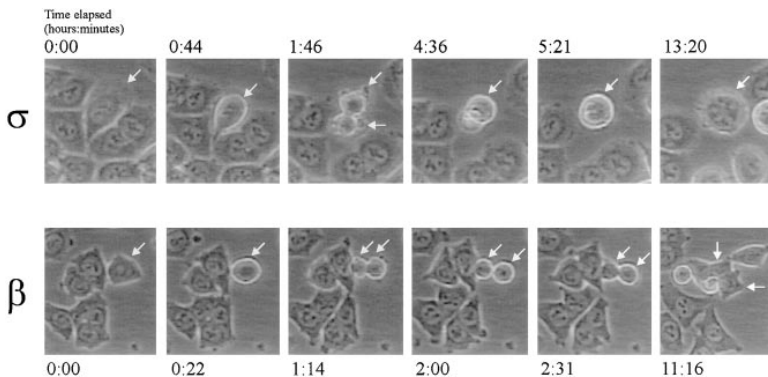

Figure 7. σ Expression Prevents the Completion of Cytokinesis

HCT116 cells were infected with Ad-σ (top row) or Ad-β (bottom row) and observed with time-lapse video microscopy. Representative examples of single cells either performing an incomplete (Ad-σ) or a complete division (Ad-β) are shown (arrows). The times indicate the elapsed time since the first picture in the sequence was taken at approximately 31 hr after adenovirus infection.
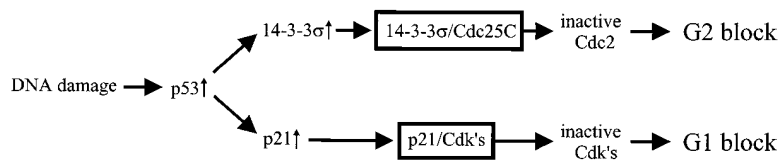
Figure 8. Model for DNA Damage–Induced Cell-Cycle Regulation by p53

DNA damage activates the sequence-specific transcriptional capacity of p53 through translational and posttranslational mechanisms (Cox and Lane, 1995; Levine, 1997; Morgan and Kastan, 1997). The p53 protein then binds to the promoters of the *p21* and σ genes, inducing their expression and resulting in a coordinated arrest in which cells are blocked in both G1 and G2 phases. The p21 gene product binds to and inhibits the cyclin–cdk complexes required for the transition from G1 to S phase (Harper and Elledge, 1996). The σ gene product is predicted to bind to and sequester phosphorylated Cdc25C, preventing Cdc25C from dephosphorylating Cdc2 and initiating the transition from G2 to M phase (Peng et al., 1997).

increasingly clear that they can proceed independently and that specific checkpoint genes link them in all eukaryotic cells (Paulovich et al., 1997). During a normal irradiation-induced cell-cycle arrest, the three phases seem to be blocked by independent effectors. Among these effectors, p21 inhibits entry into S phase, σ prevents cells that have completed S phase from entering into mitosis, and cytokinesis is presumably inhibited by other, unidentified protein(s). The expression of these proteins following radiation thereby results in a coordinated arrest in which cells are blocked in either G1 or G2 and can survive for prolonged periods. Experimental manipulation of these checkpoints results in an uncoordinated arrest. For example, σ overexpression in the absence of elevated *p21* expression does not prevent chromatids from detaching from each other (Figure 6A) or from reinitiation of S phase without an intervening mitosis (Figures 5 and 6). Similarly, expression of σ in the absence of elevated levels of p21 prevents chromosome condensation and nuclear membrane breakdown but does not prevent the onset of cytokinesis (which cannot be completed, presumably because the nucleus is still intact; Figure 7). When such uncoordinated events are occurring in cells, the terms "G1" and "G2" are no longer applicable to describe their states.

Interestingly, though exogenous expression of σ resulted in the accumulation of cells containing a DNA content of 4N in all cells tested, additional rounds of DNA synthesis in the absence of mitosis ensued in HCT116 and some other colorectal cancer cells, but not in normal prostate epithelial cells (H. H., unpublished data). Eventually, these uncoordinated arrests resulted in the apoptotic death of the cancer cells. Such results have potentially important implications for cancer therapy. The *p53* mutations that occur commonly in cancer abrogate part of the normal responses to DNA damage, i.e., induction of *p21* and σ. In combination with other genetic alterations that may alter checkpoint monitoring or execution, the *p53* mutations should result in an uncoordinated cell cycle, eventually resulting in the apoptotic death of the cancer cells. Such differences in checkpoint function between normal and cancer cells may explain why current cancer chemotherapy is successful in some patients (Hartwell and Kastan, 1994; Paulovich et al., 1997). Exploitation of these differences may in the future lead to more specific chemotherapeutic agents (Waldman et al., 1996).

Experimental Procedures

Cells
Most cell lines used in this study were obtained from the American Type Culture Collection. The RKO and Lim2405 CRC cell lines were generous gifts from M. Brattain and R. Whitehead, respectively. The derivation of the HCT116 *p21*$^{-/-}$ (Waldman et al., 1995) and DH-F (Polyak et al., 1996) CRC cell lines have been described. CRC cells were cultured in McCoy's medium supplemented with 10% fetal bovine serum. Normal human fibroblasts, endothelial cells, and prostate epithelial cells and their respective growth media were obtained from Clonetics. Irradiation was performed using a ^{137}Cs γ irradiator at 1 Gy/min for 12 min.

SAGE
SAGE was performed as previously described (Velculescu et al., 1995, 1997). In brief, polyadenylated RNA was converted to double-stranded cDNA with the inclusion of the primer biotin-5′-T$_{18}$-3′. The cDNA was cleaved with NlaIII and the 3′-terminal cDNA fragments were bound to streptavidin-coated magnetic beads (Dynal). After ligation of oligonucleotides containing recognition sites for BsmFI, tags were released from the beads by digestion with BsmFI. The released tags were ligated to one another. The resulting ditags were PCR-amplified, isolated, and concatemerized, and then cloned into the SphI site of pZero (Invitrogen). Colonies were screened with PCR using M13 forward and M13 reverse primers. PCR products containing inserts of greater than 500 bp (>25 tags) were sequenced with the TaqFS DyePrimer kit and analyzed using a 377 ABI automated sequencer (Perkin Elmer) and the SAGE Software Package (Velculescu et al., 1995).

Candidate γ-irradiation-induced genes were identified by comparing 55,429 tags from irradiated HCT116 cells to 5,266 tags isolated from exponentially growing, nonirradiated HCT116 cells. For a given number of evaluated tags, biasing the number so that more tags were derived from one library than the other allows a deeper analysis of the state wherein increased expression is expected. However, because this bias is more likely to produce false positives, any candidate tag will require independent validation of its induction. In the studies reported here, candidate irradiation-associated tags were further evaluated by comparison to those represented in a library generated after ectopic p53 expression in CRC cells (Polyak et al., 1997), as described in the text.

Northern Blot Analysis
Total RNA was prepared by CsCl gradient ultracentrifugation of guanidine-isothiocyanate-lysed cells as described (Davis et al., 1986). A 375 bp probe specific for the 3′ untranslated region of σ was generated by PCR using EST W79136 as template and the primers 5′-ACAGGGGAACTTTATTGAGAGG-3′ and 5′-AAGGGCTC CGTGGAGAGGG-3′. Probes for *p21* were generated by a restriction endonuclease digestion of pCEP-WAF1 (El-Deiry et al., 1993) with SalI and isolation of the 2.1 kb cDNA fragment. The probe for the constitutively expressed gene *EF1* was obtained by RT–PCR with the primers 5′-GAAAACTACCCCTAAAAGCC-3′ and 5′-GTTGGGTG GCAGGTATTAGG-3′. Hybridizations were performed in QuickHyb using the manufacturer's instructions (Stratagene).

σ Genomic Clones and Reporter Constructs
A BAC library (Research Genetics) was screened with PCR, using the primers 5′-GTGTGTCCCCAGAGCCATGG-3′ and 5′-ACCTTCTC CCGGTACTCACG-3′, yielding a 278 bp product corresponding to the 5′ end of the σ cDNA. A BAC containing σ was digested with BamHI, and a 9.5 kb fragment including the complete coding sequence of σ was subcloned into pBR322. The resulting construct was used for subcloning smaller fragments, which were placed into

a vector containing the luciferase reporter gene and a minimal promoter derived from the adenovirus *E1B* gene. To test the activity of presumptive p53-binding sites, the following oligonucleotide pairs were used: 5′-CCTGTAGCATTAGCCCAGACATGTCCCTACTCCG TAC-3′ and 5′-GGAGTAGGGACATGTCTGGGCTAATGCTACAGGG TAC-3′ for *BDS-2* and 5′-CCTGTAGAATTATCCCAGAAATTTCCCT ACTCCGTAC-3′ and 5′-GGAGTAGGGAAATTTCTGGGATAATTCTA CAGGGTAC-3′ for *BDS-2**, altered at critical p53-binding residues. The oligonucleotide pairs were concatemerized and subcloned into the KpnI site of pGL3-basic, containing a minimal promoter derived from the *E1B* gene and the luciferase reporter (Promega). Transfections were performed in SW480 cells using Lipofectamine (Life Sciences), using 1 μg of reporter plasmid, and either 2 μg of pCEP4 (Invitrogen) or 2 μg of pCEP4 encoding wild-type p53 or mutant p53 R175H. A β-galactosidase reporter construct (0.5 μg) was included in each transfection to control for efficiency. Luciferase and β-galactosidase activities were assessed 24 hr following transfection using reagents from Promega and ICN Pharmaceuticals, respectively.

Recombinant Adenovirus Generation

The σ-containing EST W79136 was used as template for PCR, employing the primers 5′-GCATGCGGTACCTAATACGACTCACTATAG GGCGACCACCATGGAGAGAGCCAGTCTGAT-3′ and 5′-ACCTCCG GATCCTTAGCTAGCGTAATCTGGAACATCGTAAGCGTAATCTGGA ACATCGTATCCACCGCTCTGGGGCTCCTGGGGAG-3′. The 849 bp product was inserted into the KpnI and EcoRV sites of pHRCMV, an adenoviral shuttle vector in which the expression of inserted genes is driven by a CMV promoter (T.-C. H., K. W. K., and B. V., unpublished data). Recombinant Ad-σ and Ad-β adenoviruses were generated in 911 cells (Fallaux et al., 1996). Viruses were purified via a CsCl gradient and titers determined by plaque assays. Cells were infected with adenoviruses at an MOI of $\sim$100:1.

Immunohistochemistry

Cells were fixed in Histochoice (Amresco), permeabilized with 1% NP-40 in phosphate-buffered saline (PBS), and blocked in goat serum for 1 hr. The 3F10 rat monoclonal anti-HA antibody (Boehringer Mannheim) was applied in GT (goat serum containing 0.05% Tween-20). After washing in PBST (PBS with 0.05% Tween 20), a biotin-labeled goat anti-rat Ig antibody (Pierce) was diluted in GT and applied for 30 min. After three washes in PBST for 5 min each, streptavidin-fluorescein (Molecular Probes), diluted in PBST, was used for detection. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Slides were mounted in DAPCO/glycerin and analyzed with a Nikon Eclipse E800 microscope equipped with a CCD camera (Photometrics). Images were pseudocolored using the software program IPLab (Signal Analytics Cooperation, Vienna, VI).

Flow Cytometry

Cells were rinsed in Hanks' Balanced Salt Solution (HBSS), trypsinized, collected by centrifugation, and resuspended in 40 μl of HBSS. Ten volumes of PBS containing 4% formaldehyde, 1% NP-40, and 4 μg/ml Hoechst H33528 were added, and flow cytometry was performed with a EPICS 752 instrument (Coulter, Hialeah, FL), excluding cell doublets.

FISH

P1 probes specific for chromosomes *2p* (red) and *11q25* (green) were labeled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively, by nick translation. Cells were fixed on slides and pretreated with RNase and pepsin. Multicolor FISH on interphase cells was performed following standard procedures (Lichter and Cremer, 1992). Digoxigenin-labeled probes were detected with an anti-digoxin mouse monoclonal, TRITC-conjugated rabbit-anti-mouse, and TRITC-conjugated goat-anti-rabbit antibodies, whereas biotin-ylated probes were detected with FITC Avidin-DCS (Vector). Cells were counterstained with DAPI.

For chromosomal mapping of the σ gene, a BAC containing the σ gene was labeled with biotin-16-dUTP. Human prometaphase spreads were fixed on slides and pretreated with RNase and pepsin. Hybridized probe sequences were detected as described above, and chromosomes were counterstained with DAPI. The resulting banding pattern and the hybridization signals were evaluated by standard epifluorescence microscopy (Nikon Eclipse E800). A total of 50 randomly selected prometaphases were evaluated. All of them showed hybridization signals on the distal arm of both chromatids of the homologous chromosomal regions *1p35*. In addition, fractional length measurements were performed as described (Lichter et al., 1990), confirming the mapping of σ to the *1p35* band. Photographs were taken using a CCD camera (Photometrics). The sequentially recorded gray-scale images were pseudocolored and merged using the software program IPLab (Signal Analytics Cooperation, Vienna, VI).

Time-Lapse Video Microscopy

Tissue culture flasks were sealed under 5% CO_2 and kept at 37°C on a Fryer Temperature Controller A-50–equipped Nikon inverted microscope. Video recordings were taken using a CCD camera (Optronics DEI-470) and a time-lapse video recorder (Hitachi VT-L2000 AR) recording one frame every 17 sec.

References

Agarwal, M.L., Agarwal, A., Taylor, W.R., and Stark, G.R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc. Natl. Acad. Sci. USA *92*, 8493–8497.

Aitken, A. (1995). 14-3-3 proteins on the MAP. Trends Biochem. Sci. *20*, 95–97.

Aloni-Grinstein, R., Schwartz, D., and Rotter, V. (1995). Accumulation of wild-type p53 protein upon gamma-irradiation induces a G2 arrest–dependent immunoglobulin kappa light chain gene expression. EMBO J. *14*, 1392–1401.

Blomstrom, D.C., Fahey, D., Kutny, R., Korant, B.D., and Knight, E.J. (1986). Molecular characterization of the interferon-induced 15-kDa protein: molecular cloning and nucleotide and amino acid sequence. J. Biol. Chem. *261*, 8811–8816.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552–557.

Cox, L.S., and Lane, D.P. (1995). Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. Bioessays *17*, 501–508.

Davis, L.G., Dibner, M.D., and Battey, J.F. (1986). Basic Methods in Molecular Biology (New York: Elsevier Science Publishing).

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice lacking p21*Cip/WAF1* undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675–684.

El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nat. Genet. *1*, 45–49.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B.

(1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Fallaux, F.J., Kranenburg, O., Cramer, S.J., Houweling, A., Van Ormondt, H., Hoeben, R.C., and Van Der Eb, A.J. (1996). Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum. Gene Ther. *7*, 215–222.

Ford, J.C., al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J., and Carr, A.M. (1994). 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. Science *265*, 533–535.

Furnari, B., Rhind, N., and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. Science *277*, 1495–1497.

Goi, K., Takagi, M., Iwata, S., Delia, D., Asada, M., Donghi, R., Tsunematsu, Y., Nakazawa, S., Yamamoto, H., Yokota, J., et al. (1997). DNA damage–associated dysregulation of the cell cycle and apoptosis control in cells with germ-line p53 mutation. Cancer Res. *57*, 1895–1902.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Hartwell, L.H., and Kastan, M.B. (1994). Cell cycle control and cancer. Science *266*, 1821–1828.

Harper, J.W., and Elledge, S.J. (1996). Cdk inhibitors in development and cancer. Curr. Opin. Genet. Dev. *6*, 56–64.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W., and Vogelstein, B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science *256*, 827–830.

Leffers, H., Madsen, P., Rasmussen, H.H., Honore, B., Andersen, A.H., Walbum, E., Vandekerckhove, J., and Celis, J.E. (1993). Molecular cloning and expression of the transformation sensitive epithelial marker stratifin. A member of a protein family that has been involved in the protein kinase C signaling pathway. J. Mol. Biol. *231*, 982–998.

Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323–331.

Lichter, P., Tang, C.J., Call, K., Hermanson, G., Evans, G.A., Housman, D., and Ward, D.C. (1990). High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science *247*, 64–69.

Lichter, P., and Cremer, T. (1992). In Human Cytogenetics: A Practical Approach, D.E. Rooney and B.H. Czepulkowski, eds. (Oxford: IRL), pp. 157–192.

Morgan, S.E., and Kastan, M.B. (1997). p53 and ATM: cell cycle, cell death, and cancer. Adv. Cancer Res. *71*, 1–25.

Muslin, A.J., Tanner, J.W., Allen, P.M., and Shaw, A.S. (1996). Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell *84*, 889–897.

Paulovich, A.G., Toczyski, D.P., and Hartwell, L.H. (1997). When checkpoints fail. Cell *88*, 315–321.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501–1505.

Polyak, K., Waldman, T., He, T.-C., Kinzler, K.W., and Vogelstein, B. (1996). Genetic determinants of p53 induced apoptosis and growth arrest. Genes Dev. *10*, 1945–1952.

Polyak, K., Yong, X., Zweier, J.L., Kinzler, K.W., and Vogelstein, B. (1997). A model for p53-induced apoptosis. Nature *389*, 300–306.

Prasad, G.L., Valverius, E.M., McDuffie, E., and Cooper, H.L. (1992). Complementary DNA cloning of a novel epithelial cell marker protein, HME1, that may be down-regulated in neoplastic mammary cells. Cell Growth. Differ. *3*, 507–513.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S.J. (1997). Conservation of the chk1 checkpoint pathway in mammals: linkage of DNA damage to cdk regulation through cdc25. Science *277*, 1497–1501.

Stewart, N., Hicks, G.G., Paraskevas, F., and Mowat, M. (1995). Evidence for a second cell cycle block at G2/M by p53. Oncogene *10*, 109–115.

Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995). Serial analysis of gene expression. Science *270*, 484–487.

Velculescu, V.E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M.A., Bassett, D.E., Jr., Hieter, P., Vogelstein, B., and Kinzler, K.W. (1997). Characterization of the yeast transcriptome. Cell *88*, 243–251.

Waldman, T., Kinzler, K.W., and Vogelstein, B. (1995). p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res. *55*, 5187–5190.

Waldman, T., Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1996). Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. Nature *381*, 713–716.

Wang, W., and Shakes, D.C. (1996). Molecular evolution of the 14-3-3 protein family. J. Mol. Evol. *43*, 384–398.

Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652–665.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature *366*, 701–704.

GenBank Accession Numbers