

A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model

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ABSTRACT Approximately 40% of human breast cancers contain alterations in the tumor suppressor p53. The p53 172R-H gain-of-function mutant (equivalent to the common 175R-H human breast cancer mutant) has been shown to promote aneuploidy and tumorigenesis in the mammary gland in transgenic mice and may affect genomic stability in part by causing centrosome abnormalities. The precise mechanism of action of these gain-of-function mutants is not well understood, and has been studied primarily in fibroblast cell lines. A novel p53-null mouse mammary epithelial cell line developed from p53-null mice has been used in adenovirus-mediated transient transfection experiments to study the properties of this p53 mutant. Marked centrosome amplification and an increased frequency of aberrant mitoses were observed within 72 h of introduction of p53 172R-H. However, few cells with aberrant centrosome numbers were observed in cells stably expressing the p53 172R-H mutant. Furthermore, stable expression of this p53 mutant reduced both basal and DNA damage-induced apoptosis. This result may be mediated in part through abrogation of p73 function. The p53 172R-H mutant, therefore, appears to influence tumorigenesis at the molecular level in two distinct ways: promoting the development of aneuploidy in cells while also altering their apoptotic response after DNA damage.—Murphy, K. L., Dennis, A. P., Rosen, J. M. A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. *FASEB J.* 14, 2291–2302 (2000)

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WILD-TYPE p53 ACTS as the 'guardian of the genome' (1), responding to DNA damage or checkpoint failure by either arresting cell cycle progression to facilitate DNA repair or initiating an apoptotic pathway to remove damaged cells. Wild-type p53 is critical for the maintenance of genomic stability: aberrant ploidy, gene amplification, in-

creased recombination, and centrosomal dysregulation have all been observed in cells lacking p53 (2). p63 and p73, two other proposed members of the p53 family, may compensate for wild-type p53 under some circumstances (3).

Tumorigenesis is a multistage process involving multiple genetic aberrations (4). p53 is the most commonly mutated gene in human cancers, with ~40% of tumors displaying some genetic alteration (5). Most p53 alterations are missense mutations (6) that may be accompanied by loss of the remaining wild-type allele. The p53 175 Arg-His (R-H) mutation makes up ~5% of all p53 mutations found in breast cancer and is the third most frequent p53 mutation (ref 6; and see web site at http://perso.curie.fr/Thierry.Soussi/p53_databaseWh.htm). Specific p53 mutations, including those at codon 175, have been associated with poor prognosis in breast cancer patients and also with primary resistance to chemotherapy (7).

Mutations in p53 may result in loss of wild-type function or generation of dominant-negative and gain-of-function mutants (e.g., ref 8). The 175R-H p53 protein is a dominant-negative mutant that can interact with wild-type p53, but is no longer capable of specific DNA binding (6, 9–11) and so loses many of the direct transcriptional regulatory capabilities of wild-type p53. It also appears to confer novel functions, indicating that it is a gain-of-function mutant (see, for example, ref 12). However, these studies have been performed in either fibroblasts or non-mammary epithelial cell lines usually containing multiple genetic aberrations in addition to p53 loss or mutation. To better understand the role of mutant p53 in mammary tumorigenesis, it is critical to use the appropriate cell system (mammary epithelial cells) containing a minimal number of other genetic aberrations and no competing wild-type (or other forms of mutant) p53. A novel cell system meeting

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these criteria has been developed and was used for the current experiments.

One of the gain-of-function phenotypes previously reported for the 172R-H mutant relates to centrosome number and ploidy. Primary skin tumors from mice bearing a skin-targeted p53 172R-H mutation (murine amino acid 172 is equivalent to human 175) display a much greater degree of aberrant centrosomal duplication than do tumors from p53-null mice (13). Normal nondividing cells have one centrosome, and dividing cells two, which form the poles of the spindle (14). Centrosomal amplification is implicated in two processes adversely affecting prognosis in cancer patients: 1) loss of cell polarity and tissue organization, and 2) increased occurrence of multipolar mitoses, which promotes unequal genomic segregation (15). A recent study found that centrosomes in high-grade breast adenocarcinoma cells are larger and more numerous than those from normal breast specimens (14). Centrosome amplification induces chromosomal instability (16), which is associated with tumor aneuploidy (17).

A transgenic mouse model has previously been generated to explore the role of the murine p53 172R-H protein in mammary tumorigenesis (18). A genomic minigene construct containing this mutation was targeted specifically to the murine mammary gland using a whey acidic protein (WAP) promoter (19). Transgene expression resulted in very few spontaneous tumors, but predisposed mice to the development of mammary tumors once some initiating event (e.g., carcinogen treatment or oncogene coexpression in the mammary gland) had taken place (12, 18, 20, 21). These tumors were frequently aneuploid (12, 18, 20, 21).

Studies were initiated using the unique p53-null mammary epithelial cell line in order to dissect the mechanism(s) by which the p53 172R-H mutant promotes aneuploidy and tumorigenesis in the mouse mammary gland. The results of these studies suggest that the p53 172R-H mutant may play a dual role in promoting mammary tumorigenesis by influencing genomic stability at the centrosome level as well as reducing both basal and DNA damage-induced apoptosis.

MATERIALS AND METHODS

Cells

The p53-null mouse mammary epithelial cell (MEC) line was generated in the laboratory of Dr. Daniel Medina (Baylor College of Medicine), as described in Kittrell et al. (22). This line was derived from p53-knockout mice generated by Dr. Larry Donehower (Baylor College of Medicine) that were of mixed (129/Sv × C57Bl/6) genetic background (23). Cells were maintained in DMEM:F12 (Life Technologies, Inc., Grand Island, N.Y.) containing 2% ABS, 5 µg/ml gentamicin,

10 µg/ml insulin, and 5 ng/ml EGF. Stable cell lines were selected using this media plus 650 µg/ml G418. All cells were used at the lowest possible passage numbers, typically 7–11 in transient transfection experiments and 12–16 in stable cell experiments.

Plasmids

Transient transfections

The plasmid pBL120 contains a CMV-p53 172R-H minigene construct in pBluescript SK (Stratagene, San Diego, Calif.), while the plasmid pBL106B contains the wild-type p53 construct.

Stable transfections

The retroviral plasmid pL53NRNL (24), containing p53 172R-H under the control of the Mo-MuLV LTR and a *neo* selectable marker, was used in the generation of stable cell lines. Control stables were generated using a similar plasmid lacking the p53 construct.

Transient adenoviral transfection

Cells were transiently transfected using a replication-deficient adenovirus-mediated system. The adenovirus (25) was obtained from Dr. Nancy Weigel (Baylor College of Medicine). Cells were at 50–80% confluency when infected. At the time of infection, media were removed from the plates (10 cm dishes were used in these experiments) and replaced with 2 ml of serum-free media. Adenovirus–DNA complexes were prepared by incubating adenovirus with plasmid DNA for 30 min at room temperature in the dark, followed by a 30 min incubation (as above) with polylysine at a molar concentration equivalent to 125-fold the molar plasmid DNA concentration. Adenovirus/DNA/lysine complexes were immediately added to the serum-free media on the target cells and allowed to incubate for 2 h at 37°C. The viral solution was then removed and replaced with a normal volume of growth medium. Cells were harvested for analysis at the indicated number of days after transfection. Dishes (10 cm) were transfected with a total of 1 µg of DNA each, which included *bax* reporter plasmid (obtained from Dr. Moshe Oren, Weizmann Institute, Israel) or p53 construct and internal control plasmids where appropriate.

Stable transfection

293T retroviral packaging cells were transfected with pCL-Eco (26) and the appropriate retroviral plasmid using FuGene (Boehringer Mannheim, Mannheim, Germany). After 2 days, the virus-containing media were harvested and filtered (0.45 µm filter). Polybrene (Sigma, St. Louis, Mo.) was added to a final concentration of 5 mg/ml and the media were placed on target cells. The plates of cells were spun in a clinical centrifuge for 3 × 10 min at 1800 RPM, rotating the plates 60° after each spin to ensure complete coverage of the target cell plate. The medium was then removed and replaced with normal growth medium. Two days after infection, the medium was replaced with selective medium.

Western blotting

Western blots were performed in accordance with standard protocols (27). Membranes were blocked in 5% nonfat dry milk (NFDM)/TBST [Blotto] for 1 h, then incubated in

either a 1:750 dilution of anti-p53 antibody (Santa Cruz, Santa Cruz, Calif.; sc-6243) or a 1:200 dilution of anti-p73 antibody (Novus Biologicals, NB 200–102) for 1 h at room temperature. After washing, the membrane was incubated in a 1:2000 dilution (3% NFD) of anti-rabbit (or anti-sheep, respectively) biotinylated secondary antibody, followed by incubation in a 1:2500 dilution (3% NFD) of horseradish peroxidase/streptavidin solution, treatment with ECL reagents, and exposure to film. The pan-ERK mAb used to normalize for protein content was obtained from Transduction Laboratories (Lexington, Ky.).

Immunocytochemistry

Cells to be immunostained were grown on lysine-coated circular coverslips and fixed in methanol at -20°C for 10 min, then washed and incubated in a solution of Blotto containing 1:50 polyclonal anti-p53 antibody (Santa Cruz, catalog # sc-6243) and 1:400 monoclonal anti-gamma-tubulin antibody (Sigma, catalog # T6557) for 3 h at 37°C . After washing with Blotto, they were further incubated with 1:500 Texas red-conjugated anti-rabbit secondary antibody and 1:200 FITC-conjugated anti-mouse secondary antibody (both from Molecular Probes, Eugene, Oreg.) in Blotto in the dark at 37°C for 1 h. The coverslips were then washed in TBST and mounted in DAPI solution (Vector Labs, Burlingame, Calif.; catalog # H-1200). Cells for p53/spindle double immunostaining were permeabilized in 0.5% Triton-X in PEM/PEG (80 mM Pipes, 1 mM EGTA, 1 mM MgCl_2 , (pH 6.9) + 4% [8000 MW] PEG) and then fixed in 3.7% formaldehyde (all solutions in PEM/PEG) at room temperature for 15 min. After washing in PEM/PEG, coverslips were blocked in 5% NGS and incubated in 1:15 (in 5% NGS) TU27B mouse monoclonal antibody (gift of Dr. B. R. Brinkley, Baylor College of Medicine) and 1:50 p53 antibody as above for 1 h at 37°C . After washing in PEM/PEG, the cells were incubated in 1:100 (in 5% NGS) FITC-conjugated goat anti-mouse secondary antibody and 1:500 Texas red-conjugated goat anti-rabbit secondary antibody for 1 h at 37°C , washed, and mounted as above.

Centrosome analysis

Images from stained coverslips were captured with a Sony 3CCD color video camera attached to a BX-50 Olympus microscope with Adobe Photoshop software. All images were viewed using a 100 \times oil immersion lens coupled with a 10 \times objective. The number of centrosomes in cells with red nuclei (i.e., in cells expressing the transfected p53) was observed and recorded. Centrosome numbers in apoptotic cells were difficult to determine and therefore were not included in the analysis. The number of centrosomes in nonpositive cells from the same coverslips was recorded as an internal negative control. At least 1500 cells for each transfected population were counted at various cell passage numbers to ensure no passage number effect on centrosome results. Statistical analysis (by *t* test) was performed using the JMP Statistical Visualization software from SAS Institute, Inc (Cary, N.C.).

DNA damage protocols

Mitomycin C (MMC; Sigma) was applied to cells in media for the indicated period of time at the indicated concentrations. UV irradiation of cells was accomplished using a Stratallinker (Stratagene). A Gammacell 1000 Irradiator was used for exposure of cells to ionizing radiation. Doses of DNA-damaging agents and exposure times were chosen in accordance

with accepted ranges in the literature (see, for example, ref 28).

Caspase assays

Cells were seeded into 96-well dishes at $\sim 20,000$ cells per well and treated as appropriate for each experiment. Each experimental point represents the average of at least four wells. At the time of assay, the media were aspirated and 30 μl of lysis buffer (10 mM Tris-Cl pH 7.5, 10 mM $\text{NaH}_2\text{P}_4/\text{NaHPO}_4$ pH 7.5, 130 mM NaCl, 1% Triton-X 100, 10 mM NaPi) was added to each well. The plate was then incubated for 30 min on ice. After the incubation, a mixture of 3.6 μl substrate (Ac-DEVD-AMC caspase-3 fluorogenic substrate (PharMingen, San Diego, Calif.) and 216 μl of PAB (20 mM HEPES pH 7.5, 10% glycerol, 2 mM DTT) was added to each well and the plate was incubated in darkness at 37°C for 3–6 h. The quantity of AMC liberated after cleavage of the substrate by activated caspase-3 was measured using a spectrofluorometer (excitation wavelength 380, emission wavelength 430–460 nm). The protein content of each well was determined after the assay, and the plate readout was normalized accordingly to account for cell number variability and time of final incubation. The results are presented as the relative caspase activity/ μg protein/h.

Colony assays

After irradiation, 500 cells were plated on each 10 cm dish and allowed to form colonies. After 10–14 days, colonies were stained with Coomassie blue; colony numbers ('colony' defined as >50 cells) were counted and statistical analysis was performed as above.

Luciferase assays

Cell extracts were made in 1 \times CAT assay lysis buffer (Boehringer Mannheim). Luciferase assays were performed as per manufacturer's instructions (PharMingen) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, Mich.). Internal control (β -gal activity) assays were performed in 96-well plates. Twenty microliters of extract/well was incubated at 37°C in a solution containing magnesium (0.1M MgCl_2 , 4.5M β -ME, 1.5 μl /well), 0.1M NaHPO_4 pH 7.5 (95.5 μl /well), and ONPG (4 mg/ml in 0.1 M NaHPO_4 pH 7.5, 33 μl /well) until a faint yellow color appeared (10–60 min). Plates were read at 420 nm on a spectrophotometer and units β -gal activity/ μl protein were calculated.

Chromosome staining

Cells were resuspended in 1 ml of media, to which 10 ml of 0.8% sodium citrate solution was added. The cells were then incubated at room temperature for 20 min, centrifuged, and resuspended in 1 ml of sodium citrate solution. Five milliliters of fresh 3:1 methanol:acetic acid solution (fixative) was added dropwise while gently vortexing. The cells were centrifuged and resuspended in 5 ml of fixative. Cells were dropped from a height of 4 inches onto slides slanted at a 30° angle and immediately followed with a few drops of fixative. After drying, cells were stained with a 1:1000 dilution of DAPI mount solution/phosphate-buffered saline for 1–2 min, rinsed, and mounted using solution lacking DAPI (Vector Labs catalog # H-1000). Chromosomes were imaged as described above. At least 50 metaphase spreads were counted per cell genotype.

SCE analysis

Confluent cells were split 1:5. Twenty-four hours after passage, 3 $\mu\text{g}/\text{ml}$ BrdU was added to the medium and the cells were allowed to grow for an additional 48 h. The cells were refed with fresh regular medium for 3 h, then treated with 10 $\mu\text{g}/\text{ml}$ demecolcine for 15 min to stop cell growth. Cells were washed, trypsinized, and centrifuged. The cell pellets were resuspended, and 10 ml prewarmed (37°C) 0.075M KCl solution was added dropwise while vortexing gently. One milliliter of fixative (see above) was then added and the cells were centrifuged. After aspiration of the media, the cells were resuspended in 10 ml of fixative and kept at room temperature for 30 min. The fixative was changed several times, and the cells were finally resuspended in ~ 0.5 ml of fixative. Cells were dropped onto slides as above and dried. Slides were then incubated in 0.1 mg/ml acridine orange (Sigma) solution for 5 min and rinsed well. The slides were mounted in 2.8% sodium phosphate anhydrous dibasic solution, pH 11.0, and visualized using a green (FITC) filter. For analysis, the number of breaks and the number of chromosomes were counted for each of 50 metaphase spreads per genotype, and the average break-per-chromosome ratio for each genotype was calculated.

RESULTS

Transient expression of the p53 172R-H mutant results in increased centrosome numbers

Loss of p53 expression in murine fibroblasts (29) and expression of the 172R-H p53 mutant in murine skin (13) result in aberrantly large numbers of centrosomes and aneuploidy. As aneuploidy is characteristic of mammary tumors arising in the WAP-p53 172R-H mice (12, 18, 20, 21), studies were undertaken to examine the effect of expression of this mutant p53 on centrosome number *in vitro*.

p53-null mouse MECs were transiently transfected with constructs encoding either wild-type p53 or the p53 172R-H mutant. Lipid-mediated transfection methods worked poorly with these cells, so an efficient adenovirus-mediated method (see Materials and Methods) was used. This resulted in a 20–40% transfection efficiency. Expression of wild-type or mutant p53 in transfected cells was initially verified by both immunocytochemistry and Western blotting (data not shown), and p53 expression could be detected in these cells for up to 5 days.

To determine the effect of transfected p53 on centrosome number in these cells with time, cells were transfected and populations grown on coverslips were fixed for analysis 2 h after transfection (day 0) and each day for the next 5 days (days 1–5). Fixed cells were then simultaneously immunostained for p53 and the centrosome component gamma-tubulin (see Materials and Methods). The double immunocytochemistry procedure permitted the identification of transfected cells for the analysis of centrosome number. The polyclonal p53 antibody

used (see Materials and Methods) recognizes both wild-type and mutant forms of p53, and was therefore used for the analysis of both experimental groups.

Representative examples of centrosome and p53 staining in p53-null MECs transfected with either wild-type (Fig. 1A) or mutant (Fig. 1B) p53 are shown in Fig. 1. Statistical analysis of centrosome numbers in cells transfected with wild-type (Fig. 1C) or mutant (Fig. 1D) p53 as a function of the day after transfection was performed as described in Materials and Methods, and revealed that there were significantly more cells with supernumerary centrosomes in the population transfected with mutant p53 by day 3. (Fig. 1, compare the day 3–5 bars for the '2+')

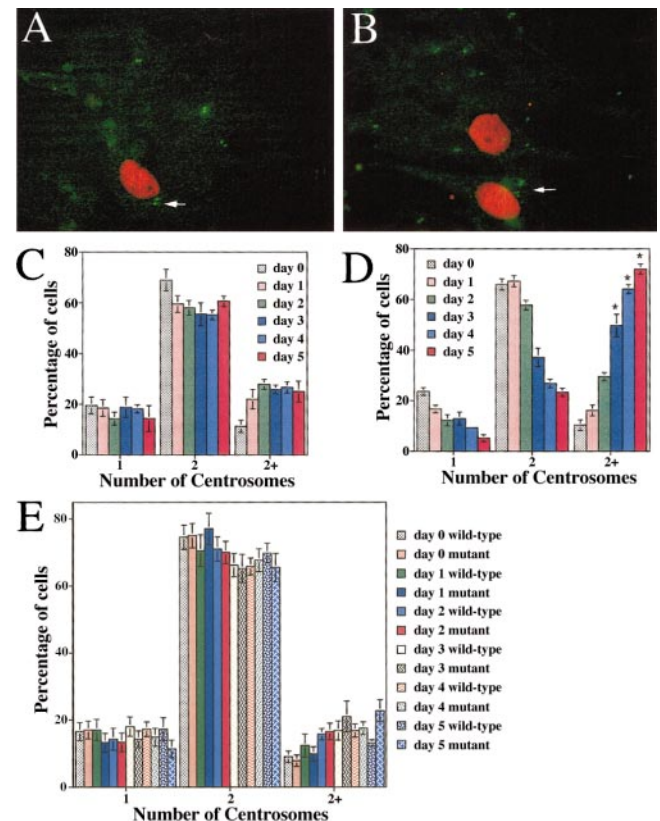


Figure 1. Transient expression of the p53 172R-H mutant results in increased centrosome numbers. *A*) Representative nucleus positive for wild-type p53 (red) and associated with two centrosomes (green) 5 days after transfection. *B*) Representative nucleus positive for mutant p53 and associated with six centrosomes at the same time point. *C*, *D*) Statistical analysis of centrosome numbers in cells transfected with either wild-type p53 or the 172R-H mutant at time points ranging from the day of transfection (day 0) to 5 days after transfection (day 5). Asterisks indicate that in each case there are significantly ($P < 0.001$) more cells with supernumerary (i.e., 2+) centrosomes in the mutant p53-transfected population than in the corresponding wild-type p53-transfected population at the same time point. *E*) Statistical analysis of centrosome numbers in untransfected cells from the same coverslips (both mutant p53- and wild-type p53-transfected coverslips). Error bars in all cases represent the standard error of the mean.

category between panels *C* and *D*). The percentage of mutant p53-transfected cells with aberrant centrosome numbers increased each day after transfection, reaching a maximum of over 70% of transfected cells by day 5. A concomitant decrease in the numbers of cells with one or two centrosomes was observed. A small percentage of cells above background contain more than two centrosomes in the wild-type p53 transfectants (normal background levels are defined as $\leq 10\%$ of cells; B. R. Brinkley, personal communication). However, the percentage of these wild-type p53-transfected cells with abnormal centrosome numbers plateaued at 20–25% by day 2 and remained relatively constant through day 5.

Centrosome numbers for nontransfected (p53-null) cells on the same coverslips at the same time points were also assessed and analyzed as an internal control (Fig. 1*E*). Nontransfected cells from wild-type and mutant p53-transfected plates exhibited very similar centrosome number profiles: $\sim 20\%$ with one centrosome, $\sim 60\%$ with two centrosomes, and $\sim 20\%$ with more than two centrosomes across all time points. This did not appear to vary significantly with cell passage number. These results essentially mirror those for wild-type p53 expressing cells depicted in Fig. 1*C* and indicate that the introduction of the gain-of-function p53 mutant into p53 null MECs results in centrosome abnormalities. Attempts were made to directly examine the effect of transfected wild-type or mutant p53 on cell ploidy by flow cytometry, but it was difficult to distinguish between transfected and nontransfected cells by this method. However, overall cell cycle profiles of populations transfected with mutant p53 and wild-type p53 were similar over time, even at time points at which centrosome number differences in the transfected populations were significantly different (data not shown).

Functional supernumerary centrosomes in cells transfected with the p53 172R-H mutant

Although it is possible for cells with more than two centrosomes to form a bipolar spindle (30; K. Murphy, unpublished observations), one predicted consequence of the multiple centrosomes seen with this p53 mutant is the formation of multipolar spindles leading to aberrant genome segregation at mitosis. As illustrated in Fig. 2, a small percentage of cells containing multipolar spindles was observed in these experiments. Double immunocytochemistry experiments were performed with simultaneous staining for p53 and α -tubulin, a component of the mitotic spindle. These experiments demonstrated that cells transfected with mutant p53 often contained multipolar spindles by the fifth day after transfection (Fig. 2*A/B*, *C/D*, and *E/F*) whereas only a small percentage

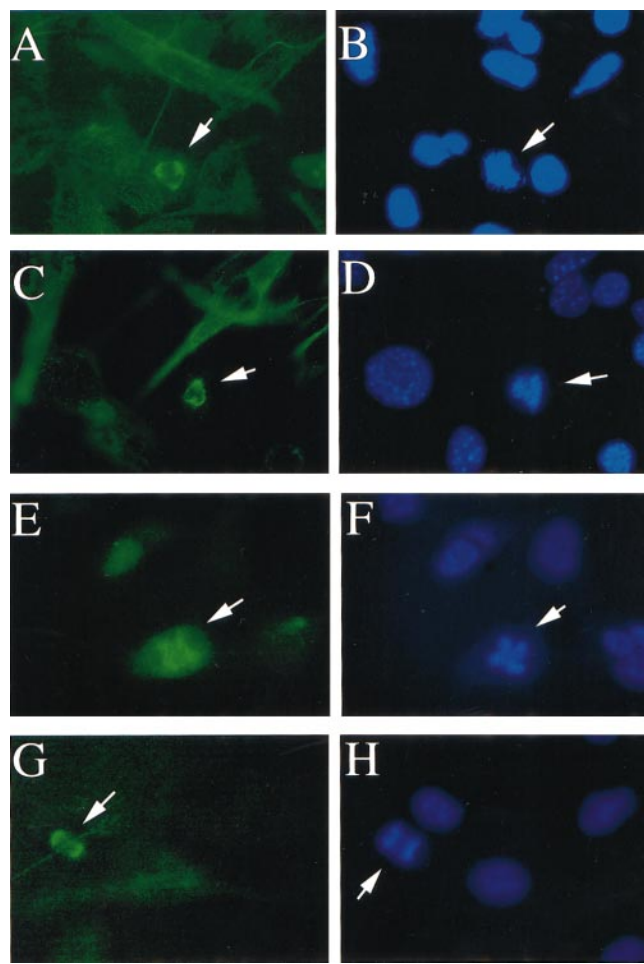


Figure 2. Functional supernumerary centrosomes in cells transfected with the p53 172R-H mutant. *A, C, E*) Aberrant mitotic spindles found in cells transiently overexpressing p53 172R-H. *B, D, F*) Aberrant segregation of the cellular DNA in these same aberrant mitoses. By comparison, panels *G* and *H* depict a normal mitosis in an untransfected (i.e., p53 null) cell.

of untransfected control (i.e., p53-null) cells had abnormal spindles at the same time point (Fig. 2*G/H*).

Moderately abnormal centrosome numbers in cells stably expressing p53 172R-H

To determine whether the abnormal centrosome numbers induced by expression of the p53 172R-H mutant persisted for longer than 5 days, cell lines stably expressing p53 172R-H or a control plasmid lacking p53 were generated as described in Materials and Methods, and their p53 status was confirmed by Western blotting (data not shown). No attempt was made to generate cells stably expressing wild-type p53, as stable overexpression of wild-type p53 leads to growth repression and/or apoptosis.

Mutant p53 was expressed at high levels in the stable cells (Fig. 3*A*) whereas, as expected, the p53-

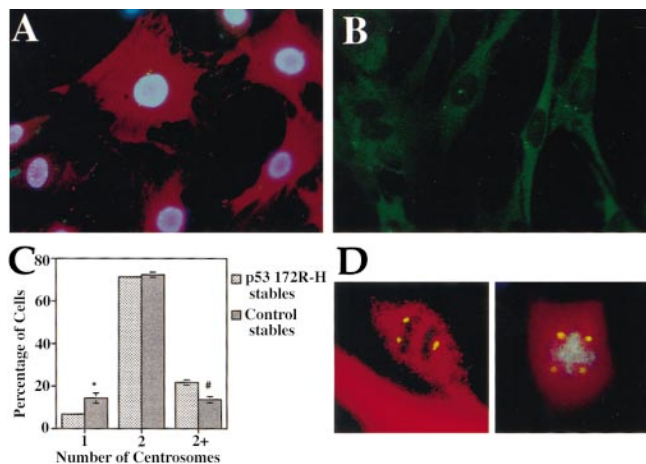


Figure 3. Moderately abnormal centrosome numbers in cells stably expressing p53 172R-H. p53 (red) and centrosome (green) staining is illustrated in p53 172R-H stable (A) and control (i.e., p53 null) stable (B) cells. C) Statistical analysis of centrosome numbers in p53 mutant stable and control stable cell lines. There were significantly more control stable cells with one centrosome ($*P < 0.05$), and significantly more p53 172R-H stable cells with more than two centrosomes ($\#P < 0.02$). D) Two examples of p53 172R-H stable cells with supernumerary functional centrosomes (tripolar and quadri-polar mitotic spindles, respectively).

null controls (Fig. 3B) displayed no detectable staining. Analysis of centrosome number in the mutant p53 stably transfected cells and controls (Fig. 3C) indicated that there were significantly more control cells containing one centrosome, and p53 172R-H cells with more than two centrosomes. Multipolar spindles were also observed to coexist with multiple centrosomes in some of the mutant p53 stable cells (Fig. 3D). However, based on the results from the transient transfection experiments, these differences in centrosome number were unexpectedly small. This raised the issue of the fate of the cells having multiple centrosomes and multipolar spindles after transient transfection with p53 172R-H.

As there were only moderately elevated numbers of stably transfected cells expressing the p53 mutant containing supernumerary centrosomes, it was hypothesized that many of the cells with multiple centrosomes nucleating multipolar spindles were undergoing apoptosis (30). To test this hypothesis, transient transfections were used to measure the level of activity of an integral apoptotic caspase, caspase-3. Some apoptosis was induced by the transfection process, but levels were equivalent between the cell types at day 1. Apoptosis was significantly higher ($P < 0.001$) in cells transfected with mutant p53 than in mock-transfected cells by day 5 post-transfection (Fig. 4), and the difference approached statistical significance ($P < 0.067$) by day 4. Caspase activity did not change significantly between day 1 and day 5 in the mock-transfected cells. These results were independently confirmed through use of a

fluorescent TUNEL assay combined with p53 immunostaining, which indicated that p53 staining frequently colocalized with TUNEL staining in day 4 and day 5 mutant p53-transfected cells (data not shown). As this coincides with the time points in which peak numbers of multicentrosomal mutant p53-positive cells were observed, these results suggest that the majority of these multicentrosomal cells were undergoing apoptosis, accounting for the observation of relatively few multicentrosomal cells in stably transfected populations.

Lower basal levels of apoptosis and increased resistance to DNA damage in p53 172R-H-expressing stable cell lines

Wild-type p53 is believed to play a role in DNA repair processes (31, 32) and p53 loss or mutation is known to have deleterious effects on these processes (33–35), although p53-deficient cells do not exhibit an increased mutation frequency after exposure to DNA-damaging agents (36). To test whether DNA repair was influenced by the presence of the p53 172R-H mutant in the stably transfected p53-null

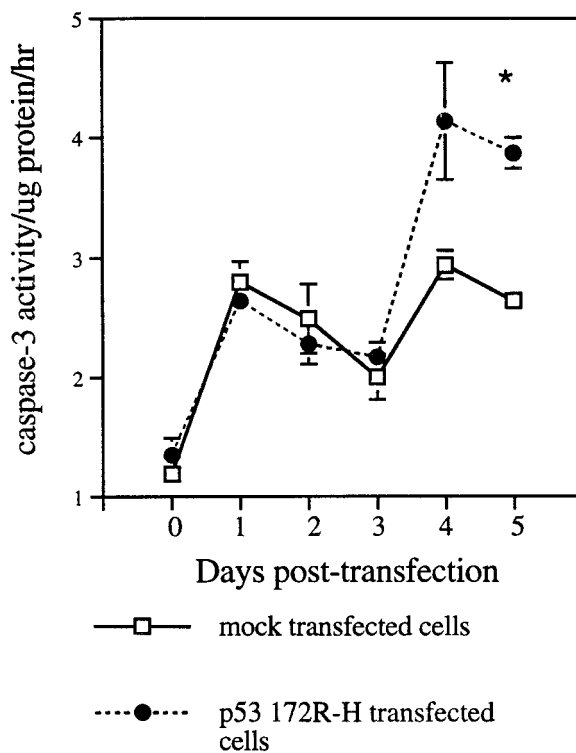


Figure 4. Cells transiently expressing p53 172R-H show substantially more apoptosis than mock-transfected cells by days 4 and 5 post-transfection. Caspase-3 assays were performed to measure apoptosis in MECs transiently transfected with p53 172R-H or control MECs on the day of transfection and each day thereafter until day 5. Numbers shown represent the average of at least four individual data points. The difference between the two cell lines is statistically significant ($*P < 0.001$) by day 5.

MECs, cells were treated with DNA-damaging agents and cell viability after treatment was ascertained by caspase-3 assays and/or colony assays as described in Materials and Methods.

Cells were treated with varying doses of mitomycin C (a DNA cross-linking agent), UV (which induces formation of cyclobutane dimers), or ionizing radiation (which induces double-strand DNA breaks) and cell survival was assessed at different subsequent time points. As shown in **Fig. 5**, treatment with all three of these DNA-damaging regimens yielded similar, if unexpected, results. Control cells treated with increasing concentrations of MMC contained significantly more caspase-3 activity (**Fig. 5A**) than correspondingly treated cells stably expressing the p53 172R-H mutant, indicating there was more apoptosis in control cells after MMC treatment. Treatment with UV radiation (**Fig. 5B**) appeared to exert a similar effect, especially at higher (30 or 50 J/cm²) doses. Control cells treated with ionizing radiation (IR) also appear to die preferentially at all doses tested (**Fig. 5C**). The differences shown were statistically significant ($P=0.05$) for all three agents and at all concentrations/doses tested, including basal levels. Colony survival assays were also performed for the latter experiment. Control or p53 172R-H-expressing stable cells were exposed to either 5 or 8 Gy

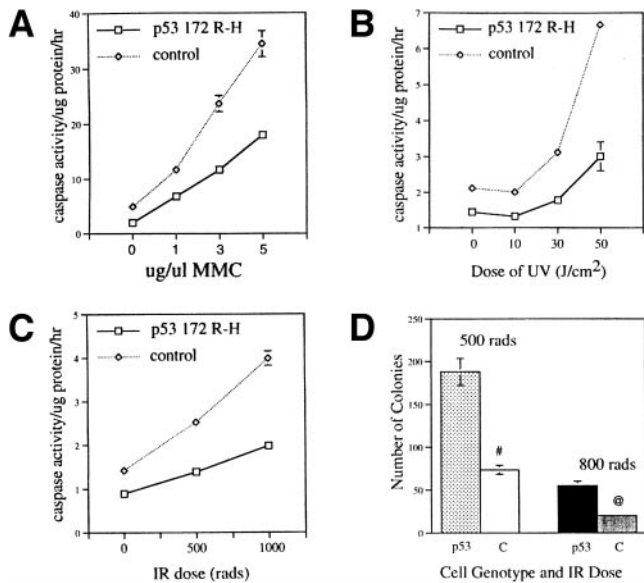


Figure 5. p53 172R-H-expressing stable cell lines have lower basal apoptosis and are more resistant to DNA damage induced by MMC (A), UV (B), and IR (C, D). A–C) The results of caspase-3 activity assays, representing time points of 2 days, 1 day, and 2 days after treatment, respectively. D) The results of colony assays, assessed 12 days after plating. Differences shown in panels A–C are statistically significant ($P<0.05$) at all doses and for all three agents. In many cases, error bars are small enough that they are not visible, although every point in each panel of this figure represents the average of at least four individual data points and has an error bar. Units are relative, therefore absolute caspase activities should not be compared from one treatment to another.

of ionizing radiation and allowed to form colonies as described in Materials and Methods. Colony counts indicated that the mutant-p53 expressing stables preferentially survived and formed more colonies at both doses tested (**Fig. 5D**). **Figure 5A–C** also shows that the p53 172R-H mutant can suppress basal apoptosis, as there is substantially more apoptosis in control than in mutant p53-expressing cells even without treatment with DNA damaging agents.

One potential explanation for these results is that other members of the p53 family are inducing apoptosis in the p53-null control cells, but that the mutant p53 protein exerts a dominant negative effect on that alternative proapoptotic pathway when stably expressed. It has previously been reported (37) that coexpression of the human p53 175R-H mutant inhibits the transcriptional activity of p73 α on the *bax* promoter, thereby reducing the ability of exogenous p73 α to promote apoptosis in p53-null H1299 cells. To examine the role of potential p73-mediated *trans*-activation of *bax* in apoptosis, a *bax*-luciferase reporter was transfected into cells stably expressing mutant p53 or control cells, which were left untreated or exposed to 30 J/cm² of UV irradiation to induce DNA damage and apoptosis. After 24 h, cells were harvested and luciferase activity was quantitated and normalized to an internal transfection control. These experiments indicated that the *bax* reporter is induced above basal levels by UV irradiation in both cell types and that there is significantly more reporter activity in both untreated and irradiated control cells than in their mutant p53-expressing counterparts (**Fig. 6B**). Slightly elevated levels of p73 protein were observed in extracts of control cells relative to those in cells expressing mutant p53 in two separate experiments, although p73 levels did not appear to increase after irradiation (**Fig. 6A**). Endogenous p73 levels were extremely low in these p53-null MECs.

Increased chromosome number, but no change in sister chromatid exchange in cells stably expressing p53 172R-H

Mammary tumors arising in mice carrying the p53 172R-H transgene are frequently aneuploid (12, 18, 20, 21). To assess the effect of this p53 mutant on ploidy *in vitro*, metaphase spreads (**Fig. 7A**) were prepared from p53 172R-H stably transfected or control cells as described in Materials and Methods, and chromosome numbers were analyzed. Both the control and p53 172R-H transfected cell lines averaged more than the normal 40 chromosomes per mouse cell. However, at equivalent passage numbers, there are significantly ($P<0.05$) more chromosomes (**Fig. 7B**) in cells stably transfected with the p53 172R-H mutant (mean=80, range=50–206) than in

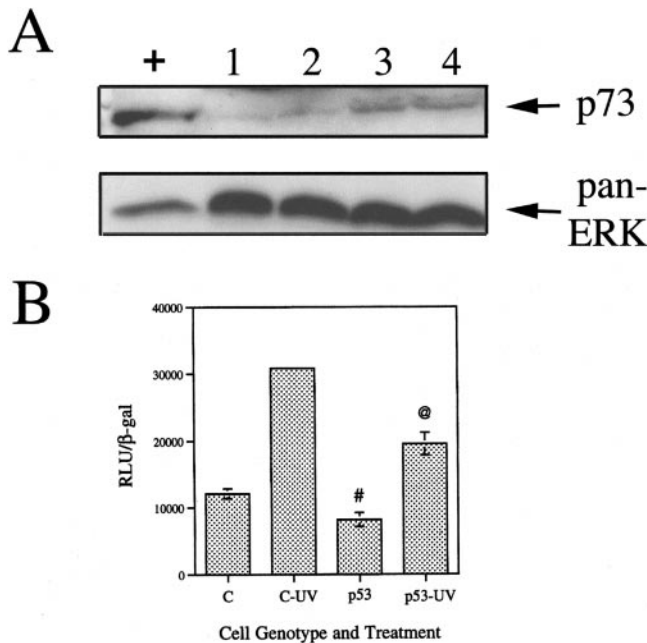


Figure 6. A) p53 172R-H-expressing stable cell lines appear to contain lower endogenous levels of p73 than control lines. A p73 Western blot with a COS cell positive control (+) followed by p53 172R-H-expressing stable cells, untreated and irradiated (lanes 1 and 2, respectively) and then untreated and irradiated control cells (lanes 3 and 4, respectively). Cells were exposed to 30 J/cm² of UV irradiation. The same blot was subsequently blotted with a pan-ERK antibody as a loading control, which showed no significant differences between lanes. B) *Bax* promoter activity is reduced in p53 172R-H-expressing stable cell lines. A *bax*-luciferase construct was transfected into mutant p53-transfected stable and control stable cells. Luciferase activity was quantitated in both cell types in the presence and absence of UV-induced (30 J/cm²) DNA damage. There is significantly more *bax* promoter activity in both untreated and irradiated control (C) cells relative to their mutant p53-expressing counterparts ($P < 0.017$ [#] and $P < 0.001$ [@], respectively). The error bar in the lane labeled C-UV is too small to be visible.

control (i.e., p53-null) cells (mean=66, range=37–142).

One mechanism thought to promote abnormalities in DNA ploidy is uncontrolled sister chromatid exchange (SCE), which is influenced by p53 loss or mutation (38–40). Specifically, it has been suggested that the p53 172R-H mutant protein may improperly promote illegitimate homologous recombination (41, 42). Accordingly, the incidence of SCE (Fig. 7C) in p53 172R-H and control cell lines was assessed as described in Materials and Methods. Surprisingly, no significant difference in the average number of SCEs per chromosome was detected between the two stable cell lines (Fig. 7D). Average frequencies of 0.270 ± 0.015 and $0.292 \pm .020$ SCE/chromosome were observed for the p53 172R-H stably transfected and control cells, respectively. Of course, this does not preclude early changes in SCE or the occurrence of other compensatory mutations by the time of analysis of these stably transfected cells.

DISCUSSION

Previous studies (12, 18, 20, 21) have shown that expression of the p53 172R-H mutant predisposes the mouse mammary gland to the development of aneuploid mammary tumors, but these studies did not address the specific mechanism(s) by which this occurs. The present studies performed in both transiently and stably transfected p53-null MECs suggest that this p53 mutant plays a dual role in mammary tumorigenesis, both negatively influencing apoptotic pathways and promoting the development of aneuploid cells.

Reduction in both basal and DNA damage-induced apoptosis

It was hypothesized that p53-null MECs stably overexpressing the p53 172R-H mutant would be defective in DNA repair and therefore more sensitive to DNA-damaging agents. However, these cells were in fact more resistant to DNA damage induced by MMC, UV, or IR over a range of doses. This suggests that, at least *in vitro*, this p53 mutant may promote cell survival after DNA damage. This p53 mutant also appeared to suppress basal apoptosis in the absence of DNA damage (Fig. 5A–C). It has been argued that p53 status does not significantly affect cellular sensitivity to DNA-damaging agents, in contrast to what is

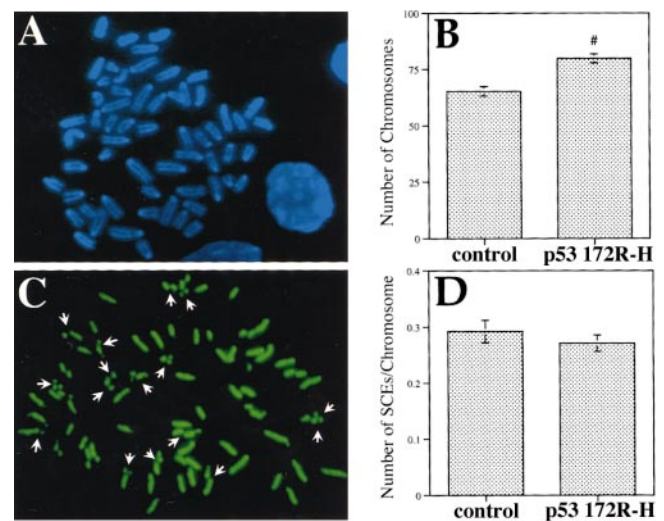


Figure 7. Increased chromosome number, but no change in sister chromatid exchange in cells stably expressing p53 172R-H. A) Representative DAPI-stained metaphase spread from a cell stably expressing p53 172R-H. The statistical analysis of chromosome numbers in p53 172R-H stable cells vs. controls is presented (B) and indicates there are significantly more chromosomes in cells stably expressing mutant p53 (#, $P < 0.001$). C) Representative metaphase spread illustrating occurrence of SCEs in the stable cell lines. Analysis of SCE frequency (number of SCEs/chromosome) indicated that there was no significant difference between the p53 172R-H and control stable cells (D).

seen in short-term DNA damage assays (43), but to date no studies have directly compared the effects of DNA-damaging agents on survival in p53-null cells vs. p53-null cells expressing this mutant p53 isoform. The short-term and 'gold standard' (43) colony survival assay results here agree, which suggests that p53 172R-H may indeed be suppressing apoptosis in this p53-null MEC system.

How the 172R-H p53 mutant might suppress apoptosis is not clear. This p53 mutant may exert a dominant-negative effect on p63 or p73, other proposed members of the p53 tumor suppressor family that can *trans*-activate proapoptotic p53 target genes such as *bax* (3). Coexpression of the human p53 175R-H mutant is known to reduce the transcriptional activity of p73 α on the *bax* promoter, thereby affecting the ability of ectopically expressed p73 α to promote apoptosis in p53-null H1299 cells (37). In accordance with these results, both basal and DNA damage-induced *trans*-activation of a *bax*-luciferase construct was significantly diminished in MECs stably expressing the p53 172R-H mutant relative to levels seen in control p53-null cells. This has not been directly proved to result from p73 inactivation by the p53 mutant, but given the concordance of these results with those of Di Como et al., it is likely that the p53 172R-H mutant in this system is reducing apoptosis, at least in part, by blocking the activity of endogenous cellular p73. An increase in cellular p73 levels was not detected after DNA damage induced by UV irradiation, in keeping with the results of Fang et al., who used mitomycin C, doxorubicin, and actinomycin D to induce DNA damage (44), but in contrast to those of Gong et al. (45), who induced DNA damage using cisplatin. This suggests that there is DNA-damaging agent and possibly cell type specificity to p73 stabilization after DNA damage.

Chromosomal consequences of p53 172R-H expression

Mammary tumors arising in mice carrying the p53 172R-H transgene are frequently aneuploid (12, 18, 20, 21), and exogenous expression of this p53 mutant in p53-null fibroblasts results in hyperdiploidy (46). In these experiments, both the control and p53 172R-H stable cell lines averaged more than the normal 40 chromosomes per cell, probably because of the relatively high passage numbers required to generate stable cell lines and the inherent tendency of p53-null cell lines to be genomically unstable. However, at equivalent passage numbers, there are significantly more chromosomes (80 vs. 66) in cells stably transfected with the p53 172R-H mutant than in control (p53-null) cells, suggesting that the p53 mutant does exhibit a gain-of-function effect on chromosome number in this system.

It is believed that induction of SCEs represents the interchange of DNA replication products at homologous loci, involving DNA breakage and reunion (47, 48). Functional wild-type p53 interacts directly with RAD51 and its bacterial homologue RecA, key factors involved with homologous chromosomal recombination, and inhibits their function when appropriate (38). Defects in p53-mediated control of homologous recombination caused by mutation (38) or loss (40) of p53 may result in inappropriate chromosomal rearrangements and genomic instability. Previous experiments (41, 42) suggested that stable expression of the human p53 175R-H mutant increases the incidence of both spontaneous and radiation-induced homologous recombination, although meiotic recombination is unaffected by p53 loss (49). This suggested that more SCE might occur in MECs stably expressing the p53 172R-H mutant than in the control p53-null MECs, but SCE levels in these cells were in fact equivalent. The most likely reason for the disparity between these results and those reported previously is the lack of wild-type p53 in the MECs; in the previous experiments, the mutant p53 was probably functioning as a dominant negative. The p53 172R-H mutant does not appear to promote SCE beyond levels resulting from the absence of wild-type p53. However, both the control and mutant p53 stably transfected MECs appear to exhibit a mildly elevated SCE frequency relative to what is seen in normal mouse cells [0.185 SCE/chromosome (50) vs. 0.270 and 0.292 SCE/chromosome in the p53 172R-H stably transfected and control cells, respectively]. By comparison, SCE frequency is 1.94 ± 0.07 SCE/chromosome in cells derived from *Xrcc1*^{-/-}/*p53*^{-/-} mouse embryos (51).

Centrosome amplification in mouse MECs expressing p53 172R-H

Transient expression of the p53 172R-H mutant protein in p53-null mouse MECs resulted in progressively increasing numbers of centrosomes per cell, reaching a maximum of over 70% of transfected cells with supernumerary centrosomes by day 5 post-transfection. By contrast, in transient transfections using wild-type p53, the percentages of cells with supernumerary centrosomes plateaued at ~20–25% by day 2 post-transfection, and similar results were seen with the internal control untransfected cells from the same coverslips at the same time points. Any cell containing more than two centrosomes is by definition aberrant (14). Loss of p53 in mouse embryo fibroblasts is known to result in extensive centrosome amplification (31–55% of cells with multiple centrosomes) by passage 2 (29). The relatively low background percentages of control p53-null MECs with aberrant centrosome numbers even after

8–12 passages highlight a significant difference between fibroblasts and MECs. There are also species-related differences within individual cell types (see ref 52).

A variety of centrosomal kinases (e.g., *STK15/BTAK*; ref 53), phosphatases and other proteins (for example, the cdk2/cyclin E complex; ref 54, 55) have been implicated in centrosome duplication (for a review, see ref 30). *STK15/BTAK* is amplified and overexpressed in ~12% of human breast cancers, and expression of this kinase leads to centrosome amplification, chromosomal instability, and transformation *in vitro* (53). Cyclin E is an important regulator of S-phase entry in the mammalian cell cycle and is often overexpressed or deregulated in tumors, leading to centrosome amplification and/or genomic instability (54, 55). Cyclin E has been localized to the centrosome (55), as have wild-type p53 (56) and *STK15/BTAK* (53). It may be that the p53 172R-H mutant deregulates centrosome amplification by somehow influencing the activity of either *STK15/BTAK* or cyclin E-cdk2 at the centrosome.

A moderate percentage of p53-null MECs stably expressing the p53 172R-H mutant also contained more than two centrosomes. Functional supernumerary centrosomes might be expected to nucleate multipolar spindles, resulting in aberrant genome segregation. As expected, this phenomenon was observed in both the transient (Fig. 2) and stable transfectants (Fig. 3).

Brinkley and Goepfert have proposed a model to account for the continuing presence of supernumerary centrosomes in established breast tumors (30). In this model, centrosome amplification is initiated at a very early stage in cell transformation, followed by clonal selection of viable tumor progenitor cells. Major mitotic spindle aberrations resulting from the presence of multiple centrosomes would therefore be characteristic of early stages of tumorigenesis, and would result in significant loss or gain of chromosomes. Most of the initial progeny cells would then be removed by apoptosis, leaving an occasional tumor progenitor cell (30). The results presented in this study are consistent with this model and demonstrate that transient expression of the p53 172R-H mutant results in dramatic centrosome amplification and mitotic spindle aberrations followed by apoptosis. Stable expression of this p53 mutant is characterized by a much smaller percentage of cells with supernumerary centrosomes. Fully transformed cells with supernumerary centrosomes may survive and assemble bipolar mitotic spindles (30; K. Murphy, unpublished observations) by an unknown mechanism. It appears that in some cases cells manage to 'cluster' multiple centrosomes into only two spindle poles (57, 58).

In summary, a novel p53-null mouse mammary epithelial cell model has been used in studies inves-

tigating the role of p53 in mammary tumorigenesis *in vitro*. The p53 172R-H mutant plays a dual role in promoting mammary tumorigenesis. Expression of this mutant leads to centrosome amplification, predominantly followed by multipolar mitotic division and apoptosis. On rare occasions, these cells that have sustained mutant p53-related genetic abnormalities survive, and then may nucleate a pretumorigenic population. The presence of this p53 mutant reduced both basal and DNA damage-induced apoptosis in stably transfected cell lines. The combination of these two pathways presents a scenario in which cells are both more likely to sustain genetic aberrations and more likely to survive the presence of these aberrations, thus providing a plausible explanation for the frequency with which this p53 mutant is found in human breast cancers. **[F]**

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