p53 Controls Low DNA Damage-dependent Premieiotic Checkpoint and Facilitates DNA Repair during Spermatogenesis

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Abstract

Previously, it was implicated that p53 plays a role in spermatogenesis. Here we report that p53 knockout mice exhibit significantly less mature motile spermatozoa than their p53(+/+) counterparts. To better understand the role of p53 in spermatogenesis, we analyzed the response of spermatogenic cells to DNA insult during prophase. It was found that although low-level γ-irradiation activated a p53-dependent premeiotic delay, higher levels of γ-irradiation induced a p53-independent apoptosis during meiosis. Furthermore, p53 knockout mice exhibited reduced in vivo levels of unscheduled DNA synthesis, indicative of compromised DNA repair. Thus, p53 provides another level of stringency in addition to other spermatogenic “quality control” mechanisms.

Introduction

Spermatogenesis involves the genetic recombination process, which takes place during the prolonged arrest at the tetraploid DNA content, termed meiotic prophase. Double- and single-strand DNA breaks, actively generated by still unknown mechanisms during the zygotene stage of the meiotic prophase, serve as substrates for the strand exchange during recombination. Strict maintenance of genomic stability and prevention of mutagenesis are essential for successful outcome of meiosis to assure the fidelity sufficient for proper heredity (1).

It is therefore not surprising that p53, the “guardian of the genome” (2), seems to play a role in spermatogenesis (3–19). Indeed, p53 mRNA and protein are highly expressed during mouse and rat spermatogenesis (4, 5, 8) and are predominantly evident in the premeiotic primary spermatocytes at the zygotene-pachytene stages, before the onset of meiotic division (5, 8). Furthermore, p53 knockout mice and mice with reduced levels of p53 exhibit germ cell degeneration during the meiotic prophase, manifested by the appearance of multinucleated giant cells (3). p53 is also suggested to mediate stress-related spermatogonial apoptosis after DNA damage (15) as well as after overheating of the testicular tissue (13). p53 knockout mice exhibit an increased incidence of testicular cancer, indicating that p53 has a role in the prevention of carcinogenesis during the mitotic stages of spermatogenesis (6, 9, 10). The role of p53 in the spermatogonial stress response is supported also by the extremely good responsiveness of testicular cancer cells expressing wild-type p53 to chemo- and radiotherapy (12, 14, 16). This was shown to be a result of activation of “normally latent” wild-type p53, which in turn induces extensive apoptotic response (11).

Several reports deal with the role of p53 protein in meiotic and premeiotic stages of spermatogenesis. Recently, it was shown that the fidelity of the meiotic crossing-over in several genomic loci is not severely affected in p53-knockout mice (17). Odorisio et al. (18) reported that whereas spermatogonial DNA-damage induced apoptosis is p53 dependent, the meiotic “quality control” chromosome-synthesis-dependent checkpoint at meiotic metaphase I is p53 independent (18).

On the other hand, it was observed that knockout mice for both p53 and ATM genes proceed to later stages of prophase than those knockout mice with the ATM gene only (20). Yin et al. (19) showed that p53(−/−) mice exhibit compromised apoptosis specially in tetraploid DNA state. These results suggest that the DNA damage-dependent checkpoint situated in meiotic prophase is p53 dependent.

p53 protein plays an important role in the maintenance of genomic stability during mitotic proliferation (21–23). These functions are carried out by composite regulation of key cellular responses to DNA damage (24). On the one hand, p53 mediates the arrest of cells at the G1–S boundary (25) and affects the G2 (26–28) and spindle (29–31) checkpoints. This permits the cells to repair the DNA damage, prior to stages of its fixation and propagation, which may lead to carcinogenic transformation (32, 33). Moreover, p53 was shown to facilitate general genomic repair of DNA damage (33) and to bind proteins involved in DNA repair like XPD (34), RPA (35), and rad51 (36, 37). On the other hand, in many cellular systems, p53 promotes apoptosis of cells harboring “irreparable” or high DNA damage (32).

Several lines of evidence suggest that p53 is involved in DNA recombination and rearrangement. p53 binds the mammalian homologue of yeast rad51 protein, which is directly involved in homologous recombination, and in repair of DNA double-strand breaks (36). Recently, it was shown that
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In this report, we compare the performance of the spermatogenic process in mice with a different p53 genotype. We found that p53(−/−) spermatocytes exhibit a deregulated progression toward meiosis after application of low-level DNA damage during the meiotic prophase. In vivo analysis of UDS, indicative of DNA repair, revealed that p53(−/−) mice exhibited lower levels of DNA repair compared with that of p53(+/+) littermates.

We also found that p53(−/−) mice have significantly less mature motile spermatozoa in comparison with their p53(+/+) counterparts. This reduction in the amount of motile sperm is, at least in part, a result of pronounced loss of cells during meiotic divisions. The p53(−/−) spermatocytes exhibit a deregulated progression toward meiosis after application of low-level DNA damage during the meiotic prophase. In vivo analysis of UDS, indicative of DNA repair, revealed that p53(−/−) mice exhibited lower levels of DNA repair compared with that of p53(+/+) littermates.

These results suggest that p53 plays a role in the low-level DNA damage-dependent premeiotic checkpoint and contributes to the efficiency of DNA repair, which in turn ensure appropriate quality and quantity of mature spermatozoa.

**Results**

**Comparison of Epididimal Sperm Motility in p53(+/+), p53(+/−), and p53(−/−) Mice.** Although not well documented, it is known that p53(−/−) mice suffer infertility (7). Previously, we observed that p53(−/−) mice or those with reduced levels of p53 protein exhibit a degenerative process of the spermatogenic tissue, termed the giant cell syndrome (3). It was, therefore, of importance to evaluate whether lack of p53 would significantly affect the overall yield of motile spermatozoa, a primary measure of male fertility. To that end, we surgically removed the cauda epididimi of C57Bl/6 mice of various p53 genotypes. The mature spermatozoa released from the epididimi were analyzed for motility using the “swim-up” assay. As shown in Fig. 1, the amount of motile spermatozoa of p53(+/−) or p53(−/−) mice was about 60% of the p53(+/+) control mice. This indicates that indeed lack of p53 reduces the yield of “functional” spermatozoa.

![Fig. 1. Reduced numbers of motile epididimal spermatozoa in p53(−/−) mice. The cauda epididi di of three mice with p53(+/+), p53(+/−), and p53(−/−) genotype were surgically removed and minced. The motility of spermatozoa was measured by the “swim-up” assay. Bars, SD.](image)

**In Vivo Analysis of Spermatogenic Progression Using BrdUrd Pulse Labeling.** To elucidate whether the reduced numbers of motile spermatozoa in p53 (+/−) and p53(−/−) mice are a result of enhanced degeneration of spermatogenic cells, we in vivo pulse labeled replicating spermatogonia with BrdUrd and followed the abundance and DNA content distribution of the cohort of labeled cells along meiosis (see chart, Fig. 2a). The proportion of the BrdUrd-labeled cells in the total population and the DNA content distribution at various days after the in vivo BrdUrd pulse were measured by FACs analysis. Fig. 2b depicts a typical example of spermatogenic population progression as a function of time. Analysis of mice with various p53 genotypes indicated no significant difference in the percentage of mitotically dividing spermatogonia (24 h after BrdUrd pulse) and of cells at the pachytene stage of the meiotic prophase (9 days after BrdUrd pulse; data not presented). This suggests that lack of p53 does not affect spermatogonial proliferation or early stages of the meiotic prophase. However, after traverse of meiotic divisions (14 and 15 days after BrdUrd pulse), the percentage of haploid labeled cells in p53(−/−) or p53(+/−) mice was significantly reduced in comparison with that of p53(+/+) controls (Fig. 2, c and d). This implies that lack of p53 in mice may cause spermatogenic degeneration during meiosis, even without subject to exogenous DNA damage.

**Effect of Low-Level γ-Irradiation on Spermatogenic Progression in the Various Mice.** To examine whether the presence of aberrant or damaged DNA in p53 knockout spermatocytes undergoing meiotic division is indeed the primary cause for observed enhancement in cell degeneration, we analyzed the meiotic progression upon induction of exogenous DNA damage at the pachytene and spermatogonial stages. For this purpose, mice with different p53 genotypes were γ-irradiated (1 or 3.5 Gy) 9 days or 24 h after in vivo BrdUrd pulse, and cell survival and DNA content distribution were analyzed by FACs. At these DNA damage time points, most of the BrdUrd-labeled cell cohort was situated in the mid-late pachytene stage or in the spermatogonial stage, respectively. Analysis of the BrdUrd-labeled cells 4 days after 1 Gy γ-irradiation at the spermatogonial stage

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3 The abbreviations used are: UDS, unscheduled DNA synthesis; BrdUrd, 5-bromo-2-deoxyuridine; FACs, fluorescence-activated cell sorter; 3AB, 3-aminobenzamide; AO, acridine orange; DDW, double-distilled water; Tdt, terminal deoxynucleotidyl transferase.
revealed no significant difference between the mice (data not presented). In contrast, the percentage of tetraploid spermatogenic cells in p53(+/-) and p53(-/-) mice was significantly increased after 1 Gy γ-irradiation at the pachytene phase (Fig. 3a). The same differences were scored when mice were exposed to 3.5 Gy of γ-irradiation. Starting from these DNA damage levels, the premeiotic delay was accompanied by p53-independent apoptosis of cells undergoing meiotic divisions (data not shown).

To examine whether other types of DNA damage will also activate the in vivo p53-dependent or -independent checkpoint controls, the BrdUrd-pulsed mice with a spermatogenic cohort situated at the pachytene (Fig. 3b) were treated with cisplatin or 3AB. Cisplatin causes interstrand DNA adducts, thus activating the nucleotide excision repair pathway, whereas 3AB is a potent inhibitor of DNA damage-dependent protein poly(ADP)-ribosylation, which facilitates the ligation of the intrinsic DNA damage. As can be seen in Fig. 3b, introduction of 3AB reduced the meiotic progression in a p53-independent manner. Introduction of cisplatin caused a p53-dependent activation of the premeiotic checkpoint in a manner similar to γ-irradiation (data regarding cisplatin is not shown). This suggests that the kind of DNA damage applied during the meiotic prophase will determine...
whether the stress response will be p53-dependent or p53-independent.

Previously, we observed an elevated expression of p53 at the pachytene phase (5). Therefore, we decided to examine the effect of γ-irradiation on the levels of p53 protein expression in the various stages of spermatogenesis. To that end, we analyzed p53 expression in well-defined, enriched spermatogenic populations obtained from p53(-/-) mice before and after exposure to γ-irradiation (3.5 Gy). p53 expression was assessed by FACS and by immunoprecipitation with monoclonal PAb-421 anti-p53 antibody. Detection of meiotic, mitotic, and interphase cells was based on differential DNA denaturability assay using the AO fluorescent DNA binding dye. The identity of each spermatogenic population was determined by the DNA content, DNA denaturability (respective to chromosomal condensation), and chromosome morphology. We verified the identity of the various populations appearing in adult spermatogenic cells using the AO assay by analyzing their appearance during the first spermatogenic round. In particular, we identified a distinct population of tetraploid cells possessing condensed chromosomes as diplotene spermatocytes, because this population first appeared in the first round just before the onset of the meiotic division (data not shown). To further confirm these results, we analyzed the p53 levels using immunoprecipitation and the AO pattern of adult spermatogenic cells, enriched for various spermatogenic stages using centrifugal elutriation. Fig. 4a shows the DNA content of the spermatogenic populations obtained by centrifugal elutriation. Analysis of DNA morphology and DNA denaturability indicated that fraction I consisted mainly (>85%) of round spermatids, whereas fraction II contained early prophase and meiotic cells. Fractions III and IV contained zygotene–early pachytene and late pachytene cells, respectively (Fig. 4b). Analysis of p53 expression in the various isolated fractions was carried out by immunoprecipitation and FACS analysis of p53 levels using p53-specific antibodies. In agreement with our previous report, we observed that p53 is expressed predominantly in fractions III and IV enriched for the zygote-pachytene spermatocytes (Fig. 4c; Ref. 5). Interestingly, however, under the present experimental conditions, no significant up-regulation of p53 levels was detected after DNA damage in p53-containing fractions. Previously, it was suggested that activation of p53 protein in testis after DNA insults is not associated with an accumulation in protein levels (11, 16).

Next we determined the outcome of DNA damage on the percentage of meiotic tetraploid cells using the AO assay (Fig. 5a). We found that γ-irradiation (1 Gy) of p53(+/-) mice caused a gradual reduction in the percentage of tetraploid meiotic cells possessing condensed chromosomes, which was detectable at ~2 days after treatment (Fig. 5b). This time course correlates with the traverse of mid/late pachytene cells through meiosis. In parallel with the reduction of the meiotic tetraploid population, the only population of tetraploid cells detectable was the high p53 expressors (data not shown). This indicates that DNA damage causes the accumulation of p53-positive premeiotic cells.

In contrast, 1 Gy of γ-irradiation of p53(-/-) or p53(+/-) mice did not significantly change the percentage of tetraploid meiotic cells. Under the same conditions, a 50% decline of this population in p53(+/-) mice was noticed. As can be
seen in Fig. 5a, similar patterns of spermatogenic populations were observed before and after DNA insult. Quantitative comparison of tetraploid meiotic cells indicated similar cell numbers in the p53$^{1/2}$ and p53$^{1/1}$ mice at different time points after exposure to $\gamma$-irradiation, thus suggesting that knockout of p53 expression releases a restraint preventing prophase spermatocytes possessing low levels of DNA damage to progress toward chromosomal condensation, an initial step of meiotic division. This implies that the presence of low levels of DNA damage at the end of the meiotic prophase activates a p53-dependent checkpoint.

Analysis of apoptosis in the various mice 4 days after 1 Gy of $\gamma$-irradiation using Tdt nick end labeling assay on testicular sections revealed a mild increase in apoptosis only in spermatogonial cells. However, this increment was observed irrespective of p53 genotype. Upon application of $\gamma$-irradiation higher than 5 Gy, dose-dependent apoptosis was also observed in spermatocytes in a p53-independent manner. This indicates that p53-independent mechanisms are responsible for elimination of spermatogenic cells containing high levels of DNA damage during and after meiosis.

Analysis of UDS after in Vivo DNA Damage in the Various Mice. In addition to controlling cell cycle checkpoints, p53 was also suggested to play a role in DNA repair, and because spermatogenesis involves such pathways extensively, we next examined the possibility that p53 participates in the DNA repair-related processes of spermatogenesis. To that end, we utilized an assay measuring the in vivo levels of UDS in testicular tissue. It should be noted that the levels of UDS reflect the repair rate of a variety of DNA repair mechanisms because the de novo nucleotide incorporation is a step shared by practically all of the DNA repair pathways. This was carried out by measurement of the in vivo BrdUrd incorporation in nonreplicating, postmitotic cells by using monoclonal anti-BrdUrd antibodies with preferential affinity toward low levels of BrdUrd incorporation in DNA, typical for DNA repair (47). Mice of different p53 genotypes were implanted s.c. with BrdUrd pills (see "Materials and Methods") several hours prior to application of in vivo DNA damage by either $\gamma$-irradiation or injection of various doses of cisplatin. The levels of BrdUrd incorporation were measured 24 h later. As can be seen in Fig. 6a, the untreated p53$^{1/1}$ and p53$^{1/2}$ mice exhibited no significant difference in the levels BrdUrd incorporation into postmitotic cells. After cisplatin treatment, the p53$^{1/1}$ mice exhibited a dose-dependent increase in BrdUrd incorporation in nonreplicating cells. A similar increment was measured in these mice after exposure to increasing doses of $\gamma$-irradiation. However, the DNA damage-dependent increase in BrdUrd incorporation measured in the p53$^{1/2}$ mice subjected to similar treatments was significantly less pronounced. Fig. 6b represents a quantitative summary of the results obtained. On the basis of the FACs analysis of BrdUrd incorporation in the various spermatogenic cell populations, it appears that the DNA repair levels measured here are significantly lower in p53$^{1/2}$ mice than in p53$^{1/1}$ littermates.

To confirm these results and to identify the cells engaged in DNA repair, we analyzed in parallel the levels of UDS in testicular sections from the same mice, using computerized quantitation of BrdUrd incorporation. To that end, testicular sections obtained from mice with different p53 genotypes were subjected to an immunohistochemical staining using
the monoclonal anti-BrdUrd antibodies that were further visualized by a secondary FITC-conjugated antibody (see “Materials and Methods”). Sections were then analyzed under the same data acquisition conditions to enable semi-quantitative analysis of the data. Exposure of animals to 10 Gy of γ-irradiation induced a significant increase in the number of the low-level BrdUrd-incorporating cells of the p53(+/+) mice. These cells were clearly situated in the regions of the seminiferous tubuli containing postmitotic cells, mapping internally to the peripheral, spermatogonial cells. No such increment in BrdUrd-incorporating cells was evident in sections obtained from the p53(−/−) mice. Similar patterns of spermatogonial cell proliferation, scored as high BrdUrd incorporators, were evident in all sections analyzed. These cells, located at the borders of the somniferous tubuli, represent the replicating spermatogonia.

Treatment of cells with cisplatin seemed to induce the same patterns of cells incorporating BrdUrd. Again, the p53(+/+) mice exhibited increased numbers of cells expressing fine grains of incorporated BrdUrd, corresponding to UDS, as compared with p53(−/−) mice. Although some differences in proliferating cell distribution could be seen, the general pattern of high BrdUrd-incorporating cells in p53(+/+) and p53(−/−) mice was comparable.

These results suggest, therefore, that at least a part of DNA repair activity measured by the UDS assay in spermatogenic cells seems to be p53 dependent.

Discussion

A role for p53 in spermatogenesis was suggested by the observation that changes in p53 expression are associated with key phases regulating meiotic progression, peaking at the pachytene stage (5), and that mice deficient in p53 exhibit a degenerative syndrome manifested by the appearance of giant cells (3). Furthermore, although not well documented, p53 knockout mice seem to suffer infertility. p53 was suggested to take part in a number of pathways that were shown to be associated essentially with the maintenance of genome stability. p53 seems to control, among others, cell growth arrest, induction of apoptosis, cell differentiation, and DNA repair. These activities were shown to be associated with the different cell cycle phases and checkpoints, most likely in a cell type-specific manner (48). It is, therefore, of interest to elucidate the contribution of p53 in spermatogenesis, a
Fig. 6. Spermatocytes of p53-knockout mice exhibit less efficient in vivo DNA repair. The UDS levels after treatment with various doses of γ-irradiation or cisplatin were measured in vivo in p53(−/−) or p53(+/+) mice by FACS or by immunohistochemistry. a, representative results obtained by FACS analysis. b, statistical analysis of UDS levels after 10 Gy of γ-irradiation and 8 mg/kg treatment with cisplatin after normalization with nonirradiated controls of p53(+/+) (■) and p53(−/−) mice (□). Bars, SD. Immunohistochemical detection of UDS after 10 Gy of γ-irradiation (c), or cisplatin treatment (d), using semiquantitative computerized analysis, is shown. Green, Hoechst 33342 staining for DNA morphology; red, BrdUrd incorporation, measured indirectly with the IU-4 anti-BrdUrd antibody.
physiological process that engages many of the p53-associated activities.

Spermatogenesis in most mouse strains, carrying either stable chromosomal translocations or deregulated premeiotic DNA recombination and DNA repair (40, 58), arrests at the pachytene stage (49). This implies that this stage harbors a checkpoint that is dependent on chromosomal aberrations. In some cases, elevated apoptosis during the mitotic divisions of spermatogonia is prevalent (18, 50). Normally, a degenerative process is known to take place during the two reduction divisions of meiosis and at the first stages of spermatogenesis (51). The primary cause of this degeneration was found to be chromosomal abnormalities (50, 52). Given this fact as well as the fact that p53 is expressed during the meiotic prophase led us to adopt the working hypothesis that p53 functions in the meiotic prophase in a similar manner as it does in DNA damage-dependent $G_2$ delay during the mitotic cell cycle.

The involvement of p53 in the DNA damage-dependent delay at the $G_2$ phase of the cell cycle and its contribution to the DNA repair at this phase were alluded to in several studies. Recently, Bunz et al. (53) demonstrated that knockout of either p53 or p21/waf1 gene expression in human colorectal cancer cell lines causes premature exit of DNA damage-dependent $G_2$ delay accompanied by failure of cytokinesis, resulting in endoreduplication of the tetraploid cells and formation of polyploid giant cells. It appears that high levels of p53 can modulate the arrest at the $G_2$ phase prior to mitotic chromosomal condensation. The duration of the DNA damage-dependent delay is also affected by the efficiency of DNA repair at the $G_2$ phase (26–28, 54–58).

There are several studies dealing with the possible involvement of p53 in spermatogenesis. The observation that no differences in recombination frequency in specific genomic loci were found upon comparison of p53 knockout mice with their intact litter mates suggested no role for p53 during and after recombination in spermatogenesis (17). Furthermore, it was observed that the DNA damage-dependent apoptosis occurring during meiotic metaphase I is p53-independent (18). Others, however, suggested that the radio sensitivity (15) and (partially) the thermo sensitivity (59) of replicating spermatogonia are p53-dependent. It should be noted that these studies did not assess the effect of p53 targeting on the general quantitative aspects of spermatogenic progression and maturation.

In this report, we compared the pattern of progression of cells along the spermatogenic differentiation in mice of different p53 genotypes. To evaluate the nature of p53 involvement in the regulation of spermatogenic process, we first pulse-labeled with BrdUrd a cohort of replicating spermatogonia and quantitated the percentage of labeled cells at key phases of spermatogenesis, i.e., mitotic proliferation, meiotic reduction divisions, and mature spermatooza. We found that without application of exogenous DNA damage, the percentage of BrdUrd-labeled replicating spermatogonia and prophase spermatocytes is comparable between p53 knockout and normal mice. Thus, in these conditions, there was no significant difference in levels of spermatogonial apoptosis or cell loss before and during prophase between these mice. However, upon completion of meiosis, p53 knockout mice had only 60–65% of the labeled haploid cells found in normal mice. This suggests that the reduction in the numbers of motile spermatozoa observed in p53 knockout mice may be attributed to cell degeneration at some stage of spermatogenesis.

The pronounced degenerative process during meiosis observed in p53-knockout mice (which is manifested by the appearance of multinucleated giant cells) can be caused by either deficient DNA repair during the meiotic prophase and/or by deregulation of the checkpoint that is supposed to prevent the entrance of cells possessing damaged DNA to meiosis. To compare the response to DNA damage at the meiotic prophase, we used BrdUrd pulse labeling, as well as the AO DNA denaturability assay, to quantify the progression of spermatogenic cells without BrdUrd labeling. Using these two methods, we found the existence of a p53-dependent checkpoint during the pachytene stage, which regulates the progression toward meiosis upon low levels of DNA damage. In addition, p53-knockout mice exhibited lower levels of $in vivo$ DNA repair, measured by semiquantitative evaluation of UDS upon insult with $\gamma$-irradiation or cisplatin.

Taken together, it appears that in p53-knockout mice, part of the postmeiotic spermatids possessing DNA damage presumably mature to spermatozoa, which in many cases are immotile because of the mutations that were not eliminated during the meiotic divisions. Some of these haploid cells may degenerate by the p53-independent pathways, as suggested by others (18). We suggest, therefore, that the end result of the p53-knockout phenotype is less efficient spermatogenesis, characterized by lower numbers of motile "functional" spermatozoa.

It was already shown that the duration of late-pachytene phase is regulated by phosphorylation, because okadaic acid treatment is sufficient to induce transfer from pachytene to dyplotene phases $in vitro$, without requirement for any de novo protein translation (60). This implies that progression toward premeiotic chromosome condensation is regulated by a typical protein-phosphorylation based "checkpoint" mechanism. We propose that among other proteins, p53 regulates this process in a specific set of conditions, which are disregarded by other DNA damage-sensing mechanisms. Our results suggest that p53 may provide an additional level of stringency, especially in low levels of DNA damage, to the existing p53-independent "quality control" mechanisms responsible for elimination or repair of spermatocytes with damaged DNA. Moreover, p53 contributes to the efficiency of DNA repair during the postmitotic stages of spermatogenesis. By these means, p53 function ensures appropriate "quality" and "yield" of mature intact and functional spermatozoa in normal mice.

**Materials and Methods**

**Mouse Maintenance and Genotype Analysis.** p53(-/-) mice of C57BL strain (61) were bred by mating p53(+/-) or p53(-/-) parents. Genotype analysis was performed by PCR analysis, which permits the specific identification of p53(+/-), p53(+/-), and p53(-/-) carriers. Tissue fragments obtained from either adult mice or embryos (tails or ears) were analyzed. Genomic DNA was prepared by incubating tissues in 0.5 ml of TE containing 0.4 mg proteinase K and 0.5% SDS overnight at 37°C. The
samples were extracted three times in phenol/chloroform/isomyl alcohol and twice in alcohol. DNA was washed in isopropanol, centrifuged, washed twice in 70% alcohol, and dissolved in DDW. Each PCR reaction contained 0.5 µg of genomic DNA. The primers used were as described before (82); for the wild-type p53 sequences, we used the 5′ SW2 primer GTC CGC GCC ATG GCC ATC TA and the 3′ 3′2W primer ATG GGA GGC TCG CAG TCG TAA CCC. For the neo p53(−/−)-specific marker, we used the 5′ ND, CAG CCC AGT GGA GTG ACA CAC ACC T (specifically designed in our lab); the sequence of the 3′ 3′2W was TTT AGG CAG CCC TGG CGC TCG ATG T. The PCR program included 5 min at 94°C, 36 cycles of 30 s at 94°C, 30 s at 69°C, 60 s at 72°C, and finally 7 min at 72°C. Typical patterns of DNA fragments were obtained for the p53(+/−) homozygous (138 bp), p53(−/−) heterozygous (180 bp), and p53(+/−) heterozygous (138 bp and 180 bp).

Spermatozoa Motility Measured by the “Swim-Up” Assay. The cauda epididimi of mice were removed surgically after sacrifice, gently cut to small (about 1-mm size) pieces using a blade, as described previously (63). The cut tissue was resuspended in modified McCoy 5A medium supplemented with glucose (2 g/l), lactate (1.8 g/l), and sodium pyruvate (0.11 g/l). The tissue was spun down at 800 × g and incubated for 1 h at 32°C. The supernatant medium containing the motile spermatozoa that swam up after centrifugation was carefully collected and counted in a hemocytometer.

Testicular Cell Suspension Preparation Using the Collagenase-Trypsin Method. Testis were surgically removed and decapsulated. To remove the extracellular tissue, the testis were incubated for 20 min under shaking in McCoy 5A medium containing 1 mg/ml of collagenase IV (Sigma). After two washes with the McCoy 5A medium, the testis were resuspended in the same medium containing 2.5 mg/ml trypsin (Sigma) and 1 mg/ml DNase I (Sigma). The tissue was incubated for another 15 min at 32°C under shaking. Then the testis were gently dispersed by pipetting with a Pasteur pipette. Subsequently, the cell suspension was washed once in McCoy 5A medium supplemented with 10% FCS, 0.5% BSA, and 0.08% w/v soybean trypsin inhibitor (Sigma; type I-A) and again with solution except the trypsin inhibitor. Finally, the cell suspension was filtered through nylon mesh and washed twice with McCoy 5A medium.

Isolation of Enriched Spermatogenic Populations by Centrifugal Elutriation. Spermatogenic cells (1–3 × 109), obtained as described above, were loaded into an elutriation rotor (J-6MI centrifuge equipped with a JE-5.0 elutriation system, including a Sanderson chamber (Beckman Instruments Inc.) and MasterFlex (Cole-Parmer Instruments)) peristaltic pump presterilized with 5% sodium hypochlorite. The separation was performed at room temperature (20–22°C) using McCoy 5A medium supplemented with lactate and pyruvate (see above) and 2% heat-inactivated fetal calf serum. The protocol is essentially as published by Selden et al. (47). In brief, the ethanol-fixed cells were treated with RNase A, resuspended in cold 0.1 × HCl 0.5% Triton X-100, and incubated on ice for 10 min. Next, the cells were resuspended in deionized H2O and incubated for 15 min at 90°C to additionally denature the DNA. Cells were incubated for an additional 1 h at room temperature with anti-BrdUrd-specific monoclonal IU-4 antibody (CALTAG Laboratories code MDS010; (Ref. 47). After washing by HBSS, the cells were incubated with secondary FITC-conjugated anti-mouse IgG antibody, washed, and resuspended in HBSS containing 5 mg/ml propidium iodide (Sigma) and analyzed by FACS, using the FACSort (Beckton Dickinson) machine operated by the CellQuest software (Beckton Dickinson).

Analysis of Meiotic and Mitotic Testicular Cells by FACS Using the AO DNA Denaturability Assay. Testicular cell suspensions (1 × 109 cells/ml) were fixed for 2–24 h in 70% ethanol-30% HBSS v/v at −20°C. Cells were washed once with HBSS and resuspended in HBSS with 0.5 mg/ml RNase A. After 1-h incubation at 37°C, cells were washed once and resuspended in HBSS at 2 × 109 cells/ml. Cell suspensions (200 µl) were added to 0.5 ml of 0.1 µ HCL. After 40-s incubation at room temperature, 2 ml of Acidine Orange AO (Molecular Probes) staining solution of pH 2.6, containing 90% v/v 0.1 sodium citrate, 10% v/v 0.2 µ Na2HP04, and 6 µg/ml AO, were added (64). Cells were analyzed by the FACS using the FACSort flow cytometer and CellQuest list mode analysis software (Beckton-Dickinson; Ref. 65).

Immunoprecipitation. Spermatogenic cell suspension was obtained by the collagenase trypsin method. The enriched populations for various spermatogenic phases were obtained by centrifugal elutriation and incubated for 1.5 h in methionine-deficient medium supplemented with 50 µg/5 × 109 cells of [35S]methionine.
([35S]Metionine-labeled proteins were immunoprecipitated with anti-p53-specific antibodies. The complexes generated were precipitated with 30 μl of Sepharose-protein A (50%) and washed three times in PLB buffer [10 mM NaH2PO4, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS]. The immune complexes were separated on SDS-PAGE.

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20. Edelmann, W., Cohen, P. E., Kane, M., Lau, K., Morrow, B., Bennett, S.,
19. Nacht, M., Strasser, A., Chan, R. Y., Harris, A. W., Schlissel, M., Bronson, T. R.,
18. Seiden, J. R., Dolbeare, F., Clair, J. H., Nichols, W. W., Miller, J. E.,
17. Wiesmuller, L., Cammenga, J., and Deppert, W. W. In vivo assay of
13. Calamera, J. C., Quiros, M. C., Brugo, S., and Nicholson, R. F. Com-
12. Clausen, O. P., Berg, K. A., Kirkhus, B., De Angelis, P., and Huitfeldt,
9. Nacht, M., Strasser, A., Chan, R. Y., Harris, A. W., Schlissel, M., Bronson, T. R.,
8. Seiden, J. R., Dolbeare, F., Clair, J. H., Nichols, W. W., Miller, J. E.,
7. Wiesmuller, L., Cammenga, J., and Deppert, W. W. In vivo assay of
3. Calamera, J. C., Quiros, M. C., Brugo, S., and Nicholson, R. F. Com-
2. Clausen, O. P., Berg, K. A., Kirkhus, B., De Angelis, P., and Huitfeldt,
1. Wiesmuller, L., Cammenga, J., and Deppert, W. W. In vivo assay of