Blackwell Publishing

Asian J Androl 2007; 9 (6): 739–750
DOI: 10.1111/j.1745-7262.2007.00339.x

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·*Original Article*·

Analysis of the genetic interactions between *Cyclin A1, Atm* and *p53* during spermatogenesis

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Abstract

Aim: To analyze the functional interactions of *Cyclin* with *p53* and *Atm* in spermatogenesis and DNA double-strand break repair. **Methods:** Two lines of double knockout mice were generated. Spermatogenesis and double strand break repair mechanisms were analyzed in *Cyclin A1* (*Ccna1*); *p53*- and *Ccna1; Atm*-double knockout mice. **Results**: The block in spermatogenesis observed in *Cyclin A1-/-* (*Ccna1-/-*) testes at the mid-diplotene stage is associated with polynucleated giant cells. We found that *Ccna1*-deficient testes and especially the giant cells accumulate unrepaired DNA double-strand breaks, as detected by immunohistochemistry for phosphorylated H2AX. In addition, the giant cells escape from apoptosis. The development of giant cells occurred in meiotic prophase I, because testes lacking ATM, which are known to develop spermatogenic arrest earlier than prophase I, do not develop giant cells in the absence of cyclin A1. Cyclin A1 interacted with p53 and phosphorylated p53 in complex with CDK2. Interestingly, *p53*-deficiency significantly increased the number of giant cells in *Ccna1*-deficient testes. Gene expression analyses of a panel of DNA repair genes in the mutant testes revealed that none of the genes examined were consistently misregulated in the absence of cyclin A1. **Conclusion:** *Ccna1*-deficiency in spermatogenesis is associated with defects in DNA double-strand break repair, which is enhanced by loss of *p53*. *(Asian J Androl 2007 Nov; 9: 739–750)*

Keywords: spermatogenesis; testis; cell cycle; meiosis; DNA double-strand break; giant cell; knockout mice

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Received 2007-06-26 Accepted 2007-07-24

1 Introduction

Tight regulation of the cell cycle machinery plays an essential role in mitotic and meiotic cell divisions. Spermatogenesis is a tightly regulated process that is governed by multiple important factors [1–4]. Cells involved in meiosis (e.g. in spermatogenesis) appear to be more sensitive to cell cycle disturbances than somatic cells. Several cell

cycle regulators initially thought to be essential for all cells, are indeed indispensable for meiosis [5]. This is apparently associated with a lower degree of redundancy among the involved factors compared to mitosis.

Deficiency of the cell cycle regulator *Cyclin A1* (*Ccna1*) leads to a block in spermatogenesis at the mid-diplotene stage in male mice [6] and coincides with the appearance of multinucleated giant cells, which usually do not occur in wildtype testes. The development of these cells is a rare testicular phenotype and was up to now only shown in mice deficient for *Ccna1*, *citron kinase,* a myotonin-related protein acting downstream of the GTPase Rho in cytokinesis control [7], or the tumor suppressor *p53* [8], which has been associated with multiple DNA repair pathways and recombination events [9]. The molecular and pathological mechanisms underlying the development of these cells remain to be elucidated.

Recently, we demonstrated that cyclin A1 is actively involved in DNA double-strand break repair through direct interaction with the repair factor Ku70 and that cyclin A1/CDK2 (cyclin-dependant kinase) complex plays an important role in DNA double-strand break repair, *Ccna1*-deficient somatic cells being impaired in DNA double-strand break repair [7]. Because many mutant mouse models for DNA repair-related proteins exhibit meiotic phenotypes (reviewed in [8]), we hypothesized that the development of multinucleated giant cells in *Ccna1*-deficient testes and the increased rate of apoptosis could result from impaired DNA repair mechanisms.

Therefore, we analyze the nature of the giant cells by investigating double knockout mice for *Ccna1* and ataxia telangiectasie mutated (*Atm*) as well as for *Ccna1* and *p53*. *Atm*-deficient testes are known as a model for the block of spermatogenesis around early pachynema of prophase I. In the absence of *Atm*, which is a major player involved in DNA repair, spermatocytes stop to differentiate at prophase I of meiosis with only few cells developing up to pachynema and diplonema [10]. P53 is known to be a major player in the DNA damage response in the context of spermatogenesis [11]. Wildtype p53 is expressed at significant levels in early spermatocytes, similar to cyclin A1. In addition, C*cna1* can be induced by p53 [12]. Therefore, we analyze physical and functional interactions of p53 in a double knockout mouse model. In addition, we determine the expression levels of a panel of genes involved in testicular cell cycle regulation and in DNA double-strand break repair using quantitative and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

2 Materials and methods

2.1 Mouse strains and genotyping

Ccna1-/- and *Atm-/-* mice were generated as previously described [13, 14]. *Ccna1*+/- mice (genetic background *BALB/c* × *Mf1*) were obtained from Dr Marc Carrington (Cambridge, UK), *p53*+/- mice (*129/Sv*) were obtained from Jackson Laboratories (Maine, USA, Stock-Nummer 2080) and *Atm*+/- mice (genetic background *C57B1/6N* × *DBA*) were obtained from the Beckman Institute, California Institute of Technology Pasadena, USA. The ethical guidelines Guide for Care and Use of Laboratory Animals [15] were followed for the mice experiments. Permission to proceed with the work was obtained from the Bezirksregierung Münster, Germany.

Tail tips from all mouse strains were digested in 500 μL NET buffer (100 mmol/L Tris [pH 8.5]; 5 mmol/L ethylene-diamine-tetra-acetic acid [EDTA], 200 mmol/L NaCl, 0.2% sodium dodecyl sulfate [SDS]) with 100 μg/mL Proteinase K overnight at 56ºC. The following primers were used to identify the different genotypes: (i) CyclinA1-wt-f: 5'-AGCAGCAGGCTGTGGCTTAC-3', CyclinA1-wt-r: 5'-TCCTTGGCATCGTTCTCCAT-3', CyclinA1-ko-r: 5'-GCGAGTGGCAACATGGAAAT-3'; (ii) p53X6.5: 5'-CAGCGTGGTGGTACCTTAT-3', p53X7: 5'-TATACTCAGAGCCGGCCT-3', Neo18.5new: 5'-CTATCAGGACATAGCGTTGG-3'; and (iii) ATM1: 5'-CCTCCTCATATTTGTAACACGCTG-3', ATM2: 5'-TGTAATGTGCCTTAAAGAACCTGG-3', ATM3: 5'-GGAAAAGCGCCTCCCCTACCCG-3'. Each PCR assay contained 1 μL of Proteinase K digest, 1/10 vol (v/v) of Biotherm Polymerase buffer (Natutec, Frankfurt/Main, Germany), 200 μmol/L dNTPs, 10 μmol/L of each primer, 1 U BiothermTaq polymerase (Natutec, Frankfurt/Main, Germany), and, for the cyclin A1 PCR 1 mol/L betaine (Sigma-Aldrich, München, Germany). Cycling conditions were 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 60ºC for 1 min, 72ºC for 2 min, and a final extension of 72ºC for 5 min. Bands of the following sizes indicated the respective alleles: *Cyclin A1*-wt: 353 bp, *CyclinA1*-ko: 695 bp, *p53*-wt: 450 bp, *p53*-ko: 615 bp, *Atm*-wt: 400 bp, *Atm*-ko: 600 bp.

2.2 Immunoprecipitation and kinase assays

To determine the physical interaction of cyclin A1 and p53, Cos-7 cells were transiently co-transfected with

an enhanced green fluorescent protein (EGFP)-tagged cyclin A1 and p53 using Superfect (Qiagen, Hilden, Germany). Immunoprecipitation with the lysate was performed as previously described [10], with either anti-p53 (Santa Cruz, Heidelberg, Germany) or an isotype Immunoglobuline G (IgG) antibody. Western blot analyses of the precipitated proteins were performed using an anti-EGFP antibody (Pharmingen, Heidelberg, Germany).

For *in vitro* kinase assays, the GST-p53 fusion protein was incubated with cell lysates of baculovirus-infected Sf9 cells expressing cyclin A1 and/or CDK2. In brief, 5 μCi [-^{32}P] labeled ATP (ICN Biomedicals, Irvine, CA, USA) were added to 15 μL of GST fusion beads (50% slurry) and 6 μg total protein from insect cell lysate expressing cyclin A1 and/or CDK2. The reactions were incubated for 30 min in 1× kinase buffer (10 μmol/L ATP, 50 mmol/L Hepes [pH 7.5], 1 mmol/L DTT, 10 mmol/L $MgCl_2$, 0.1 mmol/L Na_3VO_4, 1 mmol/L NaF). After washing and SDS-PAGE, phosphorylation was detected by autoradiography.

2.3 Immunohistochemistry and TUNEL staining

The testes were fixed overnight in 4% Paraformaldehyde/PBS (pH 7.8), washed with PBS, dehydrated and embedded in paraffin according to standard procedures. Sections of 3 μm were air-dried overnight at 37ºC and stored at room temperature. Hematoxylin-eosine (HE) stainings were performed according to standard procedures.

TUNEL stain was performed using the *In Situ* Cell Death Detection Kit-Alkaline Phosphatase (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's recommendations. As a substrate for the AP, we used NBT/BCIP (Roche Diagnostics, Mannheim, Germany).

For H2AX detection, sections were essentially treated as for the TUNEL stain, using an FITC-coupled anti-H2AX antibody (Upstate Biomol, Hamburg, Germany) instead of the TUNEL enzyme reaction step.

For immunohistochemistry, the sections were blocked with 1% BSA (New England Biolabs, Frankfurt/Main, Germany) in phosphate buffered saline (PBS) (H2AX) or 1.5% normal goat serum in PBS for 1 h at room temperature. Primary antibodies were diluted 1:200 (PCNA: mouse monoclonal, clone PC10 [DakoCytomation, Hamburg, Germany]; Ku70: mouse monoclonal [Sigma-Aldrich, München, Germany]; FITC-coupled H2AX [Upstate Biomol, Hamburg, Germany]; Cyclin D1: rabbit polyclonal, BD [Pharmingen, Heidelberg, Germany]) in the respective blocking solution and incubated at 4ºC overnight, followed by 3 × 5 min washes with PBS/0.05% Tween-20. For cyclin D1, ABC staining was performed according to the manufacturer's recommendations (Vectastain, Wiesbaden, Germany) and the signal was detected using AEC substrate (Sigma-Aldrich, München, Germany). For Ku70 and PCNA, the sections were incubated for 1 h at room temperature with Alexa 488 goat anti-mouse secondary antibody (Invitrogen Molecular Probes, Karlsruhe, Germany) diluted 1:500 in blocking solution, washed as before, and counter-stained with Hoechst dye.

All sections were finally mounted in Mowiol and documented using a Zeiss Axioskop with a digital camera system (Visitron, Puchheim, Germany) and SpotAdvanced software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.4 Quantitative and semi-quantitative RT-PCR

Primers and probes used for quantitative RT-PCR were obtained from Applied Biosystems (Foster City, CA, USA), TaqMan Gene Expression Assays *Dmc1* (Mm00494485_m1), *Brca1* (Mm00550845_m1) and *Rad51* (Mm00487905_m1) and analyses were performed as described previously [16].

For the semi-quantitative RT-PCR, 1 μg of RNA from each sample was used as a template for each reaction and 1 μL of cDNA from each sample was used for PCR. The optimal number of cycles for amplification was determined according to the cycle number that yielded the strongest band in the linear range. The range of cycles varied from 25 to 37, depending on the specific RNA target and primer set. The samples were heated to 94ºC for 2 min and then run through 25–37 cycles of 94ºC for 30 s, 60ºC for 30 s and 72ºC for 1 min, followed by 72ºC for 10 min and then 4ºC. Samples were run on a 1% agarose gel and stained with EtBr.

Primers used for the semi-quantitative RT-PCR are listed in Table 1.

For the experiments above, testes from up to four animals of each genotype were used (four wildtype testes, three testes of *Ccna1*-knockout mice, two *p53*-knockout testes, three *Atm*-knockout testes, two *Ccna1*; *p53*- and three *Ccna1*; *Atm*-double knockout testes).

2.5 Statistical analyses

For immunohistochemistry and TUNEL staining

Table 1. Primers used for the semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Gene	Sequence	GenBank accession number	Fragment length (bp)
Cyclin A1	GGTGTTGACTGAAAATGAGCAG;	NM_007628	572
	GAAACCTGTCCAGGAAGTTGAC		
p53	GGAGACATTTTCAGGCTTATGG;	NM_011640	333
	AGCTTATTGAGGGGAGGAGAGT		
DNA-PK$_{cs}$	TCAAATGGTCCATTAAGCAAACAA;	NM_011159	126
	GCTGCACCTAGCCTCTTGAAAG		
GAP-DH	ACCACAGTCCATGCCATCAC;	BC085275	451
	TCCACCACCCTGTTGCTGTA		
Mlh3	GAGAGTTGATGGAGGAGATTCG;	BC079861	356
	TAAAATGCTGGGTTCTCCAAGT		
Msh2	CCAGGGCGTGATCAAGTACA;	NM_008628	498
	TTCTGTGATCAGAATCCCTCCT		
Msh4	GCATAAAAGTTGGACACCACAA;	NM_031870	441
	AGCAGCTGCAAGGCAGTAATA		
NBS1	GAAACAGCCTCCAGATATTGAAA;	NM_013752	242
	GTTCCGGGAGCTGAAAAGAA		
XPD	GCTCTGAGCTCAAGAAAGAACC;	NM_007949	272
	CGAGTATCGAGCCAGGAAGTAG		

results, six testes of wildtype mice, three testes of *Ccna1*-knockout mice, three *p53*-knockout testes, two *Atm*-knockout testes, four *Ccna1*; *p53*- and two *Ccna1*; *Atm*-double knockout testes were analyzed.

HE-stained slides were used to count the giant cells. Only tubules, which had been sectioned perfectly vertically were taken into consideration and the number of giant cells were counted manually. For the genotype *Ccna1*-knockout, 158 tubules of three testes (60 days old) and 417 tubules of six testes (270 days old) were counted. 39 tubules of 160-day-old *Ccna1*; *p53*- double knockout testis and 158 tubules of four older *Ccna1*; *p53*-double knockout testis were taken into consideration. HE-stainings were made from eight wildtype mice, nine testes of *Ccna1*-knockout mice (three of them at the age of 60 days and six of them at the age of 270 days), six *p53*-knockout testes, four *Atm*-knockout testes, five *Ccna1*; *p53*- and six *Ccna1*; *Atm*-double knockout testes. Data of all experiments are indicated as mean and standard deviation if not indicated otherwise. Differences between groups were analyzed for statistical significance using paired *t*-test. In case several groups were compared, one-way-analysis of variance was used. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Multinucleated cells in Ccna1-deficient testes accumulate DNA double-strand breaks

Because *Ccna1* deficiency leads to a block in spermatogenesis (Figure 1A, B) which is also reported in previous studies [10], we were prompted to evaluate the function of cyclin A1 in spermatogenesis in greater detail. One prominent aspect of the spermatogenetic block in these testes is the appearance of multinucleated, so-called giant cells, which have also been described in mice deficient in *citron kinase* (*Cit-K*) [7]. In contrast to the multinucleated giant cells appearing in *Cit-K-/-* testes, *Ccna1-/-* giant cells were not cyclin D1 positive (Figure 1B, arrows). Therefore, multinucleated cells are likely to originate from different cell types and *Ccna1-/-* giant cells do not share characteristics of A-type spermatogonia or gonocytes, which express cyclin D1 [17]. The apparent higher number of cyclin D1-positive cells in the *Ccna1-/-* testes, compared to the wildtype (Figure 1A, B), might correlate with the increased mitotic activity throughout the testis, as shown by PCNA staining (Figure 1J, K).

To detect DNA double-strand breaks, immunostaining for the phosphorylated histone H2AX, γ-H2AX, was

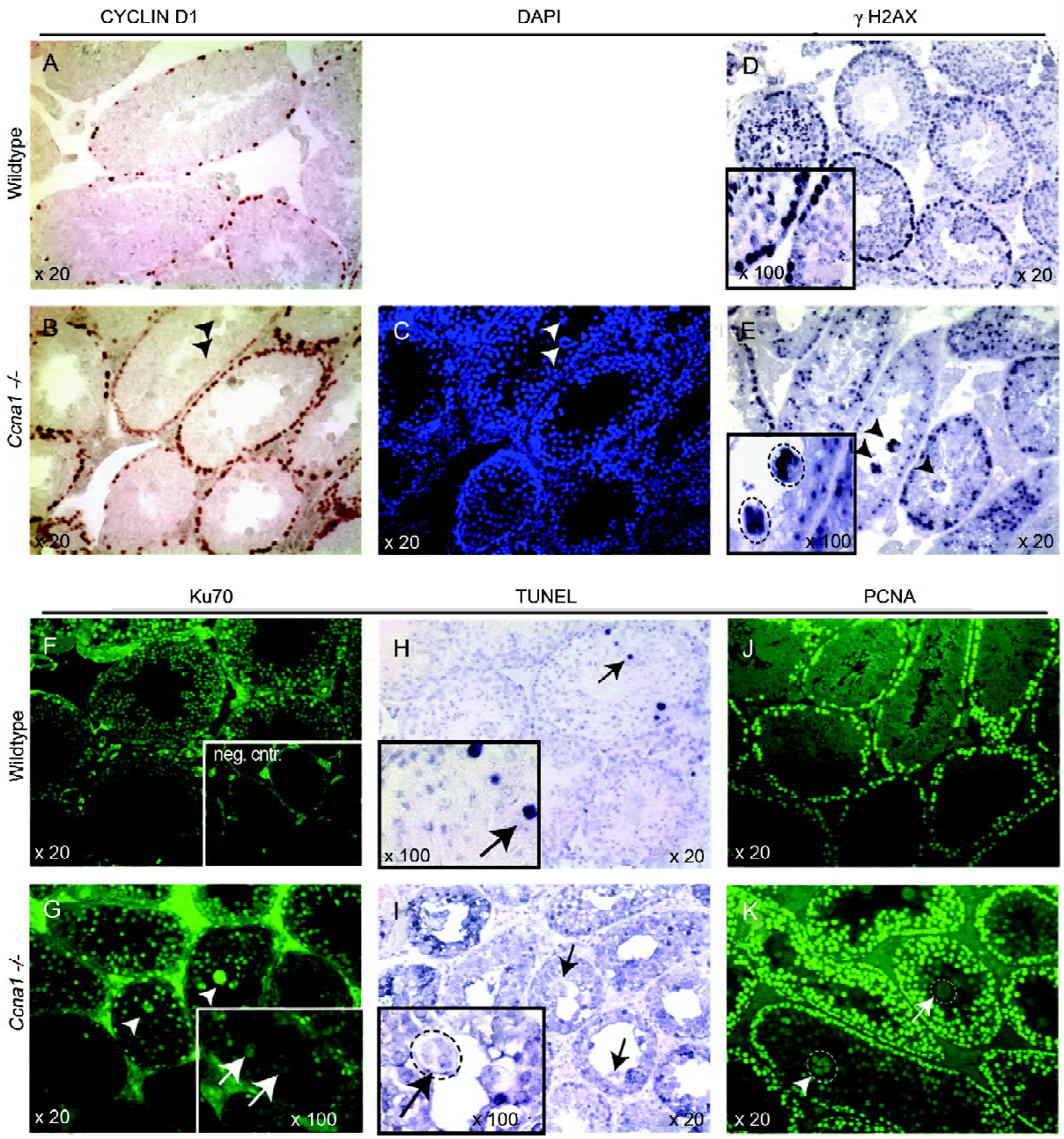

Figure 1. Giant cells in *Ccna1-/-* testes exhibit unrepaired DNA double-strand breaks. Histological sections of paraffin-embedded wildtype (WT; A, D, F, H and J) and *Ccna1-/-* testes (B, C, E, G, I and K) were stained with the antibodies indicated or for apoptotic cells by TUNEL stain, as described in Materials and methods. Original magnification × 20 (A, D, F, H and J) and × 100 (B, C, E, G, I and K). Polynucleated giant cells in *Ccna1-/-* seminiferous tubules do not express cyclin D1 (B, arrowheads), although A-type spermatogonia were positive for Cyclin D1 (B) comparable to the wildtype (A). (C): Nuclear staining of the section shown in B illustrates the abundance of polynucleated giant cells (arrowheads). These cells are positive for phosphorylated histone H2AX, which indicates unrepaired DNA double-strand breaks (E and inset in E, arrowheads). A subset of giant cells express the repair factor Ku70 (G, arrowheads), while some giant cells are negative for Ku70 (inset in G, arrows). Most giant cells are not apoptotic, as revealed by a TUNEL stain (I and inset in I, arrows). In addition, *Ccna1-/-* testes contain a higher number of PCNA-positive cells (K) compared to the wildtype testis (J). The giant cells can be PCNA-positive (K, arrowhead) or negative (K, arrow). Doted elipses in E (inset), I (inset) and K mark the cell membrane surrounding polynucleated giant cells. Magnification of all images: × 20 (if not stated otherwise).

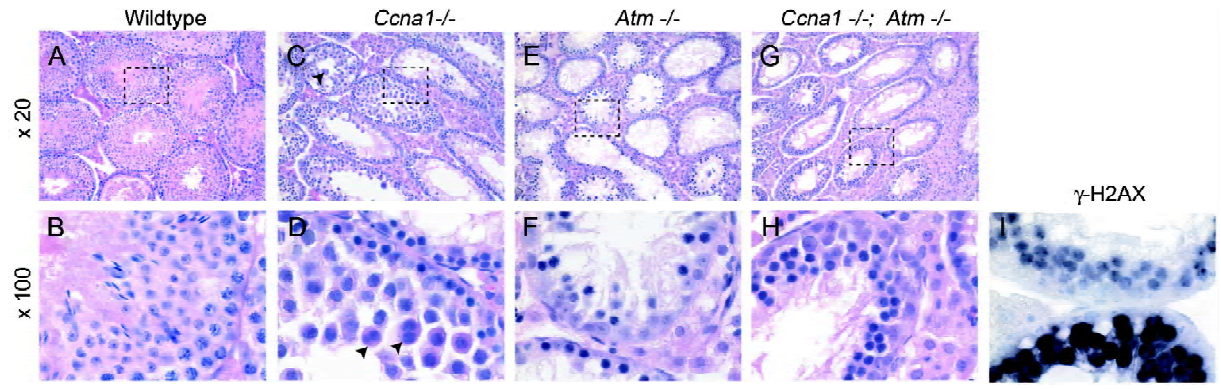

Figure 2. Infertility of male *Ccna1-/-* mice is independent of ATM function. Histological examination of HE-stained paraffin sections (original magnification × 20 and × 100) of wildtype (WT; A, B), *Ccna1-/-* (*Ccna1-/-*; C, D), *Atm-/-* (E, F) and *Ccna1-/-; Atm-/-* (G, H) testes revealed that the absence of cyclin A1 neither altered the spermatogeneic block in *Atm*-deficient testes (compare F and H) nor led to the development of multinucleated giant cells as seen in absence of cyclin A1 alone (arrowheads in C and D). Immunohistochemical staining for phosphorylated H2AX detected many positive cells in *Ccna1-/-; Atm-/-* testes (I). This is in contrast to *Ccna1-/-* testes (compare Figure 1).

performed. This histone is only phosphorylated upon DNA damage [19]. Interestingly, most of the giant cells in *Ccna1-/-* testes were γ-H2AX positive (Figure 1E and inset in E, arrows), which supports the idea that these cells occurred because of an increased number of unrepaired DNA double-strand breaks. In concordance with the detected DNA damage in these cells, most but not all giant cells were also expressing the repair factor Ku70 (Figure 1G, arrows; arrows in the inset hint at Ku70-negative giant cells). However, giant cells were mostly TUNEL-stain negative (Figure 1I and inset in I, arrows), which indicates that in spite of their persistent DNA breaks, they escaped from apoptosis. Another remarkable feature of *Ccna1-/-* testes consisted of its high proliferative activity demonstrated by the increased number of PCNA immunoreactive cells compared to the wildtype testis (Figure 1K compared to J). Some giant cells were also weakly PCNA positive (Figure 1K, arrowhead), possibly hinting to the fact that these cells performed endomitosis.

3.2 Absence of Atm and p53 promoted the development of giant cells in Ccna1-/- testes

Because the giant cells did not appear to develop from cyclin D1 positive spermatogonia or gonocytes, we analyzed whether they arose from early spermatocytes. The expression of cyclin A1 is known to be upregulated by day 11–14 of murine testis development, which is correlated with the first appearance of pachytene spermatocytes [19, 20]. Testes from *Atm-/-* mice, in which spermatogenesis is blocked around early pachynema of prophase I, do not form multinucleated cells (Figure 2E, F) and, therefore, the occurrence of giant cells in a testis that was double mutant for *Ccna1* and *Atm* expression would reveal the function of C*cna1* expression as early as pachynema of prophase I.

The *Ccna1; Atm*-double knockout mice were obtained by breeding mice, which were double heterozygous for both genes. Their offspring contained male double knockout mice at the Mendelian ratio of approximately 1:32 (data not shown). HE-stained histological sections of testes from 60-day-old *Ccna1-/-* (Figure 2C and D), *Atm-/-* (Figure 2E and F) and *Ccna1-/-*; *Atm-/-* mice (Figure 2G and H) revealed that the double knockout mice were infertile, similar to the single knockout mice. No histological differences could be observed between *Atm-/-* and *Ccna1-/-*; *Atm-/-* testes; both exhibited an earlier differential block than *Ccna1-/-* testes. The most important finding was that the *Ccna1-/-* typical giant cells were not found in *Ccna1-/-*; *Atm-/-* testes. This finding indicates that the origin of the giant cells occurred subsequent to early mid-diplotene spermatocytes when recombination events induced multiple DNA double-strand breaks.

Although the phosphorylation of histone H2AX upon DNA damage was postulated to be highly dependent on ATM function [21], we detected a high number of γ-H2AX positive cells in *Atm-/-* and *Ccna1-/-; Atm-/-* testes (Figure 2I and data not shown).

To test whether *Atm-/-* testes still express cyclin A1,

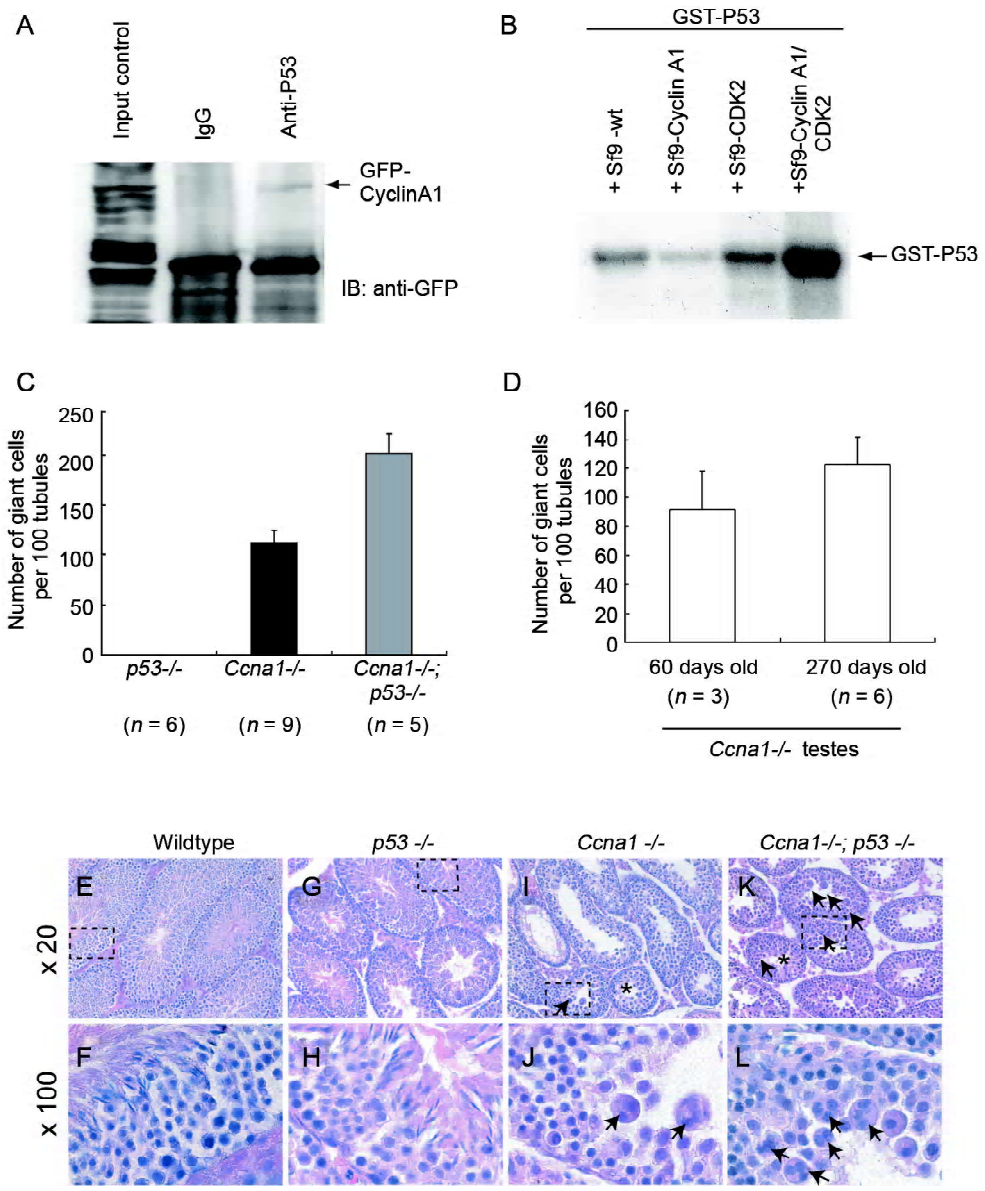

Figure 3. The function of cyclin A1 in the testis is associated with its interaction with p53. (A): Immunoprecipitation with either anti-p53 or an isotype IgG antibody using lysates of Cos-7 cells, which overexpressed p53 and enhanced green fluorescent protein (EGFP)-fused cyclin A1. Western blot analyses of the precipitated proteins was performed using an anti-EGFP antibody detecting a band of the expected size only in the input control (left lane) and after precipitation with the anti-p53 antibody (right lane), but not with IgG alone (middle lane). (B): *In vitro* kinase assay with GST-tagged p53 protein, which was incubated with control baculovirus Sf9 lysate or Sf9 cell lysates expressing cyclin A1 and/or CDK2. Cell lysates with overexpressed cyclin A1 or CDK2 alone phosphorylated p53 comparably to the wildtype lysate, while cyclin A1 and CDK2 together strongly phosphorylated p53. Hematoxylin-eosine (HE)-stained testis sections (original magnification × 20 and × 100) of wildtype (E, F), *p53-/-* (G, H), *Ccna1-/-* (I, J) and *Ccna1-/-; p53-/-* mice (K, L). Dotted areas of the × 20 magnifications in C, E, G and I are shown as × 100 magnifications in D, F, H and J, respectively. The multinucleated giant cells occurring in *Ccna1-/-* testes (arrows in I and J) were found at higher numbers in *Ccna1-/-; p53-/-* testes (arrows in K and L). Asterisks in G and I mark examples of tubules that meet the criteria for the quantification shown in K. (C) Quantification of the number of giant cells occurring in testes of *p53-/-* mice (60 to 150 days old; mean age 105 days), *Ccna1-/-* mice at an age of 60 to 270 days (mean age 210 days) and *Ccna1-/-; p53-/-* mice at 60 to 150 days (mean age 125 days) by counting the multinucleated cells in at least 20 exactly cross-sectioned seminiferous tubules (as examples: see tubules marked by asterisks in G and I) as mean ± SE. The number of giant cells as a result of *Ccna1*-deficiency increased in absence of *p53*. (D): Quantification of the giant cells as in K. The number of giant cells increases in *Ccna1-/-* testes with the age of mice, shown as mean ± SE.

we quantified its expression level using quantitative RT-PCR. Interestingly, the expression of *Ccna1* mRNA was reduced by more than 99% in *Atm-/-* testes (Figure A1).

After establishing the sequential roles of ATM and cyclin A1 in spermatogenesis, we analyzed the potential physical and genetic interactions between C*cna1* and another major player in the DNA damage response, *p53*, in the context of spermatogenesis. Wildtype p53 is expressed at significant levels in early spermatocytes similar to cyclin A1. In addition to our recent findings that cyclin A1 can be induced by p53, we hypothesized that both factors could directly interact *in vivo*. To examine this potential interaction, we co-transfected EGFP-tagged cyclin A1 along with p53 into Cos-7 cells. EGFP is a suitable tag that is clearly detectable by Western blotting but as a non-mammalian protein does not usually react with human proteins. Immunoprecipitation was performed with either anti-p53 or an isotype IgG antibody (Figure 3A). Western blot analyses with anti-EGFP antibody indicated that p53 and cyclin A1 indeed directly interacted in these cells. Consequently, we analyzed whether cyclin A1/CDK2 could utilize p53 as a substrate for phosphorylation. A GST-p53 fusion protein expressed in *Escherichia coli* was incubated with baculovirally-expressed recombinant cyclin A1 and CDK2. In these analyses, Cyclin A1 or CDK2 alone weakly phosphorylated GST-p53 (Figure 3B). In contrast, the cyclin A1/CDK2 complex strongly phosphorylated p53 (Figure 3B).

The expression pattern of p53 in the testis overlaps with the cyclin A1 expression in pachytene spermatocytes and some murine *p53-/-* testes in pure *129/Sv* background also exhibited giant cells [8]. This challenged us to determine whether cyclin A1 and p53 function was redundant during spermatogenesis by examining spermatogenesis in *Ccna1-/-*; *p53-/-* testes. These mice were obtained by breeding mice, which were double heterozygous for both genes. Their offspring contained male double knockout mice at the Mendelian ratio of approximately 1:32 (data not shown). The comparison of HE-stained histological sections from *p53-/-* (Figure 3G, H), from *Ccna1-/-* (Figure 3I, J) and from *Ccna1-/-*; *p53-/-* testes (Figure 3K, L) demonstrated that the seminiferous tubules in the *Ccna1-/-*; *p53-/-* testes contained significantly higher numbers of multinucleated giant cells compared to the *Ccna1-/-* tubules (Figure 3K and L, arrows; Figure 3C). No giant cells were observed in *p53* mutant testes of littermates (Figure 3G, H, and C). It is interesting to note that the frequency of the giant cells in the *Ccna1-/-* testes increased with the age of the animals (Figure 3D), as quantified by counting the giant cells per cross-sectioned tubule.

In summary, the development of giant cells in absence of cyclin A1 function is greatly enhanced by the absence of p53 in *Ccna1-/-* testes. These findings indicate that cyclin A1 and p53 interact with each other during spermatogenesis.

3.3 Cyclin B2 expression is partially dependent on the redundant function of cyclin A1 and p53

To further characterize the effect of loss of p53 function in *Ccna1-/-* testes, we examined the expression levels of cyclins that are highly regulated during spermatogenesis [19]. *Cyclin A2* (*Ccna2*) and *Cyclin D2* (*Ccnd2*) expression was shown to be downregulated, whereas *Cyclin B2* (*Ccnb2*) and *Cyclin K* (*Ccnk*) were strikingly upregulated during meiosis [19]. To determine the changes of cell cycle regulators involved in meiosis in the absence of *Ccna1* and *p53* expression, we performed quantitative RT-PCR for these cyclins from whole-testis cDNA of 60-day-old mice. The expression of *Ccna2* was not significantly changed in any genotype analyzed compared to the wildtype testes (Figure 4). This led to the assumption that the expression of *Ccna2* and the viability of *Ccna2* expressing cells in the testes are not dependent on cyclin A1, p53 or Atm function. Remarkably, the expression of *Ccnd2* was increased upon loss of *Ccna1* (Figure 4) and slightly decreased in *Atm*-deficient testes. We concluded that *Ccnd2* expression is partially dependent on *Atm* function, whereas the higher levels of *Ccnd2* expression in the absence of *Ccna1* might be a result of a proportionally higher number of undifferentiated and mitotically active cells, a notion that is supported by the higher number of PCNA-positive cells in *Ccna1*-deficient testes shown in Figure 1K and 1L.

Ccnb2 was differentially expressed in *Ccna1; p53*-double knockout testes compared to the wildtype and the *Ccna1*-single knockout testes (Figure 4). It is usually upregulated in later stage spermatocytes [16]. Therefore, the very low expression of *Ccnb2* detected in *Atm*-deficient testes was in accordance with its published expression pattern (Figure 4). The expression of *Ccnb2* was increased in *p53-/-* and in *Ccna1-/-* testes, but decreased in comparison to the wildtype in *Ccna1; p53*-double deficient testes (Figure 4). The obvious deregu-

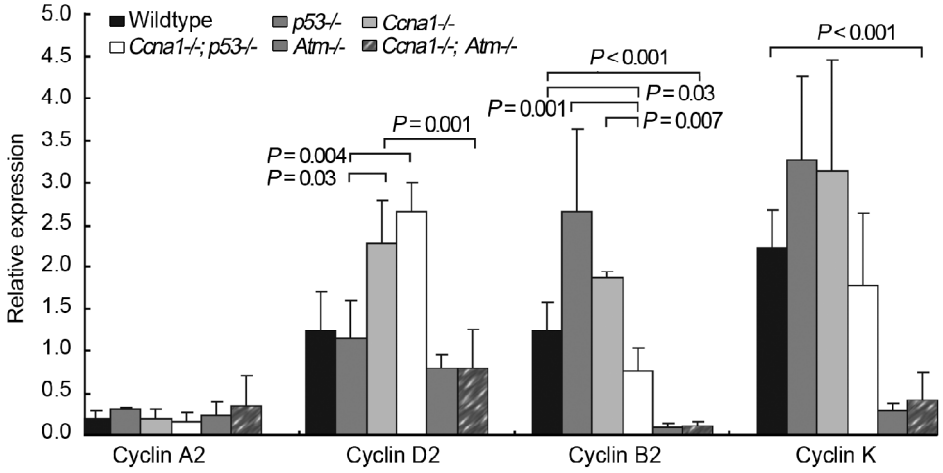

Figure 4. Expression analysis of cell cycle regulators in testes of different genotypes. Reverse-transcriptase polymerase chain reaction (RT-PCR) detecting *Ccna2, Ccnd2, Ccnb2* or *Ccnk* expression levels was performed with cDNA from two to four testes of the indicated genotypes. Relative expression is indicated as mean ± SE.

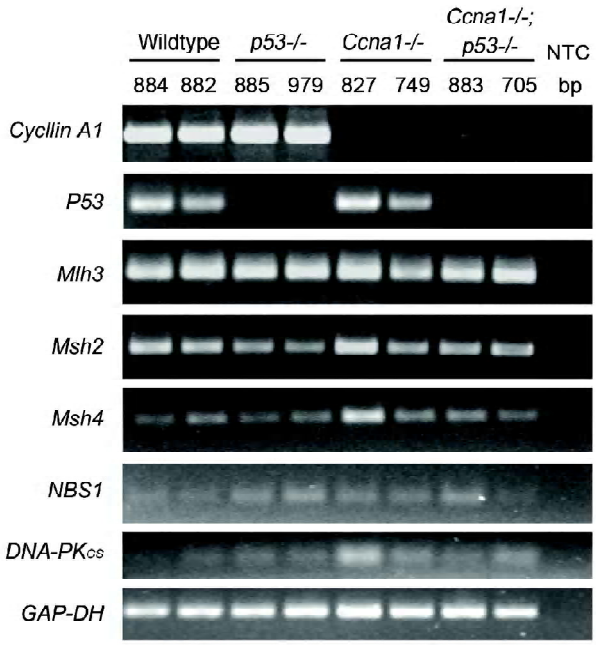

Figure 5. Gene expression of DNA repair regulators is largely unchanged in absence of cyclin A1 expression. Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) using primers, which were specific for the indicated genes was performed with two total testis RNA samples. All genes tested were still abundant in the absence of cyclin A1.

lated repression of *Ccnb2* in absence of *p53*, which was here shown for the first time for spermatogenesis, coincides with the repression of the *Ccnb2*-promoter demonstrated by Imbriano and co-workers [22, 23]. A role of cyclin A1 in the control of *Ccnb2* expression has not yet been shown. For *Ccnk*, we found an expression pattern (Figure 4) closely resembling the *Ccnb2* profile with less pronounced differences between the genotypes, corroborating their co-regulation observed in testis development [19].

3.4 Expression of DNA repair-related genes is not affected in Ccna1-/- testes

Recently, we demonstrated a role of cyclin A1 in DNA double-strand break repair via binding to and thereby regulating Ku70 after occurrence of DNA damage [10]. During meiosis, DNA double-strand breaks and their repair occur naturally to ensure homologous recombination between sister chromatides. Therefore, we hypothesized that the differential block during spermatogenesis and the abundance of multinucleated cells in *Ccna1-/-* testes might be a result of unrepaired DNA double-strand breaks, which we indeed found to be detectable at higher rates via H2AX-staining in these testes (Figure 1E), especially in the giant cells. This raised the question of whether the increased rate of

unrepaired DNA double-strand breaks was associated with further alterations in repair factor expression. We wanted to determine the regulation of a panel of genes involved in different stages in DNA repair in the testes (reviewed in [11]).

We found out that all genes that we analyzed were still expressed in absence of *Ccna1* and/or *p53* (Figure 5; Figure A2 in Appendices; data not shown). Quantitative RT-PCR for *Brca1*, *Dmc1* and *Rad51* and semi-quantitative RT-PCR for *XPD*, *Sycp-3*, *Mlh3*, *Msh2*, *Msh4*, *Msh5*, *Msh6*, *NBS1*, *Pms2* and *Blm* revealed that the expression levels of these genes were not consistently changed in the different mutant testes compared to the wildtype (Figure 5; Figure A2 in Appendices; data not shown). *DNA-PK*$_{cs}$ expression as determined by semi-quantitative RT-PCR was slightly upregulated in *Ccna1-/-* and double knockout testes (Figure 5). Therefore, the DNA repair deficiency observed in the absence of cyclin A1 could not be explained by the absence of any of the putative downstream executing factors tested here, but provides a further hint at a direct role of cyclin A1 itself in DNA repair.

4 Discussion

Recombination events occurring during spermatogenesis require controlled DNA double-strand breaks and their adequate repair. Disturbances of this tightly controlled mechanism lead to the complete abolishment of spermatogenesis, although they can mostly be compensated in somatic cells. The same phenomenon can be observed in mice, which are deficient for the cell cycle regulator cyclin A1. These mice lack spermatocytes beyond the mid-diplotene stage, but the somatic cells appear largely unaffected. Instead of spermatocytes, they develop unusual multinucleated cells, so-called giant cells, in the luminal part of the seminiferous tubules.

We recently demonstrated that cyclin A1 has a function in somatic DNA double-strand break repair by binding to the repair factor Ku70 upon cellular stress as, for example, irradiation [10]. The prominent development of the multinucleated giant cells in the *Ccna1-/-* testes, which was not explainable up to now, prompted us to investigate the role of cyclin A1 in DNA repair in spermatogenesis.

We provide evidence that the giant cells are of a different origin than the multinucleated cells occurring upon loss of *citron kinase* [7], because *Ccna1*-deficient giant cells lack cyclin D1 expression and, therefore, do not have A-type spermatogonial or gonocyte differentiation status [17]. In addition, the giant cells originate from cells later than leptonema of prophase I, because *Ccna1/Atm* double mutant testes do not develop these cells.

The *Ccna1-/-; Atm-/-* testes revealed several important insights into the role of cyclin A1 during spermatogenesis. First, *Ccna1+/+; Atm-/-* testes exhibit only a very low expression level of cyclin A1 (see Figure A1). Because *Atm-/-* testes produce some pachytene and diplotene spermatocytes [12], which usually express cyclin A1 [24], these few remaining cells explain the basal cyclin A1 expression observed in these testes. Finally, these double knockouts provide conclusive evidence that the origin of the giant cells is indeed downstream of the ATM-mediated spermatogenesis block.

Immunohistochemical detection revealed that most of the giant cells are positive for γ-H2AX, the phosphorylated histone H2AX, which only appears upon DNA damage, and for Ku70, indicating DNA double-strand breaks, but also the attempt to repair this damage. Moreover, the expression analysis of a whole panel of DNA repair factors, which are known to be required for functional spermatogenesis revealed that all genes examined here were still abundant in the absence of cyclin A1. These findings suggest that the unrepaired DNA damage observed in *Ccna1-/-* testes might depend on the specific functions of cyclin A1 in the regulation of DNA double-strand break repair.

We further support this notion by demonstrating that cyclin A1 can directly interact with p53 and in complex with CDK2 phosphorylate p53. P53 is a well-known tumor suppressor protein, which is also implicated to be involved in spermatogenesis and especially in DNA repair mechanisms in meiotic pachytene stage cells [8].

In lysates from COS-7 cells that overexpressed cyclin A1 and p53, these proteins co-immunoprecipitated. A physical interaction between cyclin A1 and p53 is possible during spermatogenesis because both proteins are expressed at high levels in pachytene spermatocytes [24].

Despite intense ongoing investigations, the role of P53 in DNA double-strand break repair is still not well understood [9]. Our finding that cyclin A1/CDK2 can phosphorylate p53 *in vitro* is in accordance with previous findings that cyclin A2/CDK2 can also phosphorylate p53 [25]. The functional consequences of p53 phosphorylation by A-type cyclins/CDK2 remain unclear, but

in the light of our data it is reasonable to assume that it modulates the p53 response to DNA damage.

Moreover, *Ccna1/p53* double deficient testes exhibit an increased number of multinucleated cells compared to *Ccna1* single knockout testes. Remarkably, the giant cells escape from apoptosis as detected in TUNEL staining, although *Ccna1-/-* testes generally exhibit a higher rate of apoptosis [6, 26]. It seems surprising that cyclin A1 might suppress apoptosis in one cell type but promote apoptosis in another spermatogenetic cell type. This phenomenon might be explained by the different stimuli and pathways of apoptosis induction. It is possible that cyclin A1 is involved in apoptosis induction by p53 after DNA double-strand breaks occur, leading to the apoptosis escape of giant cells in *Ccna1-/-* testes and the higher number of giant cells in *Ccna1-/-; p53-/-* double knockout testes. However, cyclin A1 might inhibit other pro-apoptotic pathways triggered by cell cycle dysregulation and, thereby, its depletion contributes to increased apoptosis of other cell types. We conclude that the loss of mature spermatids in *Ccna1-/-* testes might be a result of the enhanced apoptosis, while the giant cells develop because of the function of cyclin A1 in DNA double-strand break repair.

In addition, the increase of multinucleated cells with age of the animal in *Ccna1-/-* testes supports our current model in which stochastical DNA breaks, which accumulate with time in older animals, are insufficiently repaired due to lack of *Ccna1*, giving rise to the giant cells escaping apoptosis because of the cyclin A1 depletion.

In summary, we establish a specific role for cyclin A1 in conjunction with its interaction partner and substrate p53 in DNA double-strand break repair during spermatogenesis. In particular, we show for the first time that the giant cells formed in testicular tubules lacking cyclin A1 occur after leptonema of prophase I, escape apoptosis and contain unrepaired double-strand breaks, corroborating the important function of cyclin A1 in this process.

Acknowledgment

We would like to thank Mr Frank Berkenfeld (Department of Medicine, Hematology and Oncology, University of Münster, Germany) and Ms Petra Meier (Department of Pathology, University of Münster, Germany) for excellent technical assistance and Dr Marc Carrington (Cambridge, UK) for the *Ccna1*-knockout mice. This work was supported by grants from the Interdisciplinary Center for Clinical Research, University of Münster (Mül2/096/04) and the Deutsche Krebshilfe (10-2155-Mü3).

References

1 Weikert S, Christoph F, Schulze W, Krause H, Kempkensteffen C, Schostak M, *et al*. Testicular expression of survivin and human telomerase reverse transcriptase (hTERT) associated with spermatogenic function in infertile patients. Asian J Androl 2006; 8: 95–100.
2 Li ZX, Ma X, Wang ZH. A differentially methylated region of the DAZ1 gene in spermatic and somatic cells. Asian J Androl 2006; 8: 61–7.
3 Yin LL, Li JM, Zhou ZM, Sha JH. Identification of a novel testis-specific gene and its potential roles in testis development/spermatogenesis. Asian J Androl 2005; 7: 127–37.
4 Zheng Y, Zhou ZM, Min X, Li JM, Sha JH. Identification and characterization of the BGR-like gene with a potential role in human testicular development/spermatogenesis. Asian J Androl 2005; 7: 21–32.
5 Ortega S, Prieto I, Odajima J, Martín A, Dubus P, Sotillo R, *et al*. Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat Genet 2003; 35: 25–31.
6 Liu D, Matzuk MM, Sung WK, Guo Q, Wang P, Wolgemuth DJ. Cyclin A1 is required for meiosis in the male mouse. Nat Genet 1998; 20:377–80.
7 Cunto FD, Imarisio S, Camera P, Boitani C, Altruda F, Silengo L. Essential role of citron kinase in cytokinesis of spermatogenic precursors. J Cell Sci 2002; 115: 4819–26.
8 Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, *et al*. Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. Proc Natl Acad Sci U S A 1993; 90: 9075–9.
9 Sengupta S, Harris CC. p53: traffic cop at the crossroads of DNA repair and recombination. Nat Rev Mol Cell Biol 2005; 6: 44–55.
10 Barlow C, Liyanage M, Moens PB, Tarsounas M, Nagashima K, Brown K, *et al*. Atm deficiency results in severe meiotic disruption as early as leptonema of prophase I. Development 1998; 125: 4007–17.
11 de Rooij DG, de Boer P. Specific arrests of spermatogenesis in genetically modified and mutant mice. Cytogenet Genome Res 2003; 103: 267–76.
12 Müller-Tidow C, Ji P, Diederichs S, Potratz J, Bäumer N, Köhler G, *et al*. The cyclin A1-CDK2 complex regulates DNA double-strand break repair. Mol Cell Biol 2004; 24: 8917–28.
13 van der Meer T, Chan WY, Palazon LS, Nieduszynski C, Murphy M, Sobczak-Thépot J, *et al*. Cyclin A1 protein shows haplo-insufficiency for normal fertility in male mice. Reproduction 2004; 127: 503–11.
14 Xu Y, Ashley T, Brainerd EE, Bronson RT, Meyn MS, Baltimore D. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, im-

mune defects, and thymic lymphoma. Genes Dev 1996; 10: 2411–22.
15 Institute of Laboratory Animal Resource, Commission on Life Sciences, National Research Council. Guide for the care and use of laboratory animals. National Academy Press Washington D.C. 1996
16 Diederichs S, Bäumer N, Ji P, Metzelder SK, Idos GE, Cauvet T, *et al*. Identification of interaction partners and substrates of the cyclin A1-CDK2 complex. J Biol Chem 2004; 279: 33727–41.
17 Beumer TL, Roepers-Gajadien HL, Gademan IS, Kal HB, de Rooij DG. Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse. Biol Reprod 2000; 63: 1893–8.
18 Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodríguez J, *et al*. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet 2001; 27: 271–6.
19 Diederichs S, Bäumer N, Schultz N, Hamra FK, Schrader MG, Sandstede ML, *et al*. Expression patterns of mitotic and meiotic cell cycle regulators in testicular cancer and development. Int J Cancer 2005; 116: 207–17.
20 Müller-Tidow C, Readhead C, Cohen AH, Asotra K, Idos G, Diederichs S, *et al*. Successive increases in human cyclin A1 promoter activity during spermatogenesis in transgenic mice. Int J Mol Med 2003; 11: 311–5.
21 Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem 2001, 276: 42462–7.
22 Krause K, Wasner M, Reinhard W, Haugwitz U, Dohna CL, Mossner J, *et al*. The tumour suppressor protein p53 can repress transcription of cyclin B. Nucleic Acids Res 2000, 28: 4410–8.
23 Imbriano C, Gurtner A, Cocchiarella F, Di Agostino S, Basile V, Gostissa M, *et al*. Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. Mol Cell Biol 2005, 25: 3737–51.
24 Sweeney C, Murphy M, Kubelka M, Ravnik SE, Hawkins CF, Wolgemuth DJ, *et al*. A distinct cyclin A is expressed in germ cells in the mouse. Development 1996; 122: 53–64.
25 Luciani MG, Hutchins JR, Zheleva D, Hupp TR. The C-terminal regulatory domain of p53 contains a functional docking site for cyclin A. J Mol Biol 2000; 300: 503–18.
26 Salazar G, Joshi A, Liu D, Wei H, Persson JL, Wolgemuth DJ. Induction of apoptosis involving multiple pathways is a primary response to cyclin A1-deficiency in male meiosis. Dev Dyn 2005; 234: 114–23.

Edited by Dr Sidney Grimes

Appendices

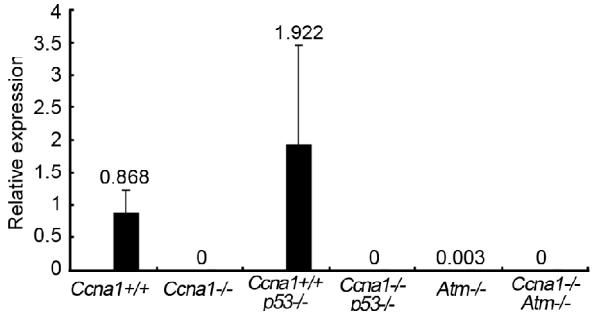

Figure A1. *Ccna1* mRNA expression is abrogated in *Atm-/-* testes. Expression analysis of *Ccna1* by reverse transcriptase-polymerase-chain-reaction using total cDNA from murine testes of different genotypes revealed undetectable expression in the *Ccna1-/-* testes, but also very low expression in testes from *Atm-/-* animals. Relative expression levels are shown as mean ± SE.

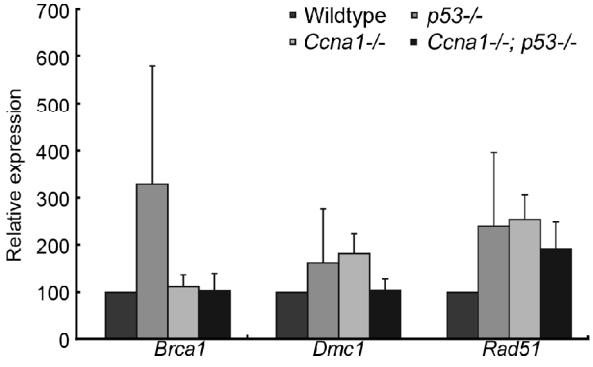

Figure A2. Expression levels of *Brca1*, *Dmc1* and *Rad51* are not significantly affected in absence of *Ccna1* in the murine testis. Expression analysis of *Brca1*, *Dmc1* and *Rad51* was performed by reverse transcriptase-polymerase-chain-reaction using total testis cDNAs of different genotypes. The expression levels are shown here relative to the respective wildtype expression (= 100%) with standard deviation.