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

Nicole Bäumer1,4*, Marie-Luise Sandstede1,*, Sven Diederichs1,9, Gabriele Köhler1, Carol Readhead3, Ping Ji1, Feng Zhang1, Etmar Bulk1, Jörg Gromoll4,5, Wolfgang E. Berdel1, Hubert Serve1,4, Carsten Müller-Tidow1,4

1Department of Medicine, Hematology and Oncology, University of Münster, Domagkstrasse 3, Münster 48149, Germany
2Department of Pathology, University of Münster, Domagstrasse 17, Münster 48149, Germany
3California Institute of Technology, 1200 East California Boulevard, Pasadena, California 91125, USA
4Interdisciplinary Center for Clinical Research, University of Münster, Domagstrasse 3, Münster 48149, Germany
5Institute of Reproductive Medicine, University of Münster, Domagstrasse 12, Münster 48129, Germany

Correspondence to: Dr. Carsten Müller-Tidow, Department of Medicine, Hematology and Oncology, University of Münster, Domagkstr. 3, D-48129 Münster, Germany. Tel: +49-251-835-6229 Fax: +49-251-835-2673 E-mail: muellerc@uni-muenster.de
#Present address: MGH Cancer Center, Harvard Medical School, 149 13th Street, Charlestown 02129-2020, MA, USA.
*The two authors contributed equally to this work.

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

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

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 myotyon-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 telangiectasia 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, Ccna1 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 × M/Fl) were obtained from Dr. Marc Carrington (Cambridge, UK), p53+/+ mice (129/Sv) were obtained from Jackson Laboratories (Maine, USA, Stock-Number 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'-AGCAGCAGGCTGCCTGTGGCTTAC-3', CyclinA1-wt-r: 5'-TCCTTGGCATCGTTCTCCAT-3', CyclinA1-ko-r: 5'-GCGAGTGGCAACAATGGAAAAT-3'; (ii) p53X6.5: 5'-CAGCGTGGTGGTACCTTAT-3', p53X7: 5'-TATACTCAGAGCCGGCCT-3', Neo18.5new: 5'-CTATCAGACATAGGCGGTTG-3'; and (iii) ATM1: 5'-CCTCCCTATATTGTAACACGCTG-3', ATM2: 5'-TGAATTCTGCTTAAAGAACCTGG-3', ATM3: 5'-GGAAAGAGCGGCTCCTCCCTACCGG-3'. Each PCR assay contained 1 µL of Proteinase K digest, 1/10 vol (v/v) of Biotherm Polymerase buffer (Natutec, Frankfurtr/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, Munich, 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

http://www.asiaandro.com; aja@sibs.ac.cn
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 [32P] 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 at 30°C in kinase buffer (10 mmol/L ATP, 50 mmol/L Hepes [pH 7.5], 1 mmol/L DTT, 10 mmol/L MgCl2, 0.1 mmol/L Na3VO4, 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 37°C and stored at room temperature. Hematoxylin-eosin (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 4°C 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 94°C for 2 min and then run through 25–37 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by 72°C for 10 min and then 4°C. 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
Interactions of Cyclin A1 with p53 and Atm

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 1</th>
<th>GenBank accession number</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A1</td>
<td>GGTGTGGACTGAAAATGAGCAG;</td>
<td>NM_007628</td>
<td>572</td>
</tr>
<tr>
<td></td>
<td>GAAACCTGTCAGGAAGAAGTGGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>GGAGACATTGTCGGCTTATGG;</td>
<td>NM_011640</td>
<td>333</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>TCAATGGTTAATACGAACTACTAA;</td>
<td>NM_011159</td>
<td>126</td>
</tr>
<tr>
<td>GAP-DH</td>
<td>ACACAGCTCATGGCAATCAC;</td>
<td>BC085275</td>
<td>451</td>
</tr>
<tr>
<td>Mih3</td>
<td>GAGACTGAGTGGAGAGGATTGAG;</td>
<td>BC079861</td>
<td>356</td>
</tr>
<tr>
<td>Msh2</td>
<td>CCAGGCGGTGATCAAGTAC;</td>
<td>NM_008628</td>
<td>498</td>
</tr>
<tr>
<td>Msh4</td>
<td>GCATAAAAGTGGGAACCGACCACAAA;</td>
<td>NM_031870</td>
<td>441</td>
</tr>
<tr>
<td>NBS1</td>
<td>GAAACAGCGTCCAGATTTGAAA;</td>
<td>NM_013752</td>
<td>242</td>
</tr>
<tr>
<td>XPD</td>
<td>GCTCTGAGCTCAAGAAAGAACC;</td>
<td>NM_007949</td>
<td>272</td>
</tr>
</tbody>
</table>

Results, six testes of wildtype mice, three testes of Ccnal-knockout mice, three p53-knockout testes, two Atm-knockout testes, four Ccnal; p53- and two Ccnal; 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 Ccnal-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 Ccnal; p53- double knockout testis and 158 tubules of four older Ccnal; p53- double knockout testis were taken into consideration. HE-stainings were made from eight wildtype mice, nine testes of Ccnal-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 Ccnal; p53- and six Ccnal; 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 Ccnal-deficient testes accumulate DNA double-strand breaks

Because Ccnal 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, Ccnal-/- giant cells were not cyclin D1 positive (Figure 1B, arrows). Therefore, multinucleated cells are likely to originate from different cell types and Ccnal-/- 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 Ccnal-/- 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 Ccnal−/− testes exhibit unrepaired DNA double-strand breaks. Histological sections of paraffin-embedded wildtype (WT; A, D, F, H and J) and Ccnal−/− 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 Ccnal−/− 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, Ccnal−/− 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 ellipses in E (inset), I (inset) and K mark the cell membrane surrounding polynucleated giant cells. Magnification of all images: × 20 (if not stated otherwise).
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 pachytenocyte 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 Ccnal and Atm expression would reveal the function of Ccnal expression as early as pachynema of prophase I.

The Ccnal; 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 Ccnal-/- (Figure 2C and D), Atm-/- (Figure 2E and F) and Ccnal-/-; 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 Ccnal-/-; Atm-/- testes; both exhibited an earlier differential block than Ccnal-/- testes. The most important finding was that the Ccnal-/- typical giant cells were not found in Ccnal-/-; 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 Ccnal-/-; 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 and CDK2 alone phosphorylated p53 comparably to the wildtype lysate, while cyclin A1 and CDK2 together strongly phosphorylated p53. Hematoxylin-eosin (HE)-stained testis sections (original magnification ×20 and ×100) of wildtype (E, F), p53-/- (G, H), Ccn1-/- (I, J) and Ccn1-/-; 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 Ccn1-/- testes (arrows in I and J) were found at higher numbers in Ccn1-/-; 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), Ccn1-/- mice at an age of 60 to 270 days (mean age 210 days) and Ccn1-/-; p53-/- mice (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 Ccn1-deficiency increased in absence of p53. (D): Quantification of the giant cells as in K. The number of giant cells increases in Ccn1-/- testes with the age of mice, shown as mean ± SE.
we quantified its expression level using quantitative RT-PCR. Interestingly, the expression of Ccnal 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 Ccnal 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 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 did not interact 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 Ccnal−/−; 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 Ccnal−/− (Figure 3I, J) and from Ccnal−/−; p53−/− testes (Figure 3K, L) demonstrated that the seminiferous tubules in the Ccnal−/−; p53−/− testes contained significantly higher numbers of multinucleated giant cells compared to the Ccnal−/− 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 Ccnal−/− 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 Ccnal−/− 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 Ccnal−/− 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 Ccnal and p53 expression, we performed quantitative RT-PCR for these cyclins from whole-testis cDNA of 60-day-old mice. The expression of Ccnb2 was not significantly changed in any genotype analyzed compared to the wildtype testes (Figure 4). This led to the assumption that the expression of Ccnb2 and the viability of Ccnal expressing cells in the testes are not dependent on cyclin A1, p53 or Atm function. Remarkably, the expression of Ccnb2 was increased upon loss of Ccnal (Figure 4) and slightly decreased in Atm-deficient testes. We concluded that Ccnb2 expression is partially dependent on Atm function, whereas the higher levels of Ccnb2 expression in the absence of Ccnal 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 Ccnal-deficient testes shown in Figure 1K and 1L.

Ccnb2 was differentially expressed in Ccnal; p53-double knockout testes compared to the wildtype and the Ccnal-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 Ccnal−/− testes, but decreased in comparison to the wildtype in Ccnal; p53-double deficient testes (Figure 4). The obvious deregu-
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 Ccnal-/- 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
Interactions of Cyclin A1 with p53 and Atm

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 Ccnal and/or p53 (Figure 5; Figure A2 in Appendices; data not shown). Quantitative RT-PCR for Bree1, Dmc1 and Rad51 and semi-quantitative RT-PCR for XPD, Syce-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-PKcs expression as determined by semi-quantitative RT-PCR was slightly upregulated in Ccnal-/- 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 Ccnal-/- 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 Ccnal-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 Ccnal/Atm double mutant testes do not develop these cells.

The Ccnal-/-; Atm-/- testes revealed several important insights into the role of cyclin A1 during spermatogenesis. First, Ccnal+/+; Atm-/- testes exhibit only a very low expression level of cyclin A1 (see Figure A1). Because Atm-/- testes produce some pachytenes 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 Ccnal-/- 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 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

14. Xu Y, Ashley T, Brainier EE, Bronson RT, Meyn MS, Baltimore D. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, im-
Interactions of Cyclin A1 with p53 and Atm


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 Brcal, Dmc1 and Rad51 are not significantly affected in absence of Ccna1 in the murine testis. Expression analysis of Brcal, 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.

http://www.asiaandro.com; aja@sibs.ac.cn