DNA DOUBLE-STRAND BREAKS & APOPTOSIS IN THE TESTIS

Voor Opa Leur,

Printing: FeboDruk BV, Enschede
ISBN: 90-901-7161-4

Cover illustration & Chapter numbering. *With some imagination or scientific creativity one might see a homunculus in the densely packed chromatin of an elongated germ cell. Such a homunculus only needs some soil and nourishment (which, of course, it will find in the womb) in order to develop into a complete human being. This is the most famous picture of a homunculus, observed and drawn by Nicolaas Hartsoeker in 1694.*

DNA DOUBLE-STRAND BREAKS & APOPTOSIS IN THE TESTIS

DNA dubbelstrengs breuken & apoptose in de testis
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
maandag 22 september 2003 des middags te 16:15 uur

Door

Geert Hamer

Geboren op 23 oktober 1973, te Oldekerk

Promotor: Prof. Dr. D.G. de Rooij
verbonden aan de faculteit Biologie van de Universiteit Utrecht

The research described has been supported by the J.A. Cohen Institute for Radiopathology and Radiation Protection, Leiden, the Netherlands

TABLE OF CONTENTS

INTRODUCTION **7**

CHAPTER 2
Intercellular bridges & apoptosis in clones of male germ cells **21**

CHAPTER 3
Role for c-Abl & p73 in the radiation response of male germ cells **31**

CHAPTER 4
DNA double-strand breaks & γ-H2AX signaling in the testis **43**

CHAPTER 5
Function of DNA-PKcs during the early meiotic prophase without Ku70 & Ku86 **57**

CHAPTER 6
ATM expression & activation in the testis **67**

SUMMARIZING DISCUSSION **79**

SAMENVATTING VOOR IEDEREEN **91**

DANKWOORD/ ACKNOWLEDGEMENTS **93**

CURRICULUM VITAE **95**

LIST OF PUBLICATIONS **96**

COLOR FIGURES **97**

DNA DOUBLE-STRAND BREAKS & APOPTOSIS IN THE TESTIS

A GENERAL INTRODUCTION

Geert Hamer[1,2] & Dirk G. de Rooij[1,2]

[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*

[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

During spermatogenesis the male genome is being prepared for reproduction and whereas most somatic cells function to maintain proper function of the individual, the germ cells hold the genetic future of a species. This has important biological consequences. In all cells genome integrity is carefully guarded as mutations can lead to cellular malfunction or, when oncogenes or tumor-suppressor genes are affected, even to cancer. However, a mutation in the germ line will be inherited by all cells of the offspring. This implies that the efficiency by which genome integrity is maintained during spermatogenesis even has evolutionary consequences.

Although proper spermatogenesis requires many biological processes that are used by many cell types, other features are unique to the male germ line. This certainly also applies for the many ways in which male germ cells are protected against genome damage. In response to DNA damage many cells stop dividing (cell cycle arrest). During this stop they either repair the damaged DNA or, when the damage is too severe, undergo a programmed cell death sequence (apoptosis). This study will specifically focus on DNA damage repair and apoptosis during spermatogenesis. During spermatogenesis, DNA damage is a naturally occurring event. At a certain stage, during the meiotic prophase, DNA breaks are even required for meiotic recombination (1). However, in order to study the germ cell response to non-physiological damage, we also induced DNA damage using ionizing radiation (IR). Spermatogenesis is a multi step process that begins with mitotically dividing diploid stem cells and ends with highly differentiated haploid elongated spermatids. Every phase of spermatogenesis is tightly regulated, and during the process the different spermatogenic cells completely change in chromatin structure and response to DNA damage. A proper response to DNA damage is essential for germ cell development and loss-of-function mutations of many DNA damage response proteins lead to abrogation of spermatogenesis (2, 3).

SPERMATOGENESIS

In the mammalian testis **(Figure 1)** spermatogenesis takes place in the seminiferous tubules **(Figure 2)**. At both ends, these tubules are connected to an excurrent duct system consisting of the rete testis, the efferent ducts, the epididymus and the defferent duct (vas deferens).

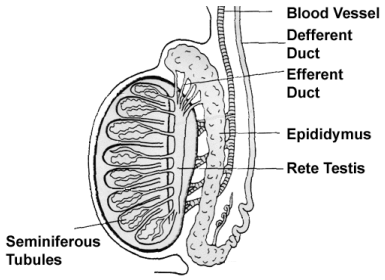


Figure 1. ***Schematic representation of a mammalian testis.*** *Adapted from www.klinika-papic.co.yu.*

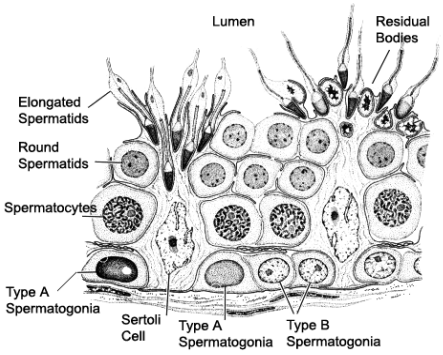


Figure 2. ***Cross-section of a seminiferous tubule.*** *Adapted from Scott Gilbert, 1997 (4) courtesy by Sinauer Associates, Inc..*

Within the seminiferous tubules the developing germ cells are supported by the somatic Sertoli cells and in between the seminiferous tubules are the Leydig cells, that play a role in the endocrine regulation of spermatogenesis, macrophages and blood and lymphatic vessels. During spermatogenesis mitotically dividing spermatogonia differentiate into the haploid spermatids. Several spermatogonial subtypes exist (5); the A single (A_s) spermatogonia are the spermatogonial stem cells which can self renew or divide into a pair of interconnected spermatogonia, the A paired (A_{pr}). These A_{pr} divide further to form A aligned (A_{al}) spermatogonia that consist of 4, 8 or 16 interconnected cells. The A_{al} divide into differentiating spermatogonia called A_1 that further divide and differentiate to form A_2 to A_4 spermatogonia. The A_4 divide into intermediate spermatogonia (In) that divide into B spermatogonia. Eventually the B spermatogonia divide and form pre-leptotene spermatocytes, in which the male genome is duplicated during the pre-meiotic S-phase, and subsequently enter the meiotic prophase. After a prolonged meiotic prophase, during which meiotic recombination takes place, and the two meiotic divisions the haploid round spermatids are formed that will elongate and eventually leave the seminiferous tubules and mature into spermatozoa. Spermatogenesis is a cyclic process that can be subdivided into several stages **(Figure 3)**. Each stage is defined by the presence of a fixed combination of specific germ cell types in a tubular cross-section.

During the subsequent mitotic and meiotic divisions the germ cells stay cytoplasmically connected by intercellular bridges leading to large “clones” of spermatids at the end of spermatogenesis (6, 7). The intercellular bridges allow communication between germ cells within such a clone (8-10) and germ cells within a clone differentiate synchronously suggesting that maintenance of synchronous development is a major function of these bridges (7-11).

Throughout spermatogenesis many germ cells “spontaneously” undergo apoptosis (15). This can be induced by DNA damage during the mitotic or meiotic cell divisions or during meiotic recombination (16), and influenced by many factors including gonadotropin levels and intra-testicular androgens (17-19). DNA damage also can be artificially induced, for instance by cytostatic drugs or radiation. Additionally, apart from DNA damage signaling, apoptosis in the testis also occurs in order to regulate germ cell density (20, 21). Remnants of apoptotic germ cells are very efficiently phagocytized by the Sertoli cells (17, 18).

IONIZING RADIATION & DNA DOUBLE-STRAND BREAKS

IR can damage DNA in several ways (22, 23). In response to irradiation the DNA can be chemically modified, for instance, bases can be eliminated or cross-linked leading to changes in the conformation of the DNA. IR can also induce cross-linking of two complementary DNA strands or DNA with proteins. However, the most severe type of DNA damage caused by IR are DNA double-strand breaks (DSBs) (24-34).

How these DSBs are dealt with differs for every germ cell type. In spermatogonia, IR mostly leads to apoptosis (35-39), but, might also lead to cell cycle arrest (40-42) **(Figure 4)**. Spermatocytes and spermatids are very resistant to IR, in the sense that they will not as easily undergo apoptosis after irradiation (43). However, in early spermatocytes, DSBs are also endogenously induced, leading to repair by meiotic recombination or apoptosis (16). In the coming chapters the subsequent male germ cell types and their response to IR and DSBs will be discussed.

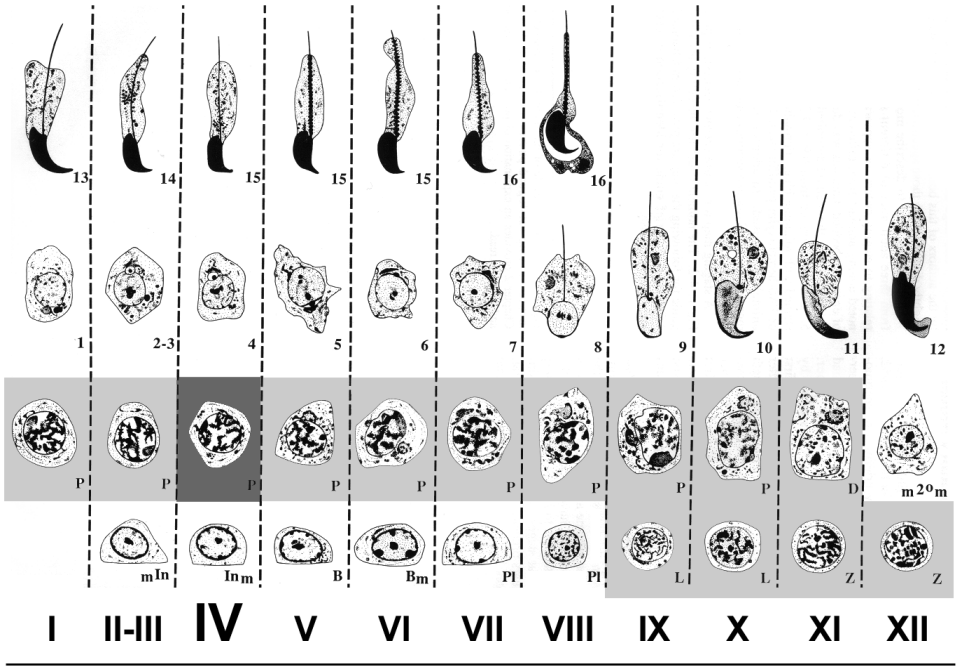
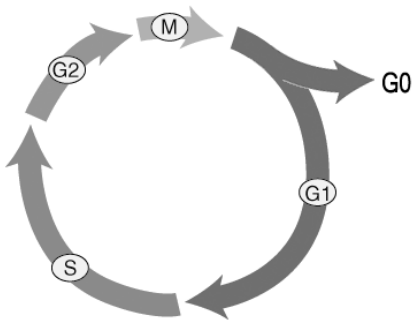


Figure 3. ***The different stages of the seminiferous epithelium of the mouse.*** *Spermatogenesis, being a cyclic process, can be subdivided into stages, each stage being defined by the presence of a fixed combination of specific germ cell types in a tubular cross-section (the broken lines). In the mouse seminiferous epithelium 12 stages can be distinguished, taking 8.6 days to complete one cycle (12, 13). Depicted are: intermediate (In) and B (B) spermatogonia; the premeiotic S-phase or preleptotene (Pl), leptotene (L), zygotene (Z) and pachytene (P) spermatocytes; the two subsequent meiotic divisions ($m^{2o}m$) and spermiation steps 1-16. Not depicted are the type A spermatogonia which are present at the basal membrane at all stages.*

Lightly shaded is the first meiotic prophase ***(Figure 6)*** *from leptotene until diplotene during which meiotic recombination takes place. Early during pachytene, at epithelial stage IV, a meiotic checkpoint exists at which DNA damage can be repaired or lead to apoptosis (14, 120). Adapted from Russell et al., 1990 (12) courtesy by Cache River Press.*

Figure 4. ***The cell cycle*** *consists of four successive phases starting with the first GAP phase (G_1). During the DNA synthesis phase (S) the cells DNA is replicated followed by the second GAP-phase (G_2). Actual cell division occurs during the mitotic phase (M). After completion of the cell cycle, the two daughter cells can proceed in a second cycle or enter a quiescent phase (G_0). Progression of the cell cycle requires strict conditions including, cell size, nutrients and DNA integrity. These conditions are checked at several cell cycle checkpoints, for instance at the G_1/S or the G_2/M transition. When a cell fails to fulfill one of these requirements, the cell cycle will be interrupted, leading to cell cycle arrest (44, 45).*



SPERMATOGONIA

Of the male germ cells the mitotically dividing spermatogonia are the most radiosensitive (46), meaning they will easily undergo apoptosis in response to IR. Also within the spermatogonial population there are differences in radiosensitivity, the spermatogonial stem cells being much more radioresistant than the differentiating spermatogonia (46, 47). Like most mitotically dividing cells (44, 45), also the spermatogonia will probably undergo cell cycle arrest in response to DNA damage as for instance caused by IR (40-42). During this arrest the spermatogonia will either repair the damaged DNA or, in most cases, undergo apoptosis (35-39).

SPERMATOGONIAL APOPTOSIS

Apoptosis of spermatogonia is not only necessary for the removal of damaged cells but also for the regulation of germ cell density (15, 20, 48). Interestingly, these different causes for spermatogonial cell death lead to different apoptotic pathways.

Life and death fate of a cell can be determent by the Bcl-2 protein family (15, 49). This protein family consists of pro- and anti-apoptotic members. Spermatogonial degeneration during germ cell density regulation has been proven to depend on a pro-apoptotic member of the Bcl-2 family (49) called Bax (37, 50, 51). However, Bax has been shown not to be involved in DNA-damage induced apoptosis of germ cells (37).

During DNA-damage induced spermatogonial apoptosis the tumor-suppressor protein p53 has been proven to play a central role (39, 52, 53) whereas this protein is not even expressed in spermatogonia before irradiation (39).

After exposure to IR, the tumor suppressor p53 is induced in spermatogonia which then can induce spermatogonial apoptosis (39, 52, 53) or perhaps regulate cell cycle arrest. p53 has been discovered almost twenty years ago and appears to be the most prominent protein involved in the response to genotoxic stress in most mitotically dividing cells. Function and regulation of p53 have been extensively reviewed (54, 55), summarized in **Figure 5**.

Although mice deficient for p53 show a high incidence in tumor development, they develop normally and have viable and fertile offspring (56). IR can even induce apoptosis in $p53^{-/-}$ spermatogonia, although most of these cells are more radioresistant than in wild type mice (39, 52). Apparently, there are alternative routes leading to spermatogonial apoptosis. Perhaps, there will even appear to be differences between the spermatogonial subtypes, like the spermatogonial stem cells and the differentiating spermatogonia, due to different apoptotic pathways or different regulation of the cell cycle.

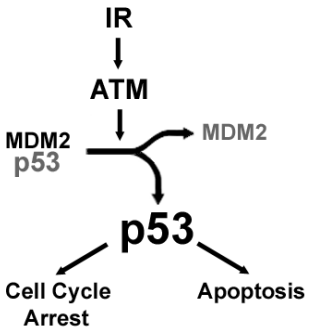


Figure 5. ***p53 signaling in response to DNA damage as induced by ionizing radiation (IR).*** *ATM (ataxia telangiectasia mutated), which belongs to a larger family of phosphatidylinositol 3-kinases also including the catalytic subunit of DNA-PK (DNA-dependent protein kinase) (57, 58) exists in the cell as an inactive dimer (59). In response to radiation induced DSBs the ATM dimer partners cross-phosphorylate each-other and are subsequently released as active, phosphorylated, monomers (59). These active monomers then phosphorylate p53 (60-62). Once phosphorylated, p53 dissociates from the protein MDM2 (63), which normally targets p53 for degradation. Activated p53 then regulates transcription of many genes involved in cell cycle arrest (e.g. p21 (64), 14-3-3σ (65) and cyclinB_1 (66)) and apoptosis (e.g. Bcl2 family proteins (49)).*

REPAIR OF DNA DOUBLE-STRAND BREAKS

Instead of leading to apoptosis, DNA damage can also be repaired. There are several ways in which damaged DNA can be repaired (24-34) of which non-homologous end joining (NHEJ) and homologous recombinational repair (HRR) are the major mechanisms involved in the repair of DSBs **(Figure 6)**.

During NHEJ the broken DNA ends are "simply" re-attached whereas during HRR homologues sister chromatids are used as a template for repair (24-34).

Although less accurate than HRR, NHEJ still predominates during the G_1 phase of the cell cycle when a second copy of the DNA is not yet available (67, 68). Apparently it is difficult to align with the homologous chromosome, maybe due to the complex organization of the genome in the G_1 nucleus. The error-free HRR dominates during the S and G_2 phase when the DNA has been replicated and a sister chromatid is present for aligning with the damaged DNA (67, 68).

In early spermatocytes, being discussed in the next chapter, HRR plays an especially important role as DSBs, required for meiotic recombination, are induced during the first meiotic prophase and resolved by meiotic recombination (1, 16, 69-72).

SPERMATOCYTES

Compared to spermatogonia, spermatocytes are very resistant to IR (43). However, independent of irradiation, some early spermatocytes "spontaneously" undergo apoptosis, mostly at pachytene stage IV (14) **(Figure 3)**. Also further developed spermatocytes can undergo apoptosis and are sensitive for low intra-testicular testosterone levels (15, 17, 18, 73).

In contrast to the radiosensitive spermatogonia, spermatocytes express p53 independent of irradiation (39, 74-76) and p53 has been shown to play a role in regulating apoptosis during the meiotic prophase (73, 77). However, by studying $p53^{-/-}$ testes, it appeared that in spermatocytes p53 is involved in DNA repair rather than apoptosis (78). Indeed, p53 has been found to interact with HRR-proteins RPA (79), RAD51 (80) and Holliday junctions (81) **(Figure 6D)** and even to promote HRR as a whole (82). However, since meiotic recombination proceeds normally in p53 deficient mice (83), its role during the meiotic prophase does not appear to be essential.

Also the chromosome-synapsis-dependent checkpoint, at the meiotic metaphase I, did not appear to depend on functional p53 (84). Still, p53 might be involved in a meiotic checkpoint, perhaps regulated via ATM (85). However, the exact nature of this checkpoint still has to be resolved. Apparently, proteins like p53, although having a comparable function during the mitotic germ cell divisions, can function differently during meiosis. Whatever the function of p53 in the "normal", non-irradiated, testis might be, it appears to be redundant (56, 83, 84).

THE MEIOTIC PROPHASE I

For a major part radiation independent apoptosis of early spermatocytes is probably due to DSBs that are generated early during the first meiotic prophase **(Figure 7)** and are necessary for meiotic recombination (1, 16, 69-72).

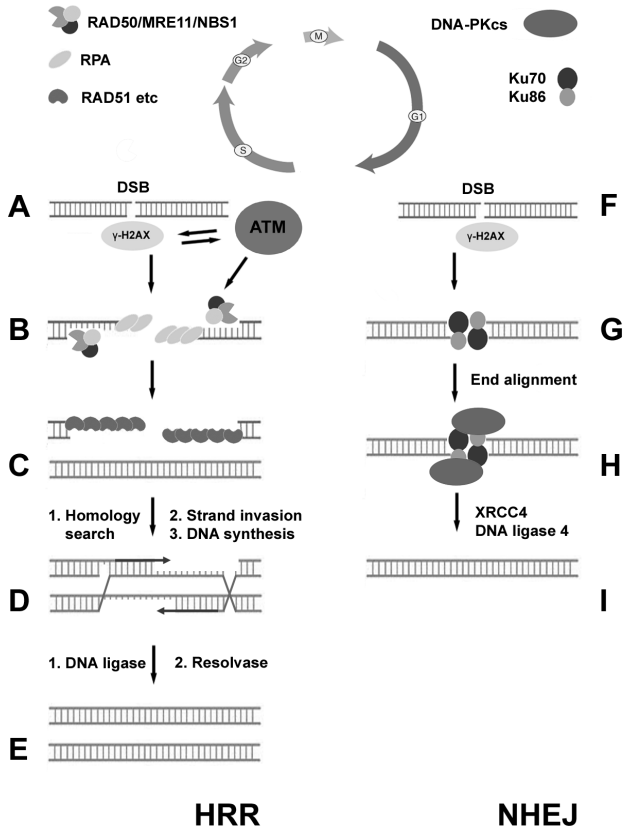


Figure 6. ***Repair of DNA double-strand breaks (DSBs) by homologous recombinational repair (HRR) or non-homologous end joining (NHEJ).*** *One of the first steps in the cellular response to DSBs is phosphorylation of histone H2AX at serine 139 (86, 87), which appears very shortly after irradiation as γ-H2AX (phosphorylated H2AX) foci at the damaged sites containing DSBs (88, 89)* ***(A&F)****. γ-H2AX has a critical function in the sequestration of DNA-repair factors and DNA damage-signaling proteins involved in NHEJ and especially HRR to the DSBs and the formation of the IR induced foci (IRIF) (89-94).*

An important part of NHEJ is carried out by DNA-PK (24-34). DNA-PK consists of a heteromeric DNA binding component, Ku70 and Ku86, and a DNA-PK catalytic subunit (DNA-PKcs), which belongs to a larger family of phosphatidylinositol 3-kinases that also includes ATM (57, 58). The Ku heterodimer binds to DSBs ***(G)*** *followed by binding and activation of DNA-PKcs* ***(H)*** *and subsequently ligation by XRCC4/DNA ligase4* ***(I)*** *(24-34). NHEJ can be very error-prone since it just ligates two blunt ends and can even lead to gain or loss of nucleotides if internal micro-homologies are used to anneal the damaged DNA before sealing (24-34).*

HRR involves the simultaneous action of many proteins which are probably recruited by γ-H2AX ***(A)*** *(87, 89, 90) and appear at DSBs visible as nuclear foci. A possible scenario for HRR could begin with activation of ATM which is necessary for phosphorylation of γ-H2AX* ***(A)*** *(95). ATM exists in the cell as an inactive dimer (59). However, in response to DSBs, the ATM dimer partners cross-phosphorylate each other and are subsequently released as active, phosphorylated, monomers (59) that are capable of interacting with DNA (96). ATM then activates the RAD50/MRE11/NBS1 complex, which has 5'-3'-exonuclease activity and thus exposes both 3'-ends* ***(B)*** *(97). To these single stranded 3'-ends the protein RPA binds* ***(B)*** *followed by assembly of large DNA/protein-complexes* ***(C)*** *that include RAD51 and other members of the RecA/RAD51-family (98, 99). These proteins are needed for the search and identification of the homologous sister chromatid and for proper positioning of the homologues for strand invasion* ***(D)****. After proper strand invasion, the non-damaged sister chromatid-DNA can be used as a template for DNA synthesis after which, finally, all DNA ends are ligated and the so-called Holliday-junctions (100-102) can be resolved* ***(D)****, eventually leading to two non-damaged copies* ***(E)****.*

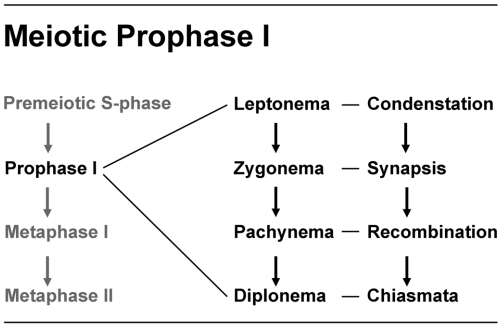


Figure 7. ***The meiotic prophase I*** *can be sub-divided in four stages; during the leptotene stage condensation of the chromosomes takes place, subsequently, during the zygotene stage, the sister-chromatids will align and form the synaptonemal complexes, then, during the pachytene stage, meiotic recombination will occur; later, during the diplotene stage, visible as Chiasmata.*

During this early prophase of meiosis, from leptotene until early pachytene, these breaks often result in activation of proteins, like ATM (103), that are also activated in mitotically dividing cells in response to DNA damage. Like during the mitotic cell cycle, during meiosis these proteins are also involved in DNA repair signaling and interruption often leads to abrogation of spermatogenesis at an early pachytene stage (104-108) at the so called epithelial stage IV meiotic checkpoint **(Figure 1)** (14).

To ensure genome integrity during meiosis, pathways leading to the error prone NHEJ should be suppressed and the DSBs should be repaired by recombination **(Figure 6)**. The meiotic prophase can be compared with the S-G_2 phase of the mitotic cell cycle (109) in which also HRR prevails over NHEJ (67, 68) **(Figure 6)**. However, meiotic recombination is not exactly the same as HRR (110, 111). During the meiotic prophase, inhibition of the error prone NHEJ needs special attention, in order to promote crossing-over and thus maintain genome integrity in the germ line. Therefore it is striking that Ku70, an essential component of the NHEJ pathway **(Figures 6F-I)**, appears to be absent exactly in early spermatocytes in which meiotic recombination takes place (112). The complete DNA-PK (including Ku70 and Ku86) has been shown to suppress homologous recombination (113) and interruption of DNA-PK stimulates homologous recombination (114, 115). Suppression of NHEJ, for instance by down regulation of the Ku proteins during the first meiotic prophase in order to promote meiotic recombination, therefore seems to be a very important event at the start of the first meiotic prophase.

SPERMATIDS

Like the spermatocytes, also the haploid spermatids are very resistant to irradiation (43) but still sometimes undergo apoptosis (17, 18). About the regulation and nature of apoptosis of spermatids very little is known, although DNA damage is not likely to be a major apoptosis inducing factor in these cells.

Before leaving the seminiferous tubules, elongated spermatids pinch of cellular remnants, called the residual bodies, which are no longer needed in matured spermatozoa. Although lacking a nucleus these residual bodies exhibit similar protein contents and morphology as do apoptotic bodies (116) and, like apoptotic germ cells, also the residual bodies are eventually phagocytized by Sertoli cells. Therefore, these processes could also be interpreted as partial apoptosis, leaving the condensed nucleus and proteins needed for maturation and fertilization unharmed.

IN THIS THESIS

In this thesis different germ cell types within the testis and their different responses to irradiation and DSBs are described, compared and discussed. Many proteins, pathways and molecular mechanisms appear to be generally used in all mammalian, or even eukaryotic, cells. However, within the testis, in the different germ cell types and especially during meiosis, proteins, generally involved in the response to DNA damage, appear to be rearranged in a testis specific manner.

In chapter two (117) we describe how spermatogonia, being connected by intercellular bridges, can individually undergo apoptosis in response IR, whereas during density regulation spermatogonia degenerate in interconnected clusters (20, 48).

In chapter three (118) we investigated whether the c-Abl-p73 pathway, and possibly p63, can be an alternative apoptotic pathway as described for p53 deficient mice (39). Therefore we studied the testicular expression patterns and interactions of these proteins before and after irradiation.

In chapter four (119) we describe the expression pattern of γ-H2AX, which is commonly used as a marker for DSBs, in the testis before and after irradiation. Furthermore we investigated whether γ-H2AX signaling is connected with p53 induced spermatogonial apoptosis and whether DNA-PK is required for p53 signaling and γ-H2AX foci formation and signaling during spermatogenesis.

In chapter five (120) we localized the three subunits of DNA-PK; Ku70, Ku86 and DNA-PKcs, in different cell types during spermatogenesis. Additionally, using DNA-PKcs deficient *scid* mice, we investigated the function of DNA-PKcs in the spermatogonial DNA damage response and during meiosis.

Finally, in chapter six, we studied expression and activation of ATM, which lies at the basis of most responses to DSBs, during spermatogenesis. Using an antibody specific for phosphorylated ATM we were able to compare ATM activation in response to IR with the presence of endogenously active ATM during the meiosis.

The different experiments performed, as described in this thesis, will hopefully help to develop an idea about the many aspects of DNA damage signaling and apoptosis during spermatogenesis.

REFERENCES

1. **Lichten M (2001)** Meiotic recombination: breaking the genome to save it *Curr Biol* **11,** R253-6.
2. **Cohen PE & Pollard JW (2001)** Regulation of meiotic recombination and prophase I progression in mammals *Bioessays* **23,** 996-1009.
3. **Tarsounas M & Moens PB (2001)** Checkpoint and DNA-repair proteins are associated with the cores of mammalian meiotic chromosomes *Curr Top Dev Biol* **51,** 109-34.
4. **Gilbert SF (1997)** Developmental Biology *Sinauer Associates, Inc.* **Fifth Edition**.
5. **de Rooij DG, Russell LD. (2000)** All you wanted to know about spermatogonia but were afraid to ask. *J Androl* **21,** 776-98.
6. **Dym M & Fawcett DW (1971)** Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis *Biol Reprod* **4,** 195-215.
7. **Huckins C (1978)** Spermatogonial intercellular bridges in whole-mounted seminiferous tubules from normal and irradiated rodent testes *Am J Anat* **153,** 97-121.
8. **Fawcett DW, Ito S & Slautterback D (1959)** The occurence of intercellular bridges in groups of cells exhibiting synchronous differentiation *J Biophys Cytol* **5,** 453-60.
9. **Fawcett DW (1961)** Intercellular bridges *Exp Cell Res* **8,** 174-87.
10. **Gondos B & Zemjanis R (1970)** Fine structure of spermatogonia and intercellular bridges in Macaca nemestrina *J Morphol* **131,** 431-46.
11. **Ren HP & Russell LD (1991)** Clonal development of interconnected germ cells in the rat and its relationship to the segmental and subsegmental organization of spermatogenesis *Am J Anat* **192,** 121-8.
12. **Russell LD, Ettlin RA, Hikim APS & Clegg ED (1990)** Histological and histopathological evaluation of the testis *Cache River Press, Clearwater, Fl. USA*.
13. **Oakberg EF (1956)** A description of spermiogenesis in the mouse and its use in analysis of the seminiferous epithelium and germ cell renewal *Am J Anat* **99,** 391-414.
14. **Ashley T (2002)** Multiple guardians of the epithelial stage IV meiotic checkpoint. In: Testicular Tangrams *Springer-Verlag, Berlin Heidelberg New York* **Ernst Schering Research Foundation Workshop Supplement 9,** 1-18.
15. **Print CG & Loveland KL (2000)** Germ cell suicide: new insights into apoptosis during spermatogenesis *Bioessays* **22,** 423-30.
16. **Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM & Burgoyne PS (2001)** Recombinational DNA double-strand breaks in mice precede synapsis *Nat Genet* **27,** 271-6.
17. **Dunkel L, Hirvonen V & Erkkilä K (1997)** Clinical aspects of male germ cell apoptosis during testis development and spermatogenesis *Cell Death Differ* **4,** 171- 79.
18. **Sinha Hikim AP & Swerdloff RS (1999)** Hormonal and genetic control of germ cell apoptosis in the testis *Rev Reprod* **4,** 38-47.
19. **Tapanainen JS, Tilly JL, Vihko KK & Hsueh AJ (1993)** Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors *Mol Endocrinol* **7,** 643-50.
20. **Huckins C (1978)** The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium *Anat Rec* **190,** 905-26.
21. **Huckins C & Oakberg EF (1978)** Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules, I. The normal testes *Anat Rec* **192,** 519-28.
22. **Nunez MI, McMillan TJ, Valenzuela MT, Ruiz de Almodovar JM & Pedraza V (1996)** Relationship between DNA damage, rejoining and cell killing by radiation in mammalian cells *Radiother Oncol* **39,** 155-65.
23. **Powell S & McMillan TJ (1990)** DNA damage and repair following treatment with ionizing radiation *Radiother Oncol* **19,** 95-108.
24. **Dasika GK, Lin SC, Zhao S, Sung P, Tomkinson A & Lee EY (1999)** DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis *Oncogene* **18,** 7883-99.
25. **Haber JE (2000)** Partners and pathways repairing a double-strand break *Trends Genet* **16,** 259-64.
26. **van Gent DC, Hoeijmakers JH & Kanaar R (2001)** Chromosomal stability and the DNA double-stranded break connection *Nat Rev Genet* **2,** 196-206.
27. **Hoeijmakers JH (2001)** Genome maintenance mechanisms for preventing cancer *Nature* **411,** 366-74.
28. **Khanna KK & Jackson SP (2001)** DNA double-strand breaks: signaling, repair and the cancer connection *Nat Genet* **27,** 247-54.
29. **Sekiguchi M (2002)** DNA repair, Review Issue 58 *Oncogene Reviews* **21,** 8885-9056.
30. **Bernstein C, Bernstein H, Payne CM & Garewal H (2002)** DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis *Mutat Res* **511,** 145-78.
31. **Rouse J & Jackson SP (2002)** Interfaces between the detection, signaling, and repair of DNA damage *Science* **297,** 547-51.
32. **Friedberg EC (2003)** DNA damage and repair *Nature* **421,** 436-40.

33. **Alberts B (2003)** DNA replication and recombination *Nature* **421,** 431-5.
34. **Cline SD & Hanawalt PC (2003)** Who's on first in the cellular response to DNA damage? *Nat Rev Mol Cell Biol* **4,** 361-73.
35. **Beumer TL, Kiyokawa H, Roepers-Gajadien HL, van den Bos AC, Lock TM, Gademan IS, Rutgers DH, Koff A & de Rooij DG (1999)** Regulatory role of p27(Kip1) in the mouse and human testis *Endocrinology* **140,** 1834-40.
36. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG (2000)** Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse *Biol Reprod* **63,** 1893-8.
37. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB & de Rooij DG (2000)** Apoptosis regulation in the testis: Involvement of Bcl-2 family members *Mol Reprod Dev* **56,** 353-59.
38. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Rutgers DH & de Rooij DG (1997)** p21(Cip1/WAF1) expression in the mouse testis before and after X irradiation *Mol Reprod Dev* **47,** 240-7.
39. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
40. **Bootsma AL & Davids JA (1988)** The cell cycle of spermatogonial colony forming stem cells in the CBA mouse after neutron irradiation *Cell Tissue Kinet* **21,** 105-13.
41. **van Buul PP, van Duyn-Goedhart A, Beumer T & Bootsma AL (2001)** Role of cell cycle control in radiosensitization of mouse spermatogonial stem cells *Int J Radiat Biol* **77,** 357-63.
42. **van Buul PP & van Duijn-Goedhart A (2002)** Enhanced recovery of radiation-induced translocations from spermatogonial stem cells of p53 null mice *Mutat Res* **508,** 29-32.
43. **Oakberg EF & DiMinno RL (1960)** X-ray sensitivity of primary spermatocytes of the mouse *Int J Radiat Biol* **2,** 196-209.
44. **Nigg EA (2001)** Mitotic kinases as regulators of cell division and its checkpoints *Nat Rev Mol Cell Biol* **2,** 21-32.
45. **Malumbres M & Barbacid M (2001)** To cycle or not to cycle: a critical decision in cancer *Nat Rev Cancer* **1,** 222-31.
46. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia *Radiat Res* **130,** 296-302.
47. **van Beek ME, Davids JA, van de Kant HJ & de Rooij DG (1984)** Response to fission neutron irradiation of spermatogonial stem cells in different stages of the cycle of the seminiferous epithelium *Radiat Res* **97,** 556-69.
48. **de Rooij DG & Lok D (1987)** Regulation of the density of spermatogonia in the seminiferous epithelium of the Chinese hamster: II. Differentiating spermatogonia *Anat Rec* **217,** 131-6.
49. **Cory S & Adams JM (2002)** The Bcl2 family: regulators of the cellular life-or-death switch *Nat Rev Cancer* **2,** 647-56.
50. **Knudson CM, Tung KS, Tourtellotte WG, Brown GA & Korsmeyer SJ (1995)** Bax-deficient mice with lymphoid hyperplasia and male germ cell death *Science* **270,** 96-9.
51. **Russell LD, Chiarini-Garcia H, Korsmeyer SJ & Knudson CM (2002)** Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis *Biol Reprod* **66,** 950-8.
52. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
53. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
54. **Levine AJ (1997)** p53, the cellular gatekeeper for growth and division *Cell* **88,** 323-31.
55. **Jenkins JR & Banks LM (1999)** p53, Review Issue 5 *Oncogene Reviews* **18,** 7617-717.
56. **Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS & Bradley A (1992)** Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours *Nature* **356,** 215-21.
57. **Smith GC & Jackson SP (1999)** The DNA-dependent protein kinase *Genes Dev* **13,** 916-34.
58. **Durocher D & Jackson SP (2001)** DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr Opin Cell Biol* **13,** 225-31.
59. **Bakkenist CJ & Kastan MB (2003)** DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation *Nature* **421,** 499-506.
60. **Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB & Siliciano JD (1998)** Activation of the ATM kinase by ionizing radiation and phosphorylation of p53 *Science* **281,** 1677-9.
61. **Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP & Lavin MF (1998)** ATM associates with and phosphorylates p53: mapping the region of interaction *Nat Genet* **20,** 398-400.
62. **Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y & Ziv Y (1998)** Enhanced phosphorylation of p53 by ATM in response to DNA damage *Science* **281,** 1674-7.

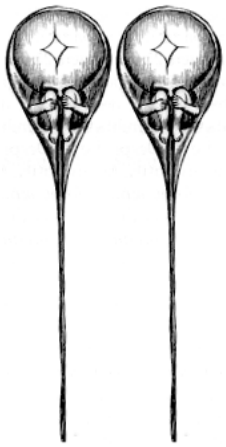
63. **Momand J, Wu HH & Dasgupta G (2000)** MDM2--master regulator of the p53 tumor suppressor protein *Gene* **242,** 15-29.
64. **el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW & Vogelstein B (1993)** WAF1, a potential mediator of p53 tumor suppression *Cell* **75,** 817-25.
65. **Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW & Vogelstein B (1997)** 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression *Mol Cell* **1,** 3-11.
66. **Innocente SA, Abrahamson JL, Cogswell JP & Lee JM (1999)** p53 regulates a G2 checkpoint through cyclin B1 *Proc Natl Acad Sci USA* **96,** 2147-52.
67. **Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A & Takeda S (1998)** Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells *Embo J* **17,** 5497-508.
68. **Yoshida M, Hosoi Y, Miyachi H, Ishii N, Matsumoto Y, Enomoto A, Nakagawa K, Yamada S, Suzuki N & Ono T (2002)** Roles of DNA-dependent protein kinase and ATM in cell-cycle-dependent radiation sensitivity in human cells *Int J Radiat Biol* **78,** 503-12.
69. **Keeney S (2001)** Mechanism and control of meiotic recombination initiation *Curr Top Dev Biol* **52,** 1-53.
70. **Baudat F, Manova K, Yuen JP, Jasin M & Keeney S (2000)** Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11 *Mol Cell* **6,** 989-98.
71. **Romanienko PJ & Camerini-Otero RD (2000)** The mouse Spo11 gene is required for meiotic chromosome synapsis *Mol Cell* **6,** 975-87.
72. **Keeney S, Giroux CN & Kleckner N (1997)** Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family *Cell* **88,** 375-84.
73. **Yin Y, Stahl BC, DeWolf WC & Morgentaler A (1998)** p53-mediated germ cell quality control in spermatogenesis *Dev Biol* **204,** 165-71.
74. **Almon E, Goldfinger N, Kapon A, Schwartz D, Levine AJ & Rotter V (1993)** Testicular tissue-specific expression of the p53 suppressor gene *Dev Biol* **156,** 107-16.
75. **Schwartz D, Goldfinger N & Rotter V (1993)** Expression of p53 protein in spermatogenesis is confined to the tetraploid pachytene primary spermatocytes *Oncogene* **8,** 1487-94.
76. **Sjöblom T & Lähdetie J (1996)** Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p53 in meiotic recombination and repair *Oncogene* **12,** 2499-505.
77. **Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, Donehower LA & Levine AJ (1993)** Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome *Proc Natl Acad Sci USA* **90,** 9075-9.
78. **Schwartz D, Goldfinger N, Kam Z & Rotter V (1999)** p53 controls low DNA damage-dependent premeiotic checkpoint and facilitates DNA repair during spermatogenesis *Cell Growth Differ* **10,** 665-75.
79. **Dutta A, Ruppert JM, Aster JC & Winchester E (1993)** Inhibition of DNA replication factor RPA by p53 *Nature* **365,** 79-82.
80. **Sturzbecher HW, Donzelmann B, Henning W, Knippschild U & Buchhop S (1996)** p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction *Embo J* **15,** 1992-2002.
81. **Lee S, Cavallo L & Griffith J (1997)** Human p53 binds Holliday junctions strongly and facilitates their cleavage *J Biol Chem* **272,** 7532-9.
82. **Wiesmuller L, Cammenga J & Deppert WW (1996)** In vivo assay of p53 function in homologous recombination between simian virus 40 chromosomes *J Virol* **70,** 737-44.
83. **Gersten KM & Kemp CJ (1997)** Normal meiotic recombination in p53-deficient mice *Nat Genet* **17,** 378-9.
84. **Odorisio T, Rodriguez TA, Evans EP, Clarke AR & Burgoyne PS (1998)** The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis *Nat Genet* **18,** 257-61.
85. **Barlow C, Liyanage M, Moens PB, Deng CX, Ried T & Wynshaw-Boris A (1997)** Partial rescue of the prophase I defects of ATM-deficient mice by p53 and p21 null alleles *Nat Genet* **17,** 462-6.
86. **Rogakou EP, Pilch DR, Orr AH, Ivanova VS & Bonner WM (1998)** DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139 *J Biol Chem* **273,** 5858-68.
87. **Modesti M & Kanaar R (2001)** DNA repair: spot(light)s on chromatin *Curr Biol* **11,** R229-32.
88. **Rogakou EP, Boon C, Redon C & Bonner WM (1999)** Megabase chromatin domains involved in DNA double-strand breaks in vivo *J Cell Biol* **146,** 905-16.
89. **Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M & Bonner WM (2000)** A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage *Curr Biol* **10,** 886-95.
90. **Rappold I, Iwabuchi K, Date T & Chen J (2001)** Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways *J Cell Biol* **153,** 613-20.
91. **Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K & Bonner W (2002)** Histone H2A variants H2AX and H2AZ *Curr Opin Genet Dev* **12,** 162-9.
92. **Bassing CH, Chua KF, Sekiguchi J, Suh H, Whitlow SR, Fleming JC, Monroe BC, Ciccone DN, Yan C, Vlasakova K, Livingston DM, Ferguson DO, Scully R & Alt FW (2002)** Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX *Proc Natl Acad Sci USA* **99,** 8173-8.

93. **Hunter N, Valentin Borner G, Lichten M & Kleckner N (2001)** γ-H2AX illuminates meiosis *Nat Genet* **27,** 236-8.
94. **Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, Bonner RF, Bonner WM & Nussenzweig A (2003)** Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks *Nat Cell Biol* **5,** 675-9.
95. **Burma S, Chen BP, Murphy M, Kurimasa A & Chen DJ (2001)** ATM phosphorylates histone H2AX in response to DNA double-strand breaks *J Biol Chem* **276,** 42462-7.
96. **Llorca O, Rivera-Calzada A, Grantham J & Willison KR (2003)** Electron microscopy and 3D reconstructions reveal that human ATM kinase uses an arm-like domain to clamp around double-stranded DNA *Oncogene* **22,** 3867-74.
97. **Petrini JH (2000)** The MRE11 complex and ATM: collaborating to navigate S phase *Curr Opin Cell Biol* **12,** 293-6.
98. **Essers J, Houtsmuller AB, van Veelen L, Paulusma C, Nigg AL, Pastink A, Vermeulen W, Hoeijmakers JH & Kanaar R (2002)** Nuclear dynamics of RAD52 group homologous recombination proteins in response to DNA damage *Embo J* **21,** 2030-7.
99. **Gasior SL, Olivares H, Ear U, Hari DM, Weichselbaum R & Bishop DK (2001)** Assembly of RecA-like recombinases: distinct roles for mediator proteins in mitosis and meiosis *Proc Natl Acad Sci USA* **98,** 8411-8.
100. **Schwacha A & Kleckner N (1995)** Identification of double Holliday junctions as intermediates in meiotic recombination *Cell* **83,** 783-91.
101. **Holliday R (1964)** A mechanism for gene conversion in Fungi *Genet Res* **5,** 282-304.
102. **Hunter N & Kleckner N (2001)** The single-end invasion: an asymmetric intermediate at the double-strand break to double-holliday junction transition of meiotic recombination *Cell* **106,** 59-70.
103. **Plug AW, Peters AH, Xu Y, Keegan KS, Hoekstra MF, Baltimore D, de Boer P & Ashley T (1997)** ATM and RPA in meiotic chromosome synapsis and recombination *Nat Genet* **17,** 457-61.
104. **Barlow C, Liyanage M, Moens PB, Tarsounas M, Nagashima K, Brown K, Rottinghaus S, Jackson SP, Tagle D, Ried T & Wynshaw-Boris A (1998)** ATM deficiency results in severe meiotic disruption as early as leptonema of prophase I *Development* **125,** 4007-17.
105. **Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC & Nussenzweig A (2002)** Genomic instability in mice lacking histone H2AX *Science* **296,** 922-7.
106. **de Vries SS, Baart EB, Dekker M, Siezen A, de Rooij DG, de Boer P & te Riele H (1999)** Mouse MutS-like protein MSH5 is required for proper chromosome synapsis in male and female meiosis *Genes Dev* **13,** 523-31.
107. **Pittman DL, Cobb J, Schimenti KJ, Wilson LA, Cooper DM, Brignull E, Handel MA & Schimenti JC (1998)** Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for DMC1, a germline-specific RecA homolog *Mol Cell* **1,** 697-705.
108. **Yoshida K, Kondoh G, Matsuda Y, Habu T, Nishimune Y & Morita T (1998)** The mouse RecA-like gene Dmc1 is required for homologous chromosome synapsis during meiosis *Mol Cell* **1,** 707-18.
109. **van Buul PP, Abramsson-Zetterberg L, Zandman IM & van Duyn-Goedhart A (1998)** Further characterization of the radiosensitivity of the scid mouse *Int J Radiat Biol* **74,** 35-41.
110. **Allers T & Lichten M (2001)** Differential timing and control of noncrossover and crossover recombination during meiosis *Cell* **106,** 47-57.
111. **Petronczki M, Siomos MF & Nasmyth K (2003)** Un Menage a Quatre. The Molecular Biology of Chromosome Segregation in Meiosis *Cell* **112,** 423-40.
112. **Goedecke W, Eijpe M, Offenberg HH, van Aalderen M & Heyting C (1999)** MRE11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis *Nat Genet* **23,** 194-8.
113. **Allen C, Kurimasa A, Brenneman MA, Chen DJ & Nickoloff JA (2002)** DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination *Proc Natl Acad Sci USA* **99,** 3758-63.
114. **Delacote F, Han M, Stamato TD, Jasin M & Lopez BS (2002)** An XRCC4 defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells *Nucleic Acids Res* **30,** 3454-63.
115. **Pierce AJ, Hu P, Han M, Ellis N & Jasin M (2001)** Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells *Genes Dev* **15,** 3237-42.
116. **Blanco-Rodriguez J & Martinez-Garcia C (1999)** Apoptosis is physiologically restricted to a specialized cytoplasmic compartment in rat spermatids *Biol Reprod* **61,** 1541-7.
117. **Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG (2003)** Intercellular bridges and apoptosis in clones of male germ cells *Int J Androl* **in press**.
118. **Hamer G, Gademan IS, Kal HB & de Rooij DG (2001)** Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.
119. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP & de Rooij DG (2003)** DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.

120. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T & de Rooij DG (2003)** Function of DNA-PKcs during the early meiotic prophase without Ku70 and Ku86 *Biol Reprod* **68,** 717-21.

INTERCELLULAR BRIDGES & APOPTOSIS IN CLONES OF MALE GERM CELLS

Geert Hamer[1,2], Hermien L. Roepers-Gajadien[1,2], Iris S. Gademan[3], Henk B. Kal[3] & Dirk G. de Rooij[1,2]



[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*
[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*
[3] *Department of Radiotherapy, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG (2003) Intercellular bridges and apoptosis in clones of male germ cells *Int J Androl* **in press**.

When an A_s spermatogonium divides to form a pair of A_{pr} spermatogonia the two daughter cells stay interconnected by an intercellular bridge. These cytoplasmic bridges form after every subsequent division leading to large clones of interconnected germ cells. Cohorts of spermatogonia maintain synchronous development throughout spermatogenesis, which has been attributed to the presence of these intercellular bridges. To examine whether apoptotic signals are transduced through the intercellular bridges we studied germ cell apoptosis in whole mounts of seminiferous tubules from non-irradiated and irradiated mouse testes, using whole mount seminiferous tubules and confocal microscopy. This allowed us to use TUNEL staining of apoptotic germ cells and at the same time to study these apoptotic germ cells in their topographical context. Our results show that in response to ionizing radiation (IR) single spermatogonia within a clone can undergo apoptosis without affecting their neighbouring cells. Additionally, also early spermatocytes were shown to undergo apoptosis individually. Both radiation-induced spermatogonial apoptosis and spontaneous apoptosis of spermatocytes are caused by DNA damage of individual cells. Degeneration of healthy spermatogonia due to regulatory signals, however, follows other death inducing mechanisms, which lead to apoptosis of chains of interconnected spermatogonia.

INTRODUCTION

During spermatogenesis germ cells undergo many mitotic divisions and meiosis but stay connected by intercellular bridges thus theoretically leading to thousands of cytoplasmically connected spermatids at the end of the process (1, 2). It is widely assumed that these bridges form cytoplasmic channels allowing open communication between germ cells within a clone (3-5). Germ cells within such a clone differentiate synchronously suggesting that maintenance of synchronous development is a major function of the intercellular bridges (2-6).

In the adult mouse testis the mitotically active spermatogonia are the most sensitive to ionizing radiation (IR) whereas spermatocytes, that undergo meiotic cell divisions, and spermatids, that develop into spermatozoa, are more resistant (7-11). In response to ionizing irradiation the tumor suppressor p53 is induced. This protein plays a central role in DNA-damage induced spermatogonial apoptosis (12-14). Also, in the cytoplasm proteins are activated in response to irradiation, like the non-receptor tyrosine kinase c-Abl and the p53 homologue p73 (15). These and other factors might diffuse through the intercellular bridges, thereby inducing apoptosis of neighbouring cells within a clone. This concept is supported by reports that spermatogonia in both normal and irradiated testes degenerate in clusters (2, 16).

We now studied whole mounts of seminiferous tubules (17) using confocal laser scanning microscopy (CLSM) allowing us to study the topography of germ cells together with a fluorescent staining of apoptotic germ cells. It was studied whether germ cells can individually undergo apoptosis or inevitably transduce the apoptotic signal to other cells of the clone. For this purpose whole mounts of seminiferous tubules were prepared from sham-irradiated testes and at various time intervals after doses of 1 or 4 Gy of X-rays.

MATERIALS & METHODS

Animals, irradiation and preparation of whole mounts of seminiferous tubules

The testes of groups of 4 male FvB/NAU mice of at least 7 weeks of age were given doses of 1 or 4 Gy of 200 kV X-rays as described previously (12, 15). Three, 12 or 24 h after irradiation the mice were sacrificed by cervical dislocation. Whole mounts of seminiferous tubules were prepared according to the method of Clermont and Buston-Obregon (17) and fixed for 1 h in 4% phosphate buffered (pH 6.6-7.2) formaldehyde, washed and stored in 70% alcohol. For negative controls the animals were sham-irradiated. The animals were used and maintained according to regulations provided by the animal ethical committee of the University Medical Center Utrecht that also approved the experiments.

TUNEL analysis and confocal microscopy

TUNEL analysis was performed on whole mounts of seminiferous tubules with some adaptations to the manufacturers protocol (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Mannheim, Germany). The tubules were washed in mQ and PBS prior to a 10 min incubation in 3% H_2O_2 in methanol. After washing in PBS the tubules were incubated in 1% Triton X-100 for 1 h. Again the tubules were washed in PBS before a 2 h incubation in diluted (1:4) TUNEL mix (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Mannheim, Germany) at 37^0C. Finally, in between washing with PBS, the tubules were counterstained for 10 min using propidium iodide (1:500 in PBS) and embedded in Vectashield (Vector Laboratories, Burlingame, CA, USA) and viewed with a 63X plan-apo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Images were recorded digitally.

RESULTS

CLSM analysis of whole mounts of seminiferous tubules

Using testis sections it is not possible to examine the topographical arrangement of interconnected germ cells. For this purpose, microscopy on whole mounts of seminiferous tubules has proven to be the most useful technique (17, 18). Although highly suitable for morphological studies, whole mounts of tubules allow only limited possibilities for immunohistochemistry or *in situ* hybridization. To circumvent this, we used red fluorescent propidium iodide to stain the nuclei instead of hematoxilin allowing the tubules to be viewed with the CLSM. With this technique we were able to both scan through the tubules and focus on every desired plane in order to analyze the topographical arrangement of the germ cells **(Figure 1)** as well as to perform a whole mount green fluorescent TUNEL assay **(Figure 2)**. As a result we were able to examine the apoptotic behaviour of the interconnected germ cells within the seminiferous tubules.

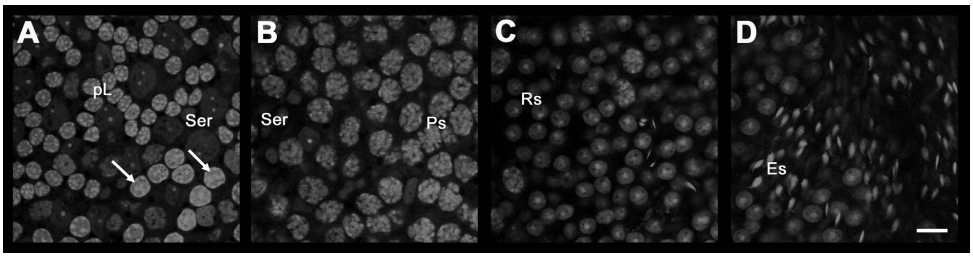


Figure 1. ***Series of confocal scans of a whole mount seminiferous tubule from the basal membrane up to the lumen,*** *showing spermatogonia (arrows), preleptotene spermatocytes (pL) and Sertoli cells (Ser)* ***(A)****, early spermatocytes (Ps)* ***(B)****, round spermatids (Rs)* ***(C)*** *and elongated spermatids (Es)* ***(D)****. Magnification: bar represents 20 µm.*

12 h after a dose of 4 Gy

In response to a dose of 4 Gy of X-rays spermatogonial apoptosis is optimally visible 12 h after irradiation (12). As expected also in the whole mount TUNEL assay many spermatogonia were TUNEL positive 12 h after a dose of 4 Gy, and were often already in the process of apoptotic degradation **(Figure 2A)**. These TUNEL positive apoptotic cells were at the basal membrane of the tubules as previously shown on sections (12). This could also be visualized by projecting sequential scans through the tubules in a XZ or YZ plane **(Figure 2A)**, although conventional cross sections have a far better resolution and morphology (12). Since so many spermatogonia become apoptotic after a dose of 4 Gy (12) it was not possible to conclude whether the apoptosis inducing signals were transduced from one cell to another. Most likely, the individual cells within apoptotic clones were all lethally "hit" and subsequently underwent apoptosis.

12 h after a dose of 1 Gy

When a dose of 1 Gy instead of 4 Gy was used not all spermatogonia were lethally damaged but only a random population of these cells (19). This did enable us to study whether the apoptotic signal was transduced through the intercellular bridges. If the apoptotic signal spreads through intercellular bridges one expects to find groups of TUNEL positive spermatogonia rather than individual apoptotic spermatogonia. However, 12 h after a dose of 1 Gy many single spermatogonia were TUNEL stained. Some of these spermatogonia could be observed being engulfed by Sertoli cells **(Figure 2B)**. A Sertoli cell that phagocytized an apoptotic spermatogonium stained green fluorescent due to uptake of the TUNEL positive cell **(Figure 2B)**.

3 and 24 h after a dose of 1 Gy

In order to study degeneration of apoptotic spermatogonia we also looked 3 and 24 h after a dose of 1 Gy. After 24 h the tubules mostly looked as if they were prepared from sham-irradiated testes although sometimes we observed a single degenerating cell that did not appear in the sham-irradiated testis **(Figure 2E)**. After 3 h many single spermatogonia were TUNEL positive and still recognizable as spermatogonia **(Figures 2C&D)**. At all time points after a dose of 1 Gy, mostly single apoptotic spermatogonia, also within clones, could be observed whereas hardly any chains of connected cells were found to be TUNEL positive **(Figures 2B-E)**.

Sham-irradiated testes

In sham-irradiated tubules only few apoptotic spermatogonia could be found **(Figure 2F)**. However, when present, it concerned large degenerating apoptotic clusters of spermatogonia **(Figure 2G)**. All metaphase germ cells were also found to be TUNEL positive **(Figure 2H)** but this was considered to be an artifact since we did not observe TUNEL negative germ cells at the metaphase stage.

Apoptosis of spermatocytes occurred independent of irradiation **(Figure 2I)**. Since spermatocytes are also interconnected one could expect apoptosis of clusters of spermatocytes. However, spontaneous apoptosis of individual spermatocytes did not always affect the neighbouring spermatocytes **(Figure 2I)** indicating that the apoptotic signal was not necessarily transduced to interconnected neighbouring spermatocytes.

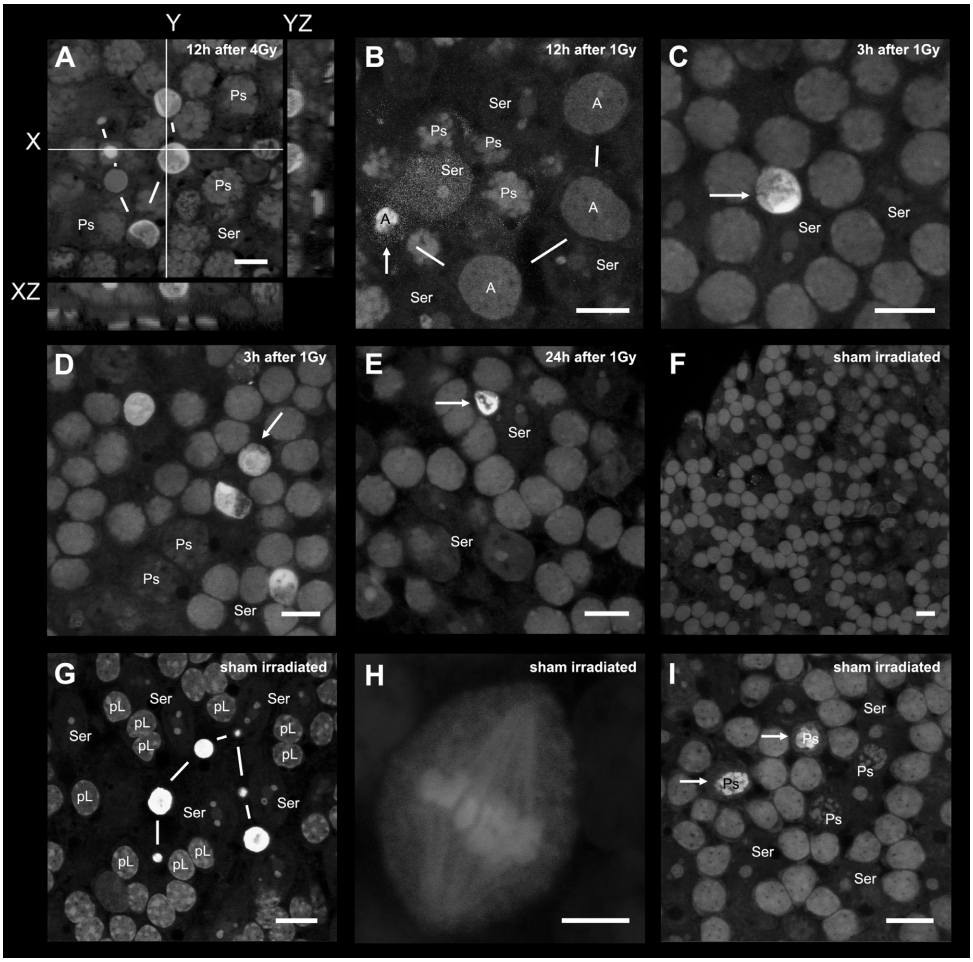


Figure 2. ***TUNEL analysis of whole mounts of seminiferous tubules.*** *12 h after a dose of 4 Gy complete spermatogonial chains are apoptotic* ***(A)****, whereas 12 h after a dose of 1 Gy mostly single spermatogonia are TUNEL positive (arrow)* ***(B)****. These TUNEL positive apoptotic cells lay at the basal membrane of the tubules as previously shown on sections (12) and visualized by projecting sequential scans through the tubules in a XZ or YZ plane* ***(A)****. After 12 h single Sertoli cells (Ser) were observed that phagocytized the apoptotic germ cells* ***(B)****. Also 3 h after a dose 1 Gy many single apoptotic spermatogonia exist within clones of unaffected spermatogonia although not as degenerated as after 12 h (arrows)* ***(C&D)****. 24 h after a dose of 1 Gy these single cells (arrow) are mostly degraded but still did not affect their neighbouring cells* ***(E)****. In the sham-irradiated testis TUNEL positive spermatogonia were very rare* ***(F)****, but were invariably found in apoptotic clusters* ***(G)****. Also cells at the metaphase stage* ***(H)*** *and apoptotic spermatocytes (arrows)* ***(I)*** *are TUNEL positive. Magnification: bar represents 20 µm or 5 µm* ***(H)****. Further depicted are: Sertoli cells (Ser), A spermatogonia (A), preleptotene spermatocytes (pL), pachytene spermatocytes (Ps).*

DISCUSSION

Using confocal laser scanning microscopy and the TUNEL assay we studied germ cell apoptosis in whole mounts of seminiferous tubules from irradiated and sham-irradiated testes.

The sham-irradiated tubules were mostly devoid of TUNEL stained spermatogonia, but the TUNEL positive spermatogonia we did find in these tubules were always present as interconnected chains of apoptotic cells. In the testis, apoptosis of spermatogonia is necessary for the regulation of germ cell density (16, 20). The number of A_1 spermatogonia varies considerably between individual animals and even between different tubule areas whereas the numbers of B spermatogonia and leptotene spermatocytes are very constant (16, 20), suggesting that the number of degenerating spermatogonia is dependent on the initial density of A_1 spermatogonia. Indeed no regulation of germ cell density has been found of undifferentiated spermatogonia (21) and apoptosis of A_1-A_3 spermatogonia has been shown to begin when spermatogonia start differentiating (20). These and the present results indicate that apoptosis necessary for germ cell density regulation occurs in whole, or at least in large parts of, clones of differentiating spermatogonia.

Chains of interconnected apoptotic spermatogonia are present in tubule areas that also contain chains of non-apoptotic spermatogonia **(Figure 2G)** (16, 20). This suggests that it is not the environment that causes apoptosis of spermatogonia within a certain tubular area but that individual clones enter apoptosis. Since clones of A_1-A_3 spermatogonia consist of many cells and cover large areas within the seminiferous tubules it seems likely that apoptosis is induced by a local stimulus and then conveyed through the intercellular bridges to other members of the spermatogonial clone.

In contrast, after a dose of 1 Gy of X-rays, many single apoptotic spermatogonia could be observed within clones of interconnected spermatogonia whereas, like in the sham-irradiated testes, there were only very few TUNEL positive clones. One could argue that these single TUNEL positive cells were A_s spermatogonia. However, within the pool of spermatogonia these are the least radiosensitive (9). Therefore we conclude that in response to a low dose of radiation single spermatogonia undergo apoptosis without affecting the interconnected cells within the spermatogonial clone. This conclusion is strongly supported by the appearance of odd numbered clones in response to a low dose of IR (19).

When Huckins studied spermatogonial apoptosis in whole mounts of seminiferous tubules, apoptotic chains of spermatogonia were found in both the non-irradiated and irradiated testes (2, 16). This seems to contradict with the present results showing single apoptotic cells after irradiation. However, the dose of X-rays used by Huckins (2) was reported to kill the entire spermatogonial population, except the A_s spermatogonia, much like the effects of a dose of 4 Gy in the present study. In that case all spermatogonia were likely lethally damaged by the irradiation resulting in seemingly clonal apoptosis. While in response to a dose of 4 Gy almost all spermatogonia underwent apoptosis, a dose of 1 Gy induced random apoptosis of single spermatogonia without affecting the neighbouring cells within the clone.

Independent of irradiation, we also observed single apoptotic spermatocytes. Clearly, also the apoptotic pathway induced in spermatocytes does not lead to the conveyance of apoptosis to interconnected cells. Apoptosis of spermatocytes occurs predominantly during the early pachytene stage (22-24) when double-stranded DNA breaks are generated during meiotic synapsis and recombination (25). Apoptosis of early spermatocytes probably occurs when these breaks are not sufficiently repaired. Whereas also radiation-induced apoptosis is predominantly induced by DNA-damage (26), apoptosis of A_1-A_3 spermatogonia in the non-irradiated testis is most likely related to cell-numbers (16, 20) and probably involves apoptosis of healthy cells without DNA-damage.

The difference in apoptotic behaviour between density regulated apoptosis and DNA-damage induced apoptosis will partly be due to different molecular pathways both processes follow. In the non-irradiated testis, p53 is already lightly expressed in spermatocytes (12, 27) but not in spermatogonia, which express p53 only after irradiation (12). p53 plays a central role in DNA-damage induced spermatogonial apoptosis (12-14). However, spermatocytes with DNA damage acquired during meiotic synapsis and recombination probably follow a different apoptotic mechanism that appears to be p53 independent (28, 29). In contrast, spermatogonial degeneration during germ cell density regulation has been proven to depend on the Bcl-2 family member Bax (30, 31), which has been shown not to be involved in DNA-damage induced apoptosis of germ cells (30, 32).

The difference in apoptotic behaviour between irradiated and non-irradiated spermatogonia might also have another interesting explanation. After irradiation the damaged spermatogonia undergo apoptosis only as they try to divide (9, 10). During cell divisions 8-12 transient parallel cisternae appear perpendicular to the long axis of the intercellular bridges (1, 33). These structures block the cytoplasmic continuum (33) and thus would isolate apoptotic spermatogonia from their interconnected neighbours, allowing them to individually undergo apoptosis. When the intercellular bridges are open at the time of induction of apoptosis this could result in clonal spermatogonial degeneration. This explanation, however, does not apply to spermatocytes since the intercellular bridges between these cells do not close until the actual meiotic division and are open at the time of apoptosis (33).

In conclusion, germ cell density regulation related apoptosis occurs in a clonal fashion. In contrast, radiation induced spermatogonial apoptosis and apoptosis of spermatocytes concerns individual cells within a clone. Apparently, although these cells are interconnected to neighbouring sister cells within the clone, it still remains possible to remove individual cells without affecting the rest of the clone.

REFERENCES

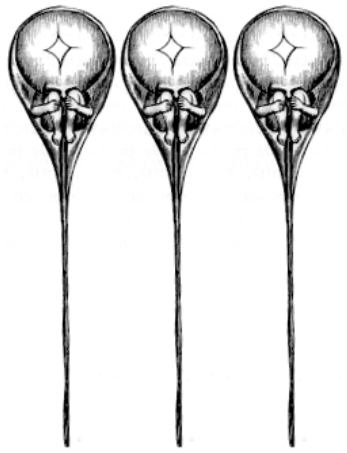
1. **Dym M & Fawcett DW (1971)** Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis *Biol Reprod* **4,** 195-215.
2. **Huckins C (1978)** Spermatogonial intercellular bridges in whole-mounted seminiferous tubules from normal and irradiated rodent testes *Am J Anat* **153,** 97-121.
3. **Fawcett DW, Ito S & Slautterback D (1959)** The occurence of intercellular bridges in groups of cells exhibiting synchronous differentiation *J Biophys Cytol* **5,** 453-60.
4. **Fawcett DW (1961)** Intercellular bridges *Exp Cell Res* **8,** 174-87.
5. **Gondos B & Zemjanis R (1970)** Fine structure of spermatogonia and intercellular bridges in Macaca nemestrina *J Morphol* **131,** 431-46.
6. **Ren HP & Russell LD (1991)** Clonal development of interconnected germ cells in the rat and its relationship to the segmental and subsegmental organization of spermatogenesis *Am J Anat* **192,** 121-8.
7. **Oakberg EF & DiMinno RL (1960)** X-ray sensitivity of primary spermatocytes of the mouse *Int J Radiat Biol* **2,** 196-209.
8. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Rutgers DH & de Rooij DG (1997)** p21(Cip1/WAF1) expression in the mouse testis before and after X irradiation *Mol Reprod Dev* **47,** 240-7.
9. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia *Radiat Res* **130,** 296-302.
10. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X irradiation *Radiat Res* **130,** 289-95.
11. **Hasegawa M, Wilson G, Russell LD & Meistrich ML (1997)** Radiation-induced cell death in the mouse testis: relationship to apoptosis *Radiat Res* **147,** 457-67.
12. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
13. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
14. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
15. **Hamer G, Gademan IS, Kal HB & de Rooij DG (2001)** Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.
16. **Huckins C (1978)** The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium *Anat Rec* **190,** 905-26.
17. **Clermont Y & Bustos-Obregon E (1968)** Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto" *Am J Anat* **122,** 237-47.
18. **de Rooij DG, Russell LD. (2000)** All you wanted to know about spermatogonia but were afraid to ask. *J Androl* **21,** 776-98.
19. **van Beek ME, Davids JA, van de Kant HJ & de Rooij DG (1984)** Response to fission neutron irradiation of spermatogonial stem cells in different stages of the cycle of the seminiferous epithelium *Radiat Res* **97,** 556-69.
20. **de Rooij DG & Lok D (1987)** Regulation of the density of spermatogonia in the seminiferous epithelium of the Chinese hamster: II. Differentiating spermatogonia *Anat Rec* **217,** 131-6.
21. **de Rooij DG & Janssen JM (1987)** Regulation of the density of spermatogonia in the seminiferous epithelium of the Chinese hamster: I. Undifferentiated spermatogonia *Anat Rec* **217,** 124-30.
22. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T & de Rooij DG (2003)** Function of DNA-PKcs during the early meiotic prophase without Ku70 and Ku86 *Biol Reprod* **68,** 717-21.
23. **de Vries SS, Baart EB, Dekker M, Siezen A, de Rooij DG, de Boer P & te Riele H (1999)** Mouse MutS-like protein MSH5 is required for proper chromosome synapsis in male and female meiosis *Genes Dev* **13,** 523-31.
24. **Barlow C, Liyanage M, Moens PB, Tarsounas M, Nagashima K, Brown K, Rottinghaus S, Jackson SP, Tagle D, Ried T & Wynshaw-Boris A (1998)** ATM deficiency results in severe meiotic disruption as early as leptonema of prophase I *Development* **125,** 4007-17.
25. **Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM & Burgoyne PS (2001)** Recombinational DNA double-strand breaks in mice precede synapsis *Nat Genet* **27,** 271-6.
26. **Nunez MI, McMillan TJ, Valenzuela MT, Ruiz de Almodovar JM & Pedraza V (1996)** Relationship between DNA damage, rejoining and cell killing by radiation in mammalian cells *Radiother Oncol* **39,** 155-65.
27. **Sjöblom T & Lähdetie J (1996)** Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p53 in meiotic recombination and repair *Oncogene* **12,** 2499-505.

28. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP & de Rooij DG (2003)** DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.
29. **Odorisio T, Rodriguez TA, Evans EP, Clarke AR & Burgoyne PS (1998)** The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis *Nat Genet* **18,** 257-61.
30. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB & de Rooij DG (2000)** Apoptosis regulation in the testis: Involvement of Bcl-2 family members *Mol Reprod Dev* **56,** 353-59.
31. **Knudson CM, Tung KS, Tourtellotte WG, Brown GA & Korsmeyer SJ (1995)** Bax-deficient mice with lymphoid hyperplasia and male germ cell death *Science* **270,** 96-9.
32. **Print CG & Loveland KL (2000)** Germ cell suicide: new insights into apoptosis during spermatogenesis *Bioessays* **22,** 423-30.
33. ***Weber JE & Russell LD (1987)*** *A study of intercellular bridges during spermatogenesis in the rat* Am J Anat ***180,*** *1-24.*

The authors thank Henk J.G. van de Kant for his assistance with the preparation of the whole mounts of seminiferous tubules.

ROLE FOR c-Abl & p73 IN THE RADIATION RESPONSE OF MALE GERM CELLS

Geert Hamer[1,2], Iris S. Gademan[3], Henk B. Kal[3], Dirk G. de Rooij[1,2]



[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*
[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*
[3] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

Hamer G, Gademan IS, Kal HB & de Rooij DG (2001) Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.

p53 plays a central role in the induction of apoptosis of spermatogonia in response to ionizing radiation (IR). In $p53^{-/-}$ testes, however, spermatogonial apoptosis still can be induced by IR, so p53 independent apoptotic pathways must exist in spermatogonia. Here we show that the p53 homologues p63 and p73 are present in the testis and that p73, but not p63, is localized in the cytoplasm of spermatogonia. Unlike p53, neither p63 nor p73 protein levels were found to increase after a dose of 4 Gy of X-rays. Although p73 protein levels did not increase, its interaction with the non-receptor tyrosine kinase c-Abl and its phosphorylation on tyrosine residues did. c-Abl and p73 co-localize in the cytoplasm of spermatogonia and spermatocytes and in the residual bodies. Furthermore, c-Abl protein levels increase after irradiation. p63 was not found to co-localize or interact with c-Abl neither before nor after irradiation. In conclusion, in the testis, IR elevates cytoplasmic c-Abl that in turn interacts with p73. This may represent an additional, cytoplasmic, apoptotic pathway. Although less efficient than the p53 route, this pathway may cause spermatogonial apoptosis as observed in p53 deficient mice.

INTRODUCTION

In the adult mouse testis the mitotically active spermatogonia are the most radiosensitive whereas spermatocytes, which undergo meiotic cell divisions, and spermatids, that develop into spermatozoa, are more resistant to IR (1-5). After exposure to IR, the tumor suppressor p53 is induced in spermatogonia and has been shown to play a central role in DNA damage induced spermatogonial apoptosis (6-8).

However, in p53 knock out mice IR still can induce apoptosis in spermatogonia, although most of these cells are more radioresistant than in wild type mice (6, 8). Apparently, there is at least one alternative route leading to spermatogonial apoptosis. In this context it is interesting to study the role of the p53 homologues p63 and p73 in the spermatogonial response to IR. Unlike p53, p63 and p73 exist in various isoforms that appear to have distinct functions not only in the response to cellular stress but also during embryonic development (9). Although some truncated isoforms may even exhibit an anti-apoptotic role in specific cell types (10, 11), full-length p73 has been shown to be able to induce apoptosis in a p53 independent manner (12).

Also p63 has the potential to transactivate p53 responsive genes and induce apoptosis (13) but probably mainly has a function during development. The p63 protein has been found to be essential in ectodermal differentiation during embryogenesis (14, 15).

The gene encoding p73 has been described as a mono-allelically expressed p53 homologue that maps on a region frequently deleted in neuroblastoma and other human cancers (16). Besides important roles in neurogenesis, sensory pathways and homeostatic control (17, 18), p73 has also been found to activate the transcription of p53-responsive genes (19, 20), to inhibit cell growth in a p53-like manner by inducing apoptosis (19) and to play a role in E2F1 induced apoptosis (21-23). Furthermore, p73 has been found to be activated by the non-receptor tyrosine kinase c-Abl and to induce apoptosis in response to DNA damage (24-27). Earlier reports already described IR to cause an increase in c-Abl kinase activity (28, 29). The exact role, however, of c-Abl in cell death signaling still remains to be elucidated and probably differs between various tissues (30).

We now have studied the localization and expression levels of p63, p73 and c-Abl in the mouse testis at different time intervals after treatment with IR. Furthermore, we studied interactions of c-Abl with p73 and p63 in the testis before and after irradiation. The results indicate a testis specific role for c-Abl and p73, but not p63, in the response to IR.

MATERIALS & METHODS

Animals, irradiation and fixation

The testes of male FvB/NAU mice (Central Laboratory Animal Institute, Utrecht, The Netherlands) of at least 7 weeks of age were locally irradiated with a single dose of 4 Gy of X-rays (Philips, 200 kV, 20 mA, 0.5 mm Cu filter). Groups of 4 mice, allowing each experiment to be repeated 3 times, were sacrificed by cervical dislocation at 1.5, 3, 6, 9, 12, 18, and 24 h after a dose of 4 Gy. Control mice were sham-irradiated. The animals were used and maintained according to regulations provided by the animal ethical committee of the University Medical Center Utrecht.

For immunohistochemistry and confocal microscopy, one testis of each mouse was fixed in 4% phosphate buffered (pH 6.6-7.2) formaldehyde for 4 h and post fixed in a diluted Bouin solution (71% picric acid (0.9%), 24% formaldehyde (37%), 5% acetic acid) for 16 h, at room temperature. Tissues were washed in 70% EtOH prior to embedding in paraffin (Stemcowax, Adamas Instruments, Ameronger, The Netherlands).

For protein isolation, the second testis of each mouse was frozen in liquid nitrogen, and stored at -80°C.

Immunohistochemistry

Five µm paraffin sections, of testes at different intervals after irradiation, were mounted together on a TESPA coated glass slide and dried overnight at 37°C. Sections were dewaxed in xylene and hydrated in a graded series of alcohols. After this and in between each following step sections were washed in PBS (phosphate buffered saline). The sections were boiled 3 times for 10 min in 0.01 M sodium citrate, using a microwave oven (H2500, Bio-Rad, CA, USA). Subsequently, the sections were incubated in 0.35% H_2O_2 in PBS for 10 min. Blocking occurred in 5% bovine serum albumin (BSA, Sigma, St. Louis, MI, USA) in PBS containing 5% goat serum (Aurion, Wageningen, The Netherlands) 5% rabbit serum (Aurion) or horse serum (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA) depending on the use of goat-anti-rabbit, rabbit anti-goat or horse-anti-mouse secondary antibodies respectively. The slides were then incubated with a rabbit polyclonal c-Abl antibody (K-12, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), a mouse monoclonal c-Abl antibody (Ab-3, Oncogene Science, Cambridge, MA, USA), a rabbit polyclonal p73 antibody (H-79, Santa Cruz Biotechnology Inc.), a goat polyclonal p73α antibody (C-17, Santa Cruz Biotechnology Inc.) or a mouse monoclonal p63 antibody (4A4, Santa Cruz Biotechnology Inc.) diluted 1:25 in PBS including 1% BSA in a humidified chamber overnight at 4°C. Incubation with a secondary biotinylated goat-anti-rabbit (Santa Cruz Biotechnology Inc.), rabbit anti-goat (Vector Laboratories) or horse-anti-mouse (Vector Laboratories) IgG diluted 1:100 in PBS including 1% BSA, was performed in a humidified chamber for 1 h at room temperature. The horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol (Vector Laboratories). Bound antibodies were visualized using 0.3 µg/µl 3, 3'-diaminobenzidine (DAB, Sigma) in PBS, to which 0.03% H_2O_2 was added. Sections were counterstained with Mayer's haematoxylin. Negative control sections were treated as described above, except that the primary antibody was omitted or incubated for 2 h with blocking peptides (sc-131P, Santa Cruz Biotechnology Inc.) or (sc-7238P, Santa Cruz Biotechnology Inc.) for the c-Abl K-12 and the p73α C-17 antibodies respectively (primary antibody: blocking peptide 1:5). Prior to mounting with Pertex (Cellpath Ltd., Hemel Hempstead, GB) sections were dehydrated in a series of graded alcohols and xylene.

Confocal microscopy

For confocal microscopy a similar protocol was used as for immunohistochemistry up until incubation with the secondary antibodies. FITC-labeled goat-anti-rabbit and Texas red-labeled goat-anti-mouse antibodies (Jackson, West Grove, PA, USA), instead of biotinylated, secondary antibodies were used ones and PBA was replaced by PBG (0.2% gelatin in PBS). Furthermore the sections were not incubated in 0.35% H_2O_2 in PBS but instead were incubated in 50 mM glycine in PBS for 30 min. The sections were mounted in VECTAshield (Vector Laboratories) and viewed with a 63X plan-apo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Images were recorded digitally. Negative control sections were treated as described above.

Western blot analysis

Total protein lysates were prepared by homogenizing the testes in a polytron device (Janke & Kunkel GmbH, Staufen, Germany) in ice-cold RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethanesulphonylfluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 mM Na_3VO_4, 1 mM Na_2MoD_4 and 10 mM NaF). Lysates were sonicated on ice for 30 min and cleared by centrifugation. Protein levels were measured using BCA analysis (Pierce Chemical Co., Rockford, IL, USA). Proteins were separated using SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane (MilliPore, Bedford, MA, USA).

Western blots were blocked using Blotto-A, containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline (10 mM Tris-HCl, pH 8.0; 150 mM NaCl), including 0.05% Tween-20 (TBT) and washed in TBT in between each step. First antibodies were diluted 1:1000 in Blotto-A. After incubation with secondary antibodies conjugated to horseradish peroxidase (DAKO A/S, Glostrup, Denmark) diluted 1:5000 in Blotto-A, the antigens were visualized using chemoluminescence (ECL, Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and exposure to X-ray film (RX, Fuji Photo Film Co., Ltd, Tokyo, Japan).

Stripping of blots was performed at 50^{o}C for 30 min in stripping buffer (0.2% SDS, 0.1 M Tris-HCl, 0.01% β-mercapto-ethanol). The stripped blots were re-analyzed using a mouse polyclonal antibody against α-tubulin (AM-2495-11, InnoGenex, San Ramon, CA, USA).

(Co-) immunoprecipitations

Volumes of testis lysates equivalent to 0.5 mg of protein were taken from whole testis lysates of sham-irradiated testes and testes 6 and 24 h post irradiation and RIPA was added to a total volume of 200 µl with a final percentage of 0.05% SDS for co-immunoprecipitations or 0.5% SDS for immunoprecipitations. 15 µl of protein A-agarose beads (50% suspension) (Repligen Co, Cambridge, MA, USA) per sample were washed 5 times with 1% BSA in PBS. 10 µl (1 µg) rabbit polyclonal anti c-Abl for co-immunoprecipitations OR 10 µl (1 µg) rabbit polyclonal anti p73 or a mouse monoclonal antibody anti PY99 (PY99, Santa Cruz Biotechnology Inc.) for immunoprecipitations was incubated with the beads in 300 µl RIPA (0.05% and 0.5% SDS respectively) for 1 h at 4^{0}C. Meanwhile, prepared tissue lysates were pre-cleared with washed beads for 1 h at 4^{0}C. Pre-cleared tissue lysates were added to the incubated beads or to washed beads for negative controls and incubated for 1 h at 4^{0}C in a total volume of 0.5 ml of RIPA. The beads were then washed three times in RIPA and dried using a 30 G needle. Finally, 20 µl of Laemli sample buffer was added and the samples were analyzed (see Western blot analysis). Immunoprecipitated c-Abl and p73 were detected using the Ab-3 (Ab-3, Oncogene Science) and H-79 (Santa Cruz Biotechnology Inc.) antibodies respectively. For detection of phosphorylated tyrosine residues the PY99 (PY99, Santa Cruz Biotechnology Inc.) antibody was used.

RESULTS

Localization of c-Abl, p63 and p73 in the testis, before and after X-irradiation

The localization of the proteins was studied by way of immunohistochemistry on sections of sham-irradiated testes and testes fixed at various intervals after a dose of 4 Gy of X-rays.

Staining for c-Abl **(Figure 1A)** in the testis was found to be cytoplasmic only. An intense staining was found in spermatogonia and residual bodies. Also spermatocytes and Leydig cells showed a clear staining, whereas other testicular cell types stained only lightly. No changes in immunohistochemical staining for c-Abl were observed after irradiation. The same results were obtained using both the Ab-3 and K-12 antibodies.

p73 staining **(Figure 1B)** was also found to be cytoplasmic and to be present in all testicular cells. Like c-Abl, p73 staining was more pronounced in spermatogonia, spermatocytes and residual bodies. Interestingly, a strong nuclear staining appeared in late pachytene spermatocytes after stage VII of the cycle of the seminiferous epithelium. This staining was also present in round spermatids but disappeared in elongating spermatids during stage IX of the seminiferous epithelium. As with c-Abl, immunohistochemical staining for p73 did not change after irradiation. These experiments were repeated using the C-17 antibody that only recognizes p73α. The same results were obtained (data not shown) as with the H-79 antibody that recognizes all p73 isoforms. Therefore it can be assumed that in the testis mainly p73α is expressed.

p63 had a totally different expression pattern **(Figure 1C)**. No p63 staining was found in germ cells until they became pachytene spermatocytes. Only a light nuclear staining was found in early pachytene spermatocytes but pachytene spermatocytes from stage VII onwards and round spermatids showed an intense nuclear staining. Elongating spermatids were negative for p63. With respect to the somatic cells in the testis, only Sertoli cells showed a very weak nuclear staining. Immunohistochemical staining for p63 did not change at different time points after irradiation.

Control sections, in which the primary antibody was omitted (c-Abl Ab-3, p73 H-79 and p63) or incubated with blocking peptide (c-Abl K-12 and p73α C-17), showed no staining except for a light interstitial staining caused by horse-anti-mouse secondary antibodies.

Co-localization of c-Abl and p73, but not p63, in the cytoplasm of spermatogonia and spermatocytes and the residual bodies

p73 and c-Abl expression patterns in the testis were also studied using confocal scanning laser microscopy. The individual expression patterns of c-Abl **(Figure 1H)** and p73 **(Figure 1G)** appeared to be the same as found using immunohistochemistry as did the negative controls. The merged pictures showed that c-Abl and p73 co-localize in the cytoplasm of spermatogonia and spermatocytes and in residual bodies **(Figure 1I)**.

The same experiments were performed using sections of testes 6 and 24 h post irradiation. No differences in expression or co-localization of c-Abl and p73 could be observed in these experiments in comparison to the non-irradiated testis.

All these experiments were also performed with c-Abl and p63 **(Figures 1D-F)**. Again the expression patterns turned out to be the same as those found using immunohistochemistry. The merged pictures revealed that p63 does not to co-localize with c-Abl **(Figure 1F)**.

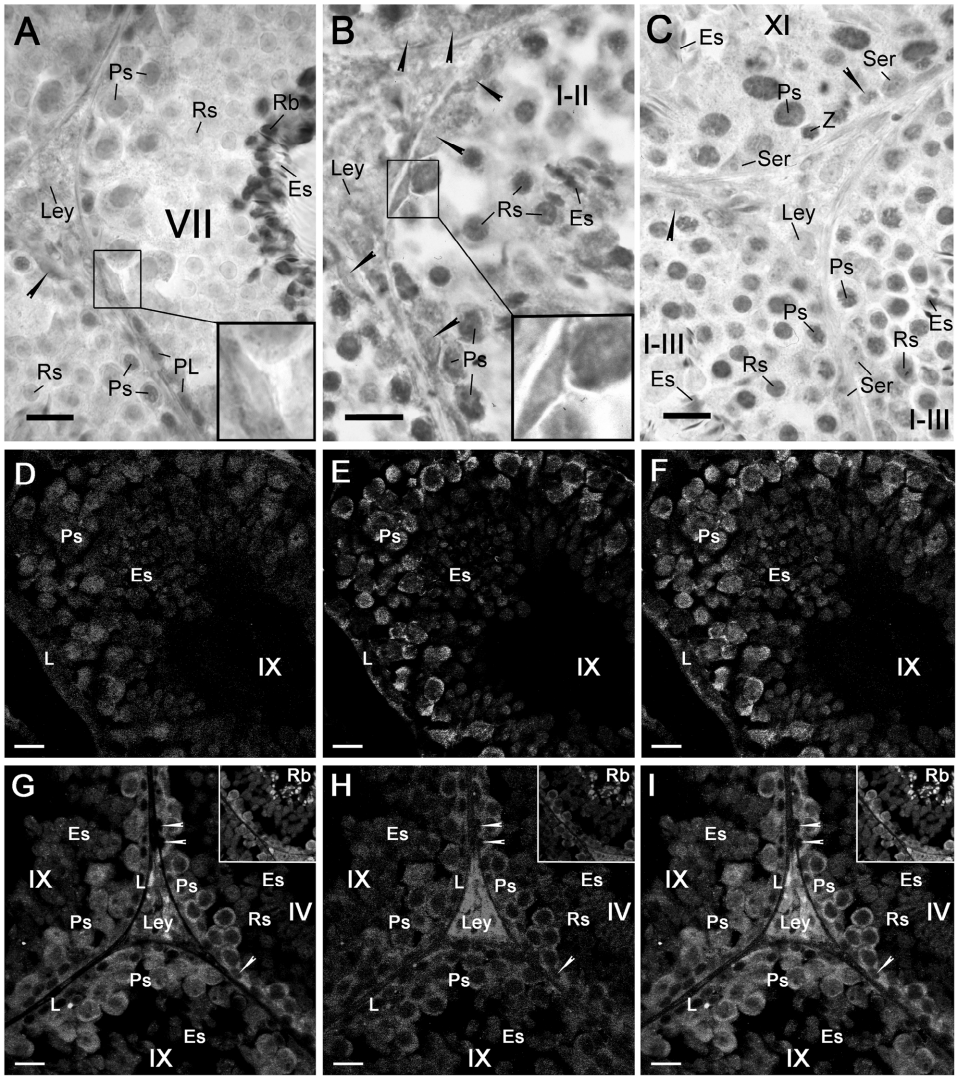


Figure 1. ***Expression of c-Abl, p73 and p63 in the testis.*** *Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, PL: pre leptotene spermatocytes, L: leptotene spermatocytes, Z: zygotene spermatocytes, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids, Rb: residual bodies. Insets show magnification of a spermatogonium* ***(A&B)*** *and a spermatocyte* ***(B)*** *or a tubule containing residual bodies* ***(G,H&I)****. Stages of the seminiferous epithelium are indicated with Roman numerals. DAB staining shows expression of c-Abl in the cytoplasm of spermatogonia, spermatocytes, Leydig cells and residual bodies* ***(A)****, p73 in the cytoplasm of spermatogonia, early spermatocytes and Leydig cells and in the nucleus of in round spermatids* ***(B)****, and nuclear p63 in spermatocytes, round spermatids and Sertoli cells* ***(C)****. Confocal images also show expression of both p63* ***(D)*** *and p73* ***(G)****, and of c-Abl* ***(E&H)****. Merged pictures show co-localization of c-Abl with p73* ***(I)*** *and not p63* ***(F)****. Note nuclear expression of p73 in late spermatocytes (stage IX)* ***(G&I)****. Magnification: bar represents 20 μm.*

Protein levels of c-Abl, p63 and p73 in the testis, before and after X-irradiation

To follow changes in protein levels in the testis before and after irradiation, Western blot analysis was performed on whole testis lysates of sham-irradiated mice and of mice at various time intervals post irradiation **(Figure 2)**.

Neither p73 nor p63 levels were found to change after X-irradiation. We detected full-length isoforms with molecular weights, round about 80 kDa, that match to the α forms (23). For p73 the same results were obtained using the C-17 antibody that only recognizes p73α (data not shown).

We detected several bands that are likely to represent c-Abl, although the bands below 85 kDa are probably degradation products. However, only one of these bands, of approximately 115 kDa, remained after immunoprecipitation of c-Abl **(Figures 1&3)**. A band of similar molecular weight has been described by Kharbanda *et al*., 1998 upon immunoprecipitations of c-Abl from isolated male rat germ cells (31). In their paper, however, they do not show the c-Abl labeling pattern of total germ cell lysates. Interestingly, we found c-Abl protein levels to increase with a maximum at 6 h post irradiation, as shown for all the detected bands **(Figure 2)**.

In order to verify that equal amounts of protein were loaded in each lane, the blots were stripped and reprobed with an antibody against α-tubulin. Equal amounts of protein were found to be loaded at each time point post irradiation.

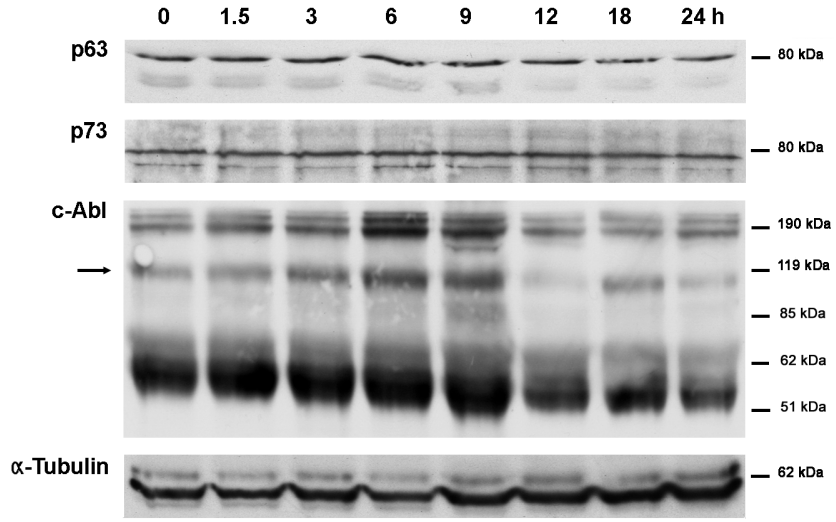


Figure 2. ***Western blot analysis of p63, p73 and c-Abl in total testis lysates before and after treatment with irradiation*** *showing an increase of c-Abl in response to ionizing irradiation. The arrow indicates bands that remain detectable when c-Abl is immunoprecipitated using K-12 and detected using Ab-3 (Figure 3). PVDF membranes stained for c-Abl were stripped and reprobed using an antibody against α-tubulin.*

Interaction of c-Abl with p73, but not with p63, in the testis

To further investigate protein interactions with c-Abl before and after treatment with IR we immunoprecipitated c-Abl from whole testis lysates using sham-irradiated testes and testes 6 and 24 h post irradiation. Co-immunoprecipitated proteins were investigated using Western blot analysis **(Figure 3A)**.

First we looked for proteins phosphorylated on tyrosine residues using the PY99 antibody against phosphotyrosine **(Figure 3A)**. One band with a molecular weight just below 80 kDa was detected that could represent p73. This band was found to be increased at 6 h and again decreased at 24 h post irradiation, sometimes even to a lesser intensity than in the non-irradiated testis. Apart from the band that possibly represents phosphorylated p73, some other bands were also detected in this experiment. Two bands that were not detected in non-irradiated precipitates, with molecular weights between 25 and 49 kDa, appeared at 6 h post irradiation and were found to decrease only slightly at 24 h. Another co-precipitated protein, smaller than 25 kDa, was found to be strongly increased at 6 h post irradiation and decreased at 24 h, still being at a higher level than in non-irradiated precipitates.

In order to confirm that the phosphorylated band with the molecular weight of p73 could represent p73, Western blot analysis was performed using an antibody against p73 **(Figure 3A)**. This resulted in a band at the same height as found using PY99. p73 bound to c-Abl was also found to increase 6 h post irradiation but did not decrease 24 h post irradiation, indicating that dephosphorylated p73 still remained bound to c-Abl.

The signal for c-Abl itself behaved similar to the c-Abl signal found in whole testis lysates with a maximum at 6 h post irradiation **(Figure 3A)**. The signal at 24 h was often even lower in intensity than the signal at 0 h, which may contribute to the low intensity of the PY99 signal at 24 h in these experiments.

p63 did not co-immunoprecipitate with c-Abl. This is in accordance with the fact that p63 and c-Abl did not co-localize. For negative controls the immunoprecipitations were performed using beads that were not incubated with an antibody. No proteins were detected in these control samples.

p73 is phosphorylated on tyrosine residues after irradiation

To confirm that p73 is phosphorylated after irradiation, immunoprecipitations under more stringent conditions (see Materials & Methods) were performed using PY99 and the antibody against p73.

p73 was found to be immunoprecipitated with PY99 **(Figure 3B)**. Only a small band was observed using sham-irradiated testes but more intense bands were found at time points 6 and 24 h post irradiation. p63 could not be detected in this experiment.

Furthermore, with anti-p73 immunoprecipitated p73 could be detected in a Western blot analysis using PY99 **(Figure 3C)**. Again only a small band was observed at time point 0 h post irradiation and again the signal was found to be increased at time points 6 and 24 h post irradiation. The signal for p73 itself was not found to change after irradiation in this experiment **(Figure 3C)**. For negative controls the immunoprecipitations were performed using beads that were not incubated with an antibody. No proteins were detected in these control samples.

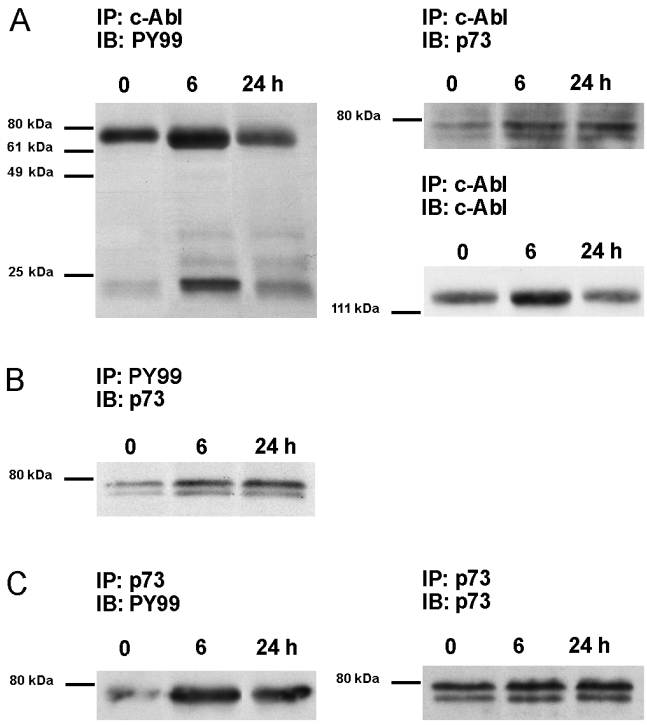


Figure 3. ***Western blot analysis of (co-) immunoprecipitations*** *showing interaction of p73 with c-Abl and phosphorylation of p73 in response to ionizing radiation (IR).* ***(A)*** *Co-immunoprecipitation (IP) with c-Abl, immunoblotted (IB) with PY99, p73 and c-Abl.* ***(B)*** *Immunoprecipitation (IP) with PY99, immunoblotted (IB) with p73* ***(C)*** *Immunoprecipitation (IP) with p73, immunoblotted (IB) with PY99 and p73.*

DISCUSSION

The present results show that in the cytoplasm of mouse male germ cells, protein levels of the non-receptor tyrosine kinase c-Abl increase in response to IR. Subsequently, the interaction of c-Abl with p73 is enhanced and p73 phosphorylation on tyrosine residues increases. Until now elevation of c-Abl levels has only been described in B cells and interestingly also in the cytoplasm (32). Although cytoplasmic c-Abl is often thought to inhibit apoptosis, for instance in muscle cells (30), our results show cytoplasmic c-Abl to be involved in the apoptotic response to IR. These data are the first that show an increase in both c-Abl levels and its interaction with p73 in the cytoplasm after irradiation.

Both c-Abl and p73 were found to be expressed in spermatogonia, spermatocytes and spermatids. While we only detected c-Abl in the cytoplasm, a nuclear localization of c-Abl has been described for isolated rat spermatocytes (31). c-Abl contains both nuclear localization sequences (33) as well as a nuclear export sequence (34). As c-Abl shuttling between nuclear and cytoplasmic compartments is regulated by cell adhesion (35) this may cause c-Abl to be nuclear in isolated spermatocytes and cytoplasmic in spermatocytes attached to Sertoli cells. From late spermatocytes onwards p73, and also p63, showed a nuclear localization suggesting a function in maintaining genome integrity during meiosis, a role already suggested for p53 (36). The function of these proteins in spermatids however remains elusive. Interestingly, a testis specific *c-Abl* mRNA was found in spermatids only (37, 38) and we also found c-Abl expression in spermatids, although weak in comparison to other cell types. In c-Abl$^{-/-}$ mice, however, only few spermatids are formed (31), indicating an indispensable function for c-Abl during meiosis or earlier in spermatogenesis.

The strong expression of c-Abl and p73 in residual bodies is intriguing. Residual bodies exhibit similar protein contents and morphology, as do apoptotic bodies (39). They are pinched off by the elongated spermatids before they leave the seminiferous tubules and are then, like apoptotic bodies in the testis, phagocytized by Sertoli cells. As residual bodies lack a nucleus, c-Abl and p73 might be involved in the apoptotic processes in these cytoplasmic remnants.

At the moment it is unknown how IR activates c-Abl in the testis. Interestingly, reactive oxygen species have been shown to activate cytoplasmic c-Abl (40) and consequently reactive oxygen species induced by IR might activate cytoplasmic c-Abl in germ cells. The effects of activated c-Abl on apoptosis vary between the different tissues and an important determinant in this context may well be the presence of different p73 isoforms (30). In the testis we found c-Abl to interact with the pro-apoptotic p73α.

How p73 induces spermatogonial apoptosis is yet unclear. Unless some of the activated p73 is transported the nucleus below our detection levels it appears to function without directly activating transcription of target genes. In the cytoplasm it might function directly via mitochondria, a route that also has been described for p53 (41). Radiation induced spermatogonial apoptosis still occurs in p53 deficient mice (6, 8), and recently various p53 independent apoptotic pathways involving p73 have been described (12). Importantly, also induction of p73 by c-Abl was found to be independent of p53 (25). Hence, in spermatogonia, cytoplasmic p73 activated by c-Abl, most likely plays a role in an additional, p53 independent, apoptotic pathway.

Regulation and function of both c-Abl and p73 appear to differ in various cell types (9, 30). Whereas most signal transduction studies are performed using cultured cells, we studied the intact testis, showing that c-Abl and p73 exhibit testis specific expression and regulation. Although studying cell lines provides invaluable fundamental knowledge, studying the various tissues *in vivo* will be required for deeper understanding of tissue specific aspects of signal transduction and responses to chemo- and radiotherapy.

REFERENCES

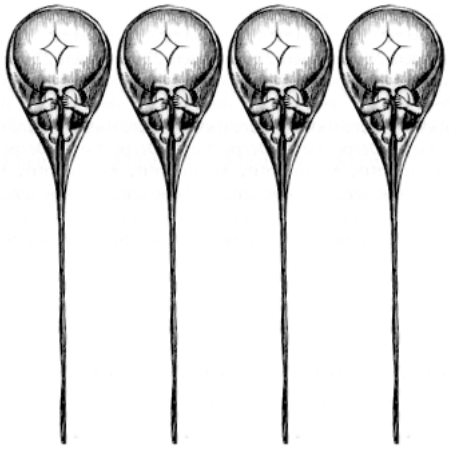
1. **Oakberg EF & DiMinno RL (1960)** X-ray sensitivity of primary spermatocytes of the mouse *Int J Radiat Biol* **2,** 196-209.
2. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Rutgers DH & de Rooij DG (1997)** p21(Cip1/WAF1) expression in the mouse testis before and after X irradiation *Mol Reprod Dev* **47,** 240-7.
3. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia *Radiat Res* **130,** 296-302.
4. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X irradiation *Radiat Res* **130,** 289-95.
5. **Hasegawa M, Wilson G, Russell LD & Meistrich ML (1997)** Radiation-induced cell death in the mouse testis: relationship to apoptosis *Radiat Res* **147,** 457-67.
6. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
7. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
8. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
9. **Levrero M, De Laurenzi V, Costanzo A, Gong J, Wang JY & Melino G (2000)** The p53/p63/p73 family of transcription factors: overlapping and distinct functions *J Cell Sci* **113,** 1661-70.
10. **Crook T, Nicholls JM, Brooks L, O'Nions J & Allday MJ (2000)** High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? *Oncogene* **19,** 3439-44.
11. **Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR & Miller FD (2000)** An anti-apoptotic role for the p53 family member, p73, during developmental neuron death *Science* **289,** 304-6.
12. **Soengas MS & Lowe SW (2000)** p53 and p73: seeing double? *Nat Genet* **26,** 391-2.
13. **Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D & McKeon F (1998)** p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities *Mol Cell* **2,** 305-16.
14. **Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR & Bradley A (1999)** p63 is a p53 homologue required for limb and epidermal morphogenesis *Nature* **398,** 708-13.
15. **Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C & McKeon F (1999)** p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development *Nature* **398,** 714-8.
16. **Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F & Caput D (1997)** Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers *Cell* **90,** 809-19.
17. **Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F & Caput D (2000)** p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours *Nature* **404,** 99-103.
18. **De Laurenzi V, Raschella G, Barcaroli D, Annicchiarico-Petruzzelli M, Ranalli M, Catani MV, Tanno B, Costanzo A, Levrero M & Melino G (2000)** Induction of neuronal differentiation by p73 in a neuroblastoma cell line *J Biol Chem* **275,** 15226-31.
19. **Jost CA, Marin MC & Kaelin WG, Jr. (1997)** p73 is a simian p53-related protein that can induce apoptosis *Nature* **389,** 191-4.
20. **Nakano K, Balint E, Ashcroft M & Vousden KH (2000)** A ribonucleotide reductase gene is a transcriptional target of p53 and p73 *Oncogene* **19,** 4283-9.
21. **Stiewe T & Putzer BM (2000)** Role of the p53-homologue p73 in E2F1-induced apoptosis *Nat Genet* **26,** 464-9.
22. **Lissy NA, Davis PK, Irwin M, Kaelin WG & Dowdy SF (2000)** A common E2F-1 and p73 pathway mediates cell death induced by TCR activation *Nature* **407,** 642-5.
23. **Irwin M, Marin MC, Phillips AC, Seelan RS, Smith DI, Liu W, Flores ER, Tsai KY, Jacks T, Vousden KH & Kaelin WG, Jr. (2000)** Role for the p53 homologue p73 in E2F-1-induced apoptosis *Nature* **407,** 645-8.
24. **White E & Prives C (1999)** DNA damage enables p73 *Nature* **399,** 734-5, 7.
25. **Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin WG, Jr., Levrero M & Wang JY (1999)** The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage *Nature* **399,** 806-9.
26. **Agami R, Blandino G, Oren M & Shaul Y (1999)** Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis *Nature* **399,** 809-13.

27. **Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R & Kufe D (1999)** p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage *Nature* **399,** 814-7.
28. **Baskaran R, Wood LD, Whitaker LL, Canman CE, Morgan SE, Xu Y, Barlow C, Baltimore D, Wynshaw-Boris A, Kastan MB & Wang JY (1997)** Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation *Nature* **387,** 516-9.
29. **Shafman T, Khanna KK, Kedar P, Spring K, Kozlov S, Yen T, Hobson K, Gatei M, Zhang N, Watters D, Egerton M, Shiloh Y, Kharbanda S, Kufe D & Lavin MF (1997)** Interaction between ATM protein and c-Abl in response to DNA damage *Nature* **387,** 520-3.
30. **Wang JY (2000)** Regulation of cell death by the Abl tyrosine kinase *Oncogene* **19,** 5643-50.
31. **Kharbanda S, Pandey P, Morris PL, Whang Y, Xu Y, Sawant S, Zhu LJ, Kumar N, Yuan ZM, Weichselbaum R, Sawyers CL, Pandita TK & Kufe D (1998)** Functional role for the c-Abl tyrosine kinase in meiosis I *Oncogene* **16,** 1773-7.
32. **Zipfel PA, Grove M, Blackburn K, Fujimoto M, Tedder TF & Pendergast AM (2000)** The c-Abl tyrosine kinase is regulated downstream of the B cell antigen receptor and interacts with CD19 *J Immunol* **165,** 6872-9.
33. **Wen ST, Jackson PK & Van Etten RA (1996)** The cytostatic function of c-Abl is controlled by multiple nuclear localization signals and requires the p53 and Rb tumor suppressor gene products *Embo J* **15,** 1583-95.
34. **Taagepera S, McDonald D, Loeb JE, Whitaker LL, McElroy AK, Wang JY & Hope TJ (1998)** Nuclear-cytoplasmic shuttling of C-Abl tyrosine kinase *Proc Natl Acad Sci USA* **95,** 7457-62.
35. **Lewis JM, Baskaran R, Taagepera S, Schwartz MA & Wang JY (1996)** Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport *Proc Natl Acad Sci USA* **93,** 15174-9.
36. **Sjöblom T & Lähdetie J (1996)** Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p53 in meiotic recombination and repair *Oncogene* **12,** 2499-505.
37. **Ponzetto C & Wolgemuth DJ (1985)** Haploid expression of a unique c-Abl transcript in the mouse male germ line *Mol Cell Biol* **5,** 1791-4.
38. **Meijer D, Hermans A, von Lindern M, van Agthoven T, de Klein A, Mackenbach P, Grootegoed A, Talarico D, Della Valle G & Grosveld G (1987)** Molecular characterization of the testis specific c-Abl mRNA in mouse *Embo J* **6,** 4041-8.
39. **Blanco-Rodriguez J & Martinez-Garcia C (1999)** Apoptosis is physiologically restricted to a specialized cytoplasmic compartment in rat spermatids *Biol Reprod* **61,** 1541-7.
40. **Sun X, Majumder P, Shioya H, Wu F, Kumar S, Weichselbaum R, Kharbanda S & Kufe D (2000)** Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species *J Biol Chem* **275,** 17237-40.
41. **Marchenko ND, Zaika A & Moll UM (2000)** Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling *J Biol Chem* **275,** 16202-12.

The authors thank René M.C. Scriwanek and Marc A. van Peski for assistance with photography and Ans M.M. van Pelt for advice and discussions.

DNA DOUBLE-STRAND BREAKS & γ-H2AX SIGNALING IN THE TESTIS

Geert Hamer[1,2], Hermien L. Roepers-Gajadien[1,2], Annemarie van Duyn-Goedhart[3], Iris S. Gademan[4], Henk B. Kal[3], Paul P.W. van Buul[4] and Dirk G. de Rooij[1,2]



[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*
[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*
[3] *MCG-Department of Radiation Genetics and Chemical Mutagenesis, Sylvius Laboratory, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands*
[4] *Department of Radiotherapy, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PPW & de Rooij DG (2003) DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.

Within minutes of the induction of DNA double-strand breaks (DSBs) in somatic cells, histone H2AX becomes phosphorylated at serine 139 and forms γ-H2AX foci at the sites of damage. These foci then play a role in recruiting DNA-repair and damage-response factors and changing chromatin structure in order to accurately repair the damaged DNA. γ-H2AX foci not only appear in response to irradiation or genotoxic stress but also during V(D)J recombination and meiotic recombination. Independent of irradiation, we find γ-H2AX in all intermediate and B spermatogonia and in preleptotene until zygotene spermatocytes. Type A spermatogonia and round spermatids do not exhibit γ-H2AX foci but show an even nuclear γ-H2AX staining while in pachytene spermatocytes γ-H2AX is only present in the sex body. In response to ionizing radiation (IR), γ-H2AX foci are generated in spermatogonia, spermatocytes and round spermatids. In irradiated spermatogonia γ-H2AX interacts with p53, which induces spermatogonial apoptosis. These events were found to occur independent of the DNA dependent protein kinase (DNA-PK). Irradiation-independent nuclear γ-H2AX staining in leptotene spermatocytes shows a function for γ-H2AX during meiosis. γ-H2AX staining in intermediate and B spermatogonia, preleptotene spermatocytes and staining of the sex bodies and round spermatids, however, indicates that the function of H2AX phosphorylation during spermatogenesis is not restricted to the formation of γ-H2AX foci at DSBs.

INTRODUCTION

In the adult mouse testis the mitotically active spermatogonia are the most radiosensitive, whereas spermatocytes, which undergo meiotic cell divisions, or spermatids, which develop into spermatozoa, are more resistant to ionizing radiation (IR) (1-5). Several spermatogonial subtypes exist (6-8), among which the spermatogonial stem cells, intermediate and B spermatogonia. The B spermatogonia divide and form preleptotene spermatocytes, which enter the meiotic prophase.

In response to IR, DNA double-strand breaks (DSBs) are generated (9). One of the first steps in the cellular response to DSBs is phosphorylation of histone H2AX at serine 139 (10), and very shortly after irradiation γ-H2AX (phosphorylated H2AX) foci appear specifically at the damaged sites containing DSBs (11, 12). At these sites of DNA damage, γ-H2AX appears to have a critical function in the recruitment of DNA-repair factors and DNA damage-signaling proteins (12, 13). Moreover, H2AX phosphorylation is a chromatin modification that is followed by apoptotic DNA fragmentation (14) and constitutes an important step in the course of mammalian apoptosis (15). Recently, the $H2AX^{-/-}$ mouse was generated (16). Although loss of H2AX did not seem to impair cell cycle checkpoints, it did lead to increased chromosomal abnormalities, deficiencies in gene targeting, impaired DNA-repair and subsequently increased radiosensitivity. Also $H2AX^{-/-}$ cells were found to exhibit increased sensitivity to IR and genomic instability (17).

After exposure to IR, the tumor suppressor p53 is induced in spermatogonia, which plays a central role in DNA damage induced spermatogonial apoptosis (18-20). In response to DNA damage, p53 can be activated by members of the large family of phosphatidylinositol 3-kinases including ATM (ataxia telangiectasia mutated) and DNA-PK (DNA-dependent protein kinase) leading to cell cycle arrest or apoptosis (9). Although some state the opposite (21, 22), most papers describe p53 induction to occur independent of DNA-PK (23-25), whereas ATM has been proven to be very important if not essential for p53 activation in response to DNA damage (26-28).

Moreover, histone H2AX has been shown to be phosphorylated by ATM in response to DSBs, whereas γ-H2AX foci formation occurs independent of DNA-PK (15, 29). After

induction of DSBs a fraction of the nuclear ATM pool relocates to the damaged DNA and co-localizes with γ-H2AX foci (30). Also the p53 binding protein 1 (53BP1) co-localizes with γ-H2AX foci and becomes hyperphosphorylated in response to DNA damage in an ATM dependent manner (13). These results suggest that γ-H2AX foci formation could be an early step in spermatogonial p53 activation and thus in spermatogonial apoptosis in response to IR.

DSBs are not only induced by IR, also V(D)J recombination in lymphoid cells (31, 32) and homologues recombination during meiosis (33) generate DSBs and subsequently γ-H2AX foci. Interestingly, loss of H2AX does not affect V(D)J recombination or non-homologues end-joining (NHEJ) but does affect class-switch recombination (16, 17), indicating that H2AX is involved in other than classical NHEJ DNA-repair pathways.

Timing of DSB induction during meiotic recombination was investigated on surface spreads of spermatogenic cells using immuno-localization of γ-H2AX together with markers specific for certain stages of meiosis (33). This study described meiotic DSBs to occur predominantly during the leptotene stage and thus before synapsis of the homologous chromosomes. During zygotene and throughout the pachytene stage, γ-H2AX staining becomes restricted to the sex body that contains the X and Y-chromosomes. In $H2AX^{-/-}$ testes preleptotene and leptotene spermatocytes were normally present and spermatogenesis arrested at the pachytene stage of meiosis I. This arrest appeared to be associated with defects in sex chromosome segregation and impaired meiotic crossover (16).

During spermatogenesis both DNA integrity and chromatin organization are essential and in both processes γ-H2AX most likely plays a role. We now have investigated in which germ cells γ-H2AX foci appear in the testis in response to IR, whether spermatogonial p53 induction is correlated with the appearance of γ-H2AX and whether DNA-PK is involved in these processes. Furthermore, independent of irradiation, we studied more closely the exact timing of the appearance of γ-H2AX during spermatogenesis.

MATERIALS & METHODS

Animals, irradiation and fixation

The testes of male FvB/NAU mice (Central Laboratory Animal Institute, Utrecht, The Netherlands) of at least 7 weeks of age were locally irradiated with a single dose of 4 Gy of X-rays (Philips, 200 kV, 20 mA, 0.5 mm Cu filter). Groups of 4 mice, allowing each experiment to be repeated 3 times, were sacrificed by cervical dislocation at 3, 6, 12, and 24 h after a dose of 4 Gy. Control mice were sham-irradiated.

For immunohistochemistry, one testis of each mouse was fixed in 4% phosphate buffered (pH 6.6-7.2) formaldehyde for 4 h and post fixed in a diluted Bouin solution (71% picric acid (0.9%), 24% formaldehyde (37%), 5% acetic acid) for 16 h, at room temperature. Tissues were washed in 70% EtOH prior to embedding in paraffin (Stemcowax, Adamas Instruments, Ameronger, The Netherlands).

Homozygous *scid* mice (*scid/scid*) on a CB-17 genetic background (34) and wildtype CB-17 mice were propagated and irradiated as described (34), receiving a dose of 4 Gy of total body X-irradiation. The testes of groups of 4 mice were fixed in 4% phosphate buffered (pH 6.6-7.2) or in a diluted Bouin's fixation at 3 and 12 h after irradiation.

Control mice were sham-irradiated. For protein isolation, the contralateral testes were frozen in liquid nitrogen, and stored at -80°C. The animals were used and maintained according to regulations provided by the animal ethical committee of the University Medical Center Utrecht that also approved the experiments.

Immunohistochemistry

Five µm paraffin sections, of testes at different intervals after irradiation, were mounted together on a TESPA coated glass slide and dried overnight at 37^{o}C. Sections were dewaxed in xylene and hydrated in a graded series of alcohols. In between each step sections were washed in PBS (phosphate buffered saline). For p53 staining, the sections were boiled 3 times for 10 min in 0.01 M sodium citrate, using a microwave oven (H2500, Bio-Rad, CA, USA). After this, sections were incubated in 0.35% H_2O_2 in PBS for 10 min. Blocking occurred in 5% bovine serum albumin (BSA, Sigma, St. Louis, MI, USA) /5% goat serum (Aurion, Wageningen, The Netherlands) in PBS. The slides were then incubated with rabbit polyclonal antibodies against γ-H2AX (Upstate Biotechnology, antiphospho-H2AX (Ser139), Lake Placid, NY, USA) or rabbit polyclonal anti p53 antibodies (Novocastra Laboratories Ltd., NCL-p53-CM5p, Newcastle, UK) diluted 1:50 in PBS including 1% BSA in a humidified chamber overnight at 4^{o}C. Incubation with secondary biotinylated goat-anti-rabbit (Santa Cruz Biotechnology Inc.) IgGs diluted 1:100 in PBS including 1% BSA, was performed in a humidified chamber for 60 min at room temperature. The horseradish peroxidase avidin-biotin complex reaction was performed according to the manufacturer's protocol (Vector Laboratories). Bound antibodies were visualized using 0.3 µg/µl 3, 3'-diaminobenzidine (DAB, Sigma) in PBS, to which 0.03% H_2O_2 was added. Sections were counterstained with Mayer's haematoxylin. For negative controls, primary antibodies were replaced by rabbit IgGs. Adjacent sections were used for a periodic acid Schiff (PAS) staining to identify the stages of the cycle of the seminiferous epithelium. Prior to mounting with Pertex (Cellpath Ltd., Hemel Hempstead, GB) sections were dehydrated in a series of graded alcohols and xylene.

Confocal microscopy

For confocal microscopy a similar protocol was used as for immunohistochemistry. For p53 antigen retrieval, the sections were boiled 3 times for 10 min in 0.01 M sodium citrate, using a microwave oven (H2500, Bio-Rad, CA, USA). For double labeling goat monoclonal antibodies against p53 (M-19, Santa Cruz Biotechnology Inc., CA, USA) were used instead of the rabbit polyclonal antibodies against p53 (Novocastra). Fluorescent secondary antibodies were used instead of biotinylated ones and PBG (0.2% gelatin in PBS) was used instead of PBS. Furthermore the sections were not incubated in 0.35% H_2O_2 in PBS but instead were incubated in 50 mM glycine in PBS for 30 min. FITC-labeled goat-anti-rabbit and Texas Red-labeled rabbit-anti-goat antibodies were obtained from Jackson (Jackson, West Grove, PA, USA). The sections were mounted in VECTAshield (Vector Laboratories) and viewed with a 63X plan-apo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Images were recorded digitally. Negative control sections were treated as described above, except that the primary antibodies were omitted.

Western blot analysis

Total protein lysates were prepared by homogenizing the testes in a polytron device (Janke & Kunkel GmbH, Staufen, Germany) after which the cells were lysed in RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethanesulphonylfluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml aprotinin, 2 mM Na_3VO_4, 1 mM Na_2MoD_4 and 10 mM NaF) for 30 min on ice. Lysates were sonicated on ice and cleared by centrifugation. Protein

levels were measured using BCA analysis (Pierce Chemical Co., Rockford, IL, USA). SDS-page was performed as described by Laemmli (35). Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (MilliPore, Bedford, MA, USA).

Western blots were blocked using Blotto-A, containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline (10 mM Tris-HCl, pH 8.0; 150 mM NaCl), including 0.05% Tween-20 (TBT) and washed in TBT in between each step. First antibodies were diluted 1:1000 in Blotto-A. After incubation with secondary antibodies conjugated to horseradish peroxidase (DAKO A/S, Glostrup, Denmark) diluted 1:5000 in Blotto-A, the antigens were visualized using chemiluminescence (ECL, Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and exposure to X-ray film (RX, Fuji Photo Film Co., Ltd, Tokyo, Japan).

Stripping of blots was performed at 50^{o}C for 30 min in stripping buffer (0.2% SDS, 0.1 M Tris-HCl, 0.01% β-mercapto-ethanol). The stripped blots were re-analyzed using a mouse polyclonal antibody against α-tubulin (AM-2495-11, InnoGenex, San Ramon, CA, USA).

Co-immunoprecipitations

Volumes of testis lysates equivalent to 0.5 mg of protein were taken from whole testis lysates of sham-irradiated testes and testes 6 and 24 h post irradiation and RIPA was added to a total volume of 200 µl with a final percentage of 0.05% SDS. 15 µl of protein A-agarose beads (50% suspension) (Repligen Co, Cambridge, MA, USA) per sample were washed 5 times with 1% BSA in PBS. 10 µl rabbit polyclonal antibodies against γ-H2AX, 10 µl rabbit polyclonal antibodies against p53, or 10 µl rabbit IgGs for negative controls were incubated with the beads in 300 µl for 1 h at 4^{0}C. The prepared tissue lysates were pre-cleared with washed beads for 1 h at 4^{0}C. Pre-cleared tissue lysates were added to the incubated beads or to washed beads for additional negative controls and incubated for 1 h at 4^{0}C in a total volume of 0.5 ml of RIPA. The beads were then washed three times in RIPA and dried using a 30 G needle. Finally, 20 µl of Laemli sample buffer was added and the samples were analyzed (see Western blot analysis).

RESULTS

γ-H2AX in the testis

The presence of γ-H2AX in the testis was studied by immunohistochemistry on sections of sham-irradiated testes and testes fixed at 1.5 to 24 h after a dose of 4 Gy of X-rays, using an antibody against phosphorylated γ-H2AX.

In the non-irradiated testis a strong even nuclear staining together with pronounced γ-H2AX foci was already present in all intermediate and B spermatogonia **(Figure 1A)**. This staining remained up until zygotene spermatocytes. In pachytene spermatocytes only the sex bodies were stained **(Figures 1A&C)**, a staining that disappeared during the meiotic divisions in stage XII of the cycle of the seminiferous epithelium. Also the type A spermatogonia **(Figure 1B)** and round spermatids **(Figures 1A&D)**, although less strongly, exhibited an even nuclear staining. Elongated spermatids **(Figure 1A)** and all somatic cells were negative for γ-H2AX.

After irradiation nuclear γ-H2AX foci also became visible in the A spermatogonia **(Figure 1F)**, pachytene spermatocytes **(Figure 1G)** and round spermatids **(Figure 1H)**. These differences in γ-H2AX staining could not be visualized using Western blot analysis, the intensity of the γ-H2AX bands remained equal at all time-points after irradiation **(Figure 1I)**. No γ-H2AX staining appeared in any of the somatic myoid, Leydig or Sertoli cells after irradiation. The controls, in which the primary antibodies were replaced by rabbit IgGs were all negative.

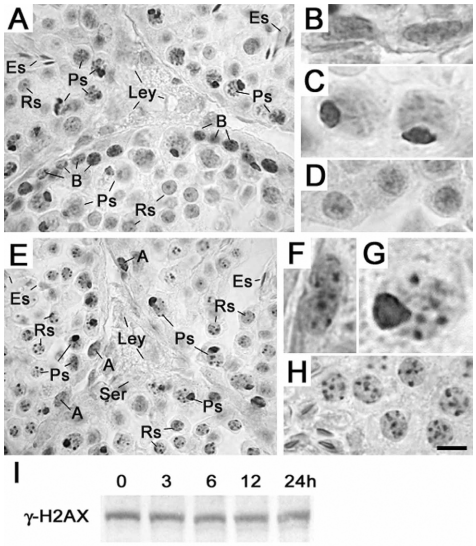


Figure 1. ***Presence of γ-H2AX in the non-irradiated (A,B,C&D) and irradiated mouse testis (E,F,G&H). (A)*** *γ-H2AX in the non-irradiated testis showing staining in B spermatogonia (B), round spermatids (Rs) and the sex bodies of pachytene spermatocytes (Ps) and γ-H2AX negative elongated spermatids (Es) and Leydig cells (Ley).* ***(B)*** *Light γ-H2AX in type A spermatogonia.* ***(C)*** *Staining of the sex bodies of pachytene spermatocytes.* ***(D)*** *Even nuclear staining of round spermatids.* ***(E)*** *γ-H2AX in the irradiated testis showing nuclear foci in type A spermatogonia (A), pachytene spermatocytes (Ps) and round spermatids (Rs).* ***(F)*** *Nuclear foci in irradiated spermatogonium,* ***(G)*** *spermatocyte and* ***(H)*** *round spermatids. Magnification: bar represents 20 μm* ***(A&E)*** *or 10 μm.* ***(I)*** *No differences in γ-H2AX levels could be detected after various time points after treatment with ionizing radiation (IR) using Western blot analysis.*

γ-H2AX interacts with tumor suppressor p53 after irradiation

The tumor suppressor p53 plays a key role in radiation-induced spermatogonial apoptosis (18-20) and is induced in these cells 3 h after irradiation (18-20). To investigate whether γ-

H2AX foci formation in response to irradiation correlates with p53 induction we co-immunoprecipitated p53 from total testis lysates using antibodies against γ-H2AX and vice versa γ-H2AX using antibodies against p53, at several time points after irradiation **(Figure 2)**. p53 binding to γ-H2AX was found to increase in response to irradiation with a maximum between 3 and 6 h after irradiation. Vice versa, also γ-H2AX binding to p53 increased 3 h after irradiation **(Figure 2)**. When p53 or γ-H2AX was immunoprecipitated and immunoblotted using antibodies against the same proteins both p53 and γ-H2AX were found to be immunoprecipitated and to behave as described previously **(Figures 1I&2)** (18-20). The negative controls in which the antibodies were omitted or replaced by rabbit IgGs were all negative.

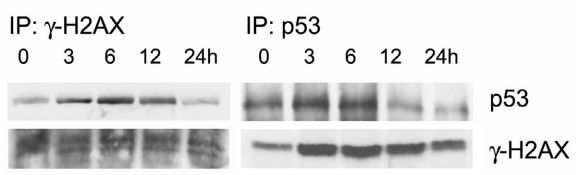


Figure 2. ***Western blot analysis of (co)-immunoprecipitations (IP)*** *using antibodies against γ-H2AX and p53 showing increased interaction between γ-H2AX and p53 after 3 and 6 h after treatment with ionizing radiation (IR).*

Interaction between p53 and γ-H2AX is spermatogonial

The mouse germ cell types in which both γ-H2AX foci and p53 expression are induced in response to irradiation and that subsequently undergo apoptosis are the spermatogonia. Therefore one would expect the interaction between γ-H2AX and p53 to be spermatogonial. To study this, we performed a p53/γ-H2AX double labeling and carried out confocal microscopy. Indeed both p53 and γ-H2AX were induced in spermatogonia after irradiation and were found to co-localize in the nucleus **(Figure 3)**. Although also other cell types express these proteins (18-20) **(Figure 1)**, co-localization was only found in the irradiated spermatogonia.

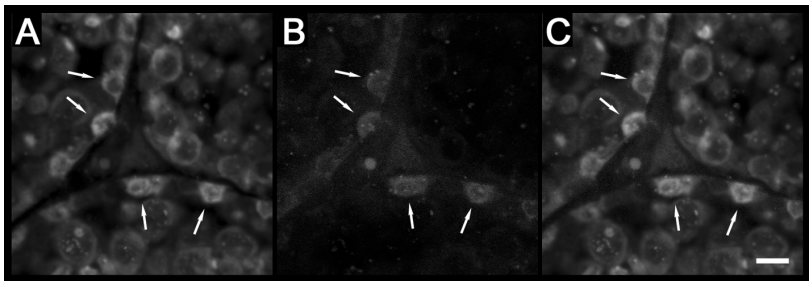


Figure 3. ***Spermatogonial co-localization of γ-H2AX and p53 in the irradiated testis.*** *Confocal images showing γ-H2AX* ***(A)*** *and p53* ***(B)*** *in the irradiated testis.* ***(C)*** *Merged picture showing co-localization of γ-H2AX and p53 in spermatogonia (arrows). Magnification: bar represents 20 μm.*

Even in spermatocytes that also lightly express p53, p53 did not co-localize with the radiation-induced γ-H2AX foci. Hence, it can be concluded that the interaction between p53 and γ-H2AX found in total testis lysates, is spermatogonial.

γ-H2AX foci formation and p53 signaling in the testis occur independent of DNA-PK

To investigate whether the DNA-dependent protein kinase DNA-PK is involved in H2AX phosphorylation and p53 signaling in the testis we repeated the localization and biochemical experiments using DNA-PKcs deficient *scid* mice on a CB-17 genetical background (34).

First, we compared p53 induction in *scid* and wildtype testes using sham-irradiated testes and testes fixed at 3 h after a dose of 4 Gy of X-rays. After 3 h p53 is known to be induced in wildtype spermatogonia (18-20). In the wildtype sham-irradiated testis, a light p53 staining was present in the nuclei of Sertoli cells **(Figure 4A)**. As expected, 3 h after irradiation nuclear p53 induction was observed in wildtype spermatogonia **(Figure 4C)** but also in *scid* spermatogonia **(Figure 4D)**. Intriguingly, in contrast to the wildtype **(Figure 4A)**, in the *scid* testes, p53 also appeared in some spermatogonia in sham-irradiated testes **(Figure 4B)**.

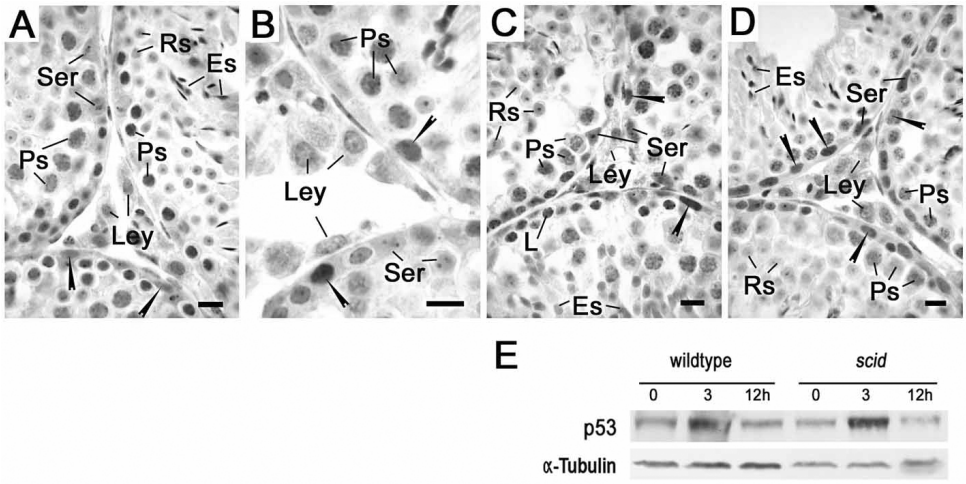


Figure 4. ***p53 induction in scid and wildtype spermatogonia.*** *p53 staining as shown in sham-irradiated wildtype **(A)** and scid **(B)** testes and in irradiated wildtype **(C)** and scid **(D)** testes. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids. Magnification: bar represents 20 μm. **(E)** Western blot analysis of p53 in total testis lysates of wildtype and scid testes before and after irradiation showing an increase of p53 in response to ionizing irradiation in both the wildtype and scid testis. PVDF membranes stained for p53 were stripped and reprobed using an antibody against α-tubulin.*

In order to compare p53 protein levels in the *scid* testis with the wildtype, whole testis lysates of sham-irradiated mice and of mice sacrificed at 3 and 12 h post irradiation were analyzed using Western blotting. In both the *scid* and the wildtype testes p53 levels were elevated 3 h after IR like described previously for the wildtype situation. No significant differences could be observed comparing *scid* to the wildtype **(Figure 4E)**. To verify that

equal amounts of protein were loaded on the gel the blots were stripped and reprobed using an antibody against α-tubulin **(Figure 4E)**.

The repeated experiments for γ-H2AX performed using *scid* mice all gave exactly the same results as the control experiments using wildtype CB-17 **(Figures 5&6)** or FvB/NAU mice **(Figures 1&2)**. In the immunohistochemical detections, no differences could be observed between formalin-fixed tissue or tissue fixed in diluted Bouin's solution or between sections that were or were not boiled in 0.01 M sodium citrate for antigen retrieval. **Figure 5** shows the staining pattern of γ-H2AX in the formalin fixed testes of wildtype and *scid* mice on a CB-17 genetic background without boiling in sodium citrate and before and after irradiation. As mentioned, the staining pattern is exactly the same as shown for FvB/NAU **(Figure 1)**. Therefore we conclude that H2AX phosphorylation and the interaction between p53 and γ-H2AX occur independent of DNA-PK.

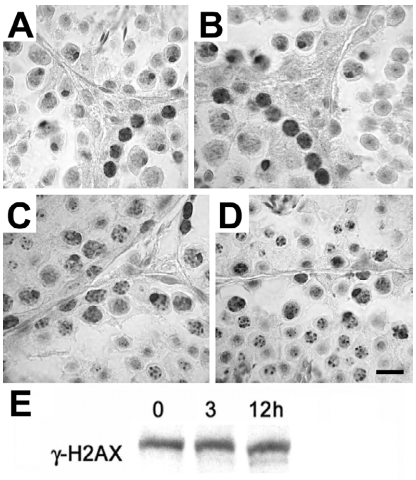


Figure 5. ***Presence of γ-H2AX in the non-irradiated (A&B) and irradiated (C&D) wildtype (A&C) and scid (B&D) mouse testis.*** *Magnification: bar represents 20 μm. Also in the scid testis no differences in γ-H2AX levels could be detected at various time points after treatment with ionizing radiation (IR)* ***(E)****.*

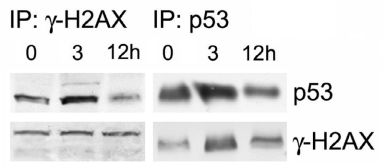


Figure 6. ***(co)-Immunoprecipitations (IP)*** *showing that the increasing interaction between γ-H2AX and p53 in response to ionizing radiation (IR) is not affected in the scid testis.*

DISCUSSION

During meiosis, Spo11 initiates DSBs that induce γ-H2AX foci in leptotene and early zygotene spermatocytes (33). However, the present data show that independent of irradiation clear γ-H2AX foci are already present in all intermediate and B spermatogonia remaining up until zygotene spermatocytes. Additionally, an even nuclear staining is present in type A spermatogonia and round spermatids whereas in pachytene spermatocytes only the sex bodies are stained. The expression pattern in the testis contradicts the assumed restriction of γ-H2AX localization to DSBs during spermatogenesis. Moreover, also for somatic cell lines, DSB-independent γ-H2AX formation already has been described, occurring at stalled replication forks (36). After irradiation, γ-H2AX foci also appeared in type A spermatogonia, pachytene spermatocytes and round spermatids.

In the testis, γ-H2AX appeared to interact with the tumor suppressor p53, which has been shown to play a key role in spermatogonial apoptosis (18-20). This interaction increased in response to IR. Although the current literature indicates the existence of a γ-H2AX-p53 pathway (12, 13), this is the first study in which an interaction between these proteins is shown. Although spermatocytes lightly express p53 (18, 37), the mouse germ cell types in which both γ-H2AX and p53 are elevated in response to irradiation **(Figures 1&3)** and subsequently undergo apoptosis are spermatogonia (18-20). Therefore one would expect the interaction between γ-H2AX and p53 to be confined to spermatogonia. This is supported by nuclear co-localization of p53 and γ-H2AX only in the irradiated spermatogonia. Apparently, spermatogonia and spermatocytes follow different apoptotic pathways, like described in a previous study that showed irradiation-induced spermatogonial apoptosis to be p53-dependent but the apoptotic elimination of spermatocytes with synaptic errors to be p53-independent (38).

As described previously for other systems (23-25), we now have proven that spermatogonial p53 induction occurs independent of DNA-PK. We also showed that phosphorylation of H2AX, γ-H2AX foci formation and the interaction of p53 with γ-H2AX in the testis are DNA-PK independent events. These results are consistent with other studies that have described DNA-PK independent phosphorylation of H2AX (15, 29) and activation of p53 (23-25). The most likely candidate for acting upstream of spermatogonial γ-H2AX foci formation and p53 activation seems to be DNA-PK's close relative ATM. ATM already was proven to be an important upstream activator of p53 (26-28) and recently also has been described to phosphorylate H2AX (29) and co-localize with γ-H2AX foci (30).

Based on our results and the literature, we propose the following sequence of events in irradiated spermatogonia: ATM phosphorylates H2AX that forms γ-H2AX foci at the sites of DSBs. At these sites γ-H2AX then recruits DNA-repair and damage-response proteins (12) including the p53 binding protein 53BP1 (13) and p53. p53 is then activated by ATM (26-28) and will induce spermatogonial apoptosis or cell cycle arrest (18-20).

Also during meiotic recombination DSBs are induced and γ-H2AX foci are formed (33). However, in spermatocytes, γ-H2AX does not co-localize with p53 and in early spermatocytes γ-H2AX appears independent of irradiation. Also after irradiation, when γ-H2AX foci appear in all pachytene spermatocytes and round spermatids **(Figures 1F&G)**, these foci do not co-localize with p53 **(Figure 3)** and indeed irradiation does not lead to apoptosis of these cells (3). Additionally, as mentioned before, irradiation-induced spermatogonial apoptosis is proven to be p53-dependent whereas the apoptotic elimination of spermatocytes with synaptic errors occurs independent of p53 (38). Apparently, γ-H2AX foci can lead to different cellular responses in different types of germ cells.

Using squashed germ cells and molecular markers, Mahadevaiah et al (33) describe γ-H2AX foci to appear in preleptotene and leptotene and to disappear in zygotene spermatocytes and

conclude that meiotic DSBs precede recombinational synapsis. However, using immunohistochemistry, we now show that, in the non-irradiated testis, γ-H2AX foci are already present in intermediate and B spermatogonia **(Figure 1A)**. If one assumes that γ-H2AX is a marker for DSBs this would mean that DSBs have to retain unrepaired during two mitotic divisions, or formed anew after each division, until the B spermatogonia divide into preleptotene spermatocytes. In this context, even γ-H2AX staining in preleptotene spermatocytes (33) is controversial as these cells represent the pre-meiotic S phase and are replicating their DNA to become 4C leptotene spermatocytes (39). Although we find γ-H2AX already present earlier in spermatogenesis, our results do not contradict the conclusion of Mahadevaiah et al (33) that DSBs precede recombinational synapsis, which is supported by using $Spo11^{-/-}$ mice. Meiotic DSBs are assumed to be generated by Spo11 (40, 41) but, although disappearing from leptotene and zygotene spermatocytes, γ-H2AX staining still remains in $Spo11^{-/-}$ preleptotene spermatocytes (33), which supports our data of H2AX phosphorylation before the leptotene stage when recombinational DSBs are induced. The detection or non-detection (33) of γ-H2AX in intermediate and B spermatogonia is most likely due to differences between the histological and cytological approach. Also the strong γ-H2AX staining of the sex body throughout the pachytene stage (33) **(Figures 1C&G)** is strange if it would be true that γ-H2AX is only present at DSBs. In the $H2AX^{-/-}$ testis the sex chromosomes fail to pair, and are fragmented or associated with autosomal chromosomes (16), indicating a specific function for H2AX in the sex body.

It has been well established that dividing cells in culture form γ-H2AX foci at DSBs (10-12). However, in the testis, various quiescent somatic cell types, like Sertoli cells, Leydig cells, myoid cells, and blood cells, do not form γ-H2AX foci, not even after treatment with IR, although DSBs are also introduced in these cells. Moreover, these cells do not induce p53 in response to irradiation (18-20) nor do they undergo apoptosis. Hence, in the testis different cell types respond differently to DSBs and the absence of γ-H2AX foci and p53 in the testicular quiescent cells very likely represents a general feature of quiescent cells *in vivo*.

A histone H2A variant from the mouse testis already has been characterized to be similar to H2AX (42) and the appearance of γ-H2AX very much coincides with the expression of this non-phosphorylated H2A in the testis (43). The absence of γ-H2AX in Leydig cells could thus be explained since these cells express H2A only very weakly. However, the non-phosphorylated H2A is distinctively expressed in Sertoli cells, again indicating that even the different somatic cell types in the testis display different chromatin organization and responses to DNA damage.

These findings raise the question whether H2AX phosphorylation in the testis is a marker for DSBs. During spermatogenesis the chromatin is constantly being reconstructed (44). These changes, like altering DNA methylation, already start in the mitotic spermatogonia (45). The major morphological difference between type A spermatogonia and intermediate and B spermatogonia is the appearance of heterochromatin in the nucleus (6-8). During the remodeling of euchromatin to heterochromatin, methylation and de-acetylation of histones play a key role (46) and perhaps in intermediate and B spermatogonia also H2AX phosphorylation is involved. In pachytene spermatocytes, when most of the linker H1 histones are replaced by the testis specific subtype H1t (44), γ-H2AX appears to have an essential function in the sex body (16, 33) **(Figure 1)**. Additionally, also histone ubiquitination is needed for chromatin modifications that occur at this stage as well as during the histone-to-protamine replacement in haploid spermatids (47, 48), whereas a function for histone phosphorylation at these stages has never even been considered. The currently known facts about histones and spermatogenesis, together with our results, indicate that the function of H2AX phosphorylation in the testis is not restricted to DSBs.

In response to IR, γ-H2AX foci appear, and in spermatogonia the interaction between γ-H2AX and p53, which induces spermatogonial apoptosis, increases. Independent of irradiation, γ-H2AX is thought to mark sites of meiotic DSBs (33). However, our results show that during spermatogenesis, γ-H2AX also has other functions that do not necessarily involve DSBs.

REFERENCES

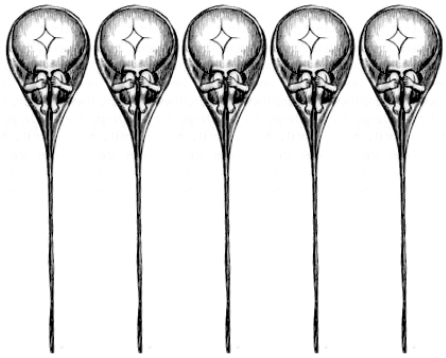
1. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Rutgers DH & de Rooij DG (1997)** p21(Cip1/WAF1) expression in the mouse testis before and after X irradiation *Mol Reprod Dev* **47,** 240-7.
2. **Hasegawa M, Wilson G, Russell LD & Meistrich ML (1997)** Radiation-induced cell death in the mouse testis: relationship to apoptosis *Radiat Res* **147,** 457-67.
3. **Oakberg EF & DiMinno RL (1960)** X-ray sensitivity of primary spermatocytes of the mouse *Int J Radiat Biol* **2,** 196-209.
4. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia *Radiat Res* **130,** 296-302.
5. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X irradiation *Radiat Res* **130,** 289-95.
6. **Chiarini-Garcia H & Russell LD (2001)** High-resolution light microscopic characterization of mouse spermatogonia *Biol Reprod* **65,** 1170-8.
7. **Chiarini-Garcia H & Russell LD (2002)** Characterization of mouse spermatogonia by transmission electron microscopy *Reproduction* **123,** 567-77.
8. **de Rooij DG, Russell LD. (2000)** All you wanted to know about spermatogonia but were afraid to ask. *J Androl* **21,** 776-98.
9. **Khanna KK & Jackson SP (2001)** DNA double-strand breaks: signaling, repair and the cancer connection *Nat Genet* **27,** 247-54.
10. **Rogakou EP, Pilch DR, Orr AH, Ivanova VS & Bonner WM (1998)** DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139 *J Biol Chem* **273,** 5858-68.
11. **Rogakou EP, Boon C, Redon C & Bonner WM (1999)** Megabase chromatin domains involved in DNA double-strand breaks in vivo *J Cell Biol* **146,** 905-16.
12. **Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M & Bonner WM (2000)** A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage *Curr Biol* **10,** 886-95.
13. **Rappold I, Iwabuchi K, Date T & Chen J (2001)** Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways *J Cell Biol* **153,** 613-20.
14. **Rogakou EP, Nieves-Neira W, Boon C, Pommier Y & Bonner WM (2000)** Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139 *J Biol Chem* **275,** 9390-5.
15. **Talasz H, Helliger W, Sarg B, Debbage PL, Puschendorf B & Lindner H (2002)** Hyperphosphorylation of histone H2A.X and dephosphorylation of histone H1 subtypes in the course of apoptosis *Cell Death Differ* **9,** 27-39.
16. **Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC & Nussenzweig A (2002)** Genomic instability in mice lacking histone H2AX *Science* **296,** 922-7.
17. **Bassing CH, Chua KF, Sekiguchi J, Suh H, Whitlow SR, Fleming JC, Monroe BC, Ciccone DN, Yan C, Vlasakova K, Livingston DM, Ferguson DO, Scully R & Alt FW (2002)** Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX *Proc Natl Acad Sci USA* **99,** 8173-8.
18. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
19. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
20. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
21. **Woo RA, McLure KG, Lees-Miller SP, Rancourt DE & Lee PW (1998)** DNA-dependent protein kinase acts upstream of p53 in response to DNA damage *Nature* **394,** 700-4.
22. **Wang S, Guo M, Ouyang H, Li X, Cordon-Cardo C, Kurimasa A, Chen DJ, Fuks Z, Ling CC & Li GC (2000)** The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest *Proc Natl Acad Sci USA* **97,** 1584-8.
23. **Jimenez GS, Bryntesson F, Torres-Arzayus MI, Priestley A, Beeche M, Saito S, Sakaguchi K, Appella E, Jeggo PA, Taccioli GE, Wahl GM & Hubank M (1999)** DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage *Nature* **400,** 81-3.
24. **Burma S, Kurimasa A, Xie G, Taya Y, Araki R, Abe M, Crissman HA, Ouyang H, Li GC & Chen DJ (1999)** DNA-dependent protein kinase-independent activation of p53 in response to DNA damage *J Biol Chem* **274,** 17139-43.
25. **Jhappan C, Yusufzai TM, Anderson S, Anver MR & Merlino G (2000)** The p53 response to DNA damage in vivo is independent of DNA-dependent protein kinase *Mol Cell Biol* **20,** 4075-83.

26. **Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB & Siliciano JD (1998)** Activation of the ATM kinase by ionizing radiation and phosphorylation of p53 *Science* **281,** 1677-9.
27. **Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP & Lavin MF (1998)** ATM associates with and phosphorylates p53: mapping the region of interaction *Nat Genet* **20,** 398-400.
28. **Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y & Ziv Y (1998)** Enhanced phosphorylation of p53 by ATM in response to DNA damage *Science* **281,** 1674-7.
29. **Burma S, Chen BP, Murphy M, Kurimasa A & Chen DJ (2001)** ATM phosphorylates histone H2AX in response to DNA double-strand breaks *J Biol Chem* **276,** 42462-7.
30. **Andegeko Y, Moyal L, Mittelman L, Tsarfaty I, Shiloh Y & Rotman G (2001)** Nuclear retention of ATM at sites of DNA double strand breaks *J Biol Chem* **276,** 38224-30.
31. **Chen HT, Bhandoola A, Difilippantonio MJ, Zhu J, Brown MJ, Tai X, Rogakou EP, Brotz TM, Bonner WM, Ried T & Nussenzweig A (2000)** Response to RAG-mediated VDJ cleavage by NBS1 and γ-H2AX *Science* **290,** 1962-5.
32. **Petersen S, Casellas R, Reina-San-Martin B, Chen HT, Difilippantonio MJ, Wilson PC, Hanitsch L, Celeste A, Muramatsu M, Pilch DR, Redon C, Ried T, Bonner WM, Honjo T, Nussenzweig MC & Nussenzweig A (2001)** AID is required to initiate NBS1/γ-H2AX focus formation and mutations at sites of class switching *Nature* **414,** 660-5.
33. **Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM & Burgoyne PS (2001)** Recombinational DNA double-strand breaks in mice precede synapsis *Nat Genet* **27,** 271-6.
34. **van Buul PP, Abramsson-Zetterberg L, Zandman IM & van Duyn-Goedhart A (1998)** Further characterization of the radiosensitivity of the scid mouse *Int J Radiat Biol* **74,** 35-41.
35. **Laemmli UK (1970)** Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature* **227,** 680-5.
36. **Ward IM & Chen J (2001)** Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress *J Biol Chem* **276,** 47759-62.
37. **Sjöblom T & Lähdetie J (1996)** Expression of p53 in normal and gamma-irradiated rat testis suggests a role for p53 in meiotic recombination and repair *Oncogene* **12,** 2499-505.
38. **Odorisio T, Rodriguez TA, Evans EP, Clarke AR & Burgoyne PS (1998)** The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis *Nat Genet* **18,** 257-61.
39. **Russell LD, Ettlin RA, Hikim APS & Clegg ED (1990)** Histological and histopathological evaluation of the testis *Cache River Press, Clearwater, Fl. USA*.
40. **Romanienko PJ & Camerini-Otero RD (2000)** The mouse Spo11 gene is required for meiotic chromosome synapsis *Mol Cell* **6,** 975-87.
41. **Baudat F, Manova K, Yuen JP, Jasin M & Keeney S (2000)** Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11 *Mol Cell* **6,** 989-98.
42. **Bhatnagar YM, Faulker RD & McCullar MK (1985)** Biochemical and immunological characterization of an H2A variant from the mouse testis *Biochim Biophys Acta* **827,** 14-22.
43. **Bhatnagar YM (1985)** Immunocytochemical localization of an H2A variant in the spermatogenic cells of the mouse *Biol Reprod* **32,** 957-68.
44. **Doenecke D, Drabent B, Bode C, Bramlage B, Franke K, Gavenis K, Kosciessa U & Witt O (1997)** Histone gene expression and chromatin structure during spermatogenesis *Adv Exp Med Biol* **424,** 37-48.
45. **Mertineit C, Yoder JA, Taketo T, Laird DW, Trasler JM & Bestor TH (1998)** Sex-specific exons control DNA methyltransferase in mammalian germ cells *Development* **125,** 889-97.
46. **Eberharter A & Becker PB (2002)** Histone acetylation: a switch between repressive and permissive chromatin: Second in review series on chromatin dynamics *EMBO Rep* **3,** 224-9.
47. **Roest HP, van Klaveren J, de Wit J, van Gurp CG, Koken MH, Vermey M, van Roijen JH, Hoogerbrugge JW, Vreeburg JT, Baarends WM, Bootsma D, Grootegoed JA & Hoeijmakers JH (1996)** Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification *Cell* **86,** 799-810.
48. **Baarends WM, Hoogerbrugge JW, Roest HP, Ooms M, Vreeburg J, Hoeijmakers JH & Grootegoed JA (1999)** Histone ubiquitination and chromatin remodeling in mouse spermatogenesis *Dev Biol* **207,** 322-33.

The authors thank Terry Ashley, Yale University School of Medicine, for her very useful advice and suggestions.

FUNCTION OF DNA-PKCS DURING THE EARLY MEIOTIC PROPHASE WITHOUT Ku70 & Ku86

Geert Hamer[1,2], Hermien L. Roepers-Gajadien[1,2], Annemarie van Duyn-Goedhart[3], Iris S. Gademan[4], Henk B. Kal[4], Paul P.W. van Buul[3], Terry Ashley[5] and Dirk G. de Rooij[1,2]



[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*

[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*

[3] *MCG-Department of Radiation Genetics and Chemical Mutagenesis, Sylvius Laboratory, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands*

[4] *Department of Radiotherapy, UMCU, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*

[5] *Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA*

Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PPW, Ashley T & de Rooij DG (2003) Function of DNA-PKcs during the early meiotic prophase without Ku70 and Ku86 *Biol Reprod* **68,** 717-21.

All components of the DNA double-strand break (DSB) repair complex DNA-PK, including Ku70, Ku86 and DNA-PKcs, were found in the radiosensitive spermatogonia. Although p53 induction is unaffected, spermatogonial apoptosis occurred faster in the irradiated DNA-PKcs-deficient *scid* testis. This suggests that spermatogonial DNA-PK functions in DNA damage repair rather than p53 induction. Despite the fact that early spermatocytes lack the Ku proteins, spontaneous apoptosis of these cells occurred in the *scid* testis. The majority of these apoptotic spermatocytes were found at stage IV of the cycle of the seminiferous epithelium where a meiotic checkpoint has been suggested to exist. Meiotic synapsis and recombination during the early meiotic prophase induce DSBs, which are apparently less accurately repaired in *scid* spermatocytes, which then fail to pass the meiotic checkpoint. The role for DNA-PKcs during the meiotic prophase differs from that in mitotic cells; it is not influenced by ionizing radiation (IR) and is independent of the Ku heterodimer.

INTRODUCTION

DNA-PK (DNA-dependent protein kinase) consists of a heteromeric DNA binding component (Ku70 and Ku86) and a DNA-PK catalytic subunit (DNA-PKcs). DNA-PKcs belongs to a larger family of phosphatidylinositol 3-kinases also including ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and RAD3 related) (1).

Apart from its function during rearrangement of the genes coding for antigen receptor molecules in lymphoid cells, DNA-PK is also involved in the cellular response to DNA damage, as caused by ionizing radiation (IR). The Ku heterodimer binds to single- and especially double-stranded DNA breaks (DSBs) followed by binding and activation of DNA-PKcs which then plays a key role in non-homologous end joining of the damaged DNA (2-6).

In the adult mouse testis the mitotically active spermatogonia are the most sensitive to IR whereas spermatocytes, which undergo meiotic cell divisions, and spermatids, that develop into spermatozoa, are more resistant (7-11).

The tumor suppressor p53 is induced in spermatogonia after irradiation and this protein plays a central role in DNA damage induced spermatogonial apoptosis (12-14). Despite the fact that DNA-PKcs also has been shown to be an upstream activator of p53 (15, 16), we found p53 induction to occur independent of DNA-PKcs (17), which is in line with several studies reporting DNA-PKcs deficient cells to have a normal response to IR with respect to p53 activation and cell cycle arrest (18-20).

However, during the early prophase of meiosis, from leptotene until early pachytene, breaks associated with meiotic synapsis and recombination (21) often also result in activation of proteins, such as ATM, that are normally activated in mitotic cells after DNA damage (22). This could also be true for DNA-PKcs, although Ku70 has been described not to be expressed in leptotene and zygotene spermatocytes (23). The absence of Ku70 at this stage might be very important to prevent non-homologous end joining to occur instead of meiotic homologous recombination. In order to understand more about the processing of DSBs during meiotic recombination, it is important to know exactly when and where DNA-PKcs and both the Ku proteins are expressed during spermatogenesis.

We have studied expression and localization of Ku70, Ku86 and DNA-PKcs in the testis before and after irradiation. Furthermore, using DNA-PKcs-deficient *scid* (24, 25) mice, we examined spermatogonial apoptosis in response to irradiation, and spontaneous apoptosis of spermatocytes.

MATERIALS & METHODS

Animals, irradiation and fixation

The testes of groups of 4 male FvB/NAU mice of at least 7 weeks of age were irradiated and fixed at various time intervals after treatment with a dose of 4 Gy of 200 kV X-rays as described previously (14, 26).

Homozygous *scid* mice (*scid/scid*) on a CB-17 genetic background (27) and wildtype CB-17 mice were propagated and irradiated as described (27), receiving a dose of 4 Gy of total body X-irradiation. Groups of 4 mice were sacrificed by cervical dislocation at 3 and 12 h after irradiation and one testis of each mouse was fixed using a diluted Bouin's fixation (14, 26) or an overnight fixation in 4% phosphate buffered (pH 6.6-7.2) formaldehyde, respectively. Control mice were sham-irradiated.

As described (14, 26), contralateral testes were used for protein isolation. The animals were used and maintained according to regulations provided by the animal ethical committee of the University Medical Center Utrecht that also approved the experiments.

Immunochemistry

Immunohistochemistry and Western blot analysis were performed as described previously (14, 26) using goat polyclonal Ku70 (M-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), goat polyclonal Ku86 (M-20, Santa Cruz Biotechnology Inc.), rabbit polyclonal DNA-PKcs (H-163, Santa Cruz Biotechnology Inc.) and goat polyclonal DNA-PKcs (C-19, Santa Cruz Biotechnology Inc) antibodies. For negative control sections 1 volume of primary antibodies was incubated with 5 volumes of blocking peptide (Santa Cruz Biotechnology Inc.) for 2 h. Adjacent sections were used for a periodic acid Schiff (PAS) staining to identify the stages of the cycle of the seminiferous epithelium.

TUNEL analysis

TUNEL analysis was performed on 5 µm paraffin sections of formalin fixed *scid* or CB-17 testes according to the manufacturers protocol (In Situ Cell Death Detection Kit, POD, Roche Diagnostics GmbH, Mannheim, Germany) (14). Adjacent sections were used for a periodic acid Schiff (PAS) staining to identify the stages of the cycle of the seminiferous epithelium.

Of every stage of the seminiferous epithelium the number of apoptotic spermatogonia/spermatocytes of at least 10 tubule cross sections were counted per mouse. The average numbers of apoptotic cells of 4 mice are depicted in **Figure 2**. Differences between wildtype and *scid* were statistically analyzed using the non-parametric Mann-Whitney test (two tailed P value < 0.05).

RESULTS

Expression of DNA-PKcs in the testis

The localization of DNA-PKcs was studied by way of immunohistochemistry on sections of sham-irradiated testes and testes fixed at 1.5 to 24 h after irradiation.

An intense, nuclear staining was found in spermatogonia, preleptotene spermatocytes and pachytene spermatocytes **(Figure 1A)**. Furthermore, all germ cells and somatic cells, including Leydig and Sertoli cells, showed a weak cytoplasmic staining for DNA-PKcs **(Figures 1A&B)**. This staining however, also appeared in the negative controls **(Figure 1B)**, just as the Golgi-like staining in round and elongated spermatids. The expression pattern did not change in response to irradiation. Negative controls were performed using antibodies that were pre-incubated with blocking peptide.

Expression of Ku70 and Ku86 in the testis

Ku70 and Ku86 were found to exhibit similar expression patterns **(Figures 1C&D)**. All cells showed a light cytoplasmic staining that also appeared in the negative controls. A stronger cytoplasmic staining, however, was seen in Leydig cells, whereas Sertoli cells were found to have a nuclear staining. Also type A spermatogonia showed a nuclear staining. However, intermediate spermatogonia and B spermatogonia appeared negative for the Ku proteins. Staining reappeared in pachytene spermatocytes of stage IV of the cycle of the seminiferous epithelium. Also round, but not elongating, spermatids showed a nuclear staining. The expression patterns of Ku70 and Ku86 did not change in response to irradiation.

Thus, all components of the DNA-PK complex are present in the nuclei of type A spermatogonia and pachytene spermatocytes from stage V onwards, whereas early spermatocytes only express DNA-PKcs and round spermatids only the Ku proteins.

Protein levels of DNA-PK do not change in response to irradiation

As immunohistochemistry is not suitable for quantification of antigens in sections, possible changes in protein levels in the testis before and after irradiation were studied using Western blot analysis of whole testis lysates of sham-irradiated mice and of mice sacrificed at 1.5 to 24 h post irradiation. Neither Ku70 and Ku86 nor DNA-PKcs levels were found to change after X-irradiation **(Figure 1G)**.

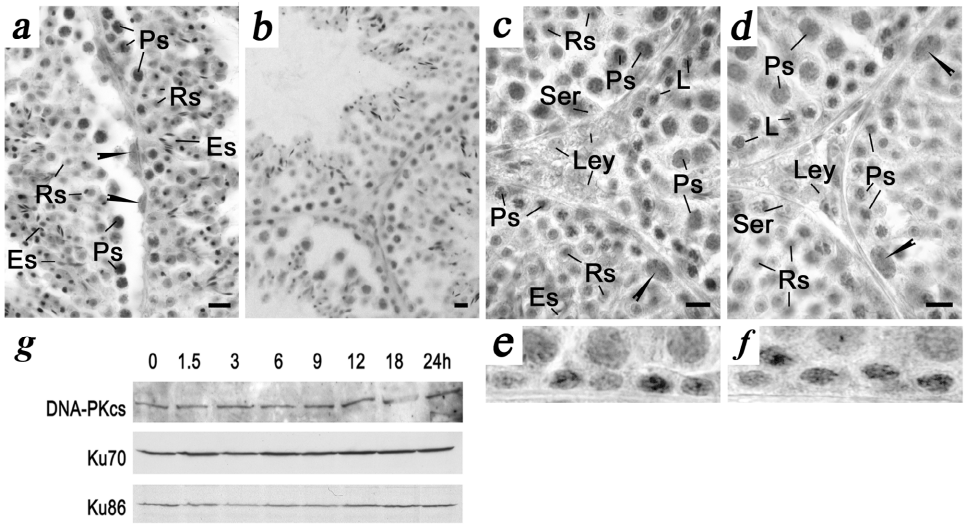


Figure 1. ***Expression and localization of DNA-PK in the testis.*** *Localization of DNA-PKcs* ***(A)****, negative control for DNA-PKcs showing cytoplasmic staining and Golgi-like staining in round and elongated spermatids* ***(B)****, Ku70* ***(C)****, Ku86* ***(D)*** *and B spermatogonia negative for Ku70* ***(E)*** *and Ku86* ***(F)****. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, L: leptotene spermatocytes, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids. Magnification: bar represents 20 μm.* ***(G)*** *Western blot analysis of DNA-PKcs, Ku70 and Ku86 in total testis lysates before and after various time intervals after irradiation showing no change in protein levels in response to ionizing radiation (IR). The results presented in this figure were obtained using FvB/NAU mice.*

Apoptosis in the scid testis

We studied the role of DNA-PK in apoptosis by comparing the number of apoptotic spermatogonia and spermatocytes before and 12 h after X-irradiation in *scid* and wildtype testes. Normally, after 12 h the most apoptotic spermatogonia are present (14), whereas after 24 h most spermatogonia will have disappeared [9,10]. In the irradiated wildtype testis many spermatogonia were TUNEL stained **(Figures 2C&2E)**. However, 12 h after irradiation, in *scid* testes the apoptotic response to irradiation of spermatogonia appeared much more severe **(Figures 2D&2E)**. Even Sertoli cells, which phagocytize apoptotic bodies, were often TUNEL stained in the *scid* testis **(Figure 2D)**, a response that was especially evident in stages V-VII of the cycle of the seminiferous epithelium where most apoptotic spermatogonia were observed **(Figure 2E)**. There are some apoptotic spermatogonia in the non-irradiated *scid* testis but when compared to the irradiated situation this is negligible. Hence, 12 h after irradiation, the increase of apoptotic spermatogonia is higher in the *scid* testis.

In sham-irradiated testes of both wildtype [14] and *scid* mice, very few TUNEL positive spermatogonia were found **(Figures 2A&2B)**. In *scid* testes, however, much higher numbers of apoptotic spermatocytes in early meiotic prophase were found than in the wildtype **(Figures 2B&2F)**. The great majority of these apoptotic spermatocytes were present in stage IV of the cycle of the seminiferous epithelium **(Figure 2F)**.

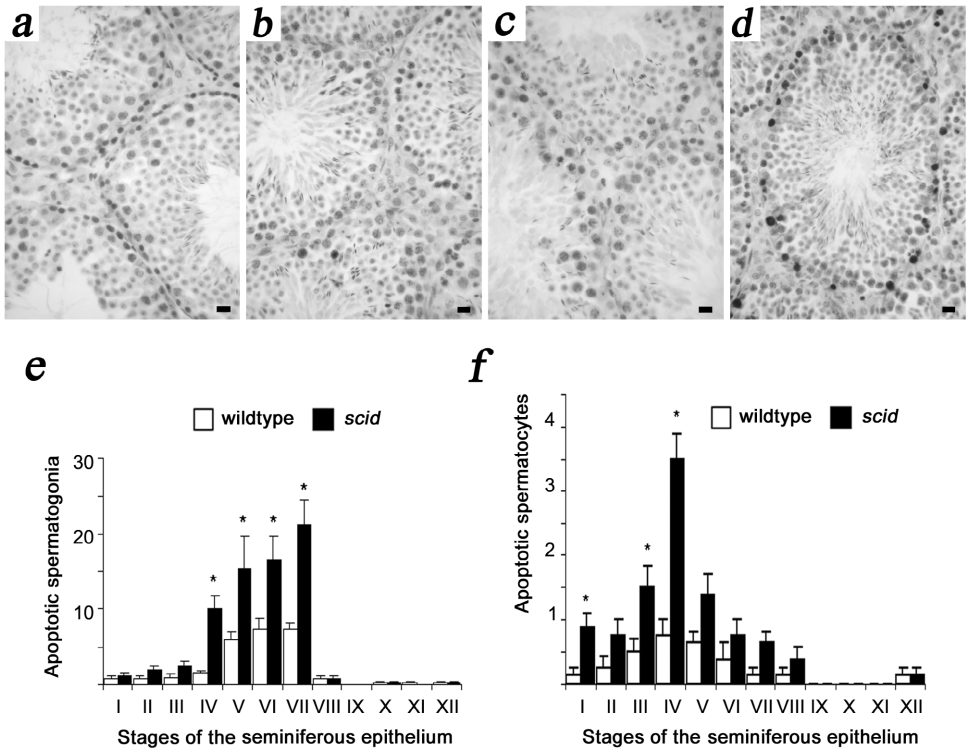


Figure 2. ***Apoptosis in scid and wildtype testes before and after irradiation.*** *TUNEL analysis is shown for sham-irradiated wildtype* ***(A)*** *and scid* ***(B)*** *testes and irradiated wildtype* ***(C)*** *and scid* ***(D)*** *testes. Average number of apoptotic spermatogonia 12 h after irradiation* ***(E)*** *or apoptotic spermatocytes independent from irradiation* ***(F)*** *per tubule cross-section at different stages of the seminiferous epithelium. Of every stage of the seminiferous epithelium the number of apoptotic spermatogonia or spermatocytes of at least 10 tubule cross sections were counted per mouse. The average numbers of apoptotic cells of 4 mice are depicted; error bars depict the standard error of the mean (SEM). Difference between wildtype and scid is statistically analyzed using the non-parametric Mann-Whitney test (two tailed P value < 0.05*). The results presented in this figure were obtained using mice on a CB-17 genetic background.*

DISCUSSION

Type A spermatogonia express all components of the DNA-PK complex, including Ku70, Ku86 and DNA-PKcs **(Figures 1&3)**. Although p53 activation has been reported to be dependent of DNA-PKcs (15, 16), we found spermatogonial p53 induction to be independent of DNA-PKcs (17), which is in line with several other studies that also describe DNA-PK independent p53 activation (18-20). High p53 levels and subsequent induction of apoptosis in *scid* spermatogonia most likely result from the persistence of DSBs, since inactive DNA-PKcs interferes with DNA repair (1).

Hence, in spermatogonia, DNA-PK detects and assists in repair of DSBs caused by IR. In the *scid* testis these DNA breaks remain non-repaired and the persistent DSBs probably activate ATM, another DSB-associated protein that can induce and activate p53 (28-30) and subsequently spermatogonial apoptosis (12-14). In wildtype spermatogonia many DSBs are more efficiently repaired, resulting in a lower number of apoptotic cells per tubule cross-section 12 h after irradiation **(Figure 2E)**.

Early spermatocytes, until stage IV pachytene, also express DNA-PKcs but do not express the Ku proteins **(Figures 1&3)**. Until now, Ku independent DNA binding and activation of DNA-PKcs only has been described *in vitro* (31). However, the significantly higher number of spontaneously apoptotic early spermatocytes in the *scid* testes **(Figure 2F)** suggests that, in the normal testis, DNA-PKcs has a function in early spermatocytes that does not include the Ku heterodimer and is not influenced by IR. DNA-PK has been shown to suppress homologous recombination (32), which would seriously interfere with meiotic recombination. Although this suppression was found to depend on functional DNA-PKcs (32), DNA-PKcs still remains present **(Figure 1A)** and functional **(Figure 2F)** in early spermatocytes. Taken together, these results suggest that the complete DNA-PK holo-enzyme is required for suppression of homologous recombination. To prevent suppression of meiotic recombination the Ku proteins are very specifically down regulated in early spermatocytes **(Figures 1C,1D&3)**. This theory is supported by findings that interruption of the DNA-PK pathway stimulates homologous recombination (33, 34).

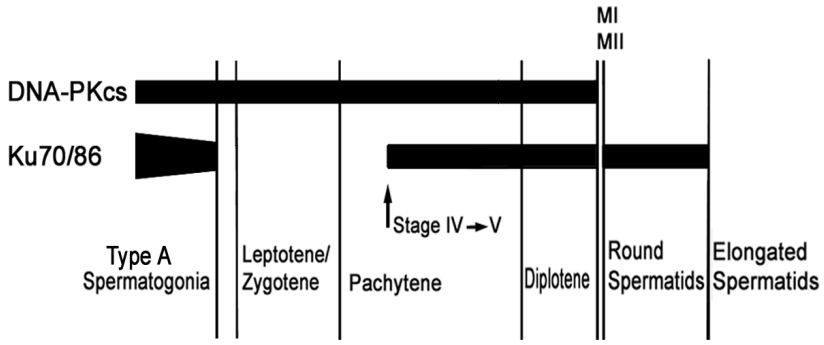


Figure 3. ***Schematic representation of the intensity of immunohistochemical staining of the different DNA-PK subunits at different stages of spermatogenesis*** *(adapted and extended from Goedecke et al., 1999) (23). The width of the lines represents the relative intensity of the staining. MI and MII represent the two meiotic divisions.*

Furthermore, round spermatids express Ku70 and Ku86 but lack DNA-PKcs **(Figures 1&3)**. Ku86 plays a key role in function and maintenance of the telomeres (35, 36), and in round spermatids the Ku proteins, without DNA-PKcs, might prevent telomeric fusions occurring in the germ line.

Although non-homologous end-joining plays a dominant role in DNA repair during the G_1 to early S-phase its role during late S to G_2 –phase is only minor (37). Since non-homologous end joining would also interfere with proper meiotic recombination it is interesting that during the meiotic prophase chromosomal radiosensitivity seems comparable with that during the somatic G_2 phase (27).

Whereas DNA-PK activity during the G_1 to early S phase has been described to correlate with IR and DSB repair, its activity during the G_2 phase is more likely required for exit from a DNA damage-induced G_2 checkpoint arrest (38). Without DNA-PKcs many spermatocytes undergo apoptosis at stage IV of the cycle of the seminiferous epithelium. This confirms and extends the notion that a meiotic checkpoint exists at stage IV, when spermatocytes with damaged DNA are induced to undergo apoptosis. At this epithelial stage spermatogenesis is known to abrogate in several knockout mice, including ATM (39)(de Rooij, unpublished data) and MSH5 (40). DSBs are associated with meiotic synapsis and recombination (21), and are not efficiently repaired in *scid* spermatocytes, resulting in more apoptosis and failure to pass epithelial stage IV.

Hence, in the testis, DNA-PKcs has a dual function; in the mitotic and radiosensitive spermatogonia it co-operates with the Ku heterodimer in order to repair radiation-induced DSBs, whereas during the meiotic prophase it appears to guard genome integrity as early spermatocytes undergo meiotic synapsis and recombination and is not influenced by IR and independent of the Ku heterodimer.

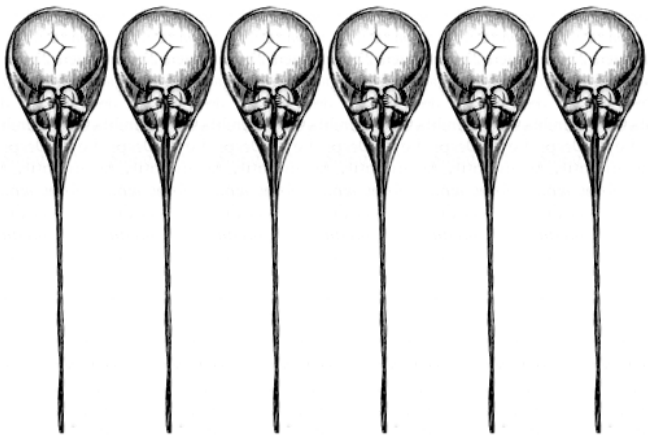
REFERENCES

1. **Smith GC & Jackson SP (1999)** The DNA-dependent protein kinase *Genes Dev* **13,** 916-34.
2. **Walker JR, Corpina RA & Goldberg J (2001)** Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair *Nature* **412,** 607-14.
3. **Hammarsten O, DeFazio LG & Chu G (2000)** Activation of DNA-dependent protein kinase by single-stranded DNA ends *J Biol Chem* **275,** 1541-50.
4. **Gottlieb TM & Jackson SP (1993)** The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen *Cell* **72,** 131-42.
5. **Suwa A, Hirakata M, Takeda Y, Jesch SA, Mimori T & Hardin JA (1994)** DNA-dependent protein kinase (Ku protein-p350 complex) assembles on double-stranded DNA *Proc Natl Acad Sci USA* **91,** 6904-8.
6. **DeFazio LG, Stansel RM, Griffith JD & Chu G (2002)** Synapsis of DNA ends by DNA-dependent protein kinase *Embo J* **21,** 3192-200.
7. **Oakberg EF & DiMinno RL (1960)** X-ray sensitivity of primary spermatocytes of the mouse *Int J Radiat Biol* **2,** 196-209.
8. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Rutgers DH & de Rooij DG (1997)** p21(Cip1/WAF1) expression in the mouse testis before and after X irradiation *Mol Reprod Dev* **47,** 240-7.
9. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia *Radiat Res* **130,** 296-302.
10. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity of quiescent and proliferating mouse spermatogonial stem cells to X irradiation *Radiat Res* **130,** 289-95.
11. **Hasegawa M, Wilson G, Russell LD & Meistrich ML (1997)** Radiation-induced cell death in the mouse testis: relationship to apoptosis *Radiat Res* **147,** 457-67.
12. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
13. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
14. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
15. **Woo RA, McLure KG, Lees-Miller SP, Rancourt DE & Lee PW (1998)** DNA-dependent protein kinase acts upstream of p53 in response to DNA damage *Nature* **394,** 700-4.
16. **Wang S, Guo M, Ouyang H, Li X, Cordon-Cardo C, Kurimasa A, Chen DJ, Fuks Z, Ling CC & Li GC (2000)** The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest *Proc Natl Acad Sci USA* **97,** 1584-8.
17. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP & de Rooij DG (2003)** DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.
18. **Jimenez GS, Bryntesson F, Torres-Arzayus MI, Priestley A, Beeche M, Saito S, Sakaguchi K, Appella E, Jeggo PA, Taccioli GE, Wahl GM & Hubank M (1999)** DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage *Nature* **400,** 81-3.
19. **Burma S, Kurimasa A, Xie G, Taya Y, Araki R, Abe M, Crissman HA, Ouyang H, Li GC & Chen DJ (1999)** DNA-dependent protein kinase-independent activation of p53 in response to DNA damage *J Biol Chem* **274,** 17139-43.
20. **Jhappan C, Yusufzai TM, Anderson S, Anver MR & Merlino G (2000)** The p53 response to DNA damage in vivo is independent of DNA-dependent protein kinase *Mol Cell Biol* **20,** 4075-83.
21. **Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM & Burgoyne PS (2001)** Recombinational DNA double-strand breaks in mice precede synapsis *Nat Genet* **27,** 271-6.
22. **Plug AW, Peters AH, Xu Y, Keegan KS, Hoekstra MF, Baltimore D, de Boer P & Ashley T (1997)** ATM and RPA in meiotic chromosome synapsis and recombination *Nat Genet* **17,** 457-61.
23. **Goedecke W, Eijpe M, Offenberg HH, van Aalderen M & Heyting C (1999)** MRE11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis *Nat Genet* **23,** 194-8.
24. **Blunt T, Finnie NJ, Taccioli GE, Smith GC, Demengeot J, Gottlieb TM, Mizuta R, Varghese AJ, Alt FW & Jeggo PA (1995)** Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation *Cell* **80,** 813-23.
25. **Kirchgessner CU, Patil CK, Evans JW, Cuomo CA, Fried LM, Carter T, Oettinger MA & Brown JM (1995)** DNA-dependent kinase (p350) as a candidate gene for the murine scid defect *Science* **267,** 1178-83.
26. **Hamer G, Gademan IS, Kal HB & de Rooij DG (2001)** Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.
27. **van Buul PP, Abramsson-Zetterberg L, Zandman IM & van Duyn-Goedhart A (1998)** Further characterization of the radiosensitivity of the scid mouse *Int J Radiat Biol* **74,** 35-41.

28. **Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB & Siliciano JD (1998)** Activation of the ATM kinase by ionizing radiation and phosphorylation of p53 *Science* **281,** 1677-9.
29. **Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP & Lavin MF (1998)** ATM associates with and phosphorylates p53: mapping the region of interaction *Nat Genet* **20,** 398-400.
30. **Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y & Ziv Y (1998)** Enhanced phosphorylation of p53 by ATM in response to DNA damage *Science* **281,** 1674-7.
31. **Hammarsten O & Chu G (1998)** DNA-dependent protein kinase: DNA binding and activation in the absence of Ku *Proc Natl Acad Sci USA* **95,** 525-30.
32. **Allen C, Kurimasa A, Brenneman MA, Chen DJ & Nickoloff JA (2002)** DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination *Proc Natl Acad Sci USA* **99,** 3758-63.
33. **Delacote F, Han M, Stamato TD, Jasin M & Lopez BS (2002)** An XRCC4 defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells *Nucleic Acids Res* **30,** 3454-63.
34. **Pierce AJ, Hu P, Han M, Ellis N & Jasin M (2001)** Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells *Genes Dev* **15,** 3237-42.
35. **Samper E, Goytisolo FA, Slijepcevic P, van Buul PP & Blasco MA (2000)** Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang *EMBO Rep* **1,** 244-52.
36. **Li G, Nelsen C & Hendrickson EA (2002)** Ku86 is essential in human somatic cells *Proc Natl Acad Sci USA* **99,** 832-7.
37. **Yoshida M, Hosoi Y, Miyachi H, Ishii N, Matsumoto Y, Enomoto A, Nakagawa K, Yamada S, Suzuki N & Ono T (2002)** Roles of DNA-dependent protein kinase and ATM in cell-cycle-dependent radiation sensitivity in human cells *Int J Radiat Biol* **78,** 503-12.
38. **Lee SE, Mitchell RA, Cheng A & Hendrickson EA (1997)** Evidence for DNA-PK-dependent and -independent DNA double-strand break repair pathways in mammalian cells as a function of the cell cycle *Mol Cell Biol* **17,** 1425-33.
39. **Barlow C, Liyanage M, Moens PB, Tarsounas M, Nagashima K, Brown K, Rottinghaus S, Jackson SP, Tagle D, Ried T & Wynshaw-Boris A (1998)** ATM deficiency results in severe meiotic disruption as early as leptonema of prophase I *Development* **125,** 4007-17.
40. **de Vries SS, Baart EB, Dekker M, Siezen A, de Rooij DG, de Boer P & te Riele H (1999)** Mouse MutS-like protein MSH5 is required for proper chromosome synapsis in male and female meiosis *Genes Dev* **13,** 523-31.

ATM EXPRESSION & ACTIVATION IN THE TESTIS

Geert Hamer[1,2], Iris S. Gademan[3], Henk B. Kal[3], Terry Ashley[4], Dirk G. de Rooij[1,2]



[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*

[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

[3] *Department of Radiotherapy, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

[4] *Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA*

Ionizing radiation (IR) and consequently induction of DNA double-strand breaks (DSBs) cause activation of ATM. Normally, ATM is present as inactive dimers, however, in response to DSBs the ATM dimer partners cross-phosphorylate each other and kinase active ATM monomers are subsequently released. We have studied the presence of both non-phosphorylated as well as active phosphorylated ATM in the testis. During spermatogenesis DSBs are not only induced by IR but are also endogenously induced during the first meiotic prophase. In spermatogonia ATM became activated by cross-phosphorylation in response to IR. However, although ATM appeared present in both spermatogonia and spermatocytes, phosphorylated ATM did not appear in spermatocytes after treatment with IR. In these cells phosphorylated ATM was only present in the sex bodies that contain the X and Y-chromosomes. These results show that spermatogonial ATM and ATM in the spermatocytes are differentially regulated. In the spermatogonia ATM is activated by cross-phosphorylation whereas in spermatocytes, during meiosis, non-phosphorylated ATM is already active. Additionally, in the sex bodies ATM is constitutively phosphorylated. In these structures it might be involved in meiotic X-chromosome inactivation and guarding integrity of the X and Y-chromosomes during the first meiotic prophase.

INTRODUCTION

ATM (ataxia telangiectasia mutated) is part of a family of phosphatidylinositol 3-kinases that includes DNA-PK (DNA-dependent protein kinase) and ATR (ataxia telangiectasia and RAD3 related) (1-6). Induction of DNA damage triggers activation of ATM, which is involved in most, if not all, cellular responses to DNA double-strand breaks (DSBs) like apoptosis, cell cycle arrest and DNA damage repair (1-6). Normally, ATM exists in the cell as inactive dimers. However, very shortly after induction of DSBs, for instance by ionizing radiation (IR), the ATM dimer partners cross-phosphorylate each other and are subsequently released as kinase active monomers (7, 8) that are capable of interacting with DNA and DSBs (9). ATM kinase activity gets induced very rapidly in response to IR (10, 11) and lies at the basis of most cellular responses to DSBs (4, 6).

ATM has been shown to be an important upstream regulator of the tumor suppressor p53 (10-13), which, in spermatogonia, plays a central role in DNA damage induced spermatogonial apoptosis (14-16). Furthermore, functional ATM is also required for cell cycle arrest at the G1, G_2/M and S phase and is responsible for phosphorylation and activation of several proteins involved in cell cycle regulation (10-13, 17-27).

ATM is also involved in DNA damage repair (28-38), especially in the repair of DSBs as caused by IR (3, 5, 6). Within minutes after induction of DSBs a substantial fraction of the nuclear ATM pool relocates to the damaged DNA and phosphorylates histone H2AX (39-41). This results in the appearance of γ-H2AX (phosphorylated H2AX) foci specifically at the sites of DSBs (42, 43). γ-H2AX foci function as docking sites for many DNA damage repair proteins and are thus essential for proper DNA damage repair (43-45). Subsequently, the proteins sequestered by γ-H2AX in IR induced foci (IRIF) are in their turn often also phosphorylated and activated by ATM that remains present at the sites of damage (3, 5, 6, 46).

DSBs are predominately repaired in two ways: the error prone non-homologous end joining (NHEJ), during which two double-stranded DNA strands are just bluntly attached and the accurate homologous recombinational repair (HRR) that uses the homologous DNA strand as a template for repair (28-38). Whereas the role for the ATM family member DNA-PK seems restricted to NHEJ, ATM itself is essential for HRR (3, 5, 6, 28-38) and activates many

proteins involved in this process like the RAD50/MRE11/NBS1 complex (47) and BRCA1 and 2 (48-51).

In the testis, HRR plays an especially important role as DSBs are also endogenously induced during the first meiotic prophase (52). These meiotic DSBs are essential for the meiotic process and are repaired by meiotic recombination, which is a meiosis specific variant of HRR (53). ATM localizes at the sites of meiotic DSBs and recombination (54) and non-functional ATM results in meiotic arrest during the meiotic prophase I (55-57) due to abnormal chromosomal synapsis and subsequent chromosome fragmentation (58) combined with altered interactions of telomeres with the nuclear matrix and distorted meiotic telomere clustering (59).

We have studied expression and localization of ATM in the testis before and after treatment with IR. Furthermore, using an antibody that specifically recognizes cross-phosphorylated ATM (7), we were able to compare ATM activation in response to IR with the presence of endogenously active ATM during meiosis. Finally, using $ATM^{-/-}$ testes, we studied in more detail at which exact stage spermatogenic arrest occurs during the meiotic prophase I.

MATERIALS & METHODS

Animals, irradiation and fixation

Testes of groups of 4 male FvB/NAU mice of at least 7 weeks of age were irradiated and fixed at various time intervals after treatment with a dose of 4 Gy of 200 kV X-rays as described previously, using the contralateral testes for protein isolation (60-62). The animals were used and maintained according to the regulations provided by the animal ethical committee of the University Medical Center Utrecht that also approved the experiments.

$ATM^{-/-}$ testis sections were kindly provided by Christoph H. Westphal, Harvard Medical School, Boston, Massachusetts, USA. $ATM^{-/-}$ mice were generated as described (55).

Immunochemistry

Immunohistochemistry and western blot analysis were performed as described previously (60-62) using goat polyclonal antibodies against total ATM (C-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rabbit monoclonal antibodies against phosphorylated ATM (pS1981-ATM, Rockland, Gilbertsville, PA, USA). For negative control sections 1 volume of primary antibodies was incubated with 5 volumes of blocking peptide (Santa Cruz Biotechnology Inc. or Rockland respectively) for 2 h. Adjacent sections were used for a periodic acid Schiff (PAS) staining to identify the stages of the cycle of the seminiferous epithelium.

RESULTS

Localization of ATM in the testis, before and after X-irradiation

In order to investigate ATM localization during spermatogenesis, tubular cross sections of mouse testes fixed before and at various time points (0-24 h) after irradiation were immunohistochemically stained using a polyclonal antibody against ATM **(Figures 1A&B)**. In the normal adult testis all germ cells showed a granular staining in the cytoplasm. Type A spermatogonia also showed a light granular staining in the nucleus, which became more pronounced and evenly spread in intermediate spermatogonia. From the B spermatogonia onwards to the pachytene spermatocytes in stage VII a clear nuclear staining was observed. No nuclear staining was observed in later spermatogenic cell types. Neither the myoid cells nor the Sertoli cells showed any staining. The Leydig cells, however, showed a heavy granular staining in the cytoplasm but no staining in the nucleus.

No changes in ATM localization were found in response to IR. Negative controls were performed using an antibody specific blocking peptide. No staining, apart from a vague cytoplasmic staining throughout the testis, could be observed in any of the negative controls.

To investigate whether ATM levels in the mouse testis change after irradiation a western blot analysis was performed on whole testis lysates of normal and irradiated testes **(Figure 1C)**. ATM levels in whole testis lysates were found not to change in response to irradiation.

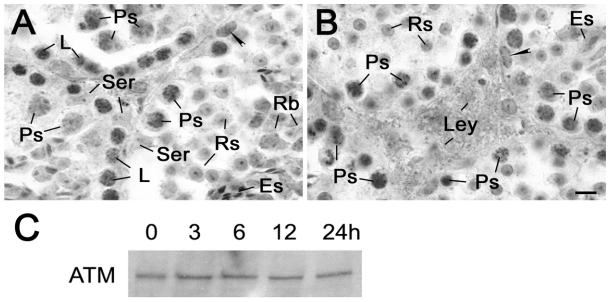


Figure 1. ***Expression and localization of ATM in the testis.*** *Localization of ATM in the testis* ***(A&B)****. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, L: leptotene spermatocytes, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids. Magnification: bar represents 20 μm.* ***(C)*** *Western blot analysis of ATM in total testis lysates before and after various time intervals after irradiation showing no change in protein levels in response to ionizing radiation (IR).*

ATM activation in the testis

To study when and where during spermatogenesis ATM gets activated we used an antibody that specifically recognizes phosphorylated ATM (7). In contrast to the staining pattern found using antibodies that recognize total ATM, phosphorylated ATM appeared only to be present in the sex bodies of pachytene spermatocytes **(Figures 2A&B)**.

However, after irradiation, active, phosphorylated ATM was observed in the nuclei of all spermatogonia and very clearly in Sertoli cells **(Figures 2C&D)**. However, no extra nuclear staining, next to the already present staining of the sex bodies, appeared in the pachytene spermatocytes. This staining pattern was found at all time points after irradiation up until 24 h.

Again, negative controls were performed using an antibody specific blocking peptide, giving no staining apart from a vague cytoplasmic staining throughout the testis and occasional cytoplasmic "dots" in Leydig cells, elongated spermatids and residual bodies.

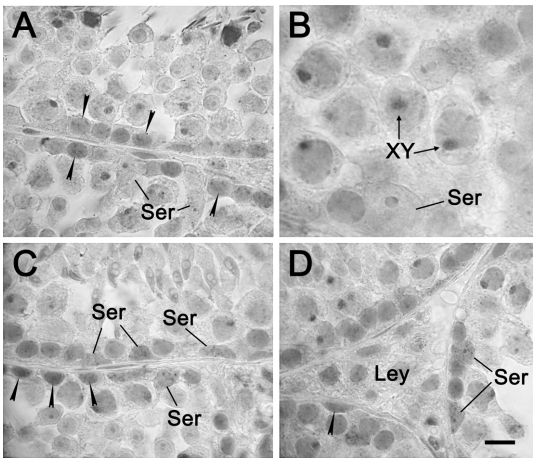


Figure 2. ***Phosphorylation of ATM in the testis.*** *Localization of phosphorylated ATM in the testis before* ***(A&B)*** *and after* ***(C&D)*** *irradiation. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, XY: sex bodies in pachytene spermatocytes. Magnification: bar represents 20 μm or 10 μm in panel B.*

Stage IV pachytene arrest in ATM$^{-/-}$ testes

Finally, in order to investigate in more detail at which exact stage spermatogenic arrest occurs during the meiotic prophase I without ATM, we studied sections of ATM$^{-/-}$ testes. In contrast to the wildtype **(Figure 3A),** spermatogenesis in the ATM$^{-/-}$ testis did not proceed any further than pachytene stage IV of the cycle of seminiferous epithelium **(Figures 3B,C&D)**. While before this point spermatogenesis appeared normal, during stage IV massive apoptosis of spermatocytes occurred in such a way that no germ cells were observed in subsequent stages.

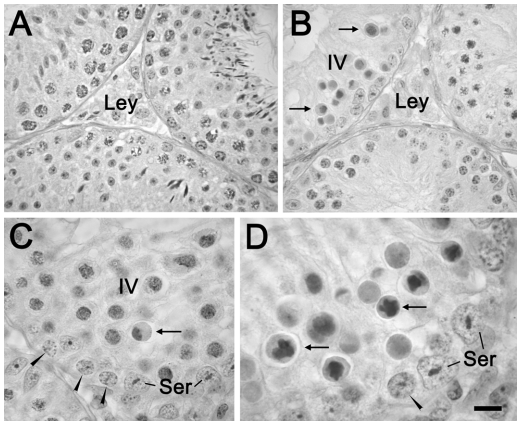


Figure 3. ***Spermatogenesis in the ATM$^{-/-}$ testis.*** *Spermatogenesis in the wildtype testis* ***(A)*** *and ATM$^{-/-}$ testis* ***(B,C&D)****. Arrows show apoptotic stage IV pachytene spermatocytes, arrowheads show intermediate spermatogonia, Ley: Leydig cells, Ser: Sertoli cells. Magnification: bar represents 40 μm* ***(A&B)*** *20 μm* ***(C)*** *or 15 μm* ***(D)****.*

DISCUSSION

In spermatogonia ATM is present before and after irradiation. However, using an antibody specific for phosphorylated ATM, we find activated ATM only in irradiated spermatogonia. Hence, as described for cultured somatic cells (7), in these germ cells "dormant" ATM dimers get activated by auto-cross-phosphorylation. Subsequently, activated ATM will phosphorylate histone H2AX (39-41) that forms γ-H2AX foci at the sites of DSBs (61). These foci then recruit DNA-repair and damage-response proteins (43), including p53, that subsequently also get activated by ATM (10-13). Finally, in spermatogonia, p53 will induce apoptosis or cell cycle arrest (14-16).

Also in Sertoli cells ATM phosphorylation occurs in response to IR, which, also in these cells, exactly coincides with induction of p53 (14, 61). Presence of phosphorylated ATM in the Sertoli cells, however, appears controversial since the antibody that should recognize all ATM never stains these cells. Additionally, the Sertoli cells do not have a regular response to IR. Although inducing both ATM phosphorylation as well as p53, they are very radio-resistant and never undergo apoptosis in response to IR (14, 62, 63). The latter is most likely due to the fact that, in the adult testis, Sertoli cells do not divide.

During spermatogenesis, DSBs are also endogenously induced and repaired by meiotic recombination (52, 53). During normal homologous recombination (HRR) ATM plays an essential role (3, 5, 6, 28-38), activating many proteins like the RAD50/MRE11/NBS1 complex (47) and BRCA1 & 2 (48-51). Therefore, it is not surprising that, when studying surface spreads of spermatocytes, ATM also localizes at the sites of meiotic DSBs and recombination (54). When ATM is not functional a meiotic arrest is found at the zygotene/pachytene stage of prophase I as a result of abnormal chromosomal synapsis and subsequent chromosome fragmentation (58) and altered interactions of telomeres with the nuclear matrix combined with distorted meiotic telomere clustering (59).

However, although non-phosphorylated ATM is present in spermatocytes until stage VII, we only find phosphorylated ATM in the sex bodies of these cells. This is surprising since ATM clearly plays an active role during meiotic recombination (54, 58, 59, 64). Additionally, in response to IR, clear γ-H2AX foci also appear in pachytene spermatocytes (61), suggesting that, in these cells, ATM phosphorylation is not essential for γ-H2AX foci formation. Apparently, at this stage of meiosis, ATM does not need to be phosphorylated in order to be activated or is perhaps phosphorylated at a different site. These are major differences in comparison to DNA repair of IR induced DSBs, like found for irradiated spermatogonia and other mitotic cells (7). Another explanation could be that, during the repair of meiotic DSBs, ATM is only present in small amounts or only needs to be phosphorylated for a very short time. This explanation, however, is not in line with the fact that IR induced ATM phosphorylation remains present for a relatively long period of time, in the testis at least until 24h after irradiation.

The staining pattern of phosphorylated ATM in the testis, before and after treatment with IR, coincides almost exactly with that of p53 (14, 61). The only difference is that p53 is not present in the sex bodies of spermatocytes that contain the X and Y-chromosomes. Although they are both induced in irradiated spermatogonia, neither p53 nor phosphorylated ATM are induced in spermatocytes in response to IR (14, 61). Moreover, whereas in spermatogonia p53 plays a central role in the induction of IR induced apoptosis (14-16), the apoptotic elimination of spermatocytes with synaptic errors has been described to be p53-independent (65). Hence, although active during meiotic recombination (54, 58), ATM in spermatocytes remains non-phosphorylated and probably does not activate p53. On the other hand, in the cells

(spermatogonia and Sertoli cells) in which p53 is induced in response to IR also ATM gets phosphorylated.

Intriguing is the presence of phosphorylated ATM in the sex bodies of spermatocytes. This presence coincides with the presence of γ-H2AX, which is not only present in the sex bodies (52, 61), but also is essential for their formation and functionality (66, 67). In the $H2AX^{-/-}$ testis pairing of the sex chromosomes fails and, as a consequence, they become fragmented or associated with autosomal chromosomes (66). Additionally, in $H2AX^{-/-}$ spermatocytes, meiotic sex chromosome inactivation is not initiated and several sex body specific proteins are not recruited (67). Possibly, as for damaged DNA (39-41), ATM is also responsible for histone H2AX phosphorylation in the sex bodies. Also in $H2AX^{-/-}$ (66, 67) testes spermatogenesis is arrested around stage V of the cycle of the seminiferous epithelium and in the $ATM^{-/-}$ testes this arrest could be partly due to failure in histone H2AX phosphorylation. Furthermore, in the sex bodies, ATM could also be responsible for activation of BRCA1 (48-51), a process that is required for γ-H2AX to appear in the sex bodies (68).

The phenotype of the $ATM^{-/-}$ testis confirms and extends the notion that a meiotic checkpoint exists at stage IV, when spermatocytes with damaged DNA are induced to undergo apoptosis (69). At this epithelial stage spermatogenesis is known to be interrupted in several transgenic mice, like the $MSH5^{-/-}$ mice, that have a spermatogenic phenotype similar to $ATM^{-/-}$ (70), and the DNA-PKcs deficient *scid* mice, in which many spermatocytes fail to pass epithelial stage IV and undergo apoptosis (62).

In conclusion, ATM is functional during the spermatogonial DNA damage response and during meiotic recombination (54, 58, 59, 64). Whereas, spermatogonial ATM is activated by cross-phosphorylation, during meiotic recombination ATM remains non-phosphorylated. Additionally, phosphorylated ATM is also present in the sex bodies of pachytene spermatocytes. In these structures it might be responsible for phosphorylation of proteins including H2AX and BRCA1. These proteins are somehow involved in meiotic X-chromosome inactivation (67, 68, 71) but are certainly also important for keeping the X and Y-chromosomes intact during the first meiotic prophase (66).

REFERENCES

1. **Westphal CH (1997)** Cell-cycle signaling: ATM displays its many talents *Curr Biol* **7,** R789-92.
2. **Smith GC & Jackson SP (1999)** The DNA-dependent protein kinase *Genes Dev* **13,** 916-34.
3. **Kastan MB & Lim DS (2000)** The many substrates and functions of ATM *Nat Rev Mol Cell Biol* **1,** 179-86.
4. **Abraham RT (2001)** Cell cycle checkpoint signaling through the ATM and ATR kinases *Genes Dev* **15,** 2177-96.
5. **Shiloh Y & Kastan MB (2001)** ATM: genome stability, neuronal development, and cancer cross paths *Adv Cancer Res* **83,** 209-54.
6. **Shiloh Y (2003)** ATM and related protein kinases: safeguarding genome integrity *Nat Rev Cancer* **3,** 155-68.
7. **Bakkenist CJ & Kastan MB (2003)** DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation *Nature* **421,** 499-506.
8. **Bartek J & Lukas J (2003)** DNA repair: Damage alert *Nature* **421,** 486-8.
9. **Llorca O, Rivera-Calzada A, Grantham J & Willison KR (2003)** Electron microscopy and 3D reconstructions reveal that human ATM kinase uses an arm-like domain to clamp around double-stranded DNA *Oncogene* **22,** 3867-74.
10. **Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y & Ziv Y (1998)** Enhanced phosphorylation of p53 by ATM in response to DNA damage *Science* **281,** 1674-7.
11. **Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB & Siliciano JD (1998)** Activation of the ATM kinase by ionizing radiation and phosphorylation of p53 *Science* **281,** 1677-9.
12. **Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP & Lavin MF (1998)** ATM associates with and phosphorylates p53: mapping the region of interaction *Nat Genet* **20,** 398-400.
13. **Maya R, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, Moas M, Buschmann T, Ronai Z, Shiloh Y, Kastan MB, Katzir E & Oren M (2001)** ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage *Genes Dev* **15,** 1067-77.
14. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
15. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
16. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
17. **Matsuoka S, Huang M & Elledge SJ (1998)** Linkage of ATM to cell cycle regulation by the Chk2 protein kinase *Science* **282,** 1893-7.
18. **Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K & Elledge SJ (2000)** Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro *Proc Natl Acad Sci USA* **97,** 10389-94.
19. **Lim DS, Kim ST, Xu B, Maser RS, Lin J, Petrini JH & Kastan MB (2000)** ATM phosphorylates p95/NBS1 in an S-phase checkpoint pathway *Nature* **404,** 613-7.
20. **Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G, Livingston DM & Weaver DT (2000)** ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response *Nature* **405,** 477-82.
21. **Zhou BB, Chaturvedi P, Spring K, Scott SP, Johanson RA, Mishra R, Mattern MR, Winkler JD & Khanna KK (2000)** Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity *J Biol Chem* **275,** 10342-8.
22. **Bao S, Tibbetts RS, Brumbaugh KM, Fang Y, Richardson DA, Ali A, Chen SM, Abraham RT & Wang XF (2001)** ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses *Nature* **411,** 969-74.
23. **Xu B, Kim S & Kastan MB (2001)** Involvement of BRCA1 in S-phase and G(2)-phase checkpoints after ionizing irradiation *Mol Cell Biol* **21,** 3445-50.
24. **Kim ST, Xu B & Kastan MB (2002)** Involvement of the cohesin protein, SMC1, in ATM-dependent and independent responses to DNA damage *Genes Dev* **16,** 560-70.
25. **Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, Kim ST, Lane WS, Kastan MB & D'Andrea AD (2002)** Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell* **109,** 459-72.
26. **Xu B, O'Donnell AH, Kim ST & Kastan MB (2002)** Phosphorylation of serine 1387 in BRCA1 is specifically required for the ATM-mediated S-phase checkpoint after ionizing irradiation *Cancer Res* **62,** 4588-91.

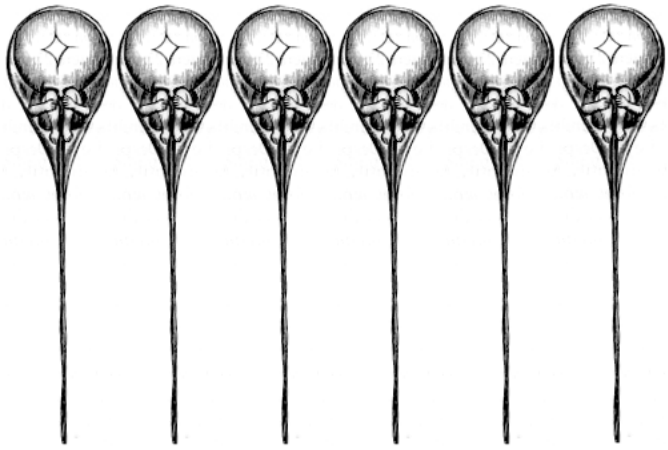
27. **Yazdi PT, Wang Y, Zhao S, Patel N, Lee EY & Qin J (2002)** SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint *Genes Dev* **16,** 571-82.
28. **Dasika GK, Lin SC, Zhao S, Sung P, Tomkinson A & Lee EY (1999)** DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis *Oncogene* **18,** 7883-99.
29. **Haber JE (2000)** Partners and pathways repairing a double-strand break *Trends Genet* **16,** 259-64.
30. **van Gent DC, Hoeijmakers JH & Kanaar R (2001)** Chromosomal stability and the DNA double-stranded break connection *Nat Rev Genet* **2,** 196-206.
31. **Hoeijmakers JH (2001)** Genome maintenance mechanisms for preventing cancer *Nature* **411,** 366-74.
32. **Khanna KK & Jackson SP (2001)** DNA double-strand breaks: signaling, repair and the cancer connection *Nat Genet* **27,** 247-54.
33. **Sekiguchi M (2002)** DNA repair, Review Issue 58 *Oncogene Reviews* **21,** 8885-9056.
34. **Bernstein C, Bernstein H, Payne CM & Garewal H (2002)** DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis *Mutat Res* **511,** 145-78.
35. **Rouse J & Jackson SP (2002)** Interfaces between the detection, signaling, and repair of DNA damage *Science* **297,** 547-51.
36. **Friedberg EC (2003)** DNA damage and repair *Nature* **421,** 436-40.
37. **Alberts B (2003)** DNA replication and recombination *Nature* **421,** 431-5.
38. **Cline SD & Hanawalt PC (2003)** Who's on first in the cellular response to DNA damage? *Nat Rev Mol Cell Biol* **4,** 361-73.
39. **Rogakou EP, Pilch DR, Orr AH, Ivanova VS & Bonner WM (1998)** DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139 *J Biol Chem* **273,** 5858-68.
40. **Andegeko Y, Moyal L, Mittelman L, Tsarfaty I, Shiloh Y & Rotman G (2001)** Nuclear retention of ATM at sites of DNA double strand breaks *J Biol Chem* **276,** 38224-30.
41. **Burma S, Chen BP, Murphy M, Kurimasa A & Chen DJ (2001)** ATM phosphorylates histone H2AX in response to DNA double-strand breaks *J Biol Chem* **276,** 42462-7.
42. **Rogakou EP, Boon C, Redon C & Bonner WM (1999)** Megabase chromatin domains involved in DNA double-strand breaks in vivo *J Cell Biol* **146,** 905-16.
43. **Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M & Bonner WM (2000)** A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage *Curr Biol* **10,** 886-95.
44. **Modesti M & Kanaar R (2001)** DNA repair: spot(light)s on chromatin *Curr Biol* **11,** R229-32.
45. **Rappold I, Iwabuchi K, Date T & Chen J (2001)** Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways *J Cell Biol* **153,** 613-20.
46. **Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, Bonner RF, Bonner WM & Nussenzweig A (2003)** Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks *Nat Cell Biol* **5,** 675-9.
47. **Petrini JH (2000)** The MRE11 complex and ATM: collaborating to navigate S phase *Curr Opin Cell Biol* **12,** 293-6.
48. **Gatei M, Zhou BB, Hobson K, Scott S, Young D & Khanna KK (2001)** Ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of BRCA1 at distinct and overlapping sites *J Biol Chem* **276,** 17276-80.
49. **Gatei M, Scott SP, Filippovitch I, Soronika N, Lavin MF, Weber B & Khanna KK (2000)** Role for ATM in DNA damage-induced phosphorylation of BRCA1 *Cancer Res* **60,** 3299-304.
50. **Li S, Ting NS, Zheng L, Chen PL, Ziv Y, Shiloh Y, Lee EY & Lee WH (2000)** Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response *Nature* **406,** 210-5.
51. **Cortez D, Wang Y, Qin J & Elledge SJ (1999)** Requirement of ATM-dependent phosphorylation of BRCA1 in the DNA damage response to double-strand breaks *Science* **286,** 1162-6.
52. **Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM & Burgoyne PS (2001)** Recombinational DNA double-strand breaks in mice precede synapsis *Nat Genet* **27,** 271-6.
53. **Lichten M (2001)** Meiotic recombination: breaking the genome to save it *Curr Biol* **11,** R253-6.
54. **Plug AW, Peters AH, Xu Y, Keegan KS, Hoekstra MF, Baltimore D, de Boer P & Ashley T (1997)** ATM and RPA in meiotic chromosome synapsis and recombination *Nat Genet* **17,** 457-61.
55. **Elson A, Wang Y, Daugherty CJ, Morton CC, Zhou F, Campos-Torres J & Leder P (1996)** Pleiotropic defects in ataxia-telangiectasia protein-deficient mice *Proc Natl Acad Sci USA* **93,** 13084-9.
56. **Barlow C, Liyanage M, Moens PB, Tarsounas M, Nagashima K, Brown K, Rottinghaus S, Jackson SP, Tagle D, Ried T & Wynshaw-Boris A (1998)** ATM deficiency results in severe meiotic disruption as early as leptonema of prophase I *Development* **125,** 4007-17.
57. **Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, Shiloh Y, Crawley JN, Ried T, Tagle D & Wynshaw-Boris A (1996)** ATM-deficient mice: a paradigm of ataxia telangiectasia *Cell* **86,** 159-71.
58. **Xu Y, Ashley T, Brainerd EE, Bronson RT, Meyn MS & Baltimore D (1996)** Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma *Genes Dev* **10,** 2411-22.

59. **Pandita TK, Westphal CH, Anger M, Sawant SG, Geard CR, Pandita RK & Scherthan H (1999)** ATM inactivation results in aberrant telomere clustering during meiotic prophase *Mol Cell Biol* **19,** 5096-105.
60. **Hamer G, Gademan IS, Kal HB & de Rooij DG (2001)** Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.
61. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP & de Rooij DG (2003)** DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.
62. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T & de Rooij DG (2003)** Function of DNA-PKcs during the early meiotic prophase without Ku70 and Ku86 *Biol Reprod* **68,** 717-21.
63. **Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG (2003)** Intercellular bridges and apoptosis in clones of male germ cells *Int J Androl* **in press**.
64. **Flaggs G, Plug AW, Dunks KM, Mundt KE, Ford JC, Quiggle MR, Taylor EM, Westphal CH, Ashley T, Hoekstra MF & Carr AM (1997)** ATM-dependent interactions of a mammalian Chk1 homolog with meiotic chromosomes *Curr Biol* **7,** 977-86.
65. **Odorisio T, Rodriguez TA, Evans EP, Clarke AR & Burgoyne PS (1998)** The meiotic checkpoint monitoring synapsis eliminates spermatocytes via p53-independent apoptosis *Nat Genet* **18,** 257-61.
66. **Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC & Nussenzweig A (2002)** Genomic instability in mice lacking histone H2AX *Science* **296,** 922-7.
67. **Fernandez-Capetillo O, Mahadevaiah SK, Celeste A, Romanienko PJ, Camerini-Otero RD, Bonner WM, Manova K, Burgoyne P & Nussenzweig A (2003)** H2AX is required for chromatin remodeling and inactivation of sex chromosomes in male mouse meiosis *Dev Cell* **4,** 497-508.
68. **Xu X, Aprelikova O, Moens P, Deng CX & Furth PA (2003)** Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice *Development* **130,** 2001-12.
69. **Ashley T (2002)** Multiple guardians of the epithelial stage IV meiotic checkpoint. In: Testicular Tangrams *Springer-Verlag, Berlin Heidelberg New York* **Ernst Schering Research Foundation Workshop Supplement 9,** 1-18.
70. **de Vries SS, Baart EB, Dekker M, Siezen A, de Rooij DG, de Boer P & te Riele H (1999)** Mouse MutS-like protein MSH5 is required for proper chromosome synapsis in male and female meiosis *Genes Dev* **13,** 523-31.
71. **Ganesan S, Silver DP, Greenberg RA, Avni D, Drapkin R, Miron A, Mok SC, Randrianarison V, Brodie S, Salstrom J, Rasmussen TP, Klimke A, Marrese C, Marahrens Y, Deng CX, Feunteun J & Livingston DM (2002)** BRCA1 supports XIST RNA concentration on the inactive X chromosome *Cell* **111,** 393-405.

DNA DOUBLE-STRAND BREAKS & APOPTOSIS IN THE TESTIS

A SUMMARIZING DISCUSSION

Geert Hamer[1,2] & Dirk G. de Rooij[1,2]



[1] *Department of Endocrinology, Faculty of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands*

[2] *Department of Cell Biology, UMCU, Heidelberglaan 100, 3584 CX Utrecht, the Netherlands*

In comparison to most other tissues the DNA damage responses in the testis are probably most divers. For instance, the mitotically dividing spermatogonia are radiosensitive and easily undergo apoptosis in response to DNA damage (1), whereas, in comparison, the meiotic and haploid germ cells appear very resistant for the direct effects of ionizing radiation (IR) (2). However, independent from any treatment with DNA damaging agents, many cells in the testis also "spontaneously" undergo apoptosis (3). Even DNA damage is a naturally occurring event in the testis as DNA double-strand breaks (DSBs) are endogenously induced during the first meiotic prophase (4). These breaks are necessary for the meiotic cell division and are repaired by meiotic recombination (5).

During male germ cell development, mitotically dividing spermatogonia differentiate into meiotic spermatocytes, into elongating haploid cells and eventually into spermatozoa. In other words: the DNA of the germ cells first has to undergo several mitotic divisions, followed by the two subsequent meiotic cell divisions, including meiotic recombination, after which it has to be condensed in a tight package upon delivery to fertilize an oocyte. These changes imply major changes in chromatin structure and as a consequence also in the molecular mechanisms by which genome integrity is maintained. Therefore, DNA damage responses in testis are not only very divers but also tightly regulated.

This thesis is mainly focused on the spermatogonial DNA damage response and meiotic recombinational repair during the first meiotic prophase. During both stages of male germ cell development different DNA damage response pathways are active. Studying expression and regulation of proteins involved in these pathways during spermatogenesis can give insight in how cells might regulate the proper DNA damage response at given moment during their development.

SPERMATOGONIAL APOPTOSIS & INTERCELLULAR BRIDGES

Spermatogonia do not only undergo apoptosis in response to DNA damage but also, when the ratio spermatogonia/ Sertoli cells is too high, as a way of germ cell density regulation (3). Although both leading to spermatogonial apoptosis, these two processes use different molecular pathways. During DNA-damage induced spermatogonial apoptosis the tumor suppressor p53 plays a central role, this protein being induced shortly after irradiation (6-8). In contrast, spermatogonial degeneration during germ cell density regulation depends on the Bcl-2 family member Bax (9, 10), which has been shown not to be involved in the spermatogonial response to DNA-damage (9).

As they divide, spermatogonia stay cytoplasmically connected by intercellular bridges leading to large spermatogonial "clones" (11, 12). However, in response to irradiation and subsequent DNA damage, single spermatogonia undergo apoptosis without affecting their neighbouring interconnected cells (13). This means that apoptotic signals induced in one damaged cell nucleus are not necessarily transmitted through the cytoplasmic bridges. In contrast, during germ cell density regulation, spermatogonia do not undergo apoptosis individually but as whole clones of interconnected cells (14). Apparently, in spermatogonia, the apoptotic effect of spermatogonial p53 signaling differs from the effect of the pathway involving Bax. Unfortunately, it is very difficult to perform immunohistochemistry using whole mount seminiferous tubules, making it hard to investigate proteins in the p53 or Bcl2-family signaling cascades in relation to intercellular connections. In order to study these aspects, a spermatogonial culture system in which intercellular relations are clearly visible would be very beneficial and probably required.

c-Abl & p73

Although very important, p53 is not always essential for spermatogonial apoptosis (15) and also p53 deficient spermatogonia are able to undergo apoptosis in response to irradiation (6). One alternative is provided by the p53 homologue p73. p73 exists in various isoforms that can be pro- and anti-apoptotic (16-20), however, we found that in the testis the full length pro-apoptotic p73α is present (21).

p73 is activated by the non-receptor tyrosine kinase c-Abl (22-25), which gets activated in response to IR (26, 27). In most cell types activated p73 then functions as a transcription factor (16-20). Also in spermatogonia c-Abl phosphorylates p73 in response to irradiation (21). However, in spermatogonia both c-Abl and p73 reside in cytoplasm, excluding direct functions as transcriptional activators.

Since c-Abl is not present in the spermatogonial nucleus, it is not very likely that, in these cells, DNA damage directly activates c-Abl. More likely reactive oxygen species, induced by IR, directly activate cytoplasmic c-Abl (28, 29). c-Abl has multiple cytoplasmic functions, including regulation of mitogenesis (30), and even directly at the mitochondria triggering the release of the apoptosis inducing cytochrome c (29, 31, 32). In the testis c-Abl also phosphorylates the pro-apoptotic p73α, giving rise to the next question: how can cytoplasmic p73 induce apoptosis?

Sub-cellular localization of p73, and also p53, are under strict regulation of both nuclear export as well as import sequences (33-37). However, most authors assume that cytoplasmic p73 is not functional. This assumption is not in line with the fact that, although phosphorylated by c-Abl, spermatogonial p73 does not translocate to the nucleus in response to IR. More likely p73 can also be active in the cytoplasm, probably acting directly via mitochondria, a route that also has been described for p53 (38, 39). The role for and regulation of c-Abl and p73 will thus differ between various tissues (40) and depends on sub-cellular localization of these proteins and the presence of specific p73 isoforms.

γ-H2AX

The most severe cellular damage induced by irradiation is the generation of DNA double-strand breaks (DSBs) (41-51), which in spermatogonia will induce p53 (6). In response to especially DSBs histone H2AX gets phosphorylated at serine 139 (52), and within minutes γ-H2AX (phosphorylated H2AX) foci appear specifically at the damaged sites containing DSBs (53, 54). Each individual focus represents a single DSB and there is a linear effect between the dose of IR and the number of γ-H2AX foci, making quantification of foci a reliable method for measuring induction and repair of DSBs (55, 56). The exact mechanisms by which γ-H2AX foci are induced remain to be elucidated, however, they are certainly essential for the sequestration of several DNA-repair factors and DNA damage-signaling proteins to IR induced foci (IRIF) (54, 57-59). Also in the testis γ-H2AX foci are induced by IR, which in spermatogonia interact with p53 (60).

Additionally, in the testis, DSBs do not only occur in response to irradiation, they are also endogenously induced during the first meiotic prophase (4, 61). These meiotic DSBs have been described to occur predominantly during the leptotene stage and thus before synapsis of the homologous chromosomes (4). However, radiation-independent γ-H2AX is already present in all intermediate and B spermatogonia, remaining up until zygotene spermatocytes and the sex bodies of pachytene spermatocytes (60). This expression pattern in the testis seems to contradict the assumed restriction of γ-H2AX localization to DSBs, at least during spermatogenesis. Otherwise DSBs would remain unrepaired during two mitotic divisions and

the pre-meiotic S phase. More likely, H2AX phosphorylation has a testis specific role in the remodeling of germ cell chromatin as during spermatogenesis chromatin is constantly being reconstructed (62).

Especially intriguing is the presence of γ-H2AX in the sex bodies that contain the X and Y-chromosomes. This presence clearly has a function since in the $H2AX^{-/-}$ testis the sex chromosomes fail to pair, and are fragmented or associated with autosomal chromosomes (63). Moreover, $H2AX^{-/-}$ spermatocytes do not initiate meiotic sex chromosome inactivation and fail to recruit several sex body specific proteins (64). As a consequence, spermatogenesis in the $H2AX^{-/-}$ mice is arrested at what, as far as can be concluded from the published figures, seems to be pachytene stage IV-V (63, 64).

Although γ-H2AX has been widely used as marker for DSBs, it clearly also has other functions during spermatogenesis. Therefore it would be very interesting to compare the presence of γ-H2AX (4, 60) with the staining pattern of another marker for DSBs in the testis. A good candidate might be the p53 binding protein 1 (53BP1). In somatic cells this protein co-localizes with γ-H2AX and also accumulates in foci at DSBs in response to IR (65-73). It would be of interest to see when and where γ-H2AX and 53BP1 overlap during spermatogenesis and to investigate whether γ-H2AX in the testis exists without co-localizing with 53BP1.

REGULATION OF DNA-PK & THE CHOICE BETWEEN HRR & NHEJ

DNA damage can be repaired in a variety of ways of which the very accurate homologous recombinational repair (HRR) and the more error prone non-homologous end joining (NHEJ) are most important for the repair of DSBs (41-51) **(Figure 6, Chapter 1)**. During every step of development or cell differentiation a "choice" between these two pathways has to be made (41). For instance, there appear to be differences in the response to IR at different developmental stages, HRR being important for DSB-repair during early mouse development and NHEJ in the adult mouse (74).

A major player in the repair of DSBs is the DNA-dependent protein kinase (DNA-PK). This protein complex consists of a heteromeric DNA binding component (Ku70 and Ku86) and a DNA-PK catalytic subunit (DNA-PKcs) (75, 76). The Ku heterodimer binds to single- and especially double-stranded DNA breaks (DSBs) and subsequently recruits DNA-PKcs that plays a key role in non-homologous end joining of the damaged DNA (77-81).

In spermatogonia, DNA-PK detects and assists in repair of DSBs by NHEJ, for instance caused by IR (82). In pachytene spermatocytes, however, DSBs have to be repaired by meiotic recombination, a specialized form of HRR. Proper meiotic recombination is essential for the entire meiotic process (5) and, moreover, NHEJ would be too error prone to repair the many DSBs induced during meiosis. The complete DNA-PK holo-enzyme has been shown to suppress homologous recombination (83), so presence of all the DNA-PK components in early spermatocytes would seriously interfere with meiotic recombination. Additionally, interruption of the DNA-PK has been found to stimulate homologous recombination (84, 85). During male meiosis, this interruption is achieved by the very specific down regulation of the Ku proteins in early spermatocytes until pachytene stage IV of the seminiferous epithelium **(Figure 3, Chapter 4)** (82, 86). Regulated expression of the Ku heterodimer during meiosis could be the major mechanism that stimulates meiotic recombination and suppresses NHEJ.

DNA-PK activity also appears to be regulated through an auto-regulatory feedback mechanism that involves *in trans* auto-phosphorylation of DNA-PKcs (87, 88). The exact characteristics of this auto-regulatory feedback loop remain to be elucidated. However, it does not seem very likely that this mechanism is involved in suppressing NHEJ during meiotic

recombination since it requires the Ku proteins, which are not present throughout the pachytene stage.

Even without the Ku heterodimer, DNA-PKcs still has a function in early spermatocytes. Until now, Ku independent DNA binding and activation of DNA-PKcs only has been described *in vitro* (89). However, the significantly higher number of apoptotic early spermatocytes in the DNA-PKcs deficient *scid* testes suggests a Ku-independent function for DNA-PKcs in these early meiotic cells (82). The majority of these apoptotic spermatocytes is present at stage IV of the seminiferous epithelium. At this epithelial stage spermatogenesis is known to abrogate at the spermatocyte level in several knockout mice deficient for DNA damage response proteins (90), which suggests that a stage IV meiotic checkpoint exists, selecting spermatocytes with damaged DNA to undergo apoptosis.

REGULATION OF ATM DURING SPERMATOGENESIS

Like DNA-PK, also ATM appears to differentially regulated during spermatogenesis. In spermatogonia ATM dimers get auto-cross-phosphorylated in response to IR (91) and initiate the spermatogonial DNA damage response, including the appearance of γ-H2AX foci at the sites of DSBs (52, 60, 92, 93) and induction of p53 (94-97), which will lead to spermatogonial apoptosis or cell cycle arrest (6-8).

However, although ATM clearly is essential for meiotic recombination (98, 99), it remains non-phosphorylated during the meiotic prophase. This in contrast to spermatogonia and other mitotic cells (91) in which ATM gets activated by phosphorylation. Hence, like DNA-PK, also ATM is differently regulated during the meiotic prophase and does not need to be phosphorylated at this stage in order to be functional.

An exception during meiosis, however, is the constitutively presence of phosphorylated ATM in the sex bodies. In these structures phosphorylated ATM might very well be responsible for phosphorylation of γ-H2AX (52, 60, 92, 93) which is, as mentioned earlier, required for meiotic X-chromosome inactivation (64, 100, 101) and keeping the X and Y-chromosomes intact during the first meiotic prophase (63). The reason why DNA damage repair proteins are present in the sex bodies in a constitutively activated state remains to be resolved. One explanation could be that the X and Y-chromosomes are in a constant state of damage. However, it seems more likely that, in the sex bodies, these proteins also can have a more preventive function rather than a reparative. The fact that some of these proteins are described to be required for meiotic X-inactivation (64, 100, 101) could also lead to another explanation. Perhaps, when transcription is being repressed during DNA damage or repair by these specific proteins, their presence at the sex chromosomes could lead to meiotic X-inactivation, even without the actual presence of DNA damage.

QUESTIONS & ANSWERS

In reality DNA repair during spermatogenesis will appear to be much more complex, involving many other proteins, like BRCA1 and 2 (101-106), the MRE11 complex (107) and the more recently discovered Artemis (108, 109). Whereas BRCA1 and 2 (110-119) and the MRE11 (107) complex function during HRR, Artemis is involved in NHEJ (108). In addition, also many proteins of the mismatch-repair system (41-51) are involved in DNA repair during spermatogenesis and especially meiotic recombination (120-141). Although its function is not entirely understood, mismatch-repair protein MHL1 is even a commonly used marker for

meiotic crossovers (129). Expression and regulation of all these and other proteins determine the “choice” between different DNA repair pathways at different steps during spermatogenesis.

Current research, using cell culture and gene over-expressing systems, shows many possibilities for a great variety of DNA damage response pathways. Also many cell cycle and apoptosis regulating proteins, like Chk2 (142, 143), the E2F family of transcription factors (143-145) and MDC1 (146-148), are continuously being discovered or further investigated. Additionally, in many cases, cross-talk between different pathways has been described and the borders between all these different routes may not be as strict as often assumed. Of many of these pathways it has not even been studied yet whether they are relevant *in vivo* in the testis, or other tissues, or involved in various cancers. Spermatogenesis provides a good model to study these processes because male germ cells are constantly developing and very precisely regulate their response to DNA damage. Therefore, also the general knowledge about DNA damage responses can benefit from testis research.

REFERENCES

1. **van der Meer Y, Huiskamp R, Davids JA, van der Tweel I & de Rooij DG (1992)** The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia *Radiat Res* **130,** 296-302.
2. **Oakberg EF & DiMinno RL (1960)** X-ray sensitivity of primary spermatocytes of the mouse *Int J Radiat Biol* **2,** 196-209.
3. **Print CG & Loveland KL (2000)** Germ cell suicide: new insights into apoptosis during spermatogenesis *Bioessays* **22,** 423-30.
4. **Mahadevaiah SK, Turner JM, Baudat F, Rogakou EP, de Boer P, Blanco-Rodriguez J, Jasin M, Keeney S, Bonner WM & Burgoyne PS (2001)** Recombinational DNA double-strand breaks in mice precede synapsis *Nat Genet* **27,** 271-6.
5. **Lichten M (2001)** Meiotic recombination: breaking the genome to save it *Curr Biol* **11,** R253-6.
6. **Beumer TL, Roepers-Gajadien HL, Gademan IS, van Buul PP, Gil-Gomez G, Rutgers DH & de Rooij DG (1998)** The role of the tumor suppressor p53 in spermatogenesis *Cell Death Differ* **5,** 669-77.
7. **Hasegawa M, Zhang Y, Niibe H, Terry NH & Meistrich ML (1998)** Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice *Radiat Res* **149,** 263-70.
8. **Gobe GC, Harmon B, Leighton J & Allan DJ (1999)** Radiation-induced apoptosis and gene expression in neonatal kidney and testis with and without protein synthesis inhibition *Int J Radiat Biol* **75,** 973-83.
9. **Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB & de Rooij DG (2000)** Apoptosis regulation in the testis: Involvement of Bcl-2 family members *Mol Reprod Dev* **56,** 353-59.
10. **Knudson CM, Tung KS, Tourtellotte WG, Brown GA & Korsmeyer SJ (1995)** Bax-deficient mice with lymphoid hyperplasia and male germ cell death *Science* **270,** 96-9.
11. **Dym M & Fawcett DW (1971)** Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis *Biol Reprod* **4,** 195-215.
12. **Huckins C (1978)** Spermatogonial intercellular bridges in whole-mounted seminiferous tubules from normal and irradiated rodent testes *Am J Anat* **153,** 97-121.
13. **Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG (2003)** Intercellular bridges and apoptosis in clones of male germ cells *Int J Androl* **in press**.
14. **Huckins C (1978)** The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium *Anat Rec* **190,** 905-26.
15. **Ohta H, Aizawa S & Nishimune Y (2003)** Functional analysis of the p53 gene in apoptosis induced by heat stress or loss of stem cell factor signaling in mouse male germ cells *Biol Reprod* **68,** 2249-54.
16. **Melino G, De Laurenzi V & Vousden KH (2002)** p73: Friend or foe in tumorigenesis *Nat Rev Cancer* **2,** 605-15.
17. **Soengas MS & Lowe SW (2000)** p53 and p73: seeing double? *Nat Genet* **26,** 391-2.
18. **Levrero M, De Laurenzi V, Costanzo A, Gong J, Wang JY & Melino G (2000)** The p53/p63/p73 family of transcription factors: overlapping and distinct functions *J Cell Sci* **113,** 1661-70.
19. **Strano S, Rossi M, Fontemaggi G, Munarriz E, Soddu S, Sacchi A & Blandino G (2001)** From p63 to p53 across p73 *FEBS Lett* **490,** 163-70.
20. **Yang A & McKeon F (2000)** p63 and p73: p53 mimics, menaces and more *Nat Rev Mol Cell Biol* **1,** 199-207.
21. **Hamer G, Gademan IS, Kal HB & de Rooij DG (2001)** Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.
22. **White E & Prives C (1999)** DNA damage enables p73 *Nature* **399,** 734-5, 7.
23. **Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin WG, Jr., Levrero M & Wang JY (1999)** The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage *Nature* **399,** 806-9.
24. **Agami R, Blandino G, Oren M & Shaul Y (1999)** Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis *Nature* **399,** 809-13.
25. **Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R & Kufe D (1999)** p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage *Nature* **399,** 814-7.
26. **Baskaran R, Wood LD, Whitaker LL, Canman CE, Morgan SE, Xu Y, Barlow C, Baltimore D, Wynshaw-Boris A, Kastan MB & Wang JY (1997)** Ataxia telangiectasia mutant protein activates c-Abl tyrosine kinase in response to ionizing radiation *Nature* **387,** 516-9.
27. **Shafman T, Khanna KK, Kedar P, Spring K, Kozlov S, Yen T, Hobson K, Gatei M, Zhang N, Watters D, Egerton M, Shiloh Y, Kharbanda S, Kufe D & Lavin MF (1997)** Interaction between ATM protein and c-Abl in response to DNA damage *Nature* **387,** 520-3.
28. **Sun X, Majumder P, Shioya H, Wu F, Kumar S, Weichselbaum R, Kharbanda S & Kufe D (2000)** Activation of the cytoplasmic c-Abl tyrosine kinase by reactive oxygen species *J Biol Chem* **275,** 17237-40.

29. **Kumar S, Mishra N, Raina D, Saxena S & Kufe D (2003)** Abrogation of the cell death response to oxidative stress by the c-Abl tyrosine kinase inhibitor STI571 *Mol Pharmacol* **63,** 276-82.
30. **Furstoss O, Dorey K, Simon V, Barila D, Superti-Furga G & Roche S (2002)** c-Abl is an effector of Src for growth factor-induced c-myc expression and DNA synthesis *Embo J* **21,** 514-24.
31. **Kumar S, Bharti A, Mishra NC, Raina D, Kharbanda S, Saxena S & Kufe D (2001)** Targeting of the c-Abl tyrosine kinase to mitochondria in the necrotic cell death response to oxidative stress *J Biol Chem* **276,** 17281-5.
32. **Ito Y, Pandey P, Mishra N, Kumar S, Narula N, Kharbanda S, Saxena S & Kufe D (2001)** Targeting of the c-Abl tyrosine kinase to mitochondria in endoplasmic reticulum stress-induced apoptosis *Mol Cell Biol* **21,** 6233-42.
33. **Gu J, Nie L, Kawai H & Yuan ZM (2001)** Subcellular distribution of p53 and p73 are differentially regulated by MDM2 *Cancer Res* **61,** 6703-7.
34. **Gu J, Nie L, Wiederschain D & Yuan ZM (2001)** Identification of p53 sequence elements that are required for MDM2-mediated nuclear export *Mol Cell Biol* **21,** 8533-46.
35. **Lohrum MA, Woods DB, Ludwig RL, Balint E & Vousden KH (2001)** C-terminal ubiquitination of p53 contributes to nuclear export *Mol Cell Biol* **21,** 8521-32.
36. **Inoue T, Stuart J, Leno R & Maki CG (2002)** Nuclear import and export signals in control of the p53-related protein p73 *J Biol Chem* **277,** 15053-60.
37. **Nikolaev AY, Li M, Puskas N, Qin J & Gu W (2003)** Parc. A Cytoplasmic Anchor for p53 *Cell* **112,** 29-40.
38. **Marchenko ND, Zaika A & Moll UM (2000)** Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling *J Biol Chem* **275,** 16202-12.
39. **Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P & Moll UM (2003)** p53 has a direct apoptogenic role at the mitochondria *Mol Cell* **Online**.
40. **Wang JY (2000)** Regulation of cell death by the Abl tyrosine kinase *Oncogene* **19,** 5643-50.
41. **Dasika GK, Lin SC, Zhao S, Sung P, Tomkinson A & Lee EY (1999)** DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis *Oncogene* **18,** 7883-99.
42. **Haber JE (2000)** Partners and pathways repairing a double-strand break *Trends Genet* **16,** 259-64.
43. **van Gent DC, Hoeijmakers JH & Kanaar R (2001)** Chromosomal stability and the DNA double-stranded break connection *Nat Rev Genet* **2,** 196-206.
44. **Hoeijmakers JH (2001)** Genome maintenance mechanisms for preventing cancer *Nature* **411,** 366-74.
45. **Khanna KK & Jackson SP (2001)** DNA double-strand breaks: signaling, repair and the cancer connection *Nat Genet* **27,** 247-54.
46. **Sekiguchi M (2002)** DNA repair, Review Issue 58 *Oncogene Reviews* **21,** 8885-9056.
47. **Bernstein C, Bernstein H, Payne CM & Garewal H (2002)** DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis *Mutat Res* **511,** 145-78.
48. **Rouse J & Jackson SP (2002)** Interfaces between the detection, signaling, and repair of DNA damage *Science* **297,** 547-51.
49. **Friedberg EC (2003)** DNA damage and repair *Nature* **421,** 436-40.
50. **Alberts B (2003)** DNA replication and recombination *Nature* **421,** 431-5.
51. **Cline SD & Hanawalt PC (2003)** Who's on first in the cellular response to DNA damage? *Nat Rev Mol Cell Biol* **4,** 361-73.
52. **Rogakou EP, Pilch DR, Orr AH, Ivanova VS & Bonner WM (1998)** DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139 *J Biol Chem* **273,** 5858-68.
53. **Rogakou EP, Boon C, Redon C & Bonner WM (1999)** Megabase chromatin domains involved in DNA double-strand breaks in vivo *J Cell Biol* **146,** 905-16.
54. **Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M & Bonner WM (2000)** A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage *Curr Biol* **10,** 886-95.
55. **Rothkamm K & Lobrich M (2003)** Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses *Proc Natl Acad Sci USA* **100,** 5057-62.
56. **Bonner WM (2003)** Low-dose radiation: Thresholds, bystander effects, and adaptive responses *Proc Natl Acad Sci USA* **100,** 4973-5.
57. **Hunter N, Valentin Borner G, Lichten M & Kleckner N (2001)** γ-H2AX illuminates meiosis *Nat Genet* **27,** 236-8.
58. **Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K & Bonner W (2002)** Histone H2A variants H2AX and H2AZ *Curr Opin Genet Dev* **12,** 162-9.
59. **Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, Bonner RF, Bonner WM & Nussenzweig A (2003)** Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks *Nat Cell Biol* **5,** 675-9.
60. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP & de Rooij DG (2003)** DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.
61. **Zenvirth D, Richler C, Bardhan A, Baudat F, Barzilai A, Wahrman J & Simchen G (2003)** Mammalian meiosis involves DNA double-strand breaks with 3' overhangs *Chromosoma* **111,** 369-76.

62. **Doenecke D, Drabent B, Bode C, Bramlage B, Franke K, Gavenis K, Kosciessa U & Witt O (1997)** Histone gene expression and chromatin structure during spermatogenesis *Adv Exp Med Biol* **424,** 37-48.
63. **Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC & Nussenzweig A (2002)** Genomic instability in mice lacking histone H2AX *Science* **296,** 922-7.
64. **Fernandez-Capetillo O, Mahadevaiah SK, Celeste A, Romanienko PJ, Camerini-Otero RD, Bonner WM, Manova K, Burgoyne P & Nussenzweig A (2003)** H2AX is required for chromatin remodeling and inactivation of sex chromosomes in male mouse meiosis *Dev Cell* **4,** 497-508.
65. **DiTullio RA, Mochan TA, Venere M, Bartkova J, Sehested M, Bartek J & Halazonetis TD (2002)** 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer *Nat Cell Biol* **4,** 998-1002.
66. **Fernandez-Capetillo O, Chen HT, Celeste A, Ward I, Romanienko PJ, Morales JC, Naka K, Xia Z, Camerini-Otero RD, Motoyama N, Carpenter PB, Bonner WM, Chen J & Nussenzweig A (2002)** DNA damage-induced G(2)-M checkpoint activation by histone H2AX and 53BP1 *Nat Cell Biol* **4,** 993-7.
67. **Kao GD, McKenna WG, Guenther MG, Muschel RJ, Lazar MA & Yen TJ (2003)** Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response *J Cell Biol* **160,** 1017-27.
68. **Anderson L, Henderson C & Adachi Y (2001)** Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage *Mol Cell Biol* **21,** 1719-29.
69. **Rappold I, Iwabuchi K, Date T & Chen J (2001)** Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways *J Cell Biol* **153,** 613-20.
70. **Schultz LB, Chehab NH, Malikzay A & Halazonetis TD (2000)** p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks *J Cell Biol* **151,** 1381-90.
71. **Wang B, Matsuoka S, Carpenter PB & Elledge SJ (2002)** 53BP1, a mediator of the DNA damage checkpoint *Science* **298,** 1435-8.
72. **Xia Z, Morales JC, Dunphy WG & Carpenter PB (2001)** Negative cell cycle regulation and DNA damage-inducible phosphorylation of the BRCT protein 53BP1 *J Biol Chem* **276,** 2708-18.
73. **Ward IM, Minn K, van Deursen J & Chen J (2003)** p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice *Mol Cell Biol* **23,** 2556-63.
74. **Essers J, van Steeg H, de Wit J, Swagemakers SM, Vermeij M, Hoeijmakers JH & Kanaar R (2000)** Homologous and non-homologous recombination differentially affect DNA damage repair in mice *Embo J* **19,** 1703-10.
75. **Smith GC & Jackson SP (1999)** The DNA-dependent protein kinase *Genes Dev* **13,** 916-34.
76. **Durocher D & Jackson SP (2001)** DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr Opin Cell Biol* **13,** 225-31.
77. **Walker JR, Corpina RA & Goldberg J (2001)** Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair *Nature* **412,** 607-14.
78. **Hammarsten O, DeFazio LG & Chu G (2000)** Activation of DNA-dependent protein kinase by single-stranded DNA ends *J Biol Chem* **275,** 1541-50.
79. **Gottlieb TM & Jackson SP (1993)** The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen *Cell* **72,** 131-42.
80. **Suwa A, Hirakata M, Takeda Y, Jesch SA, Mimori T & Hardin JA (1994)** DNA-dependent protein kinase (Ku protein-p350 complex) assembles on double-stranded DNA *Proc Natl Acad Sci USA* **91,** 6904-8.
81. **DeFazio LG, Stansel RM, Griffith JD & Chu G (2002)** Synapsis of DNA ends by DNA-dependent protein kinase *Embo J* **21,** 3192-200.
82. **Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PP, Ashley T & de Rooij DG (2003)** Function of DNA-PKcs during the early meiotic prophase without Ku70 and Ku86 *Biol Reprod* **68,** 717-21.
83. **Allen C, Kurimasa A, Brenneman MA, Chen DJ & Nickoloff JA (2002)** DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination *Proc Natl Acad Sci USA* **99,** 3758-63.
84. **Delacote F, Han M, Stamato TD, Jasin M & Lopez BS (2002)** An XRCC4 defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells *Nucleic Acids Res* **30,** 3454-63.
85. **Pierce AJ, Hu P, Han M, Ellis N & Jasin M (2001)** Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells *Genes Dev* **15,** 3237-42.
86. **Goedecke W, Eijpe M, Offenberg HH, van Aalderen M & Heyting C (1999)** MRE11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis *Nat Genet* **23,** 194-8.
87. **Chan DW, Chen BP, Prithivirajsingh S, Kurimasa A, Story MD, Qin J & Chen DJ (2002)** Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks *Genes Dev* **16,** 2333-8.
88. **Soubeyrand S, Pope L, Pakuts B & Hache RJ (2003)** Threonines 2638/2647 in DNA-PK are essential for cellular resistance to ionizing radiation *Cancer Res* **63,** 1198-201.

89. **Hammarsten O & Chu G (1998)** DNA-dependent protein kinase: DNA binding and activation in the absence of Ku *Proc Natl Acad Sci USA* **95,** 525-30.
90. **Ashley T (2002)** Multiple guardians of the epithelial stage IV meiotic checkpoint. In: Testicular Tangrams *Springer-Verlag, Berlin Heidelberg New York* **Ernst Schering Research Foundation Workshop Supplement 9,** 1-18.
91. **Bakkenist CJ & Kastan MB (2003)** DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation *Nature* **421,** 499-506.
92. **Andegeko Y, Moyal L, Mittelman L, Tsarfaty I, Shiloh Y & Rotman G (2001)** Nuclear retention of ATM at sites of DNA double strand breaks *J Biol Chem* **276,** 38224-30.
93. **Burma S, Chen BP, Murphy M, Kurimasa A & Chen DJ (2001)** ATM phosphorylates histone H2AX in response to DNA double-strand breaks *J Biol Chem* **276,** 42462-7.
94. **Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y & Ziv Y (1998)** Enhanced phosphorylation of p53 by ATM in response to DNA damage *Science* **281,** 1674-7.
95. **Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB & Siliciano JD (1998)** Activation of the ATM kinase by ionizing radiation and phosphorylation of p53 *Science* **281,** 1677-9.
96. **Khanna KK, Keating KE, Kozlov S, Scott S, Gatei M, Hobson K, Taya Y, Gabrielli B, Chan D, Lees-Miller SP & Lavin MF (1998)** ATM associates with and phosphorylates p53: mapping the region of interaction *Nat Genet* **20,** 398-400.
97. **Maya R, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, Moas M, Buschmann T, Ronai Z, Shiloh Y, Kastan MB, Katzir E & Oren M (2001)** ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage *Genes Dev* **15,** 1067-77.
98. **Plug AW, Peters AH, Xu Y, Keegan KS, Hoekstra MF, Baltimore D, de Boer P & Ashley T (1997)** ATM and RPA in meiotic chromosome synapsis and recombination *Nat Genet* **17,** 457-61.
99. **Xu Y, Ashley T, Brainerd EE, Bronson RT, Meyn MS & Baltimore D (1996)** Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma *Genes Dev* **10,** 2411-22.
100. **Ganesan S, Silver DP, Greenberg RA, Avni D, Drapkin R, Miron A, Mok SC, Randrianarison V, Brodie S, Salstrom J, Rasmussen TP, Klimke A, Marrese C, Marahrens Y, Deng CX, Feunteun J & Livingston DM (2002)** BRCA1 supports XIST RNA concentration on the inactive X chromosome *Cell* **111,** 393-405.
101. **Xu X, Aprelikova O, Moens P, Deng CX & Furth PA (2003)** Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice *Development* **130,** 2001-12.
102. **Zabludoff SD, Wright WW, Harshman K & Wold BJ (1996)** BRCA1 mRNA is expressed highly during meiosis and spermiogenesis but not during mitosis of male germ cells *Oncogene* **13,** 649-53.
103. **Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T & Livingston DM (1997)** Association of BRCA1 with RAD51 in mitotic and meiotic cells *Cell* **88,** 265-75.
104. **Blackshear PE, Goldsworthy SM, Foley JF, McAllister KA, Bennett LM, Collins NK, Bunch DO, Brown P, Wiseman RW & Davis BJ (1998)** BRCA1 and BRCA2 expression patterns in mitotic and meiotic cells of mice *Oncogene* **16,** 61-8.
105. **Cressman VL, Backlund DC, Avrutskaya AV, Leadon SA, Godfrey V & Koller BH (1999)** Growth retardation, DNA repair defects, and lack of spermatogenesis in BRCA1-deficient mice *Mol Cell Biol* **19,** 7061-75.
106. **Chen J, Silver DP, Walpita D, Cantor SB, Gazdar AF, Tomlinson G, Couch FJ, Weber BL, Ashley T, Livingston DM & Scully R (1998)** Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells *Mol Cell* **2,** 317-28.
107. **D'Amours D & Jackson SP (2002)** The MRE11 complex: at the crossroads of DNA repair and checkpoint signalling *Nat Rev Mol Cell Biol* **3,** 317-27.
108. **Moshous D, Callebaut I, de Chasseval R, Corneo B, Cavazzana-Calvo M, Le Deist F, Tezcan I, Sanal O, Bertrand Y, Philippe N, Fischer A & de Villartay JP (2001)** Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency *Cell* **105,** 177-86.
109. **Ma Y, Pannicke U, Schwarz K & Lieber MR (2002)** Hairpin opening and overhang processing by an artemis/DNA-Dependent protein kinase complex in nonhomologous end joining and V(D)J recombination *Cell* **108,** 781-94.
110. **Scully R & Livingston DM (2000)** In search of the tumour-suppressor functions of BRCA1 and BRCA2 *Nature* **408,** 429-32.
111. **Kerr P & Ashworth A (2001)** New complexities for BRCA1 and BRCA2 *Curr Biol* **11,** R668-76.
112. **Venkitaraman AR (2001)** Functions of BRCA1 and BRCA2 in the biological response to DNA damage *J Cell Sci* **114,** 3591-8.
113. **Venkitaraman AR (2001)** Chromosome stability, DNA recombination and the BRCA2 tumour suppressor *Curr Opin Cell Biol* **13,** 338-43.

114. **Venkitaraman AR (2002)** Cancer susceptibility and the functions of BRCA1 and BRCA2 *Cell* **108,** 171-82.
115. **Jasin M (2002)** Homologous repair of DNA damage and tumorigenesis: the BRCA connection *Oncogene* **21,** 8981-93.
116. **Moynahan ME, Chiu JW, Koller BH & Jasin M (1999)** BRCA1 controls homology-directed DNA repair *Mol Cell* **4,** 511-8.
117. **Moynahan ME, Pierce AJ & Jasin M (2001)** BRCA2 is required for homology-directed repair of chromosomal breaks *Mol Cell* **7,** 263-72.
118. **Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR & West SC (2001)** Role of BRCA2 in control of the RAD51 recombination and DNA repair protein *Mol Cell* **7,** 273-82.
119. **Powell SN, Willers H & Xia F (2002)** BRCA2 keeps Rad51 in line. High-fidelity homologous recombination prevents breast and ovarian cancer? *Mol Cell* **10,** 1262-3.
120. **Baker SM, Plug AW, Prolla TA, Bronner CE, Harris AC, Yao X, Christie DM, Monell C, Arnheim N, Bradley A, Ashley T & Liskay RM (1996)** Involvement of mouse MLH1 in DNA mismatch repair and meiotic crossing over *Nat Genet* **13,** 336-42.
121. **Edelmann W, Cohen PE, Kane M, Lau K, Morrow B, Bennett S, Umar A, Kunkel T, Cattoretti G, Chaganti R, Pollard JW, Kolodner RD & Kucherlapati R (1996)** Meiotic pachytene arrest in MLH1-deficient mice *Cell* **85,** 1125-34.
122. **Hassold TJ (1996)** Mismatch repair goes meiotic *Nat Genet* **13,** 261-2.
123. **Arnheim N & Shibata D (1997)** DNA mismatch repair in mammals: role in disease and meiosis *Curr Opin Genet Dev* **7,** 364-70.
124. **Hunter N & Borts RH (1997)** Mlh1 is unique among mismatch repair proteins in its ability to promote crossing-over during meiosis *Genes Dev* **11,** 1573-82.
125. **Paquis-Flucklinger V, Santucci-Darmanin S, Paul R, Saunieres A, Turc-Carel C & Desnuelle C (1997)** Cloning and expression analysis of a meiosis-specific MutS homolog: the human MSH4 gene *Genomics* **44,** 188-94.
126. **Vani RG & Rao MR (1997)** Cloning of the cDNA encoding rat homologue of the mismatch repair gene MSH2 and its expression during spermatogenesis *Gene* **185,** 19-26.
127. **Wei K, Kucherlapati R & Edelmann W (2002)** Mouse models for human DNA mismatch-repair gene defects *Trends Mol Med* **8,** 346-53.
128. **Plug AW, Peters AH, Keegan KS, Hoekstra MF, de Boer P & Ashley T (1998)** Changes in protein composition of meiotic nodules during mammalian meiosis *J Cell Sci* **111,** 413-23.
129. **Anderson LK, Reeves A, Webb LM & Ashley T (1999)** Distribution of crossing over on mouse synaptonemal complexes using immunofluorescent localization of MLH1 protein *Genetics* **151,** 1569-79.
130. **Edelmann W, Cohen PE, Kneitz B, Winand N, Lia M, Heyer J, Kolodner R, Pollard JW & Kucherlapati R (1999)** Mammalian MutS homologue 5 is required for chromosome pairing in meiosis *Nat Genet* **21,** 123-7.
131. **Geeta Vani R, Varghese CM & Rao MR (1999)** Cloning of rat MLH1 and expression analysis of MSH2, MSH3, MSH6, and MLH1 during spermatogenesis *Genomics* **62,** 460-7.
132. **de Vries SS, Baart EB, Dekker M, Siezen A, de Rooij DG, de Boer P & te Riele H (1999)** Mouse MutS-like protein MSH5 is required for proper chromosome synapsis in male and female meiosis *Genes Dev* **13,** 523-31.
133. **Kneitz B, Cohen PE, Avdievich E, Zhu L, Kane MF, Hou H, Jr., Kolodner RD, Kucherlapati R, Pollard JW & Edelmann W (2000)** MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice *Genes Dev* **14,** 1085-97.
134. **Richardson LL, Pedigo C & Ann Handel M (2000)** Expression of deoxyribonucleic acid repair enzymes during spermatogenesis in mice *Biol Reprod* **62,** 789-96.
135. **Santucci-Darmanin S, Walpita D, Lespinasse F, Desnuelle C, Ashley T & Paquis-Flucklinger V (2000)** MSH4 acts in conjunction with MLH1 during mammalian meiosis *Faseb J* **14,** 1539-47.
136. **Santucci-Darmanin S, Vidal F, Scimeca JC, Turc-Carel C & Paquis-Flucklinger V (2001)** Family of SRY/SOX proteins is involved in the regulation of the mouse MSH4 (MutS Homolog 4) gene expression *Mol Reprod Dev* **60,** 172-80.
137. **Santucci-Darmanin S, Neyton S, Lespinasse F, Saunieres A, Gaudray P & Paquis-Flucklinger V (2002)** The DNA mismatch-repair MLH3 protein interacts with MSH4 in meiotic cells, supporting a role for this MutL homolog in mammalian meiotic recombination *Hum Mol Genet* **11,** 1697-706.
138. **Eaker S, Cobb J, Pyle A & Handel MA (2002)** Meiotic prophase abnormalities and metaphase cell death in MLH1-deficient mouse spermatocytes: insights into regulation of spermatogenic progress *Dev Biol* **249,** 85-95.
139. **Lipkin SM, Moens PB, Wang V, Lenzi M, Shanmugarajah D, Gilgeous A, Thomas J, Cheng J, Touchman JW, Green ED, Schwartzberg P, Collins FS & Cohen PE (2002)** Meiotic arrest and aneuploidy in MLH3-deficient mice *Nat Genet* **31,** 385-90.
140. **Qin J, Baker S, Te Riele H, Liskay RM & Arnheim N (2002)** Evidence for the lack of mismatch-repair directed antirecombination during mouse meiosis *J Hered* **93,** 201-5.

141. **Wei K, Clark AB, Wong E, Kane MF, Mazur DJ, Parris T, Kolas NK, Russell R, Hou H, Jr., Kneitz B, Yang G, Kunkel TA, Kolodner RD, Cohen PE & Edelmann W (2003)** Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility *Genes Dev* **17,** 603-14.
142. **Bartek J, Falck J & Lukas J (2001)** Chk2 kinase--a busy messenger *Nat Rev Mol Cell Biol* **2,** 877-86.
143. **Stevens C, Smith L & La Thangue NB (2003)** Chk2 activates E2F-1 in response to DNA damage *Nat Cell Biol* **22,** 22.
144. **Ginsberg D (2002)** E2F1 pathways to apoptosis *FEBS Lett* **529,** 122-5.
145. **Trimarchi JM & Lees JA (2002)** Sibling rivalry in the E2F family *Nat Rev Mol Cell Biol* **3,** 11-20.
146. **Goldberg M, Stucki M, Falck J, D'Amours D, Rahman D, Pappin D, Bartek J & Jackson SP (2003)** MDC1 is required for the intra-S-phase DNA damage checkpoint *Nature* **421,** 952-6.
147. **Lou Z, Minter-Dykhouse K, Wu X & Chen J (2003)** MDC1 is coupled to activated CHK2 in mammalian DNA damage response pathways *Nature* **421,** 957-61.
148. **Stewart GS, Wang B, Bignell CR, Taylor AM & Elledge SJ (2003)** MDC1 is a mediator of the mammalian DNA damage checkpoint *Nature* **421,** 961-6.

SAMENVATTING VOOR IEDEREEN

Gedurende de spermatogenese, de ontwikkeling van de mannelijke voortplantingscellen, wordt het genoom voorbereid voor voortplanting. Waar de meeste cellen in het lichaam dienen voor het functioneren van het individu, bevatten de voortplantingscellen de genetische toekomst. In de lichaamscellen kan beschadiging van het genoom, oftewel DNA-schade, leiden tot de ontwikkeling van kankercellen. Echter, dergelijke beschadigingen in een voortplantingscel worden ook nog eens geërfd door alle cellen van het nageslacht. Het is dus belangrijk dat tijdens de ontwikkeling van voortplantingscellen het genoom goed beschermd wordt tegen beschadigingen.

Beschadigingen van het genoom kunnen op vele manieren optreden, bijvoorbeeld door genotoxische stoffen of straling. Tijdens de spermatogenese worden echter ook expres DNA-breuken veroorzaakt door de voortplantingscellen zelf. Dit gebeurt tijdens een zeer speciale celdeling, de meiose. In alle lichaamscellen komen de chromosomen dubbel (diploïd) voor, een paternaal en maternaal chromosoom (23 paar bij de mens). Echter, ei- of spermacellen hebben een enkelvoudig genoom (haploïd). Tijdens de bevruchting komen het maternale en paternale genoom weer samen in de eicel en onstaat een er diploïd embryo. Tijdens de meiotische celdeling worden, in het volwassen orgamisme, het maternale en paternale genoom weer opgesplitst zodat er weer haploïde voortplantingscellen onstaan. Tijdens dit opsplitsen worden delen van het maternale en paternale genoom uitgewisseld. Dit uitwisselen heet crossing-over. Crossing-over is een evolutionair belangrijk proces dat zorgt voor grotere genetische variatie in de voortplantingscellen. Om crossing-over plaats te laten vinden moeten er DNA-breuken gemaakt worden. Het onstaan en de reparatie van deze breuken is een zeer streng gereguleerd proces en bij fouten kunnen erfelijke mutaties ontstaan.

Cellen kunnen op verscheidene manieren reageren op beschadigingen van het genoom. Ten eerste moet de schade ontdekt en gemarkeerd worden. Vervolgens ontstaat er ter plaatse van een beschadigd stuk DNA een groot eiwitcomplex (eiwitten zijn grote organische moleculen die bijna al het werk in de cel verrichten, het genoom bevat het bouwplan voor alle eiwitten). Dit eiwitcomplex kan de beschadigde cel onder andere aanzetten tot het stoppen van zijn delingsactiviteit, oftwel celcyclus-arrest. Veel vormen van kanker zijn erg kwaadaardig doordat de kankercellen het vermogen tot celcyclus-arrest hebben verloren en dus ondanks alles blijven delen of woekeren.

Tijdens het celcyclus-arrest kan de cel evalueren hoe erg de schade is. Als de schade heel erg is kan een cel besluiten om er een einde aan te maken. Deze vorm van cellulaire zelfmoord heet apoptose. Veel vormen van kanker onstaan doordat cellen het vermogen tot apoptose verliezen en “onsterfelijk” worden.

Vaak valt de schade mee en besluit een cel om het DNA te repareren. Deze reparatie moet wel zeer nauwkeurig verlopen en achteraf gecontroleerd worden, anders kunnen er tijdens het repareren weer nieuwe beschadigingen optreden. Wanneer cellen het vermogen verliezen beschadigingen nauwkeurig te repareren kunnen er mutaties in het genoom optreden en kunnen er kankercellen ontstaan.

In dit proefschrift staan verschillende aspecten beschreven van de respons van mannelijke voortplantingscellen op DNA-schade. Hoofdstuk twee gaat over intercellulaire bruggetjes die na elke celdeling van de mannelijke voortplantingscellen ontstaan. Door deze bruggetjes blijven de voortplantingscellen na elke deling met elkaar verbonden. Na een aantal delingen onstaan er zo lange strengen van aan elkaar verbonden voortplantingscellen. Door de bruggetjes kunnen de cellen met elkaar communiceren en bijvoorbeeld hun celcycli op elkaar afstemmen waardoor ze synchroon blijven delen. Echter, als een van de cellen binnen een streng beschadigd raakt en in apoptose gaat heeft dit niet automatisch effect op de buurcellen.

Het derde hoofdstuk gaat over twee eiwitten, c-Abl en p73, die samen apoptose kunnen induceren. p73 lijkt erg op het eiwit p53 dat erg belangrijk is voor het induceren van apoptose. Toch kunnen cellen ook zonder p53, via alternatieve routes, in apoptose gaan. Onderzocht is of apoptose via c-Abl en p73 een goed alternatief zou kunnen zijn. In de meeste voortplantingscellen bevinden deze eiwitten zich verassend genoeg niet in de celkern, welke het DNA bevat, maar in het cytoplasma. Toch reageren deze twee eiwitten op bestraling van de cel en zetten een reactie in gang die tot apoptose kan leiden. Het genoom is niet het enige in een cel dat beschadigd kan raken en ook bestraling van andere cellulaire onderdelen kan tot apoptose leiden.

In hoofdstuk vier wordt beschreven hoe DNA breuken, die ontstaan door bestraling of tijdens de meiose, gemarkeerd kunnen worden. Het DNA ligt niet los in de celkern maar is gewikkeld om DNA-inpak-eiwitten, de histonen. Een van die histonen heet H2AX. H2AX heeft de bijzondere eigenschap dat het gemarkeerd wordt wanneer er een DNA-breuk optreedt en transformeert tot γ-H2AX. Rondom dit γ-H2AX verzamelen zich vervolgens allerlei DNA-reparatie-eiwitten en onstaan er γ-H2AX-foci. Deze foci kunnen zichtbaar gemaakt worden waardoor je kunt bestuderen waar, wanneer en hoeveel DNA-breuken er onstaan. In het algemeen is het aantonen γ-H2AX dus een goede manier om DNA-breuken aan te tonen. Gedurende de spermatogenese onstaat γ-H2AX echter niet alleen bij DNA-breuken maar ook vlak voor de meiose en op de geslachtschromosomen (X&Y).

Hoofdstuk vijf gaat over een eiwitcomplex, DNA-PK genaamd, dat betrokken is bij het repareren van breuken in het DNA. Dit complex werkt relatief eenvoudig en “plakt” gebroken DNA-uiteinden weer aan elkaar. Dit plakken gebeurt echter niet heel nauwkeurig en het kan voorkomen dat de verkeerde uiteinden aan elkaar geplakt worden. Daarom worden de DNA-breuken die onstaan tijdens de meiose via een ander mechanisme gerepareerd. Wanneer tijdens de celcyclus al het DNA in duplo aanwezig is kan de niet beschadigde “DNA-dubbelganger” als voorbeeld voor de reparatie gebruikt worden. Hiertoe worden tijdelijk DNA strengen tussen het beschadigde en niet beschadigde DNA uitgewisseld in een proces dat homologe recombinatie heet. Crossing-over tijdens de meiose is een vorm van homologe recombinatie. Om te voorkomen dat tijdens de meiotische celdeling toch het simpelere “DNA-plakken” in werking treedt zorgen de meiotische cellen ervoor dat een paar essentiele onderdelen van het DNA-PK-complex precies op het goede moment afwezig zijn. Hierdoor worden in deze cellen de DNA-breuken voornamelijk gerepareerd door relatief foutloze meiotische recombinatie.

Het laatste onderzoekshoofdstuk beschrijft waar en wanneer in de testis het eiwit ATM voorkomt en actief wordt. Dit eiwit is betrokken bij het ontdekken van DNA-schade en de initiatie van de respons van de beschadigde cel. Nadat ATM DNA-schade ontdekt heeft gaat het onmiddelijk allerlei andere eiwitten activeren die van belang zijn voor celcyclus-arrest, apoptose of DNA-reparatie. Hoogst waarschijnlijk is het ook ATM dat zorgt voor het ontstaan van de γ-H2AX-foci zoals beschreven in hoofdstuk vier. ATM is dus belangrijk voor het in gang zetten van de stralingsrespons van de voortplantingscellen. Daarnaast is ATM ook essentieel voor het goed verlopen van de meiose. Wat het tijdens deze fase precies doet en hoe is echter nog onbekend.

Juist doordat de DNA-schade-respons in voortplantingscellen zo specifiek en strak georganiseerd is vormt spermatogenese een uitstekend model voor de studie naar regulatie van verschillende DNA-schade-reparatiemechanismen. Hierdoor kan kennis over spermatogenese bijdragen aan de kennis over het ontstaan van kanker en de respons van kankercellen op stralings- of chemotherapie.

DANKWOORD/ ACKNOWLEDGEMENTS

Promoveren kan een zeer individuele bezigheid zijn. Toch hebben ook vele anderen op een zeer positieve manier bijgedragen aan dit proefschrift. Voor al deze mensen een woord van dank.

Als eerste voor mijn promotor; **Dick**, bedankt voor de ruimte die je hebt gegeven aan al mijn ideeën. De vrijheid om allerlei, niet altijd even succesvolle, experimenten uit te kunnen voeren heb ik zeer op prijs gesteld. Ook de snelheid waarmee je mijn manuscripten las en van opbouwende kritiek voorzag heb ik altijd zeer gewaardeerd.

Uiteraard ben ik ook veel dank verschuldigd aan mijn vele collega's, eerst bij celbiologie in het AZU en later, na de grote verhuizing, bij endocrinologie in het Kruytgebouw. **Hermien**, jij was officieel verbonden aan dit project en hebt dan ook vele immuno's voor mij uitgevoerd. Dit leidde bijna altijd tot zeer mooie resultaten, en ik zou haast willen beweren dat een immuno die in jouw handen niet lukt onmogelijk is. Enorm bedankt, ook voor de nodige gezelligheid. In den beginne waren er ook mijn collega-AIO's, **Tim & Bianca**. Tim, onze tijd samen was kort maar mede door jouw proefschrift kon ik snel en efficiënt van start. Bianca, nog steeds vinden we wel eens een pincet of stift met de tekst: "Bianca, afblijven!", maar ook zonder dat waren wij jou vast niet vergeten. **Ans**, je bent een enorm fijne collega, altijd kon ik bij jou terecht met praktische probleempjes of om over 'teen of 'tander te discussiëren. Het was super om met jou samen te werken. **Fari**, naast wetenschap waren er ook andere belangrijke gespreksonderwerpen, zoals wereldpolitiek, het leven (**Jean-Luc**, bedankt voor je pizza's) of gewoon het weer. **Laura**, bedankt voor je vriendschap. Onze reis naar San Francisco was geweldig en je was een ideale reisgenoot. **Majken**, jouw kritische vragen en opmerkingen werden zeker door iedereen gewaardeerd. **Pedro**, you have to work under much more difficult circumstances than most of us and the way you keep on going has my admiration. Hopefully, the situation for you and Venezuela will improve. **Henk**, keep on rockin' in the free world! **Krista**, het was een grote slag voor onze groep toen je besloot een andere baan aan te nemen, je enthousiasme was aanstekelijk. **Henriëtte**, je was een gezellige collega, ik hoop dat je je draai gevonden hebt in je nieuwe werk.

Na de grote volksverhuizing kwamen er een hoop nieuwe collega's bij. **Rüdiger**, je lijkt soms wel een wandelende endocrinologische encyclopedie, bedankt voor het delen van je kennis. **Jan**, enorm bedankt voor het helpen bij het moleculaire gedeelte van een in situ hybridizatie die verder helaas niet in dit proefschrift terug te vinden is. Ook bedankt **Wytske & Joke**, na de verhuizing voelden wij ons, mede dankzij jullie, al snel welkom. Joke, bedankt voor het rode fietstasje! **Astrid,** het was super om eens als docent in de collegezaal te staan. Ook heb ik sinds een tijdje nieuwe buren, **Canan & Wouter**, en een nieuwe kamergenote, **Maaike**. Jullie kennen de groep alleen maar zoals hij nu is, na de verhuisperikelen, en hebben dus geen last van de vergelijking met vroeger. Het was fijn om mijn laatste loodjes met jullie te delen en ik wens jullie veel succes toe. Also **Takeshi**, good luck and a pleasant stay in the Netherlands. Ook waren er de studenten: **Marrit** (mooie scriptie!), **Gregor**, **Henriëtte**, **Lydia**, **Jochem** (Coffee-man…), **Ilona** en **Anna & Maria** (the Spanish girls). Studenten vormen een belangrijk deel van een onderzoeksgroep, jullie bepalen niet alleen voor een groot deel de sfeer maar zorgen er ook voor dat wij onderzoekers niet alleen maar met onszelf bezig zijn. Ook bedankt, iedereen aan de **tien-uur-koffietafel**, dat jullie nog lang mogen genieten van jullie dagelijks bakkie pleur. **Frits & Hans**, het is heerlijk om midden in de week een uurtje hard te lopen. Hopelijk ben ik in oktober nog een beetje in vorm voor de estafetteloop waar jullie mij voor geronseld hebben.

Ook wil ik **iedereen bij celbiologie** zeer hartelijk bedanken. Ik heb tijdens mijn eerste twee jaren als AIO enorm veel aan jullie gehad, op zowel wetenschappelijk als sociaal gebied. In

jullie groep voelde ik mij als een vis in het water, wat ook leidde tot een hoge wetenschappelijke output.

Naast onze eigen onderzoeksgroepen hebben we ook samengewerkt met andere onderzoeksgroepen. Voor de stralingsexperimenten waren we afhankelijk van **Iris** en **Henk** van radiotherapie in het UMC. Enorm bedankt voor jullie hulpvaardigheid en belangstelling voor mijn onderzoek. Iris, ik hoop dat je nieuwe carriëre je weer net zoveel plezier brengt als die van analiste.

Everyone at Hannu's lab, many thanks for your great hospitality, I can recommend everyone to visit the Sariola Circus. Special thanks to **Eric,** it was nice to work with you and I appreciated the time we spend together with your friends in Helsinki and Tallinn. Also **Satu**, many thanks for your help with the in situ-robot. **Mark, Ras** and friends, thank you for your weekend entertainment. We will meet again!

Terry, I really enjoyed working with you and I'm very happy to have had the chance to visit Yale University. Also many thanks to **Adelle** for being such a good teacher and to **Ann** for being my colleague-for-one-week. My staying in New Haven would not have been the same without my beautiful room in St. Ronan Street, thank you **Will**. Terry, I'm very happy to have you in my thesis-committee and I'm looking forward to discussing my work with you in September.

Paul & Annemarie, uit Leiden, jullie zorgden voor het bestralen van de *scid* muizen en droegen derhalve bij aan maar liefst twee hoofdstukken uit dit proefschrift. Paul, je hebt me vaak geïnspireerd en een gesprek met jou leidde meestal tot een aantal nieuwe experimenten. Ik ben dan ook erg blij dat je in mijn promotiecommissie zit zodat ik in september een paar kritische en inhoudelijke vragen kan verwachten.

Also I would like to thank all the **PhD-students** of the Graduate School of Developmental Biology. Your company during the different meetings has been inspiring and useful.

Bijna aan het einde van dit dankwoord, mijn **vrienden & familie**. Ik zal jullie niet allemaal op gaan noemen want dat zou, met name in het geval van alle Hamers, een wel heel grote opsomming op gaan leveren. Ik hoop jullie nog te zien voor ik naar Stockholm vertrek. **Jelka & Klaas**, bedankt dat jullie mijn paranimfen willen zijn.

En dan nu, als slotstuk en kroon op dit dankwoord, **Else**;
Leven met jou, zeker weten!

Skål !!!

Geert

CURRICULUM VITAE

Geert Hamer was born in Oldekerk, Groningen, the Netherlands on the 23rd of October, 1973. After highschool, in 1993, he enrolled in the study Biology at the University of Utrecht, the Netherlands. As an undergraduate student he specialized in Cell Biology and performed two 9-month research projects. The first project was performed at the department of Molecular Cell Biology, Utrecht University, under supervision of Dr. M.G. Nievers en Dr. P.M.P. van Bergen en Henegouwen, investigating the molecular characteristics of "Cell-Cell" and "Cell-Extra Cellular Matrix" interactions by studying isolated Focal Adhesions. During the second project, performed at the Hubrecht laboratory (Netherlands Institute for Developmental Biology, NIOB), Utrecht under supervision of Dr. A. Beverdam en Dr. F. Meijlink, he investigated protein interactions of Paired Class Homeodomain proteins using the yeast two-hybrid system.

After obtaining his Masters degree he has been working as a PhD student at the University of Utrecht at the departments of Endocrinology and Cell Biology, Faculty of Biology and Utrecht Medical Center respectively, supervised by Prof. Dr. Dirk G. de Rooij and participating in the Utrecht Graduate School of Developmental Biology. For this graduate school he has been a member of the PhD student committee, which organizes the yearly PhD students "retreat". The results of his PhD project are described in this thesis. During the project additional laboratory work has been performed at the Biomedicum Helsinki, Department of Biomedicine, Developmental Biology, Helsinki, Finland, in the group of Prof. Dr. Hannu Sariola and at Yale School of Medicine, Department of Genetics, New Haven CT, USA, in the group of Dr. Terry Ashley.

In December 2003 he will start as a post-doc researcher in the group of Prof. Dr. Christer Höög at the Department of Cell and Molecular Biology at the Karolinska Institute in Stockholm, Sweden.

PhD COURSES

Radiation Hygiene, level 4B, Reactor Institute, Delft, the Netherlands
Laboratory Animal Science, faculty of Veterinarian Sciences, Utrecht University
Signal Transduction (theoretical course), Graduate School of Developmental Biology, Utrecht
Bio-Statistics, Center for Bio-Statistics, Utrecht University
Master Classes of the Research school of Developmental Biology, 2000-2002

CONFERENCES & LECTURES

XVIth American Testis Workshop, 2001, New Port Beach, Los Angeles, CA, USA. Poster presentation entitled, "Interaction of c-Abl with p73 in Response to Ionizing Radiation in the Cytoplasm of Germ Cells", p G-52 of the final program and abstract book.

Spring meeting 2001 of the Dutch Society of Radiation Biology, Utrecht, the Netherlands. Presentation entitled, "Interaction of c-Abl with p73 in Response to Ionizing Radiation in the Cytoplasm of Germ Cells".

Spring meeting 2002 of the Dutch Society of Radiation Biology, Leiden, the Netherlands. Presentation entitled, "Function of DNA-PKcs during the Early Meiotic Prophase without Ku70 and Ku86".

XIIth European Workshop on Molecular and Cellular Endocrinology of the Testis, 2002, Doorwerth, the Netherlands. 5 minute presentation and abstract entitled, "Apoptosis in the *Scid* Testis", p 6-9 of the final program and abstract book.

Workshop supervisor for the course "Cells & Disease" for first year medical students, 2002, Utrecht University, 6X 1 hour per week.

PhD students "retreats", 2000-2003, of the Research School of Developmental Biology, Utrecht. Abstracts and brief lectures of 5, 10 and 15 minutes respectively.

Hyytiälä meeting for Developmental Biology, 2003, Finnish Society for Developmental Biology, Hyytiälä, Finland. Presentation entitled, "DNA Double-Strand Breaks and γ-H2AX Signaling in the Testis". The 5th Hyytiälä Meeting, Development & Communication, Volume 2, p 4.

Endocrinology and Reproduction, 2003, Utrecht University College, 2x 45 minutes lecture for international premedical students entitled, "Genome Integrity during Spermatogenesis".

PUBLICATIONS

Hamer G, Gademan IS, Kal HB & de Rooij DG (2001) Role for c-Abl and p73 in the radiation response of male germ cells *Oncogene* **20,** 4298-304.

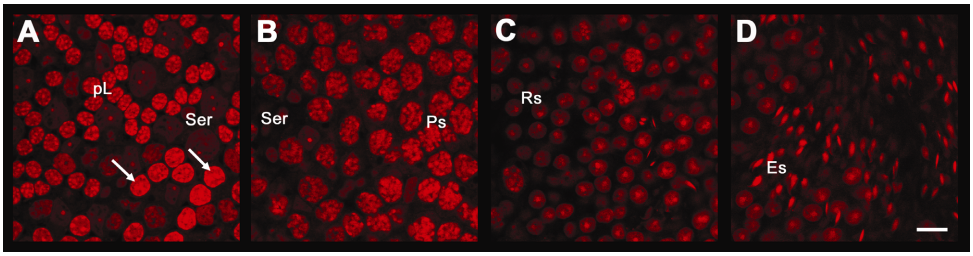
Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PPW & de Rooij DG (2003) DNA double-strand breaks and γ-H2AX signaling in the testis *Biol Reprod* **68,** 628-34.

Hamer G, Roepers-Gajadien HL, van Duyn-Goedhart A, Gademan IS, Kal HB, van Buul PPW, Ashley T & de Rooij DG (2003) Function of DNA-PKcs during the early meiotic prophase without Ku70 and Ku86 *Biol Reprod* **68,** 717-21.

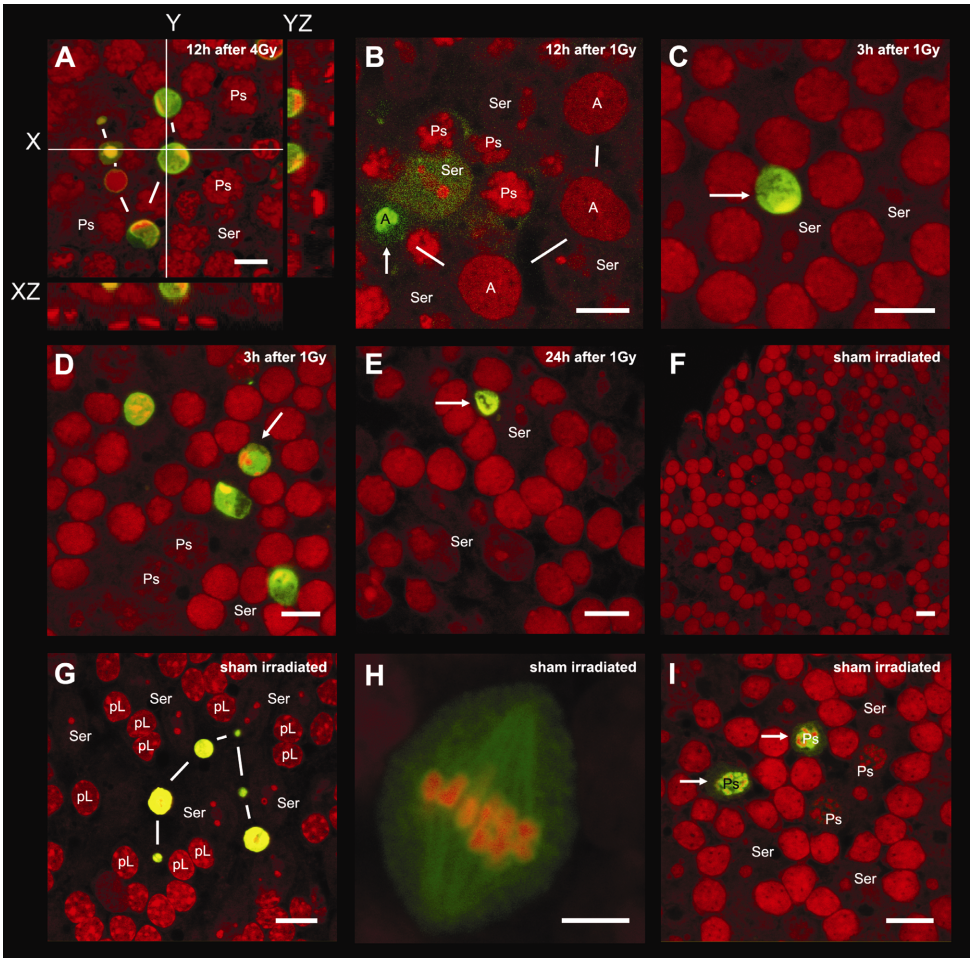
Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB & de Rooij DG (2003) Intercellular bridges and apoptosis in clones of male germ cells *Int J Androl* **in press**.

Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB, Ashley T & de Rooij DG (2003) ATM expression and activation in the testis *in preparation*

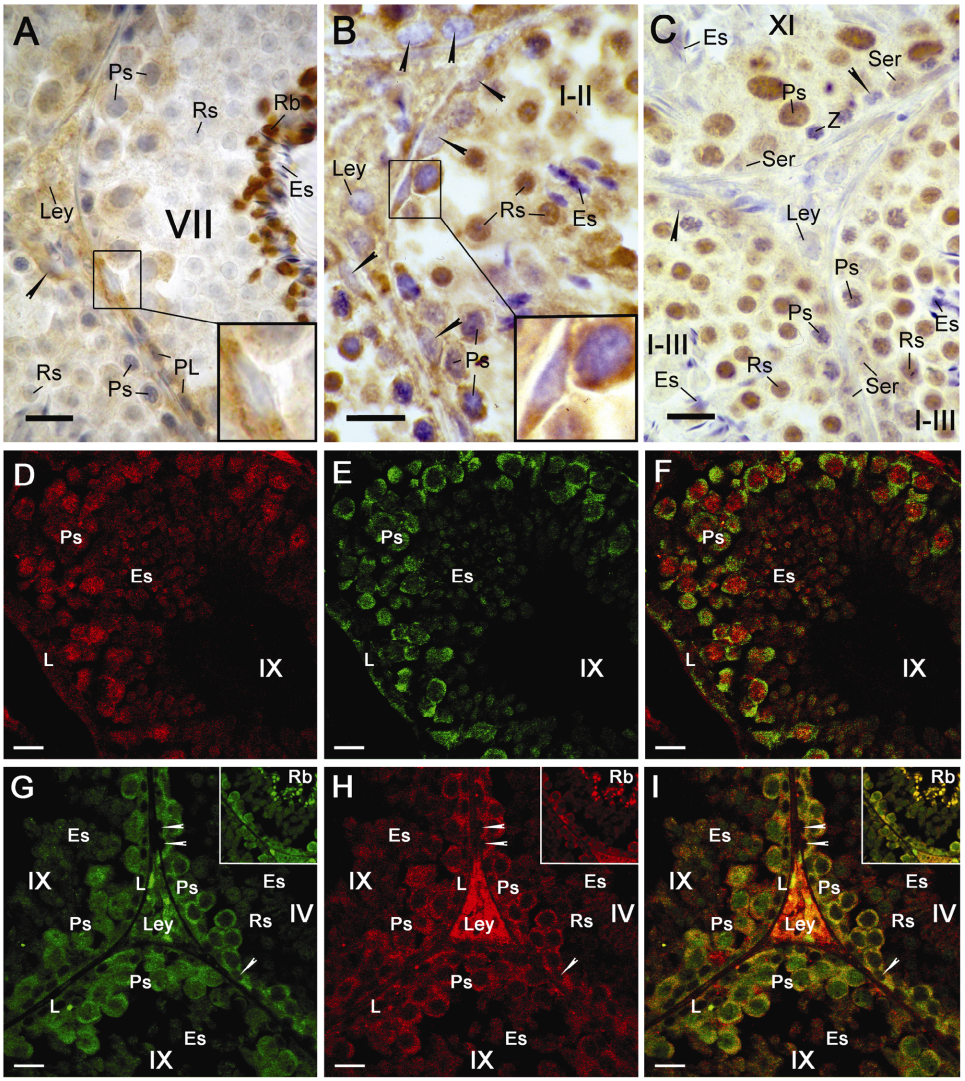
COLOR FIGURES



CHAPTER 2; FIGURE 1



CHAPTER2; FIGURE 2

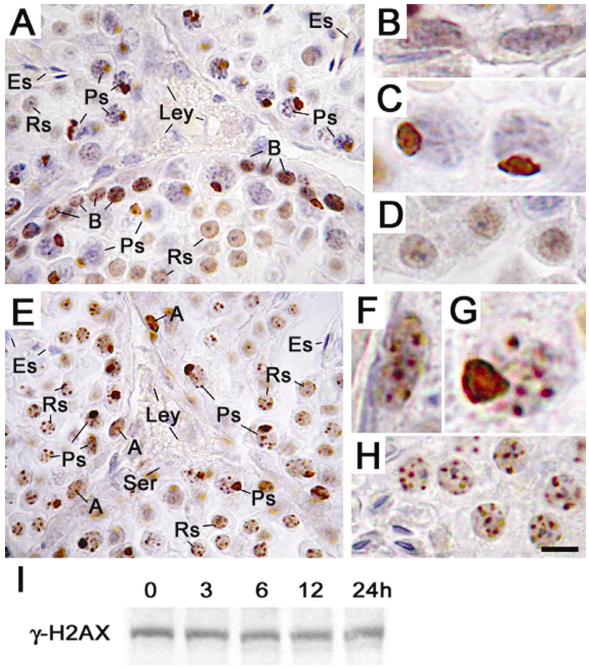


CHAPTER 3; FIGURE 1

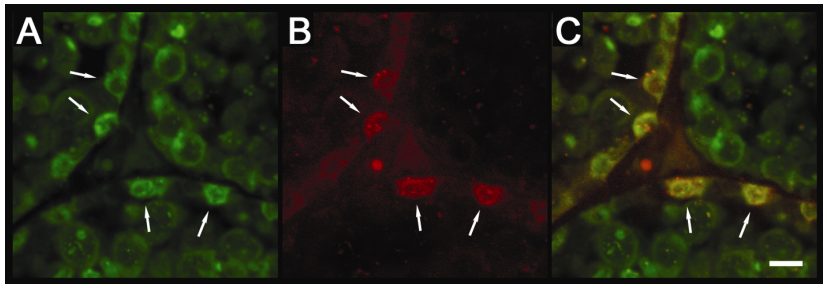
Figure 1. ***Series of confocal scans of a whole mount seminiferous tubule from the basal membrane up to the lumen,*** *showing spermatogonia (arrows), preleptotene spermatocytes (pL) and Sertoli cells (Ser)* ***(A)****, early spermatocytes (Ps)* ***(B)****, round spermatids (Rs)* ***(C)*** *and elongated spermatids (Es)* ***(D)****. Magnification: bar represents 20 µm.*

Figure 2. ***TUNEL analysis of whole mounts of seminiferous tubules.*** *12 h after a dose of 4 Gy complete spermatogonial chains are apoptotic* ***(A)****, whereas 12 h after a dose of 1 Gy mostly single spermatogonia are TUNEL positive (arrow)* ***(B)****. These TUNEL positive apoptotic cells lay at the basal membrane of the tubules as previously shown on sections (12) and visualized by projecting sequential scans through the tubules in a XZ or YZ plane* ***(A)****. After 12 h single Sertoli cells (Ser) were observed that phagocytized the apoptotic germ cells* ***(B)****. Also 3 h after a dose 1 Gy many single apoptotic spermatogonia exist within clones of unaffected spermatogonia although not as degenerated as after 12 h (arrows)* ***(C&D)****. 24 h after a dose of 1 Gy these single cells (arrow) are mostly degraded but still did not affect their neighbouring cells* ***(E)****. In the sham-irradiated testis TUNEL positive spermatogonia were very rare* ***(F)****, but were invariably found in apoptotic clusters* ***(G)****. Also cells at the metaphase stage* ***(H)*** *and apoptotic spermatocytes (arrows)* ***(I)*** *are TUNEL positive. Magnification: bar represents 20 µm or 5 µm* ***(H)****. Further depicted are: Sertoli cells (Ser), A spermatogonia (A), preleptotene spermatocytes (pL), pachytene spermatocytes (Ps).*

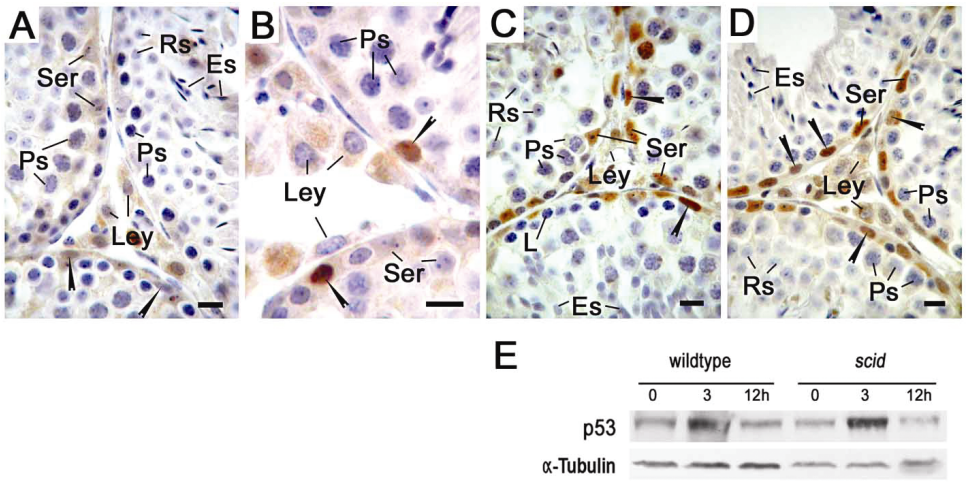
Figure 1. ***Expression of c-Abl, p73 and p63 in the testis.*** *Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, PL: pre leptotene spermatocytes, L: leptotene spermatocytes, Z: zygotene spermatocytes, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids, Rb: residual bodies. Insets show magnification of a spermatogonium* ***(A&B)*** *and a spermatocyte* ***(B)*** *or a tubule containing residual bodies* ***(G,H&I)****. Stages of the seminiferous epithelium are indicated with Roman numerals. DAB staining shows expression of c-Abl in the cytoplasm of spermatogonia, spermatocytes, Leydig cells and residual bodies* ***(A)****, p73 in the cytoplasm of spermatogonia, early spermatocytes and Leydig cells and in the nucleus of in round spermatids* ***(B)****, and nuclear p63 in spermatocytes, round spermatids and Sertoli cells* ***(C)****. Confocal images also show expression of both p63* ***(D)*** *and p73* ***(G)****, and of c-Abl* ***(E&H)****. Merged pictures show co-localization of c-Abl with p73* ***(I)*** *and not p63* ***(F)****. Note nuclear expression of p73 in late spermatocytes (stage IX)* ***(G&I)****. Magnification: bar represents 20 μm.*



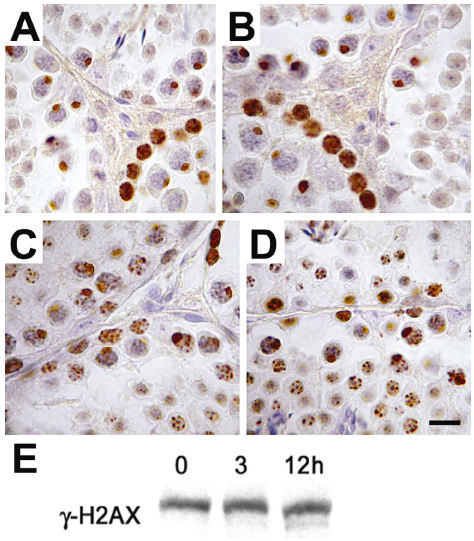
CHAPTER 4; FIGURE 1



CHAPTER 4; FIGURE 3



CHAPTER 4; FIGURE 4



CHAPTER 4; FIGURE 5

Figure 1. ***Presence of γ-H2AX in the non-irradiated (A,B,C&D) and irradiated mouse testis (E,F,G&H).*** *(A) γ-H2AX in the non-irradiated testis showing staining in B spermatogonia (B), round spermatids (Rs) and the sex bodies of pachytene spermatocytes (Ps) and γ-H2AX negative elongated spermatids (Es) and Leydig cells (Ley).* ***(B)*** *Light γ-H2AX in type A spermatogonia.* ***(C)*** *Staining of the sex bodies of pachytene spermatocytes.* ***(D)*** *Even nuclear staining of round spermatids.* ***(E)*** *γ-H2AX in the irradiated testis showing nuclear foci in type A spermatogonia (A), pachytene spermatocytes (Ps) and round spermatids (Rs).* ***(F)*** *Nuclear foci in irradiated spermatogonium,* ***(G)*** *spermatocyte and* ***(H)*** *round spermatids. Magnification: bar represents 20 μm* ***(A&E)*** *or 10 μm.* ***(I)*** *No differences in γ-H2AX levels could be detected after various time points after treatment with ionizing radiation (IR) using Western blot analysis.*

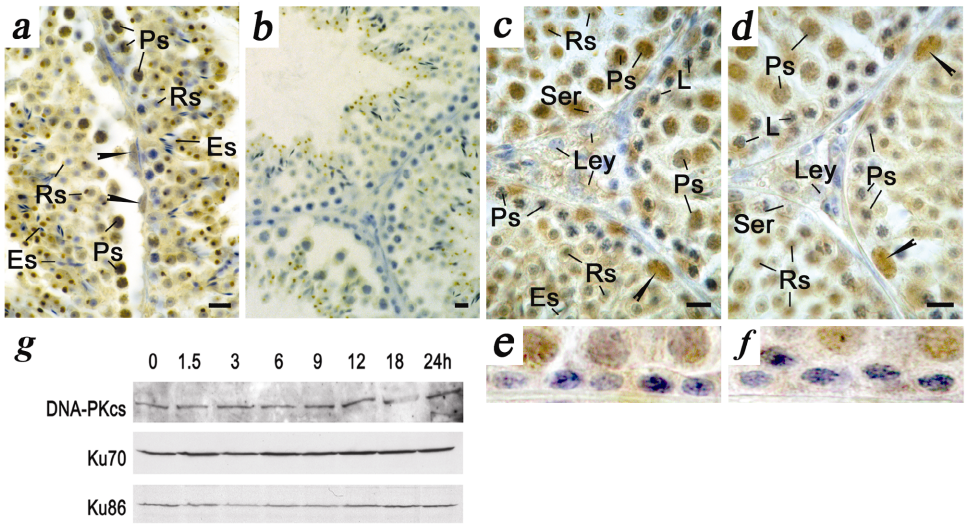
Figure 3. ***Spermatogonial co-localization of γ-H2AX and p53 in the irradiated testis.*** *Confocal images showing γ-H2AX* ***(A)*** *and p53* ***(B)*** *in the irradiated testis.* ***(C)*** *Merged picture showing co-localization of γ-H2AX and p53 in spermatogonia (arrows). Magnification: bar represents 20 μm.*

Figure 4. ***p53 induction in scid and wildtype spermatogonia.*** *p53 staining as shown in sham-irradiated wildtype* ***(A)*** *and scid* ***(B)*** *testes and in irradiated wildtype* ***(C)*** *and scid* ***(D)*** *testes. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids. Magnification: bar represents 20 μm.* ***(E)*** *Western blot analysis of p53 in total testis lysates of wildtype and scid testes before and after irradiation showing an increase of p53 in response to ionizing irradiation in both the wildtype and scid testis. PVDF membranes stained for p53 were stripped and reprobed using an antibody against α-tubulin.*

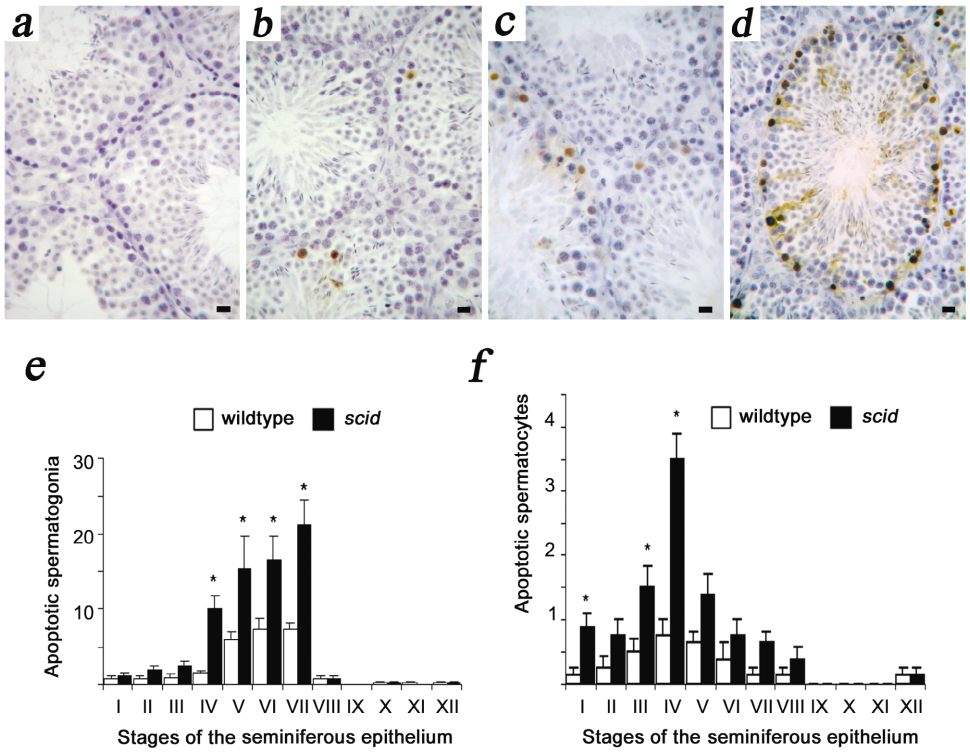
Figure 5. ***Presence of γ-H2AX in the non-irradiated (A&B) and irradiated (C&D) wildtype (A&C) and scid (B&D) mouse testis.*** *Magnification: bar represents 20 μm. Also in the scid testis no differences in γ-H2AX levels could be detected at various time points after treatment with ionizing radiation (IR)* ***(E)****.*

Figure 1. ***Expression and localization of DNA-PK in the testis.*** *Localization of DNA-PKcs* ***(A)****, negative control for DNA-PKcs showing cytoplasmic staining and Golgi-like staining in round and elongated spermatids* ***(B)****, Ku70* ***(C)****, Ku86* ***(D)*** *and B spermatogonia negative for Ku70* ***(E)*** *and Ku86* ***(F)****. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, L: leptotene spermatocytes, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids. Magnification: bar represents 20 µm.* ***(G)*** *Western blot analysis of DNA-PKcs, Ku70 and Ku86 in total testis lysates before and after various time intervals after irradiation showing no change in protein levels in response to ionizing radiation (IR). The results presented in this figure were obtained using FvB/NAU mice.*

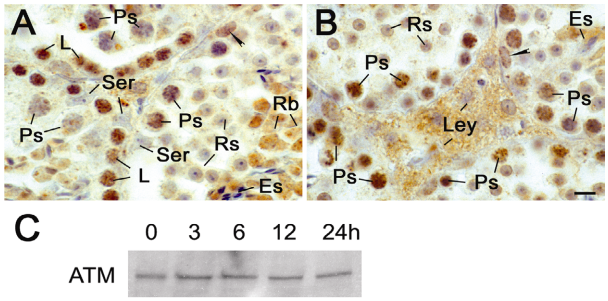
Figure 2. ***Apoptosis in scid and wildtype testes before and after irradiation.*** *TUNEL analysis is shown for sham-irradiated wildtype* ***(A)*** *and scid* ***(B)*** *testes and irradiated wildtype* ***(C)*** *and scid* ***(D)*** *testes. Average number of apoptotic spermatogonia 12 h after irradiation* ***(E)*** *or apoptotic spermatocytes independent from irradiation* ***(F)*** *per tubule cross-section at different stages of the seminiferous epithelium. Of every stage of the seminiferous epithelium the number of apoptotic spermatogonia or spermatocytes of at least 10 tubule cross sections were counted per mouse. The average numbers of apoptotic cells of 4 mice are depicted; error bars depict the standard error of the mean (SEM). Difference between wildtype and scid is statistically analyzed using the non-parametric Mann-Whitney test (two tailed P value < 0.05*). The results presented in this figure were obtained using mice on a CB-17 genetic background.*



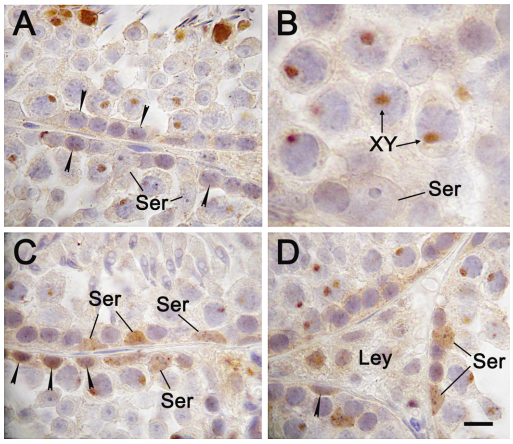
CHAPTER 5; FIGURE 1



CHAPTER 5; FIGURE 2



CHAPTER 6; FIGURE 1



CHAPTER 6; FIGURE 2

Figure 1. ***Expression and localization of ATM in the testis.*** *Localization of ATM in the testis* ***(A&B)****. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, L: leptotene spermatocytes, Ps: pachytene spermatocytes, Rs: round spermatids, Es: elongating spermatids. Magnification: bar represents 20 μm.* ***(C)*** *Western blot analysis of ATM in total testis lysates before and after various time intervals after irradiation showing no change in protein levels in response to ionizing radiation (IR).*

Figure 2. ***Phosphorylation of ATM in the testis.*** *Localization of phosphorylated ATM in the testis before* ***(A&B)*** *and after* ***(C&D)*** *irradiation. Arrowheads show spermatogonia, Ley: Leydig cells, Ser: Sertoli cells, XY: sex bodies in pachytene spermatocytes. Magnification: bar represents 20 μm or 10 μm in panel B.*