Kaarina Reini

CHARACTERISATION OF THE HUMAN DNA DAMAGE RESPONSE AND REPLICATION PROTEIN TOPOISOMERASE IIβ BINDING PROTEIN 1 (TOPBP1)
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CHARACTERISATION OF
THE HUMAN DNA DAMAGE
RESPONSE AND REPLICATION
PROTEIN TOPOISOMERASE IIβ
BINDING PROTEIN 1 (TOPBP1)

Academic dissertation to be presented, with the assent of the Faculty of Science of the University of Oulu, for public defence in Raahensali (Auditorium L10), Linnanmaa, on December 1st, 2006, at 12 noon

OULUN YLIOPISTO, OULU 2006
Genetic information is stored in the base sequence of DNA. As DNA is often damaged by radiation or reactive chemicals, cells have developed mechanisms to correct the DNA lesions. These mechanisms involve recognition of damage, DNA repair and cell cycle delay until DNA is restored. Failures in the proper processing of DNA lesions may lead to mutations, premature aging, or diseases such as cancer.

In this thesis study the human topoisomerase IIβ binding protein 1 (TopBP1) was identified as the homolog of budding yeast Dpb11 and fission yeast Cut5. TopBP1 was found to be necessary for DNA replication and to associate with replicative DNA polymerase ε. TopBP1 localised to the sites of DNA damage and stalled replication forks, which suggests a role in the DNA damage response. TopBP1 interacted with the checkpoint protein Rad9, which is a part of a protein complex whose function includes tethering proteins to sites of DNA damage. This supports a role for TopBP1 in the early steps of checkpoint activation after DNA damage. TopBP1 also interacted with the tumour suppressor protein p53 in a phosphorylation dependent manner. In addition, the data support a role for TopBP1 outside of S-phase. During M-phase, TopBP1 was found to localise to centrosomes along with the tumour suppressor proteins Brca1 and p53. Analysis of the expression of TopBP1 in mouse tissues suggested that TopBP1 may also play a role during meiosis. The localisation pattern of TopBP1 in mouse meiotic spermatocytes resembled that of many proteins functioning during meiotic recombination. For example, co-localisation of ATR kinase and TopBP1 was observed during meiotic prophase I. In accordance with the findings from mouse studies, the analysis of a cut5 mutant during yeast meiosis showed that Cut5 is essential for the meiotic checkpoint. These results strongly suggest that TopBP1 operates in replication and has checkpoint functions during both the mitotic and meiotic cell cycles.

Keywords: DNA damage response, DNA replication, meiosis, meiotic prophase I, mitosis, TopBP1
Acknowledgements

This study was carried out at the Department of Biochemistry and Biocenter Oulu at the University of Oulu.

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Oulu, October 2006

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<table>
<thead>
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<th>Abbreviations</th>
<th>Description</th>
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<tbody>
<tr>
<td>9-1-1 complex</td>
<td>Rad9-Rad1-Hus1 complex</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AT</td>
<td>ataxia telangiectasia</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3 related</td>
</tr>
<tr>
<td>ATRIP</td>
<td>ATR interacting protein</td>
</tr>
<tr>
<td>Brca1</td>
<td>breast cancer susceptibility protein 1</td>
</tr>
<tr>
<td>BRCT</td>
<td>Brca1 C-terminus</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>Cdc</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CKI</td>
<td>CDK inhibitor</td>
</tr>
<tr>
<td>Crb2</td>
<td>Cut5 repeat binding</td>
</tr>
<tr>
<td>Cut5</td>
<td>cell untimely torn 5</td>
</tr>
<tr>
<td>DDK</td>
<td>Dbf4 dependent kinase</td>
</tr>
<tr>
<td>DHJ</td>
<td>double Holliday junction</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide</td>
</tr>
<tr>
<td>Dpb</td>
<td>DNA polymerase B possible subunit</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>Gadd45</td>
<td>growth arrest and DNA damage 45</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>hsRad9</td>
<td>Homo sapiens Rad9</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>IR</td>
<td>ionising radiation</td>
</tr>
<tr>
<td>Mad2</td>
<td>mitotic arrest deficient 2</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>MCM</td>
<td>mini chromosome maintenance</td>
</tr>
<tr>
<td>MDC1</td>
<td>mediator of DNA damage checkpoint protein 1</td>
</tr>
<tr>
<td>MMS</td>
<td>methyl methane sulphonate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>Mus101</td>
<td>mutagen sensitive 101</td>
</tr>
<tr>
<td>Nbs1</td>
<td>Nijmegen breakage syndrome 1</td>
</tr>
<tr>
<td>ORC</td>
<td>origin recognition complex</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>Plk</td>
<td>polo-like kinase</td>
</tr>
<tr>
<td>Pol</td>
<td>DNA polymerase</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-replication complex</td>
</tr>
<tr>
<td>Psf</td>
<td>partner of Sld five</td>
</tr>
<tr>
<td>Rad</td>
<td>radiation sensitive</td>
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<tr>
<td>RDS</td>
<td>radioresistant DNA synthesis</td>
</tr>
<tr>
<td>RFC</td>
<td>replication factor C</td>
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<td>ribonucleic acid</td>
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<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>RTS</td>
<td>Rothmund-Thomson syndrome</td>
</tr>
<tr>
<td>SC</td>
<td>synaptonemal complex</td>
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<tr>
<td>SCcRad9</td>
<td><em>Saccharomyces cerevisiae</em> Rad9</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sld</td>
<td>synthetically lethal with <em>dpb11-1</em></td>
</tr>
<tr>
<td>Smc1</td>
<td>structural maintenance of chromosomes 1</td>
</tr>
<tr>
<td>spRad9</td>
<td><em>Schizosaccharomyces pombe</em> Rad9</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with tween-20</td>
</tr>
<tr>
<td>TopBP1</td>
<td>topoisomerase IIβ binding protein 1</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WCE</td>
<td>whole cell extract</td>
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List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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1 Introduction

The cell cycle is the universal process by which all cells reproduce. The main events during the cell cycle are duplication of the genetic material and its segregation into two daughter cells. As these processes are biochemically unrelated their correct order is ensured by elegant mechanisms called cell cycle checkpoints, which for example, halt the onset of mitosis and cell division in the presence of ongoing replication. However, versatility is required from these security systems, as the environment of the cells is rather hostile. Cells are constantly under assault from various endogenous and exogenous agents that cause DNA damage, such as ultraviolet (UV) light, ionising radiation (IR), reactive chemicals, and the byproducts of normal intracellular metabolism. Thus, cell cycle checkpoints have also evolved to respond to DNA lesions by stopping cell cycle progression and coordinating the repair of damage. In the case of severe damage, apoptosis can be induced. Failure of the proper response to DNA damage or perturbed replication can lead to the accumulation of mutations, chromosome aneuploidy, uncontrolled growth and eventually cancer. Inherited defects in DNA damage response proteins are known to cause diseases such as ataxia telangiectasia, xeroderma pigmentosum, Bloom, Werner, Seckel, Nijmegen breakage, and Li-Fraumeni syndrome.

In this study human topoisomerase IIβ binding protein 1 (TopBP1) was identified as the human homolog of the budding and fission yeast replication and checkpoint proteins Dpb11 and Cut5. TopBP1 was shown to be important for DNA replication and to associate with replicative DNA polymerase ε. TopBP1 was also shown to participate in the DNA damage response. Furthermore, the results suggest that TopBP1 plays a role in both mitotic and meiotic cell division.
2 Review of the literature

2.1 Cell cycle

One of the most important biological research articles is the description of DNA structure by Watson and Crick in 1953 (Watson & Crick 1953). In the same year Howard and Pelc published their work on cell proliferation in bean roots (Howard & Pelc 1953). In this study they showed that DNA synthesis occurs only within a certain limited period early in the cell division process. This ultimately led to the division of the eukaryotic cell cycle into four phases: G1 (G for gap), S (S for synthesis), G2, and M-phase (M for mitosis).

The overall length of the cell cycle in cultured mammalian cells is between 18 to 24 hours (Figure 1). A typical G1 phase lasts 8-10 hours, in contrast to embryonic cells that spend only a few minutes in G1. During G1, internal and external signals together determine whether the cell undergoes a round of duplication. If the cell passes the restriction point, the following S-phase and thus DNA replication must be completed, i.e. reversal is not possible. In the G2-phase, the cell confirms that it is ready to proceed to the subsequent M-phase where the duplicated chromosomes are segregated in less than one hour. If a cell lacks nutrients in the G1-phase, it enters transiently into a G0-phase. However, cells that have differentiated or have completed senescence are permanently in G0. (Golias et al. 2004, Becker et al. 2006.)

Studies in Saccharomyces cerevisiae and Schizosaccharomyces pombe have revealed the mechanisms of cell cycle regulation, and subsequent studies in metazoans have proven them to be conserved (reviewed in Nurse 2000, 2002). The main components of the regulatory machinery are cyclin-dependent kinases (CDKs), cyclins and CDK inhibitors (CKIs) (for review see Vidal & Koff 2000, Murray 2004). Unicellular eukaryotes have a single catalytic subunit (scCdc28 and spCdc2) that pairs with cell cycle specific regulatory subunit cyclin, whereas multicellular eukaryotes have several CDKs. Cyclin D and Cdk4/6 regulate the G1/S transition in mammalian cells. One important substrate of cyclin D/Cdk4 is the retinoblastoma tumour suppressor protein, Rb. Unphosphorylated Rb inhibits the E2F transcription factor, which controls the transcription of genes required for S-phase entry. Cyclin A/Cdk2 and cyclin E/Cdk2 are needed for progression through S-phase. The G2/M transition requires both cyclin
A/Cdk1 and cyclin B/Cdk1. (Sanchez & Dynlacht 2005.) Cell cycle reentry, namely the G0/G1 transition, is regulated by the cyclin C/Cdk3 complex that also inactivates Rb family members (Ren & Rollins 2004).

How is the (de)activation of different cyclin/CDK complexes organized in different cell cycle points? First, the levels of cyclins oscillate during the cell cycle. All known cyclins are targeted to the proteasome by ubiquitination. Second, spatial access is limited to some cyclins in e.g. cyclin B is excluded from the nucleus during S-phase and gains access only at the time of nuclear envelope breakdown. Third, there is a number of CKIs that are induced by anti-mitogenic signals or DNA damage. The binding of CKI to the cyclin/CDK complex inhibits the catalytic activity and induces cell cycle arrest. (Johnson & Walker 1999, Murray 2004.) The importance of CDK activity regulation is highlighted by the overactivity of the CDKs found in many human cancers (Shapiro 2006).

![Eukaryotic cell cycle](image)

**Fig. 1. Eukaryotic cell cycle.** R signifies the restriction point that is known as “start” in yeast. See text for details.

### 2.1.1 DNA replication

The basic principle of DNA replication was already suggested by the molecular structure of DNA presented by Watson and Crick in 1953. In their own words: “It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.” Although the principle of replication
seemed “obvious”, the discovery of how DNA synthesis is regulated and the different components involved has kept scientists occupied for more than 50 years.

2.1.1.1 Initiation of DNA replication

In mammalian chromosomes DNA replication begins at multiple origins, with an average spacing of 100 kb (Huberman & Riggs 1968). Unlike the origins in prokaryotes and lower eukaryotes, mammalian origins lack a specific consensus sequence. However, the initiation process of DNA replication and the components it requires are well conserved in eukaryotes. Origins direct the formation of the pre-replication complex (pre-RC) during late M-phase to early G1-phase. Pre-RC assembly begins with the binding of the origin recognition complex (ORC) to the origin. ORC binding is prerequisite for the following association of Cdc6 and Cdt1. The pre-RC complex is completed when the mini chromosome maintenance proteins (Mcm2-7) are recruited to the origin. The primary role of the pre-RC is the loading of MCM proteins, the presumptive replicative helicase, as ORC and Cdc6 can be removed from the chromatin after MCM loading without preventing the subsequent DNA replication. The pre-RC is also the master key to avoid overreplication. The pre-RC can only be assembled when CDK activity is low and anaphase promoting complex (APC) activity is high, whereas origin firing requires CDK activity and inhibition of APC activity. (Bell & Dutta 2002, Kearsey & Cotterill 2003, Diffley 2004, Blow & Dutta 2005, Machida et al. 2005, Stillman 2005.)

The next step in the initiation of DNA synthesis involves conversion of the pre-RC into an initiation complex. This process requires S-phase CDK and Cdc7/Dbf4 (Dbf4 dependent kinase, DDK) that are needed for the binding of additional factors to the pre-RC. CDK activates loading of Cdc45 onto the MCM proteins that are activated by DDK. Mcm10 possibly replaces Cdt1 and retains the other MCM proteins at the pre-RC. After Cdc45 binding, the origin unwinds and replication protein A (RPA) covers single stranded DNA followed by recruitment of DNA polymerases (Pol) α, ε, and δ. Cdc45 is also required for the loading of the proliferation cell nuclear antigen (PCNA), that is the processivity factor for Pol ε and δ. (Bell & Dutta 2002, Diffley 2004, Machida et al. 2005, Stillman 2005.) However, the picture is becoming more and more complex as new factors influencing initiation of DNA replication have been identified and as some factors also function in the progression of replication forks or in checkpoint control. For example in budding yeast, Dpb11 (DNA polymerase B possible subunit 11), Sld2 (synthetically lethal with dpb11-1), Sld3, and the GINS complex also have important roles in the conversion of the pre-RC into an initiation complex (Takayama et al. 2003, Gambus et al. 2006, Kanemaki & Labib 2006, Tak et al. 2006).

2.1.1.2 Elongation of DNA replication

The complex replication machinery that eventually forms from the initiation complex is called the replisome. The firing of the replication origin produces two bidirectional replication forks that copy the DNA in opposite directions from the origin. Because of the
anti-parallel structure of DNA and because DNA polymerase can only extend the DNA chain in the 5' → 3' direction, the cell needs different mechanisms to replicate the two DNA strands. The leading strand is synthesised continuously and the lagging strand is synthesised in short stretches called Okazaki fragments. A model for lagging strand replication exists. First, the Pol α/primase synthesises a short RNA/DNA primer. Next the replication factor C (RFC) binds the primer, displaces Pol α and loads PCNA. The polymerase switch is completed as PCNA docks on the more processive Pol δ. Okazaki fragment maturation requires RNase H1, endonucleases Fen1 and Dna2, and DNA ligase I. RNase H1, Fen1, and Dna2 participate in the removal of the RNA/DNA primer and DNA ligase I seals the gap between the Okazaki fragments. Paradoxically, the model for the much simpler leading strand machinery is incomplete. Although, the indirect evidence strongly suggests that Pol ε replicates the leading strand. (Waga & Stillman 1998, Hübscher et al. 2002, Toueille & Hübscher 2004, Garg & Burgers 2005, Johnson & O'Donnell 2005, Rossi et al. 2006.) This is also supported by the recent work of Rytkönen and coworkers (2006). The authors show that Pol ε is more active in early S-phase than Pol δ and vice versa in late S-phase. This suggests that Pol ε is the leading strand polymerase as the leading strand is copied faster than the lagging strand.

2.1.2 Cell division

Cell division, termed mitosis in somatic cells and meiosis in germ cells, consists of a series of dynamic events that involve the coordinated interactions of many components. Essential preparations for cell division begin already in S-phase as cohesion is built during the passage of the replication forks (Nasmyth 2001). In fact, accurate chromosome segregation is dependent on the cohesin complex that forms a ring around the sister chromatids (Nasmyth 2005b, Nasmyth & Haering 2005). Errors in the transmission of chromosomes during mitotic or meiotic cell division can lead to an aberrant number of chromosomes. Aneuploidy in somatic cells is associated with many forms of cancer (Jallepalli & Lengauer 2001). Meanwhile, errors in meiotic chromosome segregation may cause miscarriage or genetic disorders such as Down’s syndrome (Hassold & Hunt 2001). The survival and reproduction of all living organisms are clearly dependent on accurate and faithful chromosome segregation.

2.1.2.1 Mitosis

Mitosis, which produces two genetically identical daughter cells, was first described by Walther Fleming in the 1880s (Mitchison & Salmon 2001). He also coined the term mitosis from the Greek word for thread, depicting the shape of mitotic chromosomes. Traditionally, mitosis is classified into different phases based on the observable morphological changes (Figure 2, Mitchison & Salmon 2001, Pines & Rieder 2001, Pines 2006). During prophase in vertebrates a complex called condensin, is recruited to chromatin and the condensing chromosomes become visible. The breakdown of the nuclear envelope ends prophase and initiates prometaphase, during which the
chromosomes become attached to the mitotic spindle as it forms. The cell is in subsequent metaphase once all the chromosomes are aligned near the spindle equator. At anaphase the sister chromatids separate and move towards the opposing spindle poles (centrosomes), as the spindle elongates. During telophase both sets of sister chromatids have reached the opposite centrosomes and begin to decondense. Also the mitotic spindle disassembles and the nuclear-envelope reforms. The cell itself starts to divide (cytokinesis) during anaphase and telophase.

![Fig. 2. The phases of mitosis. The morphological changes used to define mitosis are presented for a typical vertebrate cell below the timeline (adapted from Pines and Rieder 2001).](image)

The most critical phase in mitosis is the transition from metaphase to anaphase caused by sudden dissolution of cohesion between sister chromatids (Figure 3). Indeed, the pathway affecting the stability of the cohesin ring is carefully controlled. Cohesin is composed of four subunits and cleavage of one of the subunits, Scc1, by the cysteine protease separin disrupts the structure of the cohesin ring (Nasmyth 2005b, Nasmyth & Haering 2005). Separin is kept inactive by its association with an inhibitory chaperone securin and phosphorylation by the cyclin B/Cdk1 (Nasmyth 2005a, Pines 2006). The degradation of securin is governed by the ubiquitin ligase APC that guides securin to the proteasome (de Gramont & Cohen-Fix 2005, Nasmyth 2005a, Pines 2006). Furthermore, the activity of APC requires an accessory protein called Cdc20 (Harper et al. 2002). Cdc20 is inhibited and sequestered by Mad2 (mitotic arrest deficient 2) if chromosomes have kinetochores (proteinaceous structures that assemble at the centromere), that are not connected to the mitotic spindle (Chan et al. 2005, Nasmyth 2005a). Thus, via this pathway cells are capable of monitoring the attachment of kinetochores to microtubules and prevent activation of the APC and thereby subsequently separin when the chromosomes are not yet properly attached to the mitotic spindle (Nasmyth 2005a). This mechanism is also known as the spindle assembly checkpoint (Lew & Burke 2003, Malmanche et al. 2006). In mammalian cells, this checkpoint is essential and an integral part of every mitosis (Nasmyth 2005a).
Fig. 3. The control of the metaphase-anaphase transition. Proteolytic cleavage of the cohesin subunit Scc1 disrupts cohesin ring and allows separation of sister chromatids (adapted from Nasmyth and colleagues 2000).

2.1.2.2 Meiosis

The purpose of meiosis in animals is to generate haploid gametes from diploid precursors. The reduction in chromosome number is achieved by one round of DNA replication being followed by two sequential rounds of chromosome segregation (meiosis I and meiosis II) (Petronczki et al. 2003). Pre-meiotic DNA replication is similar to pre-mitotic S-phase in many respects (Strich 2004). However, there is evidence that DNA replication is coupled differently to other cell cycle events in meiotic cells, as initiation factors Cdc6 and MCM proteins are not required for pre-meiotic S-phase in fission yeast (Forsburg & Hodson 2000). Furthermore, pre-meiotic S-phase is considerably longer compared to mitotic S-phase (Cha et al. 2000). The extra time might be necessary for establishing the interactions between homologous chromosome pairs that are essential for their faithful segregation during the first meiotic division, meiosis I (Cha et al. 2000, Marston & Amon 2004).
Fig. 4. A) Schematic model of synaptonemal complex (SC), meiotic cohesin, and chromatin in pachytene. B) Timeline of the stages and events in the meiotic prophase I. DSB is DNA double strand break and DHJ is double Holliday junction (adapted from Petronczki and colleagues 2003).

The second division of meiosis (meiosis II) resembles mitosis: sister chromatids separate and segregate (Roeder 1997). Thus, most of the meiosis specific processes take place during the meiotic prophase I (Figure 4B), as maternal and paternal homologous chromosomes align, pair and become physically connected in a process called synapsis (Roeder 1997, Petronczki et al. 2003, Cohen et al. 2006). In most organisms, synapsis is achieved via formation of the proteinaceous synaptonemal complex (SC) (Figure 4, Zickler & Kleckner 1999, Page & Hawley 2004). A more permanent link between chromosomes is established via meiotic recombination and the associated crossing over of DNA that generates chiasmata (Gerton & Hawley 2005). Chiasmata and the preservation of sister chromatid cohesion (SCC) at centromeres enable the segregation of homologous chromosomes during the first meiotic division (Petronczki et al. 2003). The
remaining cohesin at centromeres serves also another purpose: it is used for the biorientation and segregation of sister chromatids during meiosis II (Petronczki et al. 2003). Thus, like in mitotic cell division, cohesin plays an important role during meiosis. Meiotic cohesin, however, differs from its mitotic equivalent. The Scc1 subunit of the cohesin complex is replaced partly by a meiosis-specific variant Rec8 (Watanabe 2004). Furthermore, a protein called shugoshin protects centromeric cohesin Rec8 complexes from cleavage by separin (Watanabe 2005). Interestingly, meiotic cohesin also participates in the recruitment of the recombination apparatus and promotes synapsis between homologous chromosomes (Eijpe et al. 2000, Pelttari et al. 2001, Eijpe et al. 2003).

The meiotic regulatory pathways share similarity to the equivalent mitotic pathways. For example, the spindle assembly checkpoint also prevents chromosome mis-segregation during meiosis (Malmanche et al. 2006). Furthermore, Mad2 delays APC activation in response to an erroneous spindle attachment of chromosomes in meiosis as well as in mitosis (Irniger 2006). Meiotic cells also have a unique control point called the pachytene checkpoint (Roeder & Bailis 2000). It prevents exit from the pachytene stage when meiotic recombination and chromosome synapsis are incomplete. Interestingly, the pachytene checkpoint utilises the same proteins that operate in mitotic checkpoints together with meiosis specific homologs of DNA repair proteins (Roeder & Bailis 2000, Richardson et al. 2004, Marcon & Moens 2005).

2.2 DNA damage checkpoints and their components

2.2.1 Overview of DNA damage checkpoints

The fidelity of cell division is ensured by mechanisms called cell cycle checkpoints. They can be envisioned as signal transduction pathways that enforce the proper order of cell cycle events, ensuring that the next phase is not initiated before the previous one is finished (Hartwell & Weinert 1989). Cell cycle checkpoints can also delay cell cycle progression in cases of DNA damage or a DNA replication defect, thus providing a chance to restore DNA by DNA repair. These DNA damage checkpoints were discovered in mammals and yeast by Painter and Young (1980) and Weinert and Hartwell (1988), respectively. Painter and Young hypothesized that ataxia telangiectasia (AT) cells have a defect in their ability to respond to DNA damage. Weinert and Hartwell identified the RAD9 gene in *S. cerevisiae* and showed that it controls the cell cycle response to DNA damage (Weinert & Hartwell 1988). In *Escherichia coli* the functions induced by DNA damage are called collectively the SOS response (d’Ari 1985). Today, however, the historic definition of the checkpoint pathway is inadequate to describe the function of this pathway completely. Besides controlling cell cycle arrest, DNA damage checkpoint pathways control the activation of DNA repair, the movement of DNA repair proteins to the sites of DNA damage, telomere length, transcriptional response, and induction of cell death by apoptosis (Zhou & Elledge 2000). Furthermore, some checkpoint genes are essential for cell and organism survival, suggesting that checkpoint pathways are not merely surveyors of occasional damage, but integral components of cellular physiology.
(Brown & Baltimore 2000, de Klein et al. 2000, Liu et al. 2000, Takai et al. 2000). Thus, instead of a DNA damage checkpoint, Zhou and Elledge (2000) have proposed that a “DNA damage response” concept should be employed when referring to the functions generated in a cell in response to DNA damage (Figure 5). Accordingly, the term DNA damage checkpoint should be reserved for the events that delay or arrest cell cycle progression in response to DNA damage.

Fig. 5. A general outline of the DNA damage response signalling pathway. DNA damage or perturbed replication is detected by sensors that, with the aid of mediators, transmit the signal to transducers. Finally, effectors are activated by the transducers. The pathway can lead to cell cycle arrest, transcription or DNA repair. In case of severe damage, apoptosis is induced (adapted from Zhou & Elledge 2000).

It is unknown whether there are thresholds for the level of DNA damage required to elicit the checkpoint response. It is also not explicit, what is the nature of DNA structures that trigger the DNA damage checkpoints. Nonetheless, the damage signals are most likely single stranded DNA (ssDNA) and DNA DSBs (Nyberg et al. 2002, Zou & Elledge 2003, Sancar et al. 2004). It is suggested, that different types of lesions are converted to either of these common structures. Moreover, it is suggested that these structures activate different arms of the checkpoint. DNA lesions that produce DNA DSBs activate the ATM (ataxia telangiectasia mutated) kinase pathway, and ssDNA activates the ATR (ATM and Rad3 related) kinase pathway (Abraham 2001, Nyberg et al. 2002, Sancar et al. 2004). However, there is crosstalk between the ATM and ATR pathways, as ATR can at least
partially substitute for the function of ATM, and ATM regulates ATR activation in a cell cycle specific manner after IR (Abraham 2001, Jazayeri et al. 2006).

Although the DNA damage checkpoints can be named according to the cell cycle phase, many components are shared between the individual checkpoints. ATM and ATR operate both in the G1/S and G2/M checkpoints (Abraham 2001, Nyberg et al. 2002, Sancar et al. 2004). Damage sensors and transducers appear to be shared between different checkpoints to varying degrees; thus it is the effectors that finally give the checkpoints their unique identities (Sancar et al. 2004). It is also important to note that several components of DNA damage checkpoints are required for other cell cycle checkpoints, such as the pachytene checkpoint (Roeder & Bailis 2000, Richardson et al. 2004, Marcon & Moens 2005).

### 2.2.1.1 Checkpoint proteins

Proteins operating at DNA damage checkpoints are well conserved from unicellular organisms to metazoans. They were first identified from budding and fission yeast by genetic analysis (Hartwell & Weinert 1989). Because the yeast checkpoint genes, especially the RAD genes, were well characterised before their cloning, the nomenclature is not consistent (Table 1). Here, the mammalian nomenclature is used within the text unless otherwise stated.

The checkpoint proteins can be classified as sensors, mediators, or transducers according to their role in the checkpoint (Figure 5). Sensors monitor DNA for abnormalities and initiate the checkpoint signal. Mediators further promote checkpoint signalling. Transducers, many of which are protein kinases, relay and amplify the signal by phosphorylating downstream target proteins called effectors. Eventually, the effector proteins inhibit the cell cycle phase transitions and induce other responses. The present division of proteins into sensors, mediators, and transducers, however, is not absolute (Table 1). The mediator Brc1 (breast cancer susceptibility protein 1), for one, is considered to function also as an effector (Deng 2006). In addition, ATM kinase can be regarded both as a sensor and transducer, as it phosphorylates many effector proteins but is also directly activated by DNA DSBs (Abraham 2001, Bakkenist & Kastan 2003).

The sensor proteins are thought to associate either directly with damaged DNA or indirectly by interacting with other sensor protein associated with the DNA lesion. The Rad9-Rad1-Hus1 complex (dubbed the 9-1-1 complex) plays a key role in checkpoint activation and its constituents are categorised as sensor proteins (reviewed in Parrilla-Castellar et al. 2004). The 9-1-1 subunits structurally resemble the replication factor PCNA and the interactions between subunits are consistent with the PCNA-like ring structure (St Onge et al. 1999, Volkmer & Karnitz 1999, Venclovas & Thelen 2000, Burtelow et al. 2001). Like PCNA, the 9-1-1 complex is loaded onto chromatin via the RFC clamp loader, except that Rad17 substitutes for the largest RFC subunit, p140 (Rauen et al. 2000, Lindsey-Boltz et al. 2001, Bermudez et al. 2003). Thus, it is hypothesized that during the checkpoint response Rad17-RFC recruits the 9-1-1 complex to a damaged DNA structure that is analogous to RFC recruiting PCNA to
template/primer junctions (Sancar et al. 2004). Evidence also suggests that the 9-1-1 complex also participates in DNA repair systems (Toueille et al. 2004, Helt et al. 2005).

Another complex involved in damage recognition is ATR kinase and ATRIP (ATR interacting protein). This complex associates with DNA lesions via RPA coated ssDNA and independently of Rad17-RFC/9-1-1 (Nyberg et al. 2002, Zou & Elledge 2003, Sancar et al. 2004). ATM, a member of the same kinase family as ATR, is primarily activated by DNA DSBs (Abraham 2001). The recruitment mechanism of ATM is related to that of ATR. But instead of ATRIP, the MRN (Mre11-Rad50-Nbs1) complex is responsible for recruiting ATM to DNA DSBs (Falck et al. 2005, Lee et al. 2005). Both ATM and ATR execute their function by phosphorylating checkpoint proteins.

Table 1. DNA damage checkpoint protein homologs in different eukaryotes (adapted from Zhou & Elledge 2000, Melo & Toczyski 2002, Sancar et al. 2004).

<table>
<thead>
<tr>
<th>Class of checkpoint protein</th>
<th>Mammalian</th>
<th>Xenopus</th>
<th>S. pombe</th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA-like</td>
<td>Rad1</td>
<td>Rad1</td>
<td>Rad1</td>
<td>Rad17</td>
</tr>
<tr>
<td>PCNA</td>
<td>Rad9</td>
<td>Rad9</td>
<td>Rad9</td>
<td>Ddc1</td>
</tr>
<tr>
<td>RFC-like</td>
<td>Rad17</td>
<td>Rad17</td>
<td>Rad17</td>
<td>Rad24</td>
</tr>
<tr>
<td>RFC</td>
<td>Rad17</td>
<td>Rad17</td>
<td>Rad17</td>
<td>Rad24</td>
</tr>
<tr>
<td>PIKK</td>
<td>ATM</td>
<td>ATM</td>
<td>Tel1</td>
<td>Tel1</td>
</tr>
<tr>
<td>PIKK binding partner</td>
<td>ATRIP</td>
<td>ATRIP</td>
<td>Rad26</td>
<td>Ddc2</td>
</tr>
<tr>
<td>BRCT proteins</td>
<td>BRCA1, 53BP1, MDC1/NFBD1</td>
<td>53BP1</td>
<td>Crb2/Rhp9</td>
<td>Rad9</td>
</tr>
<tr>
<td>TopBP1</td>
<td>Claspin</td>
<td>Claspin</td>
<td>Mrc1</td>
<td>Mrc1</td>
</tr>
<tr>
<td>TopBP1</td>
<td>Claspin</td>
<td>Claspin</td>
<td>Mrc1</td>
<td>Mrc1</td>
</tr>
<tr>
<td>Transducers</td>
<td>Kinase</td>
<td>Kinase</td>
<td>Kinase</td>
<td>Kinase</td>
</tr>
<tr>
<td>kinase</td>
<td>Chk1</td>
<td>Chk1</td>
<td>Chk1</td>
<td>Chk1</td>
</tr>
<tr>
<td>kinase</td>
<td>Cds1</td>
<td>Cds1</td>
<td>Cds1</td>
<td>Rad53</td>
</tr>
</tbody>
</table>

1PIKK = Phosphoinositide 3-kinase related kinase.

Mediators promote checkpoint signalling from the ATM and ATR kinases to the transducer kinases Chk2 and Chk1, respectively, and provide signal transduction specificity. Many of the mediator proteins contain BRCT domains (Brca1 C-terminus) that are phospho-protein binding domains (Manke et al. 2003, Yu et al. 2003). MDC1, (mediator of DNA damage checkpoint protein 1), also called NFBD1 (nuclear factor with BRCT domains 1), is proposed to function as a true mediator in the amplification of the damage signal. MDC1 binds to γ-H2AX via its BRCT domain and recruits other checkpoint proteins for ATM phosphorylation as it interacts directly with ATM, and directly or indirectly with many checkpoint proteins (Kim et al. 2006). γ-H2AX, a phosphorylated form of the histone H2A variant, appears within seconds after DNA damage at and near the damage site. It is phosphorylated either by ATM and ATR depending on the damage type (Fernandez-Capetillo et al. 2004). γ-H2AX directly
associates with many checkpoint proteins and DNA repair proteins, such as 53BP1 (p53 binding protein 1), MCD1 and Nbs1 (Nijmegen breakage syndrome 1) (Fernandez-Capetillo et al. 2004, Kim et al. 2006, Stucki & Jackson 2006). Thus, it has been suggested that restructuring of chromatin mediated by γ-H2AX serves to concentrate and retain checkpoint proteins in the vicinity of the DNA lesion, and facilitates the tethering of DNA ends together (Fernandez-Capetillo et al. 2004, Stucki & Jackson 2006). However, γ-H2AX is not considered as an “official” checkpoint protein as there is no need to recruit an immobile chromatin protein to the damage site.

The functions of the effector proteins are discussed in the following chapters describing the individual DNA damage checkpoints.

2.2.1.2 G1/S checkpoint

The G1/S checkpoint prevents cells from entering S-phase in the presence of DNA damage by inhibiting DNA synthesis. There are two successive cell cycle checkpoint responses at G1/S; transient and sustained (Figure 6). The initial signalling events appear to be shared in both responses, and depending on the DNA damage, either the ATM-Chk2 or the ATR-Chk1 pathway is activated. The transient response operates via posttranslational modifications (Figure 6, left pathway). When active, both Chk1 and Chk2 kinases can phosphorylate the Cdc25A phosphatase, a key activator in the cell cycle progression machinery (Bartek & Lukas 2003). Phosphorylated Cdc25A is inactivated by nuclear exclusion and ubiquitin mediated proteolysis (Busino et al. 2004). The lack of active Cdc25A leads to accumulation of the phosphorylated (inactive) form of Cdk2 (Mailand et al. 2000, Falck et al. 2001). Eventually, this Cdk2 inhibition blocks the loading of Cdc45 onto chromatin, and therefore prevents initiation of DNA replication (Costanzo et al. 2000, Falck et al. 2002).

In contrast to the transient response, maintenance of the G1/S arrest is p53 dependent (Figure 6, right pathway). Both ATM and ATR phosphorylate p53 directly on Ser15 and indirectly on Ser20 via activation by phosphorylation of Chk2 and Chk1, respectively (Banin et al. 1998, Canman et al. 1998, Chehab et al. 1999, Chehab et al. 2000, Shieh et al. 2000, Abraham 2001). The phosphorylation of p53 inhibits its nuclear export and degradation, and thus leads to increased levels of the p53 protein (Zhang & Xiong 2001a, 2001b). The stabilised p53 induces transcription of p21, the inhibitor of CDKs (Ryan et al. 2001). p21 (also called WAF1 or CIP1) binds to the cyclin E/Cdk2 complex and thereby inhibits the transition to S-phase (Harper et al. 1993, Dotto 2000). Additionally, p21 inhibits the cyclin D/Cdk4, which is required for modifying the retinoblastoma protein Rb (Harper et al. 1993, Niculescu et al. 1998). In its unphosphorylated form, Rb binds the E2F transcription factor of S-phase genes and thereby prevents the transition to S-phase (Harbour & Dean 2000). In case of the persistence of DNA damage in G1, p53 can induce apoptosis or the cell can enter senescence (Artandi & Attardi 2005, Kohn & Pommier 2005).
Fig. 6. The G1/S checkpoint.Transient and rapid G1/S arrest is achieved by posttranslational modifications and maintenance of G1/S arrest via p53, see text for details (adapted from Sancar et al. 2004).
2.2.1.3 S-phase checkpoints

The S-phase checkpoints are the most complex of the cellular checkpoints (reviewed in Bartek et al. 2004, Gottifredi & Prives 2005, Andreassen et al. 2006). This feature largely stems from the task of checkpoint machinery to coordinate DNA replication and repair processes in S-phase. The cell may either repair the DNA damage before the DNA polymerase encounters the DNA lesion, or the cell may allow bypass of the lesion and restore the DNA later. In the former, DNA replication will slow down because of the DNA repair, and in the latter, cell cycle arrest will allow the DNA repair. In spite of the differences between the S-phase checkpoints and the G1/S and G2/M checkpoints, there are many common components operating in all of these pathways.

There are various checkpoints operating during S-phase: the intra S-phase checkpoint, the replication checkpoint and the S/M checkpoint. The intra S-phase checkpoint is typically activated in response to DNA DSBs and the replication checkpoint by replication fork stalling (Bartek et al. 2004). The S/M checkpoint inhibits the onset of mitosis until DNA replication has been completed (Nurse 1994). The intra S-phase checkpoint is replication independent, whereas the other S-phase checkpoints require active replication forks for their initiation (Bartek et al. 2004).

In S-phase, the main mechanism to induce cell cycle arrest after DNA damage is the inhibition of recruitment of replication initiation proteins to unfired replication origins. In fact, defects in the intra S-phase checkpoint lead to radioresistant DNA synthesis (RDS), as cells normally slow down DNA synthesis after IR (Jaspers & Zdzienicka 2006). There are numerous proteins required for RDS inhibition, including ATM, MCD1, 53BP1 (p53 binding protein 1), Brca1, and Nbs1 (Painter & Young 1980, Xu et al. 2001, Wang et al. 2002, Goldberg et al. 2003, Stewart et al. 2003). Like in the G1/S checkpoint, Cdc25A is an important target of the intra S-phase checkpoint (Busino et al. 2004). Actually, it is the same DNA damage cascade as previously described for the G1/S checkpoint: the ATM/ATR-Chk1/Chk2-Cdc25A-cyclinE(A)/Cdk2-Cdc45 pathway is also operative during S-phase. However, there are additional cascades underlying the intra S-phase checkpoint. Inhibition of Nbs1 and cohesion protein Smc1 (structural maintenance of chromosomes 1) phosphorylation by ATM also disrupt the intra S-phase checkpoint (Kim et al. 2002, Yazdi et al. 2002). The ATM/Nbs1/Smc1 pathway appears to function in parallel to the Cdc25A pathway, as prevention of Cdc25A degradation or Nbs1 phosphorylation only leads to partial RDS, whereas both defects together cause as severe an RDS as does a defect in the ATM kinase (Falck et al. 2002). The downstream events regulated by Nbs1 and/or Smc1 in this checkpoint pathway remain elusive. Cells lacking functional Brca1, 53BP1, or FANCD2 (Fanconi anemia protein D2) have a defective intra S-phase checkpoint (Xu et al. 2001, Nakanishi et al. 2002, Wang et al. 2002). All these proteins are phosphorylated by ATM in response to IR, but their position in the Cdc25A or ATM/Nbs1/Smc1 pathways hasn’t been completely resolved. It is also not known whether they form an “effector branch” of their own (Cortez et al. 1999, Rappold et al. 2001, Nakanishi et al. 2002).

The replication checkpoint can be induced chemically by hydroxyurea (HU) or aphidicolin (Bartek et al. 2004). HU causes depletion of deoxyribonucleotide (dNTP) pools and aphidicolin inhibits DNA polymerases (Pol α, Pol δ, and Pol ε) (Osborn et al.
The replication checkpoint is also induced when a replication fork encounters damaged DNA or an aberrant DNA structure (Bartek et al. 2004). The key components of this checkpoint are RPA, ATR/ATRIP, the Rad17-RFC/9-1-1 complex, Chk1, Claspin, and Cdc25A (Sancar et al. 2004). This signalling cascade inhibits initiation of DNA replication from hitherto unfired origins via targeting S-phase cyclin E/Cdk2 and Cdc7/Dbf4 kinases (Jares et al. 2000, Nyberg et al. 2002). The second function of this cascade is to protect the integrity of the replication forks and allow the recovery of the cell cycle progression after DNA repair or restoration of the dNTP pools (Bartek et al. 2004). The effectors of replication fork protection are largely unknown. Recently, however, Trenz and colleagues (2006) revealed a fundamental link between ATM, ATR, and Pol ε in the maintenance of replication fork stability. Their results showed that in *Xenopus laevis* ATM and ATR promote reloading of Pol ε onto recovering replication forks. The replication fork restart was also shown to be dependent on the MRN complex. In budding yeast, Pol ε has been shown to play a role in sensing DNA damage during S-phase (Navas et al. 1995). Actually, the checkpoint sensor function, located at the C-terminus, appears to be more important for the cell viability than its polymerase function (Kesti et al. 1999). Interestingly, in mammalian cells the C-terminus of Pol epsilon interacts with Mdm2, a down-regulator of the p53 protein (Vlatkovic et al. 2000, Asahara et al. 2003). Although, p53 accumulates after both a DNA replication block and a damage induced intra S-phase checkpoint, the transcriptional activity of p53 (in some cell lines) is impaired and p53 fails to upregulate some of its targets, including p21 (Gottifredi & Prives 2005). Therefore, the S-phase checkpoints are suggested to be p53 independent (Bartek et al. 2004). It has been proposed that inhibition of p53 is required for the DNA synthesis restart when DNA damage is repaired or the replication inhibitor is removed (Gottifredi & Prives 2005). However, as there are also differing opinions, more extensive work is required to solve the function of p53 in S-phase checkpoints (Giono & Manfredi 2006). All in all, the research concerning S-phase checkpoint pathways is still incomplete, and particularly more work is required to reveal the crosstalk between the ATR and ATM pathways in response to DNA metabolism perturbations during S-phase.

### 2.2.1.4 G2/M checkpoint

The G2/M checkpoint (also called G2 checkpoint) prevents cells from initiating mitosis in the presence of DNA damage. Similar to the G1/S checkpoint, the G2/M cell cycle delay/arrest originates from a combination of acute and sustained mechanisms, posttranslational modification of effectors and transcription, respectively. The main target of the G2/M checkpoint is the cyclin B/Cdk1 kinase (also termed cyclin B/Cdc2 or maturation promoting factor, MPF) (Figure 7). After DNA damage, the activity of the cyclin B/Cdk1 is inhibited by the ATM-Chk2-Cdc25 and/or the ATR-Chk1-Cdc25 pathway (Sancar et al. 2004, O’Connell & Cimprich 2005). In addition to the down-regulation of Cdc25, upregulation of Wee1 kinase is necessary for inhibiting the entry into M-phase. Wee1 phosphorylates and inhibits Cdk1, while Cdc25 reverses the inhibitory phosphorylation (Kellogg 2003, Boutros et al. 2006). Upon G2/M checkpoint
induction, both cyclin B and Cdk1 are also transcriptionally down-regulated, which further reinforces the cell cycle arrest (Crawford & Piwnica-Worms 2001).

Fig. 7. The G2/M checkpoint. CDC25A is kept inactive by cytoplasmic sequestration mediated by the 14-3-3 protein association. Another isoform, Cdc25C, functions during the G2/M transition, and upon DNA damage the isoform is also sequestered in the cytoplasm by 14-3-3. See text for further details (adapted from Sancar et al. 2004).
In addition to the checkpoint kinases, checkpoint mediators such as Brca1, 53BP1, and MDC1 contribute to the G2/M checkpoint. MDC1 deficient cells fail to activate G2/M checkpoints properly after exposure to IR (Stewart et al. 2003). A mutation of serine 1423 in Brca1, a target for phosphorylation by ATM, abolishes the ability of Brca1 to mediate the G2/M checkpoint without affecting its S-phase function (Xu et al. 2001). Mice lacking 53BP1 also have a defective G2-M checkpoint after exposure to IR (Fernandez-Capetillo et al. 2002).


p53 has also been shown to participate in the G2/M checkpoint (Stewart et al. 1995), and, just as in the G1/S checkpoint, it is involved in maintenance rather than in the initiation of G2 cell cycle arrest (Bunz et al. 1998). Furthermore, many p53 target genes have a role in the p53-induced G2 arrest. p21 inhibits the activity of the cyclin B/Cdk1 complex (Bunz et al. 1998, Giono & Manfredi 2006). A particular isoform of 14-3-3, 14-3-3σ, is also induced by p53 and it inhibits G2/M progression by cytoplasmic sequestration of the cyclin B/Cdk1 complexes (Hermeking et al. 1997, Hermeking & Benzinger 2006). GADD45 (growth arrest and DNA damage 45) is also a p53 target gene (Carrier et al. 1994). Gadd45 interacts physically with Cdk1, but not with cyclin B1. Presumably, Gadd45 inhibits the Cdk1 kinase activity by dissociating the cyclin B/Cdk1 complex (Zhan et al. 1999). However, many cell types lacking p53 tend to accumulate in G2 after DNA damage, indicating that additional mechanisms, such as Brca1-stimulated expression of p21 and Gadd45 can cooperate with the p53 cascade (Nyberg et al. 2002).

2.3 TopBP1 family of checkpoint proteins

Human topoisomerase IIβ binding protein 1 (TopBP1), is similar to budding yeast Dpb11, fission yeast Rad4/Cut5, Xenopus laevis Mus101/Cut5, Caenorhabditis elegans Mus101, and Drosophila melanogaster Mus101 (Table 1, reviewed in Garcia et al. 2005). All these proteins contain multiple BRCT domains. The number of BRCT domains, however, varies from the four domains found in yeast proteins to the eight domains of the Xenopus and human proteins. The BRCT domain was originally identified from Brca1 by computer analysis (Koonin et al. 1996). The BRCT domain appears to be the most common adaptor in the eukaryotic repair machinery, as it is found in over 50 proteins involved in DNA repair, recombination and cell cycle control (Bork et al. 1997, Aravind et al. 1999). The early hypothesis of the function of the BRCT domain was that it served as a site for protein-protein interactions (Koonin et al. 1996, Bork et al. 1997, Callebaut et al. 1997).
Recent studies have shown that the role of BRCT domains in checkpoint signalling cascades is even more important than first proposed, as they can specifically recognise phosphorylated proteins (Manke et al. 2003, Yu et al. 2003, Glover et al. 2004).

2.3.1 Dpb11

Dpb11 (DNA polymerase B possible subunit) was initially isolated in budding yeast as a multi-copy suppressor of a temperature sensitive mutant in the Pol ε subunit Dpb2 (Araki et al. 1995). Dpb11 also restored the growth defect of the C-terminal Pol ε mutant, but not the N-terminal mutant, implying that Dpb11 interacts both with Dpb2 and the C-terminal part of the catalytic subunit of Pol ε (Araki et al. 1995). Moreover, Dpb11 was linked to S-phase checkpoints, as mutant dpb11-1 cells entered mitosis with incompletely replicated DNA irrespective of DNA damage. Defective S-phase progression in these cells also suggested that Dpb11 is required for chromosomal replication. Masumoto and co-workers (2000) showed that Dpb11 controls the association of Pol ε and Pol α-primase to the initiation sites of DNA replication. Several other interacting factors for Dpb11 have been identified including Dpb3, Cdc45, Rad53, Sld2, Sld3, and Sld5 (Kamimura et al. 1998). Interactions with Dpb3, a subunit of the Pol ε complex, and Cdc45, further support the role of Dpb11 in DNA replication (Reid et al. 1999, Kamimura et al. 2000). The proper activation of Rad53 kinase, the homolog of human Chk2, in response to DNA damage requires both Dpb11 and Sld2 (also called Drc1, DNA replication and checkpoint protein 1) suggesting that Dpb11 functions early in the DNA damage response (Wang & Elledge 1999). The Sld2 and Dpb11 complex is also necessary for replication (Kamimura et al. 1998, Wang & Elledge 1999). Interestingly, the formation of this complex is regulated by phosphorylation of Sld2 (Masumoto et al. 2002). CDK phosphorylation of threonine residue 84 of Sld2 is essential for Dpb11 binding (Tak et al. 2006). However, this site is not accessible for CDK before it has phosphorylated several other residues of Sld2 suggesting that the Dpb11/Sld2 complex does not form until CDK activity is high (Tak et al. 2006). As pre-RC are only assembled during low CDK activity, the control of Dpb11/Sld2 complex formation could serve as a mechanism to prevent premature replication origin firing. The proposed candidates for Sld2 in metazoans are RecQL4 (a protein mutated in Rothmund-Thomson syndrome) and Xenopus, XRTS, (Sangrithi et al. 2005). However, XRTS is not the precise functional homolog of Sld2, as CDK activity is not required for its recruitment to chromatin (Sangrithi et al. 2005).

The connection between Dpb11 and the other two Sld proteins, Sld3 and Sld5, also links Dpb11 to the control of DNA replication. Like Dpb11, Sld3 interacts with Cdc45 (Kamimura et al. 2001). The Sld3/Cdc45 complex associates with the replication origin after pre-RC and participates in the unwinding of the origin (Kamimura et al. 2001). Sld5 forms the GINS complex together with three P NAT (partner of Sld five) proteins (Takayama et al. 2003). GINS does not associate with chromatin without Dpb11 and vice versa (Takayama et al. 2003). The function of the GINS is most likely to ensure the normal progression of DNA replication, as it maintains the association of MCMs and Cdc45 within the replisome (Gambus et al. 2006, Kanemaki & Labib 2006).
2.3.2 Cut5

Cut5 (cell untimely torn) was identified by two independent research groups. Fenech and co-workers (1991) reported that rad4+ was essential for viability of fission yeast cells. They also showed that the rad4 mutant was sensitive to UV and IR. A few years later Saka and Yanagida (1993) described the phenotype of the cut5 mutant, cloned the cut5+ gene, and showed it is identical to the rad4+ gene. Similar to the budding yeast homolog Dpb11, Cut5 is required for DNA replication (Saka & Yanagida 1993). Cut5 has also been shown to associate with replicative DNA polymerases α, ε, and δ (Taricani & Wang 2006). However, the precise role of Cut5 in DNA synthesis is still unknown, and it remains elusive whether the function of Cut5 is similar to that of Dpb11 in the initiation of replication. At least Cut5 is required for the Cdc45 chromatin binding, which is consistent with the retardation of Cdc45 binding in dpb11 mutants (Dolan et al. 2004, Garcia et al. 2005). The *S. pombe* Sld3 is also required for the loading and maintenance of Cdc45 on chromatin, but there are no reports concerning an interaction between Cut5 and Sld3 (Nakajima & Masukata 2002).

The dependence of mitosis onset to the completion of DNA replication is abolished in mutant cut5 cells, suggesting that Cut5 is required for the S-M checkpoint (Saka & Yanagida 1993). Furthermore, Cut5 is implicated in the DNA damage checkpoint pathway (Saka et al. 1994, McFarlene et al. 1997, Verkade & O’Connell 1998). Cut5 interacts with Chk1 kinase and the checkpoint mediator protein Crb2 (Cut5 repeat binding) (Saka et al. 1997). Crb2 resembles *S. cerevisiae* Rad9 and human 53BP1 (Saka et al. 1997). Crb2 is phosphorylated by Cdc2, the fission yeast CDK (Esashi & Yanagida 1999). This phosphorylation is required for both restarting the cell cycle from a checkpoint arrest and the putative repair function of Crb2 (Esashi & Yanagida 1999, Caspari et al. 2002). The position in the checkpoint pathway between sensors and Chk1 kinase appears to be similar for both Cut5 and Crb2. Chk1 and Cds1 (hsChk2) signaling is defective in cut5 mutants, but phosphorylation of Rad26 (hsATRIP) and the subunits of the 9-1-1 complex Rad9 and Hus1 remain unaffected (Harris et al. 2003). Phosphorylation of Chk1 after DNA damage requires functional Crb2 (Saka et al. 1997). In addition, Crb2 forms a stable complex with Chk1 after DNA lesions (Mochida et al. 2004). Moreover, a lack of Crb2 allows initiation of the checkpoint response, but the response is not sustained (Nakamura et al. 2005). Current data, thus, suggests that Cut5 potentially activates Chk1 by coordinating the assembly of Rad3 (hsATR), and the 9-1-1 and Crb2/Chk1 complex at sites of DNA damage (Garcia et al. 2005).

2.3.3 Metazoan TopBP1 proteins

The *Drosophila MUS101* (mutagen-sensitive-101) gene was first identified over 30 years ago through mutations conferring larval hypersensitivity to DNA-damaging agents (Henderson 1999). Altogether, *MUS101* has been genetically linked to DNA synthesis, DNA repair, and heterochromatin condensation (Henderson 1999). Like cut5+ and *DPB11*, *MUS101* is essential for viability (Araki et al. 1995, Saka & Yanagida 1993,

Human TopBP1 was first identified in a yeast two-hybrid screen designed to isolate interacting proteins for DNA topoisomerase II$\beta$ (Yamane \textit{et al.} 1997). Topoisomerase II$\beta$ belongs to the type II subfamily of topoisomerases that generate DNA DSBs for catalysing topological changes in DNA (Schoeffler & Berger 2005). TopBP1 has also been found to bind nicked circular and linear DNA \textit{in vitro}, but not intact circular DNA (Yamane & Tsuruo 1999). However, the functional significance of the TopBP1 and topoisomerase II$\beta$ interaction has not yet been discovered.

The \textit{Xenopus} homolog of TopBP1 is XMus101 (also known as XCut5). XMus101 functions in the initiation of replication as it recruits Cdc45 to replication origins (Van Hatten \textit{et al.} 2002). In addition, Chk1 activation in response to DNA damage requires XMus101 in a process that is independent of the replicative role of XMus101 (Parrilla-Castellar & Karnitz 2003). In \textit{C. elegans} the \textit{MUS101} gene is also essential (Holway \textit{et al.} 2005). Like the other members of the TopBP1 protein family, it is required for DNA synthesis, and it plays an important role in the DNA damage response (Holway \textit{et al.} 2005). More information especially about human TopBP1 and the \textit{Xenopus} homolog XMus101 is provided in the discussion.
3 Aims of the present work

The control of cell cycle progression is crucial for the survival of unicellular and multicellular organisms. The viability of the organisms is also closely linked to the faithful duplication of genetic material and its accurate segregation to the forming daughter cells. In case of perturbed DNA metabolism, the DNA damage response initiates and triggers the appropriate countermeasures for preservation of genetic integrity. How the cell co-ordinates normal DNA metabolism with the DNA damage response is not yet known. Therefore, proteins that harbour both replicative and DNA damage response functions such as *S. cerevisiae* Dpb11 and *S. pombe* Cut5 are of special interest.

The starting point of this study was to identify the human homolog of Dpb11 and Cut5. Subsequently, the roles of TopBP1 during cell cycle progression and after checkpoint activation were investigated.

The specific aims of the present study were:

1. To perform cDNA cloning and structural characterisation and chromosomal mapping of the *TOPBP1* gene
2. To analyse the expression of TopBP1, and its role in DNA synthesis
3. To analyse the cellular localisation of TopBP1 during both mitotic and meiotic cell cycles and after DNA damage in mitotically dividing cells
4. To identify proteins interacting with TopBP1
4 Materials and methods

The materials and methods have been described in more detail in the original articles referred to by their Roman numerals (I-IV).

4.1 cDNA cloning and chromosomal mapping (I)

Full length TopBP1 cDNA was constructed from expressed sequence tag (EST) clones AA013344, AA195149 and R54257 (Genome Systems Inc.). Nucleotides 308-1560 and 1628-2620 were amplified from human T-cell and thymus cDNA libraries because of deletions in the EST clones. The chromosomal location of the human TopBP1 gene was identified by fluorescence in situ hybridisation (FISH) using a 2 kilobase cDNA probe obtained from EST clone AA013344 (SeeDNA Biotech Inc. Canada). Human Rad9 cDNA was amplified from a human thymus cDNA library.

4.2 Cell lines and cultivation (I, II, IV)

Human breast cancer MCF-7 cells, human adenocarcinoma HeLa CCL2 cells, human embryonal kidney epithelial cell line EcR-293 (Invitrogen), and human osteosarcoma cell lines U2OS, MG-63 and SaOS-2 were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The human erythroleukemic cell line K562 was cultivated in RPMI-1640 medium supplemented with 10% FBS. Of these cell lines, MG-63, SaOS-2 and K562 were negative for the p53 protein. Human normal fibroblasts, IMR-90, were cultivated in minimum essential medium supplemented with Earle’s salts, non essential amino acids and 10% FBS. In addition all media contained 2 mM Glutamax-I and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin). All the cell lines were kept in a 5% CO₂ atmosphere at 37°C. HeLa S3 cells were cultivated in suspension as “spinner cells” in Joklik’s modification of minimal essential medium supplemented with sodium bicarbonate and 5% newborn calf serum and kept at 37°C.
4.3 Induction of DNA damage and cell cycle block (I, II, IV)

DNA synthesis was inhibited with 1 mM HU and DNA damage was induced with 50 µg/ml methyl methane sulfonate (MMS), 100 µg/ml zeocin, or 10 J/m² UV-light (Wang et al. 2000). Serum starvation was used for synchronization of IMR-90 fibroblasts (Tuusa et al. 1995) and MCF7 cells (Scully et al. 1997b) for 96 h and 24 h, respectively. Re-entry into the cell cycle from the quiescent state (G0) and S-phase progression were monitored by [³H]-thymidine incorporation (Tuusa et al. 1995) and PCNA staining patterns. To G2/M-phase cells were blocked with nocodazole (Sigma) and monastrol (Calbiochem) that also induce the spindle checkpoint. Cells were kept in normal culture medium containing 0.1 µg/ml nocodazole for 16 to 20 h or 68 µM monastrol for four hours.

4.4 Production of antibodies (I, III)

Antibodies against human TopBP1 were raised in rabbits. Peptides amino acid (aa) 792-1003 and aa 1023-1167 of TopBP1 were used as antigens and the resulting polyclonal antibodies were designated anti-TopBP1.1 and anti-TopBP1.2, respectively. Antigens were prepared as described by Uitto et al. (1995) and rabbits were immunised according to standard protocols. Antibodies were purified by protein A Sepharose CL4B (Amersham Pharmacia Biotech). The antibody against ATR was raised in sheep using aa 2341-2641 of ATR as the antigen.

4.5 Immunoprecipitation (I, IV)

The whole cell extract (WCE) for GST-pull downs and immunoprecipitation was prepared similarly. Cultivated cells were washed twice with ice cold phosphate buffered saline (PBS) and suspended into lysis buffer (100 mM Tris, 100 mM NaCl, 0.1% NP-40, 10% glycerol, pH 7.5) containing protease and phosphatase inhibitors. Cell lysates were incubated on ice for 10 min and sonicated briefly 2 x 15 s. After 10 min incubation on ice the lysates were clarified by centrifugation. Protein concentrations were determined using the Bradford assay (Bio-Rad). Prior to harvesting, when required, the cells were incubated in normal culture medium containing 1 mM caffeine for 20 h to inhibit phosphorylation of proteins, 20 mM NaF for 1 h to enrich for phosphorylated proteins, or 10 µM MG-132 for 6h, to accumulate proteins that are degraded by the proteasome pathway.

For each immunoprecipitation reaction typically 500 µg of WCE was used. Preclearing was performed with 2 µl of preimmune rabbit serum or with 5 µg of mouse IgG (Jackson ImmunoResearch Laboratories). Preimmune IgG binding proteins were collected on GammaBind G Sepharose or protein A sepharose / protein G agarose (Amersham Pharmacia Biotech). Immunoprecipitation was performed with 1-10 µg of a specific antibody. Immunocomplexes were collected on GammaBind G Sepharose or protein A sepharose / protein G agarose and washed 6 times with lysis buffer.
Alternatively, the immunoprecipitates were washed 2 times with lysis buffer, once with washing buffer II (50 mM Tris-Cl pH 7.5, 500 mM NaCl, 0.5% NP-40) and once with washing buffer I (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5% NP-40). Where applicable, ethidium bromide was added to the immunoprecipitation reaction together with the specific antibody to a final concentration of 50 μg/ml for blocking the interactions mediated by DNA.

4.6 GST-pull down (IV)

Different GST-TopBP1 constructs were made by fusing DNA fragments encoding amino acids R792-K834, R792-T861, R792-A901, K834-A1003, T861-A1003, A901-A1003, and R792-A1003 to a pGEX-vector (Amersham Pharmacia Biotech) for interaction site mapping between TopBP1 and p53. SDS-PAGE was used for analysing the expression of the recombinant peptides. Approximately 5 μg of GST or GST-TopBP1 peptides bound to Glutathione Sepharose 4B (Amersham Pharmacia Biotech) and 500 μg of WCE was used for one pull down reaction. After overnight incubation, the precipitates were collected by centrifugation and washed six times with lysis buffer (see 4.5). The pellets were boiled in SDS-loading buffer and subjected to electrophoresis in 7% or 9% SDS-polyacrylamide gels. Following transfer to an Immobilon-P PVDF membrane (Millipore) the samples were subjected to immunoblotting.

4.7 Immunoblotting (I-IV)

Samples blotted to Immobilon-P PVDF membranes were first blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20, TBST, (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h. After incubation with a specific primary antibody, the membranes were washed three times with TBST and further incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). The signals were detected by chemiluminescence, using either the SuperSignal West Pico or West Femto detection kit (Pierce) and Hyperfilm ECL (Amersham Pharmacia Biotech). Alternatively, the membrane was incubated with alkaline phosphatase conjugated anti-mouse (Schleicher & Schuell) or anti-rabbit antibodies (Bio-Rad) and the signal captured with colour developing reagents 5-bromo-4chloro-3-indolyl phosphate and nitro blue tetrazolium.

4.8 Immunostaining (I, II, IV)

Cells were grown to logarithmic phase and fixed in neutral 3% paraformaldehyde-PBS for 10 min at room temperature and permeabilised in 0.2% Triton X-100-PBS for 10 min at room temperature. Alternatively, cells were fixed in cold methanol for 10 min at -20°C or a combination of methanol and paraformaldehyde fixation was used. After fixation
nonspecific binding was blocked with 0.2% gelatin-PBS, this blocking solution was also used as diluent for the primary and secondary antibodies. Primary antibody incubation was carried out for 30 min either at 37°C or room temperature. After washes with PBS and brief blocking with 0.2% gelatin-PBS, incubation with a fluorophore conjugated secondary antibody was performed for 30 min at 37°C or room temperature. DNA was stained with 1 μg/ml Hoechst 33258 (Sigma) for 5 min at room temperature. Sample slides were mounted with IMMU-MOUNT (Shandon) and examined with an Olympus or Leitz fluorescent microscope under a 50x, 60x or 100x objective. Images were captured with a CCD-camera or on Kodak Ektachrome 400 film. Adobe Photoshop was used for image processing.

4.9 Mouse testis immunocytochemistry (II, III)

The nuclear contents of whole-mount spermocytes were laid out by surface spreading of a testicular cell suspension on a hypotonic liquid surface. The intact nuclei were attached to plastic-coated glass slides and briefly fixed in 2% paraformaldehyde-0.03% SDS solution and in 2% paraformaldehyde. The slides were then washed in 0.4% PhotoFlo 200 (Kodak) and Triton X-100 in PBS and blocked with goat serum or donkey serum. The primary antibody was incubated overnight at room temperature. After washing, the slides were incubated with a fluorophore conjugated secondary antibody for 1 h at 37°C. The cells were mounted in ProLong Antifade (Molecular Probes, USA) and with 4 μg/ml 4’, 6-diamidino-2-phenylindole (DAPI) if DNA staining was required. Slides were examined, recorded and images processed as explained in 4.8.

For electron microscopy, fixed cells were briefly treated with DNase I. Secondary antibodies were conjugated with gold particles (5 nm or 10 nm). Before transferring the samples to nickel grids the plastic was floated off. Images were recorded at 10 000 x or 20 000 x magnification on 35-mm film.

4.10 Preparation of mouse testis extracts (II)

Adult mouse testes were dissected and the capsule was removed before cutting the tubules with a razor blade. The cell suspension was collected in a 1.5 ml tube, centrifuged at low speed to remove highly insoluble material, washed once with PBS and lysed in 6 M urea, 1% SDS, 0.1% β-mercaptoethanol and 20 mM Tris-Cl, pH 8 (buffer A). To obtain extracts from mouse testes at different stages of development, testes from newborn or 1, 2, 3 and 4 week old mice were incubated directly in buffer A. Samples were sonicated briefly and the extracts were centrifuged to remove debris. The protein concentration was measured with the Bradford assay (Bio-Rad). Approximately 25 μg of protein was boiled in SDS-loading buffer, subjected to electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Immunoblotting with rabbit anti-TopBP1.2 antibody was performed as explained in 4.7.
Yeast two-hybrid assay (I, IV)

The bait for cDNA library screening was constructed in a pLex-a vector (Hollenberg et al. 1995) by fusing the LexA DNA binding domain and the BRCT domains 4 and 5 (amino acids 534-763) of human TopBP1. An activation domain tagged human peripheral lymphocyte cDNA library (Durfee et al. 1993) was transformed into an L40 yeast strain (Hollenberg et al. 1995) expressing the TopBP1 bait. Yeast colonies that grew on selective 1 mM 3-aminotriazole His - media were analysed for β-galactosidase activity. After elimination of the bait, the library plasmid was isolated from primary positive clones and retransformed into L40 together with either pLexaBRCT4+5 or a number of control baits fused to LexA. Again the colonies that grew on His - medium were tested for β-galactosidase activity.

For directed two-hybrid assays several versions of TopBP1 constructs were made including a full length TopBP1 fused to VP16 and BRCT4-6 (aa 534-991), BRCT3 (aa 348-448) and BRCT6 (aa 887-1004) fused to LexA. To verify the interaction between TopBP1 and p53, full length p53 cDNA was ligated to a pVP16 vector. Also full length Rad9 was fused to LexA to confirm the result of yeast two-hybrid cDNA library screening. Full length human Mdm2 fused to LexA was used as a positive control for the p53-VP16 fusion. Other control constructs that were used included LexA-Lamin (aa 66-230), full length human Cdc45 constructs LexA-Cdc45 and VP16-Cdc45, and LexA-Chk1 (aa 1-476).

Ecdysone-inducible expression system (I)

Full length cDNAs of human TopBP1 and Rad9 were subcloned into regular and modified pIND vectors (Invitrogen), respectively. The modified vector contained the FLAG-epitope and SV40 T-antigen like nuclear localisation signal. Constructs were linearised and transfected into EcR-293 cells (Invitrogen) with the Fugene 6 transfection reagent (Roche). The cells were next subjected to G418/geneticin selection for two weeks. G418-resistant colonies were selected, expanded and tested for 2 μM ponasterone A inducible expression by western blot.

DNA replication assay in isolated nuclei (I)

Nuclei and cytoplasmic extract were obtained from HeLa S3 cells (Stoeber et al. 1998). Lysolecithin was used to permeabilise the nuclei immediately before use and after washes the nuclei were suspended by Dounce homogeniser. Replication assays were performed in triplicate as described by Pospiech and co-authors (1999).
5 Results

5.1 TopBP1 belongs to a family of BRCT domain containing proteins (I)

In order to identify the human homolog of budding yeast Dpb11 and fission yeast Cut5, homology searches were conducted. Human cDNA, KIAA0259 (Nagase et al. 1996), was found to be similar to both of these yeast proteins. This cDNA encodes a protein of 1522 amino acids and contains eight BRCT domains. Thus, the human protein has about double the size and number of BRCT domains compared to the yeast proteins. The protein was also identified independently by another research group and named Topoisomerase IIβ-binding protein 1, TopBP1 (Yamane et al. 1997).

According to amino acid sequence analysis the two tandem BRCT domains of Dpb11 and Cut5 are most similar to the BRCT domains 1-2 and 4-5 of TopBP1. The N-terminus of TopBP1 is 35% and 44% similar to the N-terminus of Dpb11 and Cut5, respectively. The area containing BRCT domains 4-5 of TopBP1 is 27% and 41% similar to the C-terminus of Dpb11 and Cut5, respectively. Homologs for human TopBP1 were also found from Drosophila and C. elegans with seven and six BRCT domains, respectively.

In addition to the obvious homologs of TopBP1, it also shares similarity with Brca1. The C-terminus of TopBP1 shares 35% similarity to the two BRCT domains and the area upstream of the domains in Brca1.

5.2 TopBP1 is a phosphoprotein and highly expressed in S-phase (I)

The polyclonal rabbit anti-TopBP1 antibodies were used in the characterisation of the TopBP1 protein. Both anti-TopBP1.1 and anti-TopBP1.2 specifically recognised a protein of 180 kDa, which differs from the calculated molecular mass of 171 kDa. This difference may derive from phosphorylation, as in vivo phosphate labelling showed that TopBP1 is a phosphoprotein. According to the phosphoamino acid analysis, TopBP1 was
mostly phosphorylated on serine and to some extent on threonine residues. No labelling was observed on tyrosine residues.

To study the TopBP1 expression level during cell cycle progression and especially in the beginning of cell cycle, IMR-90 fibroblasts were first serum starved and then induced to proliferate again by the re-addition of serum. Western blot analysis showed that fourteen hours after serum stimulation TopBP1 protein levels started to increase. An increase in TopBP1 mRNA levels was also detected in RNase protection assays verifying that the protein increase is due to an increase in transcription. Furthermore, the mRNA levels were found to peak at 22 hours and then decrease again. Thymidine incorporation data confirms that S-phase entry is the time point when the protein levels of TopBP1 start to increase and that expression of TopBP1 is highest during S-phase.

5.3 The TOPBP1 gene is located on chromosome 3 (I)

The TOPBP1 gene was mapped to chromosome 3, region q21-q23, by FISH. According to the OMIM database there are no disease genes, which are frequently altered in human cancers, associated with this region. However, a recent study by Karppinen and colleagues (2006) links a mutation in exon 8 (Arg309Cys) of TopBPI to familial breast and ovarian cancer.

5.4 TopBP1 is required for DNA replication and interacts with human DNA polymerase epsilon (I)

The expression profile of TopBP1 and the role of its yeast homologs in DNA replication highlighted the possibility that TopBP1 may participate in replication as well. To answer this question, an in vitro DNA synthesis assay in isolated HeLa cell nuclei (Krude et al. 1997) was applied. The rate of DNA synthesis was measured by incorporation of radioactive dCMP into newly synthesised DNA. When polyclonal anti-TopBP1.1 antibody was added to the reaction, it inhibited replicative DNA synthesis by 44% whereas the preimmune serum or anti-TopBP1.2 antibody did not have any inhibitory effect. DNA synthesis in this assay was even more disturbed when a recombinant TopBP1-792-1003 peptide was added to reaction. This peptide, that was also the antigen for the anti-TopBP1.1 antibody, inhibited DNA replication by 83% suggesting that this part of TopBP1 is interacting with an important replication component.

The fact that yeast Dpb11 and DNA polymerase ε interact both physically and genetically (Araki et al. 1995, Masumoto et al. 2000) prompted an investigation of whether TopBP1 also interacts with the equivalent human replicative polymerase. Whole cell extract of EcR-293 over-expressing TopBP1 was subjected to immunoprecipitation with anti-TopBP1.2 antibodies. Western blot analysis of the immunoprecipitates revealed the presence of a 220 kDa protein that was specifically recognised by the anti-Pol epsilon antibody. This result was also repeated with HeLa S3 extracts. Furthermore, the interaction between TopBP1 and Pol ε was not cell cycle dependent nor was it affected by
DNA damage resulting from UV, MMS or HU treatment. Additional interactions between TopBP1 and other Pol, α or δ, were not detected.

5.5 TopBP1 localisation in response to replication block and DNA damage (I)

The localisation of TopBP1 in the cell and nucleus was determined by immunofluorescence studies. During S-phase, TopBP1 was mostly detected in the nucleus forming several distinct foci. However, these foci did not represent sites of ongoing replication as they did not overlap with PCNA or the nucleotide analog BrdUrd incorporated into newly synthesised DNA. As this staining pattern resembled that of Brca1 (Scully et al. 1997b), double stainings with TopBP1 and Brca1 antibodies were conducted. Interestingly, all the Brca1 foci contained TopBP1, but a number of additional TopBP1 foci lacking Brca1 were detected. However, when replication was blocked with HU TopBP1 colocalised with PCNA, BrdUrd, and Brca1, which suggests that TopBP1 responds to stalled replication forks.

DNA damage is known to cause subnuclear localisation changes for proteins involved in DNA metabolism (Wang et al. 2000), thus it was interesting to determine whether the localisation of TopBP1 is similar to Brca1 after DNA damage. For this purpose S-phase MCF-7 cells were exposed to UV light, the alkylating agent MMS, and the radio-mimetic chemical zeocin. After every treatment TopBP1 and Brca1 colocalisation was observed indicating that TopBP1 responds to DNA damage. The ability to react to DNA damage was not specific to S-phase as TopBP1 formed foci also in the G1-phase after zeocin treatment. In contrast to S-phase punctate staining, TopBP1 displayed uniform nuclear and cytoplasmic staining during unperturbed G1-phase. The localisation changes of TopBP1 during the G1-phase are very likely independent of Brca1, as its expression is very low before S-phase (Scully et al. 1997b).

5.6 TopBP1 interacts with the checkpoint protein Rad9 (I)

Yeast two-hybrid cDNA library screening was applied in the search for proteins interacting with TopBP1. The first two tandem BRCT domains were tested for their applicability as bait, because these domains are also conserved in the yeast proteins. Unfortunately, BRCT domains 1 and 2 fused to LexA could activate the marker gene LacZ alone, and therefore BRCT4-5 was used as bait. One of the several positive clones identified in the screening contained the C-terminal 148 amino acids of the human checkpoint protein Rad9. The interaction is specific for these BRCT domains as Rad9 failed to bind to BRCT domains 3 and 6 of TopBP1. This result was also repeated with full length Rad9 and TopBP1 proteins. To test whether the TopBP1 and Rad9 complex forms also in mammalian cells, co-immunoprecipitation was applied. EcR-293 cells were stably transfected with an inducible FLAG-tagged Rad9 and subjected to immunoprecipitation with both anti-FLAG and anti-TopBP1.2 antibodies. Subsequent
immunoblots probed for endogenous TopBP1 revealed the presence of TopBP1 protein in the anti-FLAG immunoprecipitate, as an identical band with a size of 180 kDa was also detected in the anti-TopBP1.2 immunoprecipitate. These data are in accordance with the two-hybrid results and confirms that TopBP1 and Rad9 interact in vivo.

5.7 TopBP1 interacts with the tumour suppressor p53 (IV)

The result from the in vitro replication assay showed that amino acids 792-1003 of TopBP1 efficiently inhibited DNA replication. To determine if there are proteins binding to this particular area of TopBP1, a GST pull-down experiment was performed. Autoradiography demonstrated that a protein of around 50 kDa appeared specifically in the GST-TopBP1-792-1003 precipitate from metabolically 35S labelled HeLa cell extract. One suitable candidate of 50 kDa and also related to the cell cycle control is the tumour suppressor p53. Thus, another set of GST pull-down experiments from proteasome inhibited MCF-7 whole cell extract was conducted. The p53 immunoblot made from the pull-down precipitates indeed showed that p53 protein was fished out from the MCF-7 cells with the TopBP1-792-1003 fragment but not with GST alone. The interaction domain of TopBP1 was further mapped by incubating a series of GST-TopBP1 fragments with MG-132 treated MCF-7 whole cell extract. Specific interactions were detected not only with the BRCT domain but also with the area before the BRCT domain.

Nevertheless, the interaction between p53 and the TopBP1 fragments appeared stronger if BRCT domain 6 was present. The results were reproducible from glioblastoma T98G cells that strongly express p53 due to an inactivating mutation (Gjerset et al. 1995).

Next, a yeast two-hybrid method was employed in the further study of the interaction domain. Full-length p53 interacted with a fragment of TopBP1 that contains the three BRCT domains 4, 5 and 6, but not with BRCT domains 4+5 alone. The functionality of these constructs was verified by the C-terminus of Rad9 that is known to interact with this area of TopBP1. Full-length Mdm2 interacted with full length p53 as expected, but no sign of interaction was observed between Mdm2 and TopBP1. These yeast two-hybrid results are consistent with the GST pull-down data that BRCT domain 6 of TopBP1 is needed for interaction with p53.

The interaction between TopBP1 and p53 was confirmed by performing co-immunoprecipitations from both the p53+/+ and p53−/− cell lines MCF7 and K562, respectively. The p53 immunoprecipitates were positive for TopBP1, but p53 was absent from TopBP1 immunoprecipitates. Surprisingly, a proteasome inhibitor did not have a notable effect on the co-immunoprecipitation of p53 and TopBP1. As expected Mdm2 and p53 immunoprecipitated each other reciprocally and no co-immunoprecipitation was detected from K562 cells either between p53 and TopBP1 or p53 and Mdm2. Similarly as in the yeast two-hybrid assay, TopBP1 and Mdm2 did not interact in these co-immunoprepitation experiments. Because both TopBP1 and p53 have the ability to bind to DNA (Yamane & Tsuruo, 1999), the effect of ethidium bromide on the immunoprecipitation reactions was examined. However, this did not abolish co-immunoprecipitation of TopBP1 and p53. Altogether, these results show that interaction
between TopBP1 and p53 is direct and that TopBP1 is in a complex with p53 alone and not with p53 and Mdm2.

Finally, the role of post-translational modification in the TopBP1 and p53 interaction was studied by treating MCF7 cells before harvesting with NaF or caffeine that inhibit serine/threonine phosphatases and serine/threonine kinases, respectively. TopBP1 and p53 co-immunoprecipitated only from NaF treated cells whereas caffeine treatment abolished the interaction. The effect of caffeine treatment was also studied by GST pull-down. In this experiment p53 was only seen in the GST-TopBP1 pull-down reactions where MG-132 treated cells were used. No p53 was present in GST precipitates. Taken together, these co-immunoprecipitation and GST-pull-down results imply that inhibition of phosphorylation affects the interaction between TopBP1 and p53.

5.8 TopBP1 localises to centrosomes with Brca1 and p53 during mitosis (II, IV)

In order to gain more information about possible functions of TopBP1 outside S-phase, additional localisation studies were carried out. D. melanogaster Mus101 is implicated in the condensation of heterochromatin during mitosis (Gatti et al. 1983, Yamamoto et al. 2000), therefore the localisation of human TopBP1 during mitosis was studied.

Immunofluorescence staining of human cultured cells revealed that TopBP1 forms numerous clear foci during prophase. These foci did not co-localise with marker proteins β-tubulin for microtubules, γ-tubulin for centrosomes, and CREST both for centromeres and kinetochores. Throughout mitosis TopBP1 was absent from the sites of kinetochores/centromeres. However, in metaphase and anaphase, the TopBP1 signal concentrated mainly into two bright foci that overlapped γ-tubulin. Staining of the spindle with β-tubulin antibodies confirmed that TopBP1 accumulates at centrosomes in late M-phase. In anaphase, the TopBP1 signal was also observed at the midbody area that is a region of cell division.

Both Brca1 and p53 are known to localise to centrosomes during mitosis (Hsu & White, 1998, Morris et al. 2000, Ciciarello et al. 2001). As TopBP1 was linked to these proteins by results from earlier experiments obtained in this study, double immunostainings were performed and especially mitotic cells were examined. In early M-phase neither Brca1 nor p53 co-localised with TopBP1, but later, in metaphase and anaphase cells, TopBP1 co-localised with both proteins to the centrosomes.

To examine the possibility that p53 governs TopBP1 localisation during M-phase, immunofluorescence stainings were repeated in MG-63 and SaOS-2 human osteosarcoma cells that lack the p53 protein. In these cells the centrosome localisation of TopBP1 during late M-phase remained unchanged as evidenced by γ-tubulin staining. Also the early M-phase TopBP1 foci persisted suggesting that mitotic localisation and recruitment to centrosomes of TopBP1 is independent of p53.
5.9 TopBP1 remains at centrosomes after spindle checkpoint activation (II, IV)

The possibility that localisation of TopBP1 to centrosomes would be disrupted by inducing the spindle checkpoint was addressed by studying the effect of monastrol and nocodazole treatments. Nocodazole depolymerises tubulin filaments disrupting the mitotic spindle and arrests the cells in pseudo-prometaphase (Kung et al. 1990). Monastrol keeps the spindle intact but prevents the action of mitotic kinesin Eg5 (Mayer et al. 1999) resulting in monoastral spindles and cell cycle arrest in prophase. Both treatments lead to Mad2 accumulation on kinetochores that signals spindle checkpoint activation (Waters et al. 1998; Kapoor et al. 2000). Overlapping of TopBP1 and $\gamma$-tubulin signals in mitotic MCF7 cells treated with monastrol or nocodazole, proved that localisation of TopBP1 to centrosome foci was not affected.

Nocodazole triggers a stable activation of p53 and furthermore interferes with its subnuclear localisation (Ciciarello et al. 2001) whereas a p53 response to monastrol has not been reported. Immunostaining results from nocodazole treated U2OS cells were in accordance with these previous findings. TopBP1 and p53 did not co-localise in cells arrested in prometaphase due to nocodazole. Astonishingly, after monastrol treatment TopBP1 and p53 did not co-localise either, which suggests that p53 is lost from the centrosomes even though tubulin filaments are intact. The spindle failure was also studied in cells lacking p53. TopBP1 remained at centrosomes after both nocodazole and monastrol treatment in MG-63 and SaOS-2 cells. Recruitment of TopBP1 to centrosomes is thus independent of spindle checkpoint activation and does not require a functional bipolar spindle or the p53 protein.

5.10 TopBP1 is highly expressed in mouse testis (II)

The striking localisation pattern of TopBP1 during mitosis raises the question of whether or not TopBP1 also plays a role in meiotic cell division. Already, the western blot analysis of different mouse tissues implied that TopBP1 could be involved in meiosis. The levels of TopBP1 were observed to be generally high in tissues containing proliferating cells, such as small intestine, testis, lung, thymus and spleen while the signal in liver and kidney was fainter. Particularly high levels of TopBP1 were found in testis tissue suggesting that TopBP1 plays a role during spermatogenesis. Therefore, TopBP1 levels were further studied in the developing mouse testis. Newborn mice lack spermatocytes and hence the development of spermatocytes is synchronous until mature sperm are formed four weeks after birth. Within the first two weeks there is an abundance of cells carrying out prophase I while at the third week the cells are also starting meiotic division II. Western blot analysis of mouse testis extracts at 0, 1, 2, 3 and 4 weeks after birth showed that TopBP1 levels increased from birth, peaking at three weeks, suggesting that TopBP1 functions in spermatogenesis both at prophase I and also at meiotic division II.
5.11 TopBP1 localises to chromosome cores in meiosis (II, III)

In order to gain more information about the role of TopBP1 during meiosis and especially during prophase I, whole mount spreads of testicular cells were immunostained with antibodies to TopBP1 and to Cor1, a structural component of the meiotic chromosome cores. TopBP1 was observed to reside in meiotic prophase cells and to localise to the autosomal cores at early meiotic prophase stages but only to the X and Y chromosome cores at later meiotic prophase stages. The female XX-pair did not accumulate TopBP1 (data not shown) indicating that staining in males is specific for the asynapsed region of the XY chromosome pair.

The number of TopBP1 foci during meiotic prophase I was also counted and the results supported the immunofluorescence data. The maximum number of TopBP1 foci, about 300, was found in the leptotene nucleus. The number steadily decreased towards the end of prophase I and a minimum was observed at diplotene when TopBP1 was only associated with the XY-pair.

The localization of TopBP1 was also studied in a case of defective recombination. The Fkbp6-/- mouse, that lacks a component of the SC, has a normal initiation of recombination but homologous chromosome pairing is faulty in meiosis (Crackover et al. 2003). During leptotene and early zygotene TopBP1 staining in Fkbp6-/- mouse spermatocytes was similar to the wild type. However, later in prophase I, TopBP1 displayed very intense staining in unsynapsed chromosomes that is most likely the result of abnormal recombination in the mutant cells. Taken together, these results suggest that TopBP1 disappears from the chromosome cores when synapsis occurs.

5.12 TopBP1 co-localises with ATR and γH2AX during meiosis (III)

Further immunostainings were made to reveal possible similarities between TopBP1 and other known proteins involved in meiosis. Dmc1 and Rad51 are RecA-type recombinases, of which Dmc1 is expressed only during meiosis (Bishop 1994). Dmc1 and Rad51 form mixed complexes, which associate with chromosome cores and SCs (Tarsounas et al. 1999). The temporal distribution of Rad51/Dmc1 foci (Tarsounas et al. 1999) resembles that of TopBP1 and thus double immunostainings were carried out. However, only a partial co-localisation was observed between TopBP1 and Dmc1 during leptotene and zygotene. Equivalent immunoelectron microscopy study confirmed that just a few TopBP1 foci co-localised with Dmc1.

In addition to Rad51/Dmc1, ATR has the same number and distribution of foci as TopBP1 during mouse spermatogenesis (Keegan et al. 1996, Moens et al. 1999). Strikingly, almost complete co-localisation of TopBP1 and ATR was observed in immunofluorescence throughout prophase I. During leptotene the proteins associated with the chromosome cores, and at later stages they were detected at unsynapsed regions. In pachytene ATR was only localised at XY-pair, whereas TopBP1 was additionally found to form some weak foci on autosomes.

As the ATR homolog in budding yeast physically localises to the sites of DNA DSBs (Kondo et al. 2001, Melo et al. 2001), doublestainings against TopBP1 and the marker of
DNA DSB, \(\gamma\)-H2AX (Rogakou et al. 1998), were performed. \(\gamma\)-H2AX was observed to co-localise completely with TopBP1 suggesting that TopBP1 resides at DNA DSBs together with ATR.

### 5.13 Cut5 is required for the meiotic checkpoint (III)

Because of the connection between human TopBP1 and ATR and because the ATR homolog in fission yeast, spRad3, is implicated in the pachytene checkpoint (Shimada et al. 2002), an analysis of the meiotic role of Cut5 was initiated. Cut5 (also called spRad4) was found to form many foci in wild type nuclear spreads during prophase I similar to its human homolog TopBP1. A yeast strain carrying a mutant rad4-116 allele was used in the further experiments, since it retains the normal replication function at the semi-permissive temperature (32°C), but is defective in the DNA damage and replication checkpoint response in vegetative cells (McFarlane et al. 1997). Furthermore, synchronous meiosis was induced by inactivating pat1 that exempts yeast cells from the prerequisites of natural meiosis: heterozygosity of the mating type loci and nitrogen starvation (Iino & Yamamoto 1985).

Flow cytometry analysis revealed that the kinetics of pre-meiotic DNA replication was normal at 32°C for rad4-116. Thus, this temperature was selected for the analysis of the meiotic recombination checkpoint function in the rad4-116 mutant. As Meu13 is required for homologous pairing and meiotic recombination (Nabeshima et al. 2001), the meu13 mutant was used to trigger the meiotic checkpoint. Kinetics of meiotic progression during synchronous meiosis of wild type, rad4-116, meu13, and rad14-116 meu13 diploid strains was examined. The rad4-116 single mutant behaved like the wild type. As noted earlier, the meu13 mutant displayed approximately a 30 min delay of entry into meiosis I (Shimada et al. 2002, Perez-Hidalgo et al. 2003). Interestingly, the rad4-116 meu13 double mutant proceeded to meiosis I with faster kinetics than the meu13 single mutant suggesting that the rad4-116 mutation partially abolishes the meu13 meiotic delay.

As the meiotic recombination checkpoint in fission yeast delays entry into meiosis by maintaining inhibitory phosphorylation of Cdc2 on tyrosine 15, the effect of a rad4 mutation on the status of Tyr15 phosphorylation was studied. Indeed, dephosphorylation of Tyr15 occurred earlier in the rad4-116 meu13 than in the meu13 mutant. Also spore viability was lower in the rad4-116 meu13 double mutant compared to the rad4-116 or meu13 single mutants. However, rad4-116 was observed to be proficient in DBSs formation, as there was no difference in the \(\gamma\)-H2AX staining pattern of meiotic nuclei spreads between wild type and rad4-116. Also recombination proceeds normally in rad4-116, as demonstrated by intergenic recombination in the leu1-his5 interval on chromosome II. Taken together this data suggests that the homolog of human TopBP1, Cut5, plays a role in the meiotic recombination checkpoint.
6 Discussion

6.1 TopBP1 in DNA replication

In our search for the human homolog for budding yeast Dpb11, a protein with eight BRCT domains was discovered. This protein had been independently identified by another research group who named the protein TopBP1, topoisomerase IIβ binding protein 1 (Yamane et al. 1997). The TopBP1 homologs both in fission and budding yeast, Cut5 and Dpb11 respectively, have been implicated in DNA replication. Although, human TopBP1 was unable to complement temperature sensitive cut5 and dpb11 mutants, the evidence obtained in this thesis study suggests that TopBP1 has functions similar to Dpb11 and Cut5.

Co-immunoprecipitation experiments indicated that TopBP1 and the replicative Pol ε associate. This interaction was also independent of the cell cycle phase. Similarly, Dpb11 has been reported to interact with Pol ε (Masumoto et al. 2000). The expression profile of TopBP1 also suggests a function during S-phase, as its expression was induced concomitantly with S-phase entry. In accordance with our data Yoshida and Inoue (2004) have described that TopBP1 is under the control of E2F, which regulates the transcription of several genes needed for S-phase entry.

The results from the in vitro DNA replication assay indicated that TopBP1 is required for DNA synthesis. The addition of an antibody against TopBP1 or the antigen itself reduced DNA synthesis dramatically in isolated nuclei, suggesting that blocking the BRCT domain 6 or a surplus of the antigen peptide disturbs the important protein-protein interaction (more discussion in 6.3). TopBP1, however, was not found to localise to the sites of ongoing replication in a cell, which implies that TopBP1 does not participate in the elongation of replication. Surprisingly, a block in DNA synthesis recruited TopBP1 to the stalled replication forks together with Brca1, which suggests that TopBP1 may be involved in the stabilisation of replication forks, damage signalling, or in the re-initiation of replication.

Studies concerning orthologs of TopBP1 strongly indicate they play a role in the initiation of DNA replication. Dpb11 controls the association of Pol ε and Pol α to the replication origins (Masumoto et al. 2000). Furthermore, Dpb11 interacts with several
proteins required for the initiation of replication. Sld2 and Dpb11 form a phosphorylation dependent complex that is essential for replication (Masumoto et al. 2002, Tak et al. 2006). Dpb11 has a genetic interaction with Cdc45, Pol ε subunit Dpb3, Sld3, and a component of the GINS initiation complex Sld5 (Kamimura et al. 1998, Takayama et al. 2003). The fission yeast Cut5 is an essential factor for Cdc45 loading to chromatin (Dolan et al. 2004). Likewise, Xenopus XMus101 is required for the chromatin binding of Cdc45 and DNA polymerases, but not for the binding of pre-RC components (Van Hatten et al. 2002, Hashimoto & Takisawa 2003). XMus101 is also dispensable for elongation of replication (Hashimoto & Takisawa 2003). In addition, the C. elegans Mus101 and Drosophila Mus101 are linked to DNA replication (Henderson 1999, Holway et al. 2005). It remains to be determined, if TopBP1 functions mechanistically like Dpb11 or XMus101 in the initiation of replication in human cells. The potential human homolog of Sld2 is already identified as the RecQL4 helicase (Sangrithi et al. 2005). Recent characterisation of XRTS, the equivalent protein in Xenopus, showed that XRTS promotes the loading of replication factors to origins after pre-RC formation (Sangrithi et al. 2005). Furthermore, Matsuno and co-workers (2006) demonstrated that XRTS performs an essential role in the assembly of the replication machinery via interaction with XMus101.

6.2 The role of TopBP1 in mitosis and meiosis

The presence of additional BRCT domains in the metazoan TopBP1 proteins compared to the number of BRCT domains in the yeast proteins suggests that the metazoan homologs have acquired additional functions. The C-terminal tandem BRCT domains and the region upstream of these domains in human TopBP1 and Brca1 share significant similarity. Interestingly, this part of Brca1 is linked to the G2/M checkpoint, as over-expression of the Brca1 C-terminus disturbs the G2/M checkpoint (Larson et al. 1997). In TopBP1 and Brca1 double-negative cells the G2/M checkpoint is even more severely abrogated (Yamane et al. 2003). Moreover, the TopBP1 homolog in Drosophila is implicated in heterochromatin condensation during M-phase (Gatti et al. 1983, Yamamoto et al. 2000).

Immunofluorescence studies revealed that TopBP1 displays a unique localisation during M-phase. Early in mitosis TopBP1 was found in multiple foci that diminished towards the end of mitosis. From metaphase onwards TopBP1 co-localised to centrosomes with Brca1 and p53. Attempts to identify other proteins located in the early M-phase TopBP1 foci failed. However, these foci do not represent centromeres, kinetochores, or telomeres. The relevance of the early TopBP1 foci in mitosis, therefore, remains to be determined.

Association with the centrosomes has been described for several cell cycle regulator proteins like p53, Brca1, Chk1, Chk2, cyclin B1, and Cdk1 (reviewed in Kramer et al. 2004), and this thesis study adds TopBP1 to the list. Both Brca1 and p53 are essential for accurate centrosome duplication and chromosome segregation (Deng 2002, Tarapore & Fukasawa 2002). Phosphorylation and dephosphorylation play a role in the regulation of centrosomal functions of Brca1 and p53 (Hsu & White 1998, Hsu et al. 2001, Tarapore et
al. 2001, Tritarelli et al. 2004). It is possible that the mitotic localisation of TopBP1 is also controlled by post-translational modifications.

Although p53 and TopBP1 were found to interact, the mitotic localisation of TopBP1 was independent of the p53 status. The proteins also behaved differently after spindle checkpoint activation. The centrosomal localisation of TopBP1 was not affected by nocodazole or monastrol treatments, whereas p53 was lost from centrosomes in response to both chemicals. The transport of p53 to the centrosomes has been suggested to be microtubule-driven (Ciciarello et al. 2001). However, p53 dislocation from centrosomes in response to mitotic kinesin inhibition indicates that additional mechanisms contributing to p53 centrosomal localisation may exist. An interesting subject for further studies is whether a lack of TopBP1 changes the mitotic localisation of p53.

Mouse TopBP1 was found to be strongly expressed in testis tissue and in late stages of spermatogenesis implying that TopBP1 may play a role in the process of meiotic cell division. Immunostaining of mouse spermatocytes undergoing meiosis prophase I revealed that TopBP1 localises to the autosomal chromosome cores at early meiotic prophase stages and to the X and Y chromosome cores at later meiotic prophase stages. The detailed characterisation of the meiotic prophase I TopBP1 foci confirmed that the appearance of these foci is very defined. The number of TopBP1 foci reaches its maximum at leptotene and then decreases gradually as homologous chromosomes begin to synapse. Almost no TopBP1 is present at aligned chromosomes, where the synaptonemal complex is fully formed. TopBP1 was also absent from the female XX chromosome cores in oocytes, indicating that staining in the male is specific for the asynapsed region of the XY chromosome pair.

Similar staining patterns during meiotic prophase I have been described for ATR and the phosphorylated histone H2AX (γ-H2AX) (Keegan et al. 1996, Moens et al. 1999, Mahadeviah et al. 2001). Immunofluorescence studies, indeed, revealed almost complete co-localisation between TopBP1 and ATR in meiotic prophase nuclei. Also TopBP1 co-localised with the DNA DSB marker γ-H2AX. Previous studies have shown that ATR localises to the sites of DNA DSBs (Kondo et al. 2001, Melo et al. 2001, Zou et al. 2002). In mitotic cells, TopBP1 has been shown to localise to nuclear foci containing γ-H2AX after γ-irradiation (Honda et al. 2002, Yamane et al. 2002). Taken together, these meiosis results and the data from the literature suggest that TopBP1 is present at the DNA DSBs in meiotic chromosomes. This in accordance with a study of Barchi and co-workers (2005) that showed that the meiotic TopBP1 foci are dependent on induction of DNA DSBs and that the TopBP1 foci persist on meiotic chromosomes when the DSBs cannot be repaired. Considering that TopBP1 can stimulate the activity of ATR (Kumagai et al. 2006), it is possible that TopBP1 is involved in the activation of the ATR-Chk1 pathway during the meiotic checkpoint.

Functional data indicating that TopBP1 could be involved in the meiotic recombination checkpoint was obtained by studying Cut5/Rad4, the fission yeast homolog of TopBP1. In the rad4-116 mutant DNA, DSBs required for meiotic recombination were generated normally. Also spore viability and recombination frequency were similar in wild type and rad4-116 strains, suggesting that homologous recombination and chromosome segregation is not altered in the rad4 mutant. However, when the rad4-116 mutant was combined with the recombination defective meu13 mutant, the delay before the onset of the first meiotic division was partially abolished.
Furthermore, the dephosphorylation of Cdc2 on tyrosine 15 occurred earlier in the rad4-116 meu13 double mutant correlating with faster entry into the first meiotic division. Interestingly, the budding yeast homolog of TopBP1, Dpb11, has also been recently linked to the recombination repair process (Ogiwara et al. 2006). In conclusion, the meiosis data and other data obtained in this study strongly suggest that TopBP1 belongs to the group of somatic DNA damage-detection and -repair proteins such as ATR, ATM, Rad51, Rad50, and Brc1 that also function in DNA damage detection and repair during meiotic recombination (Marcon & Moens 2005).

6.3 TopBP1 is part of the DNA damage response machinery

In this thesis study several pieces of evidence were obtained that point to a role for human TopBP1 in the DNA damage response and checkpoint functions. TopBP1 was found to interact with a checkpoint protein Rad9. More specifically the BRCT domains 4 and 5 of TopBP1 associated with the C-terminus of Rad9. Subsequently, it has been reported that phosphorylation of the Rad9 C-terminus is required for TopBP1 binding (St.Onge et al. 2003). Rad9 is also reported to guide TopBP1 to the sites of DNA damage (Greer et al. 2003). In addition, similar results have been obtained from both S. cerevisiae and S. pombe. Cut5 associates with phosphorylated Rad9 via BRCT domains 3 and 4 (Furuya et al. 2004). This interaction is also necessary for activation of the Chk1 DNA damage checkpoint but not for the Cds1 replication checkpoint (Furuya et al. 2004). After Rad9 phosphorylation Cut5 also co-precipitates with Rad5 (hsATR) suggesting that phosphorylation of Rad9 directs the formation of the active checkpoint complex, which is dependent on Cut5 (Furuya et al. 2004). Likewise, the C-terminus of Dpb11 interacts with the C-terminus of Ddc1 (hsRad9) (Wang & Elledge 2002), strongly suggesting that the TopBP1-Rad9 interaction is conserved within the protein family and important for the function of TopBP1.

DNA damage was found to change the cellular localisation of TopBP1. In S-phase after UV-radiation and treatment with the radiomimetic drug zeocin, TopBP1 co-localised with Brc1 implying that these proteins migrate to the sites of DNA damage (Scully et al. 1997a). Other similarities between TopBP1 and Brc1, such as centrosome localisation (Hsu & White 1998) and the localisation to unpaired X chromosome cores during meiotic prophase I (Scully et al. 1997b), observed in this study also imply that these proteins carry out similar tasks. Like mutations in Brc1, a mutation in TopBP1 has been found to predispose to familial breast and ovarian cancer (Karppinen et al. 2006). Another recent study also demonstrated a direct interaction between TopBP1 and Brc1. TopBP1 associated with Brc1 in a DNA damage induced manner and siRNA-mediated knockdown experiments showed that both Brc1 and TopBP1 contribute to the intra S-phase checkpoint (Greenberg et al. 2006).
Fig. 8. TopBP1 functions as a mediator in the DNA damage response. In this study for TopBP1 direct interactions with p53 and Rad9 and co-localisations with ATR and Brca1 were observed. Direct interactions between TopBP1 and ATR and TopBP1 and Brca1 have been reported by Kumagai et al. (2006) and Greenberg et al. (2006), respectively.

The protein level of TopBP1 appears to be under complex regulation. Without DNA lesions TopBP1 levels are kept low by ubiquitin mediated degradation (Honda et al. 2002). In case of perturbed DNA metabolism TopBP1 protein level rises due to stabilising phosphorylation (Honda et al. 2002, Yamane et al. 2002) and interaction with the promyelocytic leukemia (PML) protein (Xu et al. 2003). Interestingly, however, UV-radiation leads to down-regulation of the mRNA expression level of TopBP1 (Potter et al. 2000, Herold et al. 2002, Yoshida & Inoue 2004). As TopBP1 levels decrease, the transcription factor Miz-1 is released and Miz-1 is able to induce the transcription of the CDK inhibitor p21 (Herold et al. 2002). TopBP1 and p53 were found to interact in this study. Like Miz-1, p53 also induces the transcription of p21 after UV-radiation (Latonen & Laiho 2005). As co-immunoprecipitation of TopBP1 and p53 was observed from undamaged cells, it is possible that TopBP1, like Miz-1, can release p53 after UV-radiation. Alternatively, TopBP1 could function as a transcriptional co-activator of p53 similar to Brca1 and 53BP1 (Iwabuchi et al. 1998, Ouchi et al. 1998, Chai et al. 1999). At least the N-terminus of TopBP1 has been reported to participate in transcriptional co-activation functions (Boner et al. 2002). Interestingly, p53 associated with the BRCT
domain 6 of TopBP1. This area of TopBP1 is known to bind other proteins, such as ubiquitin ligase HYD and transcription factor E2F1 (Honda et al. 2002, Liu et al. 2003). It remains to be resolved whether these proteins compete in the binding to this particular site of TopBP1. Although, the in vitro replication assay suggested that the BRCT domain 6 of TopBP1 is essential for DNA synthesis, it is unclear if p53 and the TopBP1 complex is crucial for the replicative role of TopBP1. Further characterisation of the TopBP1 and p53 interaction suggested that post-translational modifications are required for the association of TopBP1 and p53. The fact that BRCT domains are phospho-protein binding domains (Manke et al. 2003, Yu et al. 2003) and that the GST-p53 fusion protein failed to bind to TopBP1 suggests that p53 requires phosphorylation before it can associate with TopBP1.

Several studies have indicated that TopBP1 functions in the ATR-Chk1 signalling pathway (Figure 8). Here TopBP1 was found to co-localise with ATR in mouse meiotic spermatocytes. Kumagai and co-authors (2006) have showed that TopBP1 can in fact stimulate ATR kinase activity. Also the studies of Liu and co-workers (2006) suggest that TopBP1 is a general regulator of ATR. Consistent with being an enhancer of ATR kinase, TopBP1 has been reported to be required for Chk1 activation following replication stress and DNA DSBs (Kim et al. 2005). In Xenopus, XMus101 is essential for recruitment of ATR to chromatin during checkpoint activation (Parrilla-Castellar & Karnitz 2003) and promotes phosphorylation of Chk1 by activated ATR (Yan et al. 2006). Also the fission yeast Cut5 interacts with Chk1 (Saka et al. 1997) and more specifically the Cut5-Rad9 interaction is required for the activation of the Chk1 damage checkpoint (Furuya et al. 2004). Interestingly, studies have shown that the ATR-Chk1 pathways have an active role in the control of the timing of early versus late replication origin firing also during normal S-phase (Shechter et al. 2004). Thus, TopBP1 may participate in the regulation of origin firing via the ATR-Chk1 pathway like Dpb11 (Masumoto et al. 2000).

It has been shown that it is possible to separate the checkpoint and replication functions in Cut5, Dpb11 and XMus101 (Garcia et al. 2005, Yan et al. 2006). For further studies, a TopBP1 mutant that functions normally during replication but is defective in checkpoint function would be a valuable tool for detailed examination of the checkpoint functions of TopBP1 after perturbed DNA metabolism.
7 Conclusions

In this study TopBP1, the human homolog of budding yeast Dpb11 and fission yeast Cut5, was identified and its functions characterised during both mitotic and meiotic cell cycles. TopBP1 was found to be essential for DNA replication and to associate with Pol ε. The exact role of TopBP1 in replication remains to be clarified, although the results suggest that TopBP1 is more likely to function during the initiation of replication. The localisation of TopBP1 to the sites of DNA damage and stalled replication forks suggest that it has a role in the DNA damage response. TopBP1 also associated with the checkpoint protein Rad9 that is a part of the 9-1-1 complex whose function includes tethering of proteins to the sites of DNA damage. Furthermore, during M-phase TopBP1 was found to localise to centrosomes together with the tumour suppressor proteins Brca1 and p53. Direct interaction between p53 and TopBP1 was also detected. The analysis of mouse tissues for the expression of TopBP1 suggested that TopBP1 plays a role during meiotic cell division. The localisation pattern of TopBP1 in mouse meiotic spermatocytes was similar to many proteins functioning during meiotic recombination. Co-localisation with ATR kinase and TopBP1 was observed during meiotic prophase I. In accordance with the findings from mouse, the analysis of the cut5 mutant during yeast meiosis showed that Cut5 is required for the meiotic checkpoint. Taken together the results suggest that TopBP1 participates in multiple DNA transactions including DNA replication, DNA damage signalling, and meiotic recombination/pachytene checkpoint. In conclusion, TopBP1 can be classified as both a replication protein and a DNA damage response and checkpoint protein, whose functions are closely connected to the preservation of the integrity of the genome.
References


Original articles


Original publications are not included in the electronic version of the dissertation.
459. Mutanen, Marko (2006) Genital variation in moths—evolutionary and systematic perspectives

460. Bhaumik, Prasenjit (2006) Protein crystallographic studies to understand the reaction mechanism of enzymes: α-methylacyl-CoA racemase and argininosuccinate lyase

461. Korkalo, Tuomo (2006) Gold and copper deposits in Central Lapland, Northern Finland, with special reference to their exploration and exploitation


469. Kekonen, Teija (2006) Environmental information from the Svalbard ice core for the past 800 years


Kaarina Reini

CHARACTERISATION OF THE HUMAN DNA DAMAGE RESPONSE AND REPLICATION PROTEIN TOPOISOMERASE IIβ BINDING PROTEIN 1 (TOPBP1)