Role of the DNA damage checkpoint in normal and cancer cell cycle.

Ph.D. thesis

Specialization: Molecular Biology, Genetics and Virology
Supervisor: RNDr. Michal Dvořák CSc., Department of Molecular Virology, Institute of Molecular Genetics, Academy of Science of the Czech Republic
Supervisor-consultant: Jiří Bartek M.D., Ph.D., Department of Cell Cycle and Cancer, Institute of Cancer Biology, Danish Cancer Society

Prague, 2005

Zuzana Hořejší
I would like to thank my supervisor RNDr. Michal Dvořák, CSc. and to my supervisor-consultant Jiří Bartek, M.D., Ph.D., who gave me the chance to do my Ph.D. work on extremely exciting projects in their laboratories. I would also like to thank to all people who participated on these projects. The work presented in this thesis was done in the Department of Cell Cycle and Cancer, Institute of Cancer Biology, Danish Cancer Society. The thesis was written in Prague, in the Department of Molecular Virology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic.
TABLE OF CONTENTS

ABSTRACT

1. OVERVIEW OF THE CELL CYCLE AND ITS REGULATION
   1.1 INTRODUCTION
   1.2 CELL CYCLE REGULATION
     1.2.1 Mammalian cell cycle control
     1.2.2 G1/S transition
     1.2.3 S phase progression
     1.2.4 G2/M transition
     1.2.5 Metaphase to anaphase transition
   1.3 CHECKPOINT MECHANISMS
     1.3.1 DNA damage checkpoints
     1.3.2 The G1 checkpoint
     1.3.3 The intra-S phase checkpoint
     1.3.4 G2 checkpoint
   1.4 CHECKPOINT SIGNALIZATION PATHWAYS AND CANCER

2. AIM OF THE STUDY

3. MATERIAL AND METHODS
   3.1 CELL BIOLOGY
   3.2 MOLECULAR BIOLOGY AND BIOCHEMISTRY

4. RESULTS
   4.1 PUBLICATION I
   4.2 PUBLICATION II
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3 Publication III</td>
<td>44</td>
</tr>
<tr>
<td>5. Discussion</td>
<td>48</td>
</tr>
<tr>
<td>5.1 ATM Activation is Dependent on MRN Complex</td>
<td>48</td>
</tr>
<tr>
<td>5.2 Role of the DNA Damage Signaling Pathways in Cancer Development</td>
<td>51</td>
</tr>
<tr>
<td>5.3 MDC1 and 53BP1 in Normal Cell Cycle and Tumorigenesis</td>
<td>53</td>
</tr>
<tr>
<td>6. Conclusions</td>
<td>54</td>
</tr>
<tr>
<td>7. Appendix</td>
<td>56</td>
</tr>
<tr>
<td>7.1 Publication I</td>
<td>56</td>
</tr>
<tr>
<td>4.2 Publication II</td>
<td>62</td>
</tr>
<tr>
<td>8. References</td>
<td>70</td>
</tr>
</tbody>
</table>
ABSTRACT

To maintain their genomic information unperturbed, all living organisms had to develop mechanisms that recognize diverse types of DNA damage. In cooperation with the cell cycle machinery such mechanisms ensure DNA repair and in the case of severe damage induction of cell death. These so-called DNA damage checkpoints are essential for normal cell cycle course and their alteration leads to genomic instability, severe genetic disorders and predisposition to cancer. This work is focused on further clarification of the mechanism of DNA damage checkpoint activation following introduction of DNA double strand breaks into the DNA and on the role of the checkpoint mechanisms in tumor progression.

After the DNA is exposed to any damaging agent that causes DNA double strand breaks, ATM kinase is activated by autophosphorylation on serine 1981. We showed that after exposure of human fibroblasts deficient in NBS1 to low doses of irradiation, ATM phosphorylation is delayed and its magnitude is lower than in normal fibroblasts. The kinetics can be restored by expression of wild-type NBS1 from a retroviral vector. NBS1 with mutated FHA domain or NBS1 mutant nonphosphorylatable by ATM can also restore the kinetics, contrary to the NBS1 with deleted MRE11-interaction domain. These results indicate that the MRE11 complex of which are NBS1 and MRE11 components take part in the very early stages of ATM activation and that this modulating activity requires presence of MRE11 in the nucleus.

Second problem, which has been studied in this work, is the relation between the DNA damage checkpoints and tumourigenesis. It has been shown that defects in proteins that are parts of the DNA damage checkpoints are often responsible for genetic instability of cancer cells. In this work we show, that these proteins are commonly activated in the early stages of tumourigenesis and that their activation precedes genomic instability and malignant conversion. We found that in the cell culture the checkpoint activation follows increased levels of proteins that deregulate DNA replication as E2F1, cyclin E and CDC25A. Based on our experiments we suggest that the DNA damage response could reflect deregulated DNA replication, since overexpressed cyclin E, CDC25A and E2F1 share the ability to promote unscheduled S-phase entry. As a response to the oncogene overexpression, the checkpoint pathways activate p53 pathway, which leads in the case of severe damage to apoptosis. We presume that the checkpoint pathways prevent or delay
tumour progression hence defects of these pathways may allow proliferation, survival, enhanced genomic instability and cancerogenesis.

Finally, this work also contributes to better understanding of the regulation, function, and potential involvement in human cancer of the DNA damage checkpoint mediators 53BP1 and MDC1, which are both key components of the genome surveillance network activated by DNA double strand breaks (DSBs).
1. OVERVIEW OF THE CELL CYCLE AND ITS REGULATION

1.1 INTRODUCTION

Since the first elementary discoveries have been done in the 1980s, the cell cycle field is quickly developing and attracts much attention. The knowledge of the processes ongoing during the cell cycle is of essential importance for understanding and curing cancer as well as other diseases.

Because any mutation of the genome can severely impair the ability to survive or lead to tumour transformation, the cells had to develop mechanisms that are able to detect damaged or abnormally structured DNA, arrest the cell cycle and repair the damage or direct the cell to apoptosis. The networks of signaling pathways which are part of these mechanisms are called cell cycle checkpoints. As every single cell is continually exposed to many exogenous and endogenous factors potentially damaging DNA, fully functional checkpoint signalization pathways are of vital importance. This is in agreement with the fact that loss of function of many proteins involved in these pathways leads to genomic instability, severe genetic disorders and predisposition to cancer. Therefore a detailed understanding of the process of DNA damage recognition and activation of the checkpoint response, identification and characterization of the participating proteins as well as their mutual links with other pathways that regulate the cell cycle can significantly help us to elucidate processes leading to the development of cancer.

This overview briefly summarizes the basic mechanisms of cell cycle regulation, DNA damage checkpoint signalization and their roles in tumourigenesis.

1.2 CELL CYCLE REGULATION

The ability to reproduce itself is one of the hallmarks of living organisms. It is the fundamental way to maintain their genetic information throughout long periods of time. All cells must undergo an orderly sequence of events, which results in duplication of its contents and division into two daughter cells. This process called the cell division cycle is the essential, evolutionary conserved mechanism, by which all living things reproduce.
Figure 1. The cell cycle consists of four main stages - two gap phases (G1, G2), replication phase (S), mitosis (M) and one additional resting phase (G0). At the restriction point (R) the cell becomes independent on the signals from outside and is committed to divide. The status of DNA at different cell cycle stages is indicated. Modified from Alberts, 2002.

There are two important phases of the eukaryotic cell cycle, which cannot be omitted - S and M phase. While in the S phase the DNA in each chromosome is replicated, in the M phase it is then equally distributed to the two daughter cells so that each receives an identical copy of the entire genome. The two gap phases - G1 and G2 - serve mainly for getting the cells into a good condition before the S and M phase. These two phases can be skipped or extremely shortened in some special cell types, such as embryonic cells. When circumstances are not favorable for the cell division, the cell can halt in G1 and enter a phase called G0. It is a specialized resting period (also called quiescence) where cells can remain for a long time during development, differentiation, or growth factor withdrawal. The point at which the cell makes the decision to stay in the G1 phase, enter the G0 phase or make a commitment to proceed through the cell cycle, is made at the late G1 phase and is called the restriction (R) point. Until this point, the cell needs signals from the outer environment, such as the presence of the growth factors to keep going through the G1 phase. After passing through the R point, the cell will complete the cycle regardless the external conditions (Fig.1).
1.2.1 Mammalian cell cycle control

The cell cycle and proliferation are controlled by a complex network of various signal transduction pathways, consisting of many different types of proteins. These pathways control the progression of the cell cycle and make sure that the individual events follow in an ordered and error-free manner. They also monitor conditions outside the cell. The importance of such mechanism is supported by the fact, that it is highly conserved in all eukaryotic organisms.

The cell cycle is controlled and regulated on different levels. The core of the cell cycle regulation consists from protein kinases called cyclin dependent kinases (CDKs), which are completely inactive when unmodified and monomeric\(^7,8\). Binding of small proteins called cyclins activates CDKs in specific stages of the cell cycle and determines the substrate for their kinase activity. The cyclin family is very diverse and contains proteins ranging in size and structure, which share a short domain called cyclin box. This domain is responsible for binding and activation of the CDK protein\(^9,10\).

![Diagram of the cell cycle](image)

**Figure 2.** Cyclins and cyclin dependent kinases in mammalian cell cycle. Cell proliferation is driven by the sequential activation of cyclin-CDK complexes, whose activities peak at specific points during the cell cycle. The status of DNA at different cell cycle stages is indicated. Modified from Lodish, 2004\(^11\).

As shown in (Fig. 2), mammalian cells use a family of related CDKs. The most important are CDK 1, 2, 4 and 6. Cyclin D-CDK4/6 complex are active in mid to late G1,
cyclin E-CDK2 at the G1/S transition, cyclin A-CDK2 in S phase and cyclin A/B-CDK1 complex in G2 and M phase\textsuperscript{12,13}.

During the cell cycle the levels of most of the CDKs remain constant, whereas levels of cyclins oscillate in a cell cycle-dependent manner. The oscillating protein levels of the cyclins result from a tightly controlled balance between their mRNA synthesis and protein degradation. Binding of cyclins to their catalytic subunits (the CDKs) is rate limiting and therefore it determines at which time are their associated kinases active\textsuperscript{14,15}.

In addition to cyclin binding, the fully active CDK usually requires phosphorylation at a conserved threonine residue (Thr 160 in CDK2) by CDK activating kinase (CAK)\textsuperscript{16}. This kinase is well defined in yeasts, but its identity in mammalian cells is still disputable. It could be the cyclin H-CDK7 complex, but the real mechanism is still not clear\textsuperscript{17-21}. The phosphorylation induces conformational changes in the substrate-binding site, which probably helps in better association of the cyclin-CDK complex with the substrate\textsuperscript{12}. An exception in this model, in which the activation by phosphorylation acts independently of the cyclin binding, is the cyclin A-CDK complex. In this case the phosphorylation is also required for an efficient binding of the cyclin itself\textsuperscript{22} (Fig. 3).

![Figure 3. Regulation of CDK activity. Simplified model depicting the major modes of CDK regulation. Adapted from Morgan, 1995\textsuperscript{23} and Alberts, 2004\textsuperscript{4}.](image-url)

The CDK activity is negatively regulated by inhibition phosphorylation of a tyrosine residue in the N-terminus of the CDK (Thr 15 in human CDK1 and CDK2), which is evolutionary conserved from yeast to mammals\textsuperscript{12}. In higher eukaryotes, the adjacent
threonine residue (Thr 14) is also a target for inhibitory phosphorylation\textsuperscript{24-26}. Inhibitory phosphorylation represents a preferred regulatory option for controlling and restraining activities of both CKD1 and CKD2 during normal cell cycle progression and in response to genotoxic stress (sections G1/S transition and G2/M transition). In order to generate CK activity, inhibitory phosphatases must be removed from preformed cyclin-CDK complexes thus Thr 14/Thr 15 dephosphorylation of major CKDs represents a critical rate-limiting step for cell cycle progression. This activatory function is undertaken by members of the CDC25 family of dual-specificity phosphatases, capable of dephosphorylation both Thr 14 and Tyr 15\textsuperscript{27} (section I, G1/S transition, G2/M transition and DNA damage checkpoints) (Fig. 3).

In addition to inhibitory phosphorylation, binding of CKD-inhibitory subunits (CKIs) represents the other major way to negatively regulate cyclin-CDK complexes. The two classes of CKD inhibitors in mammalian cells act not only to transiently restrain the activities of cyclin-CDKs during normal cell cycle progression, but also also an important CKD-inhibitory role to promote exit from the cell cycle and maintenance of a non-proliferative state under conditions such as differentiation and morphogenesis, where permanent cessation of cell cycle entry is required\textsuperscript{28} (chapter 1.2.2) (Fig. 3).

1.2.2 G1/S transition

After the cytokinesis occurs, the daughter cells enter the G1 phase. This phase allows the cellular growth and the cell cycle regulation by the external signals. To prevent premature entry into the S phase, the cells developed different mechanisms which hold back the diverse CKD activities.

The first one is based on the presence of APC complex, which becomes activated in the late M phase and remains active until the late G1. This complex is a part of the ubiquitin-proteasome protein degradation pathway. It functions as an ubiquitin ligase, which can specifically recognize and mark substrates destined for degradation. During the G1 phase APC is associated with an accessory factor CDH1. CDH1 activates APC by direct binding of the targets, including M and G1 cyclins\textsuperscript{29-31}.

Another mechanism is based on accumulation and binding of the CKD-inhibitory subunits (CKIs). There are two classes of CKIs-the CIP/KIP family and the INK4 proteins. The proteins of the CIP/KIP family (p21\textsuperscript{CIP1}, p27\textsuperscript{KIP1}, p57\textsuperscript{KIP2}) preferentially bind and
inhibit preassembled cyclin-CDK complexes, such as cyclin D-CDK4/6 and cyclin E/A
CDK2. Members of the INK4 family (p16INK4a, p15INK4b, p18INK4c, p19INK4a) interact with
monomeric CDK4 and CDK6, thus preventing binding of the cyclins32-34.

Figure 4. Simplified model of the G1/S transition. Cyclin E-CDK2 acts as a major interaction factor for
signals from G1/S-promoting pathways, and is engaged in several feedback loops that collectively serve to
amplify cyclin E-CDK2 activity once it appears in late G1 phase. It then stimulates DNA synthesis. Modified
from Bartek and Lukas, 200135.

The third inhibitory mechanism is based on the inhibition of cyclin gene transcription.
E2F is one of the most important transcription factors that bind to specific DNA sequences
in the promoters of many genes that encode proteins required for the S-phase entry,
including the G1/S and S cyclins. Binding of retinoblastoma (RB) protein and two related
proteins, p107 and p130, in the late M and early G1 phase converts E2F into a
transcriptional repressor. The interaction between E2F and RB is disturbed by RB
phosphorylation, which is initiated by CDK4/6-cyclin D in the mid G136,37.

If the conditions are favorable for the cell division, the cell is committed to proceed to
the S phase. In the mid G1 the levels of the D-type cyclins increase due to stimulating
extracellular signals. The higher level of the cyclin D-CDK4/6 is then capable of
phosphorylation of RB and the liberated E2F increases transcription of CDK2, cyclin E and cyclin A. cyclin E/CDK2 complex in turn enhances phosphorylation of CDH1 and p27 which leads to their rapid inactivation and destruction by the SCF complex. SCF ubiquitin ligase recruits substrates via adaptor subunits called F-box proteins. Binding of these proteins depends on the phosphorylation of the target and it enables the protein kinases to directly control the stability of substrate proteins via the ubiquitination pathway. The cyclin E-CDK2 phosphorylates cyclin E as well and this phosphorylation targets cyclin E to SCF-dependent degradation, therefore the cyclin E abundance is tightly requested.

The cyclin E-CDK2 complex also further phosphorylates RB. Since E2Fs stimulate their own expression and expression of cyclin E as well, it forms a feedback loop for phosphorylation of RB protein. When the phosphorylation of RB becomes dependent on the cyclin E-CDK2 activity, the passage through the cell cycle becomes independent of the cyclin D-CDK4/6 activity. Simultaneously the progression through the cell cycle becomes independent of the outer signals and the level of cyclin D falls. At this stage the restriction point is passed and the cell is committed to proceed to the S phase. The RB phosphorylation is maintained during S, G2 and M phases by the cyclin-CDK1/2 activities. After the mitotic cyclins are degraded by APC complex, RB is dephosphorylated and able to bind to E2Fs in the next cell cycle G1 phase.

The last major barrier in proceeding into the S phase is inhibitory phosphorylation of CDK2 on Thr 14 and Thr 15, which negatively regulates the cyclin E-CDK2 complex. The inhibitory phosphorylations probably prevent efficient phosphotransfer to substrates. Thr 15 of CDK2 (and CDK1) is phosphorylated by a tyrosine specific kinase WEE1, the identity of the kinase phosphorylating CDK2 on Thr 14 is so far unknown. Several biochemical studies showed that the inhibitory CDK phosphorylations are cyclin-dependent. Both of the phosphorylations can be removed by CDC25A phosphatase, but the mechanisms of both phosphorylation and dephosphorylation are not yet well understood. CDC25A is a member of the CDC25 phosphatases family, and it is strictly required for entry into S phase and DNA replication through its ability to activate cyclin E/CDK2. The CDC25A transcription is also stimulated by both the E2F and c-MYC transcription factors and is regulated by phosphorylation which modulates its stability.

In summary, to proceed into the S phase, the cell must activate the G1/S CDKs by allowing activation their transcription, halting their degradation, destroying of their
inhibitors and also by abrogation of their inhibitory phosphorylations. All the above-mentioned mechanisms are set so that after reaching the R point the G1/S transition is a rapid and irreversible event (Fig. 4).

1.2.3 S phase progression

All DNA is replicated during the S phase. The cell cycle machinery must ensure that each part of the genome is replicated only once during one cycle and that the replication occurs with maximal precision to avoid mutations in the next cell generation. DNA replication begins at origins of replication, which are distributed at different places in the chromosome. During the early G1 a large multiprotein complex is assembled, called origin recognition complex (ORC). ORC is essential for binding other regulatory proteins (CDC6 and MCM proteins) to form the pre-replicative complex (pre-RC), important for the initiation of replication. The assembly of pre-RC is triggered by cyclin E-CDK2 complex, which phosphorylates among others CDC6 and CDC45. Phosphorylation of CDC45 is essential for its binding to ORC and subsequently for binding of the DNA polymerase alpha into pre-RC. During the S phase, cyclin E levels decline in response to auto- and other phosphorylation events that target cyclin E for ubiquitination by SCF complex and degradation by the proteasome.

![Figure 5. The initiation of DNA replication. In early G1, CDC6 and CDC45 associate with ORC. The pre-replicative complex is formed after the MCM ring assembly is finished. The S-CDK then triggers initiation of DNA synthesis. Assembly of DNA polymerase and other replication proteins and activation of the MCM protein rings to migrate along DNA strands as a replication fork. The S-CDK also blocks the pre-replication complex by phosphorylation of CDC6 and later also MCM proteins. Modified from Aibets, 2002.](image-url)
The initiation of replication is triggered in the S phase by the cyclin E-CDK2 and cyclin A-CDK2 complexes, and the latter is most important also for the course of the S phase. Cyclin A synthesis and assembly starts at the beginning of transition from G1 to S phase\textsuperscript{62-64}. The function of the cyclin A-CDK2 complex is inhibited until the onset of S phase by inhibitory phosphorylation on Thr 14 and Thr 15. It is activated by the CDC25A phosphatase by dephosphorylation of both of these sites\textsuperscript{53}. The activity of the S phase CDK complex also ensures that the replication from one ORC occurs only once. That is thanks to CDC6 phosphorylation that causes its dissociation from the ORC after the origin has fired, which results in the disassembly of the pre-RC. The cyclin-CDK complex prevents also the CDC6 and MCM proteins from reassembling at any origin, because the phosphorylated CDC6 is polyubiquitinated and degraded by the SCF complex and phosphorylation of MCM proteins triggers their export from the nucleus. The S phase cyclin-CDK is active until early mitosis. During M phase its inactivating function is supplied by the M-phase cyclin-CDK complex. When the M phase cyclin-CDK complexes are degraded at the end of mitosis, the pre-RC can be assembled again, due to the CDC6 and MCM dephosphorylation\textsuperscript{64,65} (Fig. 5).

1.2.4 G2/M transition

The important mammalian CDK in G2 is CDK1, which associates with cyclin A and B\textsuperscript{66}. The activation of the M phase cyclin-CDK complexes begins after the DNA replication is completed and the cell enters the G2 phase. The levels of cyclin A and B rise during G2 because of their increased gene transcription. The main cyclin partner of CDK1, cyclin B, accumulates during S and G2 phases in the cytoplasm and its level peaks in early mitosis, shortly after it enters the nucleus. During G2 CDK1 also accumulates and the resulting cyclin B-CDK1 complex is inhibited by phosphorylation of Thr 14 and Thr 15, which allows the cells to accumulate a large pool of the complex. Such amount of cyclin-CDK complex enables the cell to enter rapidly and effectively the M phase upon its activation\textsuperscript{66,67} (Fig. 6).

In contrast to the CDK2 regulation, the mechanisms which control entry into mitosis by regulation of the phosphorylation state of the CDK1 complexes are well understood. In vertebrate cells, two CDK-inhibitory kinases - WEE1 and MYT1 have been identified. WEE1 is a tyrosine specific kinase and phosphorylates Thr 15 (but not Thr 14) in CDK1.
and CDK2. MYT1 can in vitro phosphorylate Thr 15 but it has much stronger preference for the Thr 14 and it targets only CDK1, not CDK2. The activities of both inhibitory kinases increase during S and G2 and are at once suppressed upon mitotic entry by hyperphosphorylation partly mediated by cyclin B-CDK1. This is a potential feedback loop which could allow irreversible commitment of the cell cycle to the mitosis.

At the late G2 phase, Thr 14 and Thr 15 phosphorylation sites have to be dephosphorylated so that CDK1 becomes active. This is achieved by members of the CDC25 phosphatase family, which can dephosphorylate both of them. It is presumed that the most important mitotic inducer is CDC25C, which has specificity only for the cyclin B-CDK1 complex and not other cyclin-CDK complexes. On the other hand, CDC25C can be substituted in its functions by some other member(s) of the CDC25 family, as shown in knockout experiments with the CDC25C gene.

CDC25s are also regulated by phosphorylation. The activity of CDC25C is low during G2 but as the cell proceeds to mitosis, it increases due to multiple phosphorylations by cyclin B-CDK1 and possibly PLK1 kinase. The phosphorylation by cyclin B-CDK1 forms a positive feedback loop, started by partial activation of CDC25 by PLK1. The phosphorylation of CDC25B and C is also important for binding of 14-3-3 proteins. This interaction regulates cellular localization and activity of both CDC25B and CDC25C.

In summary, partial activation of CDC25 by PLK1 could lead to the partial activation of a cyclin B-CDK1 subpopulation, which then phosphorylates more CDC25 and WEE1 molecules. That could quickly promote the complete activation of cyclin B-CDK1 and rapid progression into the M phase. The function of CDC25B/C is regulated by phosphorylation-dependent binding of the 14-3-3 proteins which regulate its cellular localization and activity.

During the M phase, the cyclin B-CDK1 complex phosphorylates many specific structural or regulatory proteins, involved in the rearrangements that occur in early phases of mitosis. It triggers chromosome condensation, nuclear envelope breakdown, actin cytoskeleton rearrangement, reorganization of the Golgi apparatus and endoplasmic reticulum as well as assembly of mitotic spindle ensuring that the chromosomes attach to the spindle. So far only few targets of cyclin B-CDK1 are known, such as the nuclear lamins, components of cohesin complex and proteins that regulate microtubule behavior.
1.2.5 Metaphase to anaphase transition

The separation of sister chromatids occurs in anaphase and is directed by activity of APC. This ubiquitin ligase, which is a complex of at least 13 proteins that specifically recognizes substrates destined for ubiquitination and degradation by proteasome, controls events of late mitosis to mid G1 phase\(^{29,31}\). Activation of APC in mid M phase requires presence of CDC20, which directly binds to the substrate. The binding is not dependent on the substrate phosphorylation, and the activity of is regulated via APC phosphorylation and CDC20 mRNA transcription, both of which are increasing during mitosis. APC is phosphorylated upon entry into mitosis by cyclin B-CDK1 and/or PLK1\(^{13-46}\).

Figure 6. Entry into mitosis is triggered by the activity of cyclin B-CDK1. This activation occurs when the activities of CDC25 phosphatases exceed those of the Thr 14/Thr 15 kinases, WEEl and MYT1. The commitment to mitosis then becomes irreversible as cyclin B-CDK1 phosphorylates and inactivates the inhibitory kinases, and activates CDC25 phosphatases. Cyclin B-CDK1 phosphorylates and multiple targets, thereby driving a major reorganization of the architecture of the cell. Exit from mitosis depends on the inactivation of CDK1, which occurs as a consequence of APC-mediated destruction of mitotic cyclins. Adapted from Nigg, 2001\(^{46}\).

The two sister chromatids are bound together by the cohesin complex. To allow their separation, the cohesin complex must be first degraded by a protease called separase. The target of APC is another protein called securin, which binds separese before anaphase,
thereby triggering its inhibition. After the securin is degraded by APC, separase is free to cleave the SCC1 protein, one of the subunits of the cohesin complex. The rest of the cohesin complex falls away from the chromosome and the sister chromatids can separate.

After the chromosomes have been separated to the poles of the spindle, the cell must inhibit functions of cyclin B-CDK1 to allow exit from mitosis into the next G1. The inactivation of cyclin B-CDK1 occurs mainly by ubiquitin-dependent degradation of cyclin B, mediated by CDC20-APC. Because the activity of CDC20-APC itself is dependent on the presence of cyclin B-CDK1 activity, in the late mitosis APC binds to another protein—CDH1. Until late mitosis CDH1 is repressed by phosphorylation triggered by cyclin B-CDK1 complex. The activity of the CDH1-APC further suppresses CDK activity (via degradation of mitotic cyclins and other proteins) from late M to mid G1 phase (Fig. 6).

Mechanisms which coordinate the late stages of M phase and cytokinesis are poorly understood in higher eukaryotes. Some orthologs of yeast proteins involved in these processes were found, such as CDC14 and CLP1/FLP1, but their function seems to be at least partly different in mammalian cells. It has been also shown that for a proper exit from mitosis, degradation or PLK1 and Aurora A proteins triggered by APC-CDH1 complex is important.

1.3 CHECKPOINT MECHANISMS

The cyclin-CDK network provides the basic cell cycle regulation. In principle it ensures that the events of the cell cycle follow in certain conserved order. However, the cell must have some secondary mechanisms which make sure that the cell cycle progresses to the next phase only if the previous parts have been properly completed. These mechanisms must also ensure that in case of any damage the cell cycle arrests until the damage is repaired and if it is unreparable, the cell is directed to apoptosis or senescence. The signaling pathways that are part of such mechanisms are called checkpoint pathways. There are several main checkpoints controlling cell cycle status in different stages. DNA damage is sensed mainly by the checkpoints in the G1, S and G2 phases. The checkpoint pathway which operates in the S phase also monitors the course of replication and is activated in the case of any type of DNA damage caused by the replication apparatus.
When the DNA replication is not finished, the cells are arrested by the G2/M checkpoint signaling and are not allowed to continue into mitosis. Another checkpoint mechanism ensures that the mitotic spindle is assembled properly and that the attachment of the kinetochores is completed before chromosomes separation and cytokinesis (Fig. 7).

1.3.1 DNA damage checkpoints

The DNA damage can be caused by many different ways. Exposure to UV light, gamma irradiation or certain chemicals from the environment, as well as action of several products from normal metabolic processes inside the cell can cause single or double strand breaks of the DNA backbone, modification of bases or intra and interstrand crosslinking. The checkpoint signaling pathways consist of proteins of different classes and functions, usually classified as sensors, mediators (adaptors), signal transducers and effectors. Sensors monitor DNA for presence of any kind of damage or abnormalities in structure and start signalization subsequently amplified by transducers. The mediator/adaptor

Figure 7. Cell cycle checkpoints. Adapted from Alberts, 2002 and Lodish, 2003.
proteins facilitate or mediate the interactions between sensors and transducers, typically kinases that trigger phosphorylation of broad spectrum of proteins including effectors. The effector proteins arrest cell cycle and stimulate DNA repair\cite{10,11} (Fig. 8). Although the signal cascades have generally the above-mentioned hierarchical arrangement, there are many autocata\textbackslash ytic loops that partly wipe differences between upstream and downstream positions of certain proteins as well as their functional roles. Many of the proteins involved in the checkpoints are under normal conditions part of the common mechanisms regulating and directing progression of the cell cycle. According to this, some of the checkpoints are functional only in certain stages of the cell cycle, reflecting availability of their components dependent on the cell cycle progression\cite{12}.

![Figure 8. General molecular anatomy of checkpoint pathways elicited in response to DNA damage or replicational stress. The signaling cascade is composed of sensors, which recognize abnormally structured DNA, transducers, that amplify and relay the signal and effectors, which execute the functions of the checkpoint response. If the lesions in DNA are repairable, the checkpoint response delays cell cycle progression and stimulates DNA repair. If the damage is irreparable, the checkpoint may promote apoptosis to eliminate the cell.](image)

The exact mechanisms by which the sensors recognize at least some types of damage are still not clear. They recruit within seconds to the sites of damage and are indispensable
for full activation of two apical transducing kinases – ATM and ATR. These two kinases appear to associate specifically with damaged DNA and are the proximal kinases that mediate most of the known responses to DNA damage\textsuperscript{101}. Both of them are members of the P(3)K-like protein kinase family\textsuperscript{103}. Recently it has been shown that the recruitment is mediated by a C-terminal sequence of sensor proteins which generally binds P(3)K interaction partners and that this recruitment is a requisite for P(3)K-dependent signaling\textsuperscript{104}. The ATR kinase is essential for controlling course of DNA replication in normal cells and responses to the replication stress caused by the arrest of DNA replication forks which could be a consequence of DNA damage caused by alkylating and crosslinking agents, UV light, gamma irradiation or other types of stress\textsuperscript{96,103,105}. Recent results also suggest a critical role for ATR in the normal progression of DNA replication forks and the importance of ATR function in each cell cycle is consistent with the fact, that loss of ATR causes embryonic lethality in mice\textsuperscript{106,107}. ATR is probably constitutively active because the activity of the kinase does not greatly change in the presence of DNA damage and its ability to phosphorylate substrates is regulated by its subcellular localization. The recent model presumes that ATR can be recruited to any abnormal stretch of single stranded DNA (ssDNA) by binding of its heterodimeric partner ATRIP to replication protein \( \alpha \) (RPA) which binds ssDNA regions and is involved in DNA replication\textsuperscript{108-110}. Critical importance for ATR localization and activation have also two other protein complexes, possible candidates for checkpoint sensors, RAD17-RFC clamp loading complex and RAD9-RAD1-HUS1 PCNA-like sliding clamp complex. Another protein, involved in signalization triggered by ATR is claspin. This mediator protein interacts with chromatin structures created by active replication forks, and is required for ATR-mediated activating phosphorylation of CHK1 kinase transducer\textsuperscript{111-113} (Fig.9).

In contrast to ATR, the loss of ATM is not lethal, suggesting that it is primarily activated by DNA damage, such as DNA double strand breaks, and not by side-products of activity of the replication apparatus\textsuperscript{105}. In the absence of DNA damage, ATM is inactive and homodimeric. After introduction of DNA double strand breaks, the dimer dissociates due to conformational changes and ATM monomers become autophosphorylated on Ser 1981. This phosphorylation seems to be essential for ATM activation\textsuperscript{7}. It is still not clear how does ATM dimer sense the damage. One of the possibilities is that it dissociates due to rearrangements in higher-order chromatin structure, induced by introduction of the damage to DNA and that ATM does not require close contact with the DNA breaks. On the other hand, several studies, including the present one, show that ATM activation is much...
slower and weaker in the absence of MRE11-NBS1-RAD50 complex, another potential candidate for the damage sensor\textsuperscript{114-118}. Another recent work identifies MRE11 complex to be the sensor protein complex for DNA double strand breaks, which recognizes the damage and binds ATM to broken DNA molecules\textsuperscript{104,117} (Fig. 9).

Figure 9. Inhibition of CDK activity to promote cell cycle arrest is a principal downstream effect of DNA damage checkpoint pathways. Highly simplified model showing how inactivation of different CDKs elicits cell cycle blocks in G1, S or G2 phase. p53 is a major mediator of the G1 checkpoint based on its ability to transcriptionally induce the CDK inhibitor, p21. The contribution of p53 to the G2 checkpoint arrest is less well understood. Inhibition of CDC25 activity is critical for proper checkpoint function in G1, S and G2 phases. Adapted from Lukas, 2004\textsuperscript{102}.

To the sites of DNA double strand breaks are recruited many proteins that serve as potential sensors, mediators, transducers and effector proteins that cooperate to repair the damage. The initial recruitment of at least some proteins is not exclusively dependent on
ATM activity, but after ATM relocates to the sites of damage, it starts to phosphorylate its substrates thereby initiating faster recruitment and retention at the damage sites of mediators and other proteins, and their accumulation becomes microscopically visible as foci. ATM phosphorylates histone H2AX in the DNA surrounding the breaks within a region that can be several megadaltons wide. Some of the mediator proteins, e.g., MDC1 can bind phosphorylated H2AX and simultaneous interactions with other proteins (such as components of the MRE11 complex) help to accumulate them to the foci as well. Therefore it seems that MDC1 and other mediator proteins such as BRCA1 and 53BP1, which also bind to the foci, facilitate ATM signaling by providing a large interaction interface for ATM and its substrates.

Important targets of ATR and ATM are transducing checkpoint kinases CHK1 and CHK2. Even though both of them can be phosphorylated at the same time by ATM and ATR, CHK1 is mainly target of ATR while CHK2 is in most cases phosphorylated by ATM. As with ATM and ATR, loss of CHK1 is lethal while loss of CHK2 is not. These two kinases amplify the signal and link the pathway with the cell cycle machinery.

1.3.2 The G1 checkpoint

The DNA damage checkpoint pathways slow the cell cycle in S phase or arrest it in G1 and G2 phase mainly by inhibition of CDK activity. The two main effector targets of ATM/ATR-CHK2/CHK1 pathways in G1 are p53 and CDC25A (Fig. 9). The phosphorylation of CDC25A leads to its degradation by the ubiquitin-proteasome pathway and therefore immediate cyclin E/A-CDK2 inactivation by its inhibitory phosphorylation. The inhibition of CDK activity leads to block of loading of CDC45 onto chromatin, thus preventing assembly of pre-replication complexes and initiation of DNA synthesis. This part of the checkpoint response is relatively transient and is functional only in the cells that reached the R-point in which the cell cycle progression is fully dependent on the cyclin E/A-CDK activity.

The other target (p53) is phosphorylated not only by CHK1/CHK2 but also directly by ATM/ATR kinases. They phosphorylate ubiquitin ligase MDM2 as well and its phosphorylation prevents its inhibitory binding to p53. These modifications result into stabilization and accumulation of p53 protein, which then activates transcription of p21 inhibitor of CDKs. This response is much slower than the CDC25A pathway and leads to
sustained or permanent cell cycle blockade, because it not only prevents initiation of DNA replication but also keeps the RB/E2F pathway in the growth-suppressing mode\textsuperscript{1,125,130,131}.

1.3.3 The intra-S phase checkpoint

Activation of DNA damage pathways in S phase results in transient and reversible decrease in the rate of ongoing DNA replication because, as apparent from studies with yeasts, prolongation of the intra-S phase arrest could cause recurrent initiation of once already fired origins. That would lead to inappropriate over-replication of some parts of the genome. Thus, the intra-S phase checkpoint is independent of p53\textsuperscript{128} (Fig. 9).

The activation is triggered by ATM/ATR kinases whereas full activation of ATM activity is dependent upon MRE11 nuclease activity. The kinases then activate CHK1/CHK2-CDC25A-cyclin E/A-CDK

2-CDC45 cascade which also causes the rapid and transient arrest in G1\textsuperscript{125,128}. Improper inhibition of DNA synthesis after ionizing radiation (so called radioresistant DNA synthesis-RDS) accompanies functional defects of individual components of the S phase checkpoints. Such RDS effect can be found in the cells with impaired phosphorylation of SMC1 protein. This phosphorylation is triggered by ATM and is dependent on the presence of phosphorylated NBS1 protein. It suggests that there may be another branch of ATM response to DNA damage in the S phase although the downstream components of this pathway which would cause the inhibition of DNA synthesis are not known so far\textsuperscript{122-124}.

A special part of the S phase checkpoint mechanisms is so called replication checkpoint, activated by the stalled replication, which protects the integrity of the stalled replication forks. It is indispensable also for the above mentioned pathways, because it maintains the fork stability and facilitates the subsequent recovery of DNA replication. The mechanism of this pathway is not yet discovered, but it is thought to contribute also to the G2 checkpoint pathways\textsuperscript{96}.

1.3.4 G2 checkpoint

The DNA damage checkpoint pathways activated in G2 phase protect the cell form
genetic instability and retention of potential dangerous mutations in the next generations. As in the G1 phase, these mechanisms involve pathways operating in the fast way and also pathways which are more delayed and sustained.

The key downstream target of CHK1 (CHK2) is CDC25C phosphatase which upon phosphorylation binds to 14-3-3 proteins. This interaction causes either its subcellular sequestration or inhibition, therefore leaving cyclin B-CDK1 in an inactive state. Another member of CDC25 family – CDC25A seems to be targeted as well (Fig. 9). In response to DNA damage the phosphatase is degraded probably by the same mechanism as has been described above for the acute G1 and S phase checkpoints. The role of CDC25B is not yet clear, but it seems to be also targeted in G2 after exposure to UV light. Other proteins involved in the G2 checkpoint signalization are signal mediators 53BP1, ERCA1, and kinases PLK1 and PLK3. The long-term signaling involves p53 pathway and therefore the G2 checkpoint signalization can resume into sustained cell cycle arrest. Because the cells lacking p53 can still accumulate in G2 after DNA damage, it seems that some other pathway such as BRCA1 stimulated expression of p21 and GADD45 can cooperate with the p53 pathway.

1.4 CHECKPOINT SIGNALIZATION PATHWAYS AND CANCER

As it has been mentioned earlier, every single cell is continuously exposed to endogenous and exogenous sources of DNA damage. If unrepaired, the damage contributes to tumour development and transformation. Therefore, defects in checkpoint signaling pathways increase the risk of developing cancer. It can be due to higher occurrence of mutations that can sooner or later affect any of the genes regulating cellular death, proliferation or senescence, but it is also because the checkpoints are directly connected with basic cell cycle regulation and through p53 with apoptotic and senescence pathways. Checkpoint alteration can therefore directly deregulate these pathways. Still, only some of the proteins involved in checkpoint signaling have been shown to directly contribute to tumour development. Sorting out which proteins are and which are not involved in tumourigenesis can inform us about the mechanism and help us to find new ways of cancer treatment.

Low expression or presence of mutated ATM, MRE11 and NBS1 proteins cause in
Humans ataxia telangiectasia, ataxia telangiectasia-like disorder and Nijmegen syndrome, respectively. Some of the symptoms are very similar - the patients are predisposed to develop cancer and their cells have increased genetic instability\textsuperscript{105,141-143}. Loss of ATR leads to embryonic lethality and ATR haplosufficiency in mice lacking DNA-mismatch repair causes higher occurrence of tumours\textsuperscript{144}. On the other hand, Seckel syndrome associated with human hypomorphic mutations in ATR which leads to decreased protein level does not seem to be associated with higher occurrence of cancer\textsuperscript{145,146}. Mice lacking H2AX and 53BP1 are predisposed to cancer and H2AX haplosufficiency results in genomic instability and in the absence of p33 enhances tumourigenesis\textsuperscript{122,147,148}. Inheritance of a single mutated allele of other checkpoint mediator proteins BRCA1 or BRCA2 markedly increases the incidence of breast and ovarian cancers in women\textsuperscript{149}. Knockout of BRCA1 in mice causes embryonic lethality while conditional disruptions show an increased mammary and lymphoma carcinogenesis in combination with p33 disruption\textsuperscript{150,151}. Bi-allelic hypomorphic mutations of BRCA2 gene are carried by patients with cancer prone Fanconi’s anaemia D1 complementation group and mice with homozygous truncations of BRCA2 develop thymic lymphomas\textsuperscript{152,153}.

CHK1 knockout is lethal and haplosufficiency can contribute to carcinogenesis\textsuperscript{154,155}. While CHK2 knockout mice do not develop tumours, lack of CHK2 enhances skin tumourigenesis induced by carcinogen exposure. Inherited mutations in one allele of CHK2 can be found in some families with extremely cancer-prone Li-Fraumeni syndrome and CHK2 variants predispose individuals to breast and prostate cancer\textsuperscript{125,156,157}. CDC25A and B have been found to possess oncogenic potential regarding their abilities to transform primary cells in cooperation with activated RAS. Both are also frequently overexpressed in a variety of human cancers and such increased expression often correlates with poor prognosis\textsuperscript{158-162}.

The data presented in this thesis show, that the checkpoint pathways might become activated in early stages of human tumourigenesis which could lead to cell-cycle arrest or apoptosis. This mechanism thereby constrains tumour progression, which would under such circumstances rely on selection of cells defective in their DNA damage response components such as ATM or p53, with compromised cell-cycle arrest, senescence, and apoptosis.
2. AIM OF THE STUDY

It has been shown that the checkpoint signaling pathways are essential for the proper progression of the cell cycle, maintenance of the genome integrity and moderating of the tumour progression. Even though some of the pathways have been studied in detail, the exact mechanism of DNA damage recognition is still unclear. The paper published by Bakkenist and Kastan is crucial for understanding of ATM activation but still leaves many questions that should be solved\(^1\). Because the checkpoints (including the ATM-NBS1-CHK2 pathway) are connected with the process of tumour development, detailed knowledge of the checkpoints mechanisms is important for our better understanding of carcinogenesis, which could lead to development of more efficient cancer treatment.

The aim of this Ph.D. study was to elucidate some aspects of the mechanism of ATM activation and the role played in this process by the MRE11-NBS1-RAD50 complex. In addition, an important issue of the potential involvement of the DNA damage checkpoint pathways in the early stages of human tumorigenesis has also been addressed as part of this Ph.D. project, focusing on the role of ATM and its downstream targets in particular. Finally, this work also contributes to better understanding of the regulation, function, and potential involvement in human cancer of the DNA damage checkpoint mediators 53BP1 and MDC1.
3. MATERIAL AND METHODS

3.1 CELL BIOLOGY

3.1.1 Monoclonal antibodies

Two 3alb/c mice were immunized by subcutaneous injections with 75 μg of purified GST protein (C-terminal BRCT domain of MDC1 fused to GST protein), first time mixed with complete Freund’s adjuvant and subsequently twice mixed with Freund’s incomplete adjuvant. The mouse with positive response was subjected to intravenous boost injections for three following days. Two days later the fusion was performed. The spleen cells were fused with NS-2 mouse myeloma cells in the presence of 50% polyethylene glycol and the hybridomas were selected by azarine/hypoxanthine medium. The hybridomas were cloned in 96-well microtiter plates and the supernatants were tested by dot blotting modified for fluorescence microscopy. The positive clones were further tested by western blotting on lysates with exogenously expressed MDC1 protein or with decreased mRNA level due to siRNA treatment. The positive hybridomas were recloned to ensure that the clones derived from one single cell and monoclonal antibodies were produced either as neat tissue culture supernatant or as ascitic fluid from Balb/c mice.

For different experiments we used mouse monoclonal antibodies against total and Thr 68-phosphorylated CHK2, respectively\textsuperscript{120,137}, cyclin E (Novocastra Laboratories), cyclin A (Novocastra Laboratories), γ-H2AX (Upstate), RPA p32 (Neomarkers), Ser 1981-phosphorylated ATM (from C. Bakkenist and M.B. Kastan), MRE11 (GeneTex) and MCM7\textsuperscript{133}, and rabbit antisera against ATM (from Y. Shiloh), Ser 1981-phosphorylated ATM (Rockland), Ki67 (Dako), γ-H2AX, Ser15-phosphorylated p53, Ser 317-phosphorylated CHK1, Ser 345-phosphorylated CHK1 and Ser 645-phosphorylated RAD17 (all from Cell signaling), Thr 15-phosphorylated CDK1 (Calbiochem), total SMC1 (Abcam) and Ser 966-phosphorylated SMC1 (Abcam), NBS1 (Novus Biologicals).

3.1.2 Cell lines and cell culture conditions

In this work U-2-OS-derived cell lines, normal human BJ fibroblasts, NBS telomerase (h-TERT) immortalized human fibroblasts (NBS-Tert) and Phoenix packaging cells have been used. All cell lines were grown in 37°C, 5% of CO\textsubscript{2} in DMEM supplemented with
10% Fetal Calf Serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). For selection and culturing of stable transfectants G418 (0.4 mg/ml) and puromycin (1 µg/ml) were added to the medium. To suppress transcription from the stable transfectants’ tetracycline regulatable promoters, tetracycline was added to the medium (Tet, 1 µg/ml). To induce nuclear translocation and activity of human E2F1, 4-hydroxy tamoxifen was added into the media of cells expressing ER-E2F1 fusion protein (Tam, final concentration 300 nM).

U-2-OS-derived cell lines with tetracycline-repressible expression of human CDC25A were received from N. Mailand and the U-2-OS-derived cells with tetracycline-repressible expression of wild-type, full-length human cyclin E were received from C. Lukas.

3.1.3 Transient and stable transfections and infections

DNA was introduced into cells using the calcium-phosphate transfection method, Lipofectamine 2000 (Invitrogen) and FuGENE 6 (Roche Applied Science).

The siRNA was introduced using Oligofectamine (Invitrogen) in final concentration 50 nM.

For the retroviral infection, Phoenix packaging cells were transiently transfected first and the culture medium was used for infection of the human fibroblasts. We followed protocol published on http://www.stanford.edu/group/nolan.

3.1.5 Ionising radiation

Ionising radiation was used to generate DNA double-strand breaks. Cells were irradiated with different doses of radiation in X-ray generator.

3.1.6 Immunofluorescence

To examine expression and subcellular localization of cell cycle proteins, immunofluorescence was used. Cells were grown on coverslips and following two washes in PBS they were fixed in methanol/acetone 1:1 for 10 min. The coverslips were incubated with one or two primary antibodies diluted 1:100 in DMEM containing 10% FCS for 1 hr at room temperature and washed three times briefly in PBS. Secondary anti-mouse or anti-rabbit fluorochrom conjugated antibodies or both were added and incubated for 30 min at
room temperature in the dark. After three washes in PBS and one in water the coverslips were dried, mounted in vecta shield with DAPI and examined on fluorescence or confocal microscope.

3.2 MOLECULAR BIOLOGY AND BIOCHEMISTRY

3.2.1 Bacterial media and strains
Bacteria were grown in Luria-Bertani (LB) medium, supplemented with ampicillin (0.1 g/l). Agar plates contained 15 g/l agar.

Escherichia coli TOP10: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(aral-araE) 7697 galU galK rpsL (StrR) endA1 nupG (Invitrogen) was used as the host during the cloning procedure.

Escherichia coli BL21(DE3): F- ompT' [lon] hsdSB (rB-mB-; an E.coli B strain) with DE3, a λ phage carrying the T7 RNA polymerase gene, was used as the host for the MDC1 fragment overexpression.

3.2.2 Dot blotting modified for fluorescence microscopy
For testing of the hybridomas producing antibodies specifically recognizing MDC1 protein, we grew U-2-OS cells on a 6 cm dish until they were confluent. We irradiated the cells with 10 Gy and after one hour in methanol/acetone. We performed immunofluorescence staining, using 3 μl of tested supernatants from the 96-well microtiter plates for dots as primary antibodies. We searched for those staining foci localized in the nucleus. The positive supernates were used on non-irradiated dishes while searching for homogenous nuclear staining.

3.2.3 Plasmids and Vectors
DNA manipulations were performed according to standard methodology. Restriction enzymes were purchased from New England Biolabs.

The pFLAG containing the MDC1 cDNA (pFLAG KIAA0170) was received from A.
Nakagawara\textsuperscript{48}. The protein expression vector pGEX 2TK was used for cloning the GST-MDC1 fragment. The retroviral vectors containing MYC tagged NBS1 constructs were prepared by J. Falck. The vector expressing HAER-E2FI fusion protein was received from K. Helin\textsuperscript{49}.

3.2.4 Expression of MDC1-C-terminal BRCT domain-GST fusion protein

The MDC1-C terminal BRCT domain-GST fusion protein was expressed in BL21 cells. The protein expression was induced by 1mM IPTG for 30 min. The bacteria were lysed in STE buffer (10mM Tris pH 8.0; 150 mM NaCl; 1mM EDTA) with lysozyme (100 µg/ml) and incubated on ice for 15 min before addition of 5 mM DTT (dithiothreitol), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml aprotinin and 5 µg/ml leupeptin (protease inhibitors). N-lauroylsarcosine was added to a final concentration of 1.5% and the solution was transferred to Falcon snap cap tubes. The samples were sonicated on ice at power level 4.5% duty cycle until the lysates became clear, normally three times five bursts. The samples were centrifugated for 10 min at 20000 rpm and 4°C, and the supernatant was transferred to new tubes and adjusted to 2% Triton X-100. 500 µl of glutathione sepharose beads (Sigma) were added and the solution was incubated on an end-over-end rotator at 4°C for 30-120 min. The beads were washed five times with washing buffer (20 mM Tris pH 8.0; 120 mM NaCl; 10% glycerol; 0.1% Triton -100; 5 mM DTT; 0.2 mM PMSF. The proteins were eluted two times 30 min at room temperature in 500 µl elution buffer (100 mM Tris pH 8.0; 120 mM NaCl; 10% glycerol; 20mM glutathione; 1% Triton-X-100; 5mM DTT, 0.2 mM PMSF) and dialysed over night against PBS-- (140 mM NaCl; 3mM KCl; 8mM NaH$_2$PO$_4$ pH 7.4) at 4°C. The protein concentration was measured with Bradford (BioRad) according to manufacture’s instruction using purified immunoglobulin (1 µg/ml) as an internal standard and the purity of the protein was examined by SDS-PAGE and Coomasie staining.

3.2.5 Protein extracts

In order to prepare extracts for western blotting or immunoprecipitation, cells were grown in dishes according to the needed amount of protein extract. The dishes were washed three times with cold PBS and a suitable amount of protein extraction buffer (50 mM Hapes pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 10% glycerol; 0.1%
Tween-20) containing 1mM DTT, protease inhibitors (5 µg/ml leupeptin; 2 µg/ml aprotinin; 0.1 mM PMSF) and phosphatase inhibitors (10 mM β-glycerolphosphate; 0.1 mM NaVO₄; 1mM NaF) was added. The cells were lysed and scraped off with a cell scraper. After quick-freezing in liquid nitrogen, the lysates were thawed and kept on ice with occasional shaking for 30 min. The lysates were cleared with centrifugation (10 min; 20 000 rpm) and the protein concentration was measured withBradford assay (BioRad) according to manufacturer’s instructions. Lysates were stored at -80°C.

3.2.6 Immunoprecipitation

10 µl of the rabbit antibody (Santa Cruz H-300: sc-9064) were coupled to sepharose protein-A beads for 30 min at 4°C on an end-over-end rotator. The beads were washed three times with protein extraction buffer and 800 µg lysate was added. Following incubation for 2 hr at 4°C on an end-over-end rotator, the beads were washed three times with protein extraction buffer. The IPs were lysed in Laemli sample buffer (LSB) and western blotted.

3.2.7 Other biochemical methods

SDS-PAGE, Western blotting, staining of polyacrylamide gels with Coomassie blue staining were performed according to standard methodology.¹⁶⁶
4. RESULTS

In the following two parts (Publication I and II) are in detail described only experiments elaborated by author of this Ph.D. thesis. The rest of the experiments done by other members of the research team are published in the enclosed papers (chapter 7). The third part (Publication III) is constituent of paper which is in preparation now and in the thesis includes only work done by author of this Ph.D. thesis.

4.1 PUBLICATION I


The DNA double strand breaks are one of the most toxic types of the DNA damage. The cells in the S phase activate in response to this kind of damage ATM kinase which in turn activates a complex signaling network leading to slowing of the DNA replication rate and damage repair or, in the case of severe damage, to apoptosis. The mechanism by which the DNA double strand breaks are recognized and processed is not clear. The pioneering work has been done in 2003 by Ch. Bakkenist, who postulated the ser 1981 of ATM kinase to become autophosphorylated in response to DNA damage. He has shown as well that the ATM kinase is in the inactive form a homodimer that shortly after the introduction of the DNA damage becomes autophosphorylated on ser 1981 and dissociates into two active monomers. Bakkenist and Kastan proposed that the activation of ATM could depend indirectly on the changes in chromatin structure caused by introduction of the breaks into the DNA. In this model, ATM does not need for its activation any sensor protein and its presence at the site of the damage would be required for phosphorylation of the proteins associated directly with the breaks but not for its targets which are dispersed within the nucleus.

On the other hand, some of the previously published works showed that cells lacking NBS1 or MRE11 are deficient in phosphorylation of ATM substrate proteins. Since the results differed, this has been a controversial issue for some time. The NBS1 and
MRE11 proteins are parts of the so-called MRN complex, which consists from MRE11 endonuclease, RAD50 ATPase and NBS1 protein. NBS1 brings the complex to the nucleus and to the foci arising at the sites of damage. This complex is essential for the DNA double strand break repair and its components are phosphorylated by ATM. In this work we used the monoclonal antibody which specifically recognizes the phosphorylated Ser 1981 of ATM to show whether and how is the ATM activation dependent on the presence of NBS1 protein (the antibody was prepared and provided by Ch. Bakkenist and M. Kastan). We also tested different NBS1 constructs mutated in its structural domain to clarify their role in this process (the DNA constructs were prepared by J. Faleck). The work provides some new data about the initial steps of ATM activation.

![Diagram](image)

**Figure 10.** ATM phosphorylation after ionizing radiation is impaired in NBS1-deficient cells. Human diploid BJ fibroblast strain (BJ) or NBS telomerase (h-Tert)-immortalized human fibroblasts (NES-Tert) were either untreated or treated with 2 Gy of ionizing radiation, collected at the indicated times (0 min to 8 h) after irradiation and analyzed by immunoblotting. Primary antibodies included rabbit polyclonal antibodies to anti-phospho Ser 1981 of ATM, anti-phospho Ser 966 of SMC1 and total SMC1.

To begin investigating the potential contribution of NBS1 to ATM activation, we first exposed NBS1-proficient, human diploid fibroblasts (the BJ strain) to low doses of ionizing radiation (IR) (Fig. 10). After exposure of asynchronously proliferating BJ cells to 2 Gy, ATM became rapidly autophosphorylated on Ser 1981 and therefore activated. The magnitude of this effect was stabilized between 5 and 15 min after irradiation and began to decline after additional 4 hours. Very similar kinetics was observed for phosphorylation of several ATM-downstream targets including Ser 966 of SMC1. This rapid, quantitative and transient activation of the ATM-dependent checkpoint response induced by low doses of IR indicated that our experimental system was sensitive enough to assess the kinetics of the ATM-dependent phosphorylation events.

When the same experimental protocol was applied on the human telomerase catalytic subunit (h-Tert)-immortalised NBS fibroblasts (NBS-Tert), ATM autoactivation...
Figure 11. Reconstitution of NBS1 expression in NBS-Tert fibroblasts. NBS cells containing the endogenous truncated and low-abundant form of NBS1 (657del5) were infected with pBABE-puro vector alone, with the vector expressing myc-tagged Nbs1 wild-type (NBS1 wt) or NBS1 mutant forms (R28/A, 3xS/A, Del628) (A). Adequate expression level of the constructs in selected clones was confirmed by immunoblotting (B). The ability to form foci after irradiation and to bind MRE11 were tested by immunofluorescence staining of the cells 8h after irradiation with 10 GY. The wild-type (NBS1 wt) and the phosphorylation mutant of NBS1 (3xS/A) bind MRE11 and form foci, mutation in the FHA domain (R28/A) affects foci formation while the deletion mutant (Del628) is able to form foci but cannot bind MRE11 (C). (D) The radiation sensitivity of the cells was estimated by colony forming assay. The cells were exposed to 2 Gy of IR. After 2-3 weeks, colonies per plate were counted and expressed as the percentage of colonies seen in the unirradiated control. Data shown are the mean and standard deviation of three independent experiments, each performed in triplicate.

was significantly impaired. Thus, the overall extent of Ser 1981 autophosphorylation was
weakened and it reached maximum only 1 hr after irradiation. Consistently, the kinetics of SMC1 phosphorylation was also delayed compared to the NBS1-proficient BJ fibroblasts. These results suggested that in addition to its well-established role as a genuine ATM substrate, NBS1 may have an important role in modulation of ATM activation in the IR-induced signalling cascade. To directly address this issue, we have generated a series of cellular models with distinct status of the NBS1 protein.

Proper integration of NBS1 into the IR-induced checkpoint signaling network depends on the integrity of several structural domains, phosphorylations, and protein-protein interactions. To gain insight into whether and how these NBS1 features determine its ability to modulate ATM activation we reconstituted the NBS Tert cells by retroviruses containing the wild type (wt) and several mutant forms of human NBS1 together with the gene coding for resistance to puromycin. Importantly, using the telomerase-immortalized cells eliminates the drawback of otherwise gross proliferative differences between the primary NBS1-deficient cells and their NBS1-reconstituted counterparts. The series of NBS1 mutants included an inactivating point mutation within the FHA domain (R28A), substitution of the ATM-targeted serines (S278, S343, S397) by alanines (3xS/A), and deletion of the MRE11-binding domain (Del628), respectively (Fig.11A).

Due to cell-to-cell variation in expression levels of the NBS1 proteins in the pooled cell populations, we selected puromycin-resistant clones homogenously expressing the ectopic NBS1 forms to near-physiological levels (compared to the amount of protein in the NBS1-proficient BJ cells) (Fig. 11B). While the wt, 3xS/A and Del628 forms of NBS1 readily formed the IR-induced nuclear foci (IRIF), the cells reconstituted with the FHA-deficient R28A or with the puromycin resistance gene only (pBABE) showed little or no recruitment of NBS1 to IRIF (Fig. 11C). Moreover, reconstitution with wt and 3xS/A (but not the R28A and Del628) alleles rescued survival of NBS-Tert cells after exposure to IR (Fig. 11D). All these results are consistent with earlier extensive characterization of NBS1 and its variants and suggest that we succeeded to generate a relevant model system to study the impact of NBS1 structural features on ATM activation.

Next, we subjected the above cell lines to the irradiation/time-course protocol as described in Fig.10 and assessed by immunoblotting with phospho-specific antibodies the kinetics of ATM autophosphorylation on Ser 1981 together with phosphorylation of SMC1 on Ser 966. Reconstitution of the NBS-Tert cells with wild-type NBS1 restored the delayed kinetics of both ATM autoactivation and SMC1 phosphorylation (Fig.12A) back to the profile typically observed in normal human fibroblasts (see also Fig. 10 for comparison).
Figure 12. NBS1 modulates ATM activation after low doses of IR. Human NBS-Tert cells expressing different NBS1 mutant forms and the cells infected with empty vector were irradiated with 2 Gy and lysed at the indicated times (5min to 8h) after irradiation. Phosphorylation of Ser 1981 of ATM and Ser 966 of SMC1 and the total level of SMC1 were examined by immunoblotting.

Specifically, in the NBS1 wt-reconstituted cells, phosphorylations of ATM and SMC1 were detectable already in early time-points (5-15 min) and begun to decline around 4 h after irradiation. These results strongly supported our initial hypothesis that NBS1 may modulate the timing and the amplitude of ATM activation in response to low doses of IR.

Interestingly, the three NBS1 mutants showed differential effects on the kinetics of the studied ATM phosphorylation events. Thus, expression of the FHA-deficient NBS1 (R28/A) completely rescued both ATM autoactivation and SMC1 phosphorylation defects inherent to the NBS-Tert cells (Fig. 12B). Since disruption of the FHA domain effectively abolished accumulation of NBS1 within IRIF (see Fig. 11C), it appears that the rate-limiting effect of NBS1 on the ATM phosphorylation events does not require stable integration of NBS1 into the chromosomal regions flanking the actual DNA breakage sites.

The phosphorylation-deficient mutant of NBS1 (3xS/A) also restored timely initiation of ATM activation and SMC1 phosphorylation, respectively (Fig.12C). Compared with effects of wild-type and the FHA-deficient NBS1, the NBS-Tert cells reconstituted with the phosphorylation-deficient NBS1 (3xS/A) showed a moderately protracted phosphorylation of Smc1 in some experiments, suggesting a potential delay in exit from the activated checkpoint mode in such cells. Further validation of this...
observation and elucidation of its biological significance must await future studies.

Finally, the NB31 mutant deficient in binding to MRE11 (Del628) was completely unable to rescue the ATM autoactivation and, consequently, phosphorylation of the ATM downstream targets such as SMC1 (Fig. 12D). Because the Del628 mutant was readily integrated into IRIF we concluded that some MRE11-dependent function provided jointly by the MRN complex (likely the nuclease activity) may be essential to promote rapid and massive activation of ATM-dependent signalling. Recent works from other laboratories elaborated on this topic are discussed in chapter 5.1.

4.2 PUBLICATION II


Tumourigenesis is a multistep process during which the cells in the tissue accumulate genetic and epigenetic changes that are providing conditions for development from normal tissues into benign and eventually invasive malignant tumours. Defects in proteins which are components of the checkpoint signaling cascades have been often found to be responsible for enhanced genetic instability of cancer cells. These pathways delay cell cycle progression to facilitate DNA repair and they can also eliminate the hazardous damaged cells through induction of cell death, thereby protecting the organism against cancer.

From our recent observations it is apparent that advanced carcinomas of the lung and breast have constitutively activated members of the ATM checkpoint pathway such as 53BP1 and CHK2.\textsuperscript{177} Results from other groups also show that oncogenes which can deregulate G1/S transition, such as MYC and E2F1, cause DNA damage in cultured cells.\textsuperscript{176, 177} In response to the p53 pathway becomes activated, and in the case of severe damage this leads to apoptosis. Therefore we hypothesized that over expression of the proteins inducing proliferation in the early stages of human tumourigenesis and the subsequent increase of DNA damage may activate DNA damage checkpoints. Indeed, we
showed that early precursor lesions of the human tumours of the urinary bladder, breast, lung and colon have commonly activated proteins involved in DNA damage signalling. We also showed that overexpression of several oncogenes that deregulate DNA replication in cell culture leads to phosphorylation of ATM, H2AX, CHK1, p53 and other checkpoint signaling proteins. From the genetic analysis that we performed it is possible to conclude that this ATM/ATR regulated DNA damage response network is active in the early stages of tumorigenesis and that it likely precedes genomic instability and malignant conversion. We propose that such checkpoint activation, which leads to cell-cycle blockade, senescence or apoptosis constrain tumour progression and that only the cells which become defective in their DNA damage response components, can progress in the carcinogenesis. Overall, this study provides new insights into the role of DNA damage pathways in tumorigenesis.

The author of this Ph.D. thesis has done the in vitro part of the experiments. The data from the first part of the work documented that the ATM-CHK2-p53 cascade is commonly activated at pre-invasive stages of major types of human tumours and raised a question about the identity of tumourigenic events that could evoke the observed DNA damage response in human cancer. To answer it we tested first overexpression of cyclin E (13A), an event common in carcinomas and reported to enhance genomic instability46,179-182. In our U-2-OS-derived cells (a checkpoint-proficient human osteosarcoma cell line with wild-type RB and p53) cyclin E could be overexpressed, in a tetracycline-repressible manner, to the level comparable with endogenous protein in breast cancer cell lines with amplified cyclin E gene (13B). Biochemical analysis of cells with induced cyclin E showed time-dependent appearance of Ser 15-phosphorylated p53, γH2AX, and Ser 966-phosphorylated cHesin SMIC (13A), established targets of the DNA damage response kinases ATM and ATR133,178,183,184. Analogous to cyclin E, overexpression of tetracycline-regulatable CDC25A164, another proto-oncogene overexpressed in many carcinomas161,164, evoked a DNA damage response (13A). Furthermore, a similar response was observed when estrogen receptor-tagged E2F1169, an S-phase-promoting transcription factor commonly deregulated in cancer due to aberrations of the RB pathway185, was translocated into the nucleus upon tamoxifen addition to the medium (13A).
Figure 13. Overexpressed cyclin E, CDC25A and E2F1 induce DNA damage response in U-2-OS cells. (A) Time-course immunoblotting analyses with indicated phospho-specific antibodies; MCM7: loading control; Tet: tetracycline; Tam: tamoxifen; NAC: anti-oxidant N-acetyl-L-cysteine. (B) Immunoblots documenting cyclin E abundance after 2-day induction, versus endogenous level in MDA157 cells with amplified cyclin E gene. The altered mobility of ectopic cyclin E was explained (16,17). (C) Time-course immunoblotting analyses with indicated phospho-specific antibodies; Tet, NAC: see (A). (D) Total-CHK2 immunoprecipitates were analyzed by immunoblotting for total (CHK2) and Thr 68-phosphorylated CHK2 (CHK2 Thr 68) before and 4 days after induction of cyclin E.

Interestingly, the oncogene-induced phosphorylations of CHK2-Thr 68 and ATM-Ser 1981, albeit detectable both biochemically and cytologically, were less pronounced.
than γH2AX, suggesting that the ATR-γH2AX/CHK1 pathway likely contributed to the overall response. Indeed, CHK1 was found phosphorylated on its activatory sites Ser 317 and Ser 345 (Fig. 13C), of which particularly the latter is regarded as a preferential target of ATR. Phosphorylated CHK1 was also detected by fluorescence microscopy already two days after induction of cyclin E, CDC25A or E2F1 (Fig. 14A), and another established target of ATR, Ser 645 of the checkpoint protein RAD1, was also phosphorylated (Fig. 14B). Analogous to U-2-OS cells, ectopic expression of cyclin E induced DNA damage response in human fibroblasts (Fig. 15).

Figure 14. DNA damage response to overexpressed oncogenes in U-2-OS cells. (A) Confocal immunofluorescence microscopy images of cells with cyclin E or Cdc25A induced for 4 days by removing tetracycline from the medium (-TET), or E2F1 induced by tamoxifen to be translocated to nuclei (+Tam), analyzed for the indicated markers. (B) Double-immunofluorescence colocalization of γH2AX and pS-ATM foci (top), and pT-CHK2 staining in γH2AX-foci-positive nuclei (bottom) in cells with induced cyclin E. (C) Time-course flow cytometry analysis. (D) Time-course immunoblotting analysis of MCM7 (loading marker), Tyr15-phosphorylated CDK1 and total CDK1; arrow: Tyr15-phosphorylated form of CDK1. Immunofluorescence performed by J. Bartkova.

Overall, our cell culture models show that S-phase promoting oncogenes can...
activate the ATM/ATR-regulated DNA damage network. Furthermore, unlike phosphorylations of p53 and H2AX induced by the MYC oncogene, attributed to generation of reactive oxygen radicals, the DNA damage response in our experiments was only marginally repressible by the anti-oxidant N-acetyl-L-cysteine (NAC; 13A,C; 15B,C; 16C), indicating that the bulk of the DNA damage caused by these hyperproliferative oncogenic stimuli was independent of oxidative stress.

![Figure 15](attachment:image.png)

**Figure 15.** Detection of cyclin E-activated DNA damage checkpoint markers in normal human fibroblasts. (A) Examples of immunofluorescence (confocal microscopy) and immunoperoxidase staining (light microscopy) images of early-passage human diploid fibroblasts upon retrovirus-mediated ectopic expression of cyclin E (cyc E), compared with cells infected with control virus (mock). (B,C) Detection of phosphorylated pS-ATM or γH2AX in cyclin E-overexpressing normal human fibroblasts is not altered in cells treated with an antioxidant (NAC), similarly to data with U-2-OS cells overexpressing cyclin E, CDC25A or E2F1. Note the lack of DNA damage response in mock-infected control fibroblasts (mock). Immunofluorescence and immunoperoxidase staining were performed by J. Barkova.

Rather than oxidative stress, we argued that the DNA damage response could reflect deregulated DNA replication, since overexpressed cyclin E, CDC25A and E2F1 share the ability to promote unscheduled S-phase entry. When cells experience
replication stress, they activate their replication checkpoint to delay S-phase progression and G2/M transition\textsuperscript{128}. Consistent with such a scenario, U-2-OS cells with induced cyclin E accumulated in S and G2. The G2 arrest was particularly prominent by day 4 of time-course experiments, and by day 6 many cells showed aberrantly enhanced, >4N DNA contents (Fig. 14C), likely reflecting partial re-replication of the genome uncoupled from cell division. The inability to enter mitosis correlated with massive induction of the Thr 15-phosphorylated, inactive form of the key mitosis-promoting kinase CDK1\textsuperscript{155} (Fig. 14D) a marker of an effective checkpoint.

![Figure 16. Altered dynamics of DNA replication and chromatin association of RPA upon induced overexpression of cyclin E in human U-2-OS cells. (A) DNA fiber analysis at 2 days after cyclin E induction. DNA fiber labeling was performed as described\textsuperscript{166}. (B) Cyclin E induction leads to an increase of non-extractable RPA protein, and hyperphosphorylation of RPA in U-2-OS cells (-Tet) compared with control cells grown in parallel (+Tet). Chromatin bound: cells were incubated with extraction buffer, and non-extractable, chromatin bound fraction loaded in increasing amounts (1x, 2x, 4x) and processed for...](image-url)
Western blotting. Total protein: total cell lysates analysed for RPA and MCM7 protein (loading marker). Note the pronounced mobility shift of RPA in cyclin E expressing cells, consistent with RPA hyperphosphorylation and activated checkpoint response. The presence of extended ssDNA stretches upon cyclin E induction was also verified by detectable BrDU incorporation without denaturation of the fixed cells (data not shown, method reported by Radershall et al., consistent with the enhanced chromatin association of the ssDNA-binding RPA (shown in the top panel).

Further supporting the notion that cyclin E overexpression alters replication dynamics, significantly longer replication tracks were detected using DNA fiber labelling, and increased amounts of the single-stranded-DNA-binding (ssDNA) replication protein A (RPA) bound to chromatin (16A,B; experiments performed by A. Kramer and F. Tort). RPA loading occurs during normal replication initiation, and on abnormal ssDNA intermediates that form during replicational stress, the latter scenario consistent with the hyperphosphorylation of RPA in our experiments. Importantly, RPA-coated ssDNA is a pre-requisite, and an activation signal for the ATR-dependent checkpoint, and cyclin-induced aberrant DNA replication in yeast causes DSBs, potentially deadly lesions that activate the ATM-Chk2 pathway in mammals.

Collectively, our cell culture experiments suggested that tumourigenic abnormalities that deregulate DNA replication induce DNA damage and checkpoint activation. To validate this concept for early tumourogenesis in vivo, three predictions should be verified: i) Tumourigenic abnormalities that deregulate S-phase entry must occur with high frequency; ii) There should be evidence for replicational stress; and iii) Activation of a functional checkpoint should be documented. These three postulates were indeed validated for premalignant lesions, by the analyses implicated in the last part of the paper (chapter 7.2).

4.3 PUBLICATION III

Jurina Bartikova, Zuzana Horcji, Maxwell Sehested, Jahn M. Nesland, Stephen Jackson and Lukas and Ji Bartek. DNA damage response mediators MDC1 and 53BP1 in human tissues and tumours.
MDC1 and 53BP1 are key components of the genome surveillance network activated by DNA double strand breaks. Together with BRCA1 they contain two tandem BRCT domains at their C terminus. These domains have been shown to serve as protein phosphoprotein-binding modules and are supposed to facilitate the transient interactions of ATM with its targets, thus facilitate ATM signaling and the processing/repair of the DNA lesions. MDC1 and 53BP1 become within minutes after DNA damage progressively yet transiently mobilized around the DSB-flanking chromatin. The assembly of 53BP1 lags behind MDC1 which is a key upstream determinant of 53BP1's interaction with DSBs from its dynamic assembly after DSB sites through sustained retention within the DSB-flanking chromatin up to the recovery from the checkpoint. The initial recruitment of MDC1 and 53BP1 to sites of DNA damage is largely ATM independent, but the later accumulation into the microscopically visible foci depends on ATM mediated phosphorylation of histone H2AX.

Mammalian cells that lack MDC1 due to siRNA treatment show enhanced sensitivity to DNA-damaging agents such as ionizing radiation and impaired intra-S-phase and G2/M cell cycle checkpoints. While no MDC1 knock out mice have been described so far, 53BP1-deficient mice, like ATM knockout mice, are growth retarded, immune deficient, radiation sensitive, and cancer prone. Cells lacking MDC1 are sensitive to IR and fail to activate the intra-S and G2 checkpoints.

For finding out more details about the regulation, function, and potential involvement in human cancer of the DNA damage checkpoint mediators 53BP1 and MDC1, an antibody to MDC1 suitable for tissue staining was necessary. The author of this Ph.D. work generated a monoclonal antibody to the C terminal BRCT domain of MDC1. The antibody was selected for the ability to stain MDC1 protein in the cellular nuclei before and after irradiation (see chapter 3.2.2). This monoclonal antibody showed up to be very useful for tissue staining experiments (Fig.17).

The antibody, together with antibodies to 53BP1 and Ki67 were used for immunoperoxidase detection of MDC1 and 53BP1 in normal and cancer human tissues. The staining shows their typical nuclear staining pattern in normal tissues (which is different in germ and brain cells in the case of MDC1). It is apparent that the staining pattern differs in tumours from the normal tissues e.g. the nuclear staining pattern changes, the focal staining pattern for 53BP1 appears in nonirradiated human breast cancer cell lines and there is also evidence for aberrant gross reduction of 53BP1 and MDC1 staining. These data were collected by Jirina Barkova and since the paper is in preparation, we do not
show the data in this thesis.

Figure 17. Characterization of the new mouse monoclonal antibody DCS-380.1 against MDC1. (A) Immunofluorescence staining of endogenous cellular MDC1 in nonirradiated, irradiated (15 Gy) and siRNA-treated human U-2-OS cells. Confocal microscope images of the same cells stained for DNA, MDC1 and the γH2AX marker are shown, as indicated. The published rabbit antibody 988 against MDC1 \cite{Goldberg2003} served as a positive control, and cells with siRNA-depleted MDC1 protein provided the negative control. (B) Immunoblotting analysis of endogenous MDC1 (migrating as a characteristic triplet of bands, see Goldberg \textit{et al.} 2003) \cite{Goldberg2003} in extracts of U-2-OS cells, using the 988 and DCS-380.1 antibodies. (C) Schematic representation of MDC1, with indicated protein domains, and the names of antibodies raised against three distinct domains.
5. DISCUSSION

The work presented in this thesis was focused on further clarification of the mechanism of DNA damage checkpoint activation following introduction of DNA double strand breaks into the DNA and on the role of the checkpoint mechanisms in tumour progression.

5.1 ATM ACTIVATION IS DEPENDENT ON MRN COMPLEX

The first part concentrated on the mechanism of ATM kinase activation and role of the MRN complex in this process. Our results showed that the MRN complex modulates phosphorylation and therefore activity of ATM kinase in cells treated with low doses of irradiation. We also showed that mutation of NBS1 in FHA domain and mutation of its serines phosphorylated by ATM do not affect the ATM activation. Because disruption of the FHA domain effectively abolished recruitment of NBS1 into IREF (see Fig. 11C), it appears that the rate-limiting effect of NBS1 on the ATM phosphorylation events does not require stable integration of NBS1 to the chromosomal regions flanking the actual DNA breakage sites. Consistently, two other reports demonstrated that the FHA-deficient NBS1 protein restored timely ATM-dependent phosphorylation of SMC1 and CHK2, respectively (interestingly, this happened even though the FHA-deficient NBS1 itself was a poor ATM substrate and remained un(der)phosphorylated)\textsuperscript{172}. Our new data added an important new dimension to these findings by showing that the restoration of ATM phosphorylation events (with or without the fully functional FHA domain) applies also to ATM autoactivation – in other words, to the very proximal and indeed the nodal point of the IR-induced signalling cascade. The phosphorylation-deficient mutant of NBS1 (3xS/A) also restored timely initiation of ATM activation and SMC1 phosphorylation, respectively. There is some discrepancy in the literature with regards to the requirement for NBS1 phosphorylation for targeting various ATM substrates. Our results support those by Kastan’s and Lim’s group who also concluded that phosphorylation of SMC1 does not require preceding (or concomitant) NBS1 phosphorylation. Moreover, these conclusions are also in line with the results obtained by several groups (including this study) with the FHA-deficient NBS1\textsuperscript{172}. Although this form of NBS1 cannot be efficiently phosphorylated itself, it still supports timely phosphorylation of other ATM targets including SMC1 (see
In contrast, protein with deletion of the C terminal part, which contains MRE11
binding domain cannot rescue ATM activity in cells lacking NBS1. At this point we
suggested that NBS1, through its ability to interact with MRE11 plays an important role in
regulating the timing of the IR-induced, ATM-mediated phosphorylation events. Our
hypothesis also supported broadly similar findings which have been published by Shiloh
and colleagues\textsuperscript{14} who documented that the nuclease activity of MRE11 was required for
the adequate activation of ATM in response to low doses of IR.

Recently two works about NBS C-terminal part came out. Both agreed on the fact,
that the extreme 20-50 C-terminal amino acids of NBS1 following the MRE11 binding site
contain an evolutionary conserved ATM interaction motif. You and colleagues\textsuperscript{306} used for
their work yeast and \textit{Xenopus} systems. Using these models they show that the C terminus
of Nbs1 is essential for ATM activation and for its recruitment to the sites of DNA
damage. In their experimental system, recruitment of ATM to damaged DNA precedes its
activation and autophosphorylation of ATM is not a precondition for its association with
NBS1 or its recruitment to damaged DNA. They also show that the MRE11 binding site is
for ATM activation essential as well. They propose a model, in which the DNA damage
could be first detected by the MRN complex bound to DNA. Upon binding of the MRN
complex to damaged DNA, ATP binding and hydrolysis by RAD50 induce conformational
changes in the complex\textsuperscript{207}. These conformational changes in RAD50 will be propagated
through MRE11 and NBS1 to ATM, leading to a conformational change which disengages
ATM multimers and relieves autoinhibition, leading to ATM activation and
autophosphorylation (Fig.18).

Falck and colleagues\textsuperscript{104} found that the 20-amino-acid truncation at the C-terminus
of human NBS1 abolished its interaction with ATM. When expressed in NBS-Tet cells it
fully complemented the partial defect in ATM autophosphorylation although this construct
was unable to complement defects in phosphorylation of SMC1 and CHK2. The data
suggest that although the NBS1 C terminus is not required for ATM autophosphorylation
in human cells, it is crucial for recruiting activated ATM to sites of DSBs in order to
facilitate the phosphorylation of ATM targets at such sites. These findings support a model
in which ATM is activated in a mechanism that does not require a direct interaction with
the MRN complex, although such an interaction is required for recruitment of ATM to
sites of DNA damage and subsequent phosphorylation of ATM substrates. Although the
disagreement between both works could be at least partly explained by use of different
systems e.g. NBS-tert human cells and Xenopus egg extracts, there is still left many unanswered questions for future studies.

Figure 18. Proposed mechanism of ATM activation by DSBs. In step 1, the inactive ATM/MRN complex is recruited to sites of DNA damage. In step 2, conformational changes are induced, leading to ATM dimer disengagement and autophosphorylation. Activated ATM phosphorylates the downstream effectors such as H2AX. In step 3, the checkpoint signal is amplified by recruitment of additional MRN/ATM complexes via interactions with phospho-H2AX and other adaptor proteins as MDC1.
5.2 ROLE OF THE DNA DAMAGE SIGNALING PATHWAYS IN CANCER DEVELOPMENT

The second part of this Ph.D. work has been focused on the potential involvement of the DNA damage checkpoint pathways in the early stages of human tumorigenesis.

Firstly we showed that among a large panel of clinical specimens from different stages of human tumours of the urinary bladder, breast, lung and colon, the early precursor lesions, but not normal tissues, commonly express markers of activated DNA damage response, such as phosphorylations of H2AX, ATM, CHK2, CHK1 and p53. Importantly, the immunohistochemically detectable activation (phosphorylation) of the CHK2 and ATM kinases was already maximal in the genetically most stable subset of tumours, thereby preceding the development of pronounced chromosomal instability (see chapter 7.2). We also showed that the ATM-CHK2-p53 cascade is constitutively active in human urinary bladder tumours before the occurrence of mutations in the p53 gene and/or defects in the upstream components of the DNA damage checkpoint (see chapter 7.2).

As a second step we used cultured human cells to show that similar response was induced in them upon regulatable expression of diverse oncogenes typically activated in human carcinomas. Our results also indicate that the activation of the DNA damage response network regulated by the ATM/ATR kinases alters replication dynamics and is independent of oxidative stress. Collectively, our cell culture experiments suggested that tumourigenic abnormalities that deregulate DNA replication induce DNA damage and checkpoint activation.

In the third part we performed experiments with tissue samples to validate this concept for early tumourgenesis in vivo. These studies documented that the DNA damage response in precancerous lesions is functional and indeed impacts the cell-cycle machinery, we could detect signs of replicational stress in the tissues and document frequent aberrations of G1/S control in premalignant lesions (see chapter 7.2).

Overall, our data, supported by those of Gorgoulis et al. suggest that in early precancerous lesions of major human cancer types is activated the ATM/ATR-regulated checkpoint network. Its activation is result of deregulated DNA replication, leading to rise of DNA damage. The ATM/ATR-regulated checkpoint network would in this case serve as an inducible barrier against tumour progression and genetic instability. We propose that the aberrant stimulation of cell proliferation resulting in deregulated DNA replication affect RB or impair DNA replication downstream of RB, such as deregulated E2F.
and/or overexpressed cyclin E\textsuperscript{179,182} or CDC25A\textsuperscript{161,164}, and defects in DNA repair genes such as BRCA\textsuperscript{210}. The observed DNA damage might reflect abnormalities in pre-replication complex maturation and/or stalled or collapsed replication forks. These events induce the ATR-H2AX/CHK1 cascade\textsuperscript{1,109,111,128,184}, which results in generation of DSBs\textsuperscript{198,199,210} and consequently activation of the ATM-H2AX/CHK2-p53 pathway\textsuperscript{1,126,178}.

Figure 19. Model of DNA damage response in tumorigenesis.

The activated DNA damage checkpoint limits proliferation of the expanding cell clone and we speculate that it can possibly contribute to long 'latency' periods or failure of early lesions to ever progress into malignancy. Tumour progression under such circumstances likely relies on selection of cells defective in their DNA damage response components such as ATM or p53, with compromised cell-cycle arrest, senescence, and apoptosis. The oncogene-induced DNA damage, particularly DSBs, likely contributes to the enhanced chromosomal instability, especially under conditions when p53 or other components of the checkpoint have been mutated or otherwise silenced. Whether or not the oncogene-induced...
DNA damage and the checkpoint response reported here is the predominant driving force underlying selection for mutations in genome maintenance and apoptosis inducing mechanisms remains to be seen. On the other hand, telomere dysfunction, a phenomenon that may occur in premalignant tumours\textsuperscript{211,212}, can mimic DNA damage and activate the ATM/ATR-dependent checkpoints, thereby contributing to replicative senescence in cultured cells\textsuperscript{213,214}. We propose that the oncogene-induced DNA damage analyzed here, and dysfunctional telomeres may converge to activate the DNA damage response as a common mechanism to prevent progression of pre-neoplastic lesions (fig.19).

In any case, the fact that the DNA damage response is commonly activated in precursor lesions of major types of human malignancies suggests that this apparently selective feature of cancer may provide an attractive target for future prognostic and/or therapeutic applications.

\section*{5.3 MDC1 AND 53BP1 IN NORMAL CELL CYCLE AND TUMOURGENESIS}

We prepared a monoclonal antibody to MDC1 suitable for tissue staining and used it, together with antibody against 53BP1 for studying regulation and function in normal human tissues and their relation to human cancer. The yet unpublished results show new interesting details about MDC1 and 53BP1 localization and presence in normal and tumour tissues, which could help us to elucidate their role in tumourigenesis.
6. CONCLUSIONS

The aim of this doctoral thesis was to elucidate some aspects of the mechanism of ATM activation and the role played in this process by the MRE11-NBS1-RAD50 complex. In addition, the potential involvement of the DNA damage checkpoint pathways in the early stages of human tumourigenesis has also been addressed as part of this Ph.D. project, focusing on the role of ATM and its downstream targets in particular. Finally, this work also concentrated on better understanding of the regulation, function, and potential involvement in human cancer of the DNA damage checkpoint mediators 53BP1 and MDC1.

We showed that after exposure of human fibroblasts deficient in NBS1 to low doses of irradiation, ATM phosphorylation is delayed and its magnitude is lower than in normal fibroblasts. The kinetics can be restored by expression of wild-type NBS1 from a retroviral vector. NBS1 with mutated FHA domain or NBS1 mutant nonphosphorylatable by ATM can also restore the kinetics, contrary to the NBS1 with deleted MRE11-interaction domain. These results suggested that MRN complex of which are NBS1 and MRE11 components, plays an important role in regulating the timing of the IR-induced, ATM-mediated phosphorylation events.

The relation between the DNA damage checkpoints and tumourigenesis has been studied in the second part of this work. We documented that proteins involved in the DNA damage response pathways are commonly activated in the early stages of tumourigenesis and that their activation precedes genomic instability and malignant conversion. In the second part of this doctoral thesis we showed that in the cell culture the checkpoint activation follows increased levels of proteins that deregulate DNA replication as E2F1, cyclin E and CDC25A. Based on our experiments we suggest that the DNA damage response could reflect deregulated DNA replication, since overexpressed cyclin E, CDC25A and E2F1 share the ability to promote unscheduled S-phase entry. As a response to the oncogene overexpression, the checkpoint pathways activate p53 pathway, which leads in the case of severe damage to apoptosis. We presume that the checkpoint pathways prevent or delay tumour progression hence defects of these pathways may allow proliferation, survival, enhanced genomic instability and cancerogenesis.

Finally, this work also contributes to better understanding of the regulation, function, and potential involvement in human cancer of the DNA damage checkpoint mediators.
53BP1 and MDC1, which are both key components of the genome surveillance network activated by DNA double strand breaks (DSBs).
7. APPENDIX

7.1 PUBLICATION

Distinct functional domains of Nbs1 modulate the timing and magnitude of ATM activation after low doses of ionizing radiation

Zuzana Horejší, Jacob Falck, Christopher J. Bakkenist, Michael B. Kastan, Jiri Lukas and Jiri Bartek

1Danish Cancer Society, Institute of Cancer Biology, Strandboulevarden 46, Copenhagen DK-1457 Denmark; *St Jude Children’s Research Hospital, 332 North Lauderdale Street, Memphis, TN 38109-2794, USA

The ATM kinase is a tumour suppressor and a key activator of genome integrity checkpoints in mammalian cells exposed to ionizing radiation (IR) and other insults that elicit DNA double-strand breaks (DSBs). In response to IR, autophosphorylation on serine 1981 causes dissociation of ATM dimers and initiates cellular ATM kinase activity. Here, we show that the kinetics and magnitude of ATM Ser1981 phosphorylation after exposure of human fibroblasts to low doses (2 Gy) of IR are altered in cells deficient in Nbs1, a substrate of ATM and a component of the MRN (Mre11-Rad50-Nbs1) complex involved in processing/repair of DSBs and ATM-dependent cell-cycle checkpoints. Timely phosphorylation of both ATRSer1981 and the ATM substrate S321 after IR were rescued via retroviral-mediated reconstitution of Nbs1-deficient cells by wild-type Nbs1 or mutants of Nbs1 defective in the FHA domain or nonphosphorylatable by ATM, but not by Nbs1 lacking the Mre11-interaction domain. Our data indicate that apart from its role downstream of ATM in the DNA damage checkpoint network, the MRN complex serves also as a modulator/amplifier of ATM activity. Although not absolutely required for ATM activation, the MRN nucleosome complex may help to reach the threshold activity of ATM necessary for optimal genome maintenance and prevention of cancer. Oncogene (2004) 23, 3122–3127. doi:10.1038/sj.onc.1207047

Published online 29 March 2004

Keywords: Nbs1; ATM; DNA damage; ionizing radiation; cell cycle checkpoints

One of the hallmarks of cancer cells is their genetic instability, a feature associated with malfunction of cellular responses to DNA damage due to defective cell cycle checkpoints and/or DNA repair machinery. Among defects of such genome maintenance mechanisms known to predispose to cancer are mutations of ATM, Nijmegen breakage syndrome (NBS), and MRE11 genes that cause the chromosome instability syndromes ataxia-telangiectasia (A-T), NBS, and A-T-like disease (A-TLD), respectively (Shiloh, 1997; Varon et al., 1998; Stewart et al., 1999). The clinical resemblance of these diseases, their similar cellular phenotypes including radiation sensitivity and deficient checkpoint responses, as well as the accumulating functional evidence for the biological roles of the products of these genes suggest an intimate interplay of the ATM, Nbs1, and Mre11 proteins in cellular responses to DNA double-strand breaks (DSBs) (Shiloh, 1997; Petren, 2000; Kastan and Lim, 2003; Lee et al., 2003; Shiloh, 2003).

The nucleoprotein kinase ATM becomes rapidly activated in response to ionizing radiation (IR) and other genotoxic insults that elicit DSBs, and it phosphorylates a wide range of proteins functionally implicated in the genome integrity network, including Nbs1 and Mre11 (Dong et al., 1999; Kim et al., 1999; Gatti et al., 2000; Lim et al., 2000). Additional substrates of ATM encompass the checkpoint signalling kinases Chk2 and Chk1 (Ahn et al., 2000; Gatti et al., 2003; Matsuo et al., 2006; Sereinen et al., 2003), checkpoint mediators BRCA1, 53BP1 and MDC1 (Anderson et al., 2001; Gatti et al., 2001; Lou et al., 2003, Okada and Oshiki, 2003), histone H2AX, and diverse effectors of the DNA damage network involved in DNA repair, cell cycle, and cell death control, such as RAD9, RAD17, SMCI, FANCD2, p53 etc. (reviewed in Kastan and Lim, 2000; Shiloh, 2001, 2003).

The Nbs1 and Mre11 proteins are components of the so-called MRN complex (for the core subunits: Mre11, Rad50, and Nbs1) implicated in the early processing of DSBs via its DNA-binding and nucleosome activity, contribution to DNA repair processes of homologous recombination and nonhomologous end joining, as well as participation in the intra-S phase and G2/M phase cell cycle checkpoints (D’Amours and Jackson, 2002; Thompson and Schedl, 2002; Petren and Steckler, 2003). Whereas the checkpoint functions of the MRN complex lie downstream of ATM, being regulated by the ATM-mediated phosphorylations of Nbs1 (Shiloh, 2003), the involvement of ATM in the accumulation of the MRN proteins at the sites of DNA damage seems less likely (Mirzoeva and Petren, 2001). In addition, several studies of cellular responses to IR report...
phosphorylation of some ATM targets in cells deficient in Nbs1 (Buscemi et al., 2001; Girard et al., 2002; Yandr et al., 2002; Gates et al., 2003; Lee et al., 2003), indirectly suggesting a potential role of the MRN complex in modulating some aspect(s) of ATM function. Thus, despite both ATM and the MRN complex playing critical early roles in response to DSBs and having been considered as candidate sensors of DNA damage (Petruzi, 2000; Durocher and Jackson, 2001; Khanna and Jackson, 2001), the exact temporal order and molecular basis of the interplay between ATM and MRN remain to be clarified. Elucidation of this important issue has so far been complicated, partly due to different experimental conditions used widely in previous studies, including different cellular models, doses of radiation, and the times, reagents and methods of evaluating the response to DSBs. Arguably most significantly, such studies suffered from the lack of a simple and validated approach to estimate the activity state of ATM. The latter drawback has recently been eliminated by the identification of an autophosphorylation on serine 1981 of ATM as the earliest detectable step of ATM activation (Bakkenist and Kastan, 2003).

Here, we employed the phosphospecific antibody against Ser1981-phosphorylated ATM (Bakkenist and Kastan, 2003), and generated a model system allowing for the assessment of distinct functional domains of Nbs1 in identical cellular backgrounds of telomerase-immortalized, Nbs1-deficient human fibroblasts, to gain further insights into the interplay between the MRN complex and ATM activity in response to IR.

To begin investigating the potential contribution of Nbs1 to ATM activation, we first exposed Nbs1-proficient, human diploid fibroblasts (the BJ strain) to low doses of IR. This strategy was chosen to avoid unpredictable adverse effects of cell transformation on the checkpoint kinetics, and to avoid the activation of redundant pathways by excessive DNA damage. After the exposure of asynchronously proliferating BJ cells to 2 Gy, ATM became rapidly activated as evidenced by its autophosphorylation on Ser1981. The magnitude of this effect was stabilized between 5 and 15 min after irradiation and began to decline after additional 4 h (Figure 1). Very similar kinetics was observed for the phosphorylation of several ATM downstream targets including Ser966 of Smc1, Thr68 of Chk2 and Ser15 of p53 (Figure 1; and data not shown). This rapid, quantitatively and transient activation of the ATM-dependent checkpoint response induced by low doses of IR was consistent with the recent mechanistic study of ATM activation (Bakkenist and Kastan, 2003), and it indicated that our experimental system was sensitive enough to assess the kinetics of the ATM-dependent phosphorylation events.

Strikingly, when the same experimental protocol was applied on the human telomerase catalytic subunit (hTert)-immortalized NBS fibroblasts (NBS-Tert), ATM autophosphorylation was significantly impaired. Thus, the overall extent of Ser1981 autophosphorylation was weakened and it reached maximum only 1 h after irradiation (Figure 1). Consistently, the kinetics of Smc1 phosphorylation was also delayed compared to the Nbs1-proficient BJ fibroblasts (Figure 1). These results were surprising and suggested that in addition to its well-established role as a genuine ATM substrate, Nbs1 may have an important role in the modulation of ATM activation in the IR-induced signalling cascade. To address this issue directly, we have generated a series of cellular models with distinct status of the Nbs1 protein.

Proper integration of Nbs1 into the IR-induced checkpoint signalling network depends on the integrity of several structural domains, phosphorylations, and protein-protein interactions (Desai-Mehta et al., 2001; Corossal and Concannon, 2003; Lee et al., 2003). To gain insight into whether and how these Nbs1 features determine its ability to modulate ATM activation, we reconstituted the NBS-Tert cells by retroviruses containing the wild-type (wt) and several mutant forms of human Nbs1 together with the gene coding for resistance to puromycin. Importantly, using the telomerase-immortalized cells eliminated the drawback of otherwise gross proliferative differences between the primary Nbs1-deficient cells and their Nbs1-reconstituted counterparts. The series of Nbs1 mutants included an inactivating point mutation within the FHA domain (R28A), substitution of the ATM-targeted series (S278, S343, S397) by alanine (3 × 3/12), and deletion of the Mre11-binding domain (DelM3, respectively) (Figure 2a). Owing to cell-to-cell variation in expression levels of the Nbs1 proteins in the pooled cell populations, we selected puromycin-resistant clones homogeneously expressing the epitopic Nbs1 forms to near-physiological levels (compared to the amount of protein in the Nbs1-proficient BJ cells) (Figure 2b). All results obtained with these clones correlated well with experiments performed on the pooled cell populations (data not shown). While the wt, 3 × 3/12 and DelM3 forms of Nbs1 readily formed the IR-induced nuclear foci (IRF), the cells reconstituted with the FHA-deficient R28A or
with the puropeptin resistance gene only (pBASF) showed little or no recruitment of Nbs1 to IRIF (Figure 2c). Moreover, reconstitution with wt and 3 x 5/A (but not the K38/A and Del38) alleles rescued survival of NBS-T cells after exposure to IR (Figure 2d). All these results are consistent with earlier extensive characterization of Nbs1 and its variants (Densi-Mehla et al., 2001; Zhao et al., 2002; Ceresaletti and Concannon, 2005; Lee et al., 2003), and suggest that we succeeded in generating a relevant model system to study the impact of Nbs1 structural features on ATM activation.

Next, we subjected the above cell lines to the irradiation/time-course protocol as described in Figure 1 and assayed by immunoblotting with phosphospecific antibodies the kinetics of ATM autophosphorylation on S1981 together with phosphorylation of Smc1 on S966 (the latter was chosen as a model ATM substrate). Reconstitution of the NBS-T cells with wt Nbs1 restored the delayed kinetics of both ATM autoactivation and Smc1 phosphorylation (Figure 3) back to the profile typically observed in normal human fibroblasts (Figure 1; see also Figure 1 for comparisons). Specifically, in the Nbs1 wt-reconstituted cells, phosphorylations of ATM and Smc1 were detectable already in early time points (3-15 min) and began to decline around 4 h after irradiation (Figure 3). These results strongly supported our initial hypothesis that Nbs1 may modulate the timing and the amplitude of ATM activation in response to low doses of IR.

Interestingly, the three Nbs1 mutants showed differential effects on the kinetics of the studied ATM

Figure 2. Recruitment of Nbs1 expression in NBS-T cells fibroblasts. NBS cells containing the endogenous truncated and low-abundant form of Nbs1 (Nbs1-ΔNbs1) were infected with pBASF-puro vector alone, with the vector expressing myr-tagged Nbs1 wild-type (Nbs1 wt) or Nbs1 mutant forms (R38/A, 3 x 5/A, Del38) and grown in DMEM medium with 15% FBS, 100-μM penicillin, 100-μg/ml streptomycin, and 1 μg/ml puromycin. The Phoenix A packaging cell line, grows in DMEM medium with 10% FBS, 100-μM penicillin, and 100-μg/ml streptomycin, was transfected with the retroviral DNA by the calcium phosphate method and the NBS-T cells were infected as described (Perez et al., 1994; www.stanford.edu/group/polanski). The Nbs1 constructs were generated by site-directed mutagenesis with the use of QuickChange kit (Stratagene; Fauser et al., 2002), myr-tagged, and inserted into the pBASF-puro vector (A). A detection of expression level of the constructs in selected clones was confirmed by immunoblotting (B). The ability to form foci after irradiation and to bind Mre11 were tested by immunofluorescence staining of the cells 8h after irradiation with 10 Gy (Lakes et al., 2002). The wild-type (Nbs1 wt) and the phosphorylation domain of Nbs1 (R38/A) bind Mre11 and form foci, in contrast to the R38/A domain (R38/A) affects foci formation while the deletion mutant (Del38) is able to form foci but cannot bind Mre11 (C). The antibodies used were: synon monoclonal antibodies to human Mre11 clone 1237 from Biofilix, San Antonio, TX, USA and MCM9 (DCS-14) from Sieben et al., 2009, and rabbit polyclonal antibody against Nbs1 (Novus Biologicals, AB 398, Littleton, CO, USA). (D) The radiation sensitivity of the cells was estimated by colony-forming assays. The cells were exposed to 2 Gy of IR. After 1-3 weeks, colonies per plates were counted and expressed as the percentage of colonies seen in the untreated control. Data shows the means and standard deviation of three independent experiments, each performed in triplicates.
detectable accumulation of checkpoint proteins in IRIF does not exclude their transient interaction with DSBs (Celenis et al., 2003), a feature that might be necessary and sufficient for MRN to amplify ATM activity. Consistently, two recent reports demonstrated that the FHA-deficient Nbs1 restored timely ATM-dependent phosphorylation of Smc1 and Chk2, respectively (interestingly, this occurred despite the FHA-deficient Nbs1 itself was a poor ATM substrate and remained underphosphorylated) (Cerasolletti and Concannon, 2003; Lee et al., 2003). Our present data add an important new dimension to these findings by showing that the restoration of ATM phosphorylation events (with or without the fully functional FHA domain) applies also to ATM autoactivation — in other words, to the very proximal and indeed the nodal point of the IR-induced signalling cascade.

The phosphorylation-deficient mutant of Nbs1 (3 × S/A) also restored timely initiation of ATM activation and Smc1 phosphorylation, respectively (Figure 3). There is some discrepancy in the literature with regard to the requirement of Nbs1 phosphorylation for targeting various ATM substrates. Our results support those by Kim et al. (2002), who also concluded that phosphorylation of Smc1 does not require preceding (or concomitant) Nbs1 phosphorylation. Moreover, these conclusions are also in line with the results obtained by several groups with the FHA-deficient Nbs1 (Cerasolletti and Concannon, 2003; Lee et al., 2003, this study). Although this form of Nbs1 cannot be efficiently phosphorylated, it still supports timely phosphorylation of other ATM targets including Smc1 (see Figure 3). Compared with the effects of wt and the FHA-deficient Nbs1, the NBS-Tert cells reconstituted with the phosphorylation-deficient Nbs1 (3 × S/A) showed a moderately protracted phosphorylation of Smc1 in some experiments, suggesting a potential delay in exit from the activated checkpoint mode in such cells. Further validation of this observation and elucidation of its biological significance must await future studies.

Finally, the Nbs1 mutant deficient in binding to Mre11 (Del628) was completely unable to rescue the ATM autoactivation and, consequently, phosphorylation of the ATM-downstream targets such as Smc1 (Figure 3, and data not shown). As the Del628 mutant was readily integrated into IRIF, these data suggest that some Mre11-dependent function provided jointly by the MRN complex (likely the nuclease activity) is essential to promote rapid and massive activation of ATM-dependent signalling.

Overall, our results (summarized in Table 1) support and extend several recent reports showing that Nbs1 (and/or other components of the MRN complex) may modulate phosphorylation kinetics of some ATM-downstream effectors. However, the above studies did not clarify whether this function of the MRN complex reflects its ability to convert the downstream checkpoint effectors into better substrates for ATM or whether this involves a direct influence on ATM activity. Our new data are consistent with the latter scenario and suggest that Nbs1, through its ability to interact with Mre11,


4.2 PUBLICATION II

DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis


During the evolution of cancer, the incipient tumour experiences oncogenic stress, which evokes a counter-response to eliminate such hazardous cells. However, the nature of this stress remains elusive, as does the indolent anti-cancer barrier that elicits growth arrest or cell death. Here we show that in clinical specimens from different stages of human tumours of the urinary bladder, breast, lung and colon, the early precursor lesions (but not normal tissues) commonly express markers of an activated DNA damage response. These include phosphorylated kinases ATM and Chk2, and phosphorylated histone H2AX and p53. Similar checkpoint responses were induced in cultured cells upon expression of different oncogenes that deregulate DNA replication. Together with genetic analyses, including a genome-wide assessment of allelic imbalances, our data indicate that early in tumorigenesis (before genomic instability and malignant conversion), human cells activate an ATM/ATR-regulated DNA damage response network that delays or prevents cancer. Mutations compromising this checkpoint, including defects in the ATM–Chk2–p53 pathway, may allow cell proliferation, survival, increased genomic instability and tumour progression.

Tumorigenesis is an evolutionary process that selects for genetic and epigenetic changes, allowing evasion of anti-proliferative and cell-death-inducing mechanisms that normally limit clonal expansion of somatic cells. Most tumours acquire genetic instability, but how early this occurs and whether it drives tumour development is unclear. Several mechanisms to constrain oncogenesis have been proposed, including hypoxia, telomere attrition and induced expression of the Arf tumour suppressor (which is caused by the mitochondrial overload experienced by incipient cancer cells). These are all conditions that can activate the tumour suppressor p53 (refs 1, 3–6). However, whether these mechanisms represent the major force(s) that guard against genetic instability and tumorigenesis is unknown. Recently, another possibility emerged from our observation that advanced carcinomas of the lung and breast show constitutive activation of Chk2, an effector kinase within the DNA damage network that is activated by the transferase ATM (Ataxia Telangiectasia Mutated) in response to DNA double-strand breaks. Furthermore, oncoproteins such as Myc cause DNA damage in cultured cells. These findings lead us to hypothesize that DNA damage checkpoints might become activated in the early stages of human tumorigenesis, leading to cell-cycle blockage or apoptosis and thereby restraining tumour progression.

DNA damage signalling in early bladder tumours

To determine whether Chk2 is activated in premalignant human tumours, we compared early, superficial lesions (stage Ta), early invasive (T1) and more advanced stages (T2–4) of urinary bladder cancer, all untreated by radiation or chemotherapy. Contrary to the negative staining of normal tissues, immunohistochemistry using a well-characterized antibody against activated Chk2 (phosphorylated at Thr68) showed homogeneous positive staining in the vast majority of the Ta lesions (Figs 1 and 2a). A similar pattern was seen in the T1 tumours, but the T2–4 carcinomas, although still commonly positive, showed moderately lower staining (Figs 1 and 2a).

Figure 1 Constitutive activation of the ATM–Chk2–p53 pathway in human urinary bladder cancer. Immunohistochemistry of normal urothelium, early superficial lesions (Ta), early invasive (T1) and more advanced urinary bladder carcinomas (T2–4). Chk2 and ATM antibodies are ubiquitously expressed, but Thr68-phosphorylated Chk2 (pT68-Chk2), Ser195-p53-phosphorylated Chk2 (pThr68-ATM), Ser195-p53-phosphorylated Chk2 (pThr68-p53) and the 139-phosphorylated histone H2AX (γ-H2AX) are detectable only in tumour tissues. They are all present at the early stages of tumour development, while p53 is a marker of pretreatment cells. Original magnifications, ×100.
These results support our hypothesis and raise the possibility that the upstream kinase ATM, which normally phosphorylates Chk2 (on Thr 68) in response to double-strand break-causing insults such as ionising radiation\textsuperscript{13,14}, might be constitutively activated in tumours. Indeed, parallel tissue sections showed positive staining for Ser 1981-phosphorylated ATM, a marker of ATM auto-activation\textsuperscript{2}, as well as for the ATM substrates p53 (phosphorylated on Ser 153) and histone H2AX (phosphorylated on Ser 139, γ-H2AX)\textsuperscript{10,11} (Fig. 1 and Supplementary Figs 51, S2a).

DNA damage response precedes p53 mutations

Another prediction arising from the idea that DNA damage checkpoint activation acts as a barrier against cancer and genetic instability (and a pressure selecting for p53 mutations), is that ATM–Chk2 pathway activation must precede the occurrence of pronounced genomic instability and p53 aberrations. To address these issues, we examined DNA isolated from 35 microdissected bladder tumours (stages Ta, T1, T2–4) for allelic imbalances, comparing single-nucleotide polymorphisms (SNPs) in blood and tumour DNA from each patient. This genome-wide estimate of genomic instability was obtained using SNP arrays\textsuperscript{20}, which included some 10,000 probes, of which about 2,600 were evenly heterozygous (informative). Reflecting the frequency of expected heterozygosity (present in blood DNA) versus aberrantly reduced heterozygosity in the tumour, we subdivided the tumours into groups according to their proportion of allelic imbalances (low, intermediate and high genomic instability; see Methods and Supplementary Fig. S2c). Levels of Chk2 and ATM phosphorylation (detected by immunohistochemistry) were already maximal in the most stable subset of tumours, and therefore preceded the development of pronounced genomic instability (Fig. 2b and Supplementary Fig. S2b).

We then estimated the relationship between DNA damage response activation and potential defects in the ATM–Chk2–p53 pathway using loss of heterozygosity at the respective gene loci, immunohistochemical analysis of protein abundance (Chk2 and ATM), and direct DNA sequencing (p53 and CHK2). These analyses revealed numerous abnormalities (Fig. 2c), including numerous p53 mutations and a previously unidentified splice mutation of CHK2 that was accompanied by loss of heterozygosity and lack of the Chk2 protein (Fig. 2d). Notably, whereas only one aberration was detected among the tumours classified as stable according to the SNP array data, eight defects were found in the intermediate subset and 24 aberrations were identified in the unstable lesions (Fig. 2c). These results show that the ATM–Chk2–p53 cascade\textsuperscript{1,13} is activated in human bladder tumours before the occurrence of p53 mutations and/or defects in DNA damage signalling.

Constitutive DNA damage is shared by early human lesions

Analogous to bladder carcinomas, the 68-phosphorylated Chk2 was detectable (at various levels) in the majority of 244 invasive carcinomas of the breast, colon or lung. This is in sharp contrast with negative samples from 87 normal, proliferating tissues (Fig. 3a, b, and data not shown) and 76 inflammatory lesions (Supplementary Fig. S3). Furthermore, phosphorylated Chk2, ATM and H2AX were also found in the majority of pre-invasive carcinoma in situ (CIS, n = 153 lesions of the breast) (Fig. 3a, b and data not shown), colorectal adenomas (Fig. 3b and see below) and lung hyperplasias (ref. 16 and data not shown). These data established that constitutive activation of the ATM–Chk2 pathway commonly occurs at pre-invasive stages of major types of human carcinomas.

Oncogenes induce DNA damage response in cultured cells

The above results raise a question about the identity of tumorigenic events that could evoke the observed DNA damage response in human cancer. First, we tested for the effects of cyclin E overexpression (expression increased by about 30-fold, Fig. 4a), an event common in carcinomas and reported to enhance genomic instability\textsuperscript{2,21}. In our U-2-OS-derived cells (a
checkpoint-proficient human osteosarcoma cell line with wild-type retinoblastoma (RB) and p53, cyclin E could be overexpressed in a tetracycline-repressible manner to reach levels comparable with the endogenous protein in breast cancer cell lines containing increased copies of the cyclin E gene (Fig. 4b). Biochemical analysis of cells with induced cyclin E expression showed time-dependent increases in Ser15-phosphorylated p53, γ-H2AX, and Ser960-phosphorylated cofilin (SMC2) (Fig. 4c). These are all targets of the DNA damage response ATM and ATR.24-28 Analogous to cyclin E, overexpression of a tetracycline-regulatable Cdc25A29, another proto-oncogene overexpressed in many carcinomas,30,31 evoked a DNA damage response (Fig. 4d). A similar response was also observed when overexpression was targeted to E2F1 (ref. 32), an S-phase-promoting transcription factor commonly deregulated in cancer owing to aberrations of the RB pathway,33,34 was translocated into the nucleus upon addition of tamoxifen to the medium (Fig. 4e).

Consistent with our biochemical data (Fig. 4f), the checkpoint-mediated phosphorylation markers were also detectable cytologically (Fig. 5a, b, and Supplementary Fig. 5a, b), and were reminiscent of the heterogeneous staining pattern seen in the clinical specimens (Figs 1 and 3). Although the nocodazole-induced phosphorylation of Chk2 (on Thr68) and ATM (on Ser1981) were detectable both biochemically and cytologically, they were less pronounced than γ-H2AX. This suggests that the ATM→H2AX/Chk1 pathway23-28 probably contributes to the overall response. Indeed, Chk1 was found to be phosphorylated on its activating sites, Ser317 and Ser345 (Fig. 4f), of which Ser345 is regarded as a preferable target for phosphorylation by ATR.26,29 Phosphorylated Chk1 was also detected by fluorescence microscopy two days after induction of cyclin E, Cdc25A, or E2F1 (Fig. 5b). Another established target of ATR phosphorylation, the checkpoint protein Rad17 (ref. 16, 30), was also phosphorylated (on Ser165; Fig. 4f). Analogous to U-2-OS cells, ectopic expression of cyclin E induced a DNA damage response in human fibroblasts (Supplementary Fig. 5b).

Our cell culture models show that S-phase-promoting oncogenes can activate the ATM/ATR-regulated DNA damage network. Furthermore, unlike phosphorylations of p53 and H2AX induced by the Myc oncogene, which are a consequence of oxygen radical generation,25,26 the DNA damage response in our experiments was only marginally repressed by the antioxidant N-acetyl-L-cysteine (NAC; Fig. 4a and Supplementary Fig. S4a, S5b, c). This indicates that the bulk of the DNA damage caused by the hyperproliferative oncogenic stimulus was independent of oxidative stress.

Because overexpressed cyclin E, Cdc25A, and E2F1 share the ability to promote unbridged S-phase entry32-34, we argue that the DNA damage response could reflect deregulated DNA replication rather than oxidative stress. When cells experience replicational stress, they activate their replication checkpoint to delay S-phase progression and G2/M transition35. Consistent with this scenario, U-2-OS cells with induced cyclin E expression accumulated in S and G2 phases. The G2 arrest was particularly prominent by day 4 of time course experiments, and by day 6 many cells showed aberrant enhanced DNA contents (Fig. 4c, Fig. 5c), probably reflecting partial re-replication of the genome uncoupled from cell division. The inability to enter mitosis correlated with massive induction of the Tyr 15-phosphorylated, inactive form of the key mitosis-promoting kinase Chk1 (ref. 34; Fig. 5d), which is a hallmark of the effective checkpoint.

In further support of the idea that cyclin E overexpression alters replication dynamics, significantly longer replication tracks were detected using DNA fibre labelling36, and increased amounts of the single-stranded (ss)DNA-binding replication protein A (RPA)37-39 were bound to chromatin (Supplementary Fig. 5a, b). RPA loading on ssDNA occurs during normal replication initiation36 and also on abnormal ssDNA intermediates that form during replicational stress39, the latter scenario is consistent with the hyperphosphorylation of RPA37 in our experiments. Importantly, RPA-coated ssDNA is a prerequisite (and serves as an activation signal) for the ATM/ATR-dependent checkpoint40,41, and cyclin-induced aberrant DNA replication in yeast causes double-strand breaks42,43, potentially deadly lesions that activate the ATM–Chk2 pathway in mammalian44,45.

---

**Figure 3** Phosphorylation and activation of Chk2 in early breast and ovarian tumours. A, representative haematoxylin and eosin (H&E) staining of normal breast (t) and phosphorylated Chk2 (p-Chk2) in normal breast, early breast of ductal carcinoma in situ (DCIS), and invasive ductal carcinoma. Bottom panel, normal breast shows low levels of Chk2 (p-Chk2) expression in 10% of breast tumours. B, total Chk2 and phospho-Chk2 in normal, precursor lesion of alveolar adenoma, and invasive carcinoma. Note p-Chk2 staining in the carcinoma Chk2 but not adjacent normal epithelium (b). Bottom panel, normal breast show high cell proliferation (Ki67) but lack p-ATM. Original magnification, ×1000 (a, b).
Oncogenes and checkpoint activation in early tumours in vivo

Together, our cell culture experiments suggest that tumorigenic abnormalities that deregulate DNA replication induce DNA damage and checkpoint activation. To validate this concept for early tumorigenesis in vivo, these predictions should be verified (1) tumorigenic abnormalities that deregulate entry into S phase must occur with high frequency; (2) there should be evidence for replicative stress, and (3) activation of a functional checkpoint should be documented. We were able to verify each of these three predictions for premalignant lesions, using analyses described below.

First, we found cases with RB defects among the early bladder lesions, and the majority of Tp53 lesions showed overexpressed cyclin E and/or unscheduled cyclin E expression (Fig. 6a, c). In addition, 64% of these lesions showed activating mutations in fibroblast growth factor receptor 3 (FGFR3) (ref. 45; Fig. 6a); these abnormalities might induce the DNA damage response in a manner analogous to effects of growth factor overload in human epithelial xenografts. Finally, over half of the colon adenoma samples showed unscheduled or overexpressed cyclin E (Fig. 6e–i), and this correlated well with the DNA damage response (monitored using γ-H2AX as a marker; Supplementary Fig. 5a,b). Cyclin E abundance and its unscheduled expression during cell cycle, defined as a ratio of cyclin E-positive to αE67-positive cells (Fig. 6b), provides a readout for defective RB–E2F and Myc pathways, for amplification of the cyclin E gene, and for mutations in the human CDC25C gene (the product of which controls cyclin E turnover). These results identify frequent aberrations of G1/S phase control in premalignant lesions.

Second, according to our proposed, double-strand-breaks and loss of heterozygosity would probably occur at genomic sequences that are difficult to replicate the fragile sites. Consistent with this, our SNP array data showed that among the genetically most stable early lesions, loss of heterozygosity at known fragile sites occurs 3–10 times more often than expected from random targeting (Fig. 6g).

This contrasts with only a twofold preference for loss of heterozygosity at fragile sites among the more advanced and genetically unstable bladder tumours. The exact degree of preference should be interpreted with caution, given that some fragile sites harbour tumour suppressor genes and that their loss might provide a growth advantage. However, we note that fragile sites are targeted during the period in which we observe maximal DNA damage response and that ATR is involved in the maintenance of fragile sites. Together with the emerging view that loss of heterozygosity at fragile sites is a ‘signature of stalled replication forks’, these observations support our proposed concept.

Third, to show unequivocally that the activated checkpoint machinery affects its downstream cell-cycle targets, we examined samples of normal colon and adenomas for Tyr 15-phosphorylated Cdk1 (pT/Cdk1). In normal colon, pT-Cdk1 staining was predominantly cytoplasmic, detectable within the proliferative compart-
articles

ment of the epithelial crypts in a modest fraction of cells (Fig. 6d). In contrast, over 90% of adenomas had enhanced p3-Cdk1 staining, shown as higher staining intensity and larger groups or continuous sheets of positive cells (Fig. 6d). Staining of parallel sections revealed a good correlation between p3-Cdk1 and γ-H2AX (Fig. 6d and Supplementary Fig. 5a, c), consistent with the cell-cycle checkpoint being active in the same lesions (and the same areas within a lesion) that showed the DNA damage signalling. Reflecting their somewhat attenuated DNA damage response, invasive colorectal carcinomas showed lower γ-H2AX and p3-Cdk1 staining than adenomas (Supplementary Fig. 5b). Finally, unlike in normal crypts where moderate p3-Cdk1 staining marked a subset of proliferating cells, the areas in adenomas strongly positive for p3-Cdk1 often showed only limited proliferation or were completely lacking in Ki67 staining (Supplementary Fig. 5b); this is consistent with prolonged cell-cycle arrest. These results indicate that the DNA damage response in precursor lesions is functional and indeed affects the cell-cycle machinery.

Our data and those in ref. 16 suggest that tumorigenic events early in the progression of major human cancer types activate the ATR/ATM-regulated checkpoint through deregulated DNA replication and DNA damage, and thereby activate an inducible barrier against tumour progression and genomic instability (Fig. 6f). We propose that candidate inducers of such a response include aberrations that grossly overstimulate growth factor pathways, affect RB or impact DNA replication downstream of RB (such as deregulated E2F1 (ref. 25–27, 40) and overexpressed cyclin D1 E2F1 or Cdk2A350)), and mutations in DNA repair genes such as ERCC2 (ref. 43). The observed DNA damage might reflect abnormalities in pre-replication complex maturation and/or stalled or collapsed replication forks (which are known inducers of the ATR-γ-H2AX/Chk1 cascade26–28,31–33), followed by the generation of double-strand breaks44–45 and consequent activation of the ATM-γ-H2AX/Cdk2–p53 pathway46–47. We speculate that such responses might limit the progression of lesions, possibly contributing to long latency periods before early lesions to ever become malignant. Tumour progression under such circumstances probably relies on selection of cells defective in their DNA damage response components (such as ATM or p53), with compromised cell-cycle arrest, senescence and apoptosis. Oncogene-induced DNA damage, particularly double-strand breaks, might also enhance genome instability, especially when checkpoint responses have been lost.

Whether the DNA damage response reported here represents the predominant mode for constraining tumorigenesis remains to be established. However, it is apparent that p53 induction by the ATR/ATM suppressor complex accounts for our data, because the DNA

Figure 8 DNA damage response to DNA-proliferating oncogenes in H2–G1 cells. a, Confocal immunofluorescence microscopy images of cells stimulated with γ-H2AX, p3-Cdk1, or p3-Chk1, after 4 hr induction of cycle (G1). b, Detection of E2F1-induced transactivation of γ-H2AX and p3-ATM (PDF). c, E2F1 or Chk1 staining in γ-H2AX (PDF)–positive nuclei visualised in cells with induced cycle G1. d, Time course analysis of cell cycle. e, Time course analysis of E2F1 activity. f, Time course immunodetection analysis of Mcm7 (loading marker), p3-Chk1 and total Chk1. Arrow shows the γ-H2AX-phosphorylated form of Chk1.
Figure 6. Oncogenes and DNA damage response in early lesions. a, Tumorigenic aberrations in bladder (B) and colon (C) lesions. Frequent FGF3 mutations (n = 32) were 82.8% (8/10) and 100% (10/10). Tumour lesions (A) in bladder (B) and colon (C) lesions. Frequent FGF3 mutations (n = 32) were 82.8% (8/10) and 100% (10/10). Tumour lesions (A) in bladder (B) and colon (C) lesions. Frequent FGF3 mutations (n = 32) were 82.8% (8/10) and 100% (10/10). Tumour lesions (A) in bladder (B) and colon (C) lesions. Frequent FGF3 mutations (n = 32) were 82.8% (8/10) and 100% (10/10).

**Methods**

**Articulated**

- **Antitheatrics**
- **Stirntheater**
- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antithe

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antitheatrics**

- **Stirntheater**

- **Mixture**

- **Articulated**

- **Antithe
8. REFERENCES

43. Strohmaier, H. et al. Human F-box protein hCdc4 targets cyclin E for proteolysis


65. Li, C. J., Vassilev, A. & DePamphilis, M. L. Role for Cdk1 (Cdc2)/cyclin A in
8. References


151. Xu, X. et al. Genetic interactions between tumor suppressors Brca1 and p53 in
173. Lee, J. H. et al. Distinct functions of Nijmegen breakage syndrome in ataxia telangiectasia mutated-dependent responses to DNA damage. Mol Cancer Res 1,


