Aus dem medizinischen Zentrum für Radiologie

Klinik für Strahlentherapie und Radioonkologie

Direktorin: Professor Dr. med. Rita Engenhart-Cabillic

des Fachbereichs Medizin der Philipps-Universität Marburg

in Zusammenarbeit

mit dem Universitätsklinikum Gießen und Marburg GmbH,

Standort Marburg

Investigation of the functions of 53BP1 in DNA demethylation

Inaugural-Dissertation

zur Erlangung des Doktorgrades der gesamten Humanmedizin

dem Fachbereich der Medizin der Phillips-Universität Marburg

vorgelegt von

Linfang Wang

aus

VR. China

Marburg 2008

Table of Contents

1. Background

1.1. Identification and domains of 53BP1

Using the yeast two-hybrid system, 53BP1 was identified as a protein that binds to wild type p53 (Iwabuchi et al., 1994). The 53BP1 gene localizes to chromosome 15q15-21 and encodes a protein that is 1972 amino acids long (Iwabuchi et al., 1998). A search for protein domains using relatively stringent criteria identifies consistently three protein domains: a tudor domain (aa1480-1540) and two tandem Brca1 C-terminal (BRCT) domains (aa1714-1850 and 1865-1972, respectively) (Fig. 1). The BRCT motif is firstly identified in the COOH-terminal region of BRCA1 and has been found in a large number of proteins involved in various aspects of cell cycle control, recombination, and DNA repair in mammals and yeast (Koonin et al. 1996; Bork et al. 1997; Callebaut and Mornon 1997; Manke et al., 2003). Evidence suggests that BRCT domains may mediate protein–protein interactions (Bork et al., 1997; Zhang et al. 1998) and in 53BP1, they mediate its interaction with p53 (Iwabuchi et al., 1998). The tudor domain is a conserved region of 50 amino acids firstly identified in the Tudor protein of Drosophila and found in several proteins involved in binding RNA and DNA (Ponting CP, 2004). New evidence suggests that the tudor domain containing proteins may associate with methylarginine-containing cellular proteins and modify the functions of these proteins (Côté et al., 2005; Kim et al., 2006). Two recent studies have identified the minimal region of focus formation including the conserved tudor domain in 53BP1 (Morales et al., 2003; Goldberg et al., 2003), which is critical for 53BP1 location to IR (ionizing radiation)-induced foci (Huyen et al., 2004). The 53BP1 tudor domain facilitates an interaction between p53 and 53BP1 after DNA damage to promote the localization of 53BP1-p53 complex to the sites of break and to increase the transcriptional activation of p53 (Huang et al., 2007; Kachirskaia et al., 2008).
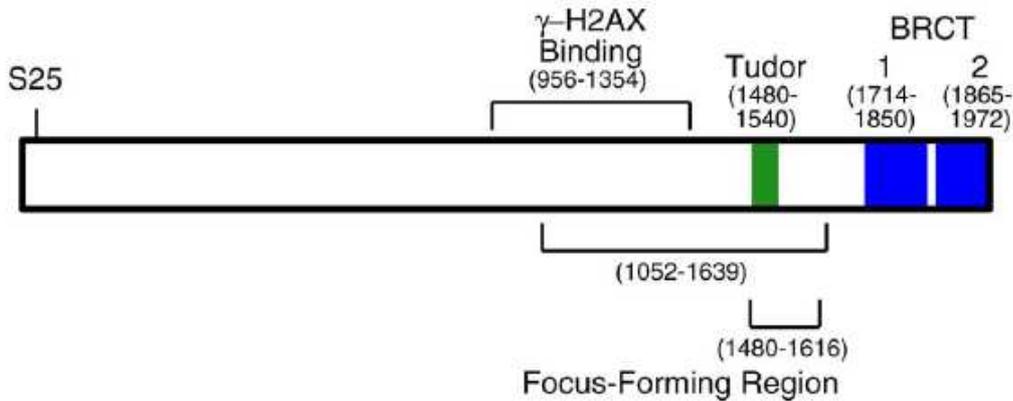
Fig. 1. Schematic diagram of human 53BP1. The functional domains including a tudor, a γ-H2AX binding, two BRCT domains, a serine phosphorylated residues (S25) and the focus-forming region are indicated.

1.2. Current models regarding 53BP1 function

1.2.1. The DNA-damage response (DDR)

DNA damage can be caused by various forms of genotoxic stress, including endogenous (reactive oxygen species, abnormal replication intermediates) and exogenous (reactive chemicals, UV and IR) sources (Shiloh Y, 2003). DNA double-strand break (DSB) is believed to be one of the most serious lesions to cells because it can result in loss or rearrangement of genetic information, leading to cell death or carcinogenesis. The DNA damage response (DDR) is crucial for cellular survival and for avoiding carcinogenesis. This DNA damage can stimulate several different components in concert to activate the cellular checkpoint that leads to cell cycle delay, DNA repair and programmed cell death (Phillips et al., 2007; d'Adda di Fagagna, 2008). These components consist of sensors that sense DNA damage, signal transducers that generate and amplify the DNA damage signal, effectors that induce cell cycle delay, programmed cell death, transcription and DNA repair (Zhou et al., 2000; Phillips et al., 2007; d'Adda di Fagagna, 2008) as shown in Fig. 2.
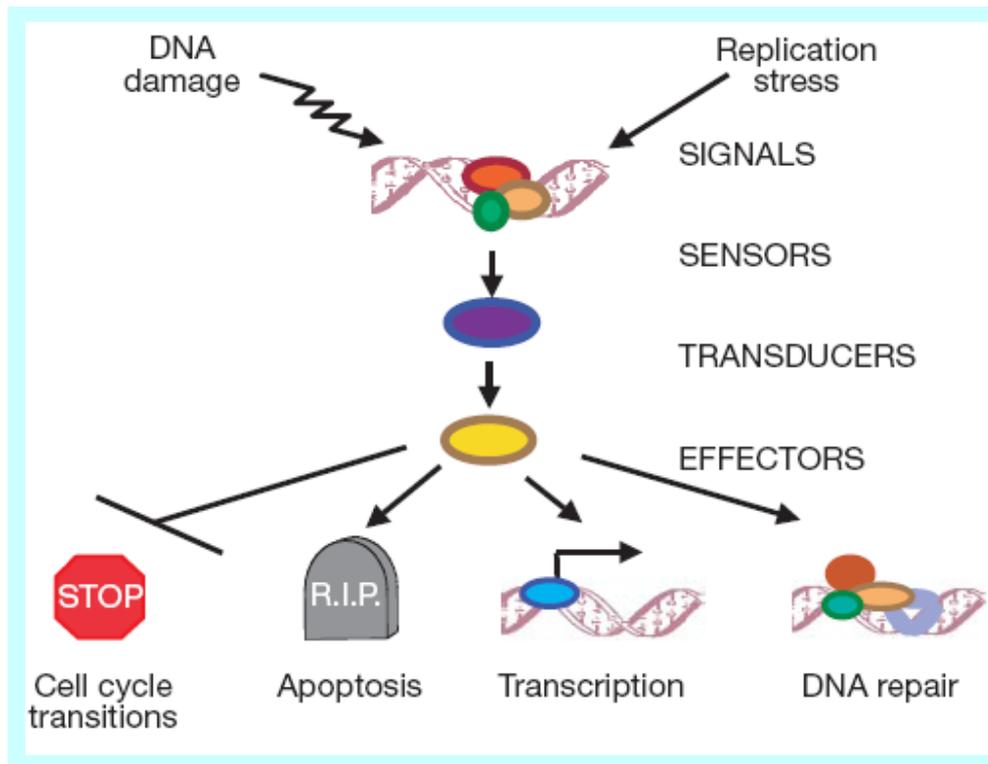
Fig. 2. A view of the general outline of the DDR signalling pathway. The network of interacting pathways is depicted as a linear pathway consisting of signals, sensors, transducers and effectors (Zhou et al., 2000). Arrowheads represent activating events and perpendicular ends represent inhibitory events.

1.2.2. 53BP1: focusing on mediating the DDR through ATM signalling pathway

Even though several candidate proteins have been implicated in DNA damage response, an official checkpoint-specific damage sensor is still unknown (Phillips et al., 2007; d'Adda di Fagagna, 2008). 53BP1 seems to be one of the key-sensors of DNA DSBs (Fig. 3), upstream of ATM (ataxia telangiectasia, mutated) (Wang et al., 2002; Ward et al., 2003; Zgheib et al., 2005). 53BP1 was found to be a nuclear protein that rapidly localizes to discrete foci following DNA damages. 53BP1 foci may represent "sites of DNA DSBs", a hypothesis further supported by the colocalization of 53BP1 with other proteins known to mark sites of DNA DSBs such as phosphorylated histone H2AX (γH2AX) and the

6

Mre11/Rad50/Nbs1 complex (Schultz et al., 2000; DiTullio et al., 2002; Adams et al., 2006). The recognition of histone H4 dimethylated at lysine 20 (H4K20me2) by the 53BP1 has been shown to be important for 53BP1 localization to at chromatin regions flanking the DSBs sites and in broader areas surrounding DSBs (Botuyan et al., 2006). Histone lysine methylation has a central role in transcriptional regulation and has recently been linked to DNA damage repair. Moreover, specific histone methylation and demethylation can both up- and downregulate the transcriptional activity of many genes (Sims et al., 2003 and 2008), suggesting the functions of 53BP1 in the transcription and cell signalling response to DNA damages.

ATM and ATR (ATM and Rad3-related) are key molecules in DDR and function as essential links between the sensors and effectors of the cellular response to DNA damage (Adams et al., 2006). Different studies of 53BP1-deficient cellular models indicate that 53BP1 function is tightly correlated with ATM. The interplay between ATM and 53BP1 may be direct, on the basis that these two proteins can be co-immunoprecipitaed in IR-damaged but not undamaged cells (DiTullio et al., 2002). Moreover, the recruitment of 53BP1 to γ-H2AX-positive nuclear foci is crucial for the phosphorylation of numerous ATM substrates, including p53, BRCA1 and the cohesin protein SMC1 (DiTullio et al., 2002; Botuyan et al., 2006). These effector proteins are responsible for halting cell cycle progression, activating transcription, initiating DNA repair mechanisms and triggering apoptosis. Interestingly, DDR pathways are not strictly linear and redundant signalling occurs. Previous evidence has suggested that 53BP1 may operate both upstream and downstream of ATM activation (Mochan et al., 2003; Huyen et al., 2004).
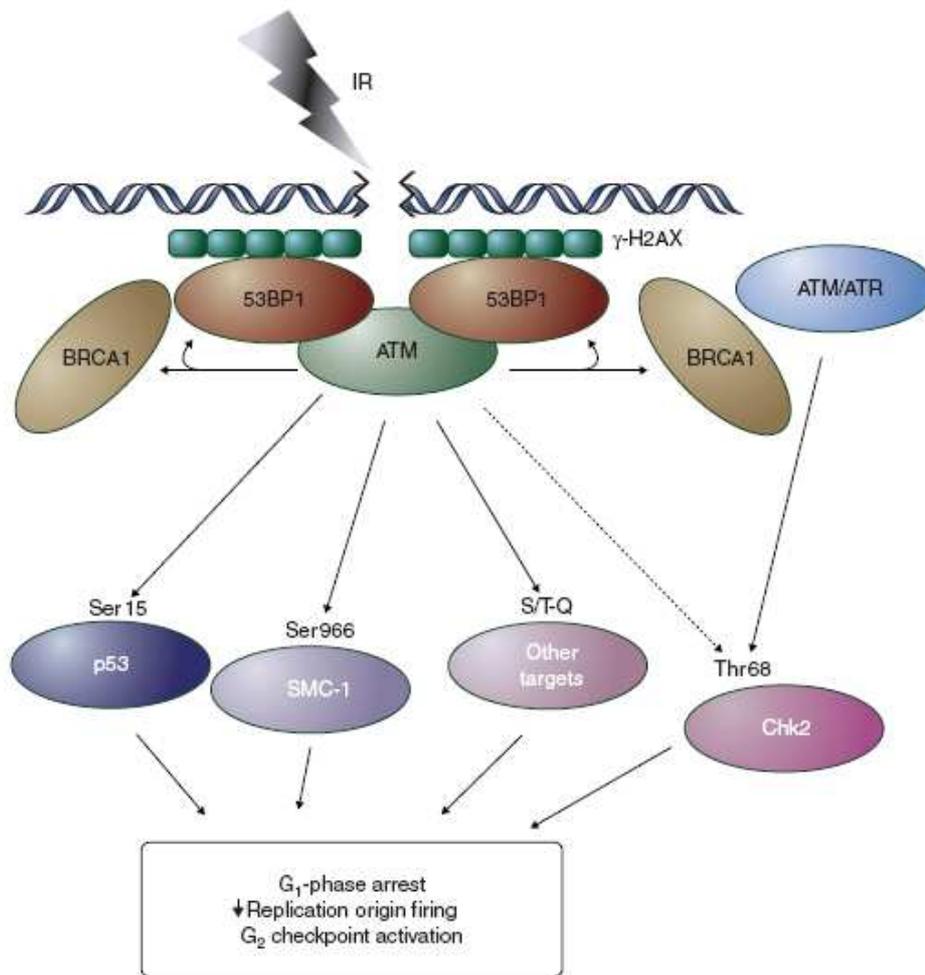
Fig. 3. Schematic representation of the 53BP1-dependent checkpoint pathway. The pathway is triggered by IR and other genotoxic events, resulting in DNA DSBs. 53BP1 accumulates at or near sites of DSBs, and is important for coupling ATM to several of its downstream targets, including p53 (Abraham et al., 2002).

1.2.3. Function of 53BP1 in Gadd45a signalling pathway

53BP1 has been functionally linked to p53 as a potential coactivator (Huang et al., 2007; Kachirskaia et al., 2008). The 53BP1 BRCT tandem repeats were shown to bind the DNA-binding domain of p53, though the physiologic context in which this interaction functions remains elusive (Adams et al., 2006). Furthermore, the tandem tudor domain of 53BP1 recognizes p53 dimethylated at lysine 382 and

facilitates an interaction between 53BP1 and p53, promoting the accumulation of p53 protein and transcriptional activation of p53 at several target genes (Huang et al., 2007; Kachirskaia et al., 2008). Subsequent transient co-transfection experiments with 53BP1 and p53 reporter plasmids suggested that 53BP1 could enhance p53-mediated transcriptional activation (Iwabuchi et al., 1998; Kachirskaia et al., 2008). Targeting 53BP1 for knockdown resulted in decreased protein levels of p21, p53 upregulated modulator of apoptosis (PUMA) and mdm2 (Zhang et al., 2006; Huang et al., 2007).

The activated p53, in turn, up-regulates many target genes that may play roles in different aspects of cellular response. Gadd45a (the growth arrest and DNA damage-inducible gene alpha) is a p53-regulated stress protein (Hollander et al., 1999). Gadd45a is implicated in the maintenance of genomic fidelity probably via its roles in the control of cell cycle G2-M checkpoint (Wang et al., 1999; Zhan et al., 1999; Jin et al., 2000), induction of cell death (Takekawa et al., 1998; Harkin et al., 1999; Zhan et al., 2002), and DNA repair process (Smith et al., 1994; Smith et al., 1996; Hollander et al., 2001). Barreto and his colleagues revealed that Gadd45a is a key regulator of active DNA demethylation at global level and it acts by promoting DNA repair (Barreto et al., 2007). This work demonstrated enhanced demethylation in the presence of overexpressed Gadd45a and XPG (xeroderma pigmentosum, complementation group G), a factor involved in NER (nucleotide excision repair).

1.3. Epigenetic modification

The genome contains information in two forms, genetic and epigenetic. The genetic information, which is inherited by the DNA replication machinery and dictated by the strict rules of Watson and Crick (Szyf et al., 1985), provides the blueprint for the manufacture of all the proteins, while the epigenetic information provides instructions on how, where, and when the genetic information should be used (Szyf et al., 1985). In the post-genomic era of cancer biology, it is becoming increasingly evident that epigenetic controls of gene expression play an important

role in determining the phenotype of cancer cells, possibly suggesting a novel approach and strategy for epigenetically restoring normal phenotype in cancer cells (Jones et al., 1999; Reik et al., 2001; Andrew et al., 2004; Esteller M, 2007 Henikoff S, 2008). Epigenetic is the regulation of changes in gene expression that do not involve changes in DNA sequence (Razin et al., 1980). Histone modifications and DNA methylation-demethylation events are central to the epigenetic regulations of development. The mechanisms controlling these events and their dynamic changes have important implications in developmental cell biology as well as carcinogenesis and tumor progression (Jones et al., 1999; Reik et al., 2001; Andrew et al., 2004; Esteller M, 2007; Henikoff S, 2008). Epigenetic technologies in cancer studies are helping increase the number of cancer candidate genes and allow us to examine changes in 5-methylcytosine DNA and histone modifications at a genome-wide level. In fact, all the various cellular pathways contributing to the neoplastic phenotype are affected by epigenetic genes in cancer (Andrew et al., 2004; Esteller M, 2007; Jones et al., 2007). They are being explored as biomarkers in clinical use for early detection of disease, tumor classification and response to treatment with classical chemotherapy agents, target compounds and epigenetic drugs. Encouraging results have been obtained with histone deacetylase and DNA methyltransferase inhibitors, leading the US Food and Drug Administration to approve several of them for the treatment of malignancies and lymphoproliferative disorders (Sansom et al., 2007; Stresemann et al., 2008)

1.3.1. DNA methylation patterns vary in time and space

DNA methylation is a conserved epigenetic modification of the genome. The presence of 5-methylcytosine (5-MeC) in DNA is an epigenetic marker and key recognition signal for the regulation of DNA-protein interactions. Methylation of cytosines within CpG dinucleotides is associated with transcriptional silencing during mammalian development and tumorigenesis (Razin et al., 1980; Jones et al., 2002). These dinucleotides are unevenly distributed throughout the genome and remain in short stretches or clusters of 500-2000 bp (base pairs), called CpG islands (Razin et al., 1980; Jones et al., 2002). CpG methylation plays an

important role in maintaining gene silencing that is necessary for tissue- and development-specific gene expression, silencing of the inactive X-chromosome, genomic imprinting, and protection against the expression of intragenomic parasitic elements such as Alu and Line sequences (Jones et al., 1999; Baylin et al., 2000; Robertson et al., 2000; Li et al., 2002). During early embryonic development, alternating waves of methylation and demethylation have programmed cellular growth and differentiation (Li et al., 2002; Haaf et al., 2006). The DNA methylation patterns established during this time remain relatively stable in normal tissue. Most CpG islands are found in the proximal promoter regions of almost half of mammalian genes and are, generally, unmethylated in normal cells (Fig. 4). In cancer cells, however, these promoter regions are usually hypermethylated, leading to their transcriptional silencing (Baylin et al., 2000; Villar-Garea et al., 2003).
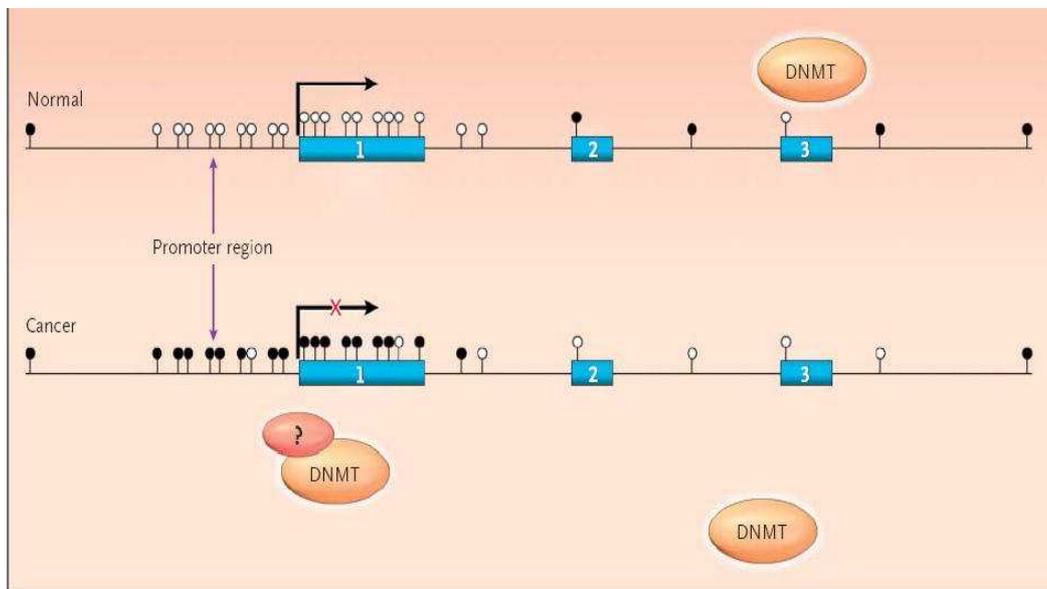
Fig. 4. Methylation patterns of CpG islands in normal and tumor cells. Most CpG islands (numerous circles) in the proximal promoter regions surrounding and within exon 1 of the sample gene are unmethylated in normal cells (white circles) and methylated in tumor cells (black circles) with associated transcriptional silencing (red X at the transcription start site) (Baylin et al., 2000).

1.3.2. DNA methyltransferases (DNMTs)

Methylation of DNA cytosine is a complex process catalyzed by DNMTs, which transfer the methyl group from Sadenosylmethionine (SAM) to generate patterns of genomic methylation (Li et al., 1992; Okano et al., 1999; Grønbaek et al., 2007) (Fig. 5).
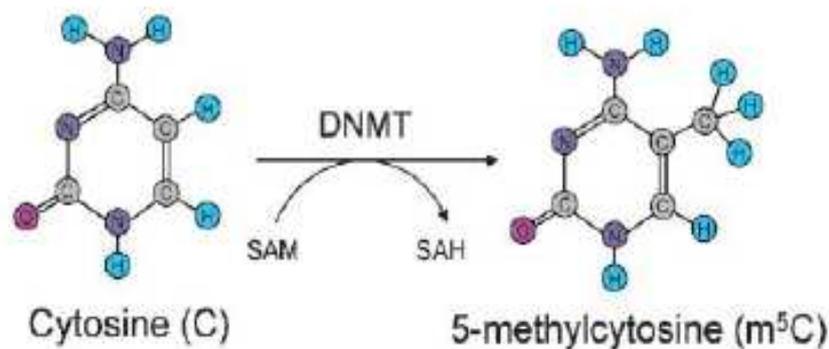
Fig. 5. Methylation of cytosine. Cytosine residues in DNA are converted to 5-methylcytosine by DNMTs. The methyl group is donated by the universal methyl donor SAM, which is converted to S-adenosylhomocysteine (SAH) (Grønbaek et al., 2007).

In mammals, DNMTs can be divided into maintenance (DNMT1) and de novo methyltransferases (DNMT3a and DNMT3b) according their functions. During DNA replication, the methylation pattern on the parental DNA strand is copied onto the newly synthesized strand mainly by DNMT1 (Okano et al., 1999; Bestor TH, 2000; Grønbaek et al., 2007) (Fig. 6). However, in early embryonic stem cells and cancer cells, methylation of previously unmethylated DNA may occur. This so-called de novo methylation is preferentially mediated via DNMT3a and DNMT3b (Okano et al., 1999; Bestor TH, 2000; Grønbaek et al., 2007) (Fig. 6).
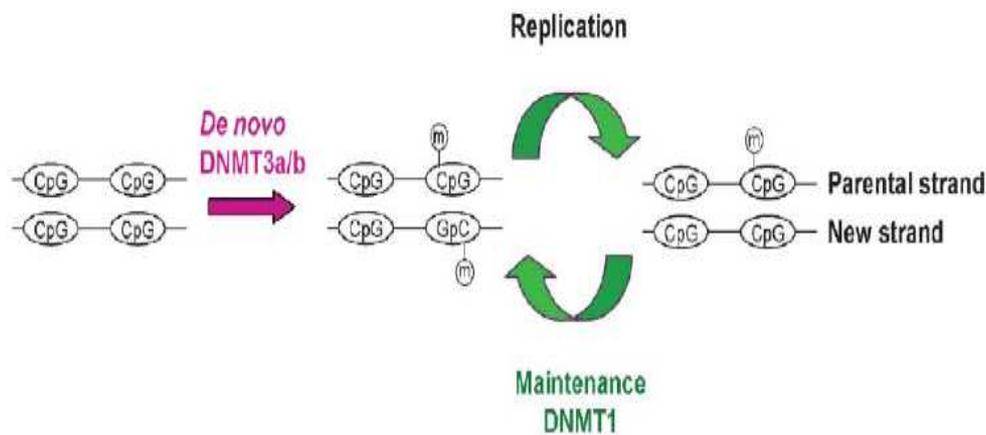
Fig. 6. Establishment and propagation of methylation patterns. De novo methylation of previously unmethylated DNA in embryonic stem cells and cancer is catalyzed mainly via DNMT3a and DNMT3b. After each replication, the methylation pattern of the parental strand is copied onto the newly synthesized strand by the maintenance methyltransferase DNMT1 (Grønbaek et al., 2007).

DNA methylation is mediated by DNMT family, which encompasses DNMT1, DNMT3a and DNMT3b. The importance of these DNMTs in mammalian development has been demonstrated in mouse knockout models in which loss of DNMT1/DNMT3b or DNMT3 was lethal at the embryonic stage and at four weeks after birth, respectively, due to hypomethylation of genomic DNA (Li et al., 1992; Okano et al., 1999).

DNA methylation is also strongly implicated in tumorigenesis (Jones et al., 1999; Baylin et al., 2000). All three DNMTs, at the mRNA and/or protein level, are modestly overexpressed in many types of tumor cells (Robertson et al., 1999; DeMarzo et al., 1999). There is considerable evidence indicating an upregulation of DNMT1 in cancer (Belinsky et al., 1996; Baylin et al., 1998). Elevated levels of DNMT1 are required to silence p16ink4a in bladder cancer cells (Fournel et al., 1999). Conversely, knock down of DNMT1 in human tumor cells causes demethylation and re-activation of tumor suppressor genes (Robert et al., 2003). Overexpression of DNMT3a, DNMT3b has also been reported in tumor cells (Robertson et al., 1999; Kanai et al., 2001). An increase methylation of DNA in

the promoter region of tumor suppressor genes correlates with downregulation of transcriptional activation (Schmutte et al., 1998). In addition to transcriptional silencing through direct methylation of CpG islands of the promoters, DNMTs proteins also have been shown to recruit other transcriptional repressors for this purpose (Aapola et al., 2002; Bachman et al., 2003).

1.3.3. The possible link between 53BP1 and DNMTs

To date, there are several lines of evidence indicating that DNA methylation mediated by DNMTs is associated with p53 signalling pathways in maintaining genome stability (Wang et al., 2005). The expression of DNMTs was found increased in certain cancers and tumor cells with constitutive 53BP1 foci (Bartkoval et al., 2005; Gorgoulis et al., 2005). 53BP1 is a central mediator of the DNA damage checkpoint and is important for coupling ATM to its downstream targets including p53 and Gadd45 (Wang et al., 2002; Adams et al., 2006). The interaction between 53BP1 and p53 facilities the stabilization of p53 protein and enhances p53-mediated transcriptional activation (Zhang et al., 2006; Huang et al., 2007; Kachirskaia et al., 2008). Furthermore, 53BP1-null mice eventually succumb to genomic instability and cancer development in a p53-dependent manner (Celeste et al., 2003; Ward et al., 2005; Morales et al., 2006). Recent studies have demonstrated that DNMT1- and DNMT3a-mediated methylation is stimulated by p53, suggesting cooperation between p53 and DNMTs in the regulation of gene methylation (Wang et al., 2005; Lai et al., 2008). Taken together, these observations implicate the possible functions of 53BP1 in regulating the DNMTs.

1.3.4. DNA demethylation

In contrast to the large amount of information accumulated on DNA methylation, relatively little is known about DNA demethylation. The DNA demethylation can be either passive or active, or a combination of both. Passive DNA demethylation occurs by inhibition or lack of maintenance of DNMTs throughout cycles of replication, whereas active DNA demethylation requires specific enzymatic

reactions (Kress et al., 2001; Bird, 2002; Patra et al., 2008). Active demethylation occurs during early development and differentiation. These mechanisms can explain active demethylation without mitotic process (Szyf et al., 1985; Kafri et al., 1993; Weiss et al., 1996). Recent studies suggested that DNA methylation pattern is a steady-state balance of methylation and demethylation (Fig. 7). In contrast to the classic model, DNA methylation pattern is in a dynamic steady state of methylation and demethylation catalyzed by DNMTs and demethylases (Szyf et al., 2005, Patra et al., 2008).
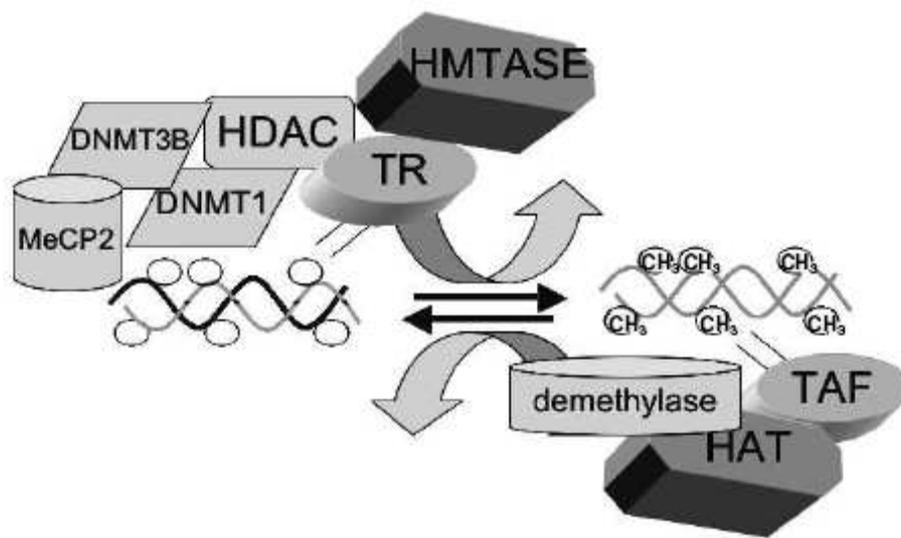
Fig. 7. The DNA methylation pattern is a steady-state balance of methylation and demethylation. Interaction of trans-acting repressors (TR) recruits histone modification enzymes such as histone deacetylases (HDAC) and histone methyltransferases (HMTASE) to specific genes. HDACs and HMTASE in turn recruit DNMTs and methylated DNA binding proteins such as MeCP2 to tilt the balance of the reaction toward DNA methylation. Trans-acting activating factors (TAF) on the other hand recruit histone acetyl transferases (HAT) to specific genes. The presence of HATs results in acetylation of histones and increased accessibility to demethylases tilting the steady state balance of the DNA methylation equilibrium toward demethylation (Szyf et al., 2005).

1.3.5. DNA demethylases

Although three active demethylation mechanisms have been proposed, none of them has gained wide acceptance (Kress et al., 2001). The first mechanism is direct replacement of the methyl moiety by a hydrogen atom. Several lines of evidence have been provided MBD2 as a demethylase by this mechanism: 1) exogenous expression of MBD2 increased replication-independent active demethylation of ectopically methylated DNA (Cervoni et al., 2001) and resulted in overexpression of the urokinase type plasminogen activator and the hexokinase type II by demethylation of their hypermethylated promoters in vitro (Guo et al., 2002; Goel et al., 2003); 2) antisense inhibition of MBD2 reduced replication-independent active demethylation (Detich et al., 2003); 3) recombinant MBD2 expressed and purified from A549 cells has been shown to possess demethylase activity in vitro (Detich et al., 2003). The second mechanism implicates a role for DNA glycosylases, which cleave the bond between the 5-methylcytosine base and the deoxyribose moiety in DNA. The abasic site is then repaired by resident repair activity resulting in replacement of a 5-methylcytosine with an unmethylated cytosine (Goel et al., 2003; Jost et al., 1995 and 1997). The third mechanism describes the methylated nucleotide as being removed by nucleotide excision, being replaced by an unmethylated cytosine (Weiss et al., 1996 and 1997). Nucleotide excision repair is an important mechanism for removing a wide spectrum of different DNA lesions, which can repair damaged DNA during DNA replication (David et al., 2007). Cells may use this repair processes as buffer, as additional localized demethylation pathways for gene transcription. Recent studies have demonstrated that the nucleotide excision mechanism induced the local targeted DNA demethylation by a transcriptional activator during development in living cells (Kapoor et al., 2005; Kress et al., 2006; Murayama et al., 2006). It has been proposed that Gadd45a, the target gene of p53, can catalyze active demethylation through this mechanism (Barreto et al., 2007).

1.4. RASSF1A and C1S2 repeats in A549 cells

The small GTP-binding protein Ras plays an important role in mediating multiple intracellular signal transductions, including growth, apoptosis, and differentiation (Vavvas et al., 1998; Khokhlatchev et al., 2002). RASSF1A (Ras association domain family 1 isoform A), a candidate tumor suppressor gene, contains the Ras-association domain that interacts with Ras and mediates K-Ras-dependent apoptosis (Vos et al., 2000; Donninger et al., 2007). Transcriptional silencing of RASSF1A by promoter methylation has been found in many types of carcinomas and tumor cells including human lung adenocarcinoma cell A549 that exhibits K-Ras activating mutation (Beaulieu et al., 2002; Dammann et al., 2005a). Recent study in non-small cell lung cancers demonstrated that RASSF1A methylation, in combination with K-ras mutation, might have an adverse synergistic effect on patient's survival (Kim et al., 2003). Re-expression of RASSF1A in various tumor cells where this gene is deleted or its promoter is methylated inhibits cell growth, invasion, stimulates apoptosis and reduces tumorigenicity in mouse models (Dammann et al., 2000; Donninger et al., 2007).

Repetitive sequences like Alu elements, α- and classic satellite DNA are strongly methylated in the human genome (Jeanpierre M 1994). Satellite 2 (Sat2) DNA sequences are found predominantly in juxtacentromeric heterochromatin of certain human chromosomes and are most abundant in the long juxtacentromeric heterochromatin region of chromosome 1 (Ehrlich et al., 2003). The chromosome 1 satellite 2 (C1S2) is methylated highly in diverse cell lines and has been used as marker for the analysis of the global methylation (Ehrlich et al., 2003; Mund et al., 2005; Wilson et al., 2007).

1.5. The aim of this study

Epigenetic silencing by hypermethylation of CpG islands in the promoter regions of tumor-suppressor genes is involved in the initiation and progression of several types of cancer (Baylin et al., 2000; Esteller M, 2007). DNA methylation changes in cancer represent innovative diagnostic markers and attractive therapeutic targets by using demethylating agents (Yoo et al., 2006; Stresemann et al., 2008). However, the mechanisms of DNA demethylation in regulation of gene expression are still unknown. 53BP1 has been suggested as a sensor and/or mediator of the DNA damage checkpoint. 53BP1 function is important for coupling ATM to its downstream targets including p53 and Gadd45. The latter has been reported to participate to active demethylation (Barreto et al., 2007). In light of these findings, we hypothesize that:

(1) 53BP1 may play an important role in DNA demethylation in cellular response to DNA damage to protect genomic stability.

(2) 53BP1 signalling is associated with DNMTs in maintaining genome stability.

(3) The possible mechanisms of 53BP1 in regulation of DNA demethylation may be involved in the activation of Gadd45a and MBD2.

The purpose of the present dissertation was to clarify the role of 53BP1 in DNA demethylation as a response to DNA damage. In order to determine the relationship between the overexpression of 53BP1 and the methylation status of DNA, the methylation status of the RASSF1A gene and C1S2 DNA repetitive elements was assessed in A549 cells transfected with different 53BP1 plasmids by methylation specific PCR (MSP) and combined bisulfite restriction analysis (COBRA). The expression of RASSF1A gene in A549 cells transfected with different 53BP1 plasmids by RT-PCR was also investigated. Additionally, the effect of 53BP1 on the expression of DNMTs, Gadd45a, and MBD2 was evaluated by quantitative real-time PCR.

2. Materials

2.1. Plasmid used in this study

The expression plasmids of full-length cDNA (pCMH6K-53BP1), N-terminal 1052 (pCMH6K-53BP1N) and C-terminal 921 residues (pCMH6K-53BP1C) of 53BP1 were kindly provided by Kuniyoshi Iwabuchi, M.D., Ph.D., Associate Professor, Department of Biochemistry, Kanazawa Medical University, Japan (Iwabuchi et al., 1998). The β-galactosidase (β-gal) -encoding plasmid pcDNA3.1/His/lacZ used to monitor transfection efficiency was purchased from Invitrogen (Kalrsruhe, Germany). The maps of these plasmids were displayed under below (Fig. 8A and 8B).
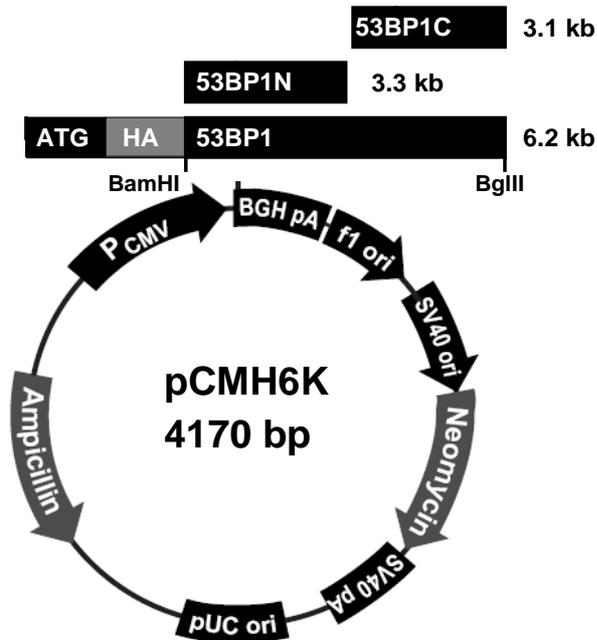
Fig. 8A. The full-length cDNA (6.2 kb), N-terminal (53BP1N, 3.3 kb) and C-terminal (53BP1C, 3.1 kb) of 53BP1 were constructed in the vector pCMH6K containing an influenza virus hemagglutinin (HA) tag at the N-terminal. Pcmv: CMV promoter, ATG: ATG initiation codon, BGH pA: BGH polyadenylation signal, Psv40: SV40 promoter and origin, Neomycin: neomycin resistance gene, Ampicillin: ampicillin resistance gene.
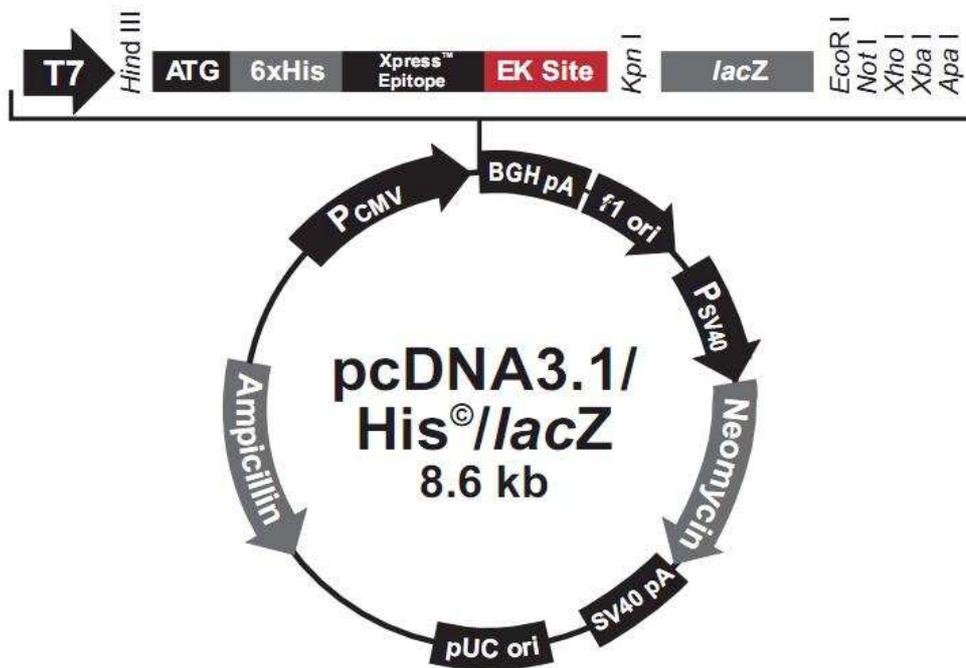
Fig. 8B The map of β-gal-encoding plasmid pcDNA3.1/His/lacZ was taken from Invitrogen, Germany. Pcmv: CMV promoter, ATG: ATG initiation codon, BGH pA: BGH polyadenylation signal, Psv40: SV40 promoter and origin, SV40 pA: SV40 polyadenylation signal, Neomycin: neomycin resistance gene, Ampicillin: ampicillin resistance gene.

2.2. Cell line

The human lung adenocarcinoma cell line A549 and cervix adenocarcinoma cell line Hela were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C in RPMI 1640 medium supplemented with 1% penicillin/streptomycin (Sigma, Germany) and 10% fetal calf serum (FBS) (Sigma, Germany).

2.3. Primers

Table. 1. **Primer sequences and PCR conditions**

Gene	Forward primer(5'-3')	Reverse primer(5'-3')	Annealing (cycles)	Product size(bp)	Reference
Reverse transcription PCR					
RASSF1A	CAGATTGCAAGTTCACCTGCCACTA	GATGAAGCCTGTGTAAGAACCGTCCT	60(37cy)	244	Dote et al., 2005
53BP1-N	AGGTGGGTGTTCTTTGGCTTCC	TTGGTGTTGAGGCTTGTGGTGATAC	60(37cy)	328	Kao et al., 2003
53BP1-C	GCAGCCTCTGTGAAGCAGCA	ATGCAAGGAATCCAGTTACACACAA	60(37cy)	303	Gorgoulis et al., 2005
GAPDH	TGGTCACCAGGGCTGCTT	AGCTTCCCGTTCTCAGCCTT	55(35cy)	150	Bussolati et al., 2005
Real time PCR					
Gadd45a	TCAGCGCACGATCACTGTC	CCAGCAGGCACAACACCAC	58(37cy)	82	Kis et al., 2006
MBD2	AACCCTGCTGTTTGGCTTAAC	CGTACTTGCTGTACTCGCTCTTC	58(40cy)	101	Fang et al., 2003
DNMT1	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTGG	60(40cy)	329	Mizuno et al., 2001
DNMT3a	CACACAGAAGCATATCCAGGAGTG	AGTGGAAACCAAATACCC	60(40cy)	550	Mizuno et al., 2001
DNMT3b	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	60(40cy)	191	Mizuno et al., 2001
Methylation-specific PCR					
RASSF1A	M: GTGTTAACGCGTTGCGTATC	M: AACCCCGCGAACTAAAAACGA	60(40cy)	93	Lo et al., 2001
	U: TTTGGTTGGAGTGTGTTAATGTG	U: CAAACCCCACAAACTAAAAACAA	60(40cy)	105	Lo et al., 2001
COBRA PCR					
C1S2	ATGGAATTTTTATGAAATTGAAATG	CATTCCATTAAATAATTCCATTC	51(40cy)	210	Mund et al., 2005

2.4. Chemicals

ABsolute SYBR Green Mixes	ABgene, Germany
Acrylamide stock 30%	Roth, Germany
Agarose	Sigma Aldrich, Germany
Ampicillin	PAA, Germany
Boric acid	Sigma Aldrich, Germany
$CoCl_2$	Sigma Aldrich, Germany
DNase I, RNase-free	Fermentas, Germany
Distilled water	Millipore, Germany
dNTPs	Fermentas, Germany
EDTA	AppliChem, Germany
Ethanol 100%	Roth, Germany
FastDigest™ buffer	Fermentas, Germany
FastDigest™ HinfI	Fermentas, Germany
GeneRuler 100bp DNA ladder	Fermentas, Germany
Glycerol (absolute)	AppliChem, Germany
Hot-star Taq-polymerase	Qiagen, Germany
Hydroquinone	Sigma Aldrich, Germany
L-glutamine	PAA, Germany
6x loading dye solution	Fermentas, Germany
$MgCl_2$	Fermentas, Germany
M-MuLV reverse transcriptase	Fermentas, Germany
NaCl	Sigma Aldrich, Germany
NaOH	Sigma Aldrich, Germany
PBS buffer	PAA, Germany
Penicillin/streptomycin	PAA, Germany
Puromycin dihydrochloride	Sigma Aldrich, Germany
Roti-fect plus	Roch, Germany
RPMI 1640 medium	PAA, Germany
Sodium bisulfite	Sigma Aldrich, Germany
Taq-polymerase	Fermentas, Germany
TEMED	Carl Roth chemical, Germany
Tris base	Sigma Aldrich, Germany

Trypan blue stain (0.4%)	Sigma Aldrich, Germany
Trypsin/EDTA	Invitrogen, Germany
Tryptone	Sigma Aldrich, Germany
Xylene cyanole	Sigma Aldrich, Germany
Yeast extract	Sigma Aldrich, Germany

2.5. Experiment Kits

First Strand cDNA synthesis kit	Fermentas, Germany
ß-gal staining kit	Invitrogen, Germany
Nucleospin extract II	Machery-Nagel, Germany
DNeasy blood & tissue kit	Qiagen, Germany
Plasmid midi kit	Qiagen, Germany
RNeasy mini kit	Qiagen, Germany

2.6. Reagents

Competent Cells

MAX Efficiency® DH5α™ Competent Cells, Invitrogen, Germany

Luria-Bertani (LB) ampicillin liquid medium (store at 4°C):

Dissolve the following in 800 ml distilled H_2O

NaCl 10 g

Tryptone 10 g

Yeast extract 5 g

Adjust the pH to 7.0 with 1 M NaOH

Adjust the volume to 1000 ml

Sterilize by autoclaving

Add ampicillin 100 mg/ml stock to 100 µg/ml final concentration

Transfer medium to plate

Cell culture medium:

RPMI 1640	PAA, Germany
10% Fetal bovine serum (FBS)	Sigma, Germany
1% Penicillin/streptomycin	PAA, Germany

10 mM hydroquinone:

Hydroquinone 11 mg

Dissolve in 5 ml distilled water

Add up to 10 ml

40.5% or 3.9 M sodium bisulfite:

Sodium bisulfite 2.835 g

Dissolve in 4 ml distilled water

Adjust pH to 5.0 with 3 M NaOH

Add the volume to 7 ml

3 M NaOH:

NaOH 1.199 g

Dissolve in 5 ml distilled water

Add up to 10 ml

1x PBS:

Dissolve the following in 800 ml of distilled H_2O

NaCl 8 g

KCl 0.2 g

Na_2HPO_4 1.44 g

KH_2PO_4 0.24 g

Adjust the pH to 7.4 with HCl and add H_2O to 1 liter

12% polyacrylamide solution:

30% Acrylamid stock solution 6 ml

Distilled water 5.9 ml

5x TBE 3 ml

10% Ammonium persulfite 0.1 ml

TEMED 10 μl

5x TBE buffer (store at room temperature):

Dissolve the following in 800 ml distilled H_2O

Tris 54 g

Boric acid 27.5 g

0.5M EDTA 20 ml

Adjust the volume to 1000 ml

TE buffer:

1M Tris-HCl (pH7.5) 10 ml

0.5M 2 ml EDTA (pH8.0)

Add H_2O to 1 liter

2.7. Consumables

Cell culture flasks 25 cm^2	Nunclon™, Denmark
Distilled water	Millipore, Germany
1.5 ml Eppendorf centrifuge tube	Eppendorf, Germany
Polypropylene tube	FALCON®, NJ, USA
6-well flat bottom culture plate	Nunclon™, Denmark
10 µl white tips	Roth, Germany
200 µl yellow tips	Roth, Germany
1000 µl blue tips	Roth, Germany

2.8. Apparatus

37°C CO_2 incubator	Heraeus, Germany
Heating block	VWR, Germany
MyQ thermal cycler	Bio-Rad, USA
Laminar flow cabinet	Heraeus, Germany
Elekta SL-25 linear accelerator	Norcross, GA
Pipettes	Eppendorf, Germany
-20°C Refrigerator	Bosch, Germany
-80°C Refrigerator	Bosch, Germany
Shaking incubators	Heraeus, Germany
Table centrifuge	Heraeus, Germany
UV spectrophotometer	Bio-Rad, USA
Water bath	Lauda, Germany

3. Methods

3.1. Bacterial transformation and plasmid recovery

3.1.1. Bacterial transformation with plasmid DNA

Plasmid DNA was transformed into MAX Efficiency® DH5α™ Competent Cells according to the manufacturer's instructions (Invitrogen, Germany). Briefly, 50 µl of competent E. coli cells were added to each pre-chilled tubes. 5 ng of plasmid DNA was added to the tubes. After incubation on ice for 30 min, the cells were heat shocked for 45 s in a water bath at 42°C. The tubes were returned to ice for 2 min. After adding 450 µl of LB medium, the tubes were incubated for 1 h at 37°C with shaking (150 rpm). 100 µl of each solution was spread onto LB plates containing 100 µg/ml ampicillin (PAA, Germany). The plates were incubated overnight at 37°C. Then a single colony was picked from the above plate and inoculated into a tube containing a starter culture volume of 5 ml of LB medium. The tubes were incubated for 8 h at 37°C with vigorous shaking (300 rpm). 50 µl of starter culture was diluted into the tubes containing 25 ml of LB ampicillin medium. The tubes were incubated at 37°C for 16 h with vigorous shaking (300 rpm).

3.1.2. Plasmid DNA recovery

The bacterial cells were harvested by centrifugation (8000 rpm) for 15 min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 4 ml of buffer P1 from the Plasmid midi kits (Qiagen, Germany). After adding 4 ml of buffer P2 and mixing by vigorously inverting the sealed tube 4 times, the tube was incubated at room temperature for 5 min. Following adding 4 ml of chilled buffer P3 and mixing immediately by vigorously inverting 4 times, the tube was incubated on ice for 15 min. Centrifugation was performed at 13000 rpm for 30 min at 4°C. The supernatant containing plasmid DNA was removed and centrifuged again at 13000 rpm for 15 min at 4°C. The supernatant was loaded onto an equilibrated QIAGEN-column and allowed it to enter the resin by gravity flow. The QIAGEN-column was washed twice with 10 ml of buffer QC. The plasmid DNA was eluted with 5 ml of buffer QF and precipitated by adding 3.5 ml of isopropanol at room temperature. The mixture was centrifuged immediately

at 13000 rpm for 30 min at 4°C. The supernatant was decanted and the pellet was washed with 2 ml of 70% ethanol. After centrifugation at 13000 rpm for 10 min, the supernatant was decanted. The purified DNA was re-dissolved in 100 μl of TE buffer (pH 8.0). The concentration and purity of DNA were determined spectrophotometrically, and required an A260/280 ratio of at least 1.8.

3.2. Cell culture

3.2.1. Thawing cultured cells

A549 cells stored in liquid nitrogen were thawed quickly in a water bath (37°C). The cells were transferred to a sterile 15ml tube containing prewarmed RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Following centrifugation at 1200 x g for 5 min, the cells were resuspended in T-25 cm^2 flask containing 5 ml prewarmed RPMI 1640 medium. The flasks were incubated overnight at 37°C in a humidified 5% CO_2 incubator. After a few days, cells grew into the gaps of the flasks, and reached confluence.

3.2.2. Subculturing cells

After trypsinizing the cells with trypsin solution, the floating cells were transferred to a centrifuge tube. Following centrifugation at 1200 x g for 7 min, the cells were resuspended in fresh growth media and seeded into a new flask. A subcultivation ratio was 1:5. The medium was re-placed 2 to 3 times per week.

3.2.3. Treatment of cells with $CoCl_2$

A549 cells (5 x 10^5) were grown in a T-25 cm^2 flask at 37°C in a humidified 5% CO_2 incubator After 24 h, the cells were replaced with prewarmed RPMI 1640 medium with and without $CoCl_2$ (100 μM) and cultured for 5 days to induce hypoxia. The cells were collected for DNA-isolation and methylation analysis.

3.3. Cell transfection

A549 cells (5 x 10^5) were grown in a T-25 cm^2 flask at 37°C in a humidified 5% CO_2 incubator for 24 h until they are 70% confluent and transfected with 53BP1 expression plasmids by using the Roti-fect plus transfection reagent. Briefly, the

following solutions were prepared in polystyrene tubes: A) 4 µg of plasmid DNA (pCMH6K-53BP1, pCMH6K-53BP1N, pCMH6K-53BP1C, pcDNA3.1, and pcDNA3.1/His/lacZ, respectively) in 300 µl of serum-free medium without antibiotics. B) 16 µl of Roti-fect plus solution in 300 µl of serum-free medium without antibiotics. Both solutions were mixed gently by pipetting several times and combined together. After incubating at room temperature for 15 min, the nucleic acid-lipid complex was added to the cells. The flask was rocked gently in order to evenly disperse the complex mixture. The medium was replaced 1 day later and the cells were harvested 72 h post transfection. The experiments were performed in triplicate.

3.3.1. Determination of transfection efficiency with ß-gal staining

At 24 h after transfection, the cells were stained to determine the transfection efficiency using the β-gal staining Kit (Invitrogen, Germany). LacZ is a bacterial gene often used as a reporter construct in eukaryotic transfection experiments because the gene product, β-gal, is resistant to proteolysis in cellular lysates and its activity is easily assayed. The β-gal enzyme catalyzes the hydrolysis of X-gal (5-bromo-3-indoyl-β-D-galactopyranoside). Following fixation and incubation with the X-gal substrate, cells transfected with a β-gal-expressing plasmid will appear blue. Briefly, the growth medium was removed from the cells transfected with plasmid pcDNA3.1/His/lacZ. After rinsing the flask with 2.5 ml of PBS, the cells were fixed using 1 x fixative solution for 10 min at room temperature. While the flask was in the fixative solution, the staining solution containing 25 µl of solution A, 25 µl of solution B, 25 µl of solution C, 125 µl of 20 mg/ml X-gal in DMF, and 2.3 ml of PBS was prepared in a polypropylene tube according to the manufacturer's protocol (Invitrogen, Germany). Staining solution was added to the flask after rinsing twice with 2.5 ml of PBS. The cells were incubated at 37°C overnight and observed under a microscope. For each flask, five visual fields were chosen randomly under 20X magnification. Cells stained blue were counted and the transfection efficiency was calculated as the percentage of the blue cells in each field.

3.4. IR of A549 cells following transfection

The cells were irradiated at 4, 6 and 10 Gy using a 6 MV Elekta SL-25 linear accelerator of 450 cGy/min at 24 h after transfection.

3.5. DNA isolation

The isolation of genomic DNA from cells was performed by using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. The cells grown in culture flasks were trypsinized, harvested and resuspended in 200 µl of PBS. After digestion with 20 µl of proteinase K, the cell pellet was incubated with 200 µl of buffer AL at 56°C for 10 min. This was followed by three times washing with 200 µl of 100% ethanol, 500 µl of buffer AW1, and 500 µl of buffer AW2, respectively. The genomic DNA was precipitated and eluted in 200 µl of buffer AE by centrifugation for 1 min at 13000 rpm. The quality and concentration of isolated DNA were determined spectrophotometrically, and required an A260/280 ratio of at least 1.8.

3.6. RNA isolation

Total RNA was isolated from each sample using RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instruction. In brief, the cell pellet was disrupted by adding 350µl of buffer RLT. The lysate was homogenized and pipetted directly into a QIAshredder spin column placed in a 2 ml collection tube. After centrifugation for 2 min at full speed, 1 volume of 70% ethanol was added to the homogenized lysates. 700 µl of the sample was transferred to the RNeasy spin column placed in a 2 ml collection tube. After centrifugation for 15 s at 13000 rpm, the flow-through was discarded. This was followed by three times washing with 700 µl of buffer RW1 and twice washing with 500 µl of buffer RPE for 15 s. The RNeasy spin column was replaced in a new 1.5 ml collection tube. The RNA was eluted in 50 µl of RNase-free water by centrifugation for 1 min at 13000 rpm. The quality and concentration of the RNA were determined spectrophotometrically, and required an A260/280 ratio of at least 1.8. All RNA samples were subjected to DNase I (Fermentas, Germany) digestion for 30 min at 37°C in order to prevent genomic DNA contamination.

3.7. cDNA synthesis

First strand cDNA was synthesized using cDNA synthesis kit (Fermentas, Germany). Briefly, one microgram of total RNA was used for synthesis reaction containing 1 μl of oligo(dT)18 primer (0.5 μg/μl) and then was added DEPC-treated water to volume of 11 μl. The reaction mixes were incubated at 70°C for 5 min and chilled on ice. After adding the following components in the indicated order: 4 μl of 5 x reaction buffer, 1 μl of RiboLock™ ribonuclease inhibitor (20 U/μl), 2 μl of 10 mM dNTP mix, the reaction mixes were incubated at 37°C for 5 min. After adding 2 μl of M-MuLV reverse transcriptase (20 U/μl), the reaction mixes were incubated at 37°C for 60 min. The reaction was inactivated by heating at 70°C for 10 min and chilled on ice. The synthesized cDNA was diluted 6-fold prior to PCR amplification.

3.8. Reverse transcription-polymerase chain reaction (RT-PCR)

The primers used in PCR reactions were summarized in Table 1. Briefly, RT-PCR reaction mixture contained 5 µl of diluted cDNA, 1.0 unit Tag-DNA polymerase, 1.5 mM MgCl2, 0.2 mM of each dNTP, and 5 pmol of each primer. PCR reaction conditions consisted of 30 s at 94°C, 30 s at anneal temperature and 1 min at 72°C for 35 cycles post initial 30 s denaturation at 94°C, and a final extension for 3 min at 72°C. The house keeping gene glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference to standardize the amount of cDNA. The PCR products (20μl) were resolved on 2% TBE agarose gels at 60V/cm for 1 h together with a 100bp marker and visualized after ethidium bromide-staining under UV illumination.

3.9. Quantitative real-time PCR

Quantitative real time PCR was carried out using SYBR Green PCR Master Mix (Abgene, UK) following manufacturer's instruction. Briefly, 5 µl of diluted cDNA was added to a total volume of 25 μl containing 12.5 µl of 2 x SYBR Green Master Mix and 0.2 μmol/L of each primer. The primer sequences, annealing temperatures, and expected PCR product size were summarized in Table 1. The PCR reaction was performed with 40 cycles of 30 s at 95°C, 30 s at anneal temperature, and 1 min at 72°C, post an initial denaturation of 15 min at

95°C. Fluorescence data was recorded at the end of each 72°C step using an iCycler apparatus (Bio-Rad). After the final cycle, melting curve analysis of all samples was conducted within the range from 58-95°C. Relative quantification of gene expression was performed by using GAPDH as an internal control. The threshold cycle and the $2^{-\Delta\Delta Ct}$ method were used for calculating the relative amount of the target RNA (Saha et al., 2001). Gene expression was firstly normalized relative to GAPDH within the same sample, and then treatment group expression was calculated by setting the controls as 100%. The quantitative real-time PCR was performed at least three times and always included a no-template sample as a negative control. For experimental comparisons, genes showing at least a 2-fold change were identified.

3.10. Bisulfite modification of genomic DNA and methylation analysis

3.10.1. Bisulfite modification of genomic DNA

Bisulfite treatment of DNA converts of unmethylated cytosine into uracil while methylcytosine remains unchanged. Bisulfite modification of genomic DNA was performed as described (Herman et al 1996). Briefly, 3 µg of genomic DNA was resuspended in 66.5µl of distilled water and denatured with 3.5 µl of 3 M NaOH at 50°C for 10 min, followed by incubation with 510 µl of 3.9 M sodium bisulfite (Sigma, Germany) and 30 µl of 10 mM hydroquinone (Sigma, Germany) at 55°C for 16 h in darkness. Afterward, modified DNA was purified using the Macherey DNA purification resin (Machery-Nagel, Germany). Briefly, 2 volume of NT-buffer was added to the bisulfite-modified DNA and the mixture was pipetted onto NucleoSpin Exact II column. After centrifugation at 9.7 rpm for 1 min, 610 µl of NT3 buffer was added to the column. After centrifugation at 9700 rpm for 1 min, the genomic DNA was eluted in 50 µl of TE buffer and stored at -20°C until ready for use.

3.10.2. Combined bisulfite restriction analysis (COBRA) of C1S2

COBRA is a sensitive and quantitative DNA methylation assay that combinates bisulfite treatment of DNA and PCR amplification followed by restriction digestion (Xiong et al., 1997). The methylation status of C1S2 elements in

transfected cells was determined by COBRA as previously described (Xiong et al., 1997). Briefly, 8 µl of the bisulfite-modified DNA was added in 50 µl reaction volume containing 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 1 x PCR reaction buffer, 5 pmol of each primer and 2.0 units of Hot-star Taq-DNA polymerase (Qiagen, Germany). The primer sequences for C1S2 used in this study are listed in Table 1. PCR was carried out using the following cycling conditions: denaturation at 95°C for 15 min, 40 cycles (95°C for 30 s, 51°C for 30 s, and 72°C for 30 s), and a final extension of 72°C for 10 min. The PCR product was further digested with FastDigest™ HinfI restriction enzyme (Fermentas, Germany), which recognizes only methylated CpG sequences. Briefly, 1 µl of FastDigest™ HinfI and 5 µl of 10 x fast digest FastDigest™ buffer were added to PCR products. The tube was incubated at 37°C for 20 min. The reaction was inactivated at 65°C for 20 min. Digested PCR products were separated by electrophoresis on 8% polyacrylamide gels. The gels were then stained with EB and visualized under UV illumination.

3.10.3. Methylation specific PCR (MSP)

The MSP is a simple, sensitive, and specific PCR method for determining the methylation status of virtually any CpG-rich region with primers specific for methylated versus unmethylated DNA (Herman et al., 1996). The methylation status of the RASSF1A promoter regions was determined by using MSP as previously described (Herman et al., 1996). PCR primers that distinguish between the methylated and unmethylated DNA sequence were used (Table 1). Negative controls without DNA were included for each set of PCR. Briefly, 8 µl of bisulfite-modified DNA was amplified in a final volume of 25 µl containing 1 x PCR reaction buffer (Qiagen, Germany), 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 5 pmol of each primer and 1.0 unit of Hot-Star Taq-DNA polymerase (Qiagen, Germany). PCR was carried out at 95°C for 15 min, 40 cycles of 95°C for 30 s, the specific annealing temperature for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 10 min. PCR products were loaded onto 8% polyacrylamide gels and visualized after ethidium bromide-staining under UV illumination.

4. Results

4.1. Establishment of cell lines with overexpression of 53BP1

To assess the biological relevance of 53BP1 on the active demethylation of DNA, A549 cells were transiently transfected with the different expression vectors of 53BP1 or control vector pcDNA3.1.. Because patterns of DNA methylation were regulated during chromatin remodeling and cell division (Meehan et al., 2001; Stirzaker et al., 2004) and the double time of A549 cells was about 42 h (www.atcc.org), we monitored transfection efficiencies of A549 cells at 24 h after transfection and analysed the effects of exogenous expression of 53BP1 on DNA demethylation at 3 days after transfection as described previously (Tawa et al., 1998). The transfection efficiencies at 24 h after transfection assessed by counting of ß-gal–stained cells were from 50 to 60%, approximately. One representative example of the ß-gal staining was shown in Fig. 9.
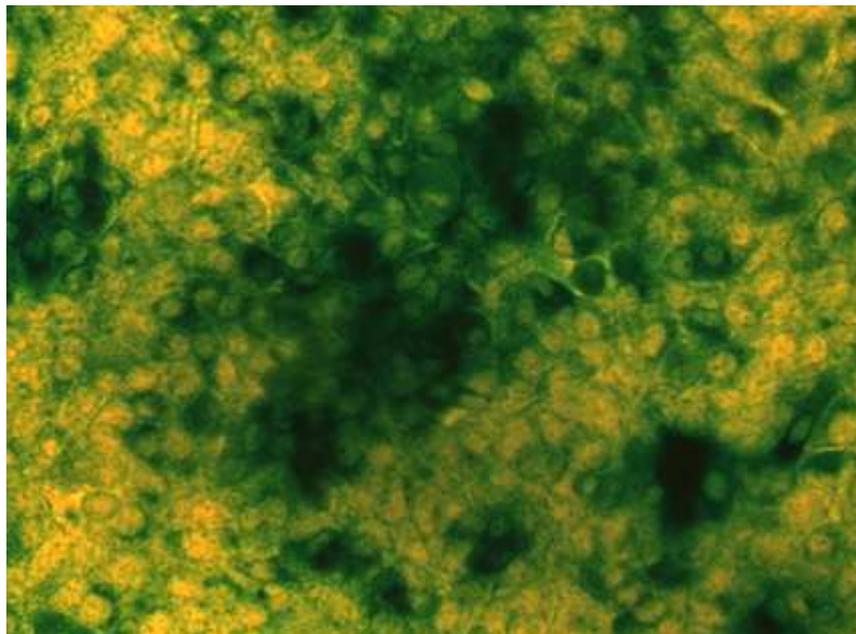
Fig. 9. The expression of ß-gal in A549 cells transfected with the plasmid pcDNA3.1/His/lacZ. The positive cells showed blue pattern after X-gal staining.

We performed real time PCR to detect the transcriptional level of 53BP1 in A549 cells at 3 days after transfection. In comparison with control, the induced expressions of 53BP1 were 374-fold, 89-fold and 98-fold in A549 cells transfected with full-length, N- and C-terminal of 53BP1 analysed by real time PCR, respectively. The expression 53BP1 and the specificity of PCR products were confirmed by RT-PCT (Figure 10).
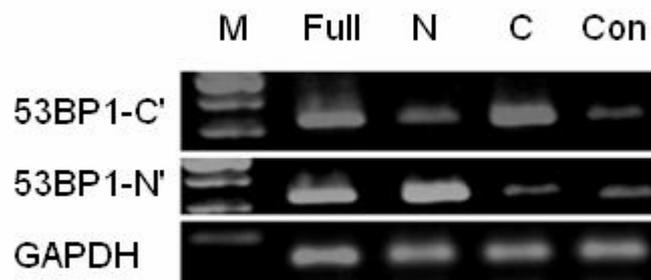
Fig. 10. Expression of 53BP1 in transfected A549 cells. The A549 cells were transfected with different expression plasmids of 53BP1 or control plasmid. The amplified PCR products were visualized on a 2% agarose gel. GAPDH served as endogenous control. 53BP1-N', primers located on N-terminal; 53BP1-C', primers located on C-terminal. M, 100 bp marker; Full, cells transfected with full-length of 53BP1; N, cells transfected with N-terminal of 53BP1; C, cells transfected with C-terminal of 53BP1; Con, cells transfected with control plasmid pcDNA3.1.

4.2. Establishment of COBRA method

Alteration in oxygenation status can induce DNA demethylation (Panayiotidis et al., 2004). $CoCl_2$ has been widely to mimic hypoxia both in vivo and in vitro studies (Wang et al., 1993). To establish COBRA for determination of the DNA methylation status of C1S2 repeats, A549 cells were incubated with $CoCl_2$ (100 μM) to induce hypoxia for 5 days as described (Wang et al., 1993; Shahrzad et al., 2007). As shown in Fig. 11A, the CpG dinucleotides in C1S2 were mainly methylated in untreated A549 cells. In contrast, $CoCl_2$ treatment for 5 days resulted in an increase in the level of unmethylated CpG dinucleotides. In line with previous study (Shahrzad et al., 2007), these data indicate that chemically induced hypoxia can promote demethylation of repetitive elements. Meanwhile, IR is also known to alter the DNA methylation in vivo and in vitro (Kalinich et al., 1989; Tawa et al., 1998; Koturbash et al., 2005). Kalinich et al. showed that different doses of X-ray reduced 5-methylcytosine levels by about 50% within a few days in cell lines (Kalinich et al., 1989). In mice irradiated with 4, 7 and 10 Gy of X-ray, the 5-methylcytosine levels were decreased at 3 days (Tawa et al., 1998). In line with previous reports, the DNA methylation levels of C1S2 in A549 cells (Fig. 11B) were downregulated at 3 days after IR with different doses of X-ray (4, 6, and 10 Gy).
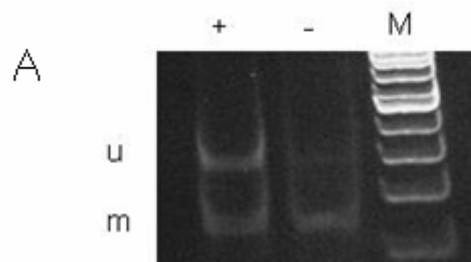
Fig. 11A. Hypoxia induces demethylation of C1S2 repetitive elements. Bisulfite-modified genomic DNA was isolated from $CoCl_2$-treated cells and amplified by PCR following digestion with HinfI, which only cuts methylated repetitive elements. +, CoCl2 treated; -, no CoCl2 treated; u, unmethylated products; m, methylated products. M, 100 bp marker.
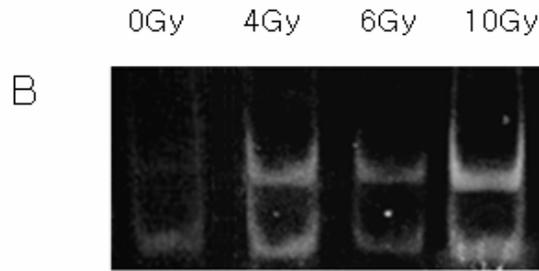
Fig. 11B. Dose-response of IR-induced reduction of DNA methylation levels in A549 cells. A549 cells were irradiated with 0, 4, 6 and 10 Gy and the DNA methylation level at 3 days after IR was determined by COBRA using specific primers for amplification of C1S2.

4.3. Effect of 53BP1 on global DNA demethylation

To test the hypothesis that 53BP1 might be involved in the induction of DNA demethylation, we employed COBRA to analyze the DNA methylation status of C1S2 in A549 cells transfected with 53BP1. As shown in Fig. 12A, high level of methylation was revealed in control A549 cells while DNA demethylation was found in A549 cells with 53BP1-overexpression, especially upon transfection with full -length 53BP1. Therefore, 53BP1 is able to induce DNA demethylation of global DNA. Furthermore, IR can induce DNA demethylation as described previously (Tawa et al., 1998; Koturbash et al., 2005) and may enhance the demethylating effect of 53BP1 in A549 cells (Fig. 12B).
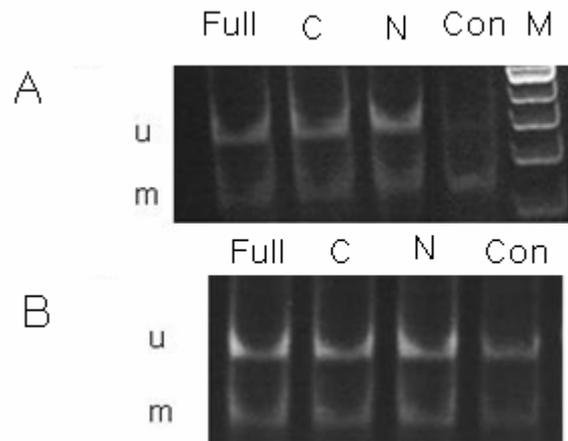
Fig. 12. 53BP1 promotes global demethylation.

COBRA analysis of C1S2 CpG dinucleotides in A549 cells transfected with indicated plasmids (A) and in irradiated cells (6 Gy) at 24 h after transfection (B). Genomic DNA was isolated 72 h later. Bisulfite-modified DNA was amplified using COBRA with specific primers for C1S2. u, unmethylated products; m, methylated products. Full, cells transfected with full-length of 53BP1; N, cells transfected with N-terminal of 53BP1; C, cells transfected with C-terminal of 53BP1; Con, cells transfected with control plasmid. M, 100 bp marker.

4.4. Effect of 53BP1 on DNA demethylation of specific gene.

The RASSF1A has been reported to be inactivated by hypermethylation in the promoter in many types of cancer and cancer cell lines including A549 cells (Dammann et al., 2002; Kuzmin et al., 2002; Mund et al., 2005). The methylation status of the promoter of RASSF1A in cells transfected with 53BP1 was analyzed by MSP. Only the methylated sequence was detected in control A549 cells (Fig. 13A). In contrast, increasing demethylation products were revealed in A549 cells transfected with 53BP1 after 3 days. Similar results were obtained from irradiated cells (6 Gy) at 24 h after transfection (Fig. 13B). These findings indicate that 53BP1 is able to induce DNA demethylation of specific gene.
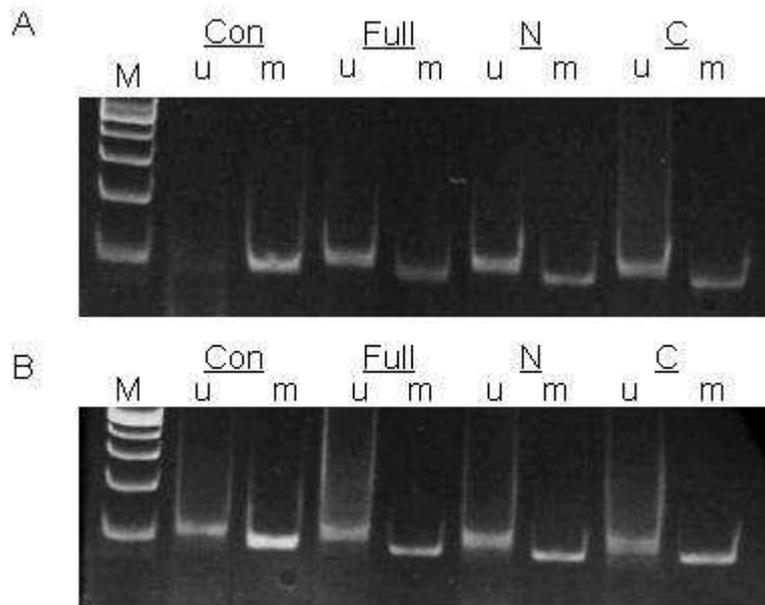
Fig. 13. 53BP1 promotes gene-specific DNA demethylation.

MSP analysis of RASSF1A in A549 cells transfected with indicated plasmids (A) and in irradiated cells (6 Gy) at 24 h after transfection (B). Genomic DNA was isolated 72 h later. Bisulfite-modified DNA was analyzed using MSP. Full, cells transfected with full-length of 53BP1; N, cells transfected with N-terminal of 53BP1; C, cells transfected with C-terminal of 53BP1; Con, cells transfected with control plasmid. M, 100 bp marker; u, unmethylated products; m, methylated products.

4.5. Effect of 53BP1 on re-expression of specific gene

Consistent with the increase in unmethylated products of its promoter, the transcriptional expression of RASSF1A gene was detectable in A549 cells after transfection with 53BP1 by RT-PCR (Fig. 14). These results confirmed that loss of RASSF1A transcription correlated with hypermethylation of its CpG islands in the promoter and indicated the effect of 53BP1 on DNA demethylation of RASSF1A.
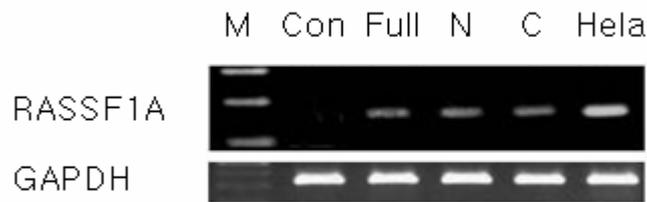
Fig. 14. Effect of 53BP1 on re-expression of RASSF1A. The expression of RASSF1A was analyzed in A549 cells transfected with indicated plasmids after 3 days. GAPDH was used as an external control. Full, cells transfected with full-length of 53BP1; N, cells transfected with N-terminal of 53BP1; C, cells transfected with C-terminal of 53BP1; Con, cells transfected with control plasmid M, 100 bp marker. Hela, Hela cells, that contains unmethylated RASSF1A gene, was used as positive control for the expression of RASSF1A.

4.6. Transcriptional levels of relative genes in 53BP1-transfected A549 cells

To gain insight into the potential mechanism responsible for 53BP1-induced demethylation, we addressed a number of DNA methylases and demethylases that can serve as determinants of DNA methylation status. The effect of 53BP1 on the transcriptional expression of human DNMT1, DNMT3a, DNMT3b, MBD2 and Gadd45a was investigated by real-time PCR. As shown in Fig. 15A, real-time PCR revealed 3-fold, 13-fold, 13.9-fold up-regulation in Gadd45a mRNA expression level in A549 cells following transfection with N-terminal, C-terminal, and full-length of 53BP1, respectively. Furthermore, IR induced a 26-fold up-regulation of Gadd45a in control A549 cells and a 36-fold up-regulation in A549 cells transfected with full-length-53BP1. These findings implicate a potential role for Gadd45a regarding 53BP1-induced demethylation in A549 cells response to DNA damage. The effect of 53BP1 on the expression of MBD2 was also determined. As shown in Fig. 15B, real-time PCR revealed a 5.1-fold, 6.5-fold up-regulation in MBD2 expression level of A549 cells following

transfection with full-length and C-terminal of 53BP1. In contrast, N-terminal of 53BP1 did not affect the expression level of MBD2 (0.95-fold). Other critical determinants of DNA methylation are DNMTs. Real time PCR analysis revealed a 0.5-fold, 0.6-fold and 0.7-fold decrease in the mRNA level of DNMT1 in A549 cells following transfection with full-length, N-terminal, and C-terminal of 53BP1, respectively (Fig. 15C). In addition, a 0.1-fold, 0.06-fold, 0.06-fold decrease in DNMT3a level was detected in A549 cells following transfection with full-length, N-terminal, and C-terminal of 53BP1, respectively (Fig. 15D). These results indicated that 53BP1 could down regulate the expression of DNMT1 and DNMT3a. In contrast, increased expression of DNMT3b (10-fold, 3-fold and 2-fold) was found in A549 cells following transfection with full-length, N-terminal, and C-terminal of 53BP1, respectively (Fig. 15E). These results suggest that DNMTs and MBD2 might have functional relevance in the pathway of 53BP1-induced DNA demethylation.

A.
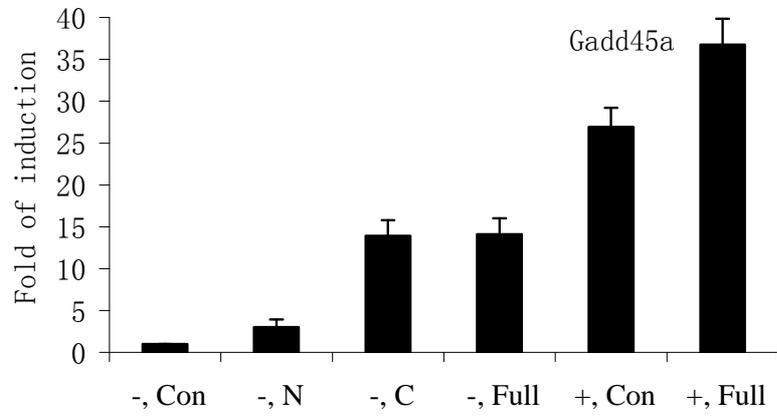
B.
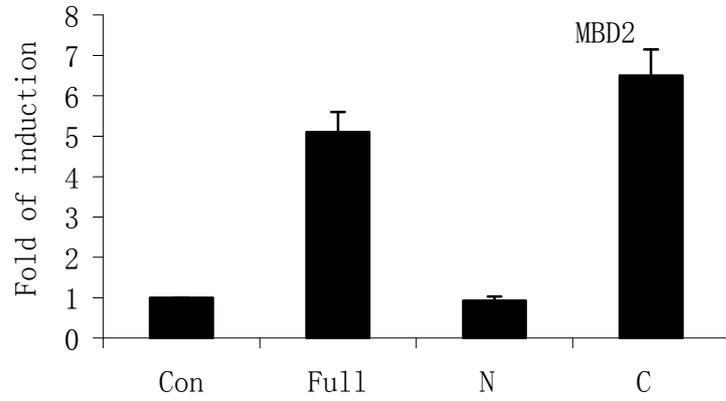
C.
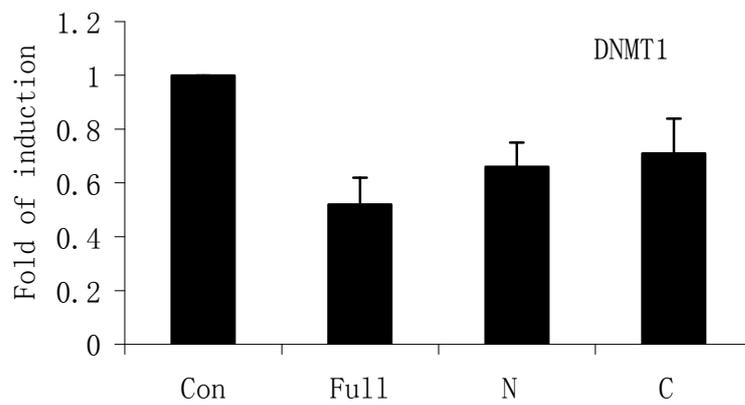
D.
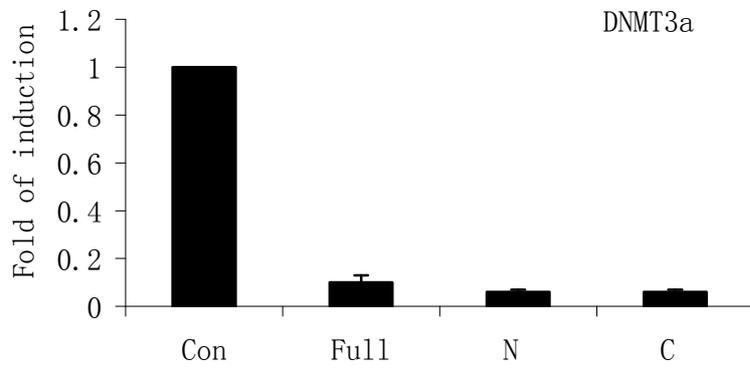
E.
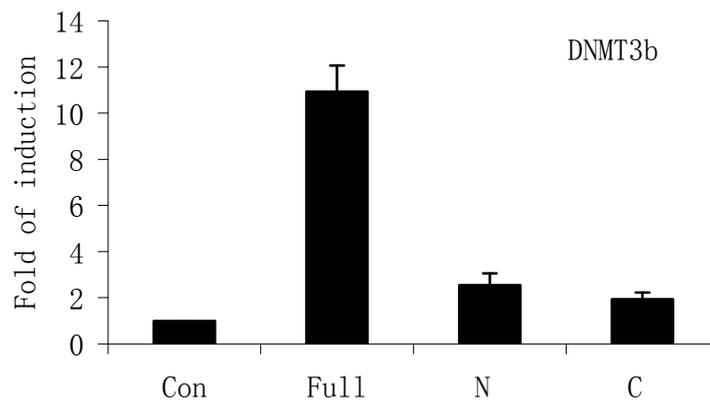
Fig. 15. The expression levels of A). Gadd45a in 53BP1-transfected and/or irradiated A549 cells. A549 cells were exposed to 6 Gy IR at 24 h after transfection. +, with IR; 一, without IR. **The expression levels of B). MBD2, C). DNMT1, D). DNMT3a and E). DNMT3b in 53BP1-transfected A549 cells.** Real time PCR was performed 72 h after transfection. Data represent mean ± SE derived from three independent experiments.

5. Discussion

In this study, the functions of 53BP1 in DNA demethylation and the expression changes of DNMTs, Gadd45a, and MBD2 induced by 53BP1 were examined in A549 cells. To the author's best knowledge, we provide the first evidence of involvement of 53BP1 in DNA demethylation process in tumor cells.

5.1. 53BP1 overexpression can promote global DNA demethylation and reactivate specific methylation-silenced gene

53BP1 has been suggested as a sensor and/or mediator of the DNA damage checkpoint in response to IR (Schultz et al., 2000; Peng et al., 2003; Wilson et al., 2008). The latter, in turn, has been previously shown to induce DNA demethylation (Tawa et al., 1998; Koturbash et al., 2005). In the present study, we focused on the possible functions of 53BP1 involved global DNA demethylation as well as gene specific demethylation. Analyzing the methylation of repetitive elements can serve as a surrogate marker for global genomic DNA methylation (Yang et al., 2004; Kim et al., 2007; Qin et al., 2007; Wilson et al., 2007). Because the repetitive elements C1S2 is frequently hypermethylated in its CpG dinucleotides in diverse cells, the effect of 53BP1 on this marker in A549 cells was analyzed using COBRA in this study. Interestingly, the overexpression of full-length cDNA as well as both N-terminal and C-terminal of 53BP1 can induce demethylation of the repetitive elements C1S2 in A549 cells.

Several recent studies have provided compelling evidence linking DNA methylation to transcriptional suppression of specific genes (Di Croce et al., 2002; Brenner et al., 2005; Esteve et al., 2005). RASSF1A is a candidate tumor suppressor gene that has recently been shown to be inactivated by methylation in many types of cancer and tumor cells including A549 cells (Beaulieu et al., 2002; Dammann et al., 2005a. b). In line with previous reports, the promoter region of RASSF1A was methylated in A549 cells resulting in transcriptional silence in this study. The overexpression of the full-length cDNA as well as both N-terminal and C-terminal of 53BP1 resulted in different intensities of DNA demethylation and restored the expression of this silenced gene analyzed by MSP and RT-PCR. Furthermore, we demonstrated that 53BP1 overexpression could

enhance DNA demethylation after DNA damage induced by irradiation, suggesting that 53BP1 may contribute to both IR induced DNA repair and DNA demethylation.

5.2. 53BP1-induced DNA demethylation is associated with DNMTs

Recent studies have demonstrated that the DNA methylation mediated by DNMTs is associated with p53 signalling in maintaining genome stability. The hypermethylation patterns in cancers have been suggested to be associated with altered changes in the DNA methylation enzymes. Several lines of evidence have suggested that 53BP1 mediated demethylation may be involved in the regulation of DNMTs activities: 1) p53 and DNMT1 cooperate to repress survivin gene expression (Esteve et al., 2005); 2) DNMT3a interacts with p53 and represses p53-mediated gene expression (Wang et al., 2005); 3) in vivo reporter assay demonstrates that 53BP1 can enhance p53-mediated transcriptional activation (Iwabuchi et al., 1998; Zhang et al., 2006; Huang et al., 2007). In this study, the expression of different DNMTs in response to overexpression of 53BP1 was investigated. The overexpression of 53BP1 resulted in upregulation of DNMT3b mRNA level. In contrast, 53BP1 gain-of-function significantly downregulated DNMT1 and DNMT3a transcriptional expression. It is surprising when considering the fact that both enzymes are de novo methyltransferases. It is noteworthy to mention that mice embryos lacking both copies of DNMT3b die before birth, whereas DNMT3a-null mice survive for about 4 weeks (Okano et al., 1999). DNMT3b has significant site selectivity that is distinct from DNMT1 (Beaulieu et al., 2002). A possible explanation for the discrepancy in the present results is that these enzymes may have distinct cell- or tissue-specific function as previously suggested (Bestor TH, 2000). In this respect, further studies of the interaction between 53BP1 and DNMTs are necessary to clarify the mechanisms of 53BP mediated DNA demethylation in different types of cells.

5.3. 53BP1 induces the activation of Gadd45a

Gadd45 plays important role in different biological processes including the maintenance of genomic stability, cell growth control, NER, chromatin accessibility and apoptosis (Smith et al., 1994; Smith et al., 2000; Hollander et al., 2001; Smith et al., 2002; Zhan et al., 2005). Barreto et al have revealed that Gadd45a is a key regulator of active DNA demethylation and it acts by promoting DNA repair, thus linking both processes (Barreto et al., 2007). The transcriptional activation of GADD45a is regulated by BRCA1 and p53, a downstream target of 53BP1, in cellular DNA damage (Wang et al., 1999). This suggests possible effects of 53BP1 in the transcriptional regulation of Gadd45a. In this study, the expression changes of Gadd45a induced by the overexpression of 53BP1 were analyzed. A remarkable increase in the transcriptional level of Gadd45a was found in cells expressing 53BP1. Furthermore, we have found that 53BP1 can enhance the up-regulation of Gadd45a in A549 cells exposed to IR. These data presented here suggest that 53BP1-induced DNA demethylation might be mediated by activation of Gadd45a.

5.4. 53BP1 induces the activation of MBD2

MBD2 has been described as a DNA demethylase (Bhattacharya et al., 1999; Detich et al., 2002, 2003). Szyf and his colleagues have reported that MBD2 purified from A549 cells is a demethylase that can transform a methyl cytosine in DNA to cytosine (Ramchandani et al., 1999; Bhattacharya et al., 1999). On the other hand, MBD2 is part of the methylated gene-repression MeCP1 complex (Stirzaker et al., 2004; David et al., 2004; Lin et al., 2003; Bakker et al., 2002, Patra et al., 2008) and has functions as a typical methylated DNA binding protein by recruitment of the chromatin remodeling complexes containing HDACs to methylated DNA (Ng et al., 1999; Wade et al., 1999). In this study, we demonstrated increased expression of MBD2 in response to overexpression of 53BP1 in A549 cells, suggesting a possible involvement of MBD2 in DNA demethylation induced by 53BP1. Noteworthy, the expression of N-terminal of 53BP1 could not regulate the transcriptional level of MBD2, whereas the expression of C-terminal and full-length cDNA of 53BP1 enhanced the mRNA expression level analyzed by RT-PCR. This discrepancy suggested that the

C-terminal 700 amino acids of 53BP1 encode important functional domains (Jullien et al., 2002; Iwabuchi et al., 2003; Koonin et al., 1996) to regulate the transcriptional level of MBD2. The genetic knock out mice with truncated 53BP1, lacking functional domains on C-terminal, has been reported to show a 53BP1 knockout phenotype (Morales et al., 2003), highlighting the importance of these domains on the C-terminal of 53BP1 and supporting our data in this study.

5.5. A suggested role of 53BP1 in DNA demethylation

Carcinogenesis can result from aberrations in genomic DNA methylation such as hypermethylation or hypomethylation of the promoter or the first exon in tumor-related genes. Hypermethylation of CpG islands associated expression silence of tumor suppressor genes has been proposed to contribute to carcinogenesis (Baylin et al., 2000; Andrew et al., 2004; Esteller M, 2007; Jones et al., 2007). The 53BP1 protein stands out as a prime model of a damage signal sensor and/or mediator in DDR (Wang et al., 2002), one of the cardinal processes for maintaining cellular homeostasis and keeping the cell from taking the neoplastic path (Phillips and McKinnon 2007; d'Adda di Fagagna, 2008). Furthermore, the DDR signifies a crossroads at which tissue development, ageing and cancer converge. Our data in present study point to 53BP1 function in DNA demethylation. However, how 53BP1 regulates different member of DNMT need to be characterized. Further experiments of the precise mechanisms of 53BP1 in DNA demethylation may clarify this association and develop therapeutic alternatives designed to promote hypomethylation and re-activation of tumor suppressor genes. These results in present study reveal a novel function of 53BP1in regulation of DNA demethylation. A model for the role of 53BP1-induced DNA demethylation is suggested.
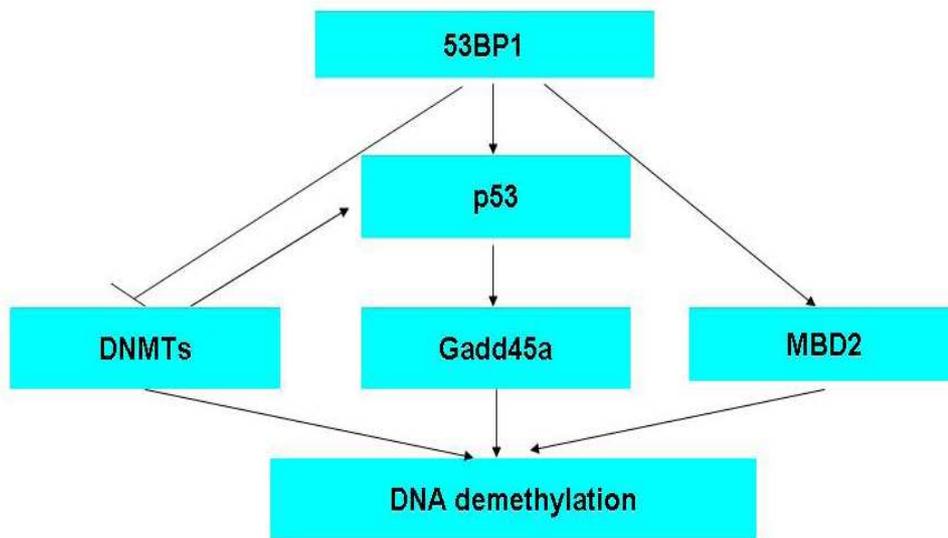
Fig. 18. A suggested model for the role of 53BP1 in DNA demethylation.
The possible signalling pathways of 53BP1 in the regulation of DNA demethylation are depicted.

6. Summary

DNA damage can be caused by various forms of genotoxic stress, including endogenous (reactive oxygen species, abnormal replication intermediates) and exogenous (UV, IR, and reactive chemicals) sources. DNA double-strand break (DSB) is believed to be one of the most serious lesions to cells because it can result in loss or rearrangement of genetic information, leading to cell death or carcinogenesis. The DNA damage response (DDR) involves multiple signal transduction pathways in that several different components act in concert to activate the cellular checkpoint. These components consist of sensors that sense DNA damage, signal transducers that generate and amplify the DNA damage signal, and effectors that induce cell cycle delay, programmed cell death, and DNA repair.

Even though several candidate proteins have been implicated in DNA damage response, an official checkpoint-specific damage sensor is still unknown. 53BP1 seems to be one of the key-sensors of DNA DSBs, upstream of ATM. The function of 53BP1 is important for coupling ATM to its downstream targets, including p53 and Gadd45a. The activation of Gadd45a as a stress protein promotes epigenetic gene *activation* by repair-mediated DNA demethylation, thus linking both processes. DNA methylation is mediated by MBD2 as well as a class of DNMTs, which encompassing DNMT1, DNMT3a and DNMT3b. Recent studies have demonstrated that the DNA methylation mediated by DNMTs is associated with p53 signalling in maintaining genome stability. Since p53 is one of the downstream targets of 53BP1, it will be of interest to investigate the functions of 53BP1 in DNA demethylation and determine the possible link between 53BP1 and these related genes.

The data presented here indicate that 53BP1 can induce DNA demethylation of single copy gene as well as repetitive elements in A549 cells. Meanwhile, the transient expression of 53BP1 can enhance DNA demethylation in combination with IR. Furthermore, the tumor suppressor gene RASSF1A was re-expressed following predominantly demethylation of CpG islands in the promoter analyzed by MSP and RT-PCR. Moreover, overexpression of 53BP1 caused a marked

decrease in DNMT1 and DNMT3a mRNA expression as well as a significant increase in Gadd45a and MBD2 mRNA expression. To our best knowledge, the present study shows for the first time the involvement of 53BP1 in DNA demethylation process. Understanding the 53BP1-mediated network will certainly have an impact on numerous fields of medicine. However, how 53BP1 regulates different member of DNMTs need to be characterized. Further experiments of the precise mechanisms of 53BP1 in DNA demethylation may clarify this association and develop therapeutic alternatives designed to promote hypomethylation and re-activation of tumor suppressor genes.

6. Zusammenfassung

Der DNA-Schaden kann durch verschiedene Formen von genotoxischem Stress, inklusive endogenen (reaktive Sauerstoffrradikale, irreguläre Replikations-produkte) sowie exogenen (UV- und ionisierende Strahlung, reaktive Chemikalien) Faktoren verursacht werden. Ein DNA-Doppelstrangbruch (DSB) ist einer der schwerwiegendsten DNA-Schäden in der Zelle und kann zur Veränderung genetischen Information, Auslösung einer Reparaturantwort oder zum Zelltod bzw. Karzinogenese führen. Die Zellantwort auf dem DNA-Schaden involviert mehrere Signaltransduktionswege, in dem mehrere verschiedene Komponenten zur Aktivierung der Zellkontrollpunkte zusammen wirken. Die Komponenten bestehen aus den Sensoren, den Signaltransduktoren, welche das DNA-Schadenssignal erkennen und amplifizieren sowie den Effektoren, die den Zellzyklusarrest, den programmierten Zelltod und die DNA-Reparatur induzieren.

Zwar sich an der Zellantwort des DNA-Schadens mehre Kandidatproteine beteiligen, ist ein spezifischer Sensor für die zelluläre Schadensantwort noch unbekannt. Das funktionelle „upstream" Protein von ATM/ATR, 53BP1, hat sich als einer der Sensoren für DNA-DSB, herausgestellt und spielt eine wichtige Rolle bei der Anbindung des ATM zu seinen abwärts Targetproteinen inklusive p53 und Gadd45. Die Aktivierung des Gadd45 Proteins führt zur epigenetischen Genaktivierung durch die Reparatur-vermittelte DNA-Demethylierung und verbindet beide Prozesse. Die DNA Methylierung wird durch MBD2 sowie eine Klasse von DNMTs, welche DNMT1, DNMT3a und DNMT3b einschließen, vermittelt. Neue Studien haben gezeigt, dass die DNMTs-vermittelte DNA Methylierung assoziiert mit dem p53 Signaltransdutionsweg zur Beibehaltung der Genomstabilität ist. Da p53 eines der abwärts gelegenen Targetgene vom 53BP1 ist, wird es von Interesse sein, um die Funktionen 53BP1 in der DNA-Demethylierung zu untersuchen und die mögliche Verbindung zwischen 53BP1 und diese zusammenhängenden Gene zu bestimmen.

Die experimentiellen Daten der vorliegenden Arbeit haben gezeigt, dass die transiente Expression des 53BP1 in A549 Zellen die DNA-Demethylierung von

einzelnen Kopiegenen sowie repetitiven Elementen induzieren konnte. Desweiteren konnte 53BP1 die DNA-Demethylierung durch ionisierte Strahlung erhöhen. mit Hilfe der MSP und RT-PCR konnte die Demethylierung dessen Promoters und Reaktivierung des silenten Tumorsuppressorgens RASSF1A untersucht werden. Ausserdem führte die Überexpression von 53BP1 zu einer signifikant reduzierten Expression von DNMT1 und DNMT3a sowie erhörten Expression von Gadd45a und MBD2. Es ist das erste Mal gezeigt worden, dass 53BP1 in der DANN-Demethylierung beteiligt sein könnte. Weitere Experimente werden durchgeführt werden müssen, um die genauen Mechanismen des 53BP1 bei der DNA-Methylierung zu klären und therapeutische Alternativen zu entwickeln, die die Demethylierung und Reaktivierung von Tumorsuppressorgenen fördern.

7. Reference

Abraham RT. (2002) Checkpoint signalling: focusing on 53BP1. Nat Cell Biol. 12: 277-279.

Adams MM, Carpenter PB. (2006) Tying the loose ends together in DNA double strand break repair with 53BP1. Cell Div. 31: 1-19.

Andrew P, Feinberg, Benjamin Tycko. (2004) The history of cancer epigenetics. Nature Reviews Cancer. 4; 143-153.

Aapola U, Liiv I, Peterson P. (2002) Imprinting regulator DNMT3L is a transcriptional repressor associated with histone deacetylase activity. Nucleic Acids Res. 30: 3602-3608.

Bachman KE, Park BH, Rhee I, Rajagopalan H, Herman JG, Baylin SB, Kinzler KW, Vogelstein B. (2003) Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. Cancer Cell. 3: 89-95.

Barreto G, Schäfer A, Marhold J, Stach D, Swaminathan SK, Handa V, Döderlein G, Maltry N, Wu W, Lyko F, Niehrs C. (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature. 445: 671-675.

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C. (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 434: 864-870.

Baylin SB and Herman JG. (2000) DNA hypermethylation in tumorigenesis: epigenetics joins genetics. TIG. 16: 168-174.

Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv. Cancer Res. 72: 141-196.

Beaulieu N, Morin S, Chute IC, Robert MF, Nguyen H, MacLeod AR. (2002) An essential role for DNA methyltransferase DNMT3B in cancer cell survival. J Biol Chem. 277: 28176-28181.

Belinsky SA, Nikula KJ, Baylin SB, Issa JP. (1996) Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. Proc. Natl. Acad. Sci. USA. 93: 4045-4050.

Bestor TH. (2000) The DNA methyltransferases of mammals. Hum Mol Genet. 9: 2395-2402.

Bhattacharya SK, Ramchandani S, Cervoni N and Szyf M. (1999) A mammalian protein with specific demethylase activity for mCpG DNA. Nature. 397: 579-583.

Bird A. (2002) DNA methylation patterns and epigenetic memory. Genes Dev. 16: 6-21.

Bork P, Hofmann K, Bucher P, Neuwald AF, Altschul SF, Koonin EV. (1997) A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. FASEB J. 11: 68-76.

Botuyan MV, Lee J, Ward IM, Kim JE, Thompson JR, Chen J, Mer G. (2006) Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. Cell. 127: 1361-1373.

Brenner C, Deplus R, Didelot C, Loriot A, Vire E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, Pelicci PG, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks F. (2005) Myc represses transcription through recruitment of DNA methyltransferase corepressor. EMBO J. 24: 336-346.

Bussolati B, Deregibus MC, Fonsato V, Doublier S, Spatola T, Procida S, Di Carlo F, Camussi G. (2005) Statins prevent oxidized LDL-induced injury of glomerular podocytes by activating the phosphatidylinositol 3-kinase/AKT-signalling pathway. J Am Soc Nephrol. 16: 1936-1947.

Carrier F, Georgel PT, Pourquier P, Blake M, Kontny HU, Antinore MJ, Gariboldi M, Myers TG, Weinstein JN, Pommier Y, Fornace AJ Jr. (1999) Gadd45, a p53-responsive stress protein, modifies DNA accessibility on damaged chromatin. Mol Cell Biol. 19: 1673-1685.

Celeste A, Difilippantonio S, Difilippantonio MJ, Fernandez-Capetillo O, Pilch DR, Sedelnikova OA, Eckhaus M, Ried T, Bonner WM, Nussenzweig A. (2003) H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. Cell. 114:371-383.

Cervoni N and Szyf M. (2001) Demethylase activity is directed by histone acetylation. J. Biol. Chem. 276: 40778-40787.

Côté J, Richard S. (2005) Tudor domains bind symmetrical dimethylated arginines. J Biol Chem. 280: 28476-28483.

d'Adda di Fagagna F (2008). Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer. 8: 512-22. Review.

Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. (2000) Epigenetic inactivation of a RAS association domain family protein from the lung tumor suppressor locus 3p21.3. Nat Genet. 25: 315-319.

Dammann R, Schagdarsurengin U, Seidel C, Trümpler C, Hoang-Vu C, Gimm O, Dralle H, Pfeifer GP, Brauckhoff M. (2005a) Frequent promoter methylation of tumor-related genes in sporadic and men2-associated pheochromocytomas. Exp Clin Endocrinol Diabetes. 113: 1-7.

Dammann R, Strunnikova M, Schagdarsurengin U, Rastetter M, Papritz M, Hattenhorst UE, Hofmann HS, Silber RE, Burdach S, Hansen G. (2005b) CpG island methylation and expression of tumour-associated genes in lung carcinoma, Eur J Cancer. 41: 1223-1236.

De Marzo AM, Marchi VL, Yang ES, Veeraswamy R, Lin X, Nelson WG. (1999) Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res. 59: 3855-3860.

Detich N, Bovenzi V, Szyf M. (2003) Valproate induce replication-independent active DNA demethylation J. Biol. Chem. 278: 27586-27592.

Detich N, Hamm S, Just G, Knox JD, Szyf M. (2003) The methyl donor S-Adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-Adenosylmethionine. J. Biol. Chem. 278: 20812-20820.

Detich N, Theberge J, Szyf M. (2002) Promoter-specific activation and demethylation by MBD2/demethylase. J. Biol.Chem. 277: 35791-35794.

Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, Fuks F, Lo Coco F, Kouzarides T, Nervi C, Minucci S, Pelicci PG. (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science. 295: 1079-1082.

DiTullio RA Jr, Mochan TA, Venere M, Bartkova J, Sehested M, Bartek J, Halazonetis TD. (2002) 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol. 4: 998-1002.

David SS, O'Shea VL, Kundu S. (2007) Base-excision repair of oxidative DNA damage. Nature. 447:941-950. Review.

Donninger H, Vos MD, Clark GJ. (2007) The RASSF1A tumor suppressor. J Cell Sci. 15: 3163-3172.

Dote H, Cerna D, Burgan WE, Carter DJ, Cerra MA, Hollingshead MG, Camphausen K, Tofilon PJ. (2005) Enhancement of in vitro and in vivo tumor

cell radiosensitivity by the DNA methylation inhibitor zebularine. Clin Cancer Res. 11: 4571-4579.

Ehrlich M, Hopkins NE, Jiang G, Dome JS, Yu MC, Woods CB, Tomlinson GE, Chintagumpala M, Champagne M, Dillerg L. (2003) Satellite DNA hypomethylation in karyotyped Wilms tumors. Cancer Genet. Cytogenet. 141: 97-105.

Esteller M. (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet; 8: 286-298.

Estève PO, Chin HG, Pradhan S. (2005) Human maintenance DNA (cytosine-5)-methyltransferase and p53 modulate expression of p53-repressed promoters. Proc Natl Acad Sci U S A. 102:1000-1005.

Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, Welsh W, Yang CS. (2003) Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res. 63: 7563-7570.

Fournel M, Sapieha P, Beaulieu N, Besterman JM, MacLeod AR. (1999) Down-regulation of human DNA-(cytosine-5) methyltransferase induces cell cycle regulators p16(ink4A) and p21(WAF/Cip1) by distinct mechanisms. J Biol Chem. 274: 24250-24256.

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA. 89: 1827-1831.

Goel A, Mathupala SP, Pedersen PL. (2003) Glucose metabolism in cancer. Evidence that demethylation events play a role in activating type II hexokinase gene expression. J Biol Chem. 278: 15333-15340.

Goldberg M, Stucki M, Falck J, D'Amours D, Rahman D, Pappin D, Bartek J, Jackson SP. (2003) MDC1 is required for the intra-S-phase DNA damage checkpoint. Nature. 421: 952-956.

Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr, Kastrinakis NG, Levy B, et al. (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 434: 907-913.

Grønbaek K, Hother C, Jones PA. (2007) Epigenetic changes in cancer. APMIS. 115: 1039-1059.

Guo Y, Pakneshan P, Gladu J, Slack A, Szyf M, Rabbani SA. (2002) Regulation of DNA methylation in human breast cancer. Effect on the urokinase-type plasminogen activator gene production and tumor invasion. J Biol Chem. 277: 41571-41579.

Haaf T. (2006) Methylation dynamics in the early mammalian embryo: implications of genome reprogramming defects for development. Curr Top Microbiol Immunol. 310: 13-22.

Hansen RS, Wijmenga C, Luo P, Stanek AM, Canfield TK, Weemaes CM, Gartler SM. (1999) The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. Proc. Natl. Acad. Sci. USA. 96: 14412-14417.

Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD, Haber DA. (1999) Induction of GADD45 and JNK/SAPKdependent apoptosis following inducible expression of BRCA1. Cell. 97: 575-586.

Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. (1996) Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA. 93: 9821-9826.

Henikoff S. (2008) Nucleosome destabilization in the epigenetic regulation of gene expression. Nat Rev Genet. 9:15-26.

Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, Fornace AJ Jr. (2002) Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signalling via MAPK and p53. Cancer Res. 62: 7305-7315.

Hollander MC, Fornace AJ Jr. (2002) Genomic instability, centrosome amplification, cell cycle checkpoints and Gadd45a. Oncogene. 21: 6228-6233.

Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-Salguero PM, Morgan WF, Deng CX, Fornace AJ Jr. (1999) Genomic instability in Gadd45a-deficient mice. Nat. Genet. 23: 176-184.

Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC, Fornace AJ Jr. (2001) Dimethylbenzanthracene carcinogenesis in Gadd45a-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Res. 61: 2487-2491.

Huang J, Sengupta R, Espejo AB, Lee MG, Dorsey JA, Richter M, Opravil S, Shiekhattar R, Bedford MT, Jenuwein T, Berger SL. (2007) 53 is regulated by the lysine demethylase LSD1. Nature. 449: 105-108.

Huyen Y, Zgheib O, Ditullio RA Jr, Gorgoulis VG, Zacharatos P, Petty TJ, Sheston EA, Mellert HS, Stavridi ES , Halazonetis TD. (2004) Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature. 432: 406-411.

Iwabuchi K, Bartel PL, Li B, Marraccino R, Fields S. (1994) Two cellular proteins that bind to wild-type but not mutant p53. Proc Natl Acad Sci USA. 91: 6098-6102.

Iwabuchi K, Li B, Massa HF, Trask BJ, Date T, Fields S. (1998) Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2, J. Biol. Chem. 273: 26061-26068.

Iwabuchi K, Basu BP, Kysela B, Kurihara T, Shibata M, Guan D, Cao Y, Hamada T, Imamura K, Jeggo PA, Date T, Doherty AJ. (2003) Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. J. Biol. Chem. 278: 36487-36495.

Jeanpierre M. (1994) Human satellites 2 and 3. Ann. Genet. 37: 163–171.

Jin S, Antinore MJ, Lung FD, Dong X, Zhao H, Fan F, Colchagie AB, Blanck P, Roller PP, Fornace AJ Jr, Zhan Q. (2000) The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J. Biol. Chem. 275: 16602-16608.

Jones PA. (1999) The DNA methylation paradox. Trends Genet. 15: 34-37.

Jones PA, Laird PW. (1999) Cancer epigenetics comes of age. Nature Genet. 21: 163-166.

Jones PA, Baylin SB. (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet. 3: 415-428.

Jones PA, Baylin SB. (2007). The epigenomics of cancer. Cell. 128: 683–692.

Jost JP, Bruhat A. (1997) The formation of DNA methylation patterns and the silencing of genes. Prog. Nucleic Acid Res. Mol. Biol. 57: 217-248.

Jost JP, Siegmann M, Sun LJ, Leung R. (1995) Mechanisms of DNA demethylation in chicken embryos purification and properties of a 5-methylcytosine-DNA glycosylase. J. Biol. Chem. 270: 9734-9739.

Jost JP, Oakeley EJ, Zhu B, Benjamin D, Thiry S, Siegmann M, Jost YC. (2001) 5-Methylcytosine DNA glycosylase participates in the genomewide loss of DNA methylation occurring during mouse myoblast differentiation. Nucleic Acids Res. 29: 4452-4461.

Jullien D, Vagnarelli P, Earnshaw WC, Adachi Y. (2002) Kinetochore localisation of the DNA damage response component 53BP1 during mitosis. J. Cell Sci. 115: 71-79.

Kachirskaia I, Shi X, Yamaguchi H, Tanoue K, Wen H, Wang EW, Appella E, Gozani O. (2008) Role for 53BP1 tudor domain recognition of p53 dimethylated at lysine 382 in DNA damage signalling. J Biol Chem. Oct 7. [Epub ahead of print]

Kafri T, Gao X, Razin A. (1993) Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo. Proc Natl Acad Sci U S A. 90: 10558-10562.

Kalinich JF, Catravas GN, Snyder SL. (1989) The effect of gamma radiation on DNA methylation. Radiat Res. 117: 185-197.

Kanai Y, Ushijima S, Kondo Y, Nakanishi Y, Hirohashi S. (2001) DNA methyltransferase expression and DNA methylation of CPG islands and peri-centromeric satellite regions in human colorectal and stomach cancers. Int J Cancer. 91: 205-212.

Kangaspeska S, Stride B, Metivier R, Polycarpou-Schwarz M, Ibberson D, Carmouche RP, Benes V, Gannon F, Reid G. (2008) Nature. 452: 112-115.

Kao GD, McKenna WG, Guenther MG, Muschel RJ, Lazar MA, Yen TJ. (2003) Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. J Cell Biol. 160: 1017-1027.

Kapoor A, Agius F, Zhu JK. (2005) Preventing transcriptional gene silencing by active DNA demethylation. FEBS Lett. 579:5889-5898.

Khokhlatchev A, Rabizadeh S, Xavier R, Nedwidek M, Chen T, Zhang XF, Seed B, Avruch J. (2002) Identification of a novel Ras-regulated proapoptotic pathway. Curr. Biol. 12: 253-265.

Kim DH, Kim JS, Park JH, Lee SK, Ji YI, Kwon YM, Shim YM, Han J, Park J. (2003) Relationship of Ras association domain family 1 methylation and K-ras mutation in primary non-small cell lung cancer. Cancer Res. 63: 6206-6211.

Kim HH, Park JH, Jeong KS, Lee S. (2007) Determining the global DNA methylation status of rat according to the identifier repetitive elements. Electrophoresis. 28: 3854-3861.

Kim J, Daniel J, Espejo A, Lake A, Krishna M, Xia L, Zhang Y, Bedford MT. (2006) Tudor, MBT and chromo domains gauge the degree of lysine methylation. EMBO Rep. 7: 397-403.

Kis E, Szatmári T, Keszei M, Farkas R, Esik O, Lumniczky K, Falus A, Sáfrány G. (2006) Microarray analysis of radiation response genes in primary human fibroblasts. Int J Radiat Oncol Biol Phys. 66: 1506-1514.

Koonin EV, Altschul SF, Bork P. (1996) BRCA1 protein products Functional motifs. Nat. Genet. 13: 266-268.

Koturbash I, Pogribny I, Kovalchuk O. (2005) Stable loss of global DNA methylation in the radiation-target tissue--a possible mechanism contributing to radiation carcinogenesis? Biochem Biophys Res Commun. 337: 526-533.

Kress C, Thomassin H, Grange T. (2001) Local DNA demethylation in vertebrates: how could it be performed and targeted. FEBS Lett. 494: 135-140.

Kress C, Thomassin H, Grange T. (2006) Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. Proc Natl Acad Sci U S A. 103:11112-11117.

Kuzmin I, Gillespie JW, Protopopov A, Geil L, Dreijerink K, Yang Y, Vocke CD, Duh FM, Zabarovsky E, Minna JD, Rhim JS, Emmert-Buck MR, Linehan WM, Lerman MI. (2002) The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. Cancer Res. 62: 3498-3502.

Lai JC, Cheng YW, Goan YG, Chang JT, Wu TC, Chen CY, Lee H. (2008) Promoter methylation of O(6)-methylguanine-DNA-methyltransferase in lung cancer is regulated by p53. DNA Repair. 2: 1352-1363

Li E, Bestor TH, Jaenisch R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell. 69: 915-926.

Li E. (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nature Rev. Genet. 3: 662-673.

Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, Chow LS, Teo PM, Johnson PJ, Huang DP. (2001) High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res. 61: 3877-3881.

Manke IA, Lowery DM, Nguyen A, Yaffe MB. (2003) BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science. 302: 636-639.

Murayama A, Sakura K, Nakama M, Yasuzawa-Tanaka K, Fujita E, Tateishi Y, Wang Y, Ushijima T, Baba T, Shibuya K, Shibuya A, Kawabe Y, Yanagisawa J.

(2006) A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. EMBO J. 25:1081-1092.

Métivier R, Gallais R, Tiffoche C, Le Peron C, Jurkowska RZ, Carmouche RP, Ibberson D, Barath P, Demay F, Reid G, et al. (2008). Nature 452: 45-50.

Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJM. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J. 19: 5194-5201.

Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, Sasaki H. (2001) Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood. 97: 1172-1179.

Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. (2003) 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. Cancer Res. 63: 8586-8591.

Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. (2004) 53BP1, an activator of ATM in response to DNA damage. DNA Repair. 3: 945-952.

Morales JC, Xia Z, Lu T, Aldrich MB, Wang B, Rosales C, Kellems RE, Hittelman WN, Elledge SJ, Carpenter PB. (2003) Role for BRCA1 Cterminal repeats (BRCT) protein 53BP1 in maintaining genomic stability. J. Biol. Chem. 278: 14971-14977.

Morales JC, Franco S, Murphy MM, Bassing CH, Mills KD, Adams MM, Walsh NC, Manis JP, Rassidakis GZ, Alt FW, Carpenter PB. (2006) 53BP1 and p53 synergize to suppress genomic instability and lymphomagenesis. Proc Natl Acad Sci U S A. 103: 3310-3315.

Mund C, Hackanson B, Stresemann C, Lübbert M, Lyko F. (2005) Characterization of DNA demethylation effects induced by 5-Aza-2'-deoxycytidine in patients with myelodysplastic syndrome. Cancer Res. 65: 7086-7090.

Murzina N, Verreault A, Laue E, Stillman B. (1999) Heterochromatin dynamics in mouse cells: interaction between chromatin assembly factor 1 and HP1 proteins. Mol. Cell. 4: 529-540.

Okano M, Bell DW, Haber DA, Li E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 99: 247-257.

Panayiotidis MI, Rancourt RC, Allen CB, Riddle SR, Schneider BK, Ahmad S, White CW. (2004) Hyperoxia-induced DNA damage causes decreased DNA methylation in human lung epithelial-like A549 cells. Antioxid Redox Signal. 6: 129-136.

Patra SK, Patra A, Rizzi F, Ghosh TC, Bettuzzi S. (2008) Demethylation of (Cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development. Cancer Metastasis Rev. 27:315-334.

Peng A, Chen PL. (2003) NFBD1, like 53BP1, is an early and redundant transducer mediating Chk2 phosphorylation in response to DNA damage. J Biol Chem.   278: 8873-8876.

Phillips ER, McKinnon PJ. (2007) DNA double-strand break repair and development. Oncogene. 26: 7799-7808. Review.

Ponting CP. (2004) Tudor domains in proteins that interact with RNA. Trends Biochem. Sci. 22:51-52.

Qin T, Youssef EM, Jelinek J, Chen R, Yang AS, Garcia-Manero G, Issa JP. (2007) Effect of cytarabine and decitabine in combination in human leukemic cell lines. Clin Cancer Res. 13: 4225-4232.

Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M. (1999) DNA methylation is a reversible biological signal. Proc. Natl. Acad. Sci. USA. 96: 6107-6112.

Rappold I, Iwabuchi K, Date T, Chen J. (2001) Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signalling pathways. J Cell Biol. 153: 613-620.

Razin A, Riggs AD. (1980) DNA methylation and gene function. Science. 210: 604-610.

Reik W, Dean W, & Walter J. (2001). Epigenetic reprogramming in mammalian development. Science. 293: 1089-1093.

Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR. (2003) DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat. Genet. 33: 61-65.

Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. (2000) DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nature Genet. 25: 338-342.

Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, Jones PA. (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic Acids Res. 27: 2291-2298.

Rudin CM, Thompson CB. (2001) Transcriptional activation of short interspersed elements by DNA-damaging agents. Genes Chromosomes Cancer. 30: 64-71.

Sansom OJ, Maddison K, Clarke AR. (2007) Mechanisms of disease: methyl-binding domain proteins as potential therapeutic targets in cancer. Nat Clin Pract Oncol. 4: 305-315.

Schmutte C, Jones PA. (1998) Involvement of DNA methylation in human carcinogenesis. Biol Chem. 379: 377-388.

Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. (2000) p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. J. Cell Biol. 151: 1381-1390.

Shahrzad S, Bertrand K, Minhas K, Coomber BL. (2007) Induction of DNA hypomethylation by tumor hypoxia. Epigenetics. 2: 119-125.

Sheikh MS, Hollander MC, Fornance AJ Jr. (2000) Role of Gadd45 in apoptosis. Biochem Pharmacol. 59: 43-45.

Shiloh Y. (2003) ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer. 3: 155-168.

Sims RJ 3rd, Nishioka K, Reinberg D. (2003) Histone lysine methylation: a signature for chromatin function.Trends Genet. 19: 629-639.

Sims RJ 3rd, Reinberg D. (2008) Is there a code embedded in proteins that is based on post-translational modifications?　Nat Rev Mol Cell Biol. 9: 815-820.

Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM, Fornace AJ Jr. (1994) Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science. 266: 1376-1380.

Smith ML, Ford JM, Hollander MC, Bortnick RA, Amundson SA, Seo YR, Deng CX, Hanawalt PC, Fornace AJ Jr. (2000) p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol. Cell. Biol. 20: 3705-3714.

Smith ML, Seo YR. (2002) p53 regulation of DNA excision repair pathways. Mutagenesis. 17: 149-156.

Stresemann C, Lyko F. (2008) Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. Int J Cancer. 123: 8-13.

Szyf M. (2005) DNA methylation and demethylation as targets for anticancer therapy. Biochemistry. 5: 533-549.

Szyf M, Eliasson L, Mann V, Klein G, Razin A. (1985) Cellular and viral DNA hypomethylation associated with induction of Epstein-Barr virus lytic cycle. Proc Natl Acad Sci. USA. 82: 8090-8094.

Takekawa M, Saito H. (1998) A family of stress-inducible GADD45- like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell. 95: 521-530.

Tawa R, Kimura Y, Komura J, Miyamura Y, Kurishita A, Sasaki MS, Sakurai H, Ono T. (1998) Effects of X-ray irradiation on genomic DNA methylation levels in mouse tissues. J Radiat Res. 39: 271-278.

Vavvas D, Li X, Avruch J, Zhang XF. (1998) Identification of Nore1 as a potential ras effector. J. Biol. Chem. 273: 5439-5442.

Villar-Garea A, Esteller M. (2003) DNA demethylating agents and chromatin-remodelling drugs: which, how and why?. Curr. Drug Metab. 4: 11-31.

Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ. (2000) Ras uses the novel tumor suppressor RASSF1A as an effector to mediate apoptosis. J. Biol. Chem. 275: 35669-35672,.

Wang B, Matsuoka S, Carpenter PB, Elledge SJ. (2002) 53BP1, a mediator of the DNA damage checkpoint. Science. 298:1435-1438.

Wang GL, Semenza GL. (1993) Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem. 268: 21513-21518.

Wang YA, Kamarova Y, Shen KC, Jiang Z, Hahn MJ, Wang Y, Brooks SC. (2005) DNA methyltransferase-3a interacts with p53 and represses p53-mediated gene expression. Cancer Biol Ther. 4: 1138-1143.

Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ Jr, Harris CC. (1999) GADD45 induction of a G2/M cell cycle checkpoint, Proc. Natl. Acad. Sci. USA. 96: 3706-3711.

Ward IM, Minn K, van Deursen J, Chen J. (2003) p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. Mol Cell Biol. 23: 2556-2563.

Ward IM, Difilippantonio S, Minn K, Mueller MD, Molina JR, Yu X, Frisk CS, Ried T, Nussenzweig A, Chen J. (2005) 53BP1 cooperates with p53 and functions as a haploinsufficient tumor suppressor in mice. Mol Cell Biol. 25: 10079-10086.

Weiss A, Keshet I, Razin A, Cedar H. (1996) DNA demethylation in vitro: involvement of RNA. Cell. 86: 709-718.

Weiss A, Cedar H. (1997) The role of DNA demethylation during development. Cells. 2: 481-486.

Wilson AS, Power BE, Molloy PL. (2007) DNA hypomethylation and human diseases. Biochim Biophys Acta. 1775:138-162.

Wilson KA, Stern DF. (2008) NFBD1/MDC1, 53BP1 and BRCA1 have both redundant and unique roles in the ATM pathway. Cell Cycle. 7: 3584-3594.

Xiong Z, Laird PW. (1997) COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 25: 2532-2534.

Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Péquignot E. (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature. 402: 187-191.

Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 32-38.

Yoo CB, Jones PA. (2006) Epigenetic therapy of cancer: past, present and future. Nat Rev Drug Discov;5: 37-50.

Zgheib O, Huyen Y, DiTullio RA Jr, Snyder A, Venere M, Stavridi ES, Halazonetis TD. (2005) ATM signalling and 53BP1. Radiother Oncol. 76: 119-122.

Zhan Q. (2005) Gadd45a, a p53- and BRCA1-regulated stress protein, in cellular response to DNA damage. Mutat Res. 569: 133-143.

Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC, Fornace AJ Jr. (1999) Association with Cdc2 and inhibition of Cdc2/cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene. 18: 2892-2900.

Zhan Q, Jin S, Ng B, Plisket J, Shangary S, Rathi A, Brown KD, Baskaran R. (2002) Caspase-3 mediated cleavage of BRCA1 during UV-induced apoptosis. Oncogene. 21: 5335-5345.

Zhang D, Zaugg K, Mak TW, Elledge SJ. (2006) A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response. Cell. 126: 529-542.

Zhou BB, Elledge SJ. (2000) The DNA damage response: putting checkpoints in perspective. Nature. 408: 433-439.

8. Attachment

8.1. Abbreviation

ATC	American Type Culture Collection
ATM	kinase ataxia-telangeictasia-mutated
ATR	ataxia telangiectasia and rad3-related
Bp	base pair
CHK1	Checkpoint Kinase 1
CDNA	complementary DNA
CHK2	Checkpoint Kinase 2
C1S2	chromosome 1 satellite 2
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMTs	DNA methyltransferases
DSBs	DNA double-strand breaks
dNTPs	deoxynucleotide triphosphates
EB	ethidium bromide
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovine serum
Gadd gene	DNA damage-inducible gene
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
HAT	histone acetyl transferases
HDACs	histone deacetylases
IR	irradiation
NER	nucleotide excision repair
Nt	nucleotide
OD	optical density
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
Rmp	round per minute
RNA	ribonucleic acid
RT	room temperature

RT-PCR	reverse transcription PCR
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
TAF	Trans-acting activating factors
TBE	Tris-boric acid, EDTA buffer
TEMED	N,N,N',N'-Tetraethylendiamin
TFs	transcription factors
TR	trans-acting repressors
Tris	Tris (hydroxymethyl) aminomethane
UV	ultraviolet radiation

8.2. Curriculum Vitae

Family Name: Wang

First Name: Linfang

Birth date: 13/11/71

Nationality: P.R. China

Contact:

Tel: 49-6421-2866270 (lab)

49-0176-65366654 (Mobile)

49-6421-2828126 (home)

Email: wang@staff.uni-marburg.de

wanglinfang2006@hotmail.com

Address: Baldingerstr., 35043, Marburg, Germany

Education Background:

07, 2006 ---- 12, 2007. Student of Dr. med.

Radiation Therapy and Radiooncology Department

Philipps-University of Marburg, Germany

Dissertation Project: Investigation of the functions of 53BP1 in DNA demethylation

09, 2001 ---- 07, 2004. Master of Clinical Medicine

Department of Emergency Surgery

Tongji Medical College, Huazhong University of Science and Technology,

Wuhan, P.R. China.

Dissertation Project: The change of toll-like receptor expression between early and delayed surgical treatment in severe acute pancreatitis

09, 1988 ----- 07, 1993. Bachelor of Clinical Medicine

Department of Clinical Medicine

Tongji Medical College, Huazhong University of Science and Technology, Wuhan, P.R. China

Working Experience:

07, 1993 ---- 06, 2006. Resident doctor and Surgeon-in-charge

Department of Emergency Surgery,

Union Hospital, Tongji Medical College, Huazhong University of Science and

Technology, Wuhan, P.R. China

8.3. Publication

Increased expression of EphA2 correlates with adverse outcome in primary and recurrent glioblastoma multiform patients. **Lin-Fang Wang,** Emmanouil Fokas, Michael Bieker, Frank Rose, Peter Rexin, Yuan Zhu, Axel Pagenstecher, Rita Engenhart-Cabillic and Han-Xiang An. Oncology reports. 2008, 19: 151-156.

Increased expression of EphA7 correlates with adverse outcome in primary and recurrent glioblastoma multiform patients. **Lin-Fang Wang**, Emmanouil Fokas, Janko Juricko, An You, Frank Rose, Axel Pagenstecher, Rita Engenhart-Cabillic and Han-Xiang An. BioMed Central Cancer 2008, 25: 8:79

EphA2 blockade reduces radiation-induced angiogenesis in non-small cell lung cancer cells. Emmanouil Fokas, **Lin-Fang Wang**, Michael Bieker, Peter Rexin, Axel Pagenstecher, Rita Engenhart-Cabillic, Han-Xiang An. 14th AEK International Cancer Congress. Frankfurt, Germany, March 2007.

Siah1 ubiquitin ligase enhances radiation response of breast cancer cells. Haitao He, Emmanouil Fokas, **Lin-Fang Wang**, Beta Kleb, Rita Engenhart-Cabillic, Han-Xiang An. 14th AEK International Cancer Congress. Frankfurt, Germany, March 2007.

8.4. Academic teacher

I would like to take this opportunity to thank all of the academic teachers.

Bo Huang, Chuanguo Xiao, Chunfang Jiang, Chunyou Wang, Guobing Wang, Guohui Liu, Heshui Wu, Hong Chen, Hong Li, Hong Wang, Jiyuan Han, Jinyuan Du, Jianshe Xiong, Jinkun Zhang, Kaifang Zhou, Lin Chen, Mingchang Zhang, Ping Zhou, Qichang zhen, Qixing Zhen, Shouxi Zhang, Xiaohua Hou, Xiaoming Lu, Xun Cui, Yancai Zhou, Yaoping Long, Yingjie Wang, Yousheng Zhou, Yuming Li, Zhehua Wang, Zhengliang Chen.

8.5. Declaration

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Humanmedizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

Investigation of the functions of 53BP1 in DNA demethylation

am medizinischen Zentrum für Radiologie，der Klinik für Strahlentherapie und Radioonkologie, unter Leitung von Frau Prof. Dr. med. R. Engenhart-Cabillic ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Ich habe bisher weder an einem in- und ausländischem medizinischem Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit oder eine andere Arbeit als Dissertation vorgelegt.

Marburg, 20. February 2008

8.6. Acknowledgement

Here, I would like to thank all people who have helped and inspired me during my doctor study. I especially want to thank my mentor, Prof. Dr. R. Engenhart-Cabillic, for her direction on my research project and helpful suggestion on my manuscript. Her perpetual energy and persistent enthusiasm in research had motivated me very much. Dr. HX An deserves special thanks as constructive guidance during my research at the Philipps-University Marburg. In addition, he was always accessible and willing to help me with my research. As a result, research life became smooth and rewarding for me.

Furthermore, my deepest gratitude goes to my family for their unflagging love and support throughout my life.

Last but not least, all my colleagues at the Labor made it a convivial place to perform research works. In particular, I would like to thank Mrs. Kleb Beate from Department of Radiology, and Mrs. H. Geißel and R. Wassmuth from Department of Neuropathology for their excellent technical assistances. I acknowledge with utmost sincerity, the friendly atmosphere I have enjoyed, with other staffs of institute. Despite my handicap in German language, they made life much easier.