UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Aus dem Labor für Strahlenbiologie und Experimentelle Radioonkologie

der Klinik für Strahlentherapie und Radioonkologie

des Onkologischen Zentrums

Leiter Prof. Dr. rer. nat. Ekkehard Dikomey

The Influence of Cadmium on the Repair of DNA Double-Strand Breaks

Dissertation

Zur Erlangung des Grades eines Doktors der Medizin

an der Medizinischen Fakultät der Universität Hamburg

Vorgelegt von:

Audrey Anne MacDougall aus Southfield, MI, USA

Hamburg 2014

Angenommen von der medizinischen Fakultät am: 28.05.2014

Veröffentlicht mit Genehmigung der medizinischen Fakultät der Universität Hamburg

Prüfungsausschuss, der Vorsitzende: Dr. med. Jochen Dahm-Daphi

Prüfungsausschuss, 2. Gutachter: Prof. Dr. rer. nat. Ekkehard Dikomey

Prüfungsausschuss, 3. Gutachter: PD Dr. H.-J. Kreienkamp

Outline

1. Topic and Working Hypothesis

Although heavy metals such as cadmium have long been known to act in a cancerogenic manner, the exact molecular mechanisms by which this can occur remain to be elucidated. As the effect of heavy metals has previously been investigated in a variety of DNA repair contexts, this work concentrates specifically on the previously unexamined topic of the influence of heavy metals, specifically cadmium, on the repair of DNA double-strand breaks. In doing so, we anticipate that cadmium will inhibit either the direct repair of DNA double-strand breaks or the associated signalling processes, potentially through interactions between the metal and cellular proteins involved in DNA repair carrying zinc- or RING-finger motives, molecular structures which have been shown to be particularly susceptible to interactions with heavy metals. Through loss of protein function, DNA repair processes may be impaired in a manner that contributes to genetic instability, thus rendering the cell susceptible to neoplastic transformation.

2. Introduction

Human DNA is continuously exposed to a variety of noxious and toxic substances capable of causing its damage. The most grievous result of DNA damage seen in the daily life of a clinician is cancer. Damage leading to cancer can be induced on a variety of levels, from the point mutations of individual bases to the gross alterations of normal chromosome structure. DNA double-strand breaks (DSBs) are the most deleterious of these damage forms, at once the starting point for mutations and chromosomal breaks as well as the biological means of killing tumors in modern radiation therapy. DSBs occur physiologically in human cells through the processes of V(D)J and class-switch recombination in the course of antibody production (Shrivastav et al. 2008, Pfeiffer et al. 2000), during meiotic and mitotic recombination, as well as as the result of natural replication errors. Additionally, a number of exogenous cytotoxic chemical and physical agents are capable of inducing DSBs, such as ionizing irradiation and various chemotherapeutic drugs such as etoposide and cisplatin. The failure of a cell to successfully repair DSBs can lead to a loss of genetic material and chromosomal aberrations including inversions, translocations and deletions, ultimately increasing genetic instability and promoting the development of cancer (Shrivastav et al. 2008). Understanding both the induction as well as the repair of DSBs, including the ways in which these mechanisms differ between “normal”, healthy tissues and those changed in a tumorous manner, has become and will remain essential to our understanding of how cancer develops and can eventually be cured.

2.1 DNA repair pathways

In order to avoid the detrimental loss of important genetic information potentially resulting from a DSB, human cells, as in all mammalian species, have evolved to develop a complex network of interlinked pathways specifically constructed to recognize different forms of damage and subsequently induce their corresponding repair pathways, all the while manipulating cell cycle and apoptotic factors to maintain the delicate balance between cells which can be saved and those which cannot. Specifically, human cells have two main DSB repair pathways at their disposal- non-homologous end-joining (NHEJ) and homologous recombination (HR), also known as gene conversion (GC). Depending on the exact nature of the lesion as well as other factors such as cell cycle phase, the cell can employ one of these mechanisms to, in the best-case scenario, completely eliminate the damage without the loss or alteration of genetic material. The repair of “clean” DSBs with complimentary break overhangs, as are generally created by nucleases or induced by ionizing irradiation, can be

facilitated by either pathway, while the repair of replication-associated DSBs as well as that of breaks occurring during the S-phase of the cell cycle in the presence of a sister chromatid are preferentially repaired via HR (Shrivastav et al. 2008). Each pathway can be said to have its own set of proteins necessary for successful repair completion, though a number of proteins are found to be active in both pathways, particularly those involved in the signalling of the initial damage event.

2.1.01. Non-homologous end-joining

Approximately 75-80% of DSBs induced in mammalian cells are assumed to be repaired by NHEJ, a repair pathway active during all phases of the cell cycle. It is often referred to as an "error-prone" pathway due to the introduction of minor sequence alterations at the break ends, though the extensive modification of genetic material through resection is not necessary to complete repair (Shrivastav et al. 2008). In the first step of the repair process, the Ku-heterodimer comprised of 70 and 80 kDa subunits identifies and binds to the "open" break ends (Fig. 1). Previous findings from our research group have shown that the binding of functional Ku80 to the breaks effectively leads to the initiation of NHEJ as the default repair pathway by shielding break ends from degradation and thus inhibiting HR, single-strand annealing and an alternative, more error-prone, PARP1-dependent version of NHEJ (Mansour et al. 2010). This binding event additionally attracts the DNA-PKcs protein (DNA-dependent protein kinase, catalytic subunit) to the break site, one member of a family of three phopho-inositide-3-kinase-related proteins (PIKKs) also including ATM and ATR, which in turn is capable of activating other downstream signalling targets including the endonucleases WRN and Artemis through phosphorylation events. In many cases, such endonucleases are necessary for the end-processing of previously un-ligatable DNA break ends. The polymerases μ and λ replenish sequence gaps and DNA ligase IV, XRCC4 and XLF are ultimately responsible for the final ligation of the damaged strands (Shrivastav et al. 2008).

2.1.02. Homologous recombination

Homologous recombination relies on the presence of homologous sequences found elsewhere in the genome to provide a template for theoretically "error-free" repair, in contrast to the often imperfect NHEJ (Shrivastav et al. 2008). As these sequences are preferentially available during specific cells cycle phases, particularly the S- or G2-phase, when the expression of essential HR-repair proteins such as RAD51 and RAD52 is up-regulated, HR

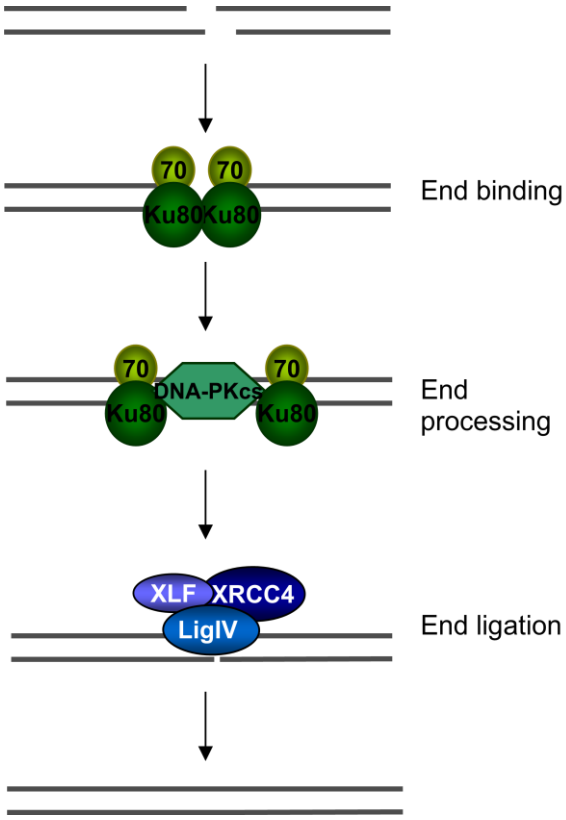

Fig 1. Model of non-homologous end-joining. After recognition of the DNA break through the Ku-heterodimer, the active catalytic subunit DNA-PKcs processes the break ends to allow for ligation by a protein complex containing XLF, XRCC4 and Ligase IV.

has been observed to be most pronounced in actively replicating cells (Shrivastav et al. 2008). The actual repair processes are preceded by a 5' to 3' end-resection step necessary for the creation of exposed 3'-ssDNA overhangs, which are then bound and stabilized by RPA (Fig. 2). Rad51, the key protein in the homologous recombination repair pathway, replaces RPA and creates filaments capable of invading the homologous chromatid, thus forming a double Holliday junction. The ensuing resolution of the junction as mediated by Gen1 or a functional complex of BLM, topoisomerase III and RMI1 can result either in crossover or non-crossover recombinants depending on the spatial orientation of the junctions to one another. BRCA2 has been shown to play a role in the stabilization of Rad51 microfilaments at the break ends (Pellegrini et al. 2002), while a role for BRCA1 has been hypothesized through interactions with Fanconi family proteins, although its exact function in the context of HR remains to be determined (Coster et al. 2010).

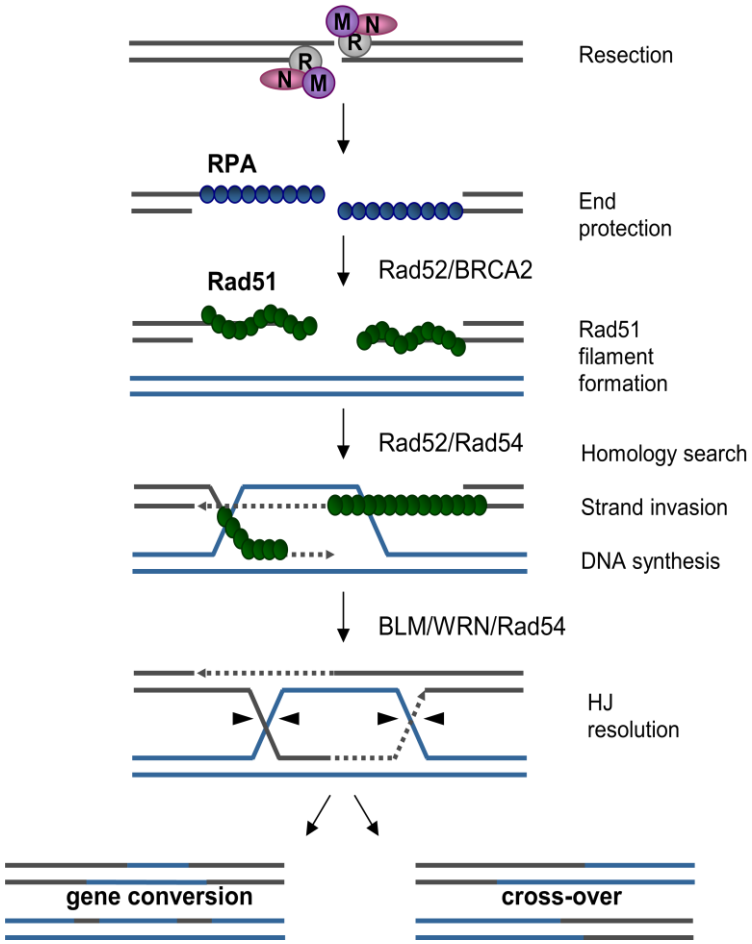

Fig 2. Model of homologous recombination. After recognition of the break by the MRN complex and end-resection by various exonucleases, RPA coats the processed ends. This protein is then replaced by Rad51 microfilaments, which with the help of Rad52 and Rad54 initiate the homology search and strand invasion, leading to the formation of a double Holliday junction. Depending on the orientation of the strands to one another, the resolution of the Holliday junctions results either in gene conversion or cross-over products.

2.1.03. Single-strand annealing

Single-strand annealing deserves a short mention at this juncture as an alternate form of homology-associated repair. This repair pathway may be activated for breaks occurring between two repetitive sequences, during the course of which one of the repeats as well as the intervening sequence are deleted (Pfeiffer et al. 2000). SSA employs a battery of repair and signalling proteins largely similar to those used in HR, with the important exception that the alignment of the break ends occurs in a Rad51-independent manner.

2.2. DNA damage signalling

The actual damage recognition response preceding the physical repair process is initiated by the so-called MRN complex, a multifunctional enzyme consisting of the proteins RAD50, MRE11 and NBS1, and which plays a number of different roles in the early phases of damage signalling (Fig 3). Serving as a structural tether to hold the two broken DNA molecules together, the MRN complex also recruits the ATM protein to the damage site and promotes its activation (Wyman et al. 2006, Kanaar et al. 2006). ATM, a member of the PIKK family, is a Ser/Thr-kinase that exists as a dimer in its inactive form; upon activation via irradiation, however, the protein autophosphorylates, monomerizes and proceeds to phosphorylate various downstream proteins involved in DNA repair and cell cycle signalling, including histone H2AX on serine 139, 53BP1 and BRCA1. Following the phosphorylation of H2AX to γH2AX by ATM or, in its absence, by ATR or DNA-PKcs, MDC1 is recruited to the break site, effectively creating a molecular platform for the pair of the E3 ubiquitin ligases RNF8 and RNF168. Through the mono- or polyubiquitinylation of various DDR proteins such as histones H2A and H2AX, the RING-finger-bearing ubiquitin ligases play a role in the recruitment of additional damage proteins to the DSB, including BRCA1 and 53BP1. Importantly, the mutation or deletion of the RING domain of RNF8 has been shown to effectively prevent the recruitment of the latter two proteins (Coster et al. 2010). It has been shown that the accumulation of 53BP1 foci is inhibited after RNF168 is knocked down by either siRNA or shRNA (Doil et al. 2009), suggesting that the recruitment of 53BP1 likely occurs as a later step following RNF168 accumulation. Finally, a complex consisting of BRCA1, ABRA1 and Rap80 is called to the break site. The assembly of large numbers of DNA damage signalling molecules such as those listed here has been shown to form molecular conglomerates which can be stained using immunoflourescent techniques and can subsequently be quantified as “damage foci” as an expression of the damage induction in or the repair capacity of a cell line.

Another signalling molecule only recently explored within the context of DSB repair is PARP1. Poly(ADP-ribose) polymerase 1 is one member of a super-family of 16 structurally related proteins that facilitate the synthesis or transfer of the polymer poly(ADP-ribose) (pADPr). PARP1 has been specifically demonstrated to be activated by ionizing radiation as well as by DNA strand breaks, the binding of which is mediated by two of PARP’s three zinc-finger domains (Tartier et al. 2003, Lord et al. 2012, Rouleau et al. 2010). As a result of the 10- to 500-fold increase in its catalytic activity, PARP1 synthesizes long chains of pADRr capable of modifying the protein function of histones, topoisomerase I and DNA-PK (Rouleau et al. 2010). Additionally, the recruitment of both MRE11 and ATM to DSBs has been shown to be influenced by pADRr synthesis, suggesting a potential role in the early steps of

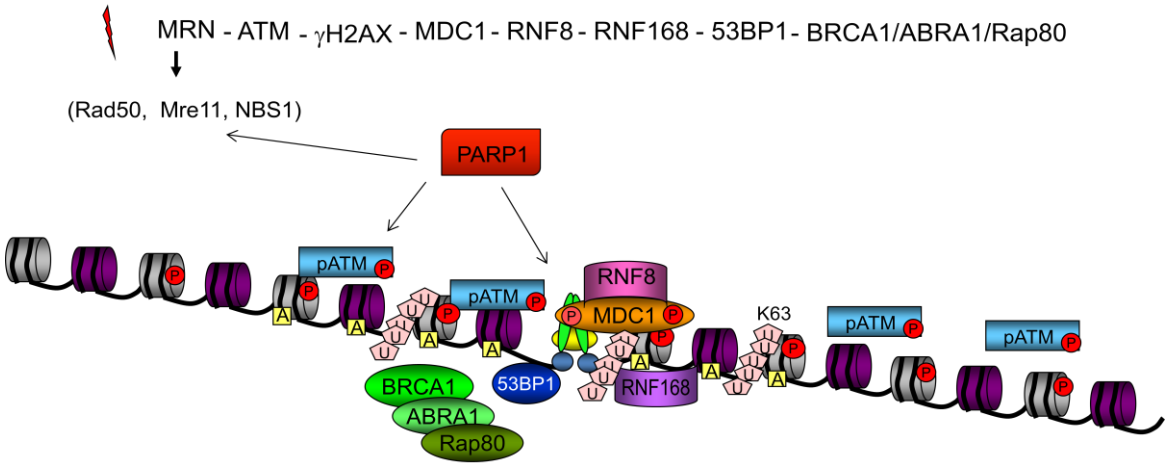

Fig 3. Model of the DNA damage response (DDR) following the induction of DSBs. After the break is recognized and bound by the MRN complex, ATM is activated to autophosphorylate and in turn phosphorylates the histone H2AX to γH2AX, in addition to a number of other targets. The activity of ATM and MRE11 is further modulated by PARP1. MDC1 binds to γH2AX and recruits additional ATM, thus allowing for the phosphorylation of H2AX over megabase distances. MDC1 further recruits RNF8 and RNF168, ubiquitin ligases that mark histones with ubiquitin chains serving as a signal for 53BP1 recruitment. The recruitment of the BRCA1/ABRA1/Rap80 constitutes the final step.

homologous recombination (Rouleau et al. 2010). This complex and the yet to be fully understood interactions between HR proteins and PARP1 build the basis for the current boom in clinical trials aiming to treat various cancers using PARP-inhibitors. Particularly in the treatment of familial forms of breast and ovarian cancer associated with loss-of-function mutations of BRAC1 and BRAC2 and thus defective HR, the application of PARP-inhibitors exerts synthetic lethality, likely through the inhibition of SSB repair. In a normal cell, these breaks would eventually encounter replication forks and form replication-associated DSBs, which would then be repaired by homologous recombination. For this reason, cells incapable of performing functional HR are particularly susceptible to PARP1 inhibition (Lord et al. 2012). In addition to damage signalling, PARP has been suggested to be a regulator of NHEJ (Davar et al. 2012). The complex interactions between PARP and various other players in damage signalling and repair have thus far allowed for the implementation of PARP-inhibitors in a number of drug treatment trials, and demonstrate the promising clinical potential in directly influencing repair processes on a molecular level in the course of individualized cancer treatments.

2.3. Cadmium

Cadmium is a heavy metal that has been classified as a group 1 carcinogen by the International Agency for Research on Cancer, indicating sufficient evidence for carcinogenicity in humans. Exposure to cadmium occurs in the industrialized world primarily through the smoking of cigarettes. One cigarette has been shown to contain between 0-6.67µg of cadmium (Smith et al. 1997), with the cadmium concentration in the blood of smokers approximately four times higher than that found in the blood of non-smokers (0.4-1.0µg/L vs. 1.4-4µg/L) (Kellen et al. 2007, Hertz-Picciotto et al. 1994). Additional sources of cadmium include occupational exposure, primarily in the areas of "cadmium production and refining, nickel-cadmium battery manufacture and zinc smelting" (Kellen et al. 2007). Various foodstuffs such as leafy vegetables, grains and particularly shellfish are also known to contain cadmium, all of which contribute to an estimated total of 30-40µg cadmium ingested daily by the average adult in the United States (Kellen et al. 2007). As the human body lacks the ability to sufficiently detoxify and/or excrete cadmium, the metal accumulates in various tissues, with the highest concentrations being measured postmortem in the renal cortex, liver, pancreas and lungs (Schwartz et al. 2000). It is in these tissues in which cadmium-associated tumors have been observed over the years. Despite an as of yet inexplicable carcinogenic mechanism, cadmium exposure has been associated with cancers of the lung, bladder (Kellen et al. 2007), pancreas (Schwartz et al. 2000), prostate (Schöpfer et al. 2010) and kidney (Kazantzis et al.1963).

2.3.01. The influence of cadmium on DNA repair processes

Though cadmium has long been known to be carcinogenic and mutagenic, the exact mechanisms by which cadmium can lead to cancer have yet to be elucidated. It has previously been demonstrated that a broad spectrum of heavy metals is capable of acting in a carcinogenic manner through a variety of mechanisms (Figure 4). Through interference in DNA repair processes, previously shown predominantly for nucleotide excision repair (Hartwig et al. 1994, Bal et al. 2003, Hartmann et al. 1998, Kopera et al. 2004), the recognition and repair of oxidative DNA damage through interactions with antioxidant enzymes such as superoxide dismutase, glutathione reductase and glutathione peroxidase (Dally et al. 1997, Beyersmann et al. 2008), the activation of mitotic signalling and the modulation of gene expression (Beyermann et al. 2008), heavy metals contribute to a general milieu of genetic instability conducive to the formation and accruement of complex mutations and thus to the development of cancer. Cadmium has specifically been demonstrated to be genotoxic in murine models, where increased frequencies of micronuclei

and chromosomal aberrations have been observed following cadmium exposure (Beyersmann et al. 2008), though it is notably merely weakly mutagenic in mammalian cells (Bertin et al. 2006). Heavy metals contribute to oxidative stress in a two-fold manner through the direct induction of reactive oxygen species as well as the inhibition of antioxidant enzymes including catalase and superoxide dismutase. In addition, it is possible that cadmium might either directly induce DNA lesions or interfere with DSB repair processes, thereby aggravating other forms of genetic damage.

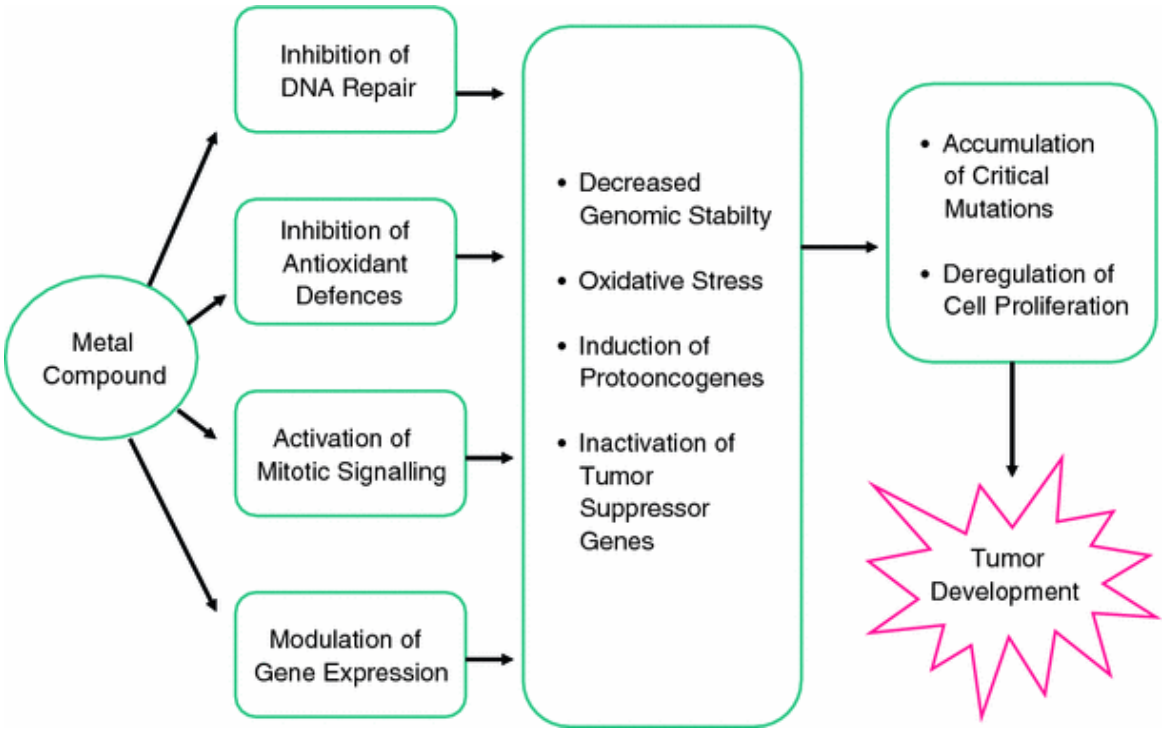

Fig 4. Mechanisms by which metal compounds can contribute to tumorigenesis. This work concentrates specifically on the inhibition of DNA double-strand break repair and its associated signalling. Figure taken from Beyersmann and Hartwig 2008.

The investigation of various levels of DNA repair has demonstrated cadmium's ability to influence particular enzymes and repair pathways. To mention a few, cadmium has been shown to inhibit a polynucleotide kinase involved in the repair of a subset of single-strand breaks (Whiteside et al. 2010), influence mismatch repair through the inhibition of repair protein binding to the DNA (Jin et al. 2003, Bertin et al. 2006), impair the initial incision step necessary for the successful completion of nucleotide excision repair through the inhibition of the xeroderma pigmentosum A (XPA) protein (Beyersmann et al. 2008, Bertin et al. 2006,

Kopera et al. 2004), as well as alter the course of base excision repair through the inhibition of proteins such as formamidopyrimidine glycosylase (Fpg) (Bertin et al. 2006). In addition, cadmium is capable of suppressing the activity of the most well known of all tumor suppressors, p53 (Méplan et al. 1999). Finally, cadmium has been shown to reduce PARP activity in response to hydrogen peroxide treatment, suggesting a specific molecular target for cadmium involved in both the signalling and repair of DSBs (Hartwig et al. 2002, 2002). Some groups claim to have established a connection between exposure to lead (Gastaldo et al. 2007) and cadmium (Viau et al. 2008) and the induction of double-strand breaks, though at biologically questionable concentrations. The goal of this work is thus to specifically investigate interactions between cadmium and the various molecular players involved in the signalling and repair of DBSs.

2.3.02. The interaction between cadmium and zinc- and RING-finger structures

Mechanistically, cadmium and other heavy metals have long been suspected to interact preferentially with zinc-bearing structures found on many proteins involved in DNA repair and signalling pathways. Despite the fact that it has slightly larger radius than Zn^{2+}, Cd^{2+} nonetheless possesses the same charge as well as the ability to effectively displace Zn^{2+} from its binding niche in a number of proteins, thus potentially altering protein structure and function (Beyersmann et al. 2008). Proteins carrying these zinc-finger motifs are known to be involved in the DNA-binding and protein-protein interactions that take place between a variety of transcription factors and have more recently been identified in the context of DNA repair in a number of proteins involved in both the damage signalling and repair of all important repair pathways (Table 1). The actual zinc-finger domain consists of a zinc ion complexed with various combinations of four histidine and cysteine residues in a motif of 30 total amino acids (Hartwig et al. 2001, 2002). RING-finger proteins, more recently discovered and identified in some 200 human proteins, have been found in a number of E3 ubiquitin ligase proteins known to play varying roles in DNA repair (Gamsjaeger et al. 2007, Joazeiro et al. 2000). Such structures, similar to zinc-finger proteins in containing complex cysteine and histidine consensus sequences building zinc-binding domains, can be found in proteins such as Rad18, KAP-1, RNF 20, RNF 40, RNF 8, RNF 168 and BRCA1. Mutations in the latter have been identified in familial breast and ovarian cancer (Joazeiro et al. 2000, Gamsjaeger et al. 2007). The structural interplay between the zinc ion complexed with the rest of the protein is essential for the maintenance of proper protein structure and thus function, as metal binding secures the structure (Hartwig et al. 2001). The high affinity of heavy metals such as cadmium towards the sulfhydryl groups contained in the cysteine residues renders these protein structures particularly susceptible to interactions with other

metals, effectively allowing them to disturb protein function by forcing the native Zn^{2+} ion from the zinc-finger structures, thus altering protein structure and potentially leading to loss of protein function (Kopera et al. 2004, Hartwig et al. 2002). It is also possible that the introduction of a heavy metal could lead to the formation of various mixed protein/metal complexes or to the oxidation of cysteine residues found in the finger structure, thus impairing the ability of the metals to bind and leading to loss of function (Hartwig et al. 2002).

Essentially all zinc- or RING-finger-bearing structures as well as metal sensitive proteins involved in the various facets of damage signalling and repair constitute potential candidates for contributing to cancerogenicity upon loss of their function. For example, the zinc-finger domain of the DNA ligase III protein is responsible for binding to DNA structures and helps to promote end-joining (Taylor et al. 2000), while the zinc-hook found in the ATPase Rad50 is necessary for the complexation of MRE11 to the DNA during repair processes (Hopfner et al. 2002), with rad50 mutants found to exhibit profound radiosensitivity in yeast. APLF (aprataxin and PNK-like factor), an endo/exonuclease containing a number of zinc-finger domains, has been shown to interact with the non-homologous end-joining proteins XRCC4 and Ku following irradiation (Macrae et al. 2008). It additionally undergoes ATM-dependent hyperphosphorylation following DNA damage induction via ionizing irradiation, thus rendering it another potential target for heavy metal-induced reactions (Macrae et al. 2008).

Rad18 is a repair protein with a RING-finger structure that promotes the monoubiquitinylation of PCNA during the S-phase and supports the chromatin retention of 53BP1 at damage sites (Watanabe et al. 2009). As previously mentioned, the RING-finger domain of RNF8 is necessary for the recruitment of BRCA1 and 53BP1, and thus for an intact DNA damage response (DDR) signal cascade. The possibilities for interactions between cadmium and proteins involved on different levels of damage signalling and repair are numerous and complex. We therefore sought to investigate the effects of cadmium on both the repair of DNA DSBs as well as the DNA damage response in detail. In doing so, we aim to elucidate specific molecular targets, likely in the form of zinc- or RING-finger-bearing proteins involved in DNA DSB damage signalling and repair, that could offer a mechanistic explanation for cadmium's carcinogenic properties. A more detailed understanding of the processes involved in the induction and progression of tumorigenesis following exposure to heavy metals will ideally lead to new insights into new and individualized therapeutic options for patients with heavy metal-associated tumors.

Table 1. Zinc-finger, RING-finger and metal sensitive proteins involved in DNA repair and damage signalling processes. Table modified according to Hartwig 2001, Hartwig et al. 2002.

DNA damage repair pathway	***Type of zinc complexation/ metal sensitivity***	***Biological function***
Nucleotide excision repair		
XPA	Cys_4	DNA damage recognition
RPA	Cys_4	DNA damage recognition
Base excision repair		
Fpg	Cys_4	Recognition and excision of oxidative DNA base modifications (procaryotes)
Ligase III	Cys_3His_1	DNA ligation
DNA damage signalling/ DSB repair		
PARP 1	Cys_3His_1	Damage signalling, DNA repair, apoptosis
P53	Cys_3His_1	Cell cycle control, DNA repair, tumor suppressor
Rad50	Zinc-hook (CysXXCys)	DSB repair
APLF	$CysX_5CysX_6HisX_5His$	DSB repair
BRCA1/2	RING-Finger	Tumor suppressor
Rad18	RING-Finger	Damage signalling
RNF8/168/20/40	RING-Finger	Damage signalling
KAP-1	RING-Finger	Chromatin modelling, DSB repair
EYA	Metal sensitive	Damage signalling, apoptosis
WSTF	Metal sensitive	Damage signalling, DSB repair

3. Materials and Methods

3.1. Materials

3.1.01. Laboratory equipment

General Equipment	
Pipetboy	Eppendorf, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Water bath	Lauda, Lauda-Königshofen, Germany
Refrigerator	Bosch, Stuttgart, Germany
Freezer -20°C	Kryotech, Hamburg, Germany
Freezer -80 °C	Fryka, Esslingen, Germany
Refrigerated microcentrifuge	Beckmann Instruments GmbH, Munich, Germany
Refrigerated centrifuge, Megafuge 1.0R	Heraeus, Hanau, Germany
Refrigerated centrifuge 5804R	Eppendorf, Hamburg, Germany
Minispin plus centrifuge	Eppendorf, Hamburg, Germany
Hot-plate thermostat 5320	Eppendorf, Hamburg, Germany
Pair of scales, AE160 / P1200	Mettler, Giessen, Germany
Magnetic stirrer, RH Basis	IKA Labortechnik, Staufen, Germany
pH-meter 300	Beckmann Instruments GmbH, Munich, Germany
Bio-photometer	Eppendorf, Hamburg, Germany
Primus Thermal cycler	MWG Biotech, Ebersberg, Germany
Cell culture	
Cell incubator Hera cell 240	Kendro, Hanau, Germany
Sterile work benches	Kendro, Hanau, Germany
Coulter Counter model Z1	Beckman Coulter, Krefeld, Germany
Olympus CK2	Olympus Optical Co., LTD, Japan
Axiovert 40CFL	Carl Zeiss, Göttingen, Germany
Microscopy	
Fluorescence microscope, Axioplan 2	Carl Zeiss, Göttingen, Germany
Apotome, AxioCam MRn	Carl Zeiss, Göttingen, Germany
Monochromator Polychrome V	TILL Photonics GmbH, Gräfeling, Germany

EM-CCD camera type DU-888	Andor Technology, Belfast, Ireland
StackReg plug-in	Philippe Thevenaz, Lausanne, Switzerland
Computer Software	
GraphPad Prism 5.0	GraphPad Software, San Diego, USA
ImageJ 1.42q	National Institutes of Health, Maryland, USA
Olympus Soft Imaging Solution	Olympus Imaging-Software, Germany
AndorIQ software	Andor Technology, Belfast, Ireland
Other	
Flow Cytometer FACScan	Beckmann Instruments GmbH, Munich, Germany
X-ray generator type RS225 research	Gulmay Medical LTD, Oxford, UK

3.1.02. Laboratory materials

General Materials	
Gloves, latex	Hartmann, Heidenheim, Germany
Gloves, nitrile	Ansell, Staffordshire, UK
Parafilm	Pechiney Plastic, Chicago, USA
Pasteur pipettes, plastic	Falkon, NJ, USA
Pipette tips	Eppendorf, Hamburg, Germany
Pipette tips, stuffed	Eppendorf, Hamburg, Germany
Wipes	Wepa, Arnsberg, Germany
Tubes 15ml, 50ml	Falkon, NJ, USA
Tubes 1.5ml, 2ml	Eppendorf, Hamburg, Germany
Pipettes, plastic (1-50ml)	Falkon, NJ, USA
Cell Culture	
6-well plates	Falkon, NJ, USA
Cell culture flasks T25, T75	Sarstedt, Nümbrecht, Germany
Pasteur pipettes, glass	Carl Roth GmbH, Karlsruhe, Germany
Cryo-tubes	Sarstedt, Nümbrecht, Germany
Sterile filter (Rotilabo 0.22 µm)	Millipore, MA, USA
Other	
Microscope slides	Karl Hecht, Sondheim, Germany
Optical Adhesive Covers	Applied Biosystems, CA, USA
Round-bottom tube (FACS)	Sarstedt, Nümbrecht, Germany
Round disks lunox film 25 (18 mm ø)	In Vitro Systems & Services, Göttingen, Germany

3.1.03. Chemicals reagents

2-propanol	Merck, Bad Soden, Germany
Ethanol	Th. Geyer, Hamburg, Germany
H_2O_2	Th. Geyer, Hamburg, Germany
Bovine serum albumin (BSA)	PAA, Pasching, Austria
Crystal violet stain	Sigma-Aldrich, Deisenhofen, Germany
Antifade mounting medium, Vectashield	Vector Laboratories, CA, USA
Formaldehyde 37%	Merck, Bad Soden, Germany
Tris-HCl	Sigma-Aldrich, Deisenhofen, Germany
Triton X	Serva, Heidelberg, Germany
Tween 20	Sigma-Aldrich, Deisenhofen, Germany

3.1.04. Solutions

Cadmium chloride ($CdCl_2$) stock solution			
Solutions for constant field gel electrophoresis			
TE-Buffer (Tris/EDTA)	10 1000	mL mL	Tris-EDTA dH_20
TBE-Buffer (10x)	86 1000	g mL	Tris-Borate-EDTA dH_20
10M NaOH	40 100	g mL	NaOH dH_20
10% SLS (sodium lauryl sulfate)	10 100	g mL	NLS dH_20
1.6% low melt agarose for plugs	0.08 5	g mL	low melt agarose DMEM
Lysis solution	0.4 2 1	M % mg/mL	EDTA Sodium-lauryl-sulfate Proteinase K
Ethidium bromide stock	100 10	mg mL	Ethidium bromide dH_20
Trichloroacetic acid	10	% (w/v)	Trichloroacetic acid
Solutions for colony assays			
PBS (phosphate buffered saline)	140 3 8	mM mM mM	NaCl KCl Na2HPO4
Crystal violet staining solution	0.1	% (w/v)	Crystal violet/ddH2O

3.1.05. Cell culture media

All media and reagents for cell culture were obtained from Invitrogen GmbH in Karlsruhe, Germany.

DMEM
Opti-MEM
Penicillin, 10,000 U/ml
Streptomycin, 10,000 µg/ml
Trypsin-EDTA
G418 Sulphate, (Geneticin, selective antibiotic)
Fetal calf serum (FCS)

3.1.06. Immunoflourescent staining

Solutions for Immunoflourescence			
Fixing solution	2	%	Formaldehyde 37% / PBS
Permeabilization solution	0.2 1	% %	Triton-X BSA / PBS
Blocking solution	3	%	BSA / PBS
PBST (0.5% Tween 20)	0.5 995	ml ml	Tween 20 PBS
Antibodies			
Primary antibodies	Monoclonal Mouse anti-γH2AX Ser139 Cell Signalling, MA, USA Polyclonal Rabbit anti-53BP1 Novus Biologicals, Cambridge, UK Monoclonal Mouse anti-PAR Trevigen Inc., MD, USA		
Secondary antibodies	Anti-mouse Alexafluor594 IgG Invitrogen, Karlsruhe, Germany Anti-rabbit fluorescein IgG Amersham Pharmacia Biotech, Freiburg, Germany		

3.1.07. Miscellaneous solutions and reagents

Plasmids	
pEGFP-N1	Clontech, BD Bioscience, Heidelberg, Germany
pEJSSA	Previously constructed by our group (Mansour et al. 2008)
pGC	Previously constructed by our group (Mansour et al. 2008)
Transfection solution	
LipoFectamin2000 transfection agent for plasmid DNA	Invitrogen, Karlsruhe, Germany
DNA staining solutions	
DAPI (4′, 6-Diamidin-2-phenylindol), 1mg/ml	
Propidum iodine; 10µg/ml	
Enzymes	
I-SceI restriction enzyme	Fermentas, St. Leon-Rot, Germany
Apal I restriction enzyme	Fermentas, St. Leon-Rot, Germany
DNA Extraction Kit	
QIAEX II Gel Extraction Kit	Qiagen, Hilden, Germany

3.1.08. Cell lines

HeLa cells are one of the commonly used human cell lines in oncological research, having been originally derived from a patient suffering from cervical cancer in 1951. HeLa cells lack functional p53 due to an HPV infection. These cells were cultivated in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

HeLa pEJSSA and pGC are HeLa cell lines containing stably integrated copies of a reporter construct designed to monitor either non-homologous end-joining and single-strand annealing (pEJSSA) or gene conversion (pGC), the construction of which is described in detail in publications from our research group (Mansour et al. 2008). These cells were cultivated in DMEM containing 10% fetal calf serum and G418 (800 µg/ml).

Human osteosarcoma cells stably expressing a NBS1-GFP construct (U2OS-NBS1-GFP) were kindly provided by Claudia Lukas (Danish Cancer Society, Copenhagen). These cells were cultured in DMEM medium (Biochrom AG, Berlin, Germany) supplemented with 10 % fetal calf serum in 75cm^2 culture flasks (BD Bioscience, Le Pont De Claix, France) at 37°C, 95 % humidity and 5 % CO_2.

3.2. Experimental Methods

3.2.01. Reporter-based repair experiments

In order to investigate the effect of cadmium on the functionally distinct NHEJ and GC repair pathways, the repair efficiency of HeLa-pEJSSA and HeLa-pGC cells was quantified following the introduction of I-SceI enzyme-induced DSBs. For this purpose, we transiently transfected these cell lines containing stably integrated chromosomal reporter constructs for the respective repair pathways with a plasmid vector expressing a single copy of the gene for the I-SceI endonuclease enzyme. The introduction of this plasmid into the cells allows the enzyme to access its specific restriction site contained within the reporter construct, thus creating an enzyme-induced DSB. Regardless of the repair pathway employed, the repair of these breaks leads to the restitution of the open reading frame (ORF) of the GFP gene. Through the expression of this gene and the subsequent transcription and translation of the functional GFP protein, the number of GFP-expressing “green” cells can be quantified via FACS analysis and understood as the percentage of cells in which DSB-repair events were successfully completed.

In the case of the pEJSSA construct for monitoring the repair of DSBs through non-homologous end-joining and single-strand annealing, the insertion of a false start codon in the 5’-untranslated region of the GFP gene located between two I-SceI digestion sites normally prevents the translation of the original GFP open reading frame (ORF). Through enzymatic digestion at the two I-SceI sites, the false start codon is removed, resulting in repair via NHEJ. The proper ORF is thus restored and the GFP gene can be expressed in its entirety (Fig. 5). Two 50-bp homologous direct repeats alternatively allow the cell to repair its damage via single-strand annealing. As the two repair products differ in length, they can be distinguished from one another via PCR and subsequent gel electrophoresis. In the case of HR, also known as gene conversion, the pGC reporter contains two non-functional copies of the GFP gene sharing 520 base pairs of homology. The first gene copy is disrupted by the insertion of a single I-SceI digestion site, while the second contains only a 3’-truncated copy of the GFP gene. When a DSB is induced in the upstream copy, the truncated downstream copy can be used as a template for homologous recombination, similarly resulting in the successful expression of the GFP gene and the production of GFP protein (Fig. 6). The commercially available pEGFP-N1 plasmid containing a single copy of the GFP gene was employed under otherwise identical experimental conditions in order to control for transfection efficiency.

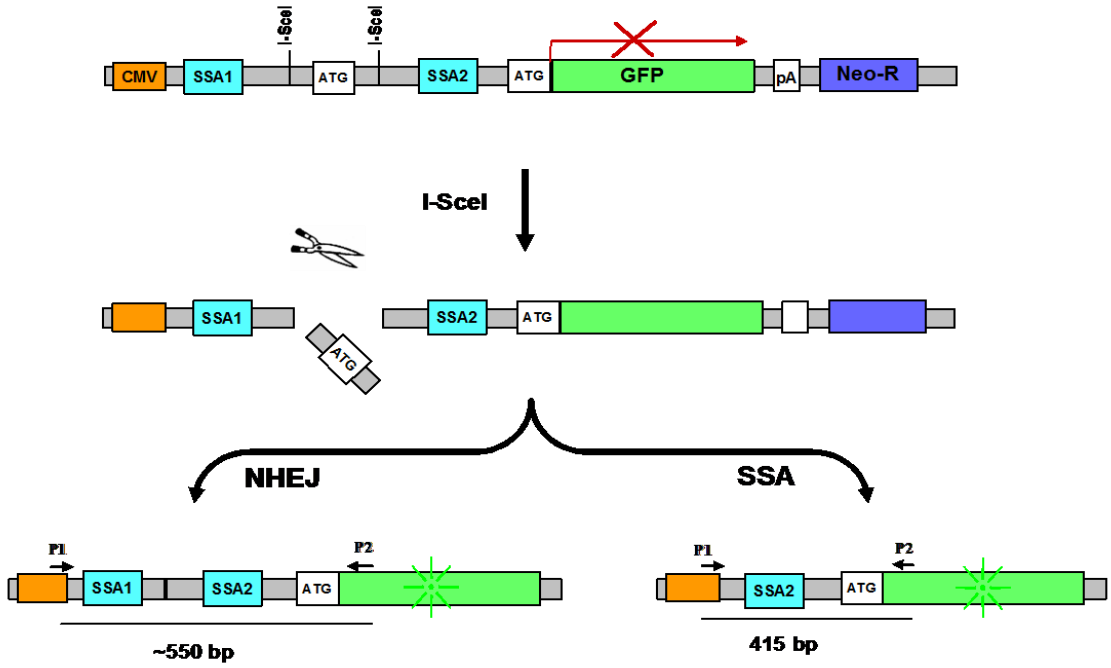

Fig 5. Schematic representation of the NHEJ repair construct. Expression of the GFP gene is normally inhibited by an insert between the CMV promoter and the ORF, which is flanked by two inverted repeat I-SceI recognition sequences. Induction of a DSB through digestion with the I-SceI endonuclease leads to the expulsion of the artificial ATG start codon. Upon successful repair of the break, GFP translation is reestablished. PCR analysis of repair fragment length using primers P1 and P2 allows for the differentiation between repair events completed via NHEJ (550 bp fragment length) and SSA (415 bp fragment). Figure taken from Mansour et al. 2008.

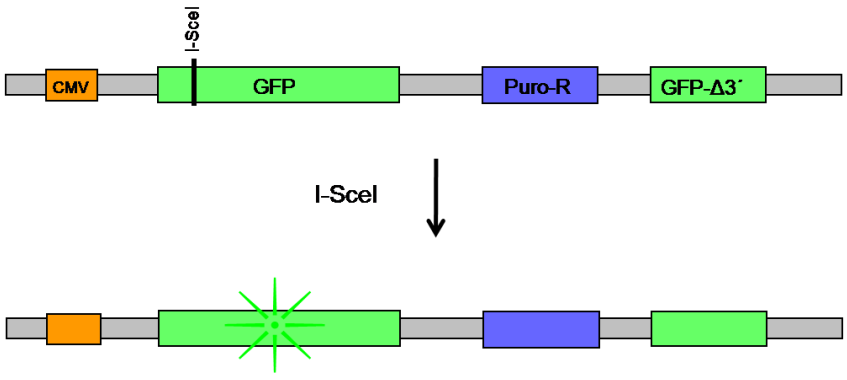

Fig 6. Schematic representation of the HR repair construct. The repair substrate contains two nonfunctional copies of the GFP gene sharing 520 bp of homology. The enzyme-induced DSB can thus be repaired using the homology found in the truncated downstream GFP sequence. As with the NHEJ/SSA construct, the successful repair of the DSB restores the integrity of the GFP gene and allows for its successful translation. Taken from Mansour et al. 2008.

For each experiment, $2x10^5$ cells were seeded into a 6-well plate. After having adhered to the plates, the cells were incubated with various doses of a cadmium-chloride solution for 24h. For each transfection (well), 2µg plasmid-DNA containing the I-SceI expression vector pCMV3xnls-I-SceI was incubated with 5µL LipoFectamin2000 transfection agent and 300µL Opti-MEM before being added to the cells growing in normal 10% FCS medium in the presence of cadmium. The cells were then incubated for up to 72h depending on the desired repair time point. Prior to measurement via FACS, the cells were trypsinized (0.5µL trypsin) and resuspended in 1.5µL medium. They were then spun down for 5 min at 4^oC (1200 rpm) and washed once with PBS before being collected for a final time and resuspended in 1mL PBS. The probes were subsequently analyzed for green fluorescence via FACS.

3.2.02. Enzyme control digestion

In order to ensure that cadmium exposure itself did not adversely affect the digestive activity of the I-SceI restriction enzyme for the range of concentrations employed in our experiments, a two-step control digestion was conducted prior to the transfection experiments. In the first digestion, 0.8µg plasmid DNA containing the pGC construct sequence was exposed to 5U of the enzyme ApaL I for 5 min in a mixture containing NE Buffer, 10X BSA and distilled water in order to generate a linearized plasmid (Fig. 7A, B).

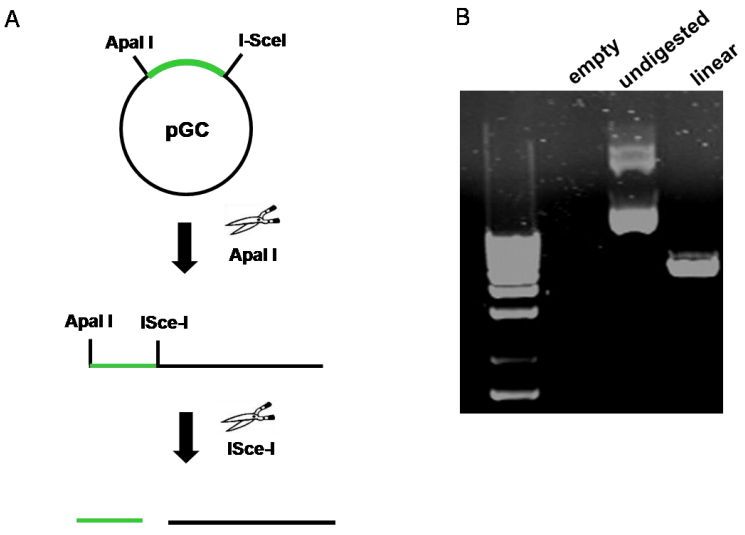

Fig 7. Control digestion to rule out a potential interaction between cadmium and the I-SceI enzyme used to induce DSBs in the repair efficiency assay. (A) Schematic representation of the digestion protocol. The pGC plasmid was initially treated with the ApaI I restriction enzyme in the absence of $CdCl_2$ to achieve linearized plasmid (B).

The digestion mixture was applied to a 1% agarose gel containing ethidium bromide and electrophoresed at 100V for 30 min. The bands corresponding to the linearized digestion product were then excised from the gel and cleaned using the QIAEX II Gel Extraction Kit according to the manufacturer's protocol. In the second digestion, the cleaned products of the excised bands were further digested using 1U I-SceI enzyme in a mixture containing buffer, 10X BSA and water for 2h in the presence of various cadmium concentrations up to 2000 times greater than the highest concentration used in the plasmid-based repair assays. The resulting digestion products were again applied to a 1% agarose gel, electrophoresed at 100V for 30 min and evaluated for band intensity.

3.2.03. Cell growth and clonogenicity

In order to quantify the impact of heavy metal treatment on the growth and clonogenic proliferation of HeLa cells, cells were seeded in T25 flasks. After the cells had had the opportunity to adhere to the bottom of the flask, the normal cell culture medium was changed to media containing cadmium in a range of concentrations from 0 to 50µM. For the analysis of cell proliferation, the cells were incubated for various amounts of time between six hours and eight days. At the end of the selected time period, the cells were washed, trypsinized and quantified using the Coulter Counter. For the colony tests, the pre-treated cells were trypsinized at the end of the 24h incubation period and subsequently re-seeded in T25 flasks in either cadmium-free medium or in medium containing the same concentration of cadmium in which the cells had already been exposed for the permanent treatment experiments. The flasks were then incubated for a further 2 weeks to allow for visible colony formation, at which time the cells were washed once with PBS, fixed with 70% ethanol, and then stained with crystal violet solution. The number of resulting colonies was determined either by visual analysis or using a computer program designed to count the number of colonies in a given flask. Only colonies containing 50 or more cells and thus representing the result of more than five cell divisions were considered in the quantification. Experiments to determine radiosensitivity were performed according to the same protocol, with the only exception being that the cells were irradiated immediately prior to reseeding. Cells were allowed to grow in cadmium-free medium for the ensuing two-week incubation period.

3.2.04. Cell cycle monitoring

In order to determine the cell cycle distribution of a heterogeneous population of HeLa cells following cadmium exposure, these cells were harvested after 24h cadmium treatment,

washed in PBS, centrifuged, resuspended in PBS, and finally fixed in 80% ethanol (-20^{o}C) for 10 min at 4^{o}C. The cells were then centrifuged at 1200 rpm for 5 min, washed again with PBS, and collected once again. For the quantification of the various cell cycle phases based on cell cycle phase-specific differences in DNA content, the DNA was stained using a PI solution (propidium iodide containing RNAse A at 1 mg/ml) for 30 min at room temperature. The relative amount of DNA was subsequently analyzed via FACS.

3.2.05. Constant field gel electrophoresis

Constant field gel electrophoresis was employed in order to determine the relative number of DNA-DSBs following exposure to cadmium and ionizing radiation on the basis of DNA fragmentation. For these experiments, HeLa cells were pre-treated for 24h with $CdCl_2$ solution or mock treated before being harvested via trypsinization and washed with PBS. The exact cell number present in each individual treatment was then determined using the Coulter Counter. These samples were centrifuged at 1000 rpm for 5 minutes (4^{o}C), the supernatant removed and the cells resuspended in enough ice-cold medium to achieve a final concentration of $6x10^6$ cells/mL. The resulting suspension was carefully mixed using a 1mL syringe and then combined with a 1% low-melt agarose solution (heated to approximately 70°C). This solution was injected into a specially formed mold to produce small gel “plugs” containing cells embedded in agarose and evenly distributed throughout. The plugs were stored in 10% DMEM medium and irradiated on ice. Immediately following irradiation and thus allowing the cells no time to repair the induced damage, the plugs were lysed in a solution containing EDTA, sodium-lauryl-sulfate and proteinase K, first on ice for 10 min and then for an additional 24h at 37°C. Experiments quantifying repair after 24h were conducted in a similar manner, with the difference that the cells were irradiated in culture flasks and then allowed up to 24h to repair the induced DSBs before being trypsinized, washed and formed into plugs. The plugs were then lysed as described above. Following the lysis step in both experimental designs, the plugs were washed thoroughly four times for 10 min in TE-buffer. They were then cut into approximately 3mm-long pieces to fit exactly into the wells of a 7.5% agarose gel (chamber filled with 0.5M TBE-buffer). An additional thin layer of agarose was poured on top of the gel to facilitate the optimal optical quantification of band intensity.

The electrophoresis was carried out at 30V (1.0 V/cm) for at least 24h. The gels were then stained in 2.5µg/mL ethidium bromide solution for 75 min in the dark and subsequently washed for 15 min. The resulting bands, indicating DNA damage fragments released following the induction of DSBs, could be visualized using a UV-transilluminator and the band

intensity quantified, thus revealing the relative amount of unrepaired DSBs/DNA damage present in a given sample. The intensity of the bands was determined using a software macro developed within our lab for this specific purpose.

3.2.06. Immunoflourescent staining and quantification of repair foci

The number of DSBs resulting from either cadmium alone or in combination ionizing irradiation was quantified using the immunoflourescent staining of γH2AX and 53BP1. Following the application of these genotoxic agents, we were able to quantify the direct induction of DSBs as well as chart the course of DSB repair kinetics over a longer period of time (up to 24h). For this purpose, HeLa cells were grown on microscope slides containing one well and were pre-treated with cadmium and/or irradiated with various doses of IR. At different time points after irradiation, the slides were fixed in 2% formaldehyde in PBS for 10 min and washed three times with PBS. The cells were then permeabilized on ice for 5 min to allow the antibody to enter the cell. The unspecific binding of antibodies was "blocked" for 1h using a 3% BSA/PBS solution. The cells were then exposed to the respective primary antibody of interest, either γH2AX (1:100) or 53BP1 (1:500), in washing solution containing 1% BSA for 1h. Following this incubation, the slides were washed three times for 10 min in PBS containing 1% BSA and 0.5% Tween on a shaker before being incubated for 1h with a 1% BSA solution containing the desired secondary antibody (anti-mouse conjugated with Alexafluor594 for γH2AX foci (1:600) or anti-rabbit conjugated with fluorescein for 53BP1 foci (1:50)). The slides were again washed four times for 10 min in PBS containing 0.5% Tween. The DNA was then stained with DAPI-Antifade mounting medium. Finally, the slides were sealed using cover slips and nail polish. All slides were evaluated and foci counted within two weeks of staining in order to ensure maximum fluorescence intensity. As both γH2AX and 53BP1 are markers of DSBs, their repair foci can be found to colocalize in microscopic analysis, a representative example of which can be seen in Fig. 8.

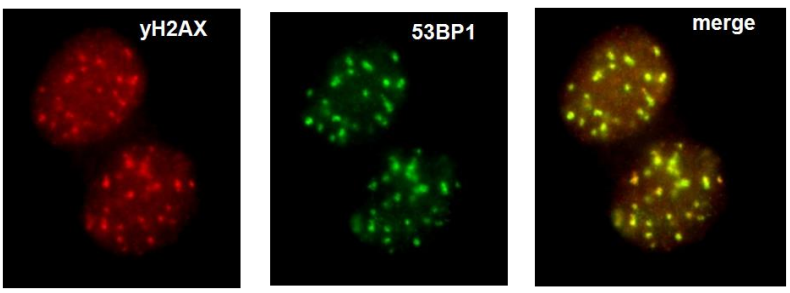

Fig 8. Examples of γH2AX and 53BP1 DNA damage foci and their colocalization (merge) at DSBs 24h after irradiation with 6Gy.

3.2.07. Immunoflourescent staining of poly(ADP-ribose)

In order to investigate the influence of cadmium exposure on the poly(ADP-ribose) polymerase, a signalling molecule involved in the early phase of damage recognition following the induction of DSBs, we detected the formation of poly (ADP-ribose), the product of this enzyme's activity. For this purpose, cells were seeded on microscope slides and incubated with cadmium as was done for the previous foci experiments. At various time points following exposure to 10Gy ionizing irradiation or H_20_2 as a control, the slides were quickly washed in PBS-buffer before being incubated in 10% trichloroacetic acid on ice for 10 min. The slides were then quickly dipped into ice-cold PBS and washed in a series of ethanol solutions with 70%, 90% and absolute ethanol all cooled to -20°C. After air-drying, the slides were blocked in BSA in PBS for 30 min at RT and subsequently incubated with anti-PAR primary antibody (1:100 in 0.5% BSA and 0.1% Tween 20 in PBS). The slides were washed and incubated in Alexafluor594 secondary antibody and washed again before the DNA was counterstained with DAPI as previously described. The slides were sealed using cover slips and nail polish and were evaluated within two weeks of staining in order to ensure maximum fluorescence intensity.

3.2.08. Particle irradiation and real time kinetics of NBS1 recruitment

The recruitment of NBS1 as a component of the MRN complex constitutes one of the earliest signalling steps in the process of DDR. In order to investigate whether cadmium might interfere with the induction of this process, we investigated the recruitment of NBS1 to the sites of DSBs following irradiation with neon ions using live cell microscopy. 90,000 human osteosarcoma cells stably expressing a NBS1-GFP construct were seeded one day before the experiment on round disks (18 mm ø) of 25µm thick lunox film 25 (In Vitro Systems & Services, Göttingen, Germany). Neon ion irradiation ($3x10^6$ p/cm²; LET: 500 keV/µm) was performed at the low energy branch of the GSI accelerator in combination with beamline microscopy as previously described (Jakob et al. 2005, 2011). Fluorescence was excited with the monochromator Polychrome V. Image acquisition was conducted using an EM-CCD camera type DU-888 and the corresponding AndorIQ software, while the quantitative image analysis was performed using ImageJ. Cell motion during acquisition was compensated using the StackReg plug-in.

4. Results

4.1. Cadmium exposure negatively affects the repair efficiency of enzyme-induced DSBs HeLa cells

Using the plasmid-based reporter construct repair assay, we were able to quantify the number of DSB repair events successfully completed in HeLa cells following the induction of enzyme-induced DSBs as well as differentially compare the repair capacities of the individual DSB-repair pathways (NHEJ/SSA and GC). Cells treated with cadmium for 24h showed inhibited repair compared to untreated cells in both of the repair scenarios investigated. For the non-homologous end-joining and single-strand annealing repair construct, a decrease in the number of GFP-positive cells could be observed with increased cadmium concentration, meaning the number of cells in which repair events were successfully completed following the induction of DSBs through I-SceI was reduced (Fig. 9A). In untreated cells, an average of approximately 2.4% of cells screened via FACS analysis were GFP-positive, compared to merely 1.9% and 1.6% at cadmium concentrations of 2 and 5µM, respectively. This repair inhibition achieved significant levels at both 2µM and 5µM cadmium (Mann-Whitney-U test, $p < 0.0041$ and $p < 0.011$ respectively). This effect constitutes the combined influence of cadmium on repair completed by both NHEJ as well as SSA, the percentual contributions of which can only be clearly elucidated following cell sorting for GFP-positive cells, the reseeding and cultivation of colonies, and finally the PCR amplification of individual repair junctions. As single-strand annealing generally plays a minor role in the repair of merely a small subset of DSBs (Belmar Campos et al. 2009), the majority of repair events we witnessed can be assumed to be completed by NHEJ. A control transfection with a plasmid containing the GFP gene (pEGPF) demonstrated that cadmium treatment has no significant effect on GFP expression itself, thus ruling out a direct interaction between cadmium and the GFP gene creating the illusion of altered repair efficiency (Fig. 9B).

We then asked if the efficiency of the second major repair pathway, HR, was affected to a comparable extent following cadmium treatment. The repair of DSBs in cells containing the reporter construct for gene conversion was indeed found to be similarly affected, with a decrease in repair capacity apparent for all cadmium concentrations (Fig. 10A, $p < 0.0159$ for 5µM $CdCl_2$). pEGFP control transfections again indicated that cadmium treatment had no significant effect on plasmid transfection efficiency (Fig. 10B), excluding the possibility that the reduced number of GFP-positive cells observed after cadmium pre-treatment is a result of a compromised uptake of I-SceI plasmid.

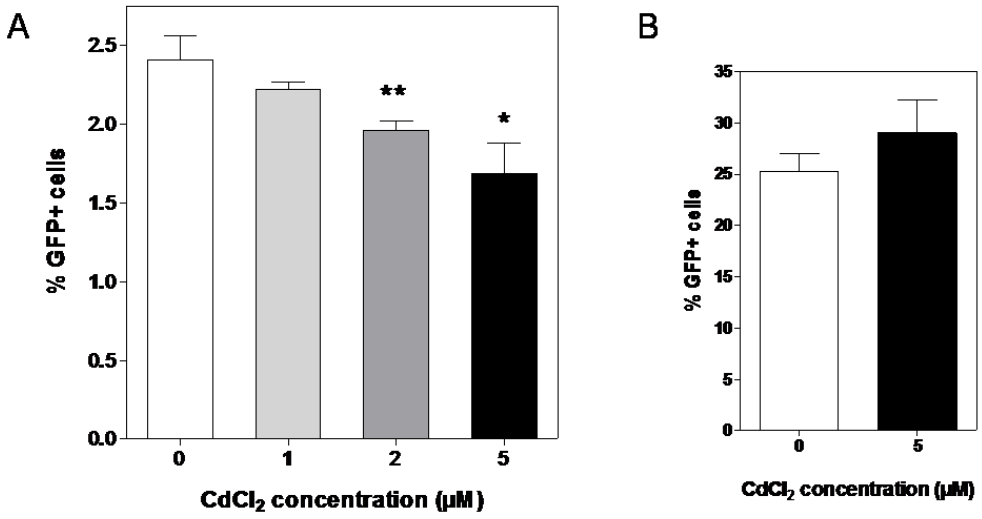

Fig 9. Treatment with cadmium leads a reduction in the repair capacity of HeLa cells via NHEJ and SSA in response to DSBs *in vitro*. (A) Cells treated with $CdCl_2$ display a dose-dependent reduction in repair capacity via NHEJ and SSA following the induction of I-SceI enzyme-induced DSBs. (B) A control transfection with the pEGPF plasmid illustrated that cadmium treatment has no significant effect on GFP expression itself.

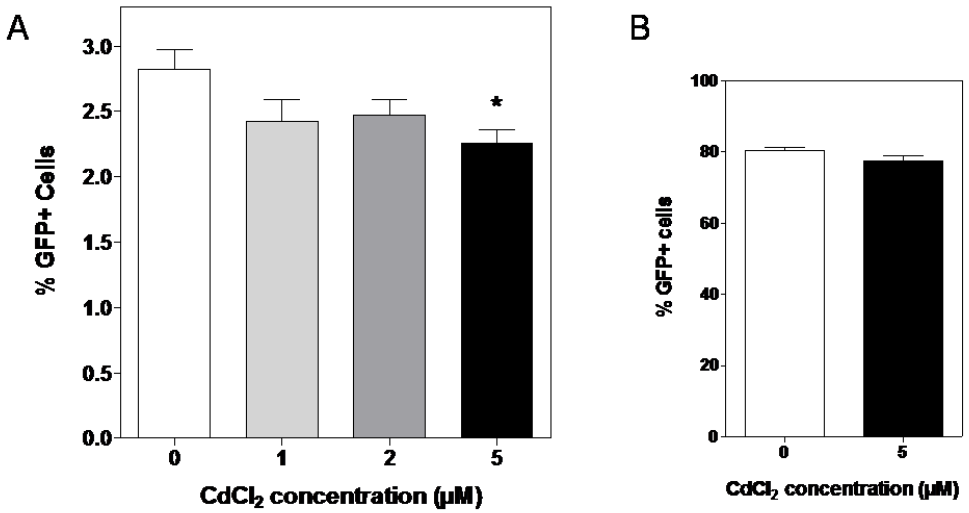

Fig 10. Treatment with cadmium leads a reduction in the repair capacity of HeLa cells via HR in response to DSBs *in vitro*. (A) Cells treated with $CdCl_2$ display a dose-dependent reduction in repair capacity via HR following the induction of I-SceI enzyme-induced DSBs. (B) A control transfection with the pEGPF plasmid showed that cadmium treatment has no significant effect on GFP expression itself.

The inhibition of repair as observed in the context of these experiments could be the result of a number of different but not necessarily separate phenomena. For example, it is possible that cadmium treatment may result in the inhibition of cell growth and in particular the growth of those cells with unrepaired damage so as to reduce the number of green cells observed after 72h. Alternatively, cadmium pre-treatment may result in the direct inhibition of the induction of DSBs despite regular plasmid uptake and I-SceI expression. In order to differentiate between these possibilities, we next investigated the direct influence of cadmium on the digestive activity of the I-SceI endonuclease as well as the ability of cadmium to directly induce DSBs.

4.2. Enzyme control digestion- cadmium does not inhibit I-SceI enzyme activity at experimentally relevant concentrations

A direct interaction between cadmium and the I-SceI endonuclease could lead to the inhibition of the latter's digestive activity, resulting in a smaller number of induced DSBs and thus creating the appearance of reduced repair efficiency. In order to exclude this possibility and confirm the validity of the repair defect observed in the transfection experiments, we conducted a control digestion experiment using concentrations of cadmium far higher than those used in any of our other experimental settings. The digestive activity of the I-SceI enzyme remained unaffected by cadmium concentrations of up to 10,000µM, a concentration 2,000 times greater than the highest used in the transfection assays (Fig. 11).

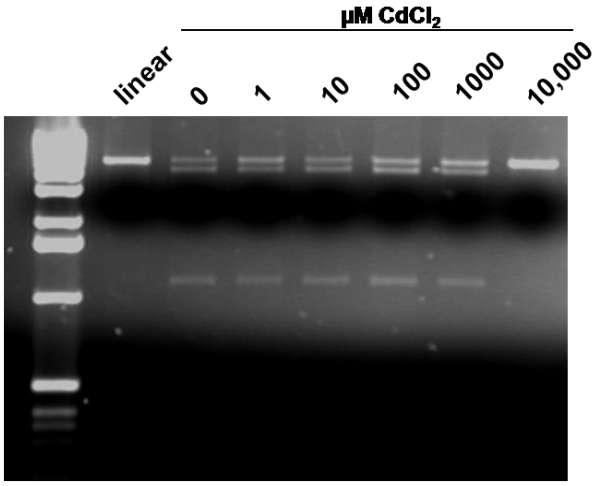

Fig 11. I-SceI restriction endonuclease control assay. pGC plasmid DNA was exposed to the I-SceI restriction enzyme in the presence of various concentrations of cadmium. An inhibition of the digestive activity of I-SceI was first observed at a concentration of 10,000µM $CdCl_2$, a concentration 2,000x greater than the highest concentration used in the repair experiments.

It can therefore be assumed that cadmium in the experimentally applied concentrations does not affect the enzyme itself in a way that could mimic impaired repair in the transfection assays.

4.3. Cadmium inhibits cell growth but does not significantly alter cell cycle distribution

A growth curve charting the number of cells present at various time points during the initial 24h of cadmium treatment shows a clear growth impediment in all cadmium-treated samples compared to the untreated control (Fig. 12A). The obvious and drastic reduction in the number of cells exposed to the highest concentration of 50µM indicates early cell death due to acute toxicity.

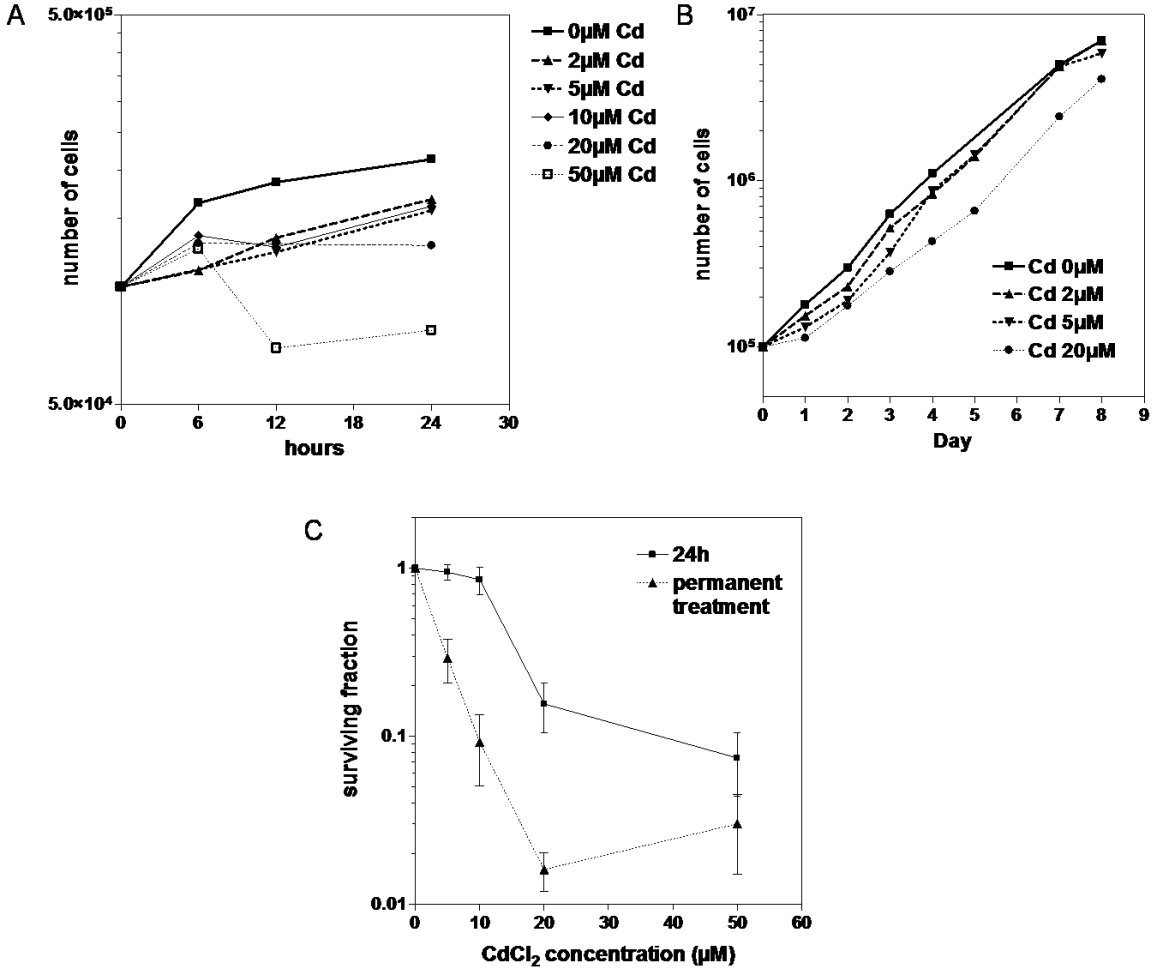

Fig 12. Inhibition of the growth and colony formation of HeLa cells through $CdCl_2$ treatment. (A) Cell growth was found to be inhibited in a dose-dependent manner within the 24h incubation period employed for all following experiments. The growth impairment could be followed throughout the course of a week-long incubation (B). (C) Colony tests revealed a decrease in clonogenic growth with increasing $CdCl_2$ concentration. Permanent treatment led to a further decrease in colony formation.

Throughout the course of long-term exposure to 2 and 5µM $CdCl_2$, the cells achieve growth rates similar to those of control cells, while notably fewer cells were present at the highest cadmium concentration of 20µM (Fig. 12B). It thus appears that cadmium acts immediately by negatively influencing cell proliferation within the first 24h of exposure, an effect which can, however, be overcome during the course of permanent treatment with lower cadmium concentrations, likely as the result of successfully completed damage repair. These findings were confirmed by the results of the colony tests, in which cells were treated either for a 24h period before being reseeded or were reseeded for the colony test in medium containing cadmium, thus constituting a form of permanent treatment (continuous exposure). The clonogenicity of the cells treated for 24h was inhibited noticeably beginning at a concentration of 20µM, while permanent treatment led to a significant reduction in clonogenicity at a concentration of merely 5µM (Fig. 12C).

In order to rule out a significant cell cycle effect that could explain the decreased proliferation of cells treated with cadmium, we investigated the cell cycle distribution of HeLa cells following 24h cadmium pre-treatment at different concentrations. An altered progression through the cell cycle following treatment with heavy metals could help to explain the differences in repair efficiency observed between the two different major pathways investigated, NHEJ and GC. If cells were found to be arrested in G1, for example, a cell cycle phase in which NHEJ is expected to repair the vast majority of DSBs, one could expect an inhibition of repair efficiency via GC, as the cell cannot carry out this type of repair without the presence of a sister chromatid as is found in the S-phase. To investigate these possibilities, the DNA content of cells stained with a PI solution was evaluated via FACS analysis, thus allowing us to differentiate between the relative amount of DNA present in the G1-phase of the cell cycle (basal DNA content), in the course of DNA replication (increased in the S/G2-phase), etc. As can be seen in Figure 13, the distribution of the cells based on total DNA content remains relatively constant between the three lowest cadmium concentrations (Fig. 13A-C). Only at the highest concentration of 50µM can a slight accumulation of cells in the G2 phase be seen, likely representative of the G2-block observed by other authors in other cell systems following heavy metal exposure (Fig. 13D, Yang et al. 2004). However, the general profile of cell cycle distribution is uniform enough to be compatible with the growth kinetics observed here, indicating that progression through the cell cycle is slowed to a similar extent in all cell cycle phases or that continuous cell death impedes the growth of the population as a whole. Taken together, these findings indicate that cell cycle influences are not likely to play a significant role in the differences in growth, clonogenicity, repair capacity or signalling observed in our data. While the distribution of cells among the individual cell cycle phases remains unaffected, the transition of these cells through all phases is delayed as a result of cadmium treatment.

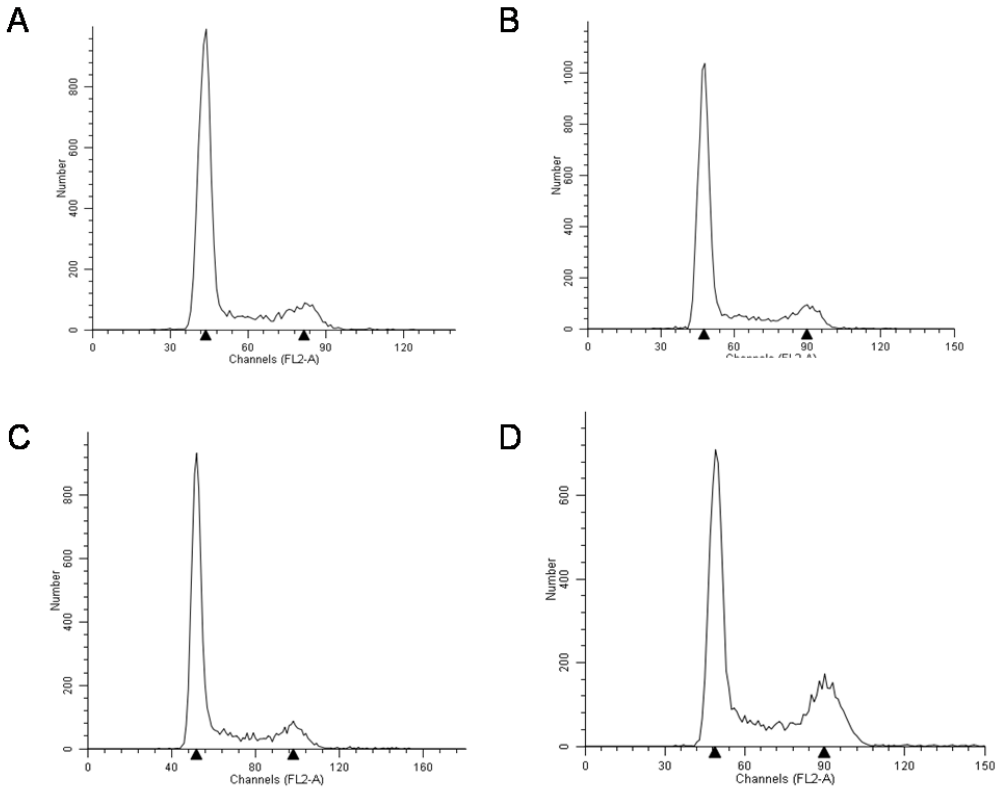

Fig 13. The cell cycle distribution of HeLa cells remains unaffected by cadmium treatment. Cells treated with 0µM (A), 5µM (B) and 20µM (C) $CdCl_2$ prior to the determination of DNA content displayed nearly identical PI-staining profiles, indicating similar progression through the cell cycle between the various concentrations. Only the highest concentration of 50µM led to a slight increase in the late-S/G2 population, indicating cell cycle arrest in the G2-phase prior to mitosis (D).

In this way, the observed results offer an explanation for the reduced number of successfully repaired DSBs observed in the I-SceI assay. Those cells likely to arrest in G2 or exhibit a slower progression through the cell cycle could be exactly those cells with unrepaired damage, thus reducing the number of green cells observed after 72h. The second possibility to explain the appearance of reduced repair capacity, that of a direct induction of DBSs or the inhibition of their repair through cadmium, was investigated in subsequent experiments.

4.4. Cadmium inhibits γH2AX and 53BP1 focus persistence after 24h

The first question to be answered with regard to the specific effects of cadmium treatment on the repair of DNA DSBs was whether or not this heavy metal is capable of directly inducing DSBs. To address this possibility, we observed the formation of γH2AX and 53BP1 damage foci as surrogate markers for DSBs. These two signals, γH2AX, a histone phosphorylated on serine residue 139 as a result of the induction of DSBs, and 53BP1, a mobile nuclear molecule directed to the sites of such damage through interactions with the MRN complex, colocalize with one another and mark the sites of DSBs. The number of γH2AX as well as 53BP1 foci remaining after a 24h period in which the cells have the opportunity to repair DSBs induced by ionizing radiation can be taken as an indication of the repair capacity of the cell line. In this way, the inhibition of DNA DSB repair is generally associated with a greater number of residual breaks after 24h and thus a larger number of damage foci.

The number of γH2AX foci observed after 24h of treatment with cadmium in unirradiated cells was nearly identical between the various concentrations used (an average of one focus per cell), meaning that control cells exhibited a very small number of DSBs. In this way, cadmium does not appear to directly induce DNA damage in the form of DSBs in unirradiated cells. A clear exception to this rule was the highest $CdCl_2$ concentration of 50µM. Visual observance of the slides treated with this concentration clearly showed that many cells had died (decreased cell density on the slides compared to other concentrations) or were actively undergoing apoptosis (visibly blebbing and DNA fragmentation), the latter of which could explain the relatively large number of foci seen at this concentration (Fig. 14A). The range in focus number was found to be especially wide at this concentration, with cells exhibiting either a large number of γH2AX foci (upwards of 40) or none at all (Fig. 14A). Interestingly, those cells with the greatest numbers of γH2AX foci did not exhibit 53BP1 foci, which otherwise colocalized with γH2AX foci at the sites of DSBs for all other cadmium concentrations (Fig. 14A, B). In conclusion, cadmium thus only leads to direct DNA damage at high concentrations through the induction of cell death.

Cells irradiated with 2Gy also showed similar numbers of foci, averaging between two and five foci pro cell at various cadmium concentrations, with slight increases in total focus number seen at the highest two concentrations (five foci pro cell at 20µM, seven at 50µM). A colocalization of γH2AX and 53BP1 foci (Fig. 14C) was observed for the lowest cadmium concentrations of 0-10µM, with disparities being observed for the two highest concentrations. The greatest difference in focus number between the two repair proteins could again be observed for the highest concentration of 50µM.

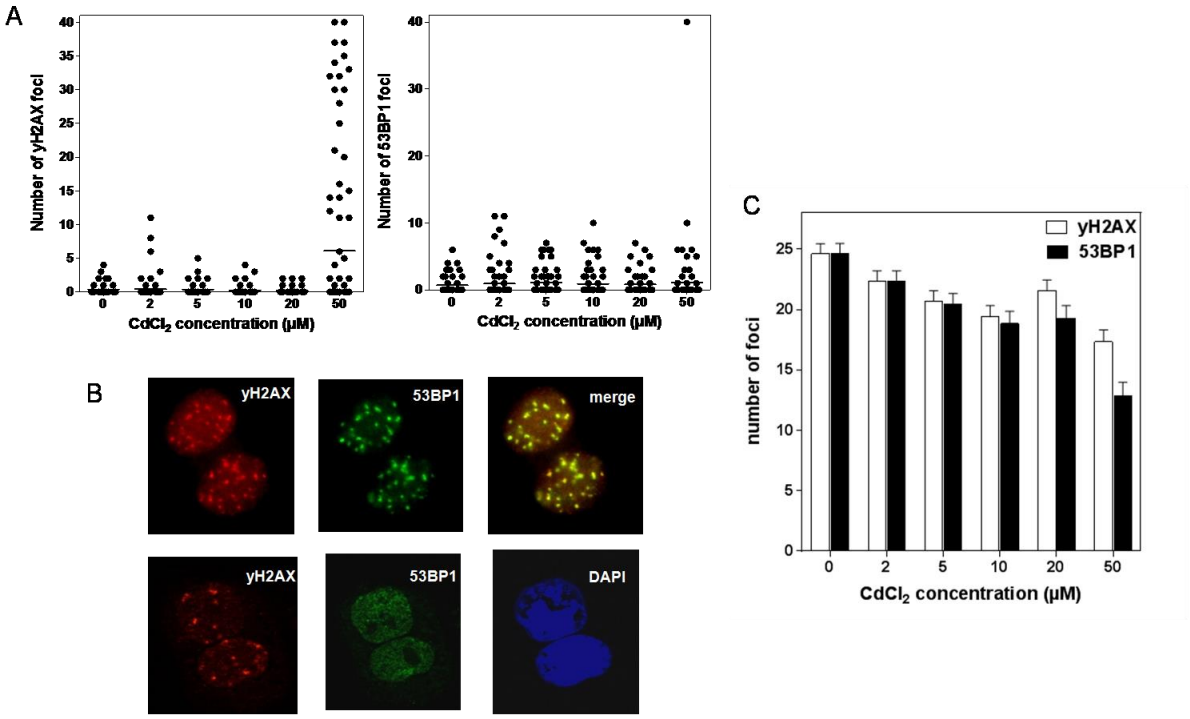

Fig 14. (A) Distribution of unirradiated cells displaying γH2AX and 53BP1 repair foci after a 24h repair period. A number of unirradiated cells treated with 50μM cadmium display γH2AX foci but lack 53BP1 foci. (B) Upper row: colocalization of γH2AX and 53BP1 in cadmium naïve cells 24h after irradiation with 6Gy, lower row: lack of colocalization in cells treated with 50μM $CdCl_2$ after irradiation with 6Gy. The same is the case for 20 and 50μM cadmium after irradiation with 2Gy. (C) Colocalization of γH2AX and 53BP1 repair foci after 24h repair period following 6Gy.

In this way, a tendency towards increasing numbers of both γH2AX and 53BP1 foci with increasing cadmium concentrations could be observed for unirradiated cells as well as for those irradiated with a relatively low dose of 2Gy. An entirely different phenomenon could be observed in cells irradiated with 6Gy, however (Fig. 15). Here, the total number of both γH2AX as well as 53BP1 foci decreased continuously with increasing cadmium concentration. Both types of foci were most evident in irradiated cells naive to cadmium treatment with a average focus number of 25 pro cell compared to the 17 γH2AX and 12 53BP1 foci seen at a $CdCl_2$ concentration of 50μM (Fig. 15). The number of 53BP1 foci closely mimicked the results for γH2AX, again with the exception of the population with a larger number of foci observed at 50μM (Fig. 14C). The smaller number of damage foci lends itself to a multitude of interpretations- it could indicate either successfully completed DSB

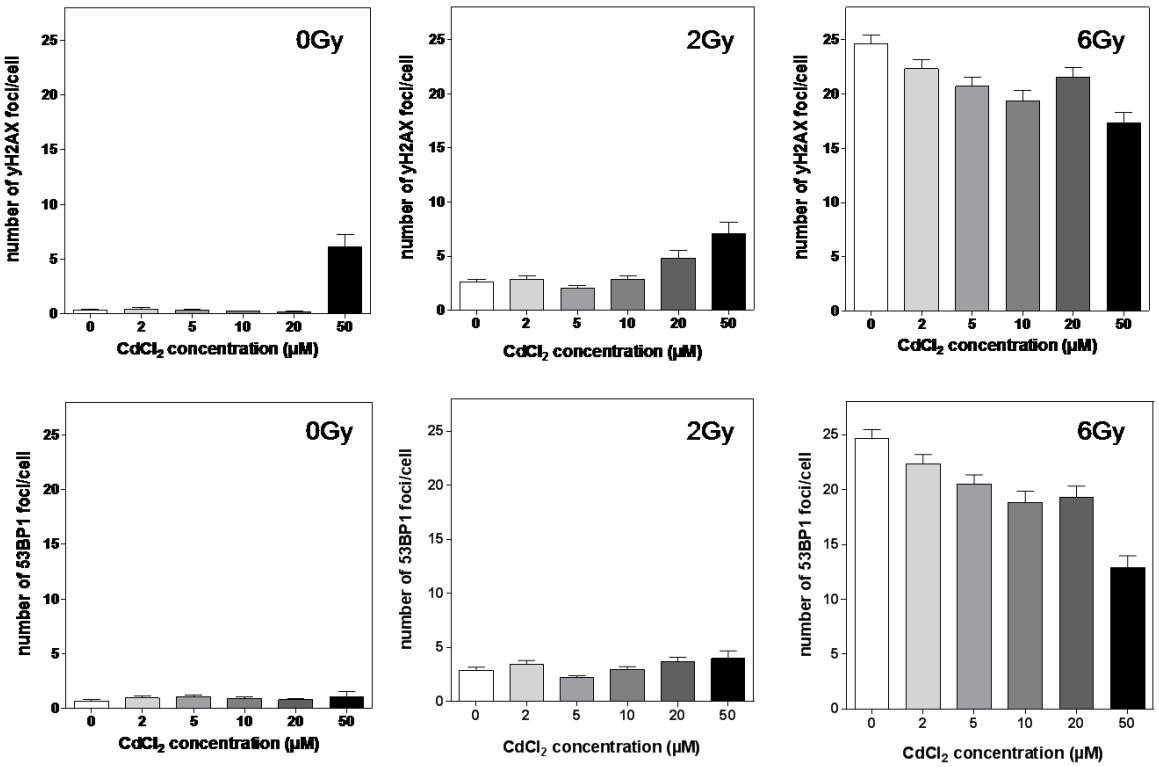

Fig 15. Cadmium treatment results in a reduced number of γH2AX as well as 53BP1 repair foci after a 24h repair period. While only minor differences in focus formation can be observed in cells irradiated with 0 or 2Gy, cells irradiated with 6Gy display a dose-dependent reduction in the number of residual DSBs 24h after damage initiation, likely indicating an impairment in damage signalling.

repair or, alternatively, an impairment in the recruitment or maintenance of DNA damage repair and signalling proteins to/at the sites of DSBs.

As an improvement in repair ability resulting from cadmium treatment seems highly unlikely in the face of our repair and growth assay results, a modulation of the damage signalling cascade appears the likely cause of this phenomenon. Both the initiation of the damage signal as well as the maintenance of the dynamic foci could potentially be altered following exposure to heavy metals. As the total number of foci present in cells irradiated with 6Gy was much larger than those observed for the other IR-doses, it is possible that the stability of a smaller number of foci but not a larger number can be maintained. In order to be able to more clearly differentiate between these possibilities, we next investigated the formation of DSBs as well as their repair through the analysis of DNA fragmentation in constant-field gel electrophoresis experiments.

4.5. Cadmium does not influence the repair of DBSs as measured by constant field gel electrophoresis

As an alternative method of quantifying absolute repair capacity independent of phosphorylation or other signalling events that may have influenced the foci data, we employed the method of constant field gel electrophoresis, in which the amount of unrepaired or fragmented DNA resulting from the induction of DSBs via irradiation can be quantified. We first investigated the ability of cadmium to directly induce DSBs in HeLa cells. For this purpose, plugs were formed as previously described, irradiated on ice and lysed immediately thereafter in order to ensure that the cells had no time to initiate repair processes. This allowed us to investigate whether cadmium may modulate the initial creation of DSBs either on its own or in combination with ionizing radiation, potentially through the induction of ROSs, which could make the DNA more susceptible towards damage induction after treatment with IR. Unirradiated plugs exposed to various doses of cadmium exhibited comparable amounts of damage, confirming our previous finding that cadmium itself does not directly induce DSBs. As expected, the amount of DNA released from the plugs increased steadily with X-ray doses of up to 60Gy (Fig 16A, B). With only slight variations, essentially no difference could be seen in the induction of DSBs following irradiation in cells pre-treated with various cadmium concentrations (Fig. 16B). The greatest amount of DNA release was observed for the highest cadmium concentrations of 10-50µM, possibly reflecting DNA fragmentation as a result of apoptosis or cell death within the 24h pre-treatment period as observed in the growth and damage focus assays. However, cadmium pre-treatment itself was not found to significantly predispose HeLa cells to damage induction prior to exposure to ionizing irradiation.

The same method was employed once again to determine whether exposure to cadmium would influence the repair capacity of cells 24h points after damage induction. For this purpose, cells were irradiated in their culture flasks and were subsequently incubated to allow for damage repair for a period of 24h. The process of DSB repair is expected to be largely completed at the end of this time period, with the damage remaining at this point representing residual or irreparable DSBs. In this setting, cadmium treatment had no significant effect on the repair of radiation-induced DSBs after 24h repair, with similar quantities of DNA being released for all cadmium concentrations and radiation doses. We therefore conclude that cadmium does not result in a defect in DSB repair following damage induction via ionizing radiation. In turn, we would expect to see this lack of influence reflected in the cellular radiosensitivity cadmium-treated, irradiated cells, an effect we sought to demonstrate in clonogenic growth assays.

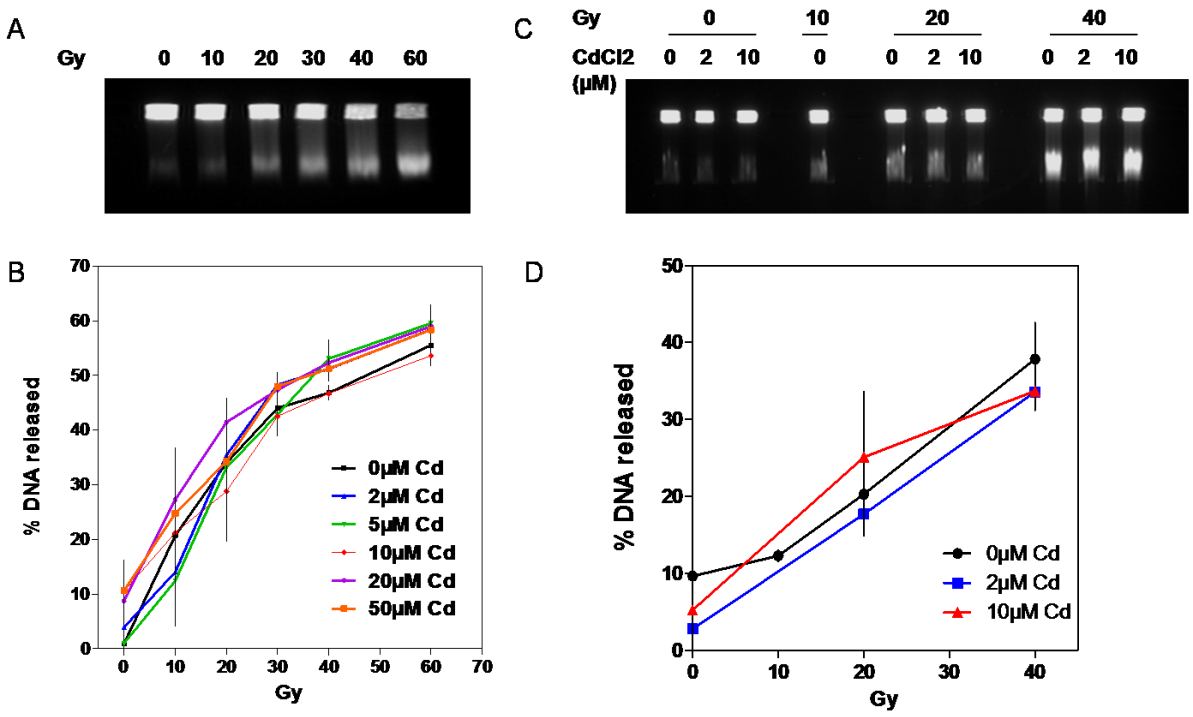

Fig 16. Constant field gel electrophoresis of cadmium-treated cells with and without repair.
(A) Representative gel for Cd-naïve and irradiated HeLa cells. (B) The induction of DSBs differed little between the individual $CdCl_2$ concentrations. Significant DNA release was observed only at the highest cadmium concentrations. (C) Representative gel for repair capacity 24h after irradiation. (D) No significant difference in the amount of residual damage present after 24h repair could be determined between the cadmium concentrations.

4.6. Cadmium only influences cellular radiosensitivity at high concentrations

The radiosensitivity of cells has classically been determined using colony tests, in which the ability of individual cells to proliferate over the course of a greater number of cells divisions serves as a marker of their sensitivity to radiation. The radiosensitivity of HeLa cells pre-treated with cadmium 24h prior to irradiation was investigated in a clonogenic growth assay. Cells treated with various cadmium concentrations showed very little difference in colony formation two weeks post-irradiation, demonstrating a similar decline in colony number with increasing doses of IR. These results suggest that cadmium exposure does not significantly modulate the radiosensitivity of HeLa cells. Somewhat unexpectedly, cells treated with lower concentrations of cadmium appeared to be slightly more radioresistant that the control, while the highest concentration of 50µM rendered the cells most radiosensitive.

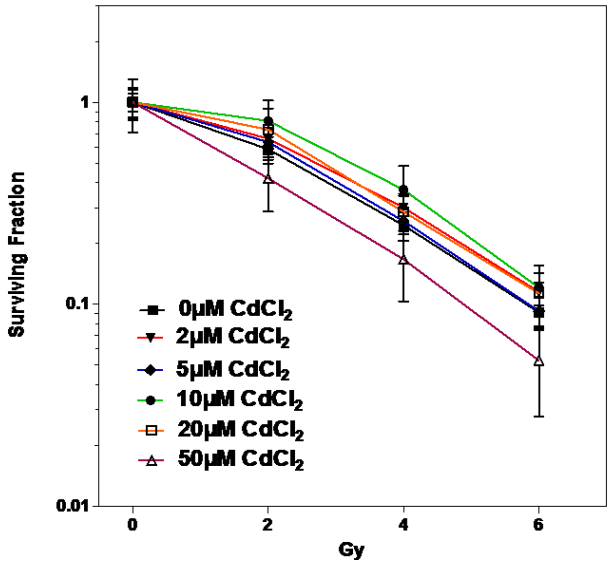

Fig 17. HeLa cells treated with varying concentrations of cadmium show no significant difference in radiosensitivity compared to the untreated control in colony formation assays.

4.7. Cadmium affects the early kinetics of γH2AX but not that of 53BP1 focus formation

The number of repair foci observed at any given time is the result of a complex equilibrium existing between the competing influences of dynamic focus formation, maintenance and ensuing repair. It becomes increasingly difficult to differentiate between these phenomena at later incubation time points after repair processes have been initiated. As we wondered whether the initial period of focus formation and specifically the recruitment of proteins associated with DNA damage recognition, repair and signalling may be particularly sensitive to the influence of heavy metals, we stained for both γH2AX as well as 53BP1 foci at several times points during the initial 2h period of focus formation following IR-damage induction. Lower concentrations of cadmium as well as a relatively low IR-dose (1Gy) were selected to ensure that focus formation was not induced so strongly that individual foci could not be differentiated at these early times points.

Somewhat unexpectedly, the kinetic profile of γH2AX focus formation showed marked differences between the various cadmium concentrations. In cells naive to cadmium, the formation of γH2AX foci occurred at a similar rate as that of 53BP1 foci, with the maximum number of foci being observed after 15 minutes. The total number of γH2AX foci at this time point was slightly lower than that observed for 53BP1 (16 γH2AX foci pro cell compared to 28 53BP1 foci, Fig. 18A, B). Following treatment with cadmium, however, the time point at which

the maximum number of γH2AX foci was formed became increasingly delayed, with the greatest number of γH2AX foci in cells treated with 1μM cadmium being observed after 30 min (approximately 17 foci), and not until 1h for those treated with 5μM cadmium (Fig. 18B). Additionally, the largest number of foci formed in cells treated with 5μM was markedly lower than that observed at lower concentrations, with a maximum of 12 γH2AX foci being observed compared to maximum focus numbers of 16 and 17 for cells treated with 0 and 1μM $CdCl_2$, respectively (Fig. 18B).

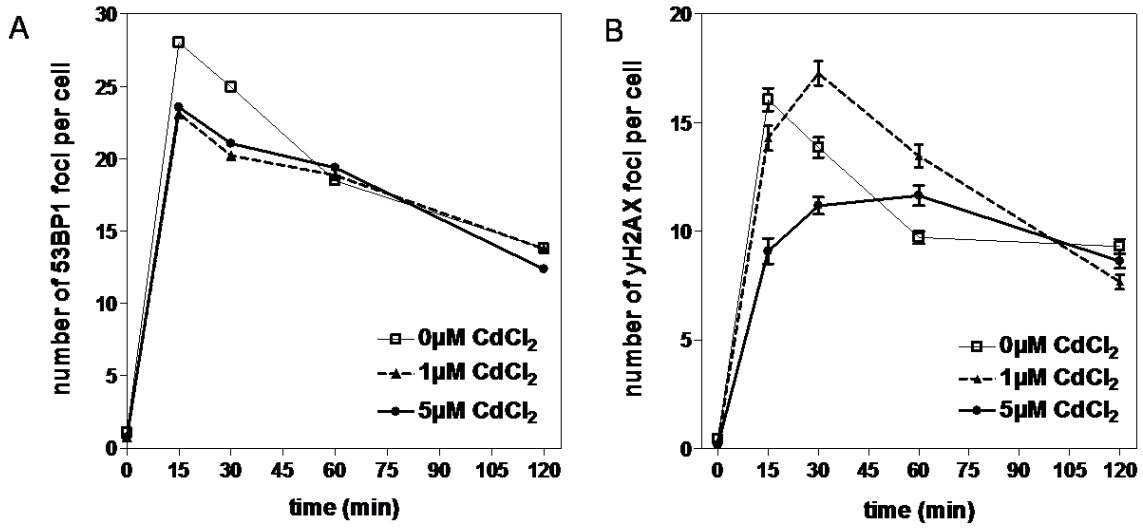

Fig 18. The initial formation of γH2AX foci is impaired following cadmium treatment, not however the formation of 53BP1 foci. Following irradiation with 1Gy, the formation of 53BP1 repair foci in cells treated with varying concentrations of $CdCl_2$ exhibited very similar kinetic profiles compared to the control (A). The kinetics of γH2AX focus formation, in contrast, were detrimentally affected both in terms of a delay in focus formation as well as in the reduction of the total number of foci formed (B).

The formation of 53BP1 foci also appears to occur very quickly following irradiation, with the maximum number of foci already being achieved at 15 min. After this time, the number sinks relatively quickly and at a nearly identical rate regardless of cadmium concentrations, again suggesting that cadmium does not influence early repair processes (Fig. 18A). A maximum focus number of 28 was found in untreated cells, compared to the 24 53BP1 foci observed in cells treated with 1 or 5μM $CdCl_2$ (Fig. 18A). In this way, the total number of 53BP1 foci formed within the first two hours following irradiation is slightly reduced in cells pre-treated with cadmium compared to the control, possibly due to the decreased stability of the γH2AX signal (see discussion). It is nonetheless surprising that the speed with which 53BP1 foci are formed remains unaffected by the relative delay in γH2AX focus formation, as the

phosphorylation of γH2AX is a molecular event generally considered to occur upstream of the recruitment of 53BP1 to the damage sites.

4.8. PARP1 Signalling

From the results of the previous experiments, we considered the possibility that cadmium could interact with proteins responsible for H2AX phosphorylation and 53BP1 recruitment. Of particular interest in this context were proteins involved in the earliest phase of damage signalling, upstream of both H2AX and 52BP1 involvement. As a zinc-finger protein involved in the early signalling of DNA damage, we asked whether the PARP response following the induction of DNA DSBs may be influenced by cadmium treatment. Hydrogen peroxide served as a positive control for the induction of PARP1 by SSBs, thus leading to the synthesis of poly-ADP-ribose. This signal was present immediately following treatment with 100µM H_2O_2 and was completely inhibited following treatment with 10µM $CdCl_2$ (Fig. 19). Unfortunately we were unable to demonstrate a reliable PARP response following ionizing irradiation, leaving the affects cadmium on the role of PARP1 in the context of DSB yet to be investigated.

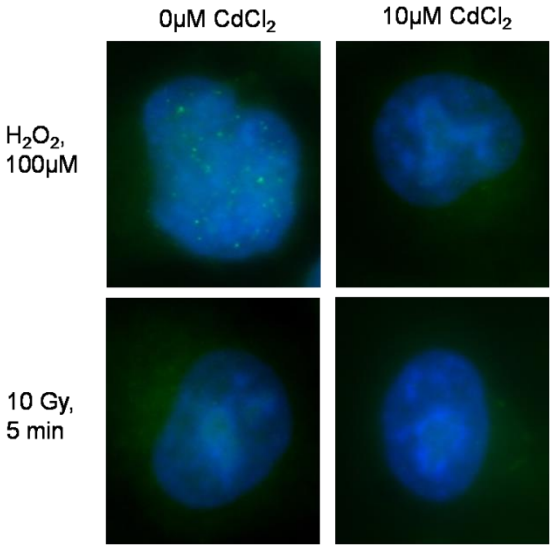

Fig 19. PARP1 signal in cells treated with cadmium and either hydrogen peroxide (H_2O_2) or IR. Cadmium treatment impairs the initiation of the PARP signal following treatment with 100µM H_2O_2. A signal following irradiation with varying IR doses at different time points (here for 5 min after exposure to 10Gy, others not shown) could not be determined.

4.9. Particle irradiation and the real time kinetics of NBS1 recruitment

Live-cell imaging experiments were performed in order to investigate the real-time recruitment of NBS1 as a component of the MRN complex immediately after the induction of DSBs via neon ions. Irradiation with heavy neon ions results in the induction of few but relatively large foci, allowing for the more exact observation of individual foci over time from their induction to their eventual disappearance through repair. In our experiments, NBS1 focus formation became visible within merely seconds following irradiation. The quantitative analyses showed, however, that the recruitment of GFP-tagged NBS1 to the sites of radiation damage was not affected by pre-treatment with 20µM cadmium. Neither the kinetics of NBS1 focus formation nor the amount of protein assembled at the damages sites was significantly altered.

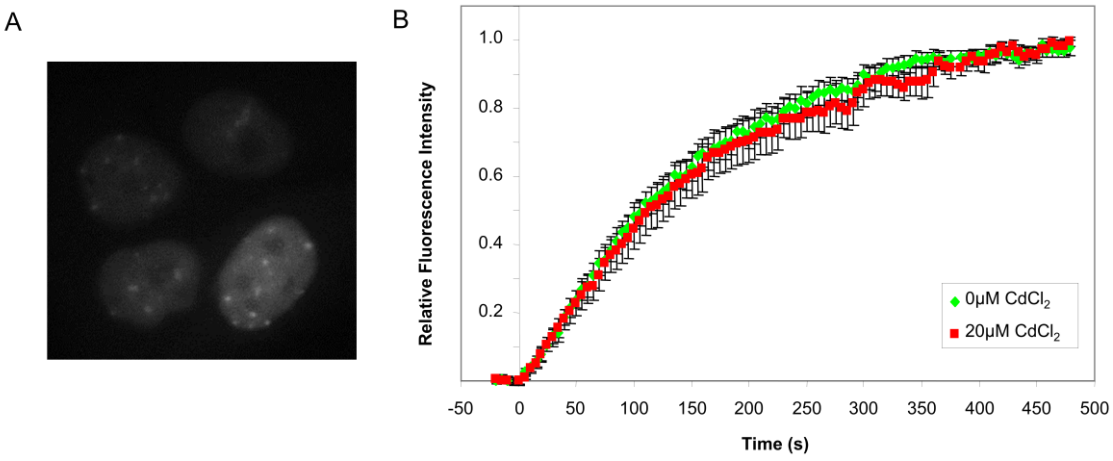

Fig 20. Human osteosarcoma cells expressing a NBS1-GFP construct treated with 20µM cadmium. (A) Focus formation appears within seconds of irradiation neon ions. (B) No significant difference was observed between the control and cadmium-treated cells in terms of either the speed of NBS1 recruitment to the DSBs following damage induction or the total amount of protein that accumulated at the damage site.

5. Discussion

The purpose of this work was to investigate the extent to and ways in which exposure to heavy metals and specifically cadmium might alter the function of various proteins involved in the complex process of DNA double-strand break repair and in this way to identify mechanisms by which heavy metals might contribute to tumorigenesis in humans. Of specific interest in this project was the potential interaction between cadmium and proteins involved in DNA damage recognition and repair containing so-called zinc-finger or RING-finger structures which, as a result of these metal-bearing protein-protein interaction domains, have been shown to be particularly susceptible to interactions with other metallic substances. Through the substitution of one metal for another, the structure of these proteins can be altered and with it, the effectiveness of their biological function.

5.1. Cadmium toxicity and resulting apoptosis

Initial experiments were conducted to determine whether cadmium exerts a directly toxic effect on HeLa cells as well as to specify a suitable range of non-toxic concentrations to use in other experimental settings. For this purpose, we investigated the growth of HeLa cells exposed to low concentrations of the metal. While cell proliferation was found to be largely unaffected at low cadmium concentrations, treatment with 20µM $CdCl_2$ consistently inhibited cell growth over the course of the eight day observation period, comparable to the growth inhibition resulting from cadmium pre-treatment observed for other cell systems (Cao et al. 2007, Yang et al. 2004, Pan et al. 2009). The highest concentration utilized in our experiments, 50µM, was found to lead to significant cell death within the first 12h of exposure, in addition to continuously inhibiting cell proliferation observed in the form of reduced colony formation in the colony assay, both after a single 24h exposure period as well as even more drastically with continuous cadmium treatment. In light of these observations, we decided to employ cadmium chloride concentrations of up 5µM for the sensitive repair efficiency experiments, while investigating exposure of up to 50µM in focus formation as well as colony assay experiments. Although apoptosis was not directly quantified in any individual experiment, the visible decrease in the number of cells growing on flask or slide surfaces as well as the observed cell morphologies resulting from higher concentrations of cadmium were consistent with those known for apoptotic cells, such as nuclear fragmentation and cell blebbing. Cadmium has been shown to induce apoptosis in human skin cells via JNK and p53-mediated pathways (Son et al. 2010), while programmed cell death following cadmium treatment has been described by a number of other authors

(Aimola et al. 2012, Yu et al. 2011, Beyersmann et al. 2008). We therefore conclude that apoptosis is indeed likely in our experimental system, but only at the highest cadmium concentration tested.

5.2. The effects of cadmium on cell cycle progression in HeLa cells

Cancer cells have evolved a number of different mechanisms in order to evade the regulatory mechanisms cells are normally subjected to in the course of their growth and proliferation. Each of these controls, when successfully carried out, constitutes an important strategy in protecting the cell against uncontrolled and potentially invasive growth. At the same time, such mechanisms, when aberrantly regulated in or completely disregarded by a growing cancer cell, provide opportunities for individual cells to win a competitive advantage over their neighbours and further evolve into invasive cancer cells. The most important regulatory mechanisms involved is this process are those of cell cycle control, the initial recognition and consequent signalling of DNA lesions and the subsequently initiated repair of this damage. All three of these aspects have been investigated in the context of this thesis in an effort to determine the potential mechanisms by which heavy metals such as cadmium, a known carcinogen, may interfere with or even impair the cell's response to damage in the form of DNA DSBs. A number of human diseases known to be associated with an increased risk of developing various cancers have been determined to result from mutations or other defects in key genes and proteins involved in the cell cycle and signalling response to DNA damage- Ataxia telangiectasia, where a defect in the ATM gene is generally associated with leukemias and lymphomas; Nijmegen breakage syndrome, in which the MRN complex member NBS1 in mutated, resulting in cancers of the blood; Fanconi's anemia, a number of related syndromes caused by various mutations in genes of the Fanconi family and associated with increased susceptibility to acute myelogenous leukemias; and familial breast and ovarian cancers as the result of BRCA1 and BRCA2 mutations, to name but a few (Kastan et al. 2004, Bolderson et al. 2009).

The cell cycle is divided into specific phases, each with its own purpose and specific criteria for entry and completion, with progression through the cell cycle dependent on the cell's ability to successfully complete replication and cell division. One of the most essential regulators of the cell cycle, the tumor suppressor and zinc-finger protein p53, would certainly constitute a potential target for heavy metals. Cadmium has been shown to inhibit the p53 response to damage by various genotoxic agents such as actinomycin D, methylmethane sulfonate and hydrogen peroxide in human breast cancer MCF7 cells expressing wild-type p53 through the structural inhibition of DNA-binding and down-regulation of a downstream

target gene (Méplan et al. 1999). The potential influence of p53 was effectively silenced in this project through the use of HeLa cells lacking functional p53. Although the differences in cell cycle progression observed in this work are relatively minor, we did observe a slight accumulation of HeLa cells in the late S- and G2-phase of the cell cycle at the highest cadmium concentration of 50µM, possibly indicating heavily damaged cells unable to enter mitosis. Similar cell cycle differences have also been observed in Chinese hamster ovary cells, where 24h cadmium-treatment with a similar range of doses (0.1-4µM) led to both a significant decrease in BrdU incorporation as well as an increased G2-fraction for a concentration of 4µM (Yang et al. 2004). Both the impaired BrdU incorporation as well as comparatively larger G2 population (approximately 25% more G2/M cells than in the untreated control), particularly at later time points (16-24h), indicate that cadmium both represses DNA synthesis as well as induces late toxicity, presumably as a reflection of DNA damage or cell death (Yang et al. 2004). The inactivation of clonogenic survival, inhibition of DNA synthesis and an accumulation of cells in G2 following cadmium treatment have also been observed in human fibroblasts, the mechanism for which the authors notably suggest to be ATM-independent due to the lack of change in Chk2 signal in western blot experiments (Cao et al. 2007). While the influence of Chk2 signalling was not specifically addressed in the context of this work, the lack of Chk2-phosphorylation does not exclude the possibility of changes to cadmium modulated ATM-signalling through other target molecules such as H2AX or other directly repair-related proteins (see below).

5.3. The induction and repair of DSBs following exposure to cadmium

The creation of double-strand breaks, the most grievous form of DNA damage, is a sufficient signal for a cell to immediately initiate repair processes or alternatively induce apoptosis or cell death. Both the persistence of unrepaired DSBs as well as those incorrectly repaired can facilitate tumorigenesis through the resulting loss or alteration of genetic material, the creation of chromosome structural abnormalities or genetic instability. In order to determine whether the observed cadmium toxicity may be the result of the direct formation of DSBs, we investigated the initial formation as well as maintenance of γH2AX and 53BP1 nuclear foci, two proteins involved in the repair of radiation-induced DSBs and known to co-localize at the sites of these breaks. Unirradiated HeLa cells were found to exhibit a small number of γH2AX foci, even without having been exposed to cadmium. A very similar number of 53BP1 foci, often with a slightly larger focus morphology than the other foci, was also found in these cells, as previously described in a number of other publications (Mochan et al. 2003, 2004; Lee et al. 2009). This number remained the same with increasing cadmium concentrations in

unirradiated cells, indicating that cadmium does not directly induce DSBs, again with the exception of the highest concentration of 50µM as the result of the initiation of apoptosis. We were therefore unable to confirm the cadmium-induced direct DSBs observed by Viau et al., who described increasing numbers of γH2AX foci in human microvascular endothelial cells at concentrations up to 100µM (Viau et al. 2008). The group also reports an increase in the percentage of cells with micronuclei as well as an inhibition of DNA-PK kinase activity in a millimolar concentration range, thus indicating an inconsistency with our observations. However, these concentrations were effectively 1,000-fold greater than normal physiological concentrations, effectively raising doubt about the practical relevance of those observations. (Viau et al. 2008).

5.3.01. I-SceI-based repair assays for NHEJ and GC

The dose-dependent decline in the number of successfully completed repair events observed for both NHEJ as well as GC in the reporter assays is not entirely consistent with the comparable numbers of DSBs determined in the constant field gel electrophoresis experiments. There are a number of mechanistic possibilities for explaining these differences. The DSBs induced in the course of our transfection experiments and measured in FACS represent a very specific type of DSB, namely restriction enzyme-induced DSBs, the repair of which may differ significantly from that of IR-induced DSBs. It has been shown that the removal of a break-inducing enzyme from the target protein upon the completion of its digestive activity is an essential step in the successful completion of the repair process. DNA and the restriction enzyme build a covalent protein DNA “cleavage complex” which is usually of a transient nature, as in the case of topoisomerases I and II in the course of DNA replication (Connelly et al. 2004). When the subsequent “resealing” of the DNA is inhibited, however, for example through application of the chemotherapeutic drug camptothecin, DNA DSBs can be the result (Connelly et al. 2004). It is therefore possible that the corresponding step in our experimental model, the removal of the I-SceI enzyme from the GFP gene following DSB induction, may be inhibited, thus creating the appearance of a DSB which cannot be completely successfully repaired. Interestingly, the tyrosyl-DNA phosphodiesterase (TDP1), a metal-sensitive DNA repair enzyme implicated in the repair of a subset of DSBs, has been found to reverse these cleavage complexes through the hydrolysis of tyrosyl-DNA phosphodiester bonds (Connelly et al. 2004, Cortes Ledesma et al. 2009, Zhou et al. 2009). Patients carrying Tdp1 mutations with the resulting genetic disorder SCAN (spinocerellar ataxia with axonal neuropathy) do not appear to be significantly more susceptible to cancer than others (Connelly et al. 2004). However, it has been demonstrated that the SCAN1 TDP1-mutant cell line lacking this functional kinase exhibits a greater

number of micronuclei as well as acentric and dicentric fragments compared to control cells following treatment with calicheamicin, an agent known to induce DNA DSBs (Zhou et al. 2009). A related publication reported an increased number of γH2AX damage foci 16h after etoposide treatment in cells depleted of TTRAP, a $Mg^{2+/}Mn^{2+}$ dependent phosphodieserase related to TDP1 (Cortes Ledesma et al. 2009). A metal-dependent interaction between cadmium and TDP1 or a functionally similar protein thus represents a possible mechanism by which this heavy metal may interfere in DSB repair by inhibiting the resolution of protein-DNA complexes during repair. This scenario would ultimately lead to a reduced number of successfully rejoined DSBs, as we observed in the reporter construct assays for enzymatically induced DSBs.

Another factor that comes into play during the execution of the reporter-based repair assays is the 48h transfection period in which the cells continue to proliferate. Given that cadmium inhibits cell growth, it is possible that especially those cells in which DSBs were induced are also those incapable of further proliferation. In this way, undamaged cells may gain a proliferative advantage over those previously damaged, thus causing the appearance of a reduced number of cells that underwent the “mutating” NHEJ or GC process leading to green fluorescence, even though the repair process per se might not be significantly affected by cadmium.

5.3.02. DSB repair and radiosensitivity

The significantly smaller number of repair events completed via the non-homologous end-joining and homologous recombination pathways observed for enzyme-induced DSBs following cadmium treatment stands in stark contrast to the reduced number of γH2AX and 53BP1 repair foci observed following exposure to both cadmium and ionizing irradiation, raising the question of whether or not cadmium may interfere with signalling and repair processes in the HeLa cell system. We thus required another experimental system in order to observe DSB induction and repair independent of signalling events such as H2AX phosphorylation, a step which itself could potentially be affected by cadmium treatment. For this purpose, we analyzed the physical fragmentation of DNA following cadmium exposure as well as in combination with ionizing irradiation by means of constant field gel electrophoresis. As the fraction of DNA released was found to increase with X-ray doses of up to 60Gy, as expected, but was not significantly altered by additional exposure to cadmium, we confirmed the earlier observation that cadmium neither directly induces DSBs, nor does it have further impact upon radiation-induced damage. Along similar lines and despite the notable differences in γH2AX and 53BP1 foci, DSB repair in the first 24h after IR

was not found to be significantly influenced by cadmium pre-treatment at the same concentrations. Together, these data demonstrate that cadmium treatment neither modulates the induction of DSBs nor their repair following exposure to ionizing irradiation.

These data correspond with those obtained from colony formation experiments in HeLa cells pre-treated with cadmium before being irradiated with various IR-doses. In this assay, the radiosensitivity of HeLa cells remaining largely unaffected by cadmium concentrations of up to 20µM. The significantly reduced colony formation observed at a concentration of 50µM, thus indicating increased radiosensitivity, was presumably due to the direct cell kill resulting from the combination of radiation and cadmium toxicity.

5.3.03. Repair fidelity

As mentioned, our research group previously established a hierarchy of DNA DSB repair pathways, with the initial step in choosing the repair pathway depending on the presence or absence of the Ku80 protein. In response to DSB induction in the presence of this functional protein, the cell will preferentially perform a high fidelity form of NHEJ facilitated by the Rad51-controlled inhibition of SSA (Mansour et al. 2008). Loss of functional Ku80, however, enables the cell to additionally perform a more error-prone form of NHEJ, resulting in larger numbers of base deletions (Mansour et al. 2008, Schulte-Uentrop et al. 2008). Liu et al. describe differences between Ku70-competent and deficient cell lines following treatment with the heavy metal salt sodium arsenite, with Ku-deficient cells showing a greater percentage of cells with longer “damage tails”, an indication of DSBs in single-cell gel electrophoresis assays (Liu et al. 2007). Although these effects are likely to be the result of direct or indirect DSB induction, the possibility that a repair defect may also play a role in this model cannot be entirely ruled out. Should cadmium in fact interact with Ku in a way that inhibits its function, the result could influence the cell’s “choice” of a DSB repair pathway. The error-prone form of NHEJ in these cells could contribute to an increase in the number of DSBs through the associated loss of genetic material. Although DSB repair fidelity was not specifically investigated in the context of this work, it nonetheless remains a possibility that the manipulation of repair pathway choice in response to cadmium exposure may preferentially activate more error-prone forms of repair and in this way increase genetic instability. This could mean that while the number of DSBs is not altered, the repair itself is less precise, thus increasing genetic instability and hence promoting carcinogenesis.

Of further interest is the fact that this alternative end-joining pathway has been shown to require PARP1 (Mansour et al. 2010). PARP1 is of decisive importance in directing the repair of DSBs through its role in the recruitment of Mre11 and NBS1 to the sites of DNA damage.

PARP1 and Mre11 have been demonstrated to physically interact with one another in response to treatment with etoposide as well as to colocalize to damage foci following micro-laser irradiation (Haince et al. 2007). In terms of repair however, Mre11 has been observed to promote efficient NHEJ, with cells depleted of Mre11 showing suppressed end-resection (Xie et al. 2009), though these data have been questioned by others (Di Virgilio et al. 2002). A functional interaction between PARP1 and cadmium could therefore inhibit the earliest step in damage recognition, thus specifically altering the efficiency or fidelity of the alternative form of NHEJ. As a decrease in the PAR-signal could be demonstrated in this work following treatment with cadmium, this possibility should certainly be further explored in the future.

5.4. DNA damage response- the effects of cadmium on γH2AX and 53BP1 signalling

The DNA damage response precedes the actual physical repair of DNA strand breaks and is essential for communicating the exact nature of the damage to be repaired by the cell's repair machinery. The complex interaction between a vast number of proteins, most involved in the damage recognition of multiple damage types of DNA damage and thus playing a role in more than one repair pathway, suggests multiple protein-protein interaction interfaces potentially susceptible to disregulation. Proteins of particular interest in the context of this work include the zinc-finger containing proteins PARP1, Rad50 and Rad18 as well as the RING-finger proteins RNF8, RNF168 and BRCA1.

We specifically investigated the formation of γH2AX and 53BP1 foci, two well established surrogate markers of DSBs, in response to cadmium treatment and in combination with IR. These proteins physically colocalize at break sites, aided in part by the physical interaction between 53BP1 and phosphorylated Ser-139 of H2AX. The phosphorylation of the histone variant H2AX is typically initiated by ATM or, in its absence, by another member of the PIKK family, within one minute of exposure to IR, a signal which is then propagated over chromatin domains extending for megabases (Rogakou et al. 1999). The phosphorylation of H2AX is delayed in cells lacking functional ATM (Stiff et al. 2004). P53 binding protein 1 (53BP1) is shown to colocalize with γH2AX foci at both early (30 min) as well as later time points (8h) after the induction of DSBs through irradiation or in response to other agents such as etoposide (Schultz et al. 2000). Focus formation could be observed at the earliest time point investigated here, 5 min, and reaches its peak approximately 30 min after irradiation (Schultz et al. 2000).

Cells naive to cadmium treatment exhibited nearly identical numbers of γH2AX and 53BP1foci following ionizing irradiation with up to 6Gy 24h after irradiation. Exposure to

higher levels of cadmium did not have a significant effect on the number of either γH2AX or 53BP1 foci formed following irradiation with up to 4Gy. Unexpectedly, however, after irradiation with 6Gy, even low cadmium concentrations significantly reduced the number of both γH2AX and 53BP1 foci in a perfectly parallel manner. These results suggest that either DSB repair was improved by increased cadmium concentrations or, again, that the stability of the damage signal was affected, but not the removal of the damage itself. Further, it is possible that the stability of a relatively smaller number of γH2AX and 53BP1 signals formed after relatively weak damage induction (2Gy) can be maintained in the presence of low cadmium concentrations, but that larger focus numbers cannot be maintained.

The possibility that cadmium may influence the formation and stability of damage signals proved difficult to address, as late repair foci after 24h constitute the result of a dynamic equilibrium between damage induction and repair, as well as the formation, stability and dissolution of the sub-nuclear protein foci. In order to effectively filter out the later effects of repair and focus resolution, we investigated the initial damage response within an early time period of up to 2h after radiation. As expected and in line with other publications (Celeste et al. 2003), the maximum number of both γH2AX and 53BP1 foci was reached within the first 15 min after irradiation before subsequently declining over the course of the 120 min observation period, presumably due to immediate early repair. Although the maximum number of 53BP1 foci induced declined slightly following cadmium treatment compared to untreated cells, the formation and repair kinetics exhibited similar profiles regardless of cadmium concentration. Interesting, however, even the very small sub-toxic concentration of 1µM cadmium delayed the phosphorylation of H2AX compared to untreated cells. Treatment with 5µM further delayed focus formation, with the maximum number of foci not being achieved until after one hour. In addition, the peak number of foci was significantly reduced by approximately 30%. This disparity in the formation of the damage foci of these two proteins was unexpected, as 53BP1 recruitment has thus far been considered to be an event downstream of H2AX phosphorylation (Doil et al. 2009). However, the early accumulation of 53BP1 has been shown to occur even in cells lacking this histone component (Celeste et al. 2003). Long-term stability cannot, however, be achieved without phospho-H2AX. The number of 53BP1 foci induced in the absence of H2AX (H2AX-/- (KO) MEFs)) was comparable to the control within the first 15 min after damage induction, but decreased rapidly in the time thereafter, indicating the participation of γH2AX in the recruitment but perhaps more importantly in the maintenance of 53BP1 foci (Celeste et al. 2003).

53BP1 focus instability was also observed in the context of a Rad18 deficiency. Rad18 knock-out cells displayed increased protein dynamics at damaged chromatin sites as indicated by enhanced FRAP signals after bleomycin treatment (Watanabe et al. 2009). It is

therefore possible that cadmium could interact with RAD18 and in this way impair not the initial recruitment of 53BP1 to DSB damage sites, but instead the stability of the damage foci or chromatin retention of 53BP1 after longer time periods.

The stability of γH2AX foci may be additionally affected by other proteins. The dephosphorylation of tyrosine residue 142 on H2AX is carried out by the EYA tyrosine phosphatase, originally identified as a transcription factor necessary in various developmental pathways. Under normal circumstances, Y142 of the H2AX histone is constitutively phosphorylated, a molecular action which is carried out by the metal-sensitive WSTF protein (Krishnan et al. 2009). Depending on the presence/absence or functional activity/inactivity of the similarly metal-sensitive EYA protein, the cell can effectively be steered down one of two pathways, with the goal of either repairing the damage or inducing apoptosis. In the presence of functional EYA, this protein is activated upon DNA damage induction, dephosphorylates Y142 and subsequently allows for the binding of proteins needed for the induction of repair events such as MDC1 and the MRN complex, both of which are necessary for building a robust γH2AX signal in response to DNA damage. If this step cannot be completed, however, the still phosphorylated tyrosine reside can attract proteins such as JNK1 and Fe65, which effectively block the recruitment of the above mentioned proteins to pS139 of γH2AX and induce apoptosis (Cook et al. 2009). If cadmium were to have an inhibitory effect on the function of the metal-sensitive EYA protein, this protein would no longer perform the dephosphorylation of Y142 necessary for MDC1 recruitment, and would thus weaken the γH2AX signal, as observed in our data. Additionally, the phosphorylation of EYA by ATM presents another molecular interface at which cadmium could exert its influence, as the heavy metal-induced inhibition of ATM function would theoretically impair EYA. The impairment of the similarly metal-sensitive WSTF protein, the activity of which is dependent on the presence of ATP and a divalent Mn^{2+} ion, could also weaken the DDR by impairing the initial phosphorylation of Y142 (Xiao et al. 2009). Cells in which WSTF had been inhibited using RNAi exhibited relatively small and decreased number of MDC1, ATM as well as γH2AX foci 8h after irradiation with 10Gy (Xiao et al. 2009). The inhibition of WSTF through cadmium treatment could therefore help to explain the reduced number of γH2AX and 53BP1 repair foci we observed with increasing concentrations of cadmium and following ionizing irradiation.

Other authors suggest that targets of ATM kinase activity may be affected by cadmium treatment (Gastaldo et al. 2007). With hundreds of known phosphorylation targets, an interaction between ATM and heavy metals could influence manifold theoretical aspects of tumorigenesis including the manipulation of cell cycle regulation, DNA damage signalling and repair. The impairment of ATM function could, for example, help to explain the decrease in

the number of γH2AX foci observed through the inhibition of the histone phosphorylation of H2AX. Though the recruitment of 53BP1 to DNA DSBs has been shown to be independent of ATM (Zgheib et al. 2005), 53BP1 has also been demonstrated to be phosphorylated on serine residue 1219 (S1219) in response to ionizing radiation (Lee et al. 2009). Interestingly, these authors also observed a weakening of the γH2AX signal in cells with S1219A mutant 53BP1, suggesting an ATM-driven signalling or feedback step downstream of γH2AX. Suppression of the 53BP1 signal response in siRNA experiments was not shown, however, to influence ATM activation (Mochan et al. 2003, 2004; DiTullio et al. 2002), though 53BP1 is required for the phosphorylation of SMC1 at Ser 966, but notably not for the phosphorylation of H2AX. It is nonetheless possible that long-term focus stability resulting from an interaction between 53BP1 and ATM protein targets could be negatively influenced by cadmium. Guo et al. recently offered a mechanistic explanation for a possible interaction between ATM and cadmium. The FATC domain of ATM contains a single cysteine residue (C2991) that is critical for protein activation in response to ROS, and thus may play a role in the early ATM signalling response following exposure to IR (Guo et al. 2010). In this way, a functional interaction between this cysteine residue and cadmium could effectively alter a myriad of signalling interactions through the modulation of ATM function. Taken together, these results suggest a potential for interaction between cadmium and ATM that could have significant effects on damage signalling as a whole and therefore warrants intensive investigation in the future.

We further addressed the question of whether early events in the DNA damage response upstream of both the γH2AX and 53BP1 signal such as the assembly of the MRN complex might potentially be affected by heavy metal treatment. Unfortunately, due to technical difficulties, we were unable to adequately evaluate the response of PARP1, known to functionally interact with the MRN complex, following cadmium treatment and DSB induction through ionizing radiation. We did, however, observe a suppression of the PAR (pADRr polymer) signal following damage induction with H_2O_2 in cadmium-treated cells, offering strong support for the hypothesis that cadmium can interact with PARP1, possibly through an association with one of the protein's zing finger domains.

The Rad50 protein contains two zinc-fingers and constitutes a central structural element of the MRN (Mre11, RAD50, NBS1) complex necessary for the initial recognition of DSBs. The binding of cadmium to these metal-bearing domains and its competition with zinc may not only critically weaken the DNA-protein binding of Rad50, but may also negatively alter the crucial interaction of the other MRN complex components. Live-cell imaging experiments indicated, however, that the recruitment of GFP-tagged NBS1 to the sites of radiation damage was not affected by exposure to cadmium. Neither the kinetics of NBS1 focus

formation nor the amount of assembled protein was altered by the relatively high concentration of 20µM. The compromised assembly of the MRN complex thus appears unlikely to be the cause of the cadmium-mediated reduction in H2AX phosphorylation. However, a potential direct effect of cadmium on the Rad50 protein cannot be ruled out based on these experiments.

5.5. Tumorigenesis through the destabilization of the DDR signalling response

In recent years, interest in recognizing early events in cancerogenesis has led to the formation of the so-called cancer barrier hypothesis, by which the progression of tumor growth and invasiveness has been associated with an early activation and later inactivation of the DNA damage response. This raises the question of whether the collapse of the DNA damage response network, as observed in the declining number of nuclear damage foci with increasing cadmium concentrations, may contribute to acceleration of the carcinogenesis process. Works by Bartek et al. document changes in the DNA damage response throughout different stages of human urinary bladder cancer from early superficial lesions in the form of stage Ta tumors through more advanced and locally invasive T4 tumors. Analyzing treatment-naive tissues samples, the authors observed a notable increase in the expression of DDR proteins such as Chk2 and ATM and their activated, phosphorylated forms as well as in pS-p53, γH2AX and Ki67 as a general proliferation marker. Interestingly, all of these proteins classically involved in protecting a cell from cancerous devolution were found to be highly active in early stages of tumorigenesis, particularly in Ta tumors, before becoming generally less prominent or only being expressed by small islands of tumor cells in T2-T4 tumors, regardless of the proliferative status of the cells. Similar results were also seen for early ductal carcinoma in situ of (DCIS) and invasive ductal carcinomas of the breast as well as for lesions of the colon (Bartkova et al. 2005) and non-small cell lung carcinomas and their precursors (Gorgoulis et al. 2005, Bartkova et al. 2005). The authors hypothesize an inhibition of oncogene-induced senescence, normally observed in cells in response to replicative stress, as the decisive mechanism in the progression of such tumors (Bartkova et al. 2006). This possibility was investigated in a further experiment. Following the infection of tumor cells with a retrovirus expressing shRNA specific for ATM, mice were inoculated with the cells and the resulting tumor growth was observed. Tumors in which ATM had been inactivated were found to be more invasive than and nearly four times as large as those containing functional ATM, thus implicating an essential role for the DNA damage response in guarding against tumor growth (Bartkova et al. 2005). The constitutive activation of the DDR could therefore represent a cell's early attempt to deal with mounting genetic instability, which however can no longer be maintained after a certain amount of damage accumulates.

The resulting decrease in DDR signal intensity would in this way represent a collapse of the barrier in the course of tumorigenesis, thus allowing for more aggressive tumor growth. The sufficient formation of 53BP1 and γH2AX foci at relatively low cadmium concentrations and IR doses may therefore be at least partially explained by this phenomenon- though cadmium itself is only weakly mutagenic, does not directly induce the formation of DSBs and does not suppress the repair of IR-induced DSBs, cells expressing mutations or various forms of unrepaired DNA damage may be particularly susceptible to heavy metal exposure and a weakening of the damage signalling response which can no longer be compensated.

This work has demonstrated that the influence of heavy metals on DNA DSB repair likely plays out on multiple levels through the subtle manipulation of cell repair and damage signalling processes and well as through influences on the cell cycle in effects potentially mediated by zinc-, RING-finger or metal sensitive proteins. While cadmium does directly inhibit cell growth through toxic effects at micromolar concentrations, it does not, however, appear to directly induce DSBs, nor does it significantly influence the quantitative repair of irradiation-induced double-strand breaks. However the stability of γH2AX and 53BP1 protein conglomerates and with it the quality of the DNA repair response has been shown to be compromised and thus perhaps the robustness of canonical repair pathways. Several potential molecular methods have been described by which cadmium might act upon the DNA damage response with the consequence of genetic instability and thus carcinogenesis.

6. Summary

This thesis explored the ways in which exposure to heavy metals, in this case cadmium, can impact upon the many complexly intertwined molecular processes involved in the repair of DNA DSBs. In order to address this question, a number of aspects of the cell's response to damage induction were investigated, including the cell cycle response, DNA damage signalling and the repair of DSBs. Though cadmium proved to be toxic and inhibit the growth and clonogenic proliferation of the HeLa cells employed in our experiments, the heavy metal did not itself induce DSBs, nor did it contribute to the induction of DSBs following irradiation with IR. Progression through the cell cycle did not prove to be significantly altered by cadmium treatment.

DNA damage signalling and repair are highly complex and intermingled processes which we investigated in many individual facets in the course of this project. Transfection-based repair experiments designed to investigate individual DSB pathways following break induction by restriction endonucleases showed a dose-dependent decrease in the number of repair events completed via both NHEJ and HR after pre-treatment with cadmium. Through the investigation of repair foci formed by proteins involved in DNA repair processes following DSB-induction, we observed a dose-dependent decrease in the number of residual γH2AX and 53BP1 observed after 24h following irradiation. This finding could create the impression of improved repair, but in reality likely reflects an impairment in damage signalling resulting from interactions between cadmium and a signalling protein functionally upstream of γH2AX, thus explaining the delayed kinetics of γH2AX focus formation at early time points following IR. Despite the fact that the recruitment of NBS1 as a component of the MRN complex was not influenced in live cell imaging experiments, other early signalling molecules such as Rad50 may still be adversely affected by cadmium. Taken together, the results of this thesis indicate that exposure to cadmium and likely that of other toxic metals may contribute to cancerogenesis through the qualitative if not quantitative inhibition of DNA damage signalling and repair. Ultimately, the weakening or eventual collapse of the DNA DDR may represent a decisive mechanism by which tumor cells progress in the course of cancerogenesis, suggesting that cadmium functions as a carcinogen by effectively accelerating this process and thus the progression of tumorous growth and invasiveness.

7. List of abbreviations

°C	degree Celsius
µ	micro (10^{-6})
53BP1	p53 binding protein 1
APLF	aprataxin and PNK-like factor
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia-Telangiectasia and rad3-related protein
BER	base excision repair
Bp	base pairs
BSA	bovine serum albumine
BrdU	5-bromo-2-deoxyuridine
BRCA1/2	breast cancer susceptibility gene 1/2
Chk1 /2	checkpoint kinase 1/2
CtIP	C-terminal binding protein interacting protein
Cys	cysteine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNA-PKc	DNA-dependent protein kinase, catalytic subunit
DDR	DNA damage response
DSB	double-strand break
EDTA	ethylenediaminetetraacetic acid
EYA	eye absent
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum, fetal bovine serum
Fig	Figure
Fpg	formamidopyrimidine glycosylase

FRAP	fluorescence recovery after photobleaching
GC	gene conversion
GFP	green fluorescent protein
Gy	Gray
h	hour
His	histidine
HR	homologous recombination
IR	ionising radiation
KAP-1	KRAB-associated protein 1
kDa	kilodaltons
l	liter
LOH	loss of heterozygosity
m	milli (10^{-3})
M	molar (mol/l)
MDC1	mediator of DNA-damage checkpoint protein 1
min	minutes
MMR	DNA mismatch repair
Mre11	meiotic recombination 11
MRN	Mre11-Rad50-NBS1
NBS	Nijmegen breakage syndrome
NBS1	Nijmegen breakage syndrome 1, Nibrin
NER	nucleotide excision repair
NHEJ	non-homologous end joining
ORF	open reading frame
pADPr	poly(ADP-ribose)
PARP1	poly (ADP-ribose) polymerase 1
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCNA	proliferating cell nuclear antigen

PCR	polymerase chain reaction
PI	propidium iodide
PIKK	phospho-inositide-3-kinase-related protein
RING	really interesting new gene
ROS	reactive oxygen species
RPA	replication protein A
rpm	rotations per minute
RT	room temperature
s	seconds
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
Ser	serine
siRNA	small interfering RNA
shRNA	small/short hairpin RNA
SLS	sodium lauryl sulfate
SNP	single-nucleotide polymorphism
SSA	single-strand annealing
SSB	single-strand break
ssDNA	single-stranded DNA
TBE	tris-borate-EDTA
TBS	tris buffered saline
TDP1	tyrosyl-DNA phosphatase 1
TG	tris-glycine
Thr	threonine
Tween 20	polyoxyethylen-sorbitanmonolaurate 20
Tyr	tyrosine
UV	ultraviolet
V	volts

V(D)J	variable (V), diversity (D) and joining (J)
v/v	volume per volume
w/v	weight per volume
WRN	Werner-syndrome ATP-dependent helicase
WSTF	Williams-Beuren syndrome transcription factor
wt	wild type
XLF	XRCC4-like factor
XPA	xeroderma pigmentosum A
XRCC4	X-ray cross complementation gene 4

8. References

Aimola P, Carmignani M, Volpe AR, Di Benedetto A, Claudio L, Waalkes MP, van Bokhoven A, Tokar EJ, Claudio PP. Cadmium induces p53-dependent apoptosis in human prostate epithelial cells. PLoS One. 2012;7(3):e33647.

Bal W, Schwerdtle T, Hartwig A. Mechanism of nickel assault on the zinc finger of DNA repair protein XPA. Chem Res Toxicol. 2003 Feb;16(2):242-8.

Bartkova J, Horejsí Z, Koed K, Krämer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Ørntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005 Apr 14;434(7035):864-70.

Bartkova J, Horejsí Z, Sehested M, Nesland JM, Rajpert-De Meyts E, Skakkebaek NE, Stucki M, Jackson S, Lukas J, Bartek J. DNA damage response mediators MDC1 and 53BP1: constitutive activation and aberrant loss in breast and lung cancer, but not in testicular germ cell tumours. Oncogene. 2007 Nov 22;26(53):7414-22.

Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Ørntoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J, Gorgoulis VG. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature. 2006 Nov 30;444(7119):633-7.

Belmar Campos C, Degenhardt S, Mansour W, Dahm-Daphi J. DNA-Doppelstrangbruchreparatur in CV1-Zellen. Exp Strahlenther Klin Strahlenbiol 2009; 18: 82-85

Bertin G, Averbeck D. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie. 2006 Nov;88(11):1549-59.

Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol. 2008 Aug;82(8):493-512.

Bolderson E, Richard DJ, Zhou BB, Khanna KK. Recent advances in cancer therapy targeting proteins involved in DNA double-strand break repair. Clin Cancer Res. 2009 Oct 15;15(20):6314-20.

Cao F, Zhou T, Simpson D, Zhou Y, Boyer J, Chen B, Jin T, Cordeiro-Stone M, Kaufmann W. p53-Dependent but ATM-independent inhibition of DNA synthesis and G2 arrest in cadmium-treated human fibroblasts. Toxicol Appl Pharmacol. 2007 Jan 15;218(2):174-85.

Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, Bonner RF, Bonner WM, Nussenzweig A. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol. 2003 Jul;5(7):675-9.

Connelly JC, Leach DR. Repair of DNA covalently linked to protein. Mol Cell. 2004 Feb 13;13(3):307-16.

Cook PJ, Ju BG, Telese F, Wang X, Glass CK, Rosenfeld MG. Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. Nature. 2009 Apr 2;458(7238):591-6.

Cortes Ledesma F, El Khamisy SF, Zuma MC, Osborn K, Caldecott KW. A human 5'-tyrosyl DNA phosphodiesterase that repairs topoisomerase-mediated DNA damage. Nature. 2009 Oct 1;461(7264):674-8.

Coster G, Goldberg M. The cellular response to DNA damage: a focus on MDC1 and its interacting proteins. Nucleus. 2010 Mar-Apr;1(2):166-78.

Dally H, Hartwig A. Induction and repair inhibition of oxidative DNA damage by nickel(II) and cadmium(II) in mammalian cells. Carcinogenesis. 1997 May;18(5):1021-6.

Davar D, Beumer JH, Hamieh L, Tawbi H. Role of PARP Inhibitors in Cancer Biology and Therapy. Curr Med Chem. 2012 Aug 1;19(23):3907-21.

DiTullio RA Jr, Mochan TA, Venere M, Bartkova J, Sehested M, Bartek J, Halazonetis TD. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol. 2002 Dec;4(12):998-1002.

Di Virgilio M, Gautier J. Repair of double-strand breaks by nonhomologous end joining in the absence of Mre11. J Cell Biol. 2005 Dec 5;171(5):765-71.

Doil C, Mailand N, Bekker-Jensen S, Menard P, Larsen DH, Pepperkok R, Ellenberg J, Panier S, Durocher D, Bartek J, Lukas J, Lukas C. RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. Cell. 2009 Feb 6;136(3):435-46.

El-Awady RA, Dikomey E, Dahm-Daphi J. Radiosensitivity of human tumour cells is correlated with the induction but not with the repair of DNA double-strand breaks. Br J Cancer. 2003 Aug 4;89(3):593-601.

Gamsjaeger R, Liew CK, Loughlin FE, Crossley M, Mackay JP. Sticky fingers: zinc-fingers as protein-recognition motifs. Trends Biochem Sci. 2007 Feb;32(2):63-70.

Gastaldo J, Viau M, Bencokova Z, Joubert A, Charvet AM, Balosso J, Foray N. Lead contamination results in late and slowly repairable DNA double-strand breaks and impacts upon the ATM-dependent signaling pathways. Toxicol Lett. 2007 Sep 28;173(3):201-14.

Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature. 2005 Apr 14;434(7035):907-13.

Guo Z, Deshpande R, Paull TT. ATM activation in the presence of oxidative stress. Cell Cycle. 2010 Dec 15;9(24):4805-11.

Jin YH, Clark AB, Slebos RJ, Al-Refai H, Taylor JA, Kunkel TA, Resnick MA, Gordenin DA. Cadmium is a mutagen that acts by inhibiting mismatch repair. Nat Genet. 2003 Jul;34(3):326-9.

Haince JF, McDonald D, Rodrigue A, Déry U, Masson JY, Hendzel MJ, Poirier GG. PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. J Biol Chem. 2008 Jan 11;283(2):1197-208.

Hartmann M, Hartwig A. Disturbance of DNA damage recognition after UV-irradiation by nickel(II) and cadmium(II) in mammalian cells. Carcinogenesis. 1998 Apr;19(4):617-21.

Hartwig A. Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxid Redox Signal. 2001 Aug;3(4):625-34.

Hartwig A, Schwerdtle T. Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications. Toxicol Lett. 2002 Feb 28;127(1-3):47-54.

Hartwig A, Mullenders LH, Schlepegrell R, Kasten U, Beyersmann D. Nickel(II) interferes with the incision step in nucleotide excision repair in mammalian cells. Cancer Res. 1994 Aug 1;54(15):4045-51.

Hartwig A, Asmuss M, Blessing H, Hoffmann S, Jahnke G, Khandelwal S, Pelzer A, Bürkle A. Interference by toxic metal ions with zinc-dependent proteins involved in maintaining genomic stability. Food Chem Toxicol. 2002 Aug;40(8):1179-84.

Hartwig A, Asmuss M, Ehleben I, Herzer U, Kostelac D, Pelzer A, Schwerdtle T, Bürkle A. Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. Environ Health Perspect. 2002 Oct;110 Suppl 5:797-9.

Hertz-Picciotto I, Hu SW. Contribution of cadmium in cigarettes to lung cancer: an evaluation of risk assessment methodologies. Arch Environ Health. 1994 Jul-Aug;49(4):297-302.

Hopfner KP, Craig L, Moncalian G, Zinkel RA, Usui T, Owen BA, Karcher A, Henderson B, Bodmer JL, McMurray CT, Carney JP, Petrini JH, Tainer JA. The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair. Nature. 2002 Aug 1;418(6897):562-6.

Jakob B, Rudolph JH, Gueven N, Lavin MF, Taucher-Scholz G. Live cell imaging of heavy-ion-induced radiation responses by beamline microscopy. Radiat Res. 2005 Jun;163(6):681-90.

Jakob B, Splinter J, Conrad S, Voss K, Zink D, Durante M, Löbrich M, Taucher-Scholz G. DNA double-strand breaks in heterochromatin elicit fast repair protein recruitment, histone H2AX phosphorylation and relocation to euchromatin. Nucleic Acids Res. 2011 Aug;39(15):6489-99.

Joazeiro CA, Weissman AM. RING finger proteins: mediators of ubiquitin ligase activity. Cell. 2000 Sep 1;102(5):549-52.

Kanaar R, Wyman C. DNA repair by the MRN complex: break it to make it. Cell. 2008 Oct 3;135(1):14-6.

Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature. 2004 Nov 18;432(7015):316-23.

Kazantzis G. Induction of sarcoma in the rat by cadmium sulphide pigment. Nature. 1963 Jun 22;198:1213-4.

Kellen E, Zeegers MP, Hond ED, Buntinx F. Blood cadmium may be associated with bladder carcinogenesis: the Belgian case-control study on bladder cancer. Cancer Detect Prev. 2007;31(1):77-82.

Kopera E, Schwerdtle T, Hartwig A, Bal W. Co(II) and Cd(II) substitute for Zn(II) in the zinc finger derived from the DNA repair protein XPA, demonstrating a variety of potential mechanisms of toxicity. Chem Res Toxicol. 2004 Nov;17(11):1452-8.

Krishnan N, Jeong DG, Jung SK, Ryu SE, Xiao A, Allis CD, Kim SJ, Tonks NK. Dephosphorylation of the C-terminal tyrosyl residue of the DNA damage-related histone H2A.X is mediated by the protein phosphatase eyes absent. J Biol Chem. 2009 Jun 12;284(24):16066-70.

Lee H, Kwak HJ, Cho IT, Park SH, Lee CH. S1219 residue of 53BP1 is phosphorylated by ATM kinase upon DNA damage and required for proper execution of DNA damage response. Biochem Biophys Res Commun. 2009 Jan 2;378(1):32-6.

Liu QZ, Jiang GF, He Y, Wang XR, Zhou JW, Zhuang ZX. Arsenite-induced alterations in Ku70-deficient cells: a model to study genotoxic effects. J Toxicol Environ Health A. 2007 Jun;70(11):938-46.

Lord CJ, Ashworth A. The DNA damage response and cancer therapy. Nature. 2012 Jan 18;481(7381):287-94.

Macrae CJ, McCulloch RD, Ylanko J, Durocher D, Koch CA. APLF (C2orf13) facilitates nonhomologous end-joining and undergoes ATM-dependent hyperphosphorylation following ionizing radiation. DNA Repair (Amst). 2008 Feb 1;7(2):292-302.

Mansour WY, Schumacher S, Rosskopf R, Rhein T, Schmidt-Petersen F, Gatzemeier F, Haag F, Borgmann K, Willers H, Dahm-Daphi J. Hierarchy of nonhomologous end-joining, single-strand annealing and gene conversion at site-directed DNA double-strand breaks. Nucleic Acids Res. 2008 Jul;36(12):4088-98.

Mansour WY, Rhein T, Dahm-Daphi J. The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies. Nucleic Acids Res. 2010 Oct;38(18):6065-77.

Méplan C, Mann K, Hainaut P. Cadmium induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. J Biol Chem. 1999 Oct 29;274(44):31663-70.

Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. Cancer Res. 2003 Dec 15;63(24):8586-91.

Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1, an activator of ATM in response to DNA damage. DNA Repair (Amst). 2004 Aug-Sep;3(8-9):945-52.

Pan Y, Yuan D, Zhang J, Xu P, Chen H, Shao C. Cadmium-induced adaptive response in cells of Chinese hamster ovary cell lines with varying DNA repair capacity. Radiat Res. 2009 Apr;171(4):446-53.

Panier S, Durocher D. Regulatory ubiquitylation in response to DNA double-strand breaks. DNA Repair (Amst). 2009 Apr 5;8(4):436-43.

Pellegrini L, Yu DS, Lo T, Anand S, Lee M, Blundell TL, Venkitaraman AR. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. Nature. 2002 Nov 21;420(6913):287-93.

Pfeiffer P, Goedecke W, Obe G. Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. Mutagenesis. 2000 Jul;15(4):289-302.

Rogakou EP, Boon C, Redon C, Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol. 1999 Sep 6;146(5):905-16.

Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. PARP inhibition: PARP1 and beyond. Nat Rev Cancer. 2010 Apr;10(4):293-301.

Schöpfer J, Drasch G, Schrauzer GN. Selenium and cadmium levels and ratios in prostates, livers, and kidneys of nonsmokers and smokers. Biol Trace Elem Res. 2010 May;134(2):180-7.

Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. J Cell Biol. 2000 Dec 25;151(7):1381-90.

Schulte-Uentrop L, El-Awady RA, Schliecker L, Willers H, Dahm-Daphi J. Distinct roles of XRCC4 and Ku80 in non-homologous end-joining of endonuclease- and ionizing radiation-induced DNA double-strand breaks. Nucleic Acids Res. 2008 May;36(8):2561-9.

Schwartz GG, Reis IM. Is cadmium a cause of human pancreatic cancer? Cancer Epidemiol Biomarkers Prev. 2000 Feb;9(2):139-45.

Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. Cell Res. 2008 Jan;18(1):134-47.

Smith CJ, Livingston SD, Doolittle DJ. An international literature survey of "IARC Group I carcinogens" reported in mainstream cigarette smoke. Food Chem Toxicol. 1997 Oct-Nov;35(10-11):1107-30.

Son YO, Lee JC, Hitron JA, Pan J, Zhang Z, Shi X. Cadmium induces intracellular Ca2+- and H2O2-dependent apoptosis through JNK- and p53-mediated pathways in skin epidermal cell line. Toxicol Sci. 2010 Jan;113(1):127-37.

Stewart GS, Stankovic T, Byrd PJ, Wechsler T, Miller ES, Huissoon A, Drayson MT, West SC, Elledge SJ, Taylor AM. RIDDLE immunodeficiency syndrome is linked to defects in 53BP1-mediated DNA damage signaling. Proc Natl Acad Sci U S A. 2007 Oct 23;104(43):16910-5.

Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Löbrich M, Jeggo PA. ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. Cancer Res. 2004 Apr 1;64(7):2390-6.

Tartier L, Spenlehauer C, Newman HC, Folkard M, Prise KM, Michael BD, Ménissier-de Murcia J, de Murcia G. Local DNA damage by proton microbeam irradiation induces poly(ADP-ribose) synthesis in mammalian cells. Mutagenesis. 2003 Sep;18(5):411-6.

Taylor RM, Whitehouse CJ, Caldecott KW. The DNA ligase III zinc finger stimulates binding to DNA secondary structure and promotes end joining. Nucleic Acids Res. 2000 Sep 15;28(18):3558-63.

Viau M, Gastaldo J, Bencokova Z, Joubert A, Foray N. Cadmium inhibits non-homologous end-joining and over-activates the MRE11-dependent repair pathway. Mutat Res. 2008 Jun 30;654(1):13-21.

Watanabe K, Iwabuchi K, Sun J, Tsuji Y, Tani T, Tokunaga K, Date T, Hashimoto M, Yamaizumi M, Tateishi S. RAD18 promotes DNA double-strand break repair during G1 phase through chromatin retention of 53BP1. Nucleic Acids Res. 2009 Apr;37(7):2176-93.

Whiteside JR, Box CL, McMillan TJ, Allinson SL. Cadmium and copper inhibit both DNA repair activities of polynucleotide kinase. DNA Repair (Amst). 2010 Jan 2;9(1):83-9.

Wyman C, Kanaar R. DNA double-strand break repair: all's well that ends well. Annu Rev Genet. 2006;40:363-83.

Xiao A, Li H, Shechter D, Ahn SH, Fabrizio LA, Erdjument-Bromage H, Ishibe-Murakami S, Wang B, Tempst P, Hofmann K, Patel DJ, Elledge SJ, Allis CD. WSTF regulates the H2A.X DNA damage response via a novel tyrosine kinase activity. Nature. 2009 Jan 1;457(7225):57-62.

Xie A, Kwok A, Scully R. Role of mammalian Mre11 in classical and alternative nonhomologous end joining. Nat Struct Mol Biol. 2009 Aug;16(8):814-8.

Xie A, Hartlerode A, Stucki M, Odate S, Puget N, Kwok A, Nagaraju G, Yan C, Alt FW, Chen J, Jackson SP, Scully R. Distinct roles of chromatin-associated proteins MDC1 and 53BP1 in mammalian double-strand break repair. Mol Cell. 2007 Dec 28;28(6):1045-57.

Yang PM, Chiu SJ, Lin KA, Lin LY. Effect of cadmium on cell cycle progression in Chinese hamster ovary cells. Chem Biol Interact. 2004 Oct 15;149(2-3):125-36.

Yu X, Sidhu JS, Hong S, Robinson JF, Ponce RA, Faustman EM. Cadmium induced p53-dependent activation of stress signaling, accumulation of ubiquitinated proteins, and apoptosis in mouse embryonic fibroblast cells. Toxicol Sci. 2011 Apr;120(2):403-12.

Zgheib O, Huyen Y, DiTullio RA Jr, Snyder A, Venere M, Stavridi ES, Halazonetis TD. ATM signaling and 53BP1. Radiother Oncol. 2005 Aug;76(2):119-22.

Zhou T, Akopiants K, Mohapatra S, Lin PS, Valerie K, Ramsden DA, Lees-Miller SP, Povirk LF. Tyrosyl-DNA phosphodiesterase and the repair of 3'-phosphoglycolate-terminated DNA double-strand breaks. DNA Repair (Amst). 2009 Aug 6;8(8):901-11.

9. Acknowledgements

There are many, many people who deserve my sincerest thanks for their help in supporting my throughout my Doktorarbeit. First and foremost I would like to thank my Doktorvater Jochen Dahm-Daphi for his unyielding and enthusiastic support in just about everything. Both in and outside of the lab. All of the other members of the Dahm-Daphi work group deserve similar thanks for their technical assistance, the countless helpful conversations and comments as well as for their senses of humor: Thorsten Rieckmann, Wael Mansour, Sabrina Köcher, Sarah Degenhardt and Fruzsina Gatzemeier. Thank you, too, to the many other members of the Laboratory for Radiobiology and Experimental Radiooncology for their support: specific mention goes to Ekkehard Dikomey, Jarob Saker, and Malte Kriegs. Thank you to Tobias Gauer for helping push me through to the end and for asking important contextual questions. I would like to specifically thank the Werner-Otto-Stiftung as well as the Rolfing-Stiftung for the financial assistance, allowing me to spend much more time in the lab than I otherwise would have been able to. Thank you to Claudia Lukas for kindly providing us with the osteosarcoma cell system as well as to Burkhard Jakob for his expertise and friendly assistance in conducting the live cell imaging experiments. And finally, my family, for their continuing support and love from afar.

10. Curriculum Vitae

Audrey Anne MacDougall

Geb. 12.11.1982, Southfield, MI, USA

Arbeit

15.07.2013-	Assistenzärztin in der Geriatrie Albertinen Haus, Hamburg, DE

Studium

10.2006-06.2013	Studium der Humanmedizin an der Universität Hamburg Erster Abschnitt der Ärztlichen Prüfung am 17.02.2009: Note: 3,0 Zweiter Abschnitt der Ärztlichen Prüfung am 14.06.2013: Note: 1,5 Gesamtnote für die Ärztliche Prüfung: 2,0 Approbation erhalten am 18.06.2013 in Hamburg
22.05.2005	Studiumabschluss in Biologie und Germanistik, Mount Holyoke College Bachelor of the Arts, *magna cum laude*
08.2003-08-2004	Austauschjahr an der Universität Hamburg
08.2001-08-2003	Studium der Biologie und Germanistik an Mount Holyoke College South Hadley, MA, USA

PJ-Tertiale

01.10.2012- 20.01.2013	Wahlfach Geriatrie Albertinen Haus, Hamburg, DE
11.06.2012- 30.09.2013	Allgemein- und Viszeralchirurgie, Unfallchirurgie Krankenhaus Buchholz, Buchholz, DE
20.02.2012- 10.06.2012	Innere Medizin- Onkologie, Kardiologie, Gastroenterologie Asklepios Klinik Altona, Hamburg, DE

Awards

12.2010	Stipendium der Rolfing-Stiftung
08.2009	Stipendium der Werner-Otto-Stiftung

11. Eidesstattliche Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift: ……