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Accumulation of single-stranded DNA in tumour cells as a result of replicative stress

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Hiermit erkläre ich, die Dissertation mit dem Titel "Accumulation of single-stranded DNA in tumour cells as a result of replicative stress" eigenständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Publication of the Results

The results of this work are published in *Proceedings of the Natural Academy of Sciences* (Köpper et al. 2013) and *Oncotarget* (Schmidt et al. 2015):

Damage induced DNA replication stalling relies on MAPK-activated protein kinase 2 activity

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Abstract

DNA damage can obstruct replication forks, resulting in replicative stress. By siRNA screening, we identified kinases involved in the accumulation of phosphohistone 2AX (γH2AX) upon UV irradiation-induced replication stress. Surprisingly, the strongest reduction of phosphohistone 2AX followed knockdown of the MAP kinase-activated protein kinase 2 (MK2), a kinase currently implicated in p38 stress signaling and G2 arrest. Depletion or inhibition of MK2 also protected cells from DNA damage-induced cell death, and mice deficient for MK2 displayed decreased apoptosis in the skin upon UV irradiation. Moreover, MK2 activity was required for damage response, accumulation of ssDNA, and decreased survival when cells were treated with the nucleoside analogue gemcitabine or when the checkpoint kinase Chk1 was antagonized. By using DNA fiber assays, we found that MK2 inhibition or knockdown rescued DNA replication impaired by gemcitabine or by Chk1 inhibition. This rescue strictly depended on translesion DNA polymerases. In conclusion, instead of being an unavoidable consequence of DNA damage, alterations of replication speed and origin firing depend on MK2-mediated signaling.

Screening analysis of ubiquitin ligases reveals G2E3 as a potential target for chemosensitizing cancer cells

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Abstract

Cisplatin is widely used against various tumors, but resistance is commonly encountered. By inducing DNA crosslinks, cisplatin triggers DNA damage response (DDR) and cell death. However, the molecular determinants of how cells respond to cisplatin are incompletely understood. Since ubiquitination plays a major role in DDR, we performed a high-content siRNA screen targeting 327 human ubiquitin ligases and 92 deubiquitinating enzymes in U2OS cells, interrogating the response to cisplatin. We quantified γH2AX by immunofluorescence and image analysis as a read-out for DNA damage. Among known mediators of DDR, the screen identified the ubiquitin ligase G2E3 as a new player in the response to cisplatin. G2E3 depletion led to decreased γH2AX levels and decreased phosphorylation of the checkpoint kinase 1 (Chk1) upon cisplatin. Moreover, loss of G2E3 triggered apoptosis and decreased proliferation of cancer cells. Treating cells with the nucleoside analogue gemcitabine led to increased accumulation of single-stranded DNA upon G2E3 depletion, pointing to a defect in replication. Furthermore, we show that endogenous G2E3 levels in cancer cells were down-regulated upon chemotherapeutic treatment. Taken together, our results suggest that G2E3 is a molecular determinant of the DDR and cell survival, and that its loss sensitizes tumor cells towards DNA-damaging treatment.

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Index of Abbreviations

°C	Degree Celsius
APS	Ammonium persulfate
ATM	Ataxia teleangiectasia mutated
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
ATR	ATM and Rad 3-related
ATRIP	ATR interacting protein
bp	Base pair
Bcl-2	B-cell lymphoma 2
BrdU	Bromdeoxyuridine
BSA	Bovine serum albumine
BSC-40	Primate kidney cell line
Cdc25	Cell division cycle 25
CDK	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CHX	Cycloheximide
Cos-7	Fibroblast-like cell line derived from primate kidney tissue
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMSO	Dimethylsulfoxid
DMEM	Dulbecco's Modified Eagle Medium
DMEM -	Dulbecco's Modified Eagle Medium without supplements
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependant protein kinase
dNTP	Deoxy nucleotide triphosphate
DSB	Double-strand break
EDTA	Ethylenediaminetetraacetic acid
Fas	First apoptotic signal
FCS	Fetal calf serum
g	Gravitational force constant
G2E3	G2 specific E3 ligase
Gem	Gemcitabine
h	Hour
H2AX	Histone variant 2AX
HCT116	Colorectal cancer cell line
HELA	Cervical cancer cell line

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HECT	Homologous to E6-associated protein
hn-RNP	Heterogeneous nuclear ribonucleoproteins
HR	Homologous recombination
HRP	Horseradish peroxidase
hsc-70	Constitutive heat shock protein 70
hsp27	Heat shock protein 27
HU	Hydroxyurea
IF	Immunofluorescence
IgG	Immunoglobulin G
kd	Knockdown
kDa	Kilodalton
LF2000	Lipofectamine 2000
MCM2-7	minichromosome maintenance2-7
MDM 2	Mouse double minute homolog 2
mec1	Budding yeast homolog of mammalian ATR
min	Minute
MK2	Mitogen-activated protein kinase-activated protein kinase 2
NHEJ	Non homologous end-joining
NLS	Nuclear localisation signal
n.s.	Not significant
NSCLC	Non small cellular lung cancer
p	Phospho-form
PARP	Poly-ADP-ribose-polymerase 1
PBS	Phosphate buffered saline
PHD/RING	Plant homeodomain/really interesting new gene
pre-RC	Pre-replicative Complex
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
ROI	Region of interest
ROS	Reactive oxygen species
RPA	Replication protein A
rpm	Rounds per minute
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SiHa	Human cervix carcinoma cell line
siRNA	Small interfering RNA
SSB	Single-strand break
ssDNA	Single-stranded DNA

TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
TRAIL	Tumour Necrosis Factor-related apoptosis-inducing ligand
Tris	Trisamine
UCN-01	Inhibitor of Chk1
U2OS	Human osteosarcoma cell line
V	Volt
γH2AX	H2AX phosphorylated on S319
Z-VAD	Caspase inhibitor

1   Introduction

1.1   DNA damage and its role in cancer

The genomic information contained in every living cell is a highly protected good that is given from one generation of cells to the next and whose integrity is a requirement for the healthy formation of tissues, organs and organisms. But even under normal circumstances, every cell is constantly subjected to DNA damage. The main part of this damage is endogenous in nature, so originating from within the cell itself. Most frequently, it is due to Reactive Oxygen Species (ROS) that are constantly generated as a by-product of a cell's metabolism (Bont and Larebeke 2004). Opposed to this, exogenous damage stems from a source outside of the cell. Examples include ultraviolet or ionising irradiation, chemicals such as formaldehyde or chemotherapeutic drugs. For them the induction of DNA damage is the very principle of their therapeutic effect. Of course, this damage needs to be sensed and somehow responded to by the cell. A signalling cascade termed the DNA damage response (DDR) does exactly this sensing and there are several repair mechanisms to rectify DNA damage once it is done. However, sometimes damage can be very severe or the sensing and repair mechanisms can fail. In these cases, either apoptosis, programmed cell death, is induced (Norbury and Zhivotovsky 2004) or the cell starts to accumulate damage in form of mutations and even passes it on to daughter cells resulting in genomic instability and eventually leading to the development of cancer.

1.1.1   The principle of chemotherapy

Chemotherapy together with surgery, radiotherapy and more recently emerging targeted approaches is one of the cornerstones of cancer therapy. It was first introduced in the middle of the 20th century and remains to be of enormous clinical significance (DeVita and Chu 2008). Nowadays, there are several different chemotherapeutics which can be divided into categories based on their chemical structure and mechanism of action and include, but are not limited to alkylating agents, topoisomerase inhibitors and antimetabolites or nucleoside analogues (Helleday et al. 2008). Most of these groups have in common that they somehow interfere with the normal replication of a cell; others possess direct toxic activity. Cancer cells are especially susceptible to perturbed replication; firstly because of their high replicational activity and secondly because they are genetically instable, particularly in the advanced stages of cancer, and have already accumulated mutations that impair the sensing and repair mechanisms employed by healthy cells (Kotsantis et al. 2015). Therefore, treatment with chemotherapeutics will lead to the death of a cell or the induction of cell cycle arrest in a higher percentage of cancer cells than it does in normal cells.

1.1.2 Nucleoside analogues and antimetabolites

Antimetabolites are drugs that interfere with other naturally occurring substances that are generated and used in the normal metabolism of a cell. Often, but not always, antimetabolites closely resemble the metabolite with which they are interfering. A subgroup of antimetabolites are nucleoside analogues that resemble either pyrimidines or purines and can be incorporated into the DNA in their place. Nucleoside analogues are S-phase-specific drugs as they only work on cells that are currently replicating their DNA (Ewald et al. 2008).

Other antimetabolites inhibit enzymes that are responsible for the generation of deoxyribonucleotides (dNTPs). Often targeted enzymes are dihydrofolate reductase or ribonucleotide reductase (RNR). Their inhibition results in imbalances within the dNTP pool of a cell and can for example lead to a shortage of nucleotides (Helleday et al. 2008). One example used in this study is hydroxyurea (HU) that is an antimetabolite but not a nucleoside analogue and acts as an inhibitor of RNR (Krakoff et al. 1968). Although, there are hints that HU may also interfere with later processes during DNA replication as cells seem to accumulate in early S phase and not at the G1/S boundary as one would assume (Wawra and Wintersberger 1983).

1.1.2.1 Gemcitabine

Gemcitabine or 2',2'-difluoro 2'-deoxycytidine is an analogue of deoxycytidine which is incorporated into the DNA during replication leading to chain termination one nucleotide after the incorporation side. Its structure is depicted in Figure 1. Gemcitabine is a prodrug, in order to be incorporated into the DNA, it needs to be phosphorylated by deoxycytidinkinase thrice to its triphosphate form. Once gemcitabine is incorporated, the DNA polymerase adds one more nucleotide to the nascent strand but then fails to elongate it any further because of the steric deformation of the strand which does no longer fit into the polymerase's catalytic site (Konerding et al. 2002). This process is called masked chain termination and gemcitabine's penultimate position in the DNA strand prevents the immediate correction by the polymerase's attached exonuclease and also hinders correction by other DNA repair mechanisms (Schy et al. 1993; Crul et al. 2003). Additionally, gemcitabine inhibits the enzyme ribonucleotide reductase (RNR) (Huang et al. 1991; Mini et al. 2006), further increasing its cytotoxic potential by causing a decrease in the number of available deoxynucleotides. This in turn leads to a shift within the available supply of deoxycytidine and gemcitabine, shifting the balance more towards the drug and allowing more gemcitabine to be incorporated, a process called self-potentiation (Ewald et al. 2008). Gemcitabine is most importantly used in pancreatic cancer (Burris et al. 1997) but is also a part of treatment protocols for advanced cases of non small cell lung cancer (NSCLC), bladder cancer and breast cancer (Ewald et al. 2008).
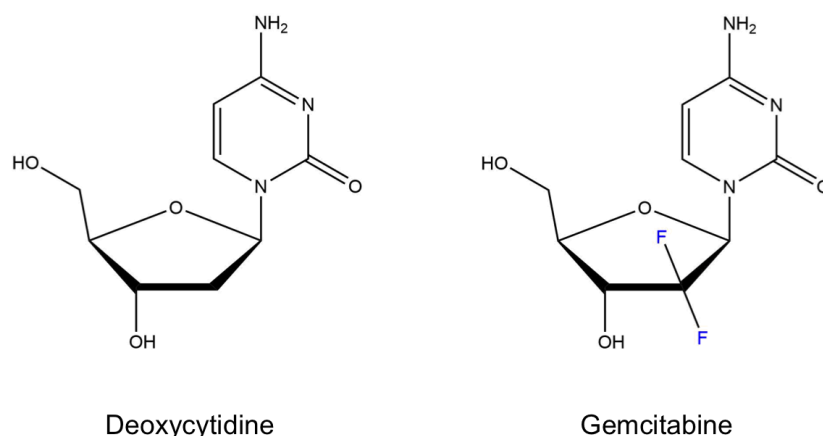
Figure 1: Structure of gemcitabine as opposed to deoxycytidine

Gemcitabine differs from deoxycytidine in its two fluorine substituents on position 2' of the furanose ring and leads to masked chain termination once it is incorporated into the DNA.

1.1.3   Sensitizing cancer cells for chemotherapy

Cancer cells are characterised by genomic instability, activation of oncogenes and inactivation of tumour suppressors, increasing for example proliferation rates but at the same time leading to an attenuated response and ability to sense and repair DNA damage. On the one hand, this favours a higher mutation rate, further increasing their malignancy; on the other hand, it abrogates sensing and repair pathways that are especially important for survival if cells are treated with chemotherapy. Therefore, cancer cells often compensate this loss by using alternative sensing and repair pathways to avoid cell death. Consequently, the externally driven disruption of other pathways in addition to standard chemotherapy can lead to a much higher percentage of cells that are no longer able to adequately react to DNA damage and are driven towards cell death, while non-cancerous cells still have alternative mechanism to avoid severe damage (Helleday et al. 2008; Tian et al. 2015). This principle is known as chemosensitisation. Promising targets for chemosensitisation are proteins involved in DNA repair or in checkpoint signalling like ATM, ATR, Chk1 and Chk2 (see 1.2.2 and 1.2.3). For two different inhibitors of Chk1 (one of them UCN-01 which was also used in this study) there are already clinical trials in patients with advanced stages of cancer (Welch et al. 2007; Helleday et al. 2008; Dobbelstein and Sørensen 2015).

1.2   Replicative stress and the DNA damage response

Replicative stress is a loosely defined term used to describe DNA damage that interferes with on-going replication, thus impeding correct and timely completion of S phase (see 1.2.5 and 1.2.6 for details). A characteristic of replicative stress is the formation of stalled and collapsed replication forks (Dobbelstein and Sørensen 2015). Sources of replicative

stress can be DNA lesions, ribonucleotides that have been incorporated instead of desoxyribonucleotides, imbalances in the dNTP pool of a cell, repetitive DNA structures, oncogene induced stress and several others. So, the obstacles posed to replication can be of various nature but all result in the inability of the replisome (see 1.2.1) to move further along the DNA strand, thus causing what is called a stalled replication fork. This stalled fork can be restarted once the obstacle, e.g. a chemically modified base, is removed but if this is not achieved in a timely manner, the replisome can dissociate from the DNA strand or be no longer functional, giving rise to a collapsed replication fork. Collapsed forks cannot be restarted even if the damage that caused them is repaired later on (Zeman and Cimprich 2014). Cells have developed several mechanisms to cope with replicative stress and in principle, three different outcomes are possible: Repair of the damaged DNA and completion of replication, cell cycle arrest or the induction of apoptosis (see Figure 2 for a schematic overview).
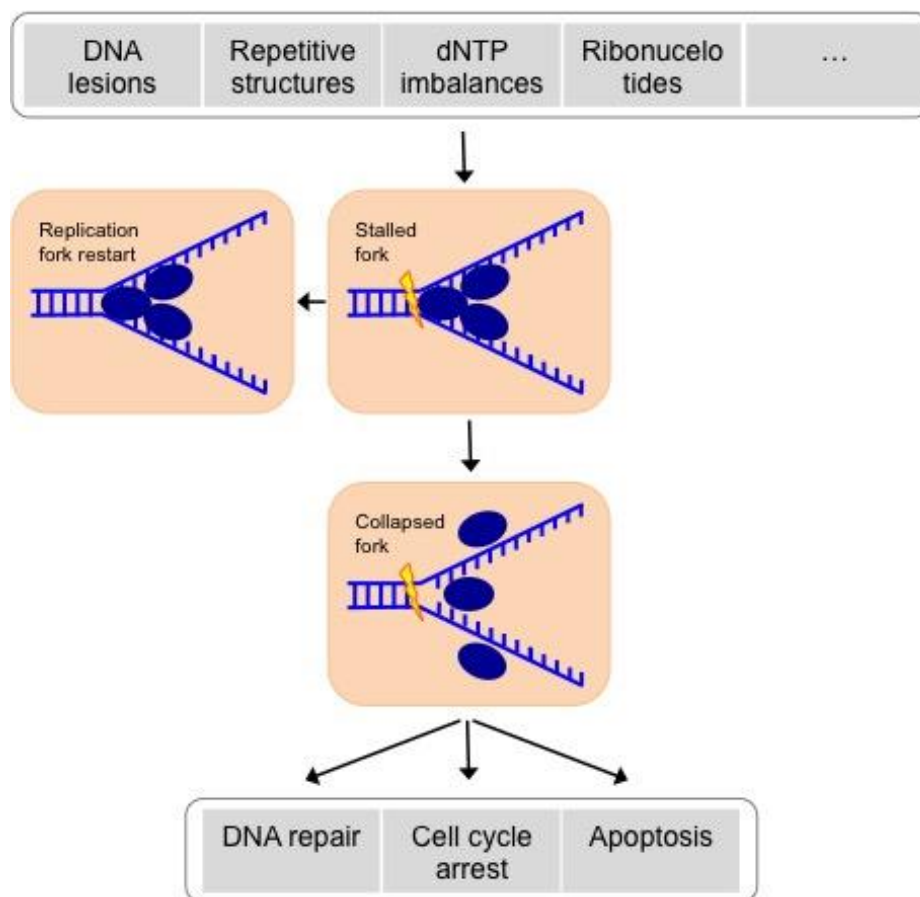
Figure 2: Sources and consequences of replicative stress

Various occurrences can pose obstacles to on-going replication, leading to fork stalling. A stalled fork can either be restarted or collapses after a short period of time. Once collapsed the DNA damage response comes into action and can lead to either DNA repair, induction of cell cycle arrest or programmed cell death. Blue ovals depict parts of the replication machinery.

To understand the causes and consequences of replicative stress, it is necessary to first look at how DNA replication is normally organised and regulated and then focus on the response to disturbances during this process. The following sections will provide a brief overview of these topics.

1.2.1   Regulation of DNA replication

In eukaryotic cells, as opposed to prokaryotes, replication does not start at a predefined point of the genome, but at several different and seemingly randomly distributed replication origins. Before S phase, these origins need to be licensed, which means that several proteins including the minichromosome maintenance2-7 (MCM2-7) helicase complex are recruited that together form the pre-Replicative Complex (pre-RC), basically a still inactive replication machinery. Upon the onset of S phase, a small portion of approximately 10 % of these origins are fired, meaning that MCM2-7 is phosphorylated and several proteins including DNA-polymerase primase α are recruited to the now active origin. The firing of an origin results in the formation of a bidirectional replication bubble with two replisomes moving away from their origin (Masai et al. 2010).

If replication at one of these forks comes to a halt and cannot be resumed, this gives rise to a collapsed replication fork. A possibility to ensure complete replication in this case is the firing of one of the previously unfired, so-called dormant origins on the other side of the lesion. The mechanisms behind this are still not completely understood and it remains unclear whether firing of dormant origins in response to replicative stress is a purely stochastic process or occurs in a regulated manner (McIntosh and Blow 2012).

1.2.2   A response to ssDNA: ATR Chk1 pathway

Upon stalling of a replication fork, helicase continues to unwind the DNA but the polymerase fails to elongate the nascent strand any further, thereby creating stretches of single-stranded DNA (as shown in Figure 5). The ssDNA is quickly coated with replication protein A (RPA) that further recruits ATR-interacting-protein (ATRIP) to these sites (Zou and Elledge 2003). ATRIP then serves as a binding partner for ATR (ATM (ataxia teleangiectasia mutated)-and-Rad3-related) (Branzei and Foiani 2009). ATR, together with ATM (discussed in detail in 1.2.3) and DNA-dependent protein-kinase (DNA-PK) is one of the key kinases of the DNA damage response. All three are serin/threonine kinases and belong to the family of nuclear phosphatidylinositol-3-kinase-related-kinases serin/threonin kinases (PIKK-family); ATM mainly mediates the response to DNA double-strand breaks and is discussed in detail below. An overview of ATR as well as ATM signalling can be found in Figure 3.

Once ATR is recruited to ssDNA it phosphorylates a wide range of substrates, one of the most important ones being checkpoint-kinase 1 (Chk1) (Zhao and Piwnica-Worms 2001). Chk1 is mostly present in S and G2 phase and even in the absence of DNA damage it is

active at a low level (Lukas et al. 2001; Bartek and Lukas 2003). ATR is the main activator of Chk1 but Chk1 can also be phosphorylated by ATM and possibly other kinases in a process known as crosstalk (Gatei et al. 2003). Chk1 then goes on to phosphorylate a plethora of substrates thereby distributing and amplifying the transduced signal. Apoptosis, chromatin remodelling, progression through the cell cycle, DNA repair and stabilisation of stalled replication forks are all subject to regulation via the ATR/Chk1 pathway. Both ATR, as well as Chk1 are essential for mammalian development and their complete loss results in early embryonic lethality in mice (Brown and Baltimore 2000; de Klein et al. 2000; Takai et al. 2000). The constant low-level activation of the ATR-Chk1 pathway seems to be necessary to suppress excessive origin firing during normal S phase (Shechter et al. 2004). If completely activated at a stalled fork, ATR-Chk1 signalling stabilises the stalled fork and allows a cell to repair the DNA damage and eventually restart the stalled fork by halting the cell's progression through the cell cycle. This function is part of the intra-S phase checkpoint and will be discussed in more detail below (1.2.6).
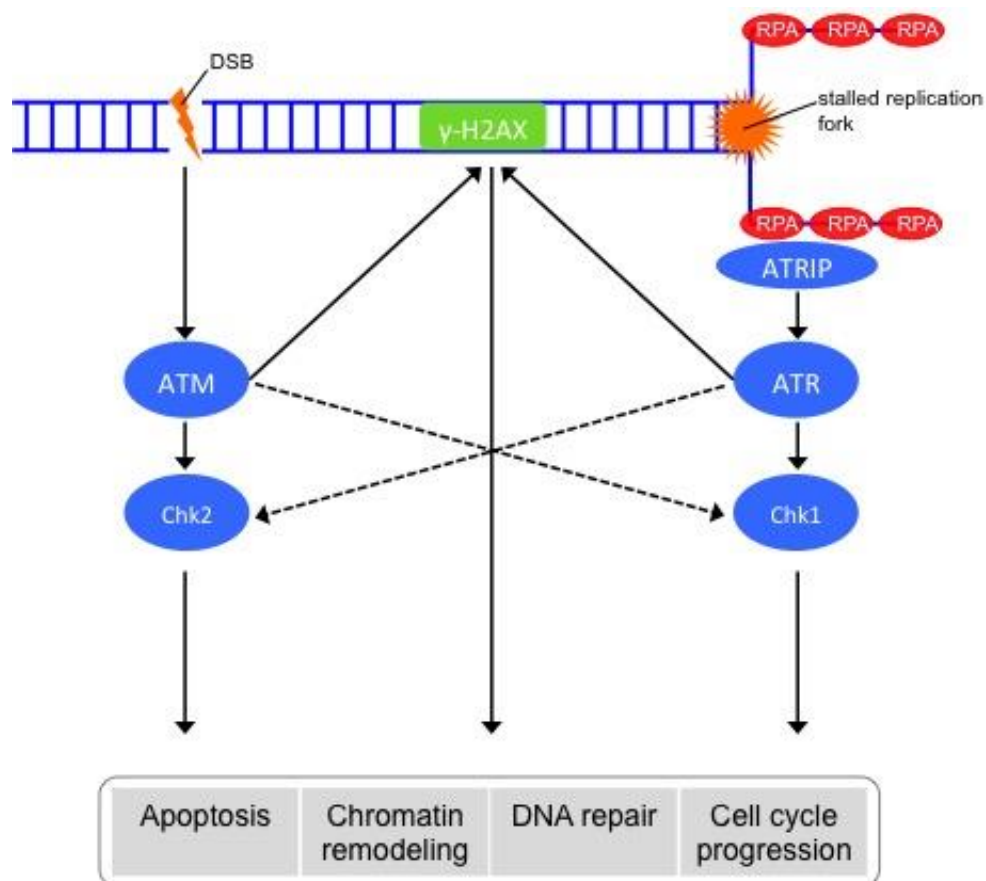
Figure 3: Schematic depiction of major signalling pathways in the DNA damage response
The ATR/Chk1 pathway mainly responds to the exposure of ssDNA at stalled replication forks, the ATM/Chk2 pathway is activated upon the detection of DNA double strand breaks. Both Chk1 and Chk2 phosphorylate a great number of targets. Their signalling can lead to apoptosis, DNA repair, the activation of cell cycle checkpoints and chromatin remodelling, depending on the type and severity of damage. Both pathways can activate and influence each other at different stages of the signalling process.

1.2.3 A response to DSBs: ATM Chk2 pathway

ATM, as already introduced above, is mainly activated upon DNA double strand breaks that can also be the product of collapsed replication forks following replicative stress (Hanada et al. 2007) but mostly occur after treatment with DNA damaging agents like topoisomerase II inhibitors or irradiation. ATM in its inactive form is a dimer that upon autophosphorylation dissociates into two active monomers and locates to the sites of DSB with the help of several adaptor proteins (Bakkenist and Kastan 2003). One protein phosphorylated by ATM is Checkpoint kinase 2 (Chk2) that largely functions in parallel to Chk1 and phosphorylates substrates like p53 and others known to be essential for cell cycle control and the induction of apoptosis. ATM itself also targets p53 via its negative regulator Mouse double minute 2 homologue (Mdm2) and further controls two pathways needed for the repair of DSBs – non-homologous end joining (NHEJ) and homologous recombination (HR) (Ciccia and Elledge 2010).

The tumour suppressor p53 has a central role in the response to DNA damage and also to more general sources of cellular stress. This key function is further illustrated by the finding that p53 is mutated in approximately 50 % of all human malignancies (Hollstein et al. 1991). It belongs to a protein family together with p73 and p63 and serves as a transcription factor for genes involved in cell-cycle-arrest, apoptosis and DNA repair (Menendez et al. 2009). P53 is always present in a cell but is normally kept at relatively low levels via ubiquitination and consequent proteosomal degradation. Upon phosphorylation by for example ATM, ATR, Chk1 or Chk2 it is stabilised and accumulates, forming a tetramer that then serves as the active transcription factor (Oren 2003).

1.2.4 Phosphorylation of H2AX

In every cell the DNA is wound around histones and thusly organised into structures that we call nucleosomes. But histones do not only package DNA, they also influence its accessibility for other proteins. Histone 2AX (H2AX) is a histone variant that is phosphorylated upon DNA damage and plays a role in the recruitment of proteins like those involved in DSB repair by homologous recombination and non homologous end-joining to the site of damage (Hartlerode and Scully 2009). The phosphorylation occurs specifically at Serine 139 of H2AX that is called γ-H2AX in its phospho-form. All three major sensing-kinases of the DNA damage response ATM, ATR and DNA-PK are shown to catalyse this posttranslational modification (Burma et al. 2001; Stiff et al. 2004; Ward et al. 2004). The phosphorylation of H2AX spreads along the DNA strand and serves as a signal amplifier for the DDR. Especially ATM is recruited to sites of γ-H2AX and further activated (Stucki and Jackson 2006; Cimprich and Cortez 2008). Due to these properties and easy staining in immunofluorescence, γ-H2AX is a well established and much used marker for DNA damage in general.

1.2.5   The cell cycle and its regulation

Most cells follow a regular and tightly controlled life cycle leading to replication of their DNA and subsequent cell division. To ensure that no vital errors occur during the process, this cell cycle includes several checkpoints that can, in case of problems, halt a cell's progression through the cell cycle and allow time for repair. A normal cell cycle consists of Gap 1 (G1) phase where cells grow and prepare for replication, S phase during which the DNA is replicated, Gap2 (G2) phase that is characterised by further cell growth and M phase or mitosis where the cell divides into two daughter cells. There are three major checkpoints that can be activated as a consequence of DNA damage: the G1/S, the intra-S phase and the G2/M checkpoint (Morgan 2006). The underlying principle of regulation for all the checkpoints is the same. In order to progress, a kinase of the so-called cyclin dependant kinases (CDKs) is needed to phosphorylate effector proteins. As suggested by their name, CDKs need cyclins to be active and transcription of cyclins is tightly controlled and only occurs during certain phases of the cell cycle and once certain requirements are met. Once CDKs and Cyclins form a complex, it needs to be phosphorylated in order to be fully active and to allow the cell to progress further through the cell cycle.

The G1/S as well as the G2/M checkpoints are mainly p53 dependant. As described above (see 1.2.3), p53 is stabilised as a consequence of DNA damage signalling for example via the ATR or ATM pathway. Upon accumulation of p53 it induces transcription of p21 and others that in turn inhibit Cyclin/CDK complexes thus hindering entry into the next cell cycle phase (el-Deiry et al. 1993; Harper et al. 1993).

1.2.6   The intra-S phase checkpoint

The intra-S phase checkpoint is crucial for maintaining genomic stability under conditions of replicative stress. As detailed above (1.2.2), ATR and Chk1 will be activated upon DNA damage that interferes with replication and leads to stalled replication forks. Chk1 phosphorylates and inactivates Cdc25 phosphatases and activates Wee1 leading to cell cycle arrest by blocking CDK1/2 (Kang et al. 2008; Reinhardt and Yaffe 2009). In addition, it also influences the stabilisation of stalled forks and origin firing. It was shown in budding yeast that mutation of mec1, the budding yeast analogue of ATR, leads to the loss of the helicase from the stalled fork thus giving rise to a collapsed replication fork that cannot be restarted (Cobb et al. 2003). The mechanisms behind restarting stalled forks are not completely understood but it is clear that in order to restart them, they first need to be stabilised and this stabilisation seems to be under the control of ATR and Chk1 (Lopes et al. 2001; Durkin et al. 2006; Sørensen and Syljuåsen 2012). In addition to fork stabilisation and delayed mitotic entry, the intra-S phase checkpoint also inhibits late origin firing and slows replication forks that are working on DNA templates that have been damaged (Lambert and Carr 2005).

1.2.7 Induction of apoptosis

Apoptosis is a form of cell death chosen by a cell upon intrinsic or extrinsic signals of extreme stress or damage beyond repair. As opposed to necrosis, apoptosis is characterised by cleavage of a cells components, resulting in fragmentation of the DNA, membrane blebbing, chromatin condensation and shrinking of the cell leading to the subsequent formation of apoptotic bodies that can be cleared by phagocytic cells without causing a spill-over and hence inflammation in the surrounding environment (Kerr et al. 1972).

There are two major ways to initiate apoptosis, an extrinsic and an intrinsic one. Both lead to the activation of caspases (cysteine aspartases), proteases with a cysteine at their active centre that cut proteins specifically at sites containing aspartic acid. An active caspase will also cleave and activate other caspases, thus increasing the apoptotic signal and reaching a point of no return. Many proteins contained in a cell are subject to caspase cleavage, including lamins that are essential for the integrity of the nuclear envelope, inhibitor proteins for DNA endonucleases, which in turn are released upon degradation of the inhibitor and cut the DNA into little pieces and PARP (poly-ADP-ribose-polymerase 1), a protein involved in DNA repair that is inactive once cleaved and was, in its cleaved form, used in this study as a marker for apoptosis in western blots (Kaufmann et al. 1993).

The extrinsic apoptotic pathway is triggered when an extracellular molecule, for example Tumour necrosis factor alpha or Fas (first apoptotic signal), binds to so-called death receptors. Death receptors consist of an extracellular ligand binding site, a transmembrane domain and intracellular death domains that recruit initiator procaspases and further proteins which can then activate executioner procaspases and spread the death signal within in the cell (reviewed in Elmore 2007).

For this study the intrinsic pathway is of more interest since it can be activated as a consequence of replicative stress and DNA damage. Normally there is a balance of pro- and anti-apoptotic stimuli within a cell. Upon certain events this balance can be shifted towards either end mostly by withdrawal of either pro- or anti-apoptotic factors. Most of these factors belong to the Bcl-2 family and are directly or indirectly controlled by p53 although the exact mechanisms of this regulation are still a matter of debate. However, if the balance of these factors is shifted towards the pro-apoptotic ones this always results in the permeabilisation of the outer mitochondrial membrane and the release of mitochondrial proteins like cytochrome c and others (Kluck et al. 2000; Saelens et al. 2004). Cytochrome c binds procaspase 9 and other proteins forming a structure called apoptosome (reviewed by Chinnaiyan 1999), leading to the activation of caspase 9 and therefore pulling the trigger for programmed cell death.

1.3   The G2 specific E3 ligase (G2E3)

G2E3, as already suggested by its name, belongs to the family of E3 ubiquitin ligases and is mainly expressed in G2 and M phase. It was first described in 2001 as a protein with maximum expression levels in G2/M and an observed down-regulation after ionizing irradiation (Crawford and Piwnica-Worms 2001). As to now, relatively little is known about G2E3 but there are strong hints that it is involved in the response to DNA damage and in the regulation of apoptosis. In this study we provide evidence that G2E3 plays a role in a cell's response to replicative stress and acts as a regulator of Chk1.

G2E3 was found to contain a HECT-domain (homologous to E6-associated protein) suggesting it might serve as an ubiquitin ligase. However, this HECT domain proved to be catalytically inactive but instead seems to control the subcellular localisation of G2E3. Nevertheless, G2E3 showed ubiquitin ligase activities in an *in vitro* assay but this activity seems to be mediated by its three PHD/RING-domains (plant homeodomain/really interesting new gene) (Brooks et al. 2008). The structure of G2E3 as described by Brooks et al. is shown in Figure 4. G2E3 is normally localised in the cell's nucleus (shown for HeLA, Cos-7, SiHa and BSC-40). It was shown that in HeLa cells G2E3 is present in the nucleolus and relocates to the nucleoplasm after DNA damage (Brooks et al. 2007). These data already suggest that G2E3 could play a role in maintaining genome stability and the DDR. But in addition, G2E3 also impacts the induction of apoptosis. It was shown that knockout of G2E3 in mice leads to early embryonic lethality mediated by massive apoptosis at the blastocyst stage. Heterozygous mice however are phenotypically normal and mainly express G2E3 in the central nervous system and limb buds (Brooks et al. 2008). Until today it is unclear how exactly these functions are achieved. It is highly likely that G2E3 indeed acts as an ubiquitin ligase but its natural substrates remain to be found.

Ubiquitination in general is a posttranslational modification that often serves as a signal for proteasomal degradation but also plays a role in regulating the localisation and activity of proteins (Komander and Rape 2012; Swatek and Komander 2016). Ubiquitin is a small protein that is covalently bound to a target protein most often by E3 ubiquitin ligases, although there are two other protein families known to catalyse this reaction. The ubiquitin ligase attaches one (monoubiquitination) to several ubiquitin molecules (polyubiquitination) to a target protein thus most often tagging it for proteasomal degradation. The 26S proteasome system recognises ubiquitin tagged proteins and subsequently degrades them. For example, p53, one of the key factors of the DDR, is normally kept at low levels by ubiquitination through its negative regulator Mdm2 and subsequent proteasomal degradation. The role of ubiquitin ligases in the development of cancer was reviewed by Nakayama and Nakayama in 2006 (Nakayama and Nakayama 2006). In addition to proteasomal degradation, ubiquitination is also known to influence chromatin remodelling by attaching ubiquitin to histones (Musselman and Kutateladze 2011). Like for example phosphorylation, ubiquitination is not an irreversible step. There are several

deubiquitinating enzymes that are able to cleave the bond between a protein and its attached ubiquitin.
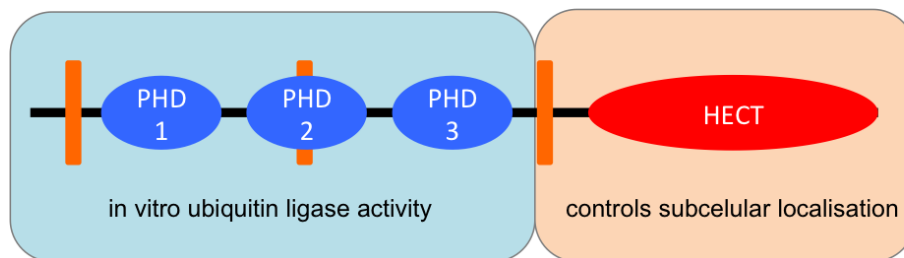
Figure 4: Domain structure of G2E3

G2E3 consists of three PHD/RING domains that possess in vitro ubiquitin ligase activity and are located at the N-terminal half and a HECT domain at the C-terminus that seems to control subcellular localisation (Brooks et al. 2007). Also located near the N-terminus are three alleged nuclear localisation signals (NLSs), depicted as orange bars.

1.4   The MAPKAP Kinase MK2

Mitogen-activated protein kinase-activated protein kinase 2 or short MK2 has for the last several years been an emerging research interest regarding its role during such fundamental processes like genome maintenance, cell proliferation, cellular stress, gene expression regulation and inflammation. MK2 has two structurally related siblings, MK3 and MK5 that together form the subfamily of MAPK-activated protein kinases. Most importantly for this study, MK2 is part of a newly identified non canonical pathway that seems to function in parallel to the Chk1 and Chk2 DDR-pathways (Bulavin et al. 2001; Manke et al. 2005; Reinhardt et al. 2007). This pathway is activated in response to general stress within a cell, including replicative stress. A kinase called p38/MAPK is activated and goes on to phosphorylate downstream proteins including p53 and MK2. In its dephosphorylated form, MK2 forms a heterodimer together with p38/MAPK within the nucleus. Upon phosphorylation, a change in conformation occurs, exposing both the catalytically active site and an export signal, leading to MK2's activation and relocalisation to the cytoplasm (Engel et al. 1998; Meng et al. 2002). MK2 itself phosphorylates a wide range of substrates, the most notable ones being hsp27 (heat shock protein 27) and Cdc25 phosphatases (see 1.2.6). Through the inhibition of Cdc25, MK2 activation leads to a cell cycle arrest during S phase or at the G2/M boundary. In this respect, MK2 functions similarly to Chk1 and Chk2 which is the reason why it is sometimes referred to as Chk3. Interestingly, MK2 also shares its minimal phosphorylation motif with Chk1 and Chk2, further supporting the theory that it could act as a third checkpoint kinase (Bulavin et al. 2001; Xiao et al. 2006). In summary, MK2 is known to be involved in cell-cycle regulation mainly via the intra-S phase checkpoint and the G2/M-checkpoint but there are also strong hints that MK2 has

further functions in the DNA damage response and replicative stress. This study aims to contribute to further understanding these functions.

1.5   Aims of this study

As detailed above, it is known that upon stalling of a replication fork ssDNA is formed which leads to the recruitment of various proteins involved in the DNA damage response. This study aims to use a new staining method for ssDNA as a read-out for replicative stress in order to identify proteins involved in the cellular response to replication fork stalling and collapse and to further elucidate their role and regulation. This will on the one hand side contribute to our general understanding of a cell's response to DNA damage and on the other hand side help to identify proteins that could in the long run be candidates for chemosensitisation, meaning that by inhibiting them with a drug administered to patients the effects of chemotherapy on cancer cells could be intensified while simultaneously sparing healthy cells from too severe side-effects. In order to achieve these goals, the study was subdivided into the following three parts, each one relying on the one before:

1. Establish the BrdU assay for ssDNA in a large, semi-automated setup as a new method to assess replicative stress.

2. Identify candidates involved in replicative stress via literature search and include them in a miniscreen using the BrdU assay for ssDNA.

3. Pick one or two candidates and investigate their role in replicative stress closer, e.g. find out more about their place in the DNA damage response and possible up- and downstream regulators and whether they could be candidates for chemosensitisation.

2 Materials and Methods

2.1 Materials

2.1.1 Buffers and Solutions

Table 1: Buffers and Solutions

Blocking solutions	
Western blot	5 % Milk powder in TBST
Western blot for phospho-antibodies	5 % BSA in TBST
Immunofluorescence	10 % FCS in PBS
BrdU Assay for ssDNA	3% BSA, 0.3 % Triton X in PBS
6x Laemmli buffer	
Tris pH 6.8	0.35 M
Glycerin	30 %
SDS	10 %
Dithiotreitol	9.3 %
Bromophenol blue	0.02 %
Cell lysis buffer	
Urea	2 M
Pefabloc	10 µM
Pepstatin A	1 µg/ml
Leupeptin/Aprotinin	1 µg/ml
dissolved in RIPA	
10x PBS, pH 7.5	
NaCl	239.9 mM
KCl	2.7 mM
Na_2HPO_4 x 2 H_2O	8.1 mM
KH_2PO_4	1.5 mM

1x Pre-extraction buffer, pH 7.5	
Triton X-100	0.5 %
HEPES	20 mM
Sucrose	300 mM
NaCl	50 mM
$MgCl_2$ x 6 H_2O	3 mM
Ponceau S solution	
Ponceau S	0.5 %
Glacial acetic acid	1 %
Ponceau S	0.5 %
RIPA, pH 7.5	
Triton-X 100	1 %
Sodium deoxycholate	1 %
SDS	0.1 %
NaCl	150 mM
EDTA	3.722 g
Tris-HCl pH 7.5	20 mM
Trasylol	100000 KIE/ l
SDS-PAGE running buffer	
Tris	25 mM
Glycin	86.1 mM
SDS	3.5 mM
10x TBS, pH 7.4	
Tris-HCl	0.5 M
NaCl	1.5 M
1x TBS-T, pH 7.4	
Tween 20	0.1 %

Dissolved in 1x TBS

10x Western salts pH 8.3	
Tris	250 mM
Glycin	1.92 M
SDS	0.02 %

If not explicitly mentioned otherwise, all buffers and solutions are dissolved in H_2O.

2.1.2 Chemotherapeutics and Inhibitors

Table 2: Chemotherapeutics and Inhibitors

Name	Target	Company
Cisplatin		Cis-Gry, Teva Germany
Cycloheximide	Ribosomes	Thermo Scientific
Gemcitabine		Eli Lilly, Indianapolis, United States
Hydroxyurea		Sigma Aldrich
MK III	MK2	Calbiochem, Merck
SB218078	Chk1	Calbiochem, Merck
UCN-01	Chk1	Sigma-Aldrich
TRAIL	Death-receptors	Sigma-Aldrich
Z-VAD	Caspases	Sigma-Aldrich

Table 3: Chemicals

Name	Company
BrdU solution	BD Pharmingen
DMSO	AppliChem

2.1.3 Kits

Table 4: Kits

Name	Company
Click-iT EdU Alexa Fluor High-throughput Imaging assay	Invitrogen, Life Technologies
Immobilion Western HRP Substrate Peroxide Solution	Millipore, Merck
Pierce, BCA Protein Assay Kit	Thermo Scientific
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Scientific

2.1.4 Small interfering RNAs

Table 5: Small interfering RNAs

Target	ID	Type
control siRNA 1 (scrambled)	4390844	not disclosed
control siRNA 2 (scrambled)	4390847	not disclosed
G2E3-A	s31128	sense 5'GAUGGUAAAUCUACAACAAtt3' antisense 5'UUGUUGUAGAUUUACCAUCtt3'
G2E3-B	s31129	sense 5'GAAGGGUCCUUGUCAAAGAtt3' antisense 5'UCUUUGACAAGGACCCCUUCaa3'
G2E3-C	s31130	sense 5'GGAUGUCUCAGACUUAUAAtt3' antisense 5'UUAUAAGUCUGAGACAUCCaa3'
hnRNP-1	s21544	
hnRNP-2	s21545	
p53-1	s605	
p53-2	s607	
p73-1	s14320	
p73-2	s14321	
p73-3	s14321	

All siRNAs are silencer select siRNAs obtained from Ambion/Life Technologies.

2.1.5   Antibodies

Table 6: Primary antibodies used in western blots

Target	Company	Cat. No.	Organism	Dilution
Chk1	Cell Signaling	2360	mouse	1:1000
Chk2	Calbiochem	CC44	mouse	1:300
hsc-70	Santa Cruz	sc-7298	rabbit	1:15000
hsp27 pS82	Cell Signaling	2401	rabbit	1:1000
MK2 pT334	Cell Signaling	3007	rabbit	1:1000
p21	Calbiochem	Op64	mouse	1:200
p53	Santa Cruz	sc-126	mouse	1:1000
phospho-Chk1 (S317)	Cell Signaling	2344	rabbit	1:1000
phospho-Chk2 (T68)	Cell Signaling	2661	rabbit	1:1000
γ-H2AX pS139	Millipore	05-636	mouse	1:4000

All primary antibodies for western blots are diluted in western blot blocking solution (5% milk powder), antibodies targeting phosphorylated Chk1 or phosphorylated Chk2 are diluted in western blot blocking solution for phospho-antibodies (5% BSA).

Table 7: Primary antibodies used in immunofluorescence

Target	Company	Cat. No.		Dilution
BrdU	Amersham/ GE Healthcare	RPN20AB	mouse	1:300
BrdU	Abcam	#Ab 8039	mouse	different
BrdU	ABD Serotec	MCA260	rat	different
γ-H2AX pS139	Millipore	05-636	mouse	1:2000
γ-H2AX pS139	Cell Signaling	#20E3	rat	1:1000

The γ-H2AX antibody is diluted in normal IF blocking solution (5% FCS), the BrdU antibody, used in the BrdU assay for ssDNA is diluted in 3 % BSA in PBS.

Table 8: Secondary antibodies used in western blots

Target	Company	Cat. No.	Dilution
HRP-coupled AffiniPure F(ab)2, fragment, anti mouse IgG	Jackson Immunoresearch	711-036-152	1:10000
HRP-coupled AffiniPure F(ab)2 fragment, anti rabbit IgG	Jackson Immunoresearch	715-036-150	1:10000

All Secondary antibodies for western blots are diluted in western blot blocking solution (5% milk powder), if the primary antibody targets a phosphorylated structure (e.g. phospho-Chk1 or phospho-Chk1), secondary antibodies are diluted in western blot blocking solution for phospho-antibodies (5% BSA).

Table 9: Secondary antibodies used in immunofluorescence

Target	Company	Cat. No.	Dilution
Alexa Fluor-488 goat anti rabbit	Invitrogen/ Life Technologies	A-11034	1:1000
Alexa Fluor-488 goat anti mouse	Invitrogen/ Life Technologies	A-11017	1:1000
Alexa Fluor-546 goat anti rabbit	Invitrogen/ Life Technologies	A-10040	1:1000
Alexa Fluor 646 goat anti mouse	Invitrogen/ Life Technologies	A-11003	1:1000

All secondary antibodies for immunofluorescence are diluted in IF blocking solution (5% FCS).

2.1.6   Cell culture

Table 10: Cell lines

Cell line	Origin	Cultured in
U2OS	human osteosarcoma	DMEM
HCT116 p53 $^{+/+}$	human colon carcinoma	McCoy's Medium
HCT116 p53 $^{-/-}$	human colon carcinoma, p53 deficient	McCoy's Medium

If not explicitly mentioned otherwise, all cell lines are cultured in media with supplements.

Table 11: Media for cell culture

Dulbecco's Modified Eagle's Medium (DMEM)	
DMEM powder	10 g/l
NaHCO$_3$	3.7 g/l
HEPES	5,96 g/l
DMEM with supplements	
DMEM	
FCS	10 %
Penicillin/Streptomycin	50 U/ml
L-Glutamine	200 µM
Ciprofloxacin	10 µg/ml
Tetracycline	2 µg/ml
McCoy's Medium with supplements	
McCoy's Medium	
FCS	10 %
Penicillin/Streptomycin	50 U/l
L-Glutamine	200 µM

All reagents are dissolved in H$_2$O.

2.2 Methods

2.2.1 Human cell culture

Human cell lines were used for all experiments, for a list of all cell lines, see Table 10. The cells were cultured at 37 °C under 5 % CO$_2$ in Dulbecco's Modified Eagle Medium (DMEM), or McCoy's Medium, both with supplements (see Table 11). All cell culture work was carried out under sterile conditions and with pre-warmed (37 °C) media and reagents. Cells were split approximately three times per week with dilutions between 1:2 and 1:10 depending on the cell line and density of cells. For splitting and experimental use, cells were washed with PBS and detached from their dish by incubating them with 0.1% Trypsin/EDTA for a couple of minutes at 37 °C. In order to stop the enzymatic activity of trypsin, cell culture medium with FCS was added and the cells were diluted and reseeded.

For experimental use, cells were seeded onto 6-, 12- or 96 well plates with a fixed amount of cells per well. To achieve this, cells were counted in a Neubauer counting chamber and diluted accordingly.

2.2.1.1 Transient transfection with siRNAs

In order to perform a selective knockdown of target proteins, such as G2E3, cells were transiently reverse transfected with siRNAs. As a transfection reagent, Lipofectamine 2000 was used and the final concentration of siRNA was 10 nM. For preparation of the transfection reagent, the required amount of Lipofectamine 2000 (see Table 12) was added to medium without supplements, vortexed and incubated for 5 min at room temperature. For U2OS, DMEM was used and for HCT116 McCoy's Medium. In parallel, a suitable amount of siRNA (see Table 12) was also diluted in medium without supplements and vortexed. These two solutions were hereafter combined, gently mixed and left to incubate for 20 min at room temperature. This mix was then given into the wells of a cell culture plate and a fixed amount of cells in medium with supplements was added. The plates were incubated for 24 h at 37 °C before the medium was first changed. A list of all siRNAs that were used in this work can be found in Table 5.

Table 12: Preparation of transfection mix and cell numbers for transient transfection with siRNAs

96 well plates	
Cell number U2OS	8000
siRNA mix	25 µl medium + 1.5 pmol siRNA
Transfection mix	14.75 µl medium + 0.25 µl LF2000
12 well plates	
Cell number U2OS	100000
Cell number HCT116	180000
siRNA mix	67.5 µl medium + 15 pmol siRNA
Transfection mix	28.65 µl medium + 1.35 µl LF2000
6 well plates	
Cell number U2OS	160000 – 22000 depending on the experiment
Cell number HCT116	360000
siRNA mix	135 µl medium + 30 pmol siRNA
Transfection mix	57.3 µl medium + 2.7 µl LF2000

2.2.1.2 Treating cells with chemotherapeutics or other chemicals

All drugs were pre-dissolved in their respective solvent and then added to pre-warmed cell culture medium. Controls were treated with an equivalent amount of DMSO or H_2O. If different amounts of DMSO or Ethanol had to be used for different inhibitors in one experiment, controls and all other treatments were adjusted to also contain the highest DMSO or Ethanol concentration.

Table 13: Concentrations of drugs and chemicals for cell treatment

Chemical or drug	Concentration	Solvent
Chk1 Inh. UCN-01	300 nM	DMSO
Chk1 Inh. SB218078	5 µM	DMSO
Cycloheximide	5 µg/ml	Ethanol
Gemcitabine	different	H_2O
Hydroxyurea	2 mM	H_2O
MK2 Inh	10 µM	DMSO
TRAIL	75 ng/ml	Ethanol
Z-VAD	50 µM	DMSO

2.2.1.3 Generation of cell lysates for SDS-PAGE

To prepare cell lysates for SDS polyacrylamide gel electrophoresis (SDS-PAGE), cells were grown and treated in 12 well plates and scraped off. The scraping as well as all other steps were performed on ice to hinder the degradation of proteins. After scraping the cells off in their medium, they were collected and centrifuged for 5 min at 845 g. The resulting pellet was washed with PBS, centrifuged again for 5 min at 845 g and then lysed in cell lysis buffer (see Table 1). The *Pierce® BCA Protein Assay Kit* was used to determine and then adjust the protein concentration of all samples to equal amounts by diluting them with RIPA buffer. These samples were then either stored at -80 °C or directly used for SDS-PAGE by adding 6x Laemmli buffer, and boiling them for 5 min at 95 °C while shaking at 1400 rpm. Before loading them onto the gel, samples were shaken with 1400 rpm for 30 min at 4 °C to shear the DNA.

2.2.2 Western blot

2.2.2.1 SDS-PAGE

SDS-PAGE is a method to separate proteins by their molecular weight and mobility in an electrophoretic field using a polyacrylamide gel. It was developed and first described by Laemmli (Laemmli 1970). The protein-samples are denatured in the presence of Laemmli buffer that contains sodium dodecyl sulphate (SDS) which is a detergent and binds to the proteins in a stoichiometric way. This leads to the proteins being negatively charged proportionally to their molecular weight. If an electric field is now applied to the gel containing the proteins, they will travel towards the anode with larger proteins moving more slowly through the pores of the resolving gel and smaller proteins moving faster. For SDS-PAGE the gel is composed of two parts, the stacking gel and the resolving gel. The stacking gel is used to focus the samples. Therefore, it contains only 5 % Polyacrylamide at a pH of 6.8. The running buffer contains chloride and glycine ions with the chloride being charged negatively due to the low pH in the stacking gel and the glycine being a zwitterion. Between these two fronts of ions, a partial electrical field is generated in which the original sample is situated and therefore focused by being repelled by both negatively charged fronts. The sample then enters the resolving gel, which was cast using 12 % or 15 % polyacrylamide and buffered to a pH of 8.8. Under these conditions glycine is now deprotonated and moves faster than the protein sample itself, therefore no partial electric field is generated anymore and the sample separates according to the molecular weight of the different proteins. The exact composition of the gels can be found in Table 14. The resolving gel was cast first in a glass chamber and covered with isopropanol to achieve an even surface. The isopropanol was removed after polymerisation, the stacking gel was cast on top of the resolving gel and a comb was inserted.

Table 14: Gels for SDS-PAGE

Reagent	5 % stacking gel	12 % resolving gel	15 % resolving gel
Acrylamide-bisacrylamide	5%	12%	15%
Tris, pH 6.8 (1 M)	126 mM	-	-
Tris, pH 8.8 (1.5 M)	-	375 mM	375 mM
SDS	0.1 %	0.1 %	0.1 %
APS	0.1 %	0.1 %	0.1 %
TEMED	0.3 %	0.4 %	0.4 %

Dissolved in H_2O

The prepared cell lysates (see 2.2.1.3) were loaded onto the gel, in the first pocket of each

gel a pre-stained protein ladder was loaded. Gels were run at 80 V for the stacking gel and 130 V for the resolving gel in SDS running buffer (see Table 1).

2.2.2.2 Immunoblotting and immunostaining

Immunoblotting or western blotting is a method that allows assessment of protein levels and post-translational modifications such as phosphorylation (Renart et al. 1979; Towbin et al. 1979). The method allows a transfer of the proteins from the gel onto a membrane where they can be detected by an antibody directed against a surface epitope. A secondary antibody, which is coupled to horseradish peroxidase (HRP), is directed against the first antibody's constant region. By applying luminol, the oxidisation of the substrate can be detected by measuring luminescence.

After the protein samples were separated by SDS-PAGE, the samples were transferred onto a nitrocellulose membrane by wet electroblotting (Bittner et al. 1980). In order to do so, the gel was put into a stack with two sponges and three Whatman papers on the cathode side, followed by the gel, the nitrocellulose membrane, three Whatman papers and a sponge on the anode side. This stack was placed in a plastic holder and put into a blotting chamber that was subsequently filled up with blotting buffer (see Table 1). An electric field with 90 V for 90 min at 4 °C was applied to achieve the transfer. As a quality control and to verify the equal transfer of proteins, membranes were stained with Ponceau S solution prior to staining them with antibodies. The membrane was then blocked in 5% milk or in the case of phospho-antibodies in 5% BSA (see Table 1) for 1 h and subsequently incubated with respective antibodies dissolved in milk or BSA for 2 h at room temperature or overnight at 4 °C. All primary antibodies used in western blots are listed in Table 6. Afterwards, the membrane was washed in TBST three times for 5 min. The secondary antibody in blocking solution was added for 1 h, followed again by three times washing in TBST. To visualize the bands *Immobilion Western HRP Substrate Peroxide Solution* (Millipore) or *SuperSignal West Femto Maximum Sensitivity Substrate* (Pierce) was added, followed by the detection of luminescence using the *Intas ChemoStar Imager* Software.

2.2.3 High content immunofluorescence microscopy

Immunofluorescence makes it possible to visualize proteins by targeting them with fluorophores coupled to antibodies. As in immunostaining, a primary antibody, directed against the protein of interest, is used together with a secondary antibody targeting the first's constant region. This secondary antibody carries a fluorescent dye. Upon excitation, the emitted fluorescence can be detected using a fluorescence microscope. Using different fluorophores, emitting different wavelengths, makes it possible to stain more than one protein per sample.

In this study, phosphorylation of H2AX was measured by high content immunofluorescence microscopy. For this purpose, 96 well plates were used to grow the

cells and each treatment was carried out in triplicates. For staining, the medium was removed and the cells were fixed with 4% Paraformaldehyd dissolved in PBS for 20 min at room temperature, rinsed twice in PBS and permeabilised with 0.5% Triton-X dissolved in PBS for 10 min at room temperature. After rinsing them again four times in PBS, blocking solution (10 % FCS) was added for 10 minutes. Hereafter, the cells were stained with the primary antibody, dissolved in blocking solution, for 1 h at room temperature or overnight at 4 °C. The primary antibody was then removed, the cells washed again three times with PBS and the secondary antibody was added and left to incubate for 45 min at room temperature. Together with the secondary antibody, 0.5 µg/ml Hoechst 33342 (Molecular Probes, Cat. No. H3570) were added to stain the cell nuclei. Afterwards, the cells were washed twice with PBS and either directly used for fluorescence microscopy or stored at 4 °C. All work involving the secondary antibody was, as far as possible, carried out in the dark to protect the fluorophores from light. A list of primary and secondary antibodies can be found in Table 7 and Table 9. Per well, nine microscopic pictures with a 10x magnification were taken automatically using the *BD pathway 855* system. With the *AttoVision* software, regions of interest (ROI) were defined by using the Hoechst channel as a mask. Since Hoechst selectively stains DNA (Latt et al. 1975) the ROIs contained only cell nuclei. Within these ROIs the nuclear fluorescence intensity of the γ-H2AX channel was quantified and a mean value per well was calculated. The data were further processed using R*: A language and environment for statistical computing, Microsoft Access* and *GraphPad Prism version 5 and 7, GraphPad Software, San Diego California USA.*

2.2.4   BrdU assay for ssDNA

The BrdU assay for single stranded DNA was employed to assess the amount of single stranded DNA per cell. The method was modified, based on a protocol published by Syljuåsen and colleagues (Syljuåsen et al. 2005). Our group published this modified protocol in 2013 (Köpper et al. 2013). Cells were exposed to 10 µM BrdU for 24 h prior to treatment; the treatment was then carried out under continued presence of BrdU. BrdU is incorporated during replication into the DNA double strand instead of thymidine. Normally, BrdU labelling is used to look at replicational activity of cells. In order to make the BrdU accessible for an antibody, the DNA double strand is normally broken down into single strands by the opening of chromatin using for example HCl, leading to a BrdU signal at all incorporation sites. Opposed to this, the BrdU assay for ssDNA only detects the BrdU at sites where the DNA itself formed single strands during the carried out treatment. To this end, no denaturation or opening of chromatin was done at any point during the staining procedure. Therefore, the antibody can only recognise the BrdU at sites of ssDNA that occur naturally for example at stalled replication forks. Therefore the BrdU assay provides additional information about the amount and specific kind of DNA damage that accumulated during the treatment. This can be combined with a parallel staining for γH2AX.

For use in the BrdU assay, two different workflows were established. Cells were either grown on coverslips in a 6 well plate for use with a confocal microscope or in 96 well plates for high content immunofluorescence microscopy. The staining procedure is similar for both. All steps were carried out on ice. After removal of the medium, cells were pre-extracted with cold (4 °C) pre-extraction buffer (see Table 1) for 5 min. They were then rinsed twice in PBS and fixed with 4 % formaldehyde in PBS for 10 min. After washing them again two times in PBS, the samples were blocked and permeabilised with 3 % BSA and 0.3 % Triton X in PBS for 30 min. The BrdU antibody was then diluted in 3 % BSA in PBS and added for 1 h at room temperature or overnight at 4 °C. Afterwards, cells were washed twice in PBS again and incubated with the secondary antibodies dissolved in 3 % BSA in PBS for 1 h at room temperature followed by washing them three times and using them either for confocal microscopy or high content immunofluorescence microscopy. For cells grown on cover slips, the cover slips were transferred into a wet-chamber after fixation and stained therein. After the staining was completed, they were briefly dried and mounted with DAPI on glass slides by placing the cover slips face down on 4 μl drops of DAPI mounting medium. Excessive mounting medium was then removed and the cover slips fixed with nail polish. A *Zeiss Confocal LSM 510 meta microscope* was used to obtain pictures of the cells. *Fiji* was used for further image processing. For cells grown in 96 well plates, 0.5 μg/ml Hoechst 33342 were added together with the secondary antibody. The plates were used for high content immunofluorescence microscopy as described above. Again the Hoechst channel was used to define ROIs wherein the BrdU signal was then quantified.

2.2.5 Statistical analysis

Statistical analysis was done in *Microsoft Excel* and *GraphPad Prism version 5 and 7*. To test whether the difference between two groups was significant, an unpaired two-tailed student's t-test was used. Variances were assessed using an F-Test, if needed variances were corrected using Welch's correction. Differences between two sets of data were considered statistically significant if $p < 0.05$. In figures asterisks indicate the following levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3   Results

3.1   The BrdU assay detects ssDNA

In order to use ssDNA as a read-out for replicative stress, a method to reliably detect it needed to be established. Therefore, a pre-existing method (Syljuåsen et al. 2005) was modified as described in the Materials and Methods part of this thesis (2.2.4). The underlying principle of the staining procedure is depicted in Figure 5. Furthermore, Figure 6 shows a comparison between the newly established BrdU assay for ssDNA and the routine method of using BrdU incorporation for an assessment of replicational activity. The Chk1 inhibitor UCN-01 was used as a positive control. UCN-01 was also used by Syljuåsen et al. and leads to a reliable, high signal in U2OS and HCT116 cells. Treatment with hydroxyurea for 4 h can also be used as a positive control (see Figure 7 and Figure 8). For the antibody test, staining with only the secondary antibody was used as a negative control, for all other experiments cells that were not treated with BrdU were employed as a negative control.
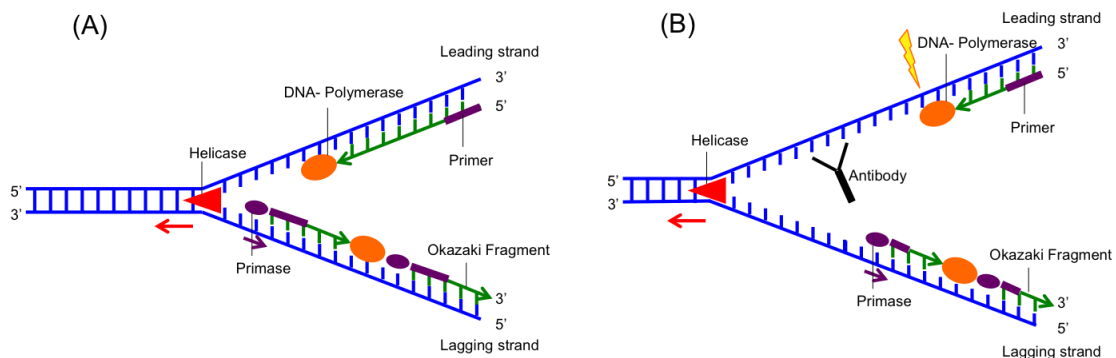

Figure 5: Schematic depiction of the BrdU assay for ssDNA

(A) During normal replication, helicase unwinds the DNA creating a replication fork, the leading strand is replicated continuously from 5' to 3', the lagging strand's replication is uncontinuously with Okazaki fragments being generated and later fused together. (B) Upon DNA damage, as indicated by the bolt, the DNA polymerase may stop replicating the DNA but the helicase continues unwinding it leading to the exposure of ssDNA which is then accessible for an antibody; in order to label the cells with BrdU they were incubated with 10 µM BrdU for 24 h prior to treatment, so the BrdU is incorporated in the S phase preceding the one during which the treatment is carried out; the BrdU is then detected by an antibody; detection is only possible if the DNA is single stranded e.g. at a stalled or collapsed replication fork.
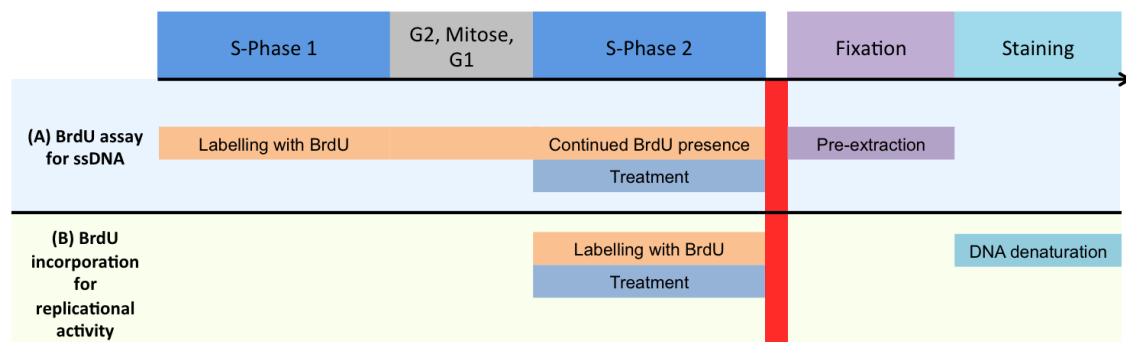
Figure 6: Comparison between BrdU assay for ssDNA and BrdU incorporation for replicational activity

(A) BrdU is incorporated into the DNA one S Phase prior to treatment; cells are incubated with 10 μM BrdU for 24 h; afterwards, the treatment is carried out under continued presence of BrdU; a pre-extraction is done during the fixation and no denaturation of the DNA is carried out, leaving only ssDNA accessible for an antibody. (B) Labelling with BrdU and treatment are done simultaneously to assess how the treatment interferes with replicational activity; after fixation the DNA is denatured to make all the BrdU accessible for an antibody.

3.1.1 Only one antibody to BrdU reliably detects its epitope upon replicative stress in a high-content semi-automated setup

Different antibodies were tested for use in the BrdU assay for ssDNA. The aim was to find an antibody that reliably detects the BrdU, can be used for costaining with γ-H2AX and works well in the automated microscopy setting. Three different antibodies were tested, two of them have a mouse constant region, one a rat constant region. For γ-H2AX there were already two antibodies that work very well, one derived from rat and one from mouse so that the desired costaining with γ-H2AX is possible by using the one with the respective other constant region. For use with the *BD pathway system* for high-content immunofluorescence, the staining needs to be very homogenous in terms of background signal and maximum signal per well so that the same exposure time can be used for all 96 wells on the plate in order to afterwards quantify and compare the fluorescence intensities within the different wells. It can be seen in Figure 7 that not all of the tested antibodies are suitable for the BrdU assay for ssDNA. #Ab 8039 produces a very high background signal since the specific signal within the nucleus is so weak that the exposure time during which the fluorescence dye is excited needed to be set to 0.77 s in the 1:100 dilution. For comparison, the final antibody RPN 20AB (Figure 8) needs an exposure time of 0.2 s for the 1:300 dilution. Another tested antibody, MCA260 also shows a weak staining within the nucleus, background signal in the cytoplasm and precipitates. These aggregates of antibody lead to a very high maximum signal intensity which makes it complicated to find the right exposure time for the much weaker fluorescence signal emitted by the antibodies bound within the cells' nuclei. Only the RPN 20AB exhibits a specific signal within the nucleus

with no high background (see Figure 8). As a result, it was decided to use the RPN 20AB in a dilution of 1:300 from here on.
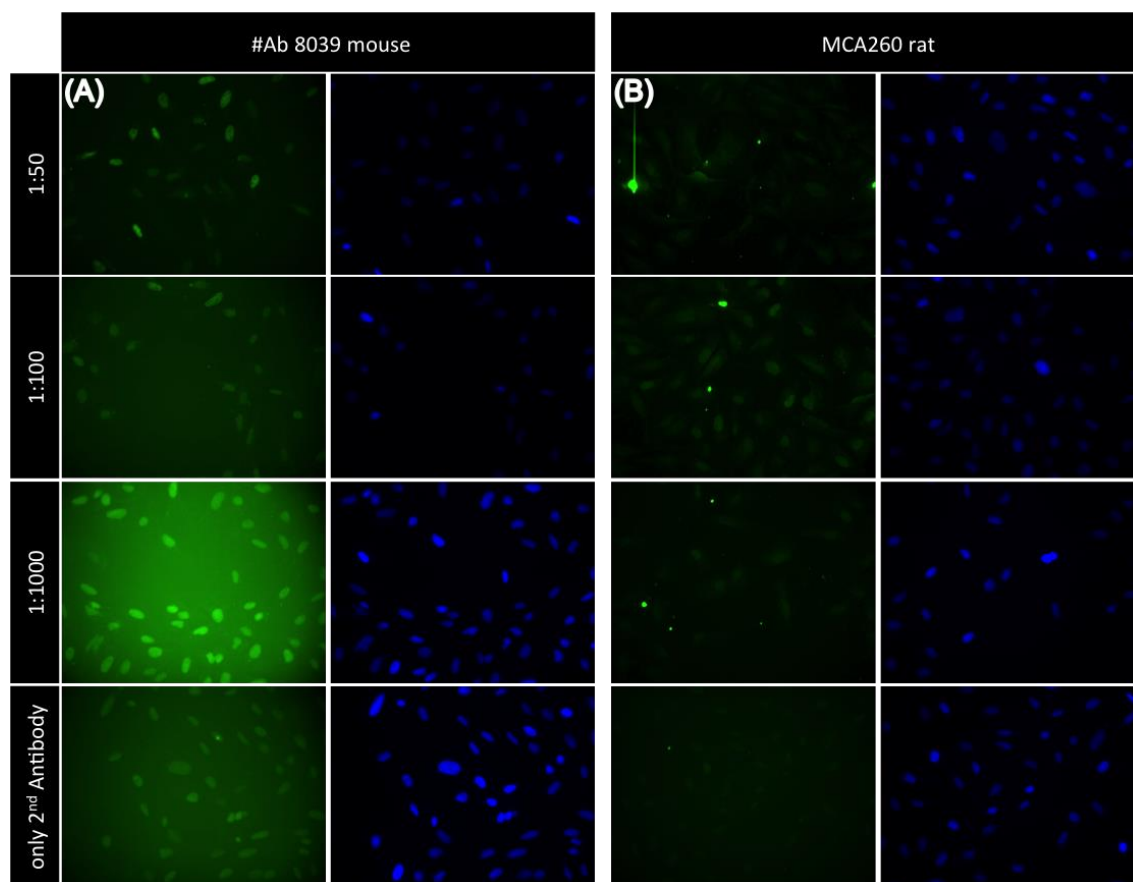

Figure 7: Not all BrdU antibodies can be used in the BrdU assay for ssDNA

Two different antibodies were tested with the established workflow for the BrdU assay for ssDNA; after incubating U2OS-cells with 10 μM BrdU for 24 h, cells were treated with 2mM hydroxyurea for 4 h under continued presence of BrdU, blue is the Hoechst signal, green the BrdU signal for ssDNA; (A) #Ab 8039 – detection of a specific BrdU signal within the nucleus only in a dilution of 1:50, higher dilutions preclude BrdU detection and overexposure only allows for the detection of a non-specific signal. (B) MCA260 – no specific BrdU signal can be detected as can be seen in the comparison between the samples stained with the primary antibody and the sample stained only with the secondary antibody; the antibody aggregates and forms precipitates, thereby impeding the setting of the right exposure time within the automated microscopy workflow. (A+B) pictures with 10x magnification, obtained using the *BD pathway system*
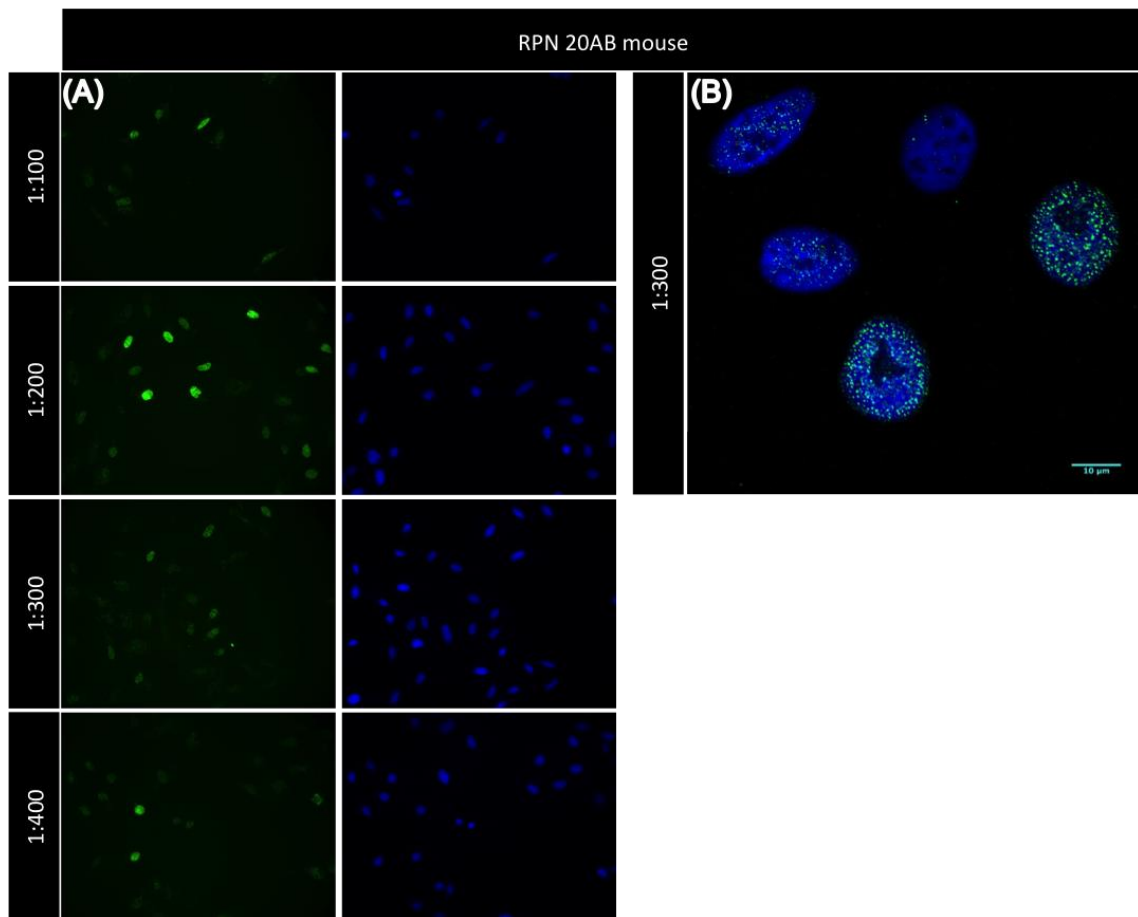
Figure 8: Staining with RPN 20AB produces a specific and selective signal within the nucleus

(A) Good staining for BrdU could be achieved at all tested dilutions; U2OS cells were labelled with 10 µM BrdU for 24 h and treated with 1 µM gemcitabine for 4 h, blue: Hoechst, green: BrdU signal for ssDNA; pictures with 10 x magnification, obtained using the *BD pathway system*. (B) Staining occurs selectively and with a high spatial resolution within the nucleus; an exemplary picture of U2OS cells treated with hydroxyurea for 4 h after labelling them with 10 µM BrdU for 24 h, blue: DAPI, green: BrdU signal for ssDNA; picture with 63x magnification, obtained using a *Zeiss Confocal LSM 510 meta microscope*

3.1.2   ssDNA is not mainly generated as a result of increased apoptosis

The aim of this study is to use the accumulation of ssDNA as a readout for replicative stress occurring in tumour cells and to apply this method, once established, to gain more knowledge about different enzymes known or suspected to be involved in replicative stress and the DNA damage response. However, following DNA damage, tumour cells can also undergo apoptosis if damage is too severe to be repaired by the cell's repair mechanisms. Therefore, it was necessary to make sure that the accumulation of ssDNA observed with the BrdU assay is really a function of replicative stress and not mainly due to the release of endonucelases during apoptosis (Zhang and Xu 2000). To achieve this, cells were intentionally driven towards apoptosis and stained for ssDNA. For the initiation of

apoptosis, cells were incubated with TRAIL or TRAIL in combination with Cycloheximide. TRAIL binds to the death receptors of a cell thereby inducing programmed cell death (Wiley et al. 1995). Cycloheximide inhibits protein biosynthesis, providing an additional stimulus for apoptosis. Figure 9 depicts the results of these experiments. A 1.2 fold increase in BrdU intensity for cells treated with TRAIL compared to the control and a 1.3 fold increase for cells treated with TRAIL and Cycloheximide can be observed. Regarding γ-H2AX intensities, the data show a very slight (1.09 fold) increase after treatment with TRAIL and a 1.7 fold increase after treatment with TRAIL and Cycloheximide. This leads to the conclusion that although some ssDNA seems to be generated when a cell induces apoptosis the main amount of ssDNA does not accumulate via this pathway. Hence the BrdU assay is suitable to provide an additional read-out for replicative stress and is not significantly confounded via apoptosis.
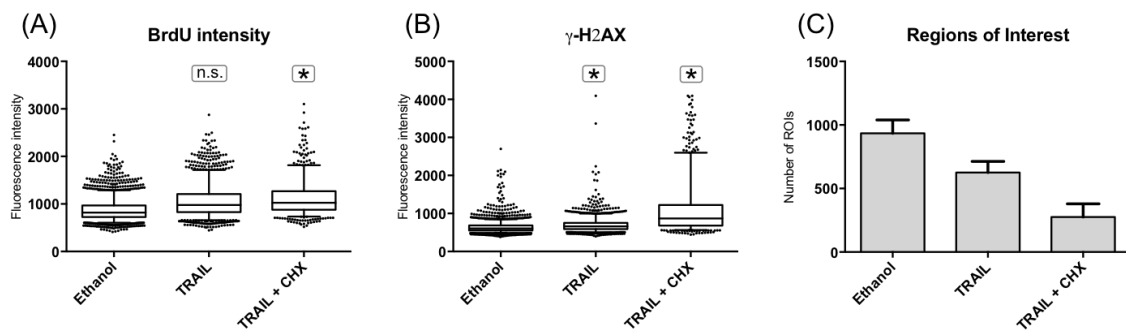

Figure 9: An increased signal for ssDNA in the BrdU assay and for γ-H2AX in immunofluorescence is not mainly mediated via apoptosis

U2OS cells were treated with 75 ng/ml TRAIL, 75 ng/ml TRAIL and 5 μg/ml Cycloheximide (CHX) or Ethanol as a negative control for 5.5 h (A, B). Only a slight induction of BrdU-signal as a read-out for ssDNA or γ-H2AX-signal upon apoptosis can be detected; Boxplots show the median, 25th and 75th percentile, whiskers indicate 5th and 95th percentile (C). As a control for the induction of programmed cell death, the number of ROIs, corresponding to the number of nuclei, is shown; indicated are mean value and SEM.

3.1.3   The BrdU signal correlates with γ-H2AX

Upon DNA damage the histone-variant H2AX is phosphorylated on Serine 139 by ATM, ATR and DNA-PK (Burma et al. 2001) and then called γ-H2AX (see also 1.2.4). As γ-H2AX is an established hallmark of the DNA damage response (Stucki and Jackson 2006) and also occurs after the exposure of ssDNA (Kinner et al. 2008) it is a good validation of the BrdU assay for ssDNA to test whether the BrdU signal correlates with the γ-H2AX signal. Indeed, there is a high correlation between the BrdU signal for ssDNA and γ-H2AX as can be seen in Figure 10. In addition, it was previously shown that the signal generated by the BrdU assay also colocalises with RPA that is recruited to stretches of ssDNA (Syljuåsen et al. 2005).

In summary, all the data show that the BrdU assay for ssDNA provides a reliable read-out for replicative stress that is not mediated via apoptosis and correlates with well established other markers of the DNA damage response. It was concluded that the BrdU assay for ssDNA is a useful tool to shed more light on the accumulation of ssDNA and its regulation after DNA damage.
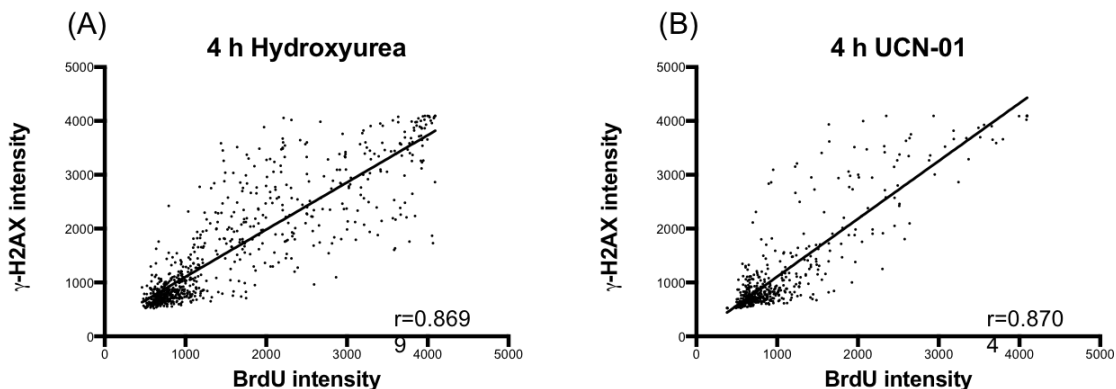

Figure 10: Accumulation of ssDNA correlates with phosphorylation of H2AX
BrdU labelled U2OS cells were used for the BrdU assay for ssDNA and simultaneously stained for γ-H2AX; fluorescence intensity of γ-H2AX is plotted against the respective BrdU intensity of the same ROI; the correlation coefficient r is given. (A) Cells were treated with hydroxyurea for 4 h after labelling them, r= 0.8699, p <0.001. (B) Cells were treated with Chk1 inhibitor UCN-01 for 4 h after labelling, r = 0.8704, p <0.001.

3.2   The ubiquitin ligase G2E3 plays a role in replicative stress

The intent of this study is to identify possible targets for chemosensitisation by using the accumulation of ssDNA as a read-out and to gain more insight in the mechanisms involved in replicative stress and mainly the generation of ssDNA. Therefore, the BrdU assay together with a costaining for γ-H2AX was employed for a mini-screen, which included proteins known to be involved in the DNA damage response and replicative stress. Gemcitabine was used to induce DNA damage as it is incorporated into the nascent DNA strand, leading to replication fork stalling and replicative stress (see 1.1.2.1). The proteins included were p53 as one of the most essential tumour suppressor genes, its homologue p73 (see 1.2.3), the Ubiquitin Ligase G2E3 (see 1.3) and hnRNP A.0, a member of the hnRNP A/B subfamily (Myer and Steitz 1995) which is known to be involved in the binding of pre m-RNA and found to induce γ-H2AX in a genome-wide siRNA screen (Paulsen et al. 2009). The results of this mini-screen are shown in Figure 11. Interestingly the knockdown of p53 or p73 did not lead to a relevant induction of ssDNA. The used siRNAs for p73 knock down all isoforms of p73 including the one lacking the transactivation domain. It would therefore be possible that by knocking down all p73 isoforms regardless of their activity, more promoters are freed and hence could lead to an induction of p53, which would be able to rescue the initial effects that may have been there

from the p73 knockdown. To exclude this possibility a double knockdown of p53 and p73 was performed which, although statistically significant showed just a slight additional accumulation of ssDNA, see Figure 12. This suggests that the accumulation of ssDNA mainly occurs in a p53 and p73 independent manner. For hnRNP the results were inconclusive between the two siRNAs used and therefore interpreted as most likely being off-target effects. Opposed to this, the knockdown of G2E3 led to a strong induction of ssDNA and this effect was consistent between two different siRNAs with a 4.02 fold increase for siRNA G2E3 A and a 3.84 fold increase for siRNA G2E3 C. G2E3 was heretofore investigated in our group in terms of its role during apoptosis, cell proliferation and the DDR upon cisplatin-treatment. As a result of the mini-screen G2E3 was identified as a potential regulator of stalled replication forks and single strand breaks which can lead to the exposure of ssDNA.
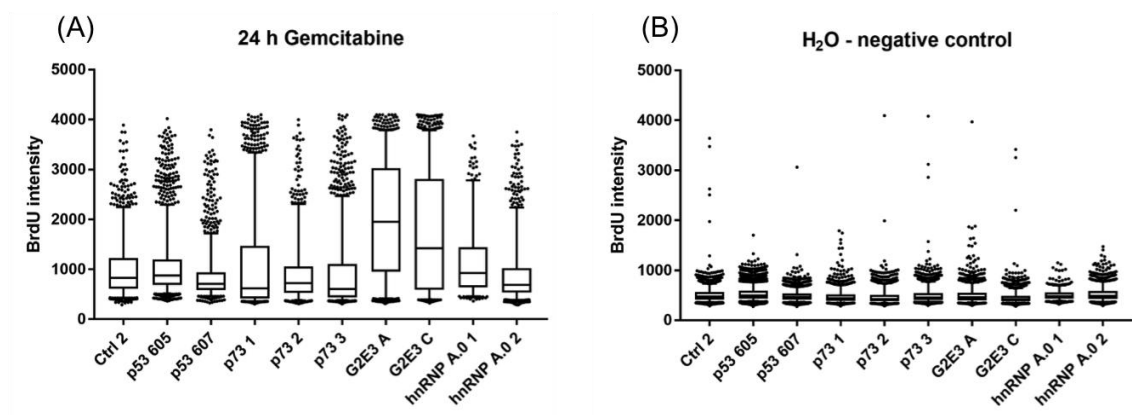

Figure 11: Knockdown of G2E3 shows the strongest ssDNA-induction of all tested DDR components

To identify components of the DNA damage response that influence the formation of ssDNA, a mini-screen in U2OS cells including p53, p73, hnRNP A and G2E3 was performed, each protein was targeted by at least two different siRNAs to minimise off-target effects, cells were labelled with 10 μM BrdU for 24 h and treated with either 300 nM gemcitabine for 24 h under continued presence of BrdU (A) or H2O as a negative control (B). Boxplots show the median, 25th and 75th percentile, whiskers indicate 5th and 95th percentile. (A+B): A baseline increase of 2.01 fold after 24 h gemcitabine treatment for the siRNA Control (Ctrl 2) is observed and a 4.02 fold increase after 24 h gemcitabine for G2E3 knockdown with G2E3 A and 3.84 fold increase after 24 h gemcitabine for G2E3 knockdown with G2E3 C.
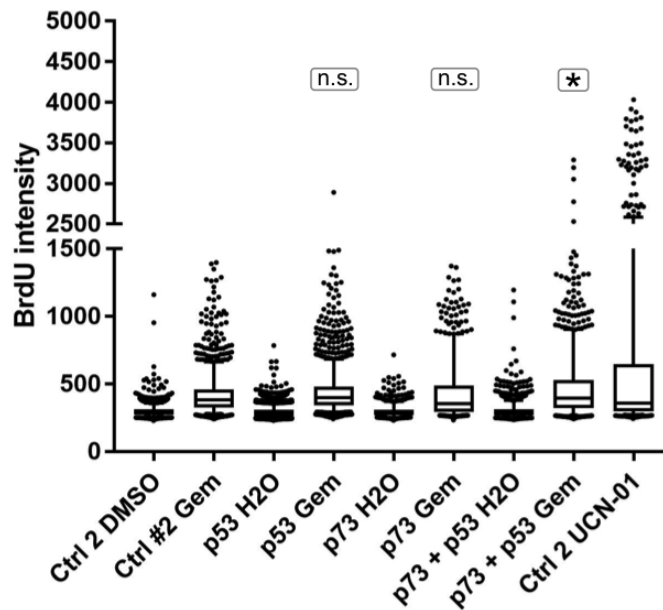
Figure 12: Double knockdown of p53 and p73 does not lead to an increase in ssDNA

In U2OS cells a knockdown of p53, p73 and a double-knockdown of both proteins was performed, followed by labelling the cells with BrdU for 24 h and treating them with 300 nM gemcitabine for further 24 h under continued BrdU presence. The Chk1 inhibitor UCN-01 was included as a positive control; boxplots show the median, 25th and 75th percentile, whiskers indicate 5th and 95th percentile. Neither knockdown of p53 nor p73 leads to a significant induction of ssDNA. Double-knockdown of p53 and p73 leads to a very slight, although statistically significant 1.13 fold increase of ssDNA as opposed to the gemcitabine treated control.

3.2.1 Knockdown of G2E3 leads to an accumulation of ssDNA

As found in the mini-screen knockdown of G2E3 leads to an increased signal in the BrdU assay. This finding was consistent and reproducible throughout a series of experiments. To further clarify at which stage of DNA damage the ssDNA was generated the temporal resolution of the observed phenomenon needed to be determined. To do so, cells in which G2E3 was temporarily knocked down via siRNA were treated with gemcitabine for only 4 h in addition for the previously used 24 h. Strikingly after 4 h gemcitabine treatment, this led to the reduction of γ-H2AX and only a very slight increase in ssDNA. The data is shown in Figure 13.
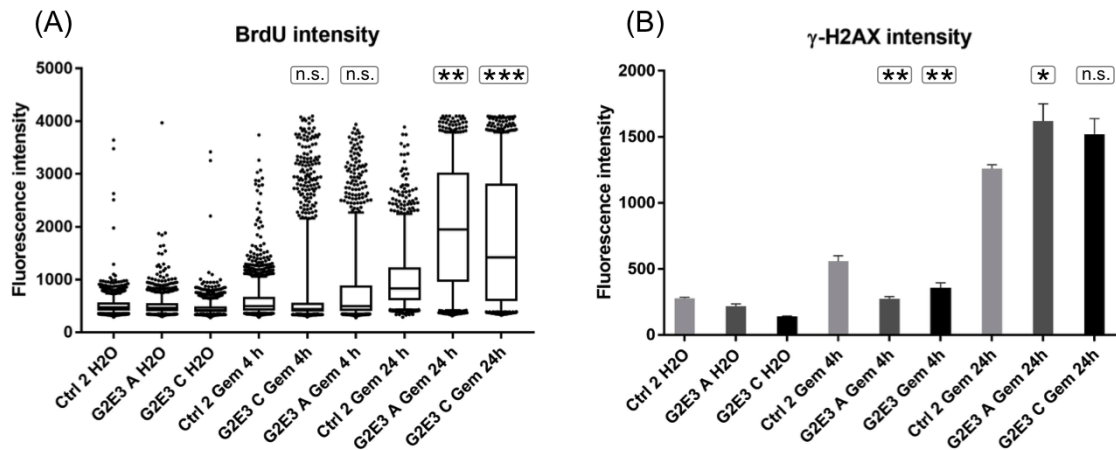
Figure 13: Gemcitabine induces strong induction of ssDNA and γ-H2AX in G2E3 knockdown cells after 24 h but only a slight, non-significant increase in ssDNA and even a reduction of γ-H2AX after 4 h

U2OS cells were transfected with two different siRNAs against G2E3 and treated with 300 nM gemcitabine for 24 h or 1 µM gemcitabine for 4 h after labelling them with 10 µM BrdU for 24 h; boxplots show the median, 25th and 75th percentile, whiskers indicate 5th and 95th percentile. After 24 h both siRNAs targeting G2E3 lead to a highly significant induction of ssDNA as observed in the BrdU assay as well as an induction of γ-H2AX. After 4 hours of gemcitabine treatment with a high dose, no significant change in the amount of ssDNA is detected although the data clearly show that some cells seem to accumulate a high degree of ssDNA as expressed by the increased outliers and high fluorescence intensities after G2E3 knockdown; for γ-H2AX treatment with a high dose of gemcitabine even leads to a highly significant reduction of γ-H2AX.

A possible explanation for the increase in ssDNA and γ-H2AX after 24 h but the decrease after 4 h could be that the knockdown of G2E3 together with gemcitabine treatment induces apoptosis. If this were true, G2E3 knockdown could first prevent an adequate response to DNA damage generated by gemcitabine and indicated by the decrease in ssDNA and γ-H2AX after 4 h but then lead to such a high amount of unaddressed damage that the cell induces apoptosis within 24 h thusly leading to an increase in ssDNA and γ-H2AX. Although normally ssDNA is not generated as a result of apoptosis (see 3.1.2) this could still be true for γ-H2AX and could also not generally be excluded for ssDNA in cells with knocked down G2E3. To clarify this, an experiment was conducted during which cells with knocked down G2E3 were treated with gemcitabine and Z-VAD an inhibitor of caspases to prevent apoptosis. No difference between the cells treated with Z-VAD and the control was found. Therefore, it was concluded that the decrease is not due to apoptosis.
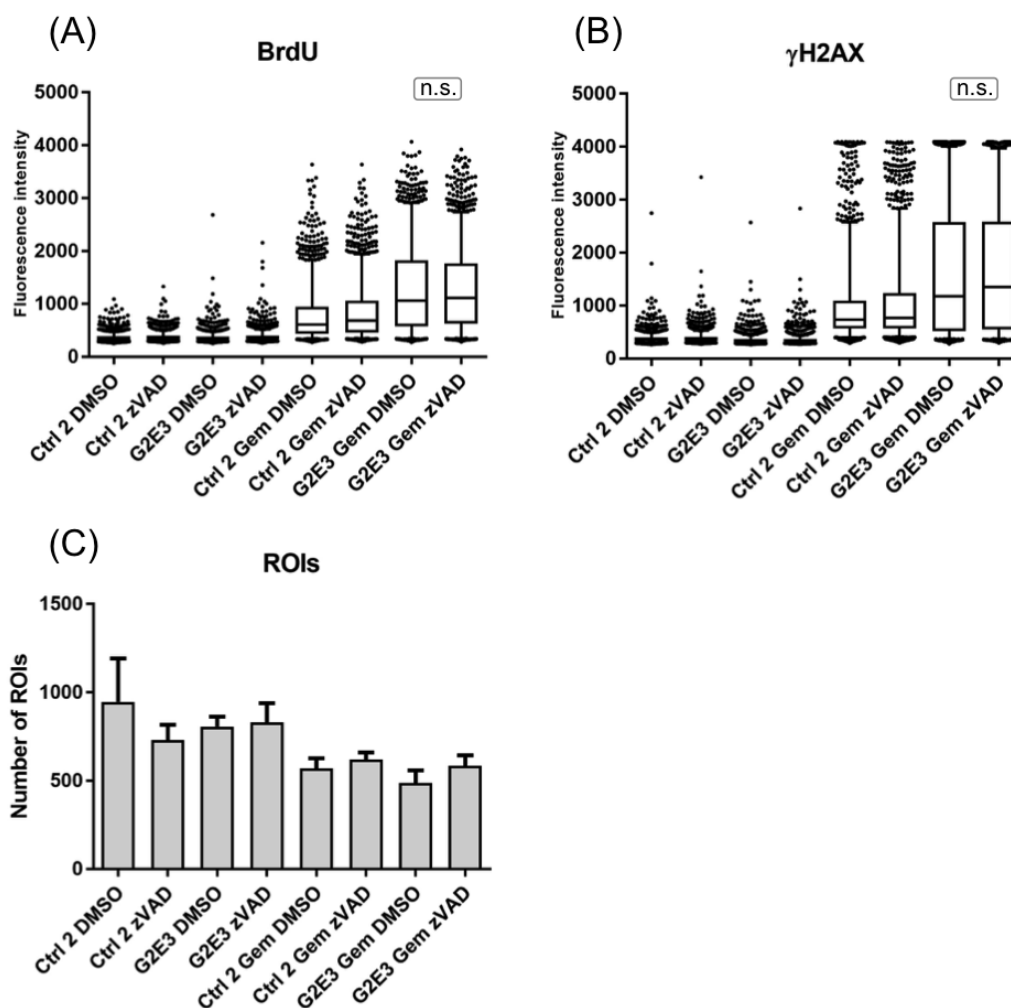
Figure 14: G2E3 knockdown in combination with gemcitabine treatment induces ssDNA and γ-H2AX independent of apoptosis

U2OS cells were transiently transfected with siRNA against G2E3, labelled with 10 μm BrdU for 24 h and treated with 300 nM gemcitabine for 24 h and either 50 μM Z-VAD or DMSO as control under continued presence of BrdU; after fixation, cells were processed for use in the BrdU assay for ssDNA and co-stained for γ-H2AX; boxplots show the median, 25th and 75th percentile, whiskers indicate 5th and 95th percentile. There is no significant difference in either ssDNA accumulation or γ-H2AX levels if apoptosis is inhibited via Z-VAD. The numbers of ROIs are included as a control and show that upon Z-VAD treatment indeed apoptosis seems to be inhibited as more cells survived treatment.

3.2.2 Knockdown of G2E3 decreases levels of phosphorylated Chk1

Having found out that G2E3 plays a role in replicative stress and impacts the accumulation of ssDNA as well as the generation of γ-H2AX the question arose whether this could be due to a process occurring in direct proximity to the replicating strand of DNA. It was therefore investigated whether G2E3 also influences the signalling cascade known as the DNA damage response and if so at which point during the cascade does this interference

occur? To this end, a series of western blot experiments were conducted to assess protein levels of key players involved in the DDR. As a cellular system U2OS cells were used to start with, since they are well established in cancer research and have been used for all previous BrdU assay experiments during this study. Another treatment duration of 6 h in addition to the normally used 24 h was included because preliminary work in our lab showed the strongest pChk1 signal upon gemcitabine treatment to occur after 5 − 6 h. The results of these experiments can be found in Figure 15. In the control cells, treatment with gemcitabine induces phosphorylation of Chk1 to pChk1 after 6 h and 24 h with no apparent difference regarding the signal intensity between these two time points. An induction of p53 can also be observed as well as a signal increase for γ-H2AX after 24 h. For both samples in which two different siRNAs were used to knockdown G2E3 there is a reduction in phosphorylated Chk1 after 6 h and 24 h gemcitabine treatment which is especially strong after 6 h, suggesting that G2E3 is not only involved in the early response to DNA damage but also directly or indirectly regulates the phosphorylation and hence activation of Chk1 one of the most important regulators of the DDR after single-strand breaks. Furthermore the western blots confirmed previous findings from immunofluorescence that knockdown of G2E3 leads to an increase in γ-H2AX after gemcitabine treatment. Previous experiments in our lab showed that G2E3 negatively regulates p53 (Schmidt et al. 2015). This leads to the question if G2E3's effect on pChk1 could somehow be mediated or influenced via p53. Consequently the immunoblot experiments were repeated in a system well suited to answer this question: a colorectal cancer cell line that exists in two forms, one possessing two copies of p53 (HCT116 p53$^{+/+}$) and one possessing none of them (HCT116 p53$^{-/-}$). The results of these experiments are again depicted in Figure 15. In both cell lines, the transient knockdown of G2E3 leads to a reduction of pChk1 after 6 h and 24 h; again this effect is stronger after 6 h. However, total levels of Chk1 in its dephosphorylated form remain unchanged and as in U2OS cells, an induction of p53 upon gemcitabine treatment, unchanged by G2E3 knockdown, can be seen in the HCT116 p53$^{+/+}$. Taken together, these results strongly support the hypothesis that G2E3 is an early regulator of pChk1 in a p53 independent manner and make G2E3 a potential candidate for sensitising cancer cells towards chemotherapy by alleviating a cell's response to DNA damaging agents such as chemotherapy, thus making DNA repair less likely to occur and shifting the balance more towards apoptosis.
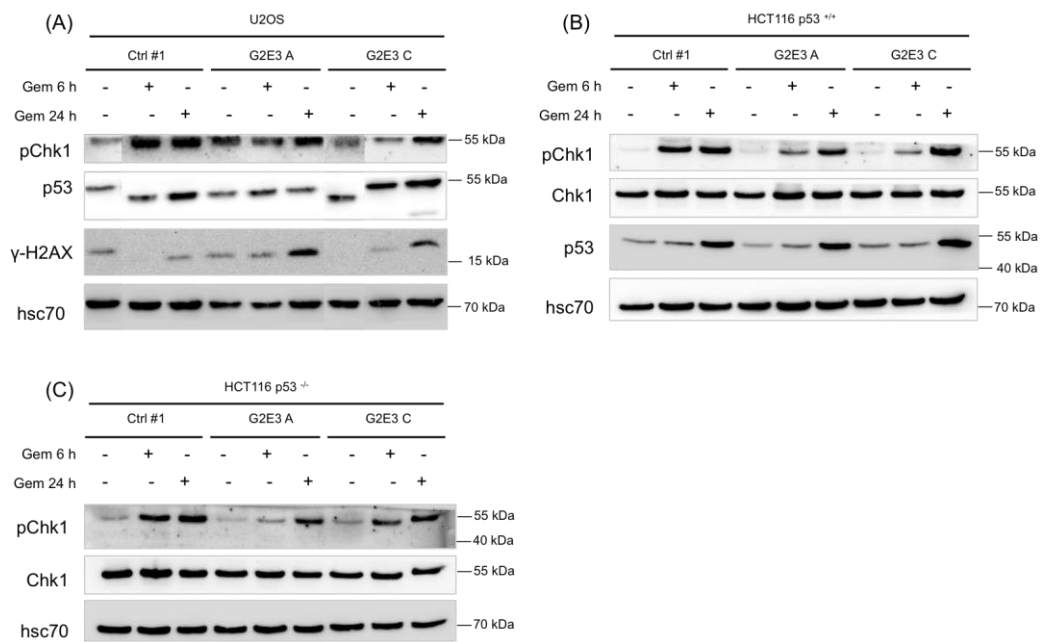
Figure 15: Phospo-Chk1 levels decrease after knockdown of G2E3 and gemcitabine treatment

U2OS (A), HCT116 p53$^{+/+}$(B) and HCT116 p53$^{-/-}$(C) were transiently transfected with two different siRNAs against G2E3 or a control siRNA, then treated with 300 nM gemcitabine for either 6 h or 24 h and processed for western blot analysis. Hsc70 staining was included as a loading control.

3.3 MK2 inhibition rescues accumulation of ssDNA and γ-H2AX upon gemcitabine treatment

Previous work in our group (mainly conducted by Frederik Köpper and Cathrin Bierwirth) provided evidence for a direct impact of the p38/MK2 pathway on a cell's response to replicative stress. The results of this work as well as results acquired as part of this thesis were published in 2013 in *PNAS* (Köpper et al. 2013), where it was shown that MK2 promotes the stalling of replication forks upon DNA damage. After inhibition or siRNA knockdown of MK2 and treatment with gemcitabine the normally observed induction of γ-H2AX and decreased cell-survival is rescued. DNA fiber assays showed that MK2 is required for two key features of replicative stress, namely the slow-down of fork speed and the increase in late origin firing. These effects were dependent on translesion synthesis, a mechanism where cells use another polymerase that has a lower fidelity and is more error-prone but able to continue synthesising if there are obstacles within the DNA strand that lead to fork stalling (Köpper et al. 2013). One of the main polymerases involved in translesion synthesis is polymerase η.

Based on these results, we were interested if this function of MK2 could also be observed when using the accumulation of ssDNA as an indirect measure of stalled replication forks. Following this idea, a series of experiments was conducted were we indeed found that the

inhibition of MK2 leads to a significant reduction of both ssDNA and γ-H2AX in U2OS cells treated with gemcitabine for 24 (see Figure 16). Interestingly, when we used hydroxyurea as a chemotherapeutic drug instead of gemcitabine and conducted the same experiment, the inhibition of MK2 had no effect at all. The results of these experiments are shown in Figure 16.
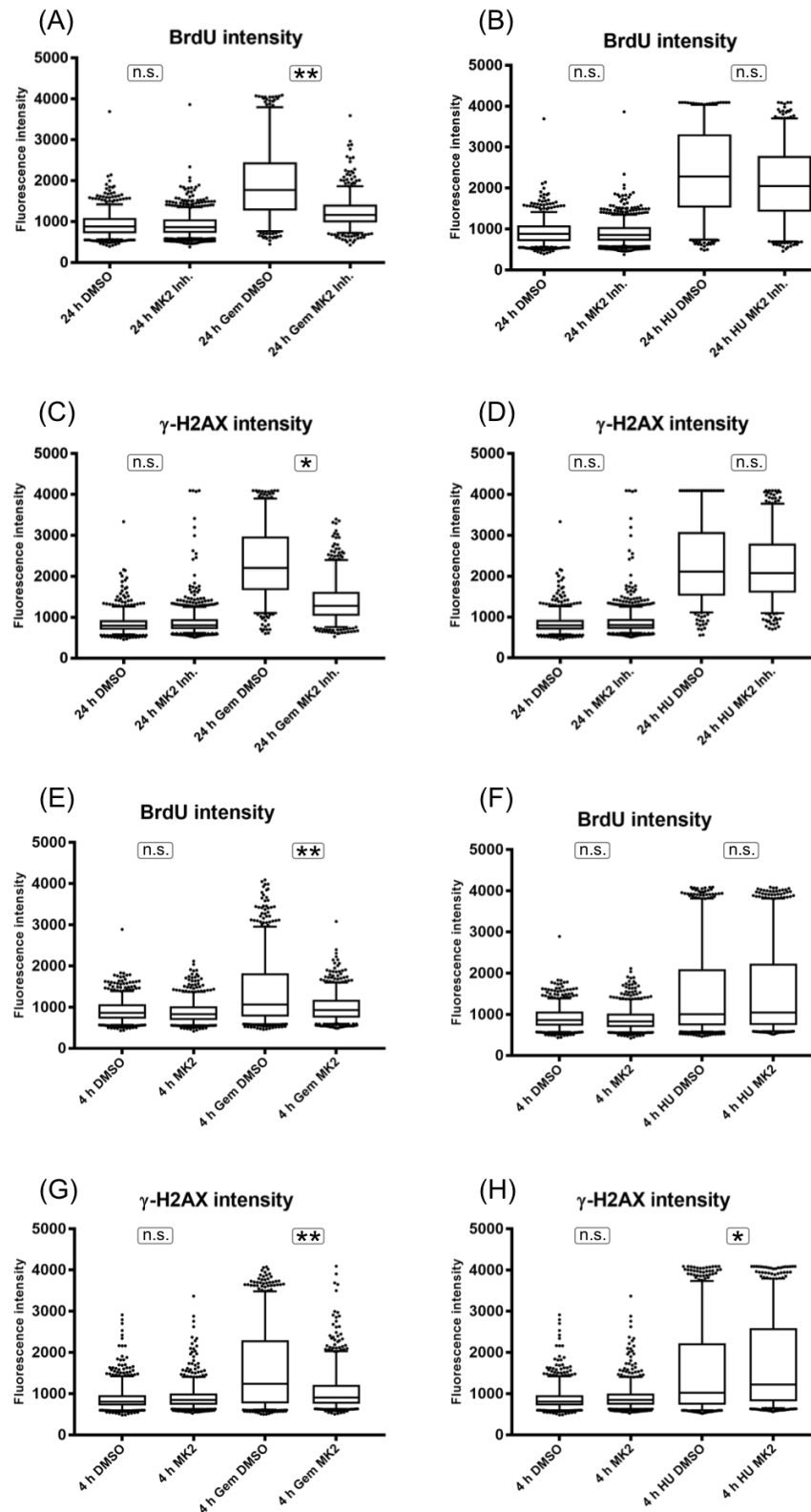
Figure 16: Inhibition of MK2 rescues ssDNA and γ-H2AX after gemcitabine treatment but not after hydroxyurea treatment

(A)-(E): Cells were labelled with 10 μM BrdU for 24 h and then treated with 300 μM gemcitabine or 2 mM hydroxyurea together with either MK2 inhibitor or DMSO as control for 24 hours. Cells were then harvested and co-stained for BrdU and γ-H2AX. For gemcitabine treatment, the inhibition of MK2 leads to a significant decrease in the amount of ssDNA as well as γ-H2AX as opposed to DMSO. In case of hydroxyurea the inhibition of MK2 does not significantly change the amount of ssDNA or BrdU. (F)-(G): All these effects could also be observed after 4 hours of gemcitabine or hydroxyurea treatment.

A possible explanation for this phenomenon lies in the different mechanisms of action used by hydroxyurea and gemcitabine. Hydroxyurea inhibits ribonucleotide reductase, thereby leading to an imbalance and most importantly shortage of available nucleotides. While gemcitabine also to a small degree inhibits RNR, it is a nucleoside analogue that first and foremost leads to misincorporation and chaintermination without influencing nucleotide pools as much (see 1.1.2). As it has previously been shown that polymerase η depends on high nucleotide levels (Washington et al. 2003), we hypothesise that the shortage of nucleotides caused by hydroxyurea impedes translesion synthesis as much as it impedes normal DNA replication and therefore abolishes the target via which MK2 exerts its effect (Köpper et al. 2013).

Nevertheless, the fact that the presence of MK2 together with gemcitabine treatment promotes replication fork stalling and accumulation of γ-H2AX makes it an interesting candidate to investigate in terms of resistance to chemotherapy.

4   Discussion

The stalling of replication forks and slow-down of replication itself is one of the key features of replicative stress and can pose great obstacles that need to be overcome in order to maintain genome integrity and prevent malignant transformation of cells. In case of tumour cells that already underwent malignant transformation, the induction of replicative stress is one of the very principles of chemo- and radiotherapy. The process of replicating DNA is the most vulnerable phase during the cell cycle and the importance, implications and extent of replicative stress have only started to be acknowledged and understood during recent years.

In this context, the work presented in this study aims to contribute to our understanding of the cellular processes that occur in times of replicative stress by establishing and refining a staining method for ssDNA in a large semi-automated setting that can also be used for screening analysis. This method was then employed to identify proteins that interfere with the accumulation of ssDNA and further investigate them. The two proteins picked were G2E3 and MK2. Both seem to be involved in signalling upon replicative stress but in a very different manner.

We found that the knockdown of the ubiquitin ligase G2E3 leads to a much higher accumulation of ssDNA upon treatment with the nucleoside analogue gemcitabine. Moreover G2E3 seems to be a regulator of one of the most important kinases involved in the DNA damage response, namely Chk1. Knockdown of G2E3 reduces the phosphorylation and hence activation of Chk1 upon gemcitabine treatment. We also provide evidence that this regulation occurs in a p53 independent manner.

We also investigated the effects of the inhibition of MK2, a kinase known to be involved in p38 signalling and an increasingly recognised regulator of replicative stress, on the accumulation of ssDNA. Inhibition of MK2 rescues the accumulation of ssDNA and γ-H2AX that is normally observed after treatment with gemcitabine. These results provide further evidence that MK2 positively regulates replication fork stalling.

4.1   The BrdU assay as a method to assess replicative stress

At the core of this study stands a further development of the BrdU assay for ssDNA that was in its first basic form originally described in 2005 (Syljuåsen et al. 2005). We could show that our refinements make it a suitable and relatively easy method to detect ssDNA at sites of replication fork stalling even in a high-content, semi-automated setup.

Due to its characteristics the BrdU assay for ssDNA stands between the immunofluorescence-based detection of indirect markers of replicative stress like γ-H2AX

that are often used for screening analysis because of the relatively easy staining method that can be applied to a large sample sizes and more in-depth tools like DNA fiber-assays that are much more complicated and time consuming and are therefore more useful for investigating proteins that are already known to be involved in processes surrounding the replication fork. In this study we introduced the BrdU assay in a refined form, making it possible to be applied in a high-content, semi-automated setup for the first time (see 3.1). This makes it a new and very useful tool to get a more direct readout regarding the amount of stalled replication forks already as part of the screening process. Additionally, it can easily be combined with a co-staining for γ-H2AX on the same plate. We could show that the BrdU signal correlates well with γ-H2AX as an established but less specific marker of DNA damage (Figure 10).

It is conceivable that the induction of apoptosis could also induce an accumulation of ssDNA and therefore produce false positive results in the BrdU assay as apoptosis leads to the release of endonucleases that cut the DNA into oligonucleotide sized pieces. Apoptosis can be triggered from within a cell as a consequence of replicative stress or more general DNA damage; this makes it difficult to differentiate between accumulation of ssDNA caused by stalled replication forks and possible accumulation caused by apoptosis. But apoptosis can also be triggered via the extrinsic pathway without any form of DNA damaging treatment, thereby avoiding the difficulties in differentiating a signal caused by replicative stress from one possibly caused by apoptosis. We therefore induced apoptosis in U2OS cells via stimulation of their death receptors and processed them for use in the BrdU assay. As Figure 9 shows there is no significant increase in BrdU intensities. Beyond that, cells were also treated with TRAIL to stimulate death receptors and Cycloheximide to inhibit protein biosynthesis in parallel to intensify apoptotic stimulation. Following this treatment we could observe a very slight but statistically significant increase in ssDNA accumulation (see Figure 9). Based on these results it can be said that a small amount of ssDNA seems to accumulate following massive induction of apoptosis but this does not relevantly confound the results of the BrdU assay as it only accounts for a very minor part of the observed signal intensities and only occurred after massive induction of apoptosis and not after sole stimulation of the death receptors using TRAIL. To make sure that assumption is still correct in the case of G2E3 knockdown cells, we treated cells as normally with gemcitabine and half of them additionally with Z-VAD, an inhibitor of caspases to suppress apoptosis (see Figure 14). There was no significant difference between the cells treated with Z-VAD and the control. It can be concluded that massive apoptosis in general may lead to a slight increase in ssDNA as detected with the BrdU assay but under normal circumstances and also in the case of G2E3 this does not significantly confound the results. Rather, the observed accumulation of ssDNA seems for the very major part due to replicative stress resulting in stalled or even collapsed replication forks.

4.1.1   Limitations and outlook

In order to lead to a positive signal in the BrdU assay, there need to be high enough levels of ssDNA. However, the assumption that replicative stress always causes enough ssDNA to be detectable may not be true and also the comparison with γ-H2AX levels does not eliminate this problem because for the phosphorylation of γ-H2AX a global response in the cell is necessary which may not be there but the cell may just react locally to a low level of replicative stress (Zeman and Cimprich 2014). Therefore, we cannot exclude that low levels of replicative stress may lead to too few stalled replication forks to be detectable. Nevertheless, this limitation is true for nearly all immunofluorescence-based assays regardless which target structure is stained and looked at.

In this study, we always quantified the BrdU intensities by defining ROIs comprised of the cells' nuclei, assessing intensities within them and then calculating a mean intensity per well. It should also be possible to actually count the number of BrdU spots within a nucleus as opposed to looking at intensities. This will most probably not be possible in the automated setting, as it requires high-resolution confocal images. Before we adapted the BrdU assay for use in high-content immunofluorescence, we conducted studies using cells grown on cover slips and stained as normal for microscopy with a *Zeiss Confocal LSM 510 meta microscope* using 63x magnification (see Figure 8 for an example picture). With these microscopic pictures or even higher magnification and resolution it should technically be possibly to segment the pictures and count individual BrdU spots that exceed a certain threshold signal intensity. This could provide a read-out that could allow for an even more direct assessment of stalled replication forks by actually counting them.

4.2   G2E3 in the response to DNA damage

Our results strongly suggest that G2E3 plays a role in the regulation of the ATR/Chk1 pathway. Depletion of G2E3 via siRNA mediated knockdown leads to reduced phosphorylation and hence activation of Chk1 in response to replicative stress induced by treatment with gemcitabine. Another study conducted by Franziska Schmidt in our group, also found this effect after treatment with the chemotherapeutic drug cisplatin (Schmidt et al. 2015). To this point, the mechanism how G2E3 influences pChk1 levels remains unclear. It is possible that G2E3 influences upstream regulators of the ATR/Chk1 pathway or ATR itself but further research is needed to unveil the exact mechanism. Based on the results obtained so far, we propose that G2E3 normally sustains the ATR/Chk1 pathway, thereby avoiding replicative stress and promoting the stabilisation and restart of stalled replication forks, see Figure 17 for a schematic depiction. After gemcitabine or cisplatin treatment, G2E3 mRNA-levels significantly decrease, suggesting a down-regulation of G2E3 following replicative stress (Schmidt et al. 2015). This down-regulation could then tip the balance more towards a decrease in proliferation or even apoptosis instead of stabilising stalled forks, halting the cell cycle and trying to repair the damage, thereby

possibly generating mutations. This fits well together with the findings that G2E3 plays a role in cell survival and G2E3 knockout leads to early embryonic lethality based on massive apoptosis at the blastocyst stage in mice (see 1.3) (Brooks et al. 2008; Schmidt et al. 2015).
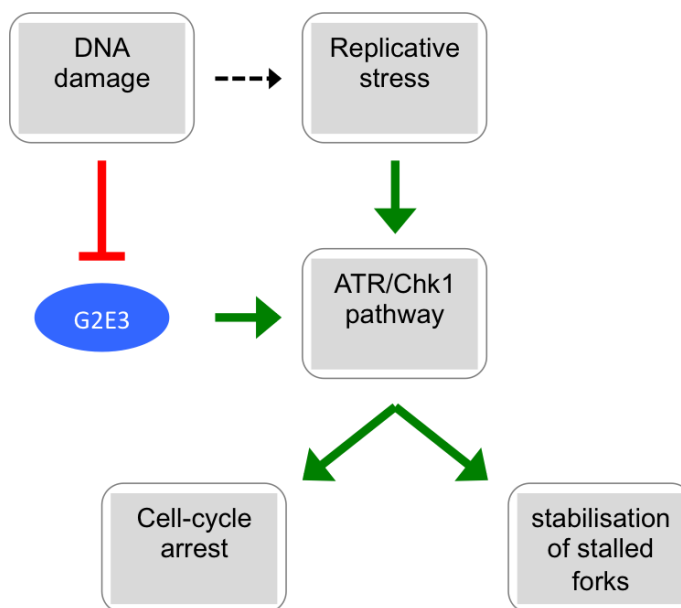

Figure 17: Proposed model of G2E3 action in the context of replicative stress

G2E3 normally sustains ATR/Chk1 signalling, thereby promoting stabilisation of stalled replication forks and halting the cell cycle in order to give the cell time to repair the damage and finish replication without accumulating too many mutations. Upon severe DNA damage though, G2E3 is downregulated and no longer positively influences ATR/Chk1 signalling, leading to more collapsed replication forks and DNA damage in general and possibly shifting the balance more towards apoptosis.

This model also explains the increased levels of ssDNA as observed in the BrdU assay. Chk1 is an important factor involved in the DNA damage response and also in the intra-S phase checkpoint. It is known that abrogation of Chk1 function leads to a massive accumulation of ssDNA, increased DNA synthesis that is at least partially mediated by increased origin firing and breakage of the DNA (Feijoo et al. 2001; Syljuåsen et al. 2005). This is fully compatibly with our results that G2E3 knockdown induces stalled replication forks upon gemcitabine treatment and also promotes phosphorylation of H2AX.

4.2.1 G2E3 as a potential target for chemosensitisation

Despite advancements in cancer therapy that were made during the last years, chemotherapy is still a key treatment for most malignancies. One way to further strengthen the effect of chemotherapy while simultaneously sparing healthy cells from too severe side effects is chemosensitisation. There are already clinical trials investigating the possibility of inhibiting Chk1 itself or its regulators like for example ATR in combination with chemotherapy (McNeely et al. 2014; Sausville et al. 2014; Daud et al. 2015; Morgan and Lawrence 2015). Often, these studies use gemcitabine as part of the chemotherapeutic

regime, the same drug that was used in this study. The fact that G2E3 knockdown leads to increased accumulation of ssDNA and γ-H2AX and lowers the level of phospho-Chk1 makes it a promising target for chemosensitisation. If G2E3 is inhibited, cells accumulate more damage and are less likely to survive treatment.

4.2.2   Open questions and outlook

Based on our findings in the BrdU assay that knockdown of G2E3 in U2OS cells lead to an increase in ssDNA and γ-H2AX we conducted western blot experiments that showed the same effect for γ-H2AX. To assess the p53 dependence of this process we repeated these western blots in a cell-line called HCT116 that exists in a $p53^{+/+}$ and a $p53^{-/-}$ form. Again, we observed an increase in γ-H2AX in G2E3 knockdown cells after treatment with gemcitabine, see Figure 15 for details. This is a strong hint that also the accumulation of ssDNA that, as we show in Figure 10, highly correlates with γ-H2AX, is not dependant on p53. In this context it would be very interesting to perform the BrdU assay in HCT116 cells. Unfortunately, this was technically not possible due to the morphology of HCT116 cells. The cell line is derived from colorectal cancer cells and the cells are very small and round and not very well attached to the surface they are growing on. All these features make them difficult to use in the BrdU assay since they are easily washed away during the staining procedure and the few cells that survive are so small that it is technically very difficult to reliably segment them and define ROIs in an automated manner. We tried HCT116 cells for the BrdU assay but finally could not get technically satisfying results. A compromise for further experiments could be to perform a double knockdown of G2E3 and p53 in U2OS cells and assess BrdU levels there.

We hypothesise that G2E3 influences pChk1 levels via regulation of ATR or its upstream regulators. To validate this assumption it will be beneficial to conduct western blot analyses including a staining for ATR-substrates. Antibodies that bind to ATR's phosphorylation motif are commercially available and could be used additionally to pChk1 and γ-H2AX. If our hypothesis is correct, we would expect a decrease in phosphorylated ATR substrates following G2E3 knockdown and DNA damaging treatment. These studies could also help in the goal of finding G2E3's natural substrates that to this point remain unknown. It is for example conceivable that G2E3 ubiquitinates a yet to be identified negative regulator of ATR that is subsequently degraded. But as of now, this remains speculation.

Another interesting question is whether G2E3 influences progression through the cell cycle. It is known that depletion of G2E3 leads to a slower replication rate (Schmidt et al. 2015) and G2E3 itself is expressed in a cell-cycle dependent manner (Crawford and Piwnica-Worms 2001). If G2E3 does indeed lead to a slow-down or even arrest of the cell-cycle, this could explain the only slight induction of ssDNA and even decrease in γ-H2AX levels in G2E3 knockdown cells after 4 h of gemcitabine but strong increase after 24 h of treatment (see Figure 13).

4.3 MK2 and chemoresistance

Replicative stress can lead to stalling and eventually collapse of replication forks but this process is not only a result of replicative stress but also tightly regulated itself. The data presented in this study, as well as further work conducted in our group by Frederik Köpper and Cathrin Bierwirth, suggest that MK2 positively regulates replication fork stalling, meaning that the presence of MK2 leads to a higher number of stalled forks, resulting in fork collapse and subsequent strand breaks, see Figure 16. Therefore, it was suggested that MK2 favours stalling and possible repair and restart of forks under low levels of replicative stress and is down regulated after DNA damage tipping the balance more towards translesion synthesis, thereby introducing replication errors and making it more likely for a cell to gain mutations (Köpper et al. 2013). The results obtained in this study further support this hypothesis as we could show that inhibition of MK2 rescues the normally observed accumulation of ssDNA as well as γ-H2AX after gemcitabine treatment. However, this is not true for treatment with hydroxyurea. We suggest that this is due to the different mechanism of action used by both drugs. Hydroxyurea leads to imbalances and shortages in the dNTP pool by inhibiting RNR and this likely impairs translesion synthesis, the very mechanism that the rescue of fork stalling upon MK2 inhibition relies on (see 3.3).

In this context, MK2 could be a potential factor in the resistance to chemotherapy. It is known that tumours treated with chemotherapy often already accumulated mutations, or continue to do so during therapy, that impede the effect of chemotherapy. Gemcitabine, the drug used in this study, is part of treatment protocols for advanced solid tumours, especially pancreatic tumours. But even with chemotherapy the clinical outcome with these malignancies is poor. For pancreatic cancer, over 75 % of patients show no or only a very slight response with the commonly used treatment protocols and nearly all patients with pancreatic cancer eventually develop metastases letting the mean 5-year survival rate drop below 5 % (Li et al. 2004; Dhayat et al. 2011). There is already extensive research regarding resistance mechanisms to gemcitabine and a multitude of proteins and microRNAs involved in the DDR, apoptotic signalling and cell growth have been identified to play a role in this context. Hence, loss of MK2 could be another factor how resistance to gemcitabine is achieved. At least within our cell culture model the effects are highly significant. But more research is needed to determine whether this is also true in a more clinical setting.

5 Abstract

Cells are constantly subjected to DNA damaging agents like reactive oxygen species, UV light or, in the case of cancer cells, chemotherapeutic drugs. All these agents can lead to replicative stress, meaning that the most sensitive part of the cell cycle, namely S phase where cells replicate their DNA, is compromised. Cells developed several mechanisms to respond to replicative stress. One of the most prominent is the stalling of replication forks. Stalled forks can either be rapidly stabilised and eventually restarted or they collapse, leaving behind unfinished sites of replication that later on need to be dealt with. The stalling of forks leads to the exposure of stretches of single-stranded DNA (ssDNA). In this study we use a new DNA staining method to detect these stretches of ssDNA and refine it for use in a high-content, semi-automated setup. With this method we then conduct a mini-screen including proteins known to be involved in the DNA damage response and identify two candidates that are investigated further. Knockdown of G2E3, an E3 ubiquitin ligase, together with gemcitabine treatment leads to much higher levels of ssDNA as a read-out for stalled replication forks and γ-H2AX, a well established marker of DNA damage. We further provide evidence that G2E3 is a novel regulator of ATR/Chk1 signalling as it inhibits the phosphorylation and hence activation of Chk1, one of the key kinases of the DNA damage response. Based on these results, G2E3 is a potential new drug target for sensitising cancer cells for chemotherapy. The second protein investigated is MK2, a kinase known to be involved in signalling following DNA damage. We show that inhibition of MK2 rescues the normally observed induction of stalled forks and γ-H2AX accumulation upon gemcitabine treatment defining it as a regulator of replication fork stalling during replicative stress.

6   Literature

Bakkenist CJ, Kastan MB (2003): DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421, 499–506

Bartek J, Lukas J (2003): Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 3, 421–429

Bittner M, Kupferer P, Morris CF (1980): Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal Biochem 102, 459–471

Bont RD, Larebeke N van (2004): Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19, 169–185

Branzei D, Foiani M (2009): The checkpoint response to replication stress. DNA Repair 8, 1038–1046

Brooks WS, Banerjee S, Crawford DF (2007): G2E3 is a nucelocytoplasmic shuttling protein with DNA damage responsive localization. Exp Cell Res 313, 665–676

Brooks WS, Helton ES, Banerjee S, Venable M, Johnson L, Schoeb TR, Kesterson RA, Crawford DF (2008): G2E3 Is a Dual Function Ubiquitin Ligase Required for Early Embryonic Development. J Biol Chem 283, 22304–22315

Brown EJ, Baltimore D (2000): ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 14, 397–402

Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ (2001): Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 411, 102–107

Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ (2001): ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem 276, 42462–42467

Burris HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, et al. (1997): Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol Off J Am Soc Clin Oncol 15, 2403–2413

Chinnaiyan AM (1999): The Apoptosome: Heart and Soul of the Cell Death Machine. Neoplasia N Y N 1, 5–15

Ciccia A, Elledge SJ (2010): The DNA damage response: making it safe to play with knives. Mol Cell 40, 179–204

Cimprich KA, Cortez D (2008): ATR: an essential regulator of genome integrity. Nat Rev

Mol Cell Biol <u>9</u>, 616–627

Cobb JA, Bjergbaek L, Shimada K, Frei C, Gasser SM (2003): DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1. EMBO J <u>22</u>, 4325–4336

Crawford DF, Piwnica-Worms H (2001): The G(2) DNA damage checkpoint delays expression of genes encoding mitotic regulators. J Biol Chem <u>276</u>, 37166–37177

Crul M, van Waardenburg RCAM, Bocxe S, van Eijndhoven MAJ, Pluim D, Beijnen JH, Schellens JHM (2003): DNA repair mechanisms involved in gemcitabine cytotoxicity and in the interaction between gemcitabine and cisplatin. Biochem Pharmacol <u>65</u>, 275–282

Daud AI, Ashworth MT, Strosberg J, Goldman JW, Mendelson D, Springett G, Venook AP, Loechner S, Rosen LS, Shanahan F, et al. (2015): Phase I dose-escalation trial of checkpoint kinase 1 inhibitor MK-8776 as monotherapy and in combination with gemcitabine in patients with advanced solid tumors. J Clin Oncol Off J Am Soc Clin Oncol <u>33</u>, 1060–1066

DeVita VT, Chu E (2008): A History of Cancer Chemotherapy. Cancer Res <u>68</u>, 8643–8653

Dhayat S, Mardin WA, Mees ST, Haier J (2011): Epigenetic markers for chemosensitivity and chemoresistance in pancreatic cancer--a review. Int J Cancer <u>129</u>, 1031–1041

Dobbelstein M, Sørensen CS (2015): Exploiting replicative stress to treat cancer. Nat Rev Drug Discov <u>14</u>, 405–423

Durkin SG, Arlt MF, Howlett NG, Glover TW (2006): Depletion of CHK1, but not CHK2, induces chromosomal instability and breaks at common fragile sites. Oncogene <u>25</u>, 4381–4388

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993): WAF1, a potential mediator of p53 tumor suppression. Cell <u>75</u>, 817–825

Elmore S (2007): Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol <u>35</u>, 495–516

Engel K, Kotlyarov A, Gaestel M (1998): Leptomycin B‑ sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. EMBO J <u>17</u>, 3363–3371

Ewald B, Sampath D, Plunkett W (2008): Nucleoside analogs: molecular mechanisms signaling cell death. Oncogene <u>27</u>, 6522–6537

Feijoo C, Hall-Jackson C, Wu R, Jenkins D, Leitch J, Gilbert DM, Smythe C (2001): Activation of mammalian Chk1 during DNA replication arrest. J Cell Biol <u>154</u>, 913–924

Gatei M, Sloper K, Sörensen C, Syljuäsen R, Falck J, Hobson K, Savage K, Lukas J, Zhou B-B, Bartek J, Khanna KK (2003): Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent Phosphorylation of Chk1 on Ser-317 in Response to Ionizing Radiation. J Biol

Chem <u>278</u>, 14806–14811

Hanada K, Budzowska M, Davies SL, van Drunen E, Onizawa H, Beverloo HB, Maas A, Essers J, Hickson ID, Kanaar R (2007): The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. Nat Struct Mol Biol <u>14</u>, 1096–1104

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993): The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell <u>75</u>, 805–816

Hartlerode AJ, Scully R (2009): Mechanisms of double-strand break repair in somatic mammalian cells. Biochem J <u>423</u>, 157–168

Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA (2008): DNA repair pathways as targets for cancer therapy. Nat Rev Cancer <u>8</u>, 193–204

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991): p53 mutations in human cancers. Science <u>253</u>, 49–53

Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W (1991): Action of 2',2'-difluorodeoxycytidine on DNA synthesis. Cancer Res <u>51</u>, 6110–6117

Kang T, Wei Y, Chiang Y-C, Yamaguchi H, Appella E, Hung M-C, Piwnica-Worms H (2008): GSK-3β Targets Cdc25A for Ubiquitin-Mediated Proteolysis and GSK-3β Inactivation Correlates with Cdc25A Overproduction in Human Cancers. Cancer Cell <u>13</u>, 36–47

Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG (1993): Specific Proteolytic Cleavage of Poly(ADP-ribose) Polymerase: An Early Marker of Chemotherapy-induced Apoptosis. Cancer Res <u>53</u>, 3976–3985

Kerr JF, Wyllie AH, Currie AR (1972): Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer <u>26</u>, 239–257

Kinner A, Wu W, Staudt C, Iliakis G (2008): Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res <u>36</u>, 5678–5694

de Klein A, Muijtjens M, van Os R, Verhoeven Y, Smit B, Carr AM, Lehmann AR, Hoeijmakers JH (2000): Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. Curr Biol CB <u>10</u>, 479–482

Kluck RM, Ellerby LM, Ellerby HM, Naiem S, Yaffe MP, Margoliash E, Bredesen D, Mauk AG, Sherman F, Newmeyer DD (2000): Determinants of Cytochrome c Pro-apoptotic Activity the role of lysine 72 trimethylation. J Biol Chem <u>275</u>, 16127–16133

Komander D, Rape M (2012): The ubiquitin code. Annu Rev Biochem <u>81</u>, 203–229

Konerding D, James TL, Trump E, Soto AM, Marky LA, Gmeiner WH (2002): NMR structure of a gemcitabine-substituted model Okazaki fragment. Biochemistry (Mosc) <u>41</u>,

839–846

Köpper F, Bierwirth C, Schön M, Kunze M, Elvers I, Kranz D, Saini P, Menon MB, Walter D, Sørensen CS, et al. (2013): Damage-induced DNA replication stalling relies on MAPK-activated protein kinase 2 activity. Proc Natl Acad Sci U S A 110, 16856–16861

Kotsantis P, Jones RM, Higgs MR, Petermann E: Chapter Three - Cancer Therapy and Replication Stress: Forks on the Road to Perdition; in: Advances in Clinical Chemistry, Band 69; hrsg. v. Makowski GS; Elsevier 2015, 91–138

Krakoff IH, Brown NC, Reichard P (1968): Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res 28, 1559–1565

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685

Lambert S, Carr AM (2005): Checkpoint responses to replication fork barriers. Biochimie 87, 591–602

Latt SA, Stetten G, Juergens LA, Willard HF, Scher CD (1975): Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. J Histochem Cytochem 23, 493–505

Li D, Xie K, Wolff R, Abbruzzese JL (2004): Pancreatic cancer. The Lancet 363, 1049–1057

Lopes M, Cotta-Ramusino C, Pellicioli A, Liberi G, Plevani P, Muzi-Falconi M, Newlon CS, Foiani M (2001): The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412, 557–561

Lukas C, Bartkova J, Latella L, Falck J, Mailand N, Schroeder T, Sehested M, Lukas J, Bartek J (2001): DNA Damage-activated Kinase Chk2 Is Independent of Proliferation or Differentiation Yet Correlates with Tissue Biology. Cancer Res 61, 4990–4993

Manke IA, Nguyen A, Lim D, Stewart MQ, Elia AEH, Yaffe MB (2005): MAPKAP Kinase-2 Is a Cell Cycle Checkpoint Kinase that Regulates the G2/M Transition and S Phase Progression in Response to UV Irradiation. Mol Cell 17, 37–48

Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N, Oda M (2010): Eukaryotic Chromosome DNA Replication: Where, When, and How? Annu Rev Biochem 79, 89–130

McIntosh D, Blow JJ (2012): Dormant Origins, the Licensing Checkpoint, and the Response to Replicative Stresses. Cold Spring Harb Perspect Biol 4

McNeely S, Beckmann R, Bence Lin AK (2014): CHEK again: revisiting the development of CHK1 inhibitors for cancer therapy. Pharmacol Ther 142, 1–10

Menendez D, Inga A, Resnick MA (2009): The expanding universe of p53 targets. Nat Rev Cancer 9, 724–737

Meng W, Swenson LL, Fitzgibbon MJ, Hayakawa K, Haar E ter, Behrens AE, Fulghum JR, Lippke JA (2002): Structure of Mitogen-activated Protein Kinase-activated Protein (MAPKAP) Kinase 2 Suggests a Bifunctional Switch That Couples Kinase Activation with Nuclear Export. J Biol Chem 277, 37401–37405

Mini E, Nobili S, Caciagli B, Landini I, Mazzei T (2006): Cellular pharmacology of gemcitabine. Ann Oncol 17, v7–v12

Morgan DO: The Cell Cycle: Principles of Control. New Science Press, London, 2006

Morgan MA, Lawrence TS (2015): Molecular Pathways: Overcoming Radiation Resistance by Targeting DNA Damage Response Pathways. Clin Cancer Res 21, 2898–2904

Musselman CA, Kutateladze TG (2011): Handpicking epigenetic marks with PHD fingers. Nucleic Acids Res 39, 9061–9071

Myer VE, Steitz JA (1995): Isolation and characterization of a novel, low abundance hnRNP protein: A0. RNA 1, 171–182

Nakayama KI, Nakayama K (2006): Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 6, 369–381

Norbury CJ, Zhivotovsky B (2004): DNA damage-induced apoptosis. Oncogene 23, 2797–2808

Oren M (2003): Decision making by p53: life, death and cancer. Cell Death Differ 10, 431–442

Paulsen RD, Soni DV, Wollman R, Hahn AT, Yee M-C, Guan A, Hesley JA, Miller SC, Cromwell EF, Solow-Cordero DE, et al. (2009): A Genome-wide siRNA Screen Reveals Diverse Cellular Processes and Pathways that Mediate Genome Stability. Mol Cell 35, 228–239

Reinhardt HC, Yaffe MB (2009): Kinases that Control the Cell Cycle in Response to DNA Damage: Chk1, Chk2, and MK2. Curr Opin Cell Biol 21, 245–255

Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB (2007): p53-Deficient Cells Rely on ATM- and ATR-Mediated Checkpoint Signaling through the p38MAPK/MK2 Pathway for Survival after DNA Damage. Cancer Cell 11, 175–189

Renart J, Reiser J, Stark GR (1979): Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. Proc Natl Acad Sci U S A 76, 3116–3120

Saelens X, Festjens N, Walle LV, Gurp M van, Loo G van, Vandenabeele P (2004): Toxic proteins released from mitochondria in cell death. Oncogene 23, 2861–2874

Sausville E, LoRusso P, Carducci M, Carter J, Quinn MF, Malburg L, Azad N, Cosgrove D, Knight R, Barker P, et al. (2014): Phase I dose-escalation study of AZD7762, a checkpoint kinase inhibitor, in combination with gemcitabine in US patients with advanced

solid tumors. Cancer Chemother Pharmacol 73, 539–549

Schmidt F, Kunze M, Loock A-C, Dobbelstein M, Schmidt F, Kunze M, Loock A-C, Dobbelstein M (2015): Screening analysis of ubiquitin ligases reveals G2E3 as a potential target for chemosensitizing cancer cells. Oncotarget 6, 617–632

Schy WE, Hertel LW, Kroin JS, Bloom LB, Goodman MF, Richardson FC (1993): Effect of a template-located 2',2'-difluorodeoxycytidine on the kinetics and fidelity of base insertion by Klenow (3'-->5'exonuclease-) fragment. Cancer Res 53, 4582–4587

Shechter D, Costanzo V, Gautier J (2004): ATR and ATM regulate the timing of DNA replication origin firing. Nat Cell Biol 6, 648–655

Sørensen CS, Syljuåsen RG (2012): Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. Nucleic Acids Res 40, 477–486

Stiff T, O'Driscoll M, Rief N, Iwabuchi K, Löbrich M, Jeggo PA (2004): ATM and DNA-PK Function Redundantly to Phosphorylate H2AX after Exposure to Ionizing Radiation. Cancer Res 64, 2390–2396

Stucki M, Jackson SP (2006): gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. DNA Repair 5, 534–543

Swatek KN, Komander D (2016): Ubiquitin modifications. Cell Res 26, 399–422

Syljuåsen RG, Sørensen CS, Hansen LT, Fugger K, Lundin C, Johansson F, Helleday T, Sehested M, Lukas J, Bartek J (2005): Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. Mol Cell Biol 25, 3553–3562

Takai H, Tominaga K, Motoyama N, Minamishima YA, Nagahama H, Tsukiyama T, Ikeda K, Nakayama K, Nakanishi M, Nakayama K (2000): Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. Genes Dev 14, 1439–1447

Tian H, Gao Z, Li H, Zhang B, Wang G, Zhang Q, Pei D, Zheng J (2015): DNA damage response – A double-edged sword in cancer prevention and cancer therapy. Cancer Lett 358, 8–16

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76, 4350–4354

Ward IM, Minn K, Chen J (2004): UV-induced ataxia-telangiectasia-mutated and Rad3-related (ATR) activation requires replication stress. J Biol Chem 279, 9677–9680

Washington MT, Johnson RE, Prakash L, Prakash S (2003): The Mechanism of Nucleotide Incorporation by Human DNA Polymerase η Differs from That of the Yeast Enzyme. Mol Cell Biol 23, 8316–8322

Wawra E, Wintersberger E (1983): Does hydroxyurea inhibit DNA replication in mouse cells by more than one mechanism? Mol Cell Biol <u>3</u>, 297–304

Welch S, Hirte HW, Carey MS, Hotte SJ, Tsao M-S, Brown S, Pond GR, Dancey JE, Oza AM (2007): UCN-01 in combination with topotecan in patients with advanced recurrent ovarian cancer: A study of the Princess Margaret Hospital Phase II consortium. Gynecol Oncol <u>106</u>, 305–310

Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA (1995): Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity <u>3</u>, 673–682

Xiao Z, Xue J, Sowin TJ, Zhang H (2006): Differential roles of checkpoint kinase 1, checkpoint kinase 2, and mitogen-activated protein kinase–activated protein kinase 2 in mediating DNA damage–induced cell cycle arrest: implications for cancer therapy. Mol Cancer Ther <u>5</u>, 1935–1943

Zeman MK, Cimprich KA (2014): Causes and consequences of replication stress. Nat Cell Biol <u>16</u>, 2–9

Zhang JH, Xu M (2000): DNA fragmentation in apoptosis. Cell Res <u>10</u>, 205–211

Zhao H, Piwnica-Worms H (2001): ATR-Mediated Checkpoint Pathways Regulate Phosphorylation and Activation of Human Chk1. Mol Cell Biol <u>21</u>, 4129–4139

Zou L, Elledge SJ (2003): Sensing DNA Damage Through ATRIP Recognition of RPA-ssDNA Complexes. Science <u>300</u>, 1542–1548

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