Modulation of the Replication Stress Response by Histone Deacetylases is connected to Suppression of the PP2A regulatory Subunit PR130

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„Es liegt in der Natur der Sache,

dass man aus schönen und unschönen Erfahrungen lernen muss, um Reife zu erlangen.“

Nelson Rolihlahla Mandela (* 18.07.1918 ; † 05.12.2013)

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LIST OF ABBREVIATIONS

A_{595}	Absorbance at specific wave length (here: 595 nm)
Ac	Acetylation
APS	Ammonium persulfate
ASK	Activator of S phase kinase
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
ATRIP	ATR interacting protein
BAK	BCL2-antagonist/killer
BAX	BCL2-associated X protein
BCL2	B cell lymphoma 2
BRCT	Breast cancer 1 C terminus
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCNB1	gene encoding for cyclin B1
CDC7	Cell division cycle 7-related protein kinase
CDC25	Cell division cycle 25 (family of dual specific phosphatases)
CDK	Cyclin dependent kinase
cDNA	complementary DNA
CG-NAP	Centrosome and Golgi-localized protein kinase N-associated protein
CHK	Checkpoint kinase
CIP1	CDK-interacting protein 1
Co-IP	Co-Immunoprecipitation
conc.	Concentration
CoREST	Corepressor of RE1-silencing transcription factor
Ctrl	Control
DDB2	Damage specific DNA binding protein 2
DDR	DNA damage response
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonulceic acid
DNA-PKc	DNA-dependent protein kinase catalytic subunit
DR4	Death receptor 4 (also TRAIL-R1)
ds	Double-stranded
DSB	Double-strand break
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
DYRK2	Dual specificity tyrosine(Y)-phosphorylation-regulated kinase 2
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
et al.	et alteri or at altii (and others)
FAS	Fas cell surface death receptor (also CD95)
FAT	FRAT-ATM-TRRAP (protein domain)
5-FU	5-Fluorouracil
FCS	Fetal calf serum
FDA	Food and Drug Administration
fwd	forward

G_1	gap 1, stage of the cell cycle during interphase
G_2	gap 2, stage of the cell cycle during interphase
GADD45A	growth arrest and DNA damage-inducible 45 alpha
H_2O	Water
HCl	Hydrogen chloride
HDM2	Human double minute 2
IP	Immunoprecipitation
IR	Ionizing radiation
KCl	Potassium chloride
kDa	kilo Dalton (1 kDa = 1,000 g/mol)
KH_2PO_4	Potassium dihydrogen phosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor(s)
HDM2	Human homolog of mouse double minute
HEK TER	Human embryonic kidney cells expressing SV40LT, hTERT, and hRas-V12
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIPK2	Homeodomain-interacting protein kinase 2
HRP	Horseradish peroxidase
HU	Hydroxyurea
HUS1	HUS1 checkpoint homolog (*Schizosaccharomyces pombe*)
M	Mitosis, cell division stage of the cell cycle
MCM	Mini-chromosome maintenance
MDC1	Mediator of DNA-damage checkpoint 1
MDM2	Mouse double minute 2
MEF	Mouse embryonic fibroblast
MRE11	Meiotic recombination 11 homolog (*Sacchoromyces cerevisiae*)
mSIN3	Mammalian SIN3
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
Na_2HPO_4 x 2 H_2O	Disodium hydrogen phosphate dihydrate
NaN_3	Sodium azide
Na_3VO_4	Sodium orthovanadate
NBS1	Nibrin (also NBN)
N-CoR	Nuclear receptor corepressor
NETN	NaCl-EDTA-Tris-Nonidet P-40
NHEJ	Non-homologous end-joining
Nonidet P-40	Octylphenoxypolyethoxyethanol
NuRD	Nucleosome remodeling deacetylase
OA	Okadaic acid
OD_{230}	Optical density at specific wavelength (here: 230 nm)
PBS	Phosphate buffered saline
PARP-1	Poly(ADP-ribose)-polymerase-1
PCNA	Proliferating cell nuclear antigen
PI	Propidium iodide
PIC	Protease inhibitor cocktail
PIKK	Phosphatidylinositole-3 kinase related kinase
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2A
PPM	Metal-dependent protein phosphatase

PPP	Phospho-protein phosphatase
PPP2R3A	gene encoding for PR130 protein
PSP	Protein serine/threonine phosphatase
PTM	posttranslational modification
PTP	Protein tyrosine phosphatase
PUMA	p53-upregulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RAD	RAD homolog (*Schizosaccharomyces pombe*)
rev	reverse
RFC	Replication factor C
RPA	Replication protein A
rpm	Revolutions per minute (unit of frequency)
RNA	Ribonucleic acid
RNase	Ribonuclease
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
RT	Room temperature
S	Synthesis, stage of the cell cycle during interphase
SAHA	Suberoylanilide hydroxamic acid (also 'vorinostat')
s.d.	Standard derivation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	Serine residue
Sir2	Silent information regulator 2
SIRT	Sirtuin
SMRT	Silencing mediator for retinoid/thyroid hormone receptors
ssDNA	Single-stranded DNA
TEMED	N,N,N,N Tetramethylethylendiamin
Thr	Threonine residue
TIP60	Tat-interacting protein 60 kDa
TOPBP1	Topoisomerase 2-binding protein 1
Tris	Tris-(hydroxymethyl)-aminomethan
TSA	Trichostatin A
UV	Ultraviolet light
VPA	Valproic acid
WEE1	Wee1 homolog (*S. pombe*)
WIP1	Wild-type p53-induced phosphatase 1
53BP1	tumor protein p53 binding protein 1
9-1-1	RAD9-RAD1-HUS1 heterotrimer

1 ZUSAMMENFASSUNG

Das zelluläre Genom ist fortwährend durch genotoxische Einflüsse und ebenso durch die DNA Duplikation in jedem Zellzyklus gefährdet. Folglich haben sich Signalwege entwickelt, die Schäden in der DNA erkennen und reparieren. Im Zentrum der DNA Schadensantwort stehen die Kinasen ATR und ATM, die Signale an *Checkpoint*-Kinasen und andere Zielproteine weiterleiten. Antimetabolite wie Hydroxyurea werden häufig in der Krebstherapie eingesetzt und blockieren gezielt die DNA Replikation, um Krebszellen abzutöten. Obwohl bekannt ist, dass die Kombination mit Histondeacetylase (HDAC)-Inhibitoren das Ergebnis solcher Behandlungen verbessert, waren die molekularen Mechanismen dazu unklar.

In der vorliegenden Arbeit wird zum ersten Mal gezeigt, dass die Aktivität von Klasse I HDACs notwendig für eine anhaltende DNA Schadensantwort nach Hydroxyurea- und 5-Fluorouracil induziertem Replikationsstress ist. Diese Beobachtungen wurden mit dem Klasse I HDAC-Inhibitor (HDACi) MS-275 erzielt. Genauer führte der zeitgleiche Verlust von HDAC1 und HDAC2 Aktivität zu einer verringerten Phosphorylierung der Kinasen ATR/ATM und CHK1/CHK2. Dies ging mit einer schwächeren Aktivierung und Zielgenexpression von p53 einher. Trotz einer verstärkten DNA-Schädigung konnten Zellen unter dem Einfluss des HDACi keinen stabilen Zellzyklusarrest in der S Phase ausbilden, was letztlich zu einer erhöhten Apoptoserate führte. Bei Experimenten mit selektiven Kinaseinhibitoren zeigte sich, dass die verringerte Aktivität nur einer Kinase nicht ausreichte, um die für MS-275 beschriebenen Effekte zu imitieren. Die Ergebnisse legen nahe, dass der HDACi die Aktivität von sowohl ATR als auch ATM negativ reguliert.

Diese Arbeit schlägt die unterdrückte Expression der PP2A B‘‘ regulatorischen Untereinheit PR130 als zugrunde liegenden Mechanismus vor, die durch HDAC1 und HDAC2 geregelt wird. MS-275 induzierte einen verzögerten Anstieg an PR130 auf transkriptioneller wie auch auf Proteinebene. Es wurde zudem gezeigt, dass PR130 mit phosphoryliertem ATM interagiert. Dies markiert die Kinase für die PP2A-vermittelte Dephosphorylierung, da ein *Knockdown* von PR130 die ATM Phosphorylierung wiederherstellte.

Die Studie beschreibt damit eine neue molekulare Verknüpfung zwischen den epigenetischen Regulatoren HDAC1/2 und einer Phosphatase-abhängigen Modulation der DNA Schadensantwort in Zellen unter Replikationsstress. Dabei wurde PR130 als potenzieller

Regler der DDR identifiziert, welcher der Kinaseaktivität entgegenwirkt und somit die normale zelluläre Physiologie wiederherstellen kann.

2 SUMMARY

The integrity of the genome is constantly challenged by genotoxic factors and also DNA duplication during every cell cycle. Hence, signaling pathways have evolved to sense and repair sites of damage. At the center of the DNA damage response (DDR) are the apical kinases ATR and ATM, which delegate signaling to checkpoint kinases and various other downstream targets. Anti-metabolites like hydroxyurea are commonly used in cancer therapy and intentionally block replication to eliminate cancer cells. Although it was found that HDAC inhibitors favorably combine with these treatments, the molecular mechanisms were unknown.

The present study shows for the first time that class I HDAC activity is necessary to keep up a sustained DDR after replication stress induced by hydroxyurea and 5-fluorouracil. These observations were made with the class I HDAC inhibitor (HDACi) MS-275. Specifically, simultaneous loss of HDAC1 and HDAC2 activity was required to diminish phosphorylation of the kinases ATR/ATM and CHK1/CHK2. This was accompanied by reduced p53 activation and target gene expression. Despite the higher incidence of DNA damage, cells were unable to establish a stable cell cycle arrest in S phase in the presence of the HDACi, which ultimately resulted in elevated rates of apoptosis. Experiments with selective kinase inhibitors revealed that the attenuated activity of only one apical or checkpoint kinase was insufficient to imitate all signaling and cellular effects of MS-275. They rather demonstrated that the HDACi negatively regulates both, ATR and ATM activity.

This work proposes the suppressed expression of the PP2A B'' regulatory subunit PR130 as the underlying molecular mechanism governed by HDAC1 and HDAC2. MS-275 induced a delayed increase in PR130 at the transcriptional and protein level. Furthermore, PR130 protein was shown to physically associate with phosphorylated ATM. This targets the kinase for PP2A-mediated dephosphorylation, since PR130 knockdown restored ATM phosphorylation.

Taken together, this study describes a novel molecular link between the epigenetic regulators HDAC1/2 and a phosphatase-dependent modulation of DDR signaling intensity in cells suffering from replication stress. PR130 was identified as a potential modulator of the DDR to terminate kinase activity and restore normal cellular physiology.

3 INTRODUCTION

Genetic information needs to be preserved from one cell generation to the next and genomic stability is a prerequisite for the survival of proliferating cells, while genomic instability promotes and is a common feature of cancer [1]. However, cells are constantly exposed to genotoxic stress originating from environmental sources as well as from within. Merely duplication of the DNA repeatedly generates unstable replication forks that are prone to breakage. Thus, a sophisticated and highly conserved network has evolved in eukaryotes to detect alterations in the DNA and ultimately coordinate their repair. It engages several signaling cascades, which are largely regulated by posttranslational modifications (PTMs), the most common of which is phosphorylation. These mechanisms are also known as the 'safeguards of genome integrity' [2].

3.1 Genomic lesions induce DNA damage response signaling

The term 'DNA damage response' (DDR) describes a network of numerous signaling pathways that is initiated in a cell after alterations of the genome and which regulates cell cycle transition, DNA replication, DNA repair, and apoptosis [3-6]. These signaling cascades are specifically regulated by PTMs. They enable cells to cope with endogenous challenges like reactive oxygen species (ROS) or stalled replication forks, as well as with environmental threats like ionizing and ultraviolet radiation (IR and UV, respectively). DNA damage response signaling is organized in successive stages during which the signal is amplified: the damage is recognized by sensor proteins that transmit the message to transducers, which in turn activate effector proteins. The first line of transducer molecules belongs to the family of phosphatidylinositol 3-kinase (PI3K)-like kinases (PIKKs). In mammals, the family consists of six members who possess serine/threonine protein kinase activity except one. They include ATM (Ataxia telangiectasia mutated), ATR (ATM and Rad3-related), DNA-PKc (DNA-dependent protein kinase catalytic subunit), mTOR (mammalian target of rapamycin), and SMG1 (suppressor of mutagenesis in genitalia 1). The last member is TRRAP (transformation/transcription associated protein) that lacks kinase activity. All of them share a PI3K-like, a FAT (FRAP-ATM-TRRAP), and a FATC (FAT C-terminal) domain but only three PIKKs play major roles in DDR signaling [7].

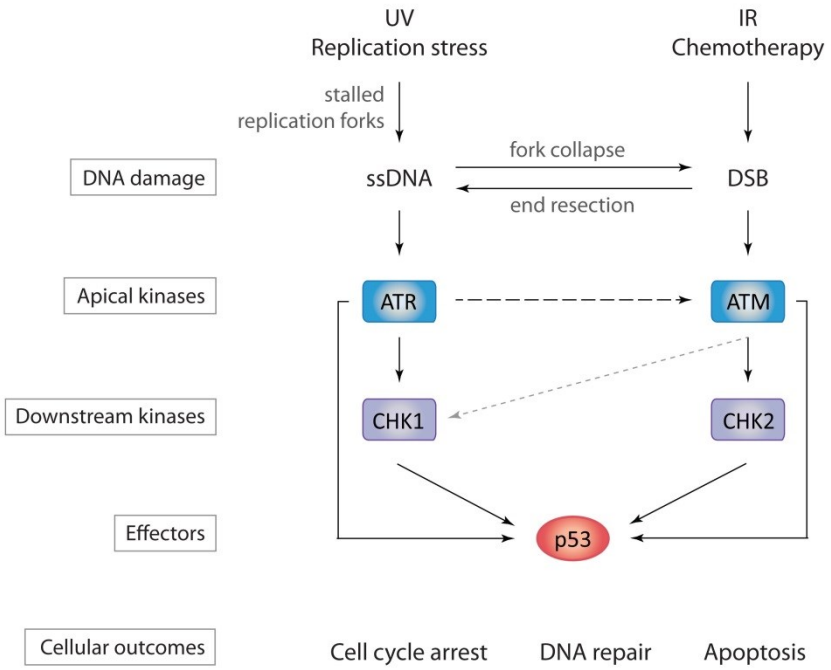

Figure 3.1: Induction of the DNA damage response – a simple overview.

UV-induced DNA lesions, replication stress (e.g. by depletion of deoxyribonucleotides), or end resection of DNA DSBs lead to the exposure of single-stranded DNA stretches, which in turn induce ATR signaling with subsequent phosphorylation of CHK1 and p53. Double-strand breaks (DSB) of the DNA induced by ionizing radiation or chemotherapeutic substances stimulate ATM activation. Cross-activation of ATM by ATR is indicated with a black dashed arrow. ATM signaling activates CHK2 and p53. To a lesser extent, also CHK1 might be phosphorylated by ATM (grey dotted arrow). After [8-11].

DNA-PK's catalytic subunit forms an active heterotrimer with KU70-KU80 in response to DNA double-strand breaks (DSB) and is critically involved in coordinating DNA repair by non-homologous end-joining (NHEJ) [12]. The apical kinases ATM and ATR are at the center of the DNA damage response and initiate comprehensive phospho-protein cascades following their catalytic activation. They are both important for the maintenance of genomic stability. In humans, mutation of the *ATM* gene causes the disease 'Ataxia telangiectasia' associated with severe genomic instability [13]. Mutations of ATR result in a rare and serious developmental disorder called 'Seckel syndrome' [14]. However, ATR is essential for the sustained survival of replicating cells and a complete loss of ATR activity is embryonically lethal [15,16]. ATM and ATR not only show sequence similarity, but also have a common SQ/TQ (a serine or threonine residue followed by glutamine) phospho-site consensus motif. That explains the considerable redundancy among their hundreds of substrates [3]. The key difference between both kinases is their response to different kinds of genotoxic stress (Figure 3.1). Rarely occurring DSBs initiate DDR signaling through ATM with subsequent phosphorylation of its downstream kinase checkpoint kinase 2 (CHK2) and of the tumor suppressor p53. ATR on the other hand is activated in every cell cycle during DNA replication

or after UV radiation and phosphorylates checkpoint kinase 1 (CHK1) and p53 [17]. Yet, both signaling pathways are not strictly separated since processing of DSB sites and replication fork collapse may trigger activation of the other kinase. A direct cross-activation of ATM by ATR was also reported [9,18]. In each case, the locally acting apical kinases delegate stress signaling to diffusing downstream molecules and thereby pave the way to a global cellular response [10].

3.1.1 Initiation of the DNA damage response – ATR and ATM

Activation of ATR

The kinase activity of ATR is stimulated in response to many genomic insults like replication stress, base adducts, cross-links, or DSBs. All these damages ultimately create stretches of single stranded DNA (ssDNA), which seem to be the key structure for ATR activation in most cases [11,19]. For example, stalled replication forks directly generate ssDNA when polymerases are stopped due to depleted dNTP pools or lesions in the DNA while the leading helicase complex proceeds to unwind DNA [6,17]. Also, nuclease activity may form ssDNA stretches during end resection of DSBs. In any case, replication protein A (RPA) rapidly coats exposed DNA single strands and creates a complex further referred to as ssDNA-RPA [20] (Figure 3.2 A). ssDNA-RPA is crucial for the recruitment of ATR to sites of DNA damage [19]. Nevertheless, ATR alone is unable to recognize RPA and depends on ATR interacting protein (ATRIP). ATRIP is not only important for ATR localization as it directly binds RPA [21], but also for ATR stability [16]. It has been shown that the assembly of ATRIP-ATR complexes on ssDNA-RPA alone already promotes ATR phosphorylation in *trans* at threonine 1989 (Thr1989) [22,23]. This phosphorylation event is indispensable yet insufficient to activate ATR kinase function (Figure 3.2 B). In an independent cascade, the heterotrimer RAD9-RAD1-HUS1 (9-1-1) is brought to the site of DNA damage. The clamp loader RAD17-replication factor C (RAD17-RFC) first recognizes a 5´-junction of dsDNA, which is adjacent to ssDNA-RPA [24]. In a second step, it loads the ring shaped 9-1-1 onto the junction, which resembles a PCNA (proliferating cell nuclear antigen)-like sliding clamp [25]. Importantly, the 9-1-1 element RAD9 is constitutively phosphorylated in its C-terminal tail at serine 397 (Ser397) [26]. This creates a binding site for topoisomerase 2-binding protein 1 (TOPBP1), which recognizes the phosphorylation with its BRCT domains [27,28] (Figure 3.2 C). TOPBP1 additionally contains an ATR activation domain and binds to the phosphorylated Thr1989 residue of ATR via other BRCT domains. The close proximity of TOPBP1 to ATRIP-ATR is crucial to trigger full

activation of ATR, although the detailed mechanisms are poorly understood and remain to be defined [29,30]. Prior to activation of downstream signaling, ATR phosphorylates a number of mediator molecules including RAD17 and Claspin [31]. The mediator Claspin associates with RAD17 and serves as a platform for the recruitment and ATR-dependent phosphorylation of CHK1 [32]. Downstream activation of CHK1 is currently the best readout of ATR activity except for Thr1989 ATR auto-phosphorylation. Once phosphorylated, CHK1 is released from chromatin and targets its soluble substrates [33] (Figure 3.2 D). Moreover, ATR phosphorylates the histone variant H2A.X at Ser139 (γH2A.X) in the chromatin flanking the damage site [34]. γH2A.X foci formation facilitates the recruitment of repair proteins or other DNA damage mediators and thereby participates in the orchestration of the DNA damage response [2].

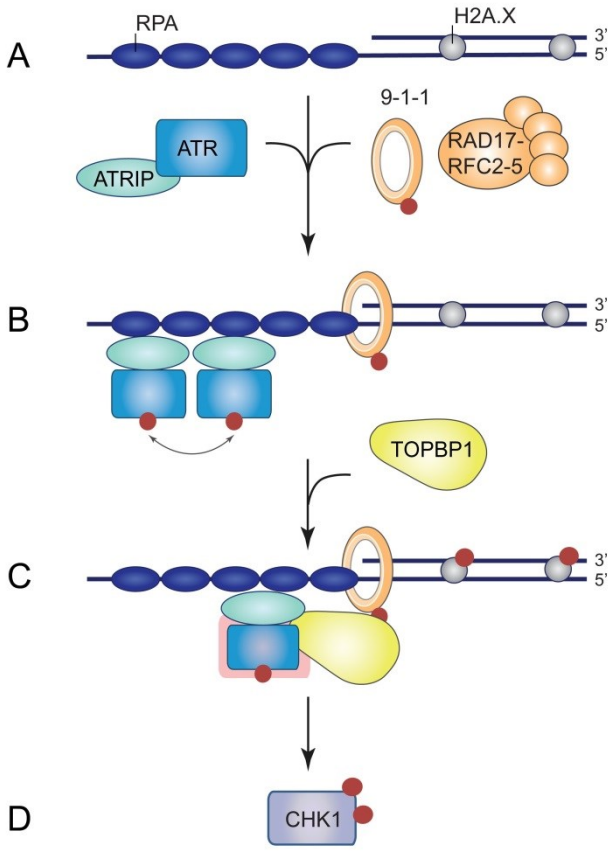

Figure 3.2: Activation of ATR by DNA damage.

(**A**) ATR is recruited to Replication protein A (RPA)-coated ssDNA through its interaction partner ATRIP. RPA also independently recruits the clamp loader complex RAD17-Replication factor C (RFC) to the ssDNA junction, where RAD17-RFC loads the RAD9-RAD1-HUS1 (9-1-1) clamp onto the DNA. (**B**) Congregation of ATRIP-ATR complexes on ssDNA-RPA enables *trans*-phosphorylation of ATR molecules. The independent loading of 9-1-1 facilitates recruitment of the Topoisomerase-binding protein 1 (TOPBP1). (**C**) Subsequent binding of TOPBP1 activates ATR (red shadow) and thereby triggers phosphorylation of histone 2A.X (H2A.X) and (**D**) ATR downstream targets like CHK1. Red bullets indicate phosphorylation. After [1,22,35].

Activation of ATM

In contrast to ATR, ATM is primarily activated in response to DSBs of the DNA [36]. Undamaged cells contain homodimerized ATM to hold the kinase inactive. Rapidly after the occurrence of DSBs, the molecules auto-phosphorylate each other at Ser1981 within the FAT domain and are released as monomers [37]. This first step of activation can be used as a marker of ATM activity since it is essential to develop complete catalytic activity of ATM in humans [38] (Figure 3.3 A). At least four additional phosphorylation sites of ATM were identified since then. Three of the residues (Ser367, Ser189, Ser2996) are auto-phosphorylated by ATM [39,40]. In parallel to phosphorylation, the histone acetyltransferase (HAT) TIP60 acetylates ATM in a DNA damage-dependent manner on a single site at Lys3016 and thereby contributes to ATM activation [41].

What exactly triggers ATM auto-phosphorylation is still a matter of debate. In addition to a conformational change in chromatin structure after DSBs, direct interaction with free linear DNA at sites of damage or simple association with chromatin were suggested [37,42,43]. However, the need of the MRE11-RAD50-NBS1 (MRN) complex for complete catalytic activation of ATM is beyond question. Initially, the MRN sensor complex directly recognizes and binds to the site of damage where it recruits ATM monomers to achieve complete activation of the kinase [44,45]. One of the earliest phosphorylation targets of ATM is H2A.X. This creates γH2A.X foci that can extend over mega-base pairs around the DSB and set on an enormous positive feedback loop for ATM signaling [2,46] (Figure 3.3 B). In the beginning, γH2A.X molecules next to the DNA lesion serve as anchor points for mediator of DNA damage checkpoint 1 (MDC1) [47,48] (Figure 3.3 C). This mediator protein is phosphorylated by ATM itself [7,38]. Phosphorylated MDC1 now generates a binding site for NBS1 of the MRN complex [49]. ATM is then indirectly tethered to γH2A.X by MRN, which stimulates its catalytic activity [50]. Moreover, direct binding of ATM to MDC1 increases the retention of the kinase at DNA damage sites [50]. This amplifies H2A.X phosphorylation and facilitates further accumulation of DNA damage mediators, transducers, and repair proteins [7,51].

Any of the multiple ATM substrates in the signaling cascade spatially and timely fine-tunes the DNA damage response. For example, 53BP1 was found to directly interact with MRN and ATM. This association not only optimizes the ATM-dependent phosphorylation of NBS1. It also promotes 53BP1 phosphorylation that in turn serves as a mediator of the DDR positive feedback loop [45,52]. Besides NBS1, also MRE11 and RAD50 of the MRN complex are targeted

by ATM [7,38]. Phosphorylation of MRE11 and RAD50 was found to affect their role in regulating DNA repair [53,54]. The functional consequences for phosphorylated NBS1 are better known. As mentioned above, it firstly assembles ATM at sites of damage. Furthermore, it is evident that phospho-NBS1 facilitates DDR downstream signaling by serving as an adapter for ATM substrates [55,56]. One of the best documented targets of ATM is the cell cycle regulator CHK2 [1] (Figure 3.3 D). Initial phosphorylation of CHK2 at Thr68 by ATM results in transient homodimerization of the checkpoint kinase, followed by auto-phosphorylation to gain complete catalytic activity [57-59].

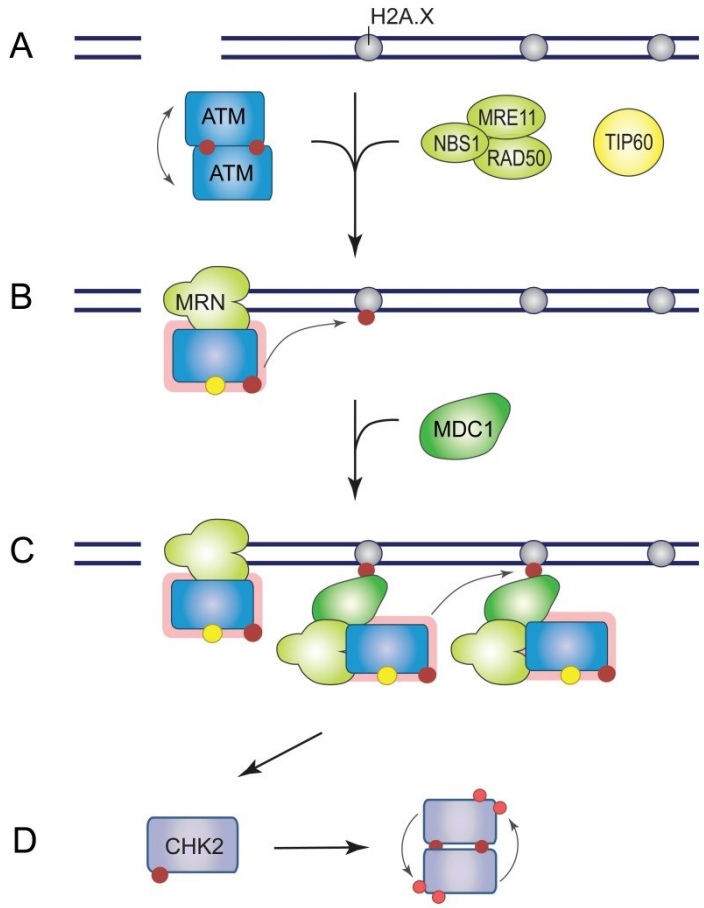

Figure 3.3: Activation of ATM by DNA double-strand breaks.

(**A**) Occurrence of DSBs causes auto-phosphorylation of inactive ATM dimers *in trans* and independently recruits the MRN complex to sites of damage. (**B**) TIP60 acetylates ATM and the MRE11-RAD50-NBS1 (MRN) complex fully activates ATM monomers (red shadow). Subsequently, ATM phosphorylates adjacent H2A.X molecules which in turn recruit the mediator of DNA damage checkpoint 1 (MDC1) to γH2A.X. (**C**) Binding of MRN complexes and ATM to MDC1 amplify the DNA damage signaling at damage foci. (**D**) The downstream kinase CHK2 is phosphorylated by ATM at Thr68, which triggers homodimerization and *trans*-phosphorylation of CHK2 molecules. Red bullets indicate phosphorylation. Yellow bullets indicate acetylation. After [5,35,37].

3.1.2 Checkpoint kinases and cell cycle control

Downstream of the apical kinases ATM and ATR phylogenetically conserved signaling cascades are activated. Two major players in this process are the serine/threonine kinases checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2). Despite being structurally unrelated and activated upon diverse stressors, they show overlapping functions [60]. CHK2 is primarily activated by ATM in response to DSB. ATM phosphorylates CHK2 at Thr68, which promotes homodimerization and subsequent intramolecular *trans*-phosphorylation of the checkpoint kinase molecules at Thr383 and Thr387 [57,61,62] (Figure 3.3 D). CHK1, on the other hand, is phosphorylated by ATR (and to a lesser extent maybe also by ATM) at Ser317 and Ser345 [6,63,64]. This phosphorylation event is dependent on Claspin (see section 3.1.1) and directly triggers catalytic activity of CHK1. There are currently two mutually non-exclusive models for CHK1 kinase activation. Firstly, phosphorylation by ATR would block an inhibiting intramolecular interaction of CHK1 whereby the kinase domain becomes exposed [65]. In a second model, phosphorylation rapidly releases CHK1 from chromatin and thereby enables the kinase to phosphorylate its soluble substrates [33]. Once activated, CHK1 and CHK2 phosphorylate specific as well as overlapping effectors implicated in DNA damage-induced transcription, replication fork stabilization, DNA repair, or apoptosis signaling. Especially CHK1 appears as a key regulator in the initiation and surveillance of cell cycle checkpoints in response to DNA damage [11,66].

Cell cycle progression is a tightly regulated process and proceeds in an ordered sequence of cell cycle phases. It is governed by cyclin-dependent kinases (CDKs), which require binding of cyclins for their temporal activation. On a second layer of control, specific phosphorylation events act as positive or negative regulators of CDK activity. In the end, CDK/cyclin complexes rule progression from one cell cycle stage to the next and are an important part of cell cycle checkpoints (Figure 3.4). Activation of these checkpoints in response to DNA damage is generally mediated by two mechanisms. The ATM/ATR-CHK1/CHK2-CDC25 pathways mediate a fast arrest of the cell cycle. Induction of the p53-dependent pathway, on the other hand, is much slower, yet indispensable to keep up sustained checkpoint control (see section 3.1.3).

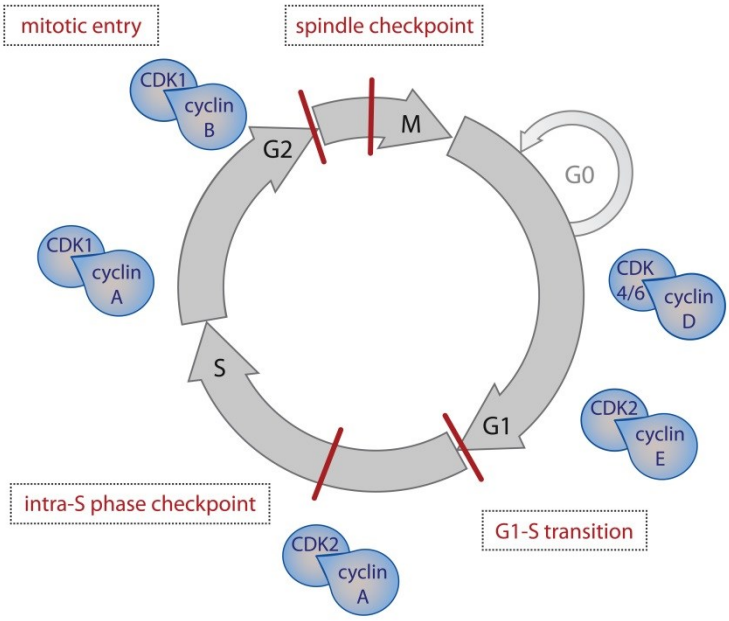

Figure 3.4: Regulation of the cell cycle by CDK/cyclin complexes.

Cell division is controlled by the regulated progression through sequential cell cycle stages (indicated by grey arrows). Specific CDK/cyclin complexes govern the passage through these phases and the transitions between them. Cell cycle checkpoints are indicated in red. After [11,67-69].

Activated CHK1 indirectly interferes with the formation of CDK/cyclin complexes as it stabilizes inhibitory phosphorylations of CDKs. In an undisturbed cell cycle, these phosphorylations are removed by the family of CDC25 phosphatases. Upon DNA damage, CDC25 phosphatase activity is impeded by CHK1 (Figure 3.5). Phosphorylation of CDC25A by CHK1 tags the phosphatase for proteasomal degradation. This inhibits formation of CDK2/cyclin E and CDK2/cyclin A complexes and consequently results in arrest at the G1/S transition or in S phase. In contrast, phosphorylation of the CDC25C isoform creates a binding site for 14-3-3 proteins, which sequester the phosphatase in the cytoplasm. The resulting lack of active CDK1/cyclin B dimers halts the cell cycle at the transition from G2 phase to mitosis [67,70]. Of note, CHK2 was also reported as a possible kinase for CDC25 phosphatases [60]. The checkpoint kinase CHK1 further contributes to CDK inactivation by targeting WEE1. Upon phosphorylation, the kinase WEE1 becomes stabilized and catalyzes inhibiting phosphorylation of CDK1 and CDK2 which prevents their interaction with cyclins [71]. CHK1 further supports the arrest of cells in S phase by directly targeting members of the DNA replication machinery. CHK1 inactivates the cyclin-like protein ASK (also called DBF4), which normally complexes with CDC7 kinase to initiate DNA replication. CDC7-dependent phosphorylation of the MCM helicase complex is essential for the recruitment of CDC45 to

sites of replication origin. A loss of CDC7/ASK activity therefore stops replication origin firing and blocks S phase progression [72,73].

Moreover, transcription of cell cycle-regulating genes is repressed by DNA damage-induced CHK1. A slight basal kinase activity enables chromatin-bound CHK1 to phosphorylate Thr11 on histone H3, which in turn correlates with the expression of e.g. *CCNB1* (cyclin B1) and *CDK1*. The dissociation of activated CHK1 from chromatin reduces expression of these genes and contributes to cell cycle arrest [74].

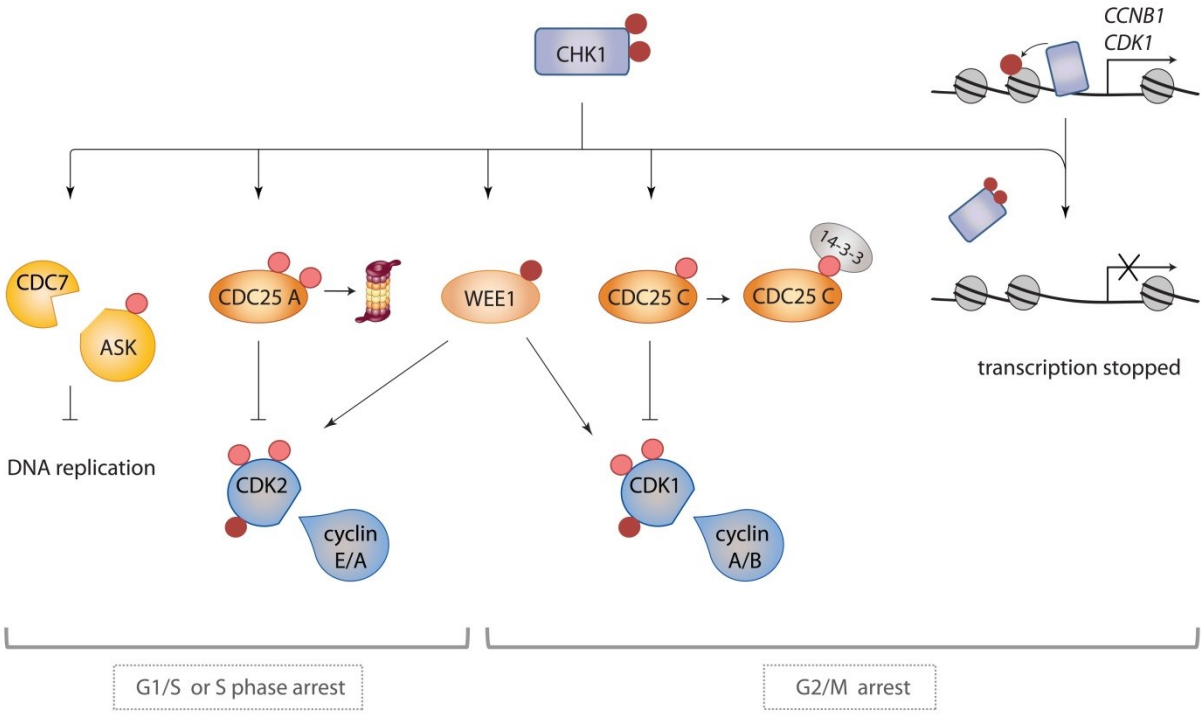

Figure 3.5: CHK1 signaling mediates cell cycle arrest.

Upon activation, CHK1 phosphorylates several downstream effectors involved in cell cycle checkpoint regulation and thereby mediates cell cycle arrest. Phosphorylation of CDC25 phosphatases results in their degradation (in case of CDC25A) or sequestration in the cytosol by binding of 14-3-3 (in case of CDC25C). Hence, cyclin-CDK complexes cannot be established and the cell cycle stops. Phosphorylated WEE1 contributes to CDK inhibition by direct negative phosphorylation of CDK proteins. CHK1 further phosphorylates ASK, which is, in complex with CDC7, an important factor for DNA replication initiation during S phase. In addition, activated CHK1 dissociates from chromatin. The resulting lack of basal histone H3 phosphorylation stops transcription of cell cycle regulatory genes like *CCNB1* (cyclin B1) or *CDK1*. Dark red bullets indicate activating phosphorylation. Light red bullets indicate inhibitory phosphorylation. After [6,67,69,71,73,74].

3.1.3 P53 as a downstream effector of the DNA damage response

The tumor suppressor p53 is strongly engaged in DDR signaling as a downstream effector, whose activity as specific transcription factor is triggered in response to a variety of stresses. Actually, p53 was the first identified substrate of ATM [75-77]. In order to keep its actions under control, p53 is subject to a number of positive and negative regulators. The level of p53

protein in unperturbed cells is kept low mainly by interaction of the tumor suppressor with its E3 ubiquitin ligase mouse double minute 2 (MDM2, in humans also 'HDM2'). This association mediates constant proteasomal degradation of p53 [78,79]. Upon DNA damage, the protein p53 is rapidly stabilized by many PTMs. Phosphorylation in the N-terminal transactivation domain at Ser15 and Ser20 by ATM/ATR and CHK1/CHK2, respectively, reduces the affinity of HDM2 for p53 [75,80-82] (Figure 3.6). In turn, these phosphorylation sites convey increased acetylation of lysines in the C-terminal regulatory domain excluding HDM2 ubiquitination at the same residues [83,84]. Furthermore, HDM2 activity is also directly regulated by ATM/CHK1/CHK2-dependent inhibiting phosphorylation at Ser395 [85,86].

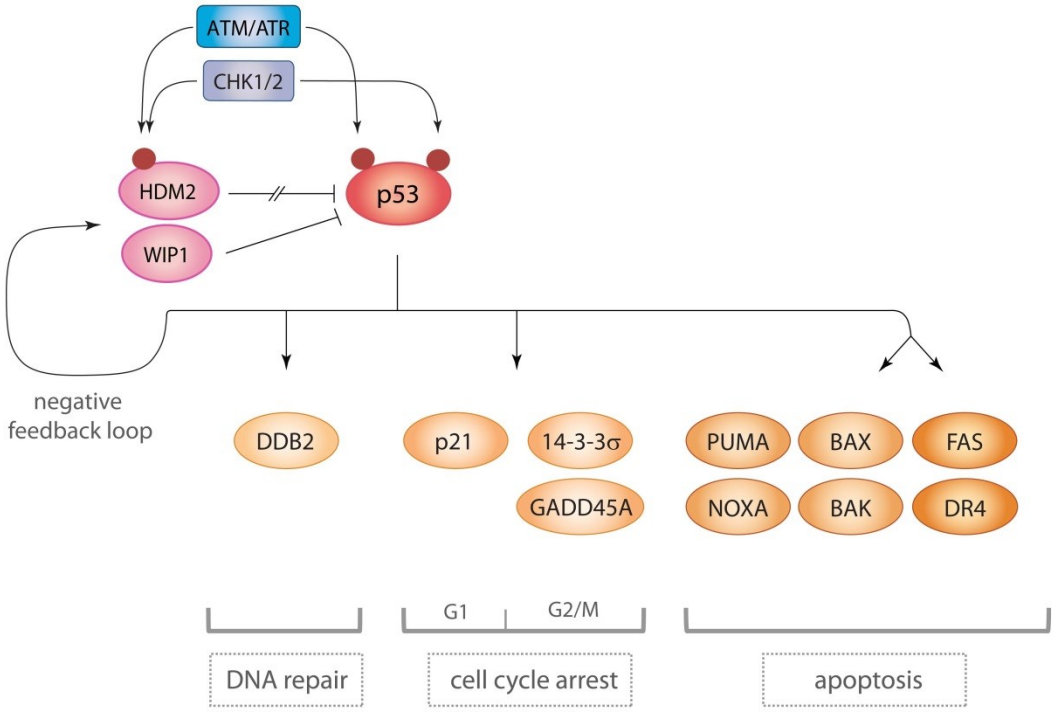

Figure 3.6: The effector p53 participates in the cellular response to DNA damage.

Activated DDR kinases ATM/ATR and CHK1/CHK2 phosphorylate p53 at the residues Ser15 and Ser20, respectively. Furthermore, ATM and CHK1/CHK2 inhibit the p53 negative regulator HDM2 via phosphorylation. Transcriptionally active p53 induces expression of its target genes, including those depicted. They are implicated in DNA repair and cell cycle checkpoint activation (e.g. the CDK inhibitor p21), but also convey induction of apoptosis if damage is too severe. In a disabling feedback loop, p53 expresses its negative regulators HDM2 and WIP1. Red bullets indicate phosphorylation. After [87-89].

Transcriptional activity of p53 originates from homotetramerization and enables the expression of its various target genes. Among those, the cyclin-dependent kinase inhibitor 1 (CDKN1 or p21) is critical to keep up sustained cell cycle arrest in G1 after DNA damage [90]. P21 is a negative regulator of CDK2 activity and prevents the transition of cells from G1 to S phase. The proteins 14-3-3σ and growth arrest and DNA damage 45 A (GADD45A) contribute

to G2/M cell cycle arrest by inhibition of CDC25C [87] (see also Figure 3.4). This allows cells to eliminate damage and p53 in parallel supports repair of DNA through expression of DDB2 [91]. Simultaneous induction of the negative regulators HDM2 and wild-type p53-induced phosphatase 1 (WIP1) tends to (i) prevent escalating and potentially fatal performance of p53 and (ii) to reduce and finally turn off p53 activity if cells recover from stress [92]. In severely damaged cells, p53 initiates the intrinsic pathway of apoptosis by expression of e.g. p53 upregulated modulator of apoptosis (PUMA) and BCL2-associated X protein (BAX). They induce permeabilization of the outer mitochondrial membrane by inhibition of anti-apoptotic proteins like B-cell lymphoma 2 (BCL2) and formation of pore complexes, respectively [93,94]. Also, cytosolic p53 takes action in this process as it can bind to and inactivate BCL2-like anti-apoptotic proteins at the mitochondrial membrane [95]. Subsequent release of cytochrome c from mitochondria is the ultimate trigger of apoptotic cell death. For completion it should be noted that p53 also transactivates genes of extrinsic apoptosis factors (e.g. FAS) [87].

3.2 Removing phosphorylation – the protein phosphatase 2A family

Protein phosphorylation as posttranslational modification is a central regulator of cellular signaling. It occurs on about 30% of all proteins and regulates their stability, enzymatic activity, interaction with other proteins, or subcellular localization [96]. In an enzymatic reaction catalyzed by kinases, a phosphate is mostly transferred to a hydroxyl group of the amino acid residues serine, threonine, or tyrosine (in order of frequency). The task of phosphatases is to hydrolytically remove phosphoryl groups in an opposing reaction, which makes phosphorylation a highly dynamic PTM. Based on their sequence, structure, and catalytic mechanism, phosphatases are classified into three families: protein serine/threonine phosphatases (PSP), protein tyrosine phosphatases (PTP), and aspartate-based protein phosphatases. About 40 PSPs are encoded by the genome and they are further categorized in phospho-protein phosphatases (PPP) and metal-dependent phosphatases (PPM; including WIP1) [97]. This rather small number of phosphatases faces a superiority of over 400 known Ser/Thr kinases [97]. However, PSPs are not promiscuous in choosing their substrates, but convey target specificity via assembly of phosphatase subunits. The class of PP2A phosphatases from the PPP family is a good example of this mechanism [98,99].

PP2A accounts for the majority of dephosphorylation events at serine and threonine residues accomplished by soluble phosphatases [100]. It regulates many biological processes like cell metabolism, G1/S transition, mitotic exit, DNA replication, transcription, translation, and predominantly displays pro-apoptotic function [101,102]. Active forms of PP2A exist as trimeric complexes. The core enzyme is composed of a catalytic subunit C and a structural subunit A. They associate with a regulatory subunit B to build the functional holoenzyme (Figure 3.7). The structural and especially the catalytic subunits are very abundant and ubiquitously expressed. Together, all PP2A subunits account for up to 1% of cellular protein [100]. Subunit A serves as a scaffolding base to bind the C-terminus of the catalytic and to facilitate the dynamic interaction with a regulatory subunit. Whilst subunit A and C have two conserved isoforms each, the structure of B subunits is far more diverse. Consequently, regulatory subunits were classified in four groups - B, B', B'', and B'''. They are encoded by 15 genes, which give rise to at least 20 B-type isoforms due to alternative splicing or translation [103]. Combinatorial assembly of all isoforms yields at least 75 active variants of PP2A phosphatase [102]. Among the three subunits, the regulatory B-type was described to act as a master regulator of PP2A. It is the B subunit that affects phosphatase activity, defines subcellular localization, and mediates target specificity [102,104]. Moreover, some B subunits are specific for certain tissues or distinct developmental stages controlling the temporal and spatial availability of PP2A activity [100]. Also, PTMs play a role in modulating the holoenzyme. The C subunit can be modified by methylation and phosphorylation, which controls catalytic efficiency [105,106]. Phosphorylation of B subunits might additionally regulate PP2A activity [102,107].

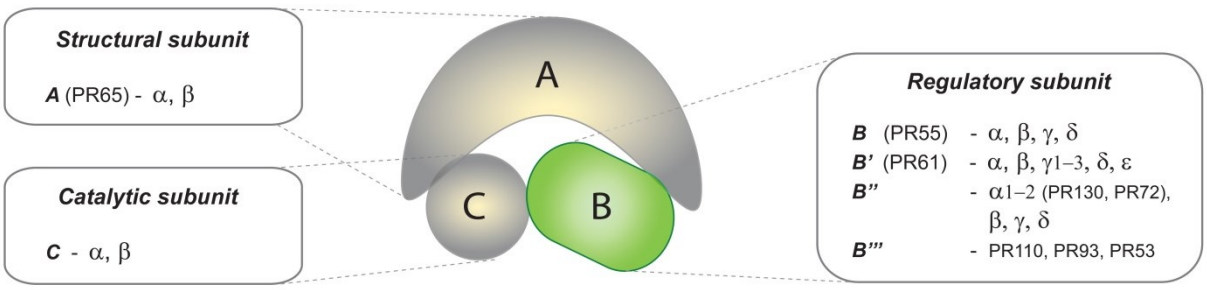

Figure 3.7: Composition of PP2A holoenzymes.

Catalytically active PP2A represents a heterotrimer, consisting of a structural subunit (A), a catalytic subunit (C), and a regulatory subunit (B). B-type subunits can be divided into four unrelated families: B, B', B'', and B'''. Each subunit (A, C) and family (B) comprises several isoforms (e.g. α, β) which are encoded by distinct genes and may give rise to several proteins (e.g. α1, α2). After [100,102].

3.3 Histone deacetylases and their inhibitors

3.3.1 The family of histone deacetylases

Protein acetylation is one of the most abundant posttranslational modifications found in eukaryotic cells. The highly dynamic state of protein acetylation is regulated by the interplay of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the covalent binding of an acetyl group from acetyl-CoA to the ε-amino group of lysine residues, while HDACs are able to remove this modification in an opposing hydrolytic reaction [108] (Figure 3.8 A). Transfer of acetyl groups to the N-terminal tails of core histones, for example, is crucial for chromatin remodeling (Figure 3.9 B). Lysine acetylation lowers the positive charge of histone tails and hence reduces their interaction with the negatively charged DNA backbone. This loss of internucleosomal affinity yields an open chromatin structure and correlates with gene transcription [109]. In contrast, a more condensed conformation of chromatin as caused by HDACs was reported to repress transcription [110]. Next to histones, HATs and HDACs can also modify a continuously growing number of non-histone proteins [111-113]. This clearly extends the role of acetylation beyond altering gene expression. The modification of non-histone substrates may regulate protein stability, activity, interaction, or signaling and thereby affects many pivotal cellular processes [111].

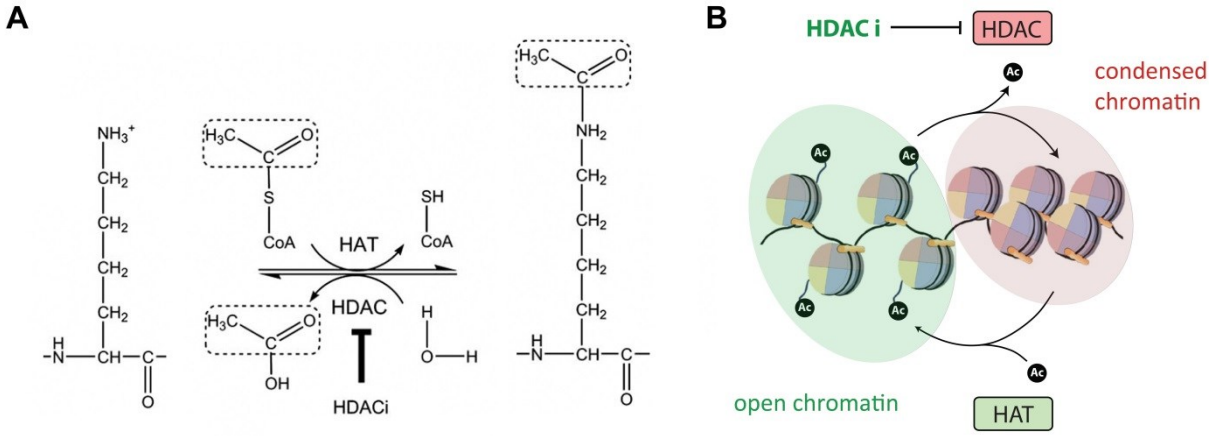

Figure 3.8: The balance of protein acetylation.

(**A**) Histone acetyltransferases (HATs) catalyze the addition of an acetyl group from acetyl-CoA to lysine residues. The opposing reaction is performed by HDACs, which remove acetyl groups by a hydrolytic reaction. Pharmacological inhibitors of HDACs (HDACi) impede deacetylation and hence stabilize protein acetylation. (**B**) N-terminal tails of core histones can be reversibly modified by HATs. Resulting acetylated histones create an open chromatin conformation. Deacetylation by HDACs conversely leads to a tightly packed chromatin state. Ac (black spot) – acetyl-group. Circles indicate histone octamers. Modified from [108] and after [113].

In humans, 18 HDACs have been identified and are grouped into two separate families based on their enzymatic activity and homology to yeast deacetylases. So called 'classical' HDACs

are zinc-dependent enzymes [114] while the second family of sirtuins (SIRT) depends on NAD^+ for catalytic activity [115] (Figure 3.9). Classical HDACs are further divided into three classes. Class I HDACs share sequence homology with the yeast deacetylase Rpd3. Included HDAC1, HDAC2, HDAC3, and HDAC8 enzymes are ubiquitously expressed and mainly localize in the nucleus [116]. The yeast Hda1 orthologues of class II HDACs are grouped into two subclasses, class IIa (HDAC4, HDAC5, HDAC7, HDAC9) and class IIb (HDAC6, HDAC10). These isoforms show tissue-specific expression and are able to shuttle between the cytoplasm and nucleus. Class IIb HDACs though predominantly localize in the cytoplasm [117]. Since HDAC11 shares sequence homology with both, Rpd3 and Hda1 yeast deacetylases, it has been grouped into a distinct class IV [118]. Finally, sirtuins represent class III of human HDACs. The seven members (SIRT1-SIRT7) show homology to the yeast silent information regulator 2 (Sir2) [119].

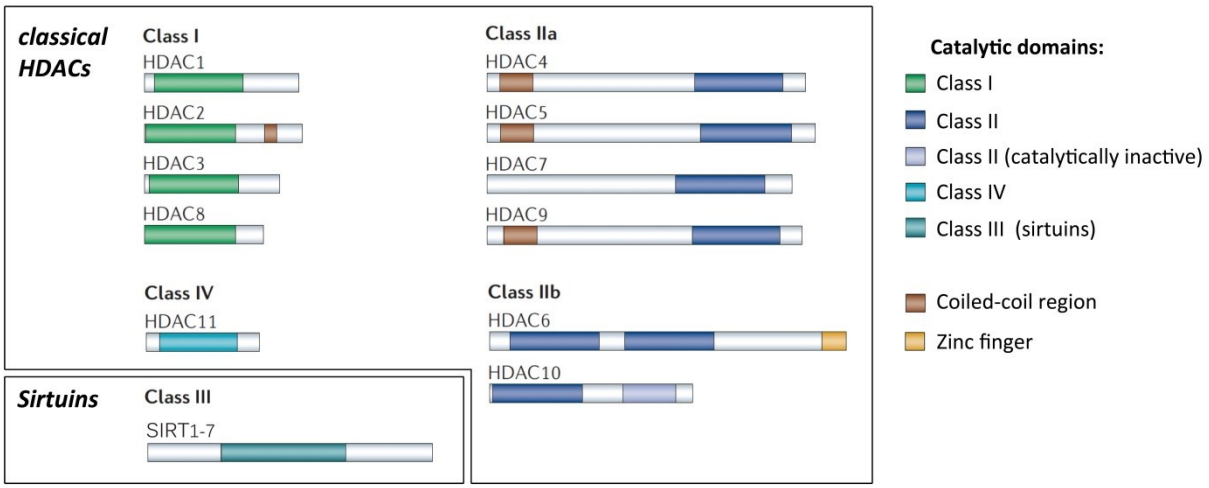

Figure 3.9: The histone deacetylase superfamily.

The human histone deacetylase (HDAC) family is divided into four classes based on their sequence homology to yeast proteins. HDACs class I, IIa/b and IV are Zn^{2+}-dependent enzymes and are also referred to as "classical" HDACs. The NAD^+-dependent class III of sirtuins includes seven members. Modified from [120].

3.3.2 Histone deacetylases 1, 2, and 3 in focus

Aberrant expression of individual HDACs has been found in a wide range of human cancers and expanded the 'classical' causes of carcinogenesis as gene mutations or deletions by epigenetic alterations. It was shown that modifications of the histone acetylation pattern alter the cellular gene expression profile as they influence chromatin conformation and affect the accessibility of transcription factors and other effector proteins to DNA [121,122]. For example, HDAC1 overexpression was demonstrated in gastric, prostate, breast, and colon cancer; HDAC2 is overexpressed in gastric and colorectal cancer, while increased levels of

HDAC3 are frequently observed in chronic lymphoblastic leukemia (CLL), ovarian, or colorectal cancer [113,120,123]. In many cases, the elevated amount of HDAC1, HDAC2, or HDAC3 in tumor tissue correlates with a poor patient prognosis and lower survival rates [123,124].

All three deacetylases are recruited to the transcriptional machinery with specific transcription factors and corepressors. As catalytic subunits, they form the core of multi-protein complexes, which are involved in chromatin modification and mediate transcriptional repression. HDAC1-HDAC2 heterodimers are components of the corepressor of RE1-silencing transcription factor (CoREST), nucleosome remodeling deacetylase (NuRD), and mammalian Sin3 corepressor complexes [116]. Both enzymes are highly related with 85% sequence identity and show redundant functions in cellular regulation [123]. Loss of one HDAC variant can be compensated by the other during neuronal differentiation, cardiac development, and hematopoiesis [125-127]. HDAC1 and HDAC2 further repress expression of the CDK inhibitor p21 and therefore play a pivotal role in cell cycle regulation [128,129]. After exposure of cells to IR, HDAC 1 and HDAC 2 deacetylate histone H3 at lysine 56 (H3K56) at DNA damage sites and thereby promote DNA repair via NHEJ [130]. Nevertheless, both deacetylases must also have specific functions, most likely dependent on developmental stage and tissue type. Knockout experiments in mice revealed that HDAC1 deprivation is embryonically lethal and HDAC2 knockout mice die soon after birth [125,128].

HDAC3 is an essential element of nuclear receptor corepressor (N-CoR) and silencing mediator for retinoic acid/thyroid hormone receptor (SMRT) complexes [131,132]. Several studies suggest an important impact of HDAC3 during mitosis and S phase. It was shown that HDAC3 complexes are localized with mitotic spindles and regulate sister chromatid adhesion as well as kinetochore-microtubule attachment [133,134]. HDAC3 seems to deacetylate the N-terminal tail of histone H3, which in turn guides the mitotic kinase Aurora B to phosphorylate H3 on serine 10 and drives progression through mitosis [135]. Moreover, conditional depletion of HDAC3 in mouse embryonic fibroblasts (MEFs) causes cell cycle delay in S phase and cells develop DSBs due to impaired DNA repair. This was also associated with reduced chromatin compaction, heterochromatin content, and apoptotic cell death showing that HDAC3 activity is required for cell viability [136,137]. Accordingly, germline deletion of HDAC3 in knockout mice caused early embryonic lethality whereas sole cardiac-specific deletion evoked severe defects in heart function [138].

3.3.3 Histone deacetylase inhibitors and their implication in cancer therapy

When epigenetic changes caused by dysregulation of HDACs were found to correlate with the onset and progression of malignancies, it was a rational strategy to establish histone deacetylase inhibitors (HDACi) as anti-cancer drugs [108]. Nowadays, a large number of naturally derived and synthetic HDACi is known. They are compounds with various structure, specificity, and biological activity even though all of them target classical HDACs [113].

HDACi are classified by their structure into hydroxamic acids, short fatty acids, benzamides and cyclic peptides. Hydroxamic acids like trichistatin A (TSA) or vorinostat (SAHA) are rather unspecific inhibitors affecting all classes of HDACs. Short fatty acid (e.g. valproic acid – VPA) and benzamide compounds (e.g. MS-275) exert their properties more specifically on class I and IIa HDACs or only class I HDACs, respectively. Cyclic peptides like depsipeptide are most selective and target only a subset of class I HDACs [108,113]. All inhibitors share a comparable structure composed of three pharmacophore domains. A metal-binding moiety interacts with the catalytical Zn^{2+} of the active site, whereas a hydrophobic linker and surface recognition domain connect with the active side cavity and its periphery [139]. Application of HDACi shows a multitude of biological effects, which origin from their ability to promote acetylation of histone and non-histone protein. This alters chromatin structure and affects expression of a variety of genes, in which about half of the genes are upregulated and the second half is suppressed [140]. On the biological level, HDACi can induce DNA damage, ROS, cell cycle arrest, differentiation, and apoptosis and reduce angiogenesis or metastasis [141]. Interestingly, they possess high tumor cell-directed toxicity whereas normal cells are relatively resistant to HDACi [142]. Both were good prerequisites for their use in clinic (Table 3.10).

Table 3.10: HDACi in clinical trials (partial list). [143]

Hydroxamic acids	Vorinostat (SAHA) Panobinostat (LBH589) Belinostat AR-42 Pracinostat (SB-939)
Short fatty acids	Valproic acid (VPA)
Benzamides	Entinostat (MS-275, SNDX-275) MGCD0103
Cyclic peptides	Romidepsin (Depsipeptide)

Many clinical studies demonstrated the potential of HDACi as efficient monotherapy for hematological malignancies and solid tumors [120,144]. However, results often indicated a more favorable outcome of HDACi treatment in combination regimens with other antitumor agents or radiotherapy [145]. So far, the inhibitors vorinostat (SAHA) and depsipeptide were approved by the Food and Drug Administration (FDA) for treatment of refractory cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) in the USA [146,147]. Many trials with several other HDACi are still ongoing [113]. One of the inhibitors in clinical development is the benzamidine MS-275 (also called Entinostat or SDNX-275), which specifically inhibits HDAC1, HDAC2, and HDAC3 [148]. It is currently applied in Phase 1 to Phase 3 studies. A benefit of MS-275 in co-treatment with aromatase inhibitors is tested in patients with recurrent or refractory breast cancer. Also, combination studies are conducted with tyrosine kinase inhibitors in non-small cell lung cancer (NSCLC) and breast cancer patients as well as with the nucleoside analogue 5-Azacytidine in patients with metastatic colorectal cancer [149].

4 AIMS OF THE WORK

Chemotherapeutic drugs are a standardized regimen in the treatment of cancer. In order to increase the efficacy of the treatment and to reduce the likelihood for the development of resistances, these compounds are often administered in combination therapies. For example, several pharmacological inhibitors for histone deacetylases (HDACi) were shown to sensitize cancer cells for the DNA-damaging, cytotoxic effects of chemotherapeutics and to act highly tumor cell-specific.

Histone deacetylases (HDACs) are key modulators of gene expression and intracellular signaling pathways as they target histones and non-histone proteins. However, it was largely unknown how the reduction of their catalytic activity by HDACi might regulate the cellular response to DNA stress. Among others, a previous study revealed that HDAC1 and HDAC2 are involved in the DNA damage response (DDR) by promoting double-strand break repair. These studies mostly concentrated on the relevance of HDACs in the context of DNA double-strand lesions. Whether and to what extent HDACi affected the S phase stress response upon drugs blocking DNA replication remained unclear.

The aim of the present study was to investigate the consequences of HDAC inhibition for the replication stress-induced DDR. In the beginning, the potential impact of the HDAC class I-specific inhibitor MS-275 on DDR signaling via ATR and ATM should be examined. The resulting extent of DNA damage and apoptosis as well as the implementation of cell cycle arrest should be additionally monitored as readouts for the final cellular outcome. If the results suggested that MS-275 modulated the DDR following perturbed DNA synthesis, the putative roles of HDAC1, HDAC2, and HDAC3 should be addressed by specific siRNA-mediated knockdown. Furthermore, the molecular mechanism underlying modulation of the replication stress response via increased intracellular acetylation by HDACi should be elucidated.

5 MATERIALS

5.1 Chemicals

Chemical	company
Albumin Fraction V (BSA)	Sigma-Aldrich
Antipain	Roth
Ammonium persulfate (APS)	AppliChem
Aprotinin	Roth
Benzamidine	Sigma-Aldrich
Bromophenol blue	Serva
Calcium chloride ($CaCl_2$)	Roth
Chloroform	Roth
Diethylpyrocarbonate (DEPC)	Roth
Dimethyl sulfoxide (DMSO)	Roth
Dithiothreitol (DTT)	Roth
ECL (Super Signal® West Pico Chemiluminescent Substrate)	Pierce (Thermo Scientific)
ECL (Immobilon Western Chemiluminescent HRP Substrate)	Millipore
Ethylenediaminetetraacetic acid (EDTA)	Roth
Ethanol (MEK denatured)	Mineralöle Albert
Ethanol (≥ 99.5%)	Roth
Gentamicin	PAA (GE Healthcare)
Glycerol	Roth
β-Glycerophosphate	Calbiochem
Glycine	Roth
Hydrogen chloride (HCl)	Roth
HEPES	Roth
Isopropyl alcohol	Roth
Leupeptin	Roth
Lipofectamine® 2000	Life Technologies
β-Mercaptoethanol	Serva
Nonidet P-40 (IGEPAL® CA-360)	Sigma-Aldrich
Okadaic acid (OA)	Abcam
Paraformaldehyde	Sigma-Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Roth
Potassium chloride (KCl)	Roth
Potassium dihydrogen phosphate (KH_2PO_4)	Riedel-DeHaën
Propidium iodide	Sigma-Aldrich
Protein A Sepharose™ CL-4B	Amersham (GE Healthcare)
Protein G Sepharose™ 4 Fast Flow	Amersham (GE Healthcare)
Roti®-Nanoquant 5x	Roth
Rotiphorese® Gel 30	Roth

Skimmed milk powder	Saliter
Sodium azide (NaN_3)	Sigma-Aldrich
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Roth
Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O)	Roth
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich
TEMED	Roth
Tris	Roth
Triton™ X-100	Sigma-Aldrich
Tween® 20	Roth

5.2 Enzymes, proteins and inhibitors

Enzyme/protein/inhibitor	company
Accutase	PAA (GE Healthcare)
Annexin V FITC-conjugated	Immunotools
Cantharidin	Sigma-Aldrich
ETP-46464	from Z-Q Wang (FLI, Jena)
5-Fluorouracil (5-FU)	Enzo Life Sciences
Hydroxyurea (HU)	Sigma-Aldrich
KU-55933	Selleck Chemicals
MS-275 (Entinostat)	Selleck Chemicals
RNase A	Serva
Trypsin-EDTA 1x	PAA (GE-Healthcare)
UCN-01	from Z-Q Wang (FLI, Jena)

5.3 Antibodies

Primary antibody	host	company	order number	dilution
anti Actin	rabbit	Sigma-Aldrich	A2066	1 : 10,000
anti ATM	rabbit	abcam	ab32420	1 : 1,000
anti ATM phospho-Ser1981	rabbit	abcam	ab81292	1 : 1,000
anti CHK1	mouse	Santa Cruz	sc-8408	1 : 2,000
anti CHK1 phospho-Ser317	rabbit	Bethyl	A300-163A	1 : 1,000
anti CHK2	rabbit	Santa Cruz	sc-9064	1 : 1,000
anti CHK2 phospho-Thr68	rabbit	abcam	ab32148	1 : 1,000
anti CHK2 phospho-Thr68	rabbit	Acris antibodies	AP01556PU-N	1 : 500
anti H2A.X phospho-Ser139	mouse	Millipore	05-636-K	1 : 5,000

anti HDAC1	mouse	abcam	ab46985	1 : 1,000
anti HDAC2	rabbit	Santa Cruz	sc-7899	1 : 5,000
anti HDAC3	rabbit	abcam	ab16047	1 : 1,000
anti HDM2	mouse	Santa Cruz	sc-56154	1 : 5,000
anti HSP90	mouse	Santa Cruz	sc-13119	1 : 400,000
anti mSIN3A	rabbit	Santa Cruz	sc-994	1 : 20,000
anti p21	mouse	Santa Cruz	sc-6246	1 : 2,000
anti p53	mouse	Calbiochem	OP03	1 : 1,000
anti p53	mouse	Santa Cruz	sc-81168	1 : 50,000
anti p53 phospho-Ser15	rabbit	Cell Signaling	9284	1 : 5,000
anti p53 phospho-Ser20	rabbit	Cell Signaling	9287	1 : 2,000
anti p53 phospho-Ser46	rabbit	Cell Signaling	2521	1 : 1,000
anti PARP-1	mouse	BD Pharmingen	556362	1 : 1,000
anti PARP-1 (cleaved)	mouse	BD Pharmingen	552596	1 : 5,000
anti PPM1D (WIP1)	rabbit	Bethyl	A300-664A	1 : 5,000
anti PPP2R3A (PR72/PR130)	rabbit	Novus	NBP1-87233	1 : 5,000
anti α-Tubulin	mouse	Sigma-Aldrich	T5168	1 : 50,000
anti Vinculin	mouse	Biozol	BZL-03106	1 : 5,000
normal rabbit IgG (pre-immune)		Santa Cruz	sc-2027	for IP
Secondary antibody				
anti mouse IgG HRP-conjugated	goat	Pierce	32430	1 : 5,000
anti rabbit IgG HRP-conjugated	goat	Pierce	32460	1 : 5,000
anti mouse IgG Alexa Fluor® 488	goat	Life	A11001	for IF

5.4 Buffers and solutions

Buffer		conc.	ingredient
Binding buffer	10x	0.1 M	HEPES, pH 7.4
		1.4 M	NaCl
		25 mM	$CaCl_2$
Blocking buffer	1x	5%	Skimmed milk powder in PBS-T
Co-IP lysis buffer	1x	50 mM	Tris-HCl, pH 8
		120 mM	NaCl
		0.5%	Nonidet P-40
Co-IP wash buffer	1x	20 mM	Tris-HCl, pH 8
		900 mM	NaCl
		1 mM	EDTA, pH 8
		0.5%	Nonidet P-40

Co-IP final wash buffer	1x	10 mM	Tris-HCl, pH 8
		100 mM	NaCl
		1 Mm	EDTA
		0.5%	Nonidet P-40
Crosslink solution for Co-IP	1x	1%	Formaldehyde
			in 1x PBS
Crosslink quencher for Co-	1x	1 M	Glycine
			in 1x PBS
Hypotonic buffer	1x	20 mM	HEPES, pH 7.9
		10%	Glycerol
		0.2%	Nonidet P-40
		10 mM	KCl
		1 mM	EDTA
Laemmli sample buffer	6x	350 mM	Tris-HCl, pH 6.8
		4.3 M	β-Mercaptoethanol
		10%	SDS
		33%	Glycerol
NETN	1x	10 mM	Tris-HCl, pH 7.4
		100 mM	NaCl
		1 mM	EDTA
		10%	Glycerol
		0.5%	Nonidet P-40
PBS, pH 7.25	10x	1.37 M	NaCl
		27 mM	KCl
		65 mM	Na_2HPO_4 x 2 H_2O
		14 mM	KH_2PO_4
PBS-T	1x	1x	PBS
		0.05%	Tween® 20
PIC	1x	1 mg/ml	Leupeptin
		2 mg/ml	Antipain
		100 mg/ml	Benzamidin
		10 kUnits/	Aprotinin
Protein A/G sepharose			Protein A/G sepharose (in equal parts)
			NETN (same volume as beads)
		0.1%	BSA
		0.01%	NaN_3
Resolving gel buffer	1x	1 M	Tris-HCl, pH 8.8
Running buffer	10x	250 mM	Tris
		2.5 M	Glycine
		1%	SDS
Strip buffer	1x	62.5 mM	Tris-HCl, pH 6.8
		2%	SDS

		100 mM	β-Mercaptoethanol
Stacking gel buffer	1x	1 M	Tris-HCl, pH 6.8
Towbin buffer, pH 8.9	10x	250 mM	Tris
		1.92 M	Glycine

5.5 Oligonucleotides

5.5.1 Primer for qRT-PCR

gene	orientation	sequence
PPP2R3A	fwd	5´- GCGGTCCAGAAGGATGTTGAGA-3´
	rev	5´- TCCTGGAATTGTGCTTGGGC-3´
GAPDH	fwd	5´-TGCACCACCAACTGCTTAGC-3´
	rev	5´- GGCATGGACTGTGGTCATGAG-3´
HMBS	fwd	5´-GGCAATGCGGCTGCAA-3´
	rev	5´- GGGTACCCACGCGAATCAC-3´

5.5.2 Small interfering RNAs (siRNAs)

siRNA target	sequence	company
Control	5'-AGGUAGUGUAAUCGCCUUGTT-3	Eurofins MWG Operon
PR72/PR130	5´-GAAAGUUGCUGAAUAACCTT-3´	Life technologies (s10975)
HDAC1	5´-CUAUGGUCUCUACCGAAAATT-3´	Life technologies (4390824)
HDAC2		Santa Cruz (sc-29346)
HDAC3	5´-GGAGCUUCCCUAUAGUGAATT-3´	Life technologies (s16878)

5.6 Kits and marker

PageRuler™ Plus Prestained Protein Ladder	Thermo Scientific
peqGOLD RNAPure™	Peqlab
Power SYBR® Green PCR Master Mix	Applied Biosystems
ProLong® Gold Antifade Reagent	Life technologies
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific

5.7 Medium and serum

DMEM (Dulbecco´s Modified Eagle Medium), high Glucose (4.5 g/l)	PAA, now GE Healthcare
FCS (Fetal Calf Serum)	PAA, now GE Healthcare
Opti-MEM® I	Life technologies

5.8 Cell lines

HCT116	human	colorectal carcinoma cell line (verified by DSMZ)
HCT116 p53 $^{-/-}$	human	p53-negative counterpart of HCT116
RKO	human	colorectal carcinoma cell line
MEF p53 $^{+/+}$	murine	Mouse embryonic fibroblasts, p53 positive; from Z.-Q. Wang (FLI, Jena)
MEF p53 $^{-/-}$	murine	Mouse embryonic fibroblasts, p53 negative; from Z.-Q. Wang (FLI, Jena)

6 METHODS

6.1 Methods of cell culture and whole cell analysis

6.1.1 Cultivation and long-term storage of adherent cells

All eukaryotic cell lines were handled with sterile buffers, media and glass equipment. Any handling, including passaging, seeding, freezing, and thawing was performed under sterile working benches in one separate room. Here, cells were stored in specific incubators ensuring stable conditions with humidified air containing 5% CO_2 and a temperature of 37°C. The used cell lines were all adherent and maintained in DMEM (supplemented with 10% FCS) in cell culture flasks until they reached a critical confluence of nearly 100%. To passage them, old medium was removed, cells were washed with sterile PBS and incubated with Trypsin/EDTA until all cells detached from the bottom of the cell culture flask. Then, they were taken up and carefully resuspended in 5 ml fresh medium. For further cultivation, cell suspension was diluted 1:10 or 1:20 in a new flask or seeded in cell culture dishes at the desired dilution. The cell density per milliliter medium was determined with the help of a Neubauer chamber.

Long-term storage was at -150°C. For this, cells were taken up in freezing medium, consisting of FCS and 10% DMSO. The cell suspension was distributed to cryo vials, which were then stored in a freezing box at -80°C. The isopropyl alcohol in the freezing container allowed a slow cooling of the cells over night, after which vials were transferred to -150°C.

6.1.2 Transient transfection

Transient transfection is defined as the temporary incorporation of exogenous DNA or RNA into a eukaryotic cell. In this study, Lipofectamin^{TM}2000 was used as transfection reagent to insert small interfering RNAs (siRNAs) into mammalian cells. The reagents cationic lipids spontaneously interact with the negatively charged backbone of DNA or RNA and form small vesicles. These can bind to and fuse with the cell´s plasma membrane and release the nucleic acid into the cytoplasm.

The day before transfection, cells were seeded in 6 or 12 well plates (Greiner Bio-One) so that confluence would be about 30-40% at the time of transfection. Cells were transfected with 10-40 pmol or 20-60 pmol siRNA in 12 and 6 well plates, respectively. For transfection,

the appropriate amount of Lipofectamin™2000 was incubated in 100 µl Opti-MEM® I reduced serum medium for 5 min. In the meantime, siRNAs were diluted in 100 µl Opti-MEM® I as well. Both dilutions were united and further incubated for 10 min. Afterwards, 200 µl of siRNA-lipid complexes were added right into a well´s growth medium. Transfection reagent remained on the cells for up to 48 h without changing the medium.Flow cytometry

Flow cytometry is a biophysical method to characterize cell populations in high speed on single cell level. For this, a constant stream of fluid with suspended cells runs through the capillary system of the FACS (Fluorescence activated cell sorter) and passes a laser. Thereby, the incident laser beam is refracted and scattered by the surface and nature of the passing cell, which directly correlates with cell size (forward light scatter) and granularity (side light scatter). Additional fluorescence labeling with antibodies or dyes allows investigation of further characteristics (see below).

Quantification of apoptosis

One of the early events of apoptosis is the loss of membrane asymmetry, which leads to the exposure of phosphatidylserine on the outer layer of the plasma membrane. Annexin V, a natural anticoagulant, binds phosphatidylserine with high affinity in a calcium-dependent manner. If conjugated with a fluorophore like Fluorescein isothiocyanate (FITC), it can be used to detect apoptotic cells by flow cytometry.

Cells were seeded in 6 well plates and treated 24 h later as required for the experiment. The medium was collected; cells were washed with PBS, and detached from the dish with 300 µl Accutase. After incubation for about 5 min at room temperature (RT), cell suspension was taken up in 1 ml PBS and transferred to the medium. They were centrifuged at 700xg for 5 min at RT and the supernatant was aspirated. The cell pellet was washed once with PBS and centrifuged as described. In the end, each sample was resuspended in 800 µl 1x binding buffer and 5 µl Annexin V-FITC were added. The following incubation for 15 min was carried out at RT and in the dark. Sampled were measured using the BD FACSCanto™ with the 488 nm excitation laser and BD FACSDiva Software (both BD Biosciences).

Cell cycle analysis

The DNA content of a cell can be determined by staining with propidium iodide (PI). PI is an intercalating agent, which can be excited to fluoresce in its DNA or RNA bound form. The fluorescence intensity of a cell correlates linearly with its DNA amount. The DNA content,

however, varies during the cell cycle. While cells in G_1 (gap 1) show a haploid set of chromosomes, G_2 (gap 2) and mitotic cells contain diploid genomes. Therefore, comparison of the fluorescence signals within a cell population allows the assignment of cell cycle stages.

For harvesting, the medium was collected. Cells were washed with PBS, and detached from the culture dish with 300 µl Accutase. After incubation for about 5 min at RT, cell suspension was taken up in 1 ml PBS and transferred to the medium. They were centrifuged at 700xg for 5 min at RT and the supernatant was aspirated. The cell pellet was washed with 1 ml PBS and centrifuged as described above. For fixation and permeabilization the remaining cell pellet was resuspended in 1 ml of ice-cold 70% Ethanol (EtOH) in a dropwise manner while vortexing. Cells were then stored at 4°C at least overnight. For final preparation, the suspension was centrifuged at 700xg for 5 min and EtOH was aspirated. The cell pellet was washed twice with 1 ml cold PBS and each centrifuged. After the last washing step, cells were again resuspended in 1 ml PBS with the addition of 5 µl PI (final concentration: 12.5 µg/ml) and 10 µl RNase A (final concentration: 100 µg/ml). To remove all double-stranded (ds) RNA by RNase A, samples were incubated at 37°C for 30 min in the dark. Sampled were measured using the BD FACSCanto™ with the 488 nm excitation laser and BD FACSDiva Software (both BD Biosciences). Evaluation of data was also carried out with FlowJo 7.6.5 (TreeStar Inc.).

6.1.4 Fluorescence microscopy

HCT116 cells were grown on sterile coverslips in a 6 well plate. At the end of the treatment, medium was vacuumed and cells were fixed in 4% formaldehyde in PBS for 10 min. After washing they were stored at 4°C in PBS or subsequently permeabilized with 0.25% TritonX-100 for 3 min at RT. After washing three times with PBS the coverslips were incubated with 25 µl of primary antibody dilution (γH2AX 1: 1,000) for 1 h in the dark. Then, coverslips were rinsed three times for 5 min each with PBS, covered with secondary anti-mouse IgG AlexaFluor®488 and stored in the dark at RT for 1 h followed by washing with PBS. All remaining PBS was removed and the coverslips were fixed on microscope slides with liquid mountant ProLong® Gold Antifade Reagent containing DAPI. Cell staining was analyzed with the confocal laser scanning microscope LSM710 (Carl Zeiss).

6.2 Methods of mRNA analysis

6.2.1 Isolation of RNA

Total cellular RNA was isolated by Trizol extraction with peqGOLD RNAPure™ according to the manufacturer´s protocol. The precipitated and washed RNA was dissolved in 50 µl RNase-free DEPC-H_2O.

6.2.2 Determination of concentration and purity for isolated RNA

The amount and quality of the isolated RNA was analyzed with the help of a NanoDrop 1000 spectrophotometer (Thermo Scientific) at a wavelength of 260 nm. One unit of OD_{260} is equivalent to a RNA concentration of 40 ng/µl. To assess the purity of the RNA, ratios of OD_{260}/OD_{230} and OD_{260}/OD_{280} were calculated. The samples were considered as not contaminated with proteins and phenol, if the ratios were $OD_{260}/OD_{280} \geq 1.8$ and $OD_{260}/OD_{230} \geq 2.2$.

6.2.3 Reverse transcription

A total of 2 µg RNA was transcribed to cDNA by means of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), using random hexamer primers. Therefore, 2 µg of total RNA and 1 µl of random hexamer primer were filled up with DEPC-H_2O to a final volume of 11 µl and incubated at 70°C for 5 min. Then, 4 µl 5x reaction buffer, 1 µl RiboLock™ RNase Inhibitor, 2 µl dNTP Mix (10 mM) and , finally, 1 µl RevertAid Reverse transcriptase (M-MuLV RT) were added. After incubation at 25°C for 5 min the reverse transcription (RT) was carried out at 42°C for 60 min and terminated at 70°C for 10 min.

6.2.4 Quantitative real-time PCR

Quantitative or real-time polymerase chain reaction (qPCR) is a method based on conventional PCR. It is able to amplify specific DNA in a sample and at the same time detect and quantify the PCR products, which are displayed by software in real-time. Specific primers mediate the amplification of desired DNA sequences. One method to detect newly synthesized DNA is using non-specific fluorescent dyes like SYBR Green, which intercalate in dsDNA. If excited, their emitted fluorescence directly correlates with the DNA amount.

Quantitative RT PCR was performed in triplicates with the SYBR® Green Real-Time PCR Master Mix on the StepOnePlus™ Real Time PCR System (both Applied Biosystems). The

mixture consisted of 7.5 µl SYBR® Green RT-PCR Master Mix, 200 nM of each specific forward and revers oligonucleotide primer and 1.5 µl of cDNA. The following program was used: activation of the polymerase for 10 min at 95°C was followed by 40 cycles of cDNA denaturation at 95°C for 15 sec, and annealing and elongation at 60°C for 1 min. In a last step, melting curves were created to define PCR products. Therefore, samples were heated gradually from 55°C to 95°C. A single peak at a specific melting temperature ensured amplification of a specific product.

Obtained data for C_t values were analyzed with the ΔC_q quantification model to determine the relative expression levels. The gene of interest (GOI) was then normalized to two reference genes, *HMBS* and *GAPDH*. Both housekeeping genes were verified as stably expressed genes with the geNorm program.

6.3 Methods of protein analysis

6.3.1 Preparation of whole cell extracts

All steps during the preparation of whole cell extracts were performed on ice. For harvesting, medium was collected and cells were scraped off the plate bottom in 1 ml cold PBS. Cell suspension was transferred to the medium and the tubes were centrifuged at 700xg and 4°C for 3 min. Supernatant was vacuumed and the cells were resuspended in NETN (supplemented with protease and phosphatase inhibitors) with 5 times the volume of the pellet. A complete lysis was achieved by incubation of the mixture for 15 min on ice and subsequent sonification with Branson sonifier (Danbury). Then, tubes were spinned at 14,000 rpm at 4°C for 15 min to remove cell debris. The supernatant was transferred to a fresh tube and stored at -80°C.

6.3.2 Preparation of cytosolic and nuclear extracts

Like in preparation of whole cell lysates, all steps were performed on ice. First, medium was aspirated from 6 well plates and cells were washed and scraped off in 1 ml cold PBS. After centrifugation, the cell pellet was carefully resuspended in about 80 µl of hypotonic buffer. Its hypotonic conditions lead to swelling and bursting of cells. In order to prevent lysis of nuclei, cells were incubated for 5 min at the most and subsequently centrifuged at 14,000 rpm and 4°C for 2 min. The supernatant which represents the cytosolic lysate was transferred to a fresh tube. Remaining nuclei were washed and resuspended in about 80 µl

hypotonic buffer. They were sonicated and nuclear as well as cytosolic lysates were stored at -80°C.

6.3.3 Bradford assay

The photometric determination of protein concentration by Bradford is based on the interaction of Coomassie Brilliant Blue G-250 with charged and hydrophobic amino acid residues of a protein. This interaction leads to a shifted absorbance maximum of the dye, which is measured as absorbance of the solution at 595 nm (A_{595}).

For the assay, lysates were diluted (1:10) and mixed with 1:5-diluted Roti®-Nanoquant in a 96 well plate. After incubation for 5 min at RT, absorbance at 595 nm was measured with the plate reader FLUOstar Optima (BMG labtech). Simultaneously, a concentration series of bovine serum albumin (BSA: 25; 50; 100; 250; 500; 1,000 µg/ml) was measured to obtain a standard curve.

6.3.4 Immunoprecipitation

The method of immunoprecipiation is used to isolate and concentrate a protein of interest out of a cell lysate and, in extension, to study protein-protein interactions. Since some interactions are very transient, like those of a phospho-protein and its phosphatase, the contact needs to be stabilized by crosslinking for detection.

For this, medium was removed from 10 cm culture dishes and cells were washed once with PBS. Then, cells were covered with crosslink solution and incubated for no longer than 10 min at RT. Crosslinking was stopped by adding a quencher solution of 1 M glycine for 5 min. Cells were washed with PBS and lysed in 1 ml Co-IP lysis buffer for 30 min on ice. Per sample, the lysates of two 10 cm dishes were united, sonicated for 5 seconds at 20% amplitude and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into a new tube. This lysate was used for Co-IP and for input control.

For Immunoprecipitation, at least 500 µl of lysate were incubated with 0.6 µg of primary antibody (pATM, rabbit monoclonal ab81292). The same volume of lysate was incubated with 0.6 µg IgG from rabbit (pre-immune serum) or no antibody as negative controls. All mixtures were rocked at 4°C for 1-2 h. After addition of 50 µl protein A/G sepharose beads, rotation was continued overnight. While protein A and G bind F_c regions of mammalian antibodies with high specificity, the sepharose beads allow easy sedimentation and thereby

purification of antibody-protein complexes. The next day, beads were washed five times with Co-IP wash buffer and once with Co-IP final wash buffer. The beads were pelleted by centrifugation at 14,000 rpm for 1 min. After the last washing step, remaining buffer was removed with an insulin syringe and 60 µl of 2x Laemmli buffer were added. Samples were heated (95°C, 5 min) and directly loaded to SDS gels.

6.3.5 SDS-PAGE by Laemmli

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) enables the separation of proteins according to their molecular weight in an electric field. The discontinuous, denaturing gels are composed of a resolving gel and a stacking gel poured on top [150]. Acrylamide concentration of the resolving gel was chosen depending on the molecular weight of the proteins, which should be separated. Gels were casted with the help of the Mini-PROTEAN® 3 System (Bio-Rad) according to the following scheme. Casted gels could be stored for up to three weeks at 4°C wrapped in wet tissue.

(for 3 gels)	**Stacking gel**	**Resolving gel**			
		7%	**8%**	**10%**	**12%**
Rotiphorese® Gel 30	1.64 ml	3.5 ml	4 ml	5 ml	6 ml
Resolving gel buffer	-	5.6 ml	5.6 ml	5.6 ml	5.6 ml
Stacking gel buffer	1.25 ml	-	-	-	-
SDS 20%	50 µl	75 µl	75 µl	75 µl	75 µl
H_2O	6.98 ml	5.74 ml	5.24 ml	4.24 ml	3.24 ml
APS 10%	50 µl	75 µl	75 µl	75 µl	75 µl
TEMED	10 µl	10 µl	10 µl	10 µl	10 µl

Prior to loading, protein samples were mixed with 6x Laemmli sample buffer and heated for 5 min at 95°C for complete denaturation. Then, they were loaded into gel pockets with a microliter syringe. A pre-stained protein marker (PageRuler™ plus prestained protein ladder) was used to estimate molecular weights of separated proteins. SDS-PAGE´s were run in a Mini-PROTEAN® 3 system (Bio-Rad) with 1x running buffer at a constant current of 20 mA per gel and a maximum voltage of 120 V.

6.3.6 Western blot and immunostaining

Gel electrophoresis was followed by western blotting, to electrophoretically transfer the separated proteins from inside the gel onto a PVDF membrane (Roti®-PVDF). The gels were blotted using a tank blot system (Mini-PROTEAN® 3 blotting chamber, Bio-Rad) and SDS-free

Towbin blot buffer. Transfer was performed either for 2 h at 150 mA per gel, or for 18 h overnight at a current of 20 mA per gel. Then, the PVDF membrane was placed in blocking buffer (5% skimmed milk powder in PBS-T) to block residual non-specific binding sites. After 1 h, membranes were incubated in primary antibody solution (diluted in 5% non-fat milk in PBS-T) overnight at 4°C. The next day, they were washed three times in PBS-T and incubated for 1 h with secondary antibody coupled to horseradish peroxidase (HRP; diluted in 2% non-fat milk in PBS-T). Afterwards, membranes were washed again three times for 10 min each in PBS-T and prepared for immunodetection.

Protein-antibody complexes are indirectly detected by a chemiluminescence reaction of the HRP with ECL (enhanced chemiluminescence) solution. The enzyme catalyzes the oxidation of luminol and phenol additionally enhances the emission of light. Therefore, membranes were covered with ECL solution for 1 min (Super Signal® West Pico Chemiluminescent Substrate; Immobilon Western Chemiluminescent HRP Substrate). In a dark chamber they were exposed to X-ray films (Fuji Super RX, Fujifilm; Euromed EC-plus Grünfilm), which were developed using an automatic film processor (Optimax, PROTEC®).

Detection of additional proteins required the removal of bound antibodies from the membrane. For this, membranes were incubated in strip buffer for 15 to 45 min on a role mixer. Afterwards, they were washed in ddH_2O and PBS-T, until the smell of the denaturing agent β-Mercaptoethanol had completely vanished.

6.4 Statistical analysis

Dispersion around the mean of a data set is always given as standard derivation (s.d.). To evaluate the difference of two data sets, and thereby their mean values, two-tailed unpaired student´s t-test was applied. Samples were considered as significantly different if the respective *P*-value was below a threshold of 0.05. The level of significance is represented by the number of added asterisks: * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

7 RESULTS

7.1 Histone deacetylases modulate the hydroxyurea-induced DNA damage response

7.1.1 The histone deacetylase inhibitor MS-275 attenuates replication stress signaling

During replication of the cellular genome in the synthesis phase (S phase) of the cell cycle, single stranded DNA is extremely susceptible to insults. One strategy in cancer therapy is the application of chemotherapeutic substances called anti-metabolites, which specifically interfere with the progression of DNA replication in S phase and generate DNA lesions in order to eliminate cancer cells. The drug hydroxyurea (HU) belongs to this family of anti-metabolites and is commonly used in the treatment of leukemia [151,152] and various other malignant carcinomas [153,154]. As an inhibitor for ribonucleotide reductase (RNR), HU cuts off the supply of deoxyribonucleotides for the synthesis of DNA [155]. Thereby, HU treatment leads to stalled replication forks as well as ruptures in the DNA during S phase and ultimately causes death of damaged cells. Recently, it has been shown that this cytotoxic effect of HU treatment on cancer cells could be further enhanced by co-treatment with histone deacetylase inhibitors (HDACi) [156,157].

The human colon carcinoma cell line HCT116 is a suitable model system for studying the DDR. HCT116 cells possess intact DNA damage-inducible cell cycle checkpoints and accomplish effective DNA repair by homologous recombination (HR) [158-160]. The cells were treated with HU and the HDACi MS-275, either alone or in combination, to decipher single effects of both drugs as well as the impact of joint administration on cellular signaling. Phosphorylation levels of DDR kinases ATM, CHK1, and CHK2 were detected by western blotting as readout for DDR pathway activation (Figure 7.1 A). Treatment with 1 mM HU led to the activation of ATM (phosphorylation at Ser1981), CHK2 (phosphorylation at Thr68), and the ATR target CHK1 (phosphorylation at Ser317) of the DDR signaling cascade. Incubation with 2 µM MS-275 on the other hand did not trigger phosphorylation of these kinases. However, MS-275 had a time-dependent impact on the HU-induced DDR in co-treated cells. After six hours, no differences were obtained in the phosphorylation of ATM, CHK1 and CHK2. A longer treatment period of 24 hours though resulted in diminished to nearly abrogated ATM/CHK2 and ATR/CHK1 signaling. This loss of phosphorylation could not be explained by reduced expression of the respective kinases.

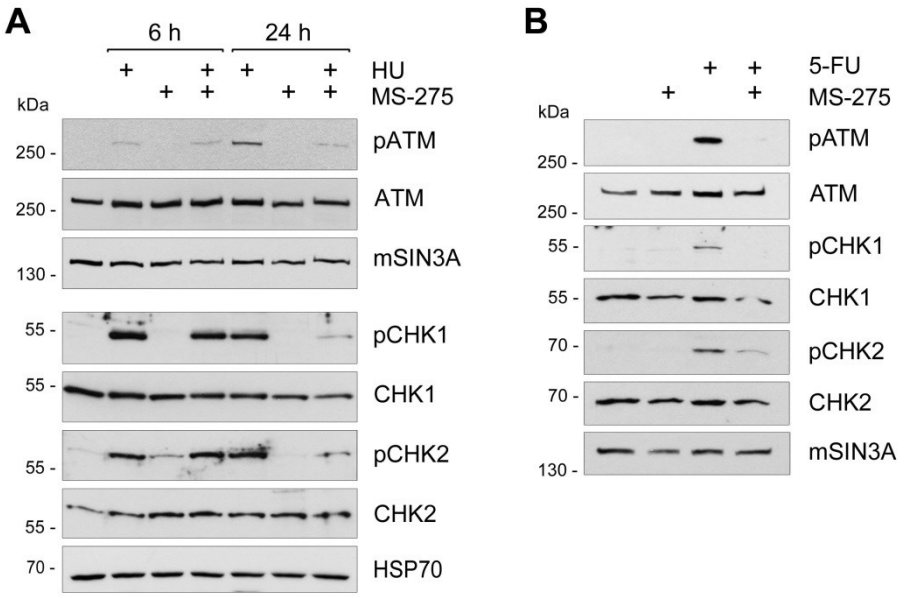

Figure 7.1: DNA damage response signaling after replication stress relies on HDAC activity.

(**A**) HCT116 cells were treated with 1 mM hydroxyurea (HU) and 2 µM MS-275 for 6 and 24 hours, respectively. Whole cell lysates were blotted to two membranes for protein detection. HSP70 and mSIN3A were used as individual loading controls. (**B**) Cells were incubated with 2 µM MS-275 and/or 5 µM 5-FU and harvested after 24 h of treatment. Protein phosphorylation and expression were assessed with specific antibodies by western blot. Experiment (B) was carried out by Maria Schreiber, former Master student of AG Krämer, FSU Jena.

This experiment was repeated with 5-Fluorouracil (5-FU), which is another commonly used anti-metabolite and also applied in the treatment of colorectal cancer [161]. It acts in a similar way like HU by reducing the pool of deoxyribonucleotides. More specifically, it inhibits the enzyme thymidylate synthase and finally depletes the amount of available deoxythymidine triphosphate (dTTP) and other deoxynucelotides [162,163]. Treatment of HCT116 cells with 5-FU resulted in the activation of DDR signaling (Figure 7.1 B). Again, simultaneous application of MS-275 for 24 hours diminished the phosphorylation of apical and checkpoint kinases. These data point to the relevance of HDAC1-3 activity for a sustained DNA damage response in cells suffering from replicative stress.

To further investigate whether this effect relies on p53, the same experimental setup was performed in p53-negative HCT116 and RKO cells, a second p53-positive colorectal carcinoma cell line. In this context, the treatment was also applied to wild-type and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs). All studied cell lines showed reduced HU-induced phosphorylation of ATM when they were exposed to the HDACi at the same time (Figure 7.2). Moreover, this effect was visible independently of the cell´s p53 status (Figure 7.2 A).

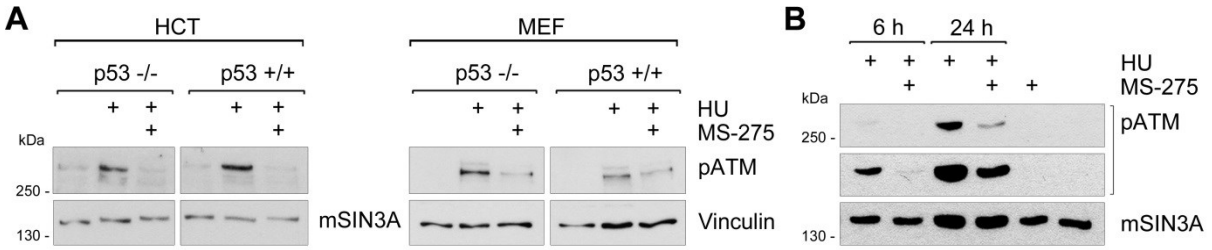

Figure 7.2: Activation of the DNA damage response in presence of MS-275 in several cell lines.

(**A**) HCT116 and MEF cells (each p53 $^{+/+}$ and p53 $^{-/-}$) were incubated with 1 mM HU alone or together with 2 µM MS-275 for 24 h or were left untreated for control. Phosphorylation of ATM was determined by western blot analysis. Vinculin (MEFs) and mSIN3A (HCT´s) served as loading controls. (**B**) RKO cells were incubated with HU and MS-275 for 6 and 24 h. Single MS-275 treatment was carried out only for 24 h. ATM phosphorylation as well as mSIN3A expression were detected by western blot.

7.1.2 DDR downstream activation of p53 and its target genes is impaired by MS-275

The protein p53 acts as an important effector downstream of the DDR signaling pathway [164]. Treatment with HU led to the phosphorylation of the residues serine 15 (Ser15) and serine 20 (Ser20) within the N-terminus of p53 by apical and checkpoint kinases, respectively [75,76,80,81] (Figure 7.3 A). At the same time, application of HU increased the amount of total p53 protein due to the role of these particular phosphorylations for p53 stabilization [165]. Single treatment with MS-275 had no effect on p53 phosphorylation (Figure 7.3 A). If MS-275 was applied simultaneously with HU though, a dramatic decrease in p53 phosphorylation of Ser15 and Ser20 could be detected. However, phosphorylation of the residue serine 46 (Ser46) was increased compared to single HU treatment (Figure 7.3 A). Both kinases reported to target this residue, HIPK2 (homeodomain-interacting protein kinase 2) and DYRK2 (dual specificity Y-phosphorylation-regulated kinase 2) can be activated upon exposure to genotoxic stress [166,167]. Total p53 protein was still increased compared to the control, although the protein was less stabilized than in cells treated with HU alone.

In a next step, the MS-275-related attenuation of p53 activation should be verified by densitometric analysis of western blots. Several blots of Ser15 phosphorylated and total p53 were measured. One representative example is shown in the right part of Figure 7.3 B. The signal intensities for treatment with either MS-275 or HU+MS-275 treatment were determined and related to that of single HU treatment (Figure 7.3 B). A statistical analysis of

the values showed, that MS-275 significantly diminished the HU-induced Ser15 phosphorylation of p53 and the increase in p53 protein itself. Although p53 Ser15 phosphorylation is reduced by about 70%, there is only 40% less p53 protein present in co-treated cells compared to the effects of HU alone.

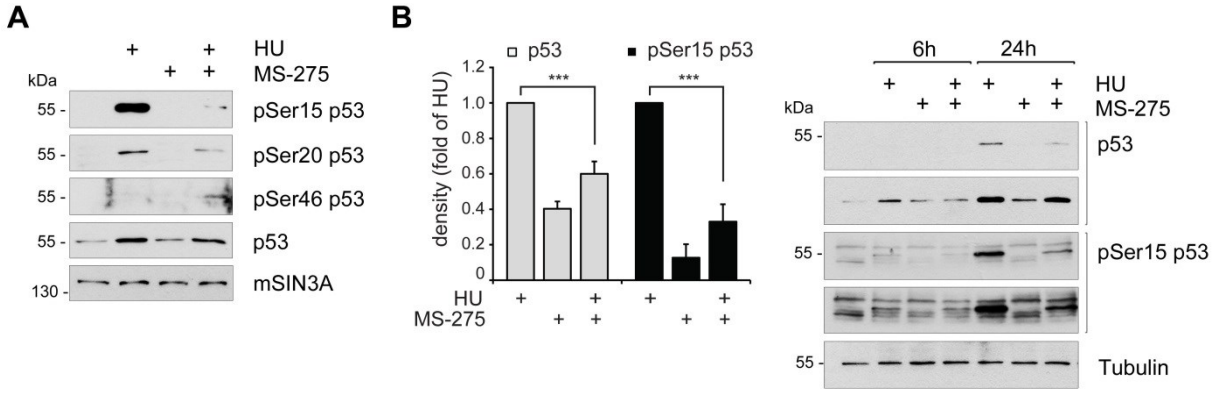

Figure 7.3: Impact of HDAC activity on the activation of p53 after HU treatment.

(**A**) Analysis of three serine phosphorylation sites within the N-terminus of p53, which are phosphorylated after DNA damage. HCT116 cells were treated for 24 h with 1 mM HU and/or 2 µM MS-275. Phosphorylation of the indicated serine residues as well as the expression of total p53 and mSIN3A was analyzed with specific antibodies. (**B**) Left panel: Densitometric evaluation of p53 phosphorylation on serine 15 and total p53 levels (after 24 h treatment) from western blots. For treatment, same concentrations were used as in (A). Data were normalized to the individual loading control being used. The respective amount of (phosphorylated) protein is compared to that of HU-treated cells. Error bars represent the standard derivation (s.d.) of three independent experiments (*** P<0.001). Right panel: Western blots show one representative experiment used for densitometric analysis. α-Tubulin (henceforth referred to only as Tubulin) was used as loading control.

The phosphorylation of p53 is inseparably linked to its function as a transcription factor. S phase stress, such as dNTP reduction caused by HU, triggers the expression of p53 target genes [168,169]. Transcription of the genes encoding for HDM2, p21 (also known as CIP1/WAF1), and WIP1 proteins is positively regulated by p53. While p21 is an important cell cycle regulator [170], both the E3 ubiquitin ligase HDM2 and the phosphatase WIP1 are engaged in a negative feedback loop to downregulate p53 [171,172] (see also Figure 3.6). As shown in Figure 7.4, the expression of these proteins was induced upon HU treatment. However, this expression was sensitive to MS-275. The levels of HMD2 and WIP1 were repressed after a treatment period of 24 hours, if HU was added in combination with the HDACi. In case of p21, MS-275 alone showed a strong induction of the protein. This effect has been described before [173]. Nevertheless, joint application with HU impaired the MS-275-supported p21 expression.

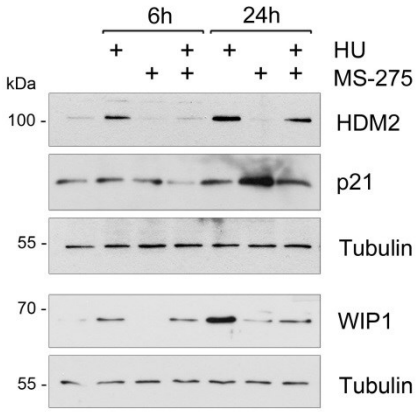

Figure 7.4: Expression of p53 target genes in HCT116 cells.

Cells were incubated with 1 mM HU and/or 2 µM MS-275 for 6 and 24 h. Western blots show detection of the p53 target genes HDM2, p21, and WIP1 from two membranes. In both cases, Tubulin was used as loading control.

7.2 Class I HDACs affect cellular physiology and the sensitivity towards DNA damage

7.2.1 Loss of HDAC activity amplifies hydroxyurea-induced DNA damage

A very rapid event after the occurrence of genomic lesions is phosphorylation of the histone variant H2A.X on serine 139, commonly referred to as γH2A.X [174]. Both, ATR and ATM are among the reported kinases for H2A.X [34,46]. In a cellular context, the formation of γH2A.X foci can be used as a marker for DNA damage [175].

In a first approach, the amount of γH2A.X in cells was analyzed by western blot (Figure 7.5 A). Treatment with HU for 24 hours showed an accumulation of γH2A.X, whereas no phosphorylation of H2A.X could be found in cells incubated with MS-275 alone. If HCT cells were incubated with HU and the HDAC inhibitor together, an even stronger γH2A.X signal was detectable compared to single HU treatment. To further analyze whether the increased amount of γH2A.X was only randomly distributed in the genome or localized in defined foci, confocal fluorescence microscopy was performed. As shown in Figure 7.5 B, control cells incubated with DMSO as solvent control and MS-275 treated cells generated no foci. In contrast, incubation with HU resulted in the formation of γH2A.X. This amount of γH2A.X within the nucleus varied among single cells and a range from less to more damaged cells was visible. When MS-275 was applied together with HU, the same variability occurred.

Additionally, some cells with an extremely high amount of γH2A.X appeared. Single HU treatment did not create cells with such severe damage.

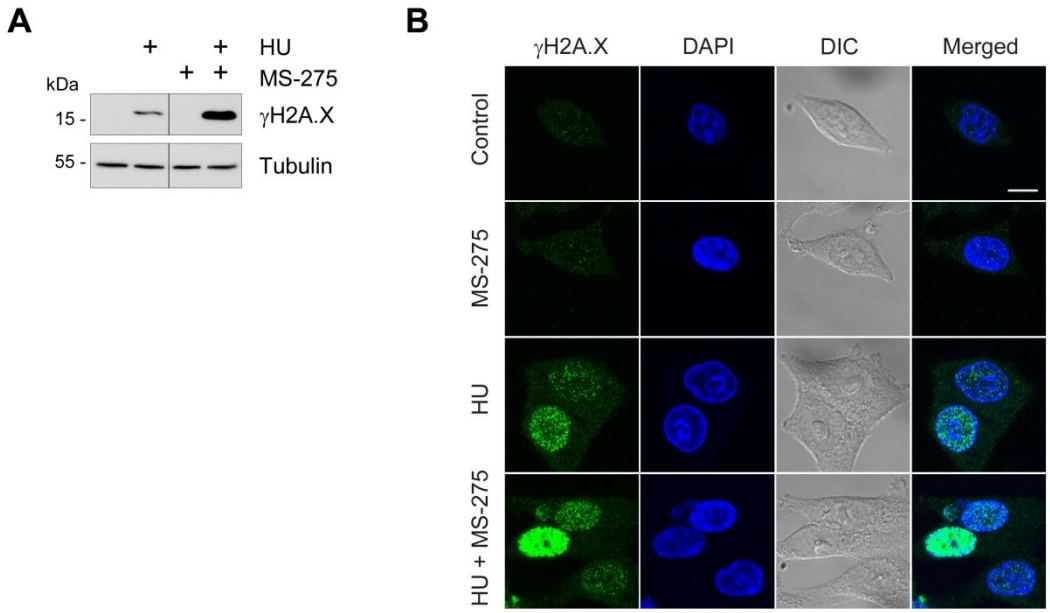

Figure 7.5: Incidence of γH2A.X in HCT116 cells.

(**A**) Cells were treated with 1 mM HU, 2 µM MS-275 or both for 24 h, whereas control cells were incubated only with DMSO. Whole cell lysates were analyzed for H2A.X phosphorylation (γH2A.X) by western blot. Some lines of the blot were cut out, which is denoted by a vertical line. (**B**) Treatment of HCT cells was carried out as in (A). After 24 h, cells were fixed and stained for γH2A.X (green). Corresponding DAPI (blue) and DIC (Differential interference contrast) images are shown, too. Scale bar = 10 µm.

7.2.2 Hydroxyurea-induced S phase arrest is counteracted by MS-275

In order to repair damaged DNA and to prevent new lesions, the cell cycle becomes arrested by a variety of pathways during replication stress [70,176]. Hydroxyurea has been shown to halt the cell cycle in S phase [177,178]. After 24 hours of treatment with HU, cell cycle analysis indeed showed an arrest of cells in S phase and therefore a reduced percentage of cells in G2 phase and mitosis (G2/M) compared to the control (Figure 7.6 A). The HDAC inhibitor MS-275 generated an arrest in G1 with depletion of the S phase (Figure 7.6 A lower part). Combined application of HU and MS-275 resulted in a cell cycle profile that also showed an accumulation of cells in S phase. However, unlike with HU treatment, far more cells have progressed towards and in G2/M. The bar diagram in Figure 7.6 A shows that the relative amount of cells in G2/M after this treatment is nearly equal to that of untreated cells.

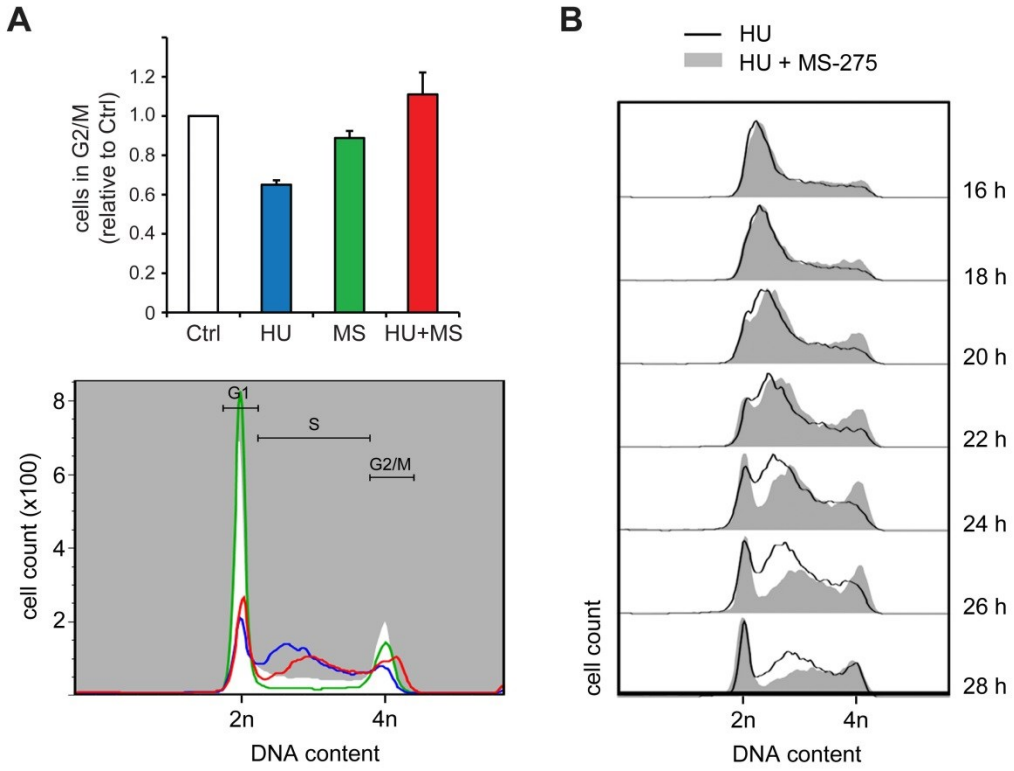

Figure 7.6: MS-275 abbreviates the HU-induced S phase arrest.

(**A**) Upper part: Cells were incubated with DMSO as solvent control, 1 mM HU and/or 2 µM MS-275 for 24 h, and subsequently fixed for PI staining. Cell cycle distribution was analyzed by flow cytometry. Amount of cells in G2/M is depicted in the diagram and is represented as fold of control. Results show the mean of three independent experiments. Error bars indicate s.d. values. Lower part: Cell cycle profile of one representative experiment used for generation of the bar diagram above. Profiles display cell cycle distribution of 10,000 cells each. Color coding for treatments: see upper bar diagram. DNA content 2n = G1 phase; DNA content 4n = G2/M phase; DNA content in between = S phase (**B**) Treatment with 1 mM HU alone or in combination with 2 µM MS-275 was carried out for the indicated time periods (16-28 h). Cell cycle analysis revealed the progression of cells through the cell cycle. Profiles display cell cycle distribution of 10,000 cells each. Image shows one representative out of three independent experiments.

In a next step, cell cycle progression should be investigated in greater detail, to gain more information about the differences between HU and HU+MS-275 treatment on cell replication. HCT116 cells were incubated with the respective drugs for a period of 16 to 28 hours and cell cycle profiles were determined every two hours (Figure 7.6 B). After 16 hours, cell cycle profiles for both treatments were still very similar and displayed an accumulation of cells at the transition from G1 to S phase. From a period of 18 hours, cell cycle progression through S phase remained strongly decelerated in cells incubated with HU. In contrast, cells treated with HU and MS-275 overcame HU-induced S phase arrest much faster. This is demonstrated by a more rapid increase of DNA content during S phase and the increasing amount of cells in G2/M (DNA content 4n) compared to single HU application. Therefore, HDAC inhibition seems to counteract the HU-induced retention of cells in S phase and promotes their progression towards G2 phase and mitosis.

7.2.3 MS-275 increases the cytotoxicity of hydroxyurea

Hydroxyurea as a chemotherapeutic substance induces apoptosis in cancer cells [156,179,180]. Since it has been reported that HDACi are able to increase this effect [156,157], initiation of apoptotic cell death was examined. Initial western blot experiments were performed with an antibody that specifically recognizes the 89kDa cleavage product of poly(ADP-ribose)-polymerase-1 (PARP-1). This fragment results from site-specific cleavage of full-length PARP-1 protein by Caspase 3 [181,182]. The presence of cleaved PARP is considered as a marker of apoptosis [183]. Only long-time exposure to HU for 24 hours resulted in a signal for cleaved PARP protein by western blot analysis (Figure 7.7 A). The same time-period of incubation with MS-275, on the other hand, only showed a very faint band for PARP cleavage. Of note, combined treatment resulted in markedly enhanced cleavage of PARP compared to HU application alone.

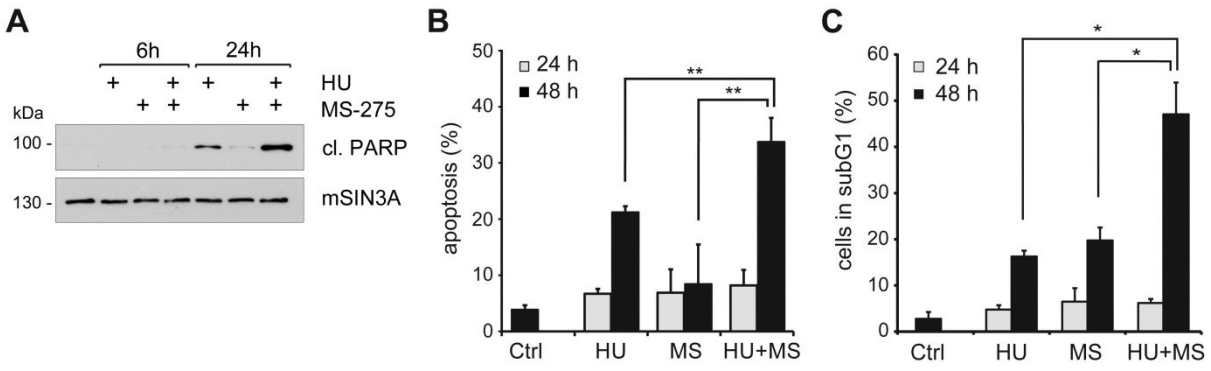

Figure 7.7: MS-275 enhances apoptosis in cells with HU-induced replicative stress.

(**A**) HCT116 cells were treated with 1 mM HU and 2 µM MS-275 for 6 and 24 h. Whole cell lysates were analyzed for PARP-1 cleavage (cl. PARP) and mSIN3A by western blot. (**B**) FACS analysis of HCT116 cells with Annexin V. Bars indicate the amount of apoptotic cells within a sample. Cells remained untreated or were treated with 1 mM HU, 2 µM MS-275 or both for 24 and 48 hours. Error bars represent the s.d. of three independent experiments (** $P<0.01$) (**C**) Fraction of HCT116 cells in subG1 was determined by FACS analysis after PI staining. Cells were treated as in (B). Error bars represent the s.d. of three independent experiments (* $P<0.05$).

Early apoptotic cell death was further quantified by staining cells with Annexin V and subsequent flow cytometry analysis (FACS). Annexin V exclusively binds to phosphatidylserine, which becomes exposed on the cell surface due to loss of membrane asymmetry after induction of apoptosis [184]. After 48 hours of incubation with HU, about 20% of cells had become apoptotic. If HU-stressed cells were co-stimulated with MS-275, the rate of apoptosis significantly increased and approximately doubled (Figure 7.7 B). The same results were obtained with subG1 fractions after cell cycle analysis (Figure 7.7 C). Samples

treated with both agents, HU and MS-275, showed a significantly increased amount of cells in subG1 compared to the single treatment schedules. For single MS-275 treatment nearly the same percentage of subG1 fraction was determined as for HU incubation. Taken together, additional application of the HDACi MS-275 sensitized HCT116 cells for the cytotoxic effect of HU.

7.3 Mimicking the effects of MS-275 through inhibition of individual DDR kinases

7.3.1 Depletion of checkpoint kinase activity during S phase stress

Stalled replication forks, like induced by HU, primarily cause activation of ATR and subsequently of the whole DDR [185]. ATR not only activates its direct downstream target CHK1, but also cross-activates the ATM-CHK2 axis and thereby lays the foundation for a global cellular reaction to stress [9,17]. To decipher the DDR after HU+MS-275 treatment, it was tested how inhibition of individual DDR kinases affects the signaling outcome. First, the putative role of checkpoint kinases CHK1 and CHK2 was evaluated. The inhibitor UCN-01 shows a much higher specificity for CHK1 than for CHK2 and was chosen to abrogate CHK1 activity [186]. In combination with HU, UCN-01 enhanced cleavage of the repair protein PARP-1 after 24 hours (Figure 7.8 A). Cells also showed increased levels of p53 phosphorylation at Ser15 and a little less at Ser20, which consequently stabilized p53 protein. These results do not completely match with the effects of MS-275 on HU-induced DDR signaling and suggested that CHK1 function is not the essential target of MS-275.

The role of CHK2 was examined in CHK2-negative HCT116 cells, which were compared to their wild-type counterparts (Figure 7.8 B). The induction of apoptosis during HU treatment was monitored by PARP cleavage and revealed that induction of apoptotic cell death after 24 hours was much stronger in wild-type cells than in cells lacking CHK2. Also, p53 accumulated to the same extent in both cell lines after application of 1 mM HU, whereas the basal level of p53 protein in $CHK2^{-/-}$ cells was a little higher. Both events are in contrast to the MS-275-dependent increase of apoptosis and downregulation of p53 (Figure 7.3 and 7.7). Taken together, neither CHK1 nor CHK2 depletion shows the same consequences for cells like adding MS-275 during exposure to HU.

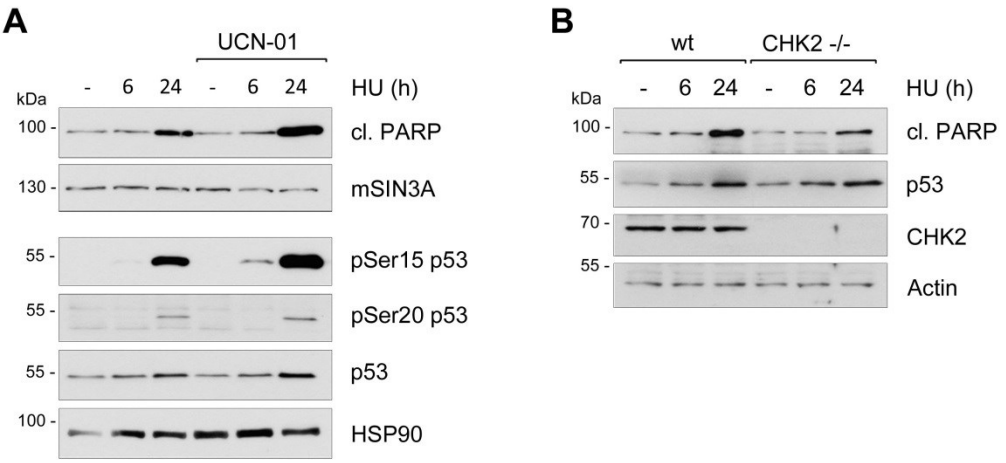

Figure 7.8: Effects of absent checkpoint kinase activity during HU treatment.

(**A**) HCT116 cells were pre-incubated with 100 nM UCN-01 for 1 h and subsequently treated with 1 mM HU for 24 h. The same whole cell lysate was blotted to two membranes for detection of PARP-1 cleavage as well as total p53 and p53 phosphorylation by western blot. mSIN3A and HSP90 were used as independent loading controls. (**B**) HCT116 cells and their comparative $CHK2^{-/-}$ cell line were treated with 1 mM HU for 6 and 24 h. PARP-1 cleavage, p53, CHK2, and Actin were detected.

7.3.2 Selective inhibition of ATR and ATM during S phase stress

On the next step, the apical kinases ATR and ATM were selectively inhibited to investigate whether repression of their catalytic activity during replication stress is determining for the effects observed with MS-275. Inhibition of ATR was achieved by means of the novel and highly ATR-selective compound ETP-46464 [187]. ETP-46464 was administered alone for 24 hours or combined with HU for six and 24 hours to HCT116 cells. The ATR inhibitor efficiently abrogated CHK1 phosphorylation after treatment with HU thus abolished ATR kinase activity for up to 24 hours (Figure 7.9 A). Western blot analysis indicated that basal amounts of cleaved PARP fragment were comparable in control cells cultured with and without ETP-46464. However, HCT116 cells had already triggered PARP cleavage after six hours if ETP-46464 was applied together with HU. Additionally, longer co-administration with the ATR inhibitor for 24 hours generated a much higher level of the apoptotic PARP fragment than single HU application. This was accompanied by a strongly enhanced Ser1981 phosphorylation of ATM at both time points, which did not occur in cells treated with ETP-46464 alone. The ATR inhibitor further on decreased the HU-induced phosphorylation of p53 at Ser15, but increased phospho-Ser46 resulting in an overall lower amount of total p53 protein compared to single exposure to HU.

For depletion of ATM activity, the ATP-competitive inhibitor KU-55933 was employed. KU-55933 is able to inhibit all PIKK family members, but shows the highest specificity for ATM [188]. In the experimental setup, HCT116 cells were exposed to HU and KU-55933 either alone or together for 24 hours. KU-55933 was administered at previously described concentrations [189,190]. When phospho-Ser1981 ATM signals were detected by western blot, addition of the ATM inhibitor to HU did not show suppressed ATM auto-phosphorylation (Figure 7.9 B). However, Ser15 phosphorylation of p53 was impaired and also total p53 protein was less present if KU-55933 was applied together with HU. Rather unexpected, also HU-induced CHK1 activation was diminished by KU-55933. Phosphorylation of CHK2 after replication stress though was intensified if ATM was inhibited, which is contrary to CHK2 as an important downstream target of the ATM kinase. Finally, a stronger signal for apoptotic PARP conversion was observed when HU was jointly applied with KU-55933.

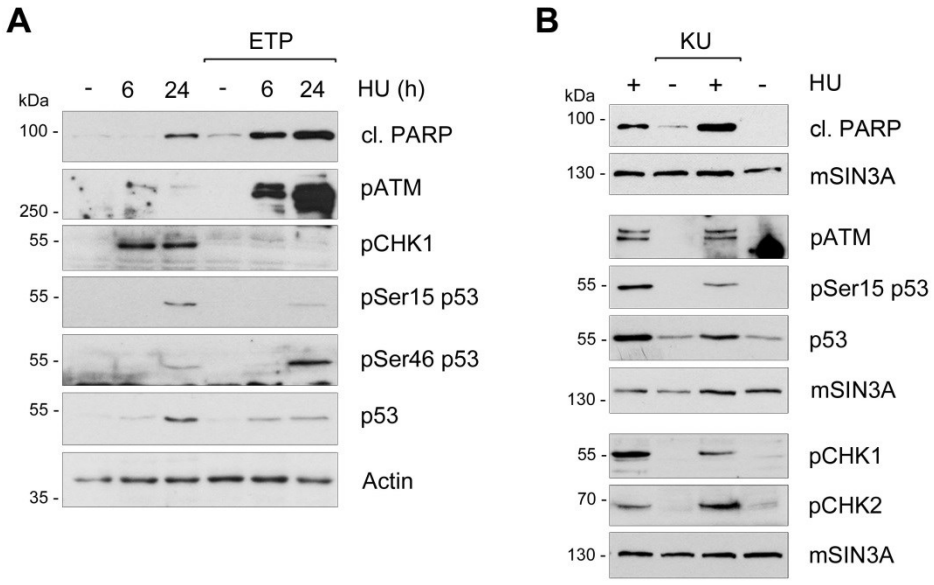

Figure 7.9: Effects of exogenous inhibitors for ATR and ATM during hydroxyurea treatment.

(**A**) HCT116 cells were pre-incubated with the specific ATR inhibitor ETP-46464 (3 µM) for 1 h and subsequently treated with 1 mM HU for additional 6 and 24 h. Control cells incubated with solvent or ETP-46464 alone were harvested after 24 h. Whole cell lysates were analyzed with specific antibodies by western blot. Actin was used as loading control (**C**) Cells were pre-incubated with 10 µM KU-55933 for 1 h and treated with 1 mM HU for additional 24 h. Total and phosphorylated proteins were detected on three different membranes from the same whole cell lysate with mSIN3A as loading control in each case.

The results obtained with both, ATM and ATR kinase inhibitors resemble the effects of MS-275 on HU-induced signaling to a large extent. Since ATR is the initial kinase of replication stress signaling, its role in apoptosis induction and cell cycle control was more closely investigated by PI and Annexin V staining for treatment with HU, ETP-46464, or both

compounds (Figure 7.10 A). Single application of ETP-46464 only marginally increased the relatively low basal level of apoptosis. HU alone evoked apoptosis in only 15% of cells after 48 hours whereas simultaneous loss of ATR activity resulted in a significantly higher rate with 50% Annexin V-positive cells (Figure 7.10 A).

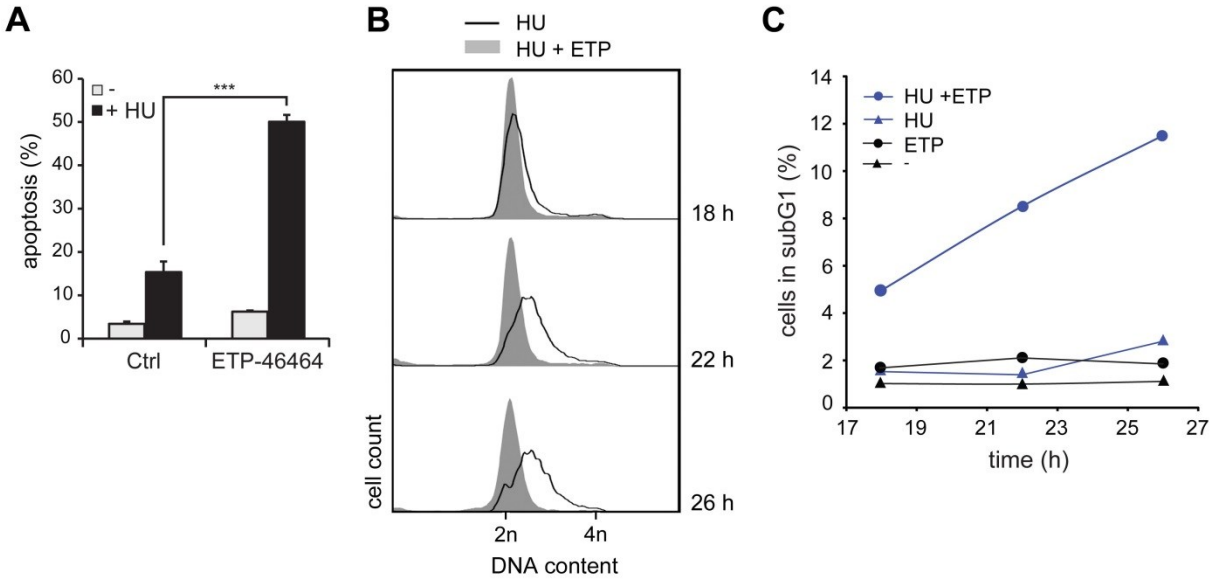

Figure 7.10: Effect of the ATR inhibitor ETP-46464 on apoptosis and cell cycle progression.

(**A**) Apoptotic cell death was determined by staining with Annexin V and subsequent measurement by flow cytometry. 3 µM ETP-46464 (3 µM) or DMSO as solvent control were pre-incubated for 1 h, then 1 mM HU was added for further 48 h. Diagram shows the mean ± s.d. of three independent experiments (*** *P*<0.001). (**B**) Pre-treatment with 3 µM ETP-46464 or DMSO as solvent control for 1 h was followed by incubation with 1 mM HU for the indicated time periods (16-26 h). FACS analysis with PI staining revealed the progression of cells through the cell cycle. Profiles display cell cycle distribution of 10,000 cells each. The shown experiment was only conducted once. (**C**) Diagram depicts the amount of subG1 cells after the indicated treatments. Results were obtained during the experiment shown in (B).

Cell cycle progression of HCT116 cells was analyzed for three time points in a period of 18 to 26 hours. The respective cell cycle profiles for HU treated cells with active (HU) and inhibited ATR kinase (HU + ETP) are shown in Figure 7.10 B. Exposure to HU for 16 hours caused accumulation of cells in G1 and S phase and such cells slowly migrated towards and through S phase in the following eight hours. In contrast, HCT cells with ETP-46464-inactived ATR did not develop this typical S phase arrest in presence of HU. The majority of these cells stayed in G1 phase over the entire treatment period. Yet, with time, an increasing amount of subG1 was detected indicating ongoing cell death (Figure 7.10 C). Incubation with HU or ETP-46464 alone caused amounts of subG1 fraction comparable to control samples and varying from 1 to 3%. When HCT cells were exposed to both substances at the same time, though, the percentage of subG1 considerably increased. About 5% of cells were present in subG1 after

16 hours of co-treatment and this rate further increased to 9% after 22 hours and almost 12% subG1 fraction after 26 hours. The results can be considered as a hint for the time-dependent increase of dead cells during the respective treatments.

Thus, inhibition of ATR kinase activity by ETP-46464 disrupted the usual S phase arrest in cells suffering from replicative stress and in the same context potentiated the induction of apoptosis. Both processes were similarly evoked in HU-treated HCT116 cells by addition of MS-275 (see Figures 7.6 and 7.7).

7.4 A link between HDAC1/2 and PP2A phosphatase activity

7.4.1 Time-dependent inactivation of the DNA damage response by MS-275

At the beginning it was shown that HU-induced phosphorylation of DDR kinases and downstream p53 was diminished or even lost after 24 hours, but not after six hours of co-treatment with MS-275 (Figures 7.1 A, Figure 7.3). This suggested a time-dependent disabling effect of the applied HDACi on replication stress signaling.

Based on this finding, MS-275 pre-treatment for 16 hours was included in a next experiment. After MS-275 incubation overnight, HU was added for six, ten and 14 hours. Whole cell lysates were compared to those with simultaneous HU+MS-275 and single HU application for the same time periods (see treatment regimen in Figure 7.11). Stimulation with HU resulted in a stable activation of ATM, CHK1 and p53 (Figure 7.11). Additional treatment with MS-275, however, strongly decreased the phosphorylation of ATM over time, which was nearly gone after 14 hours. Also, activation of CHK1 and downstream generation of Ser15-phosphorylated p53 was reduced. Strikingly, pre-treatment with MS-275 prior to HU application almost completely blunted activation of the DDR. This is illustrated by the very low levels of phosphorylated ATM and CHK1, which were detected by western blot. Furthermore, stabilizing Ser15 phosphorylation of p53 was strongly inhibited.

These data strongly suggested that MS-275 modifies the cellular gene expression pattern, which could be causal for downregulation of the DDR in cells exposed to HU. Thus, HDAC activity is necessary to maintain replication stress signaling.

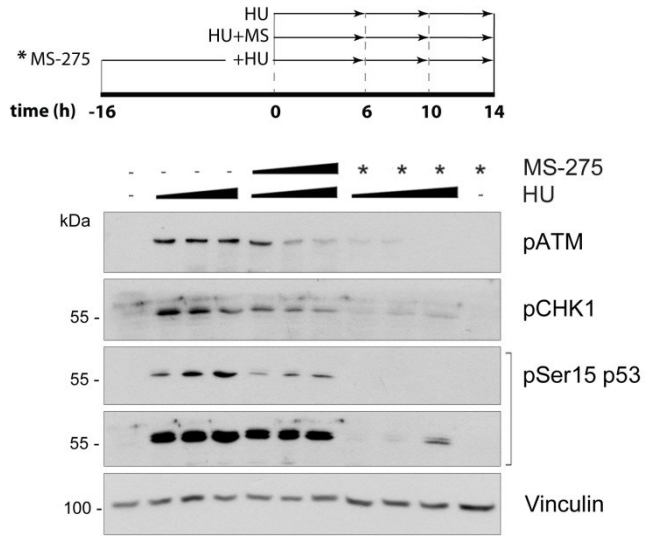

Figure 7.11: Loss of HDAC activity diminishes hydroxyurea-induced DDR signaling in a time-dependent manner.

Upper part illustrates the applied treatment regimen. HCT116 cells were stimulated with 1 mM HU alone or together with 2 µM MS-275. Wedges indicate increasing incubation periods of 6, 10 and 14 h, respectively. Asterisks mark pre-incubation with MS-275 for 16 h. Whole cell lysates were analyzed for phosphorylation of shown proteins by western blot, with Vinculin used as loading control.

7.4.2 HDACs suppress expression of the PP2A regulatory subunit B´´ PR130

HDAC inhibitors are able to activate or repress the transcription of various genes by direct and indirect mechanisms [113,120]. In order to investigate the potential impact of MS-275 on the proteome of HCT116 cells, microarray analysis was performed at the *European Molecular Biology Laboratory* (EMBL, Heidelberg). The dataset obtained was searched for genes whose transcription was altered by the HDACi and which could possibly be involved in S Phase signaling pathways. One of the genes that were identified to be affected by MS-275 was *PPP2R3A*. *PPP2R3A* encodes for specific regulatory subunits of the serine/threonine protein phosphatase 2A (PP2A). Since all of the phosphorylation sites of checkpoint kinases and p53 detected in this study are phosphorylated serine or threonine residues, PP2A was considered as a potential regulator of their phosphorylation level.

In a first approach, the general impact of this phosphatase on DDR kinase phosphorylation was assessed by using specific PP2A inhibitors. One inhibitor with high selectivity for PP2A is Cantharidin [191]. HCT cells were treated with HU alone or in combination with MS-275. After 24 hours, Cantharidin was added in two concentrations for the following four hours. Analysis of whole cell extracts by western blot revealed that Cantharidin had almost no impact on the activity of DDR kinases in control cells (Figure 7.12 A). Induction of the DDR by HU was

shown by the phosphorylation of ATM and CHK1. Additional exposure to Cantharidin further boosted these phosphorylation levels and also enhanced the signal for pCHK2. As shown before, the addition of MS-275 reduced the basal amount of phosphorylated ATM and CHK1 during simultaneous incubation with HU. Intriguingly, Cantharidin was able to reverse this effect within four hours. It counteracted the effect of MS-275 and elevated the phosphorylation of all three shown kinases to levels comparable with those of HU-treated cells.

A second highly specific PP2A inhibitor, Okadaic acid (OA), was applied in a similar experimental setup [192]. Cells were left untreated or were incubated with HU and MS-275, either alone or in combination, for 24 hours. Then, OA was applied only to co-treated cells and incubation was continued for further four hours. Western blots revealed that OA almost completely abrogated the negative effect of MS-275 on DDR signaling (Figure 7.12 B). Phosphorylation of ATM and also CHK1 was restored in presence of the PP2A inhibitor. The total amount of ATM protein remained the same throughout all treatment regimens.

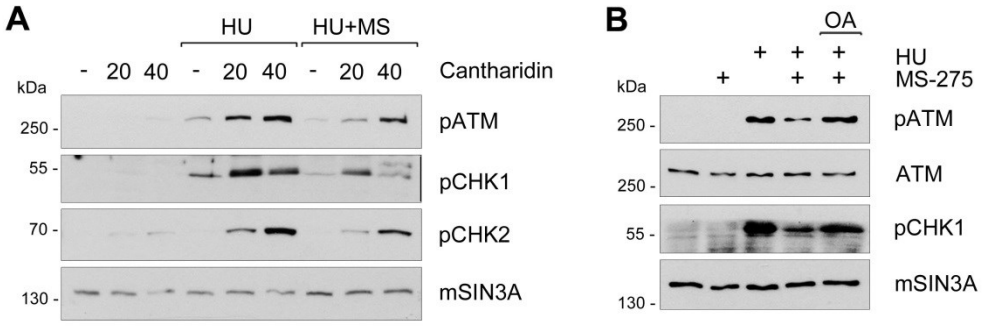

Figure 7.12: Inhibitors of PP2A restore DNA damage and checkpoint signaling.

(**A**) HCT116 cells were cultured without treatment for control, with 1 mM HU alone or in combination with 2 µM MS-275 for 24 h. Afterwards, cells were incubated with the PP2A inhibitor Cantharidin (20; 40 µM) for additional 4 h. Phosphorylated ATM, CHK1, CHK2, and mSIN3A as loading control were detected with specific antibodies by western blot. (**B**) Cells were left untreated as control or were incubated with 1 mM HU and/or 2 µM MS-275 for 24 h. PP2A inhibitor Okadaic acid (OA; 25 nM) was added for further 4 h. Proteins were detected by western blot.

The results obtained with both phosphatase inhibitors, Cantharidin and OA, pointed to a role of PP2A in the orchestration of the replication stress response. Therefore, the contribution of *PPP2R3A* to DDR signaling and its putative control by HDACs was investigated in greater detail. Since microarray analysis showed an induction of *PPP2R3A* by MS-275, this should be validated by quantitative RT-PCR. After six and 24 hours, mRNA levels of HU, MS-275 and co-treated cells were analyzed and compared to that of untreated control cells (Figure 7.13 A).

Hydroxyurea did not alter the mRNA amount of *PPP2R3A*. Also additional application of MS-275 and single exposure to the HDACi for six hours had not affected mean *PPP2R3A* mRNA levels. Administration of MS-275 for 24 h, on the other hand, led to a significant 2.5-fold increase of *PPP2R3A* transcript level. This effect was even more pronounced and highly significant for cells co-treated with HU.

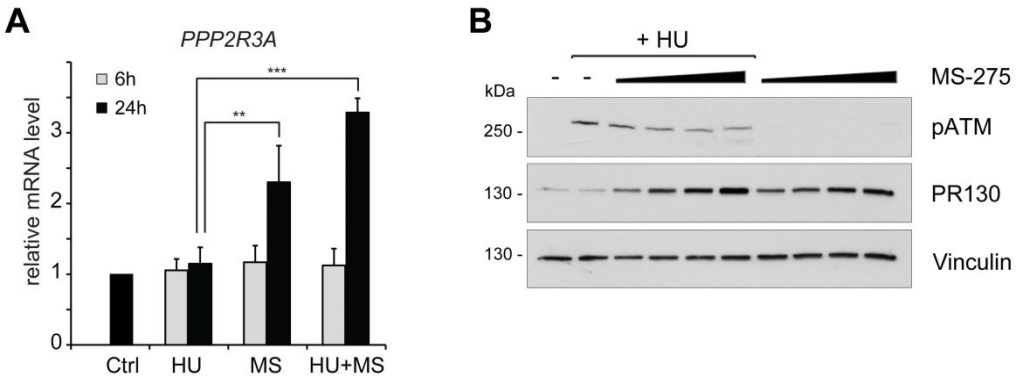

Figure 7.13: PP2A regulatory subunit PR130 is upregulated by MS-275.

(**A**) Mean relative mRNA expression levels of *PPP2R3A* in HCT116 cells evaluated by quantitative RT-PCR. Cells were treated with 1 mM HU and 2 µM MS-275 for 6 and 24 h. Error bars represent the s.d. of three independent experiments. (** $P<0.01$; *** $P<0.001$). (**B**) HCT116 cells were cultured without treatment or with increasing amounts of MS-275 (0.5; 1; 2; 5 µM) alone or in combination with 1 mM HU for 24 h. Respective proteins were detected by western blot.

Whether elevated mRNA levels of *PPP2R3A* also increased the amount of encoded protein was analyzed by western blot. A total of three transcript variants have been reported for *PPP2R3A*, which are generated by alternative splicing or alternative initiation of translation [193]. Only two of them though, PR72 and PR130, were studied so far. PR72 is present exclusively in heart and skeletal muscle, while PR130 is expressed ubiquitously in all tissues [101,194] (see also Figure 3.7). Thus, HCT116 cells were probed for PR130 protein. Western blot analysis showed that PR130 levels were elevated by MS-275, which is consistent with the increase of *PPP2R3A* mRNA (Figure 7.13 B). Moreover, this effect was concentration-dependent. PR130 progressively accumulated with rising doses of MS-275 and this increase was also detected together with HU treatment. Intriguingly, HU-induced phosphorylation of ATM diminished as PR130 protein accelerated. ATM kinase is located and acts predominantly in the nucleus of a cell [195,196]. To be directly connected with ATM phosphorylation levels, PR130 would have to be present in the nucleus as well. Therefore, cytosolic and nuclear extracts of HCT cells were analyzed for PR130 distribution. Figure 7.14 reveals that the regulatory subunit was mainly located in the cytosol. Only 5 to 10% of total

PR130 protein appeared in the nuclear fraction. Yet, its protein level was increased by MS-275 in both compartments to the same extent. Phosphorylated ATM was detected almost exclusively in the nucleus and the inhibitory effect of MS-275 on HU-induced ATM activation was observed again. mSIN3A as nuclear and α-Tubulin as cytosolic loading control remained equal.

Taken together, MS-275 elevates expression of the PP2A regulatory subunit PR130 on both mRNA and protein level. Upregulation of PR130 correlated with the loss of HU-induced ATM phosphorylation on Ser1981, while addition of specific inhibitors for PP2A was able to restore this phosphorylation.

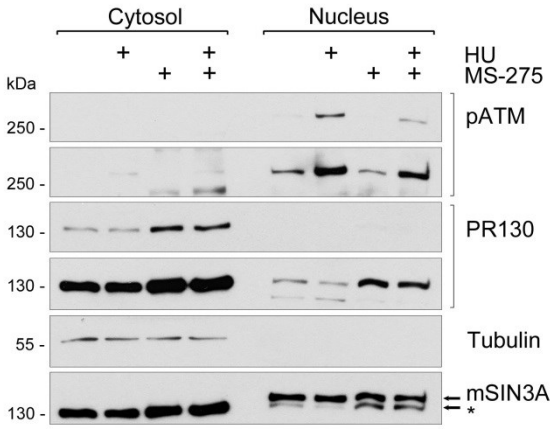

Figure 7.14: PR130 protein is mainly located in the cytoplasm.

HCT116 cells were left untreated for control or were incubated with 1 mM HU and 2 µM MS-275 alone or in combination for 24 h. Nuclear and cytosolic extracts were analyzed for the shown proteins by western blot. Tubulin was used as cytosolic and mSIN3A as nuclear loading control. (* a band which reflects remaining PR130 antibody on the membrane)

7.4.3 Simultaneous knockdown of HDAC1 and HDAC2 is sufficient to mimic the effects of MS-275

The histone deacetylase inhibitor MS-275 specifically impedes the activity of HDAC1, HDAC2 and HDAC3 [120]. Since replication stress signaling induced by HU is attenuated by MS-275, either the activity of a single or more than one of these HDACs is necessary to maintain the DDR cascade. Hence, it was studied which HDACs mediate the effects observed with MS-275. In a first attempt, transfection protocols for the knockdown of these HDACs by siRNAs were established. Simultaneous double and triple knockdowns were performed in all possible combinations of HDAC1, HDAC2 and HDAC3 in HCT116 cells. The final protocols for siRNAs

application resulted in high knockdown efficiencies of at least 70 to 90% for HDAC2 and almost 100% for HDACs 1 and 3 (Figure 7.15 A). These efficiencies were stable in all setups. Also, treatment with HU for 24 hours did not affect the knockdown of HDACs.

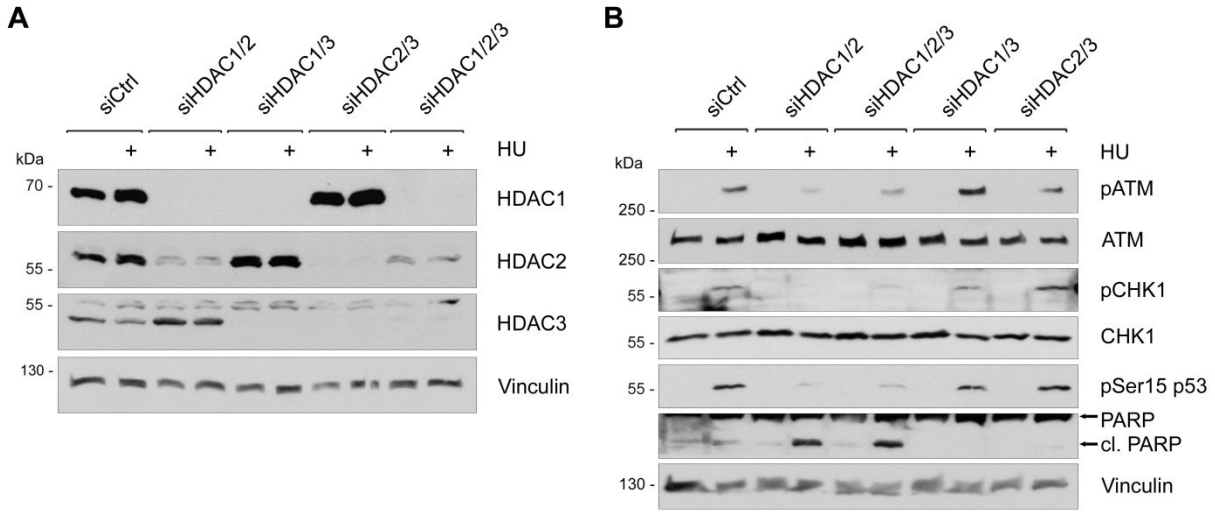

Figure 7.15: Simultaneous knockdown of HDAC1 and HDAC2 is necessary to attenuate HU-induced DDR signaling.

(**A**) Efficiencies for double and triple knockdown with siRNAs against HDAC1, 2 and/or 3. HCT116 cells were transiently transfected with siRNA against HDACs 1-3. After 48 h of incubation, 1 mM HU was added for further 24 h. (**B)** Whole cell lysates were used for evaluation of the shown proteins and their phosphorylation by western blot. Time schedule of transfection and treatment was the same as in (A). Both experiments were carried out by Maria Schreiber, former Master student of AG Krämer, FSU Jena.

With the established protocols for siRNAs, the putative role of HDACs1-3 in the modulation of DDR signaling was tested. Therefore, activation of ATM-CHK2 and ATR-CHK1 pathways was evaluated by the detection of pATM, pCHK1 and pSer15 p53 via western blot (Figure 7.15 B). Transfection of cells with scrambled siRNA and knockdown of HDACs itself did not cause activation of DDR signaling. No signals for phosphorylated ATM, CHK1 or p53 were obtained. Replication stress triggered by HU resulted in regular phosphorylation of all three proteins in control cells. Double knockdown of HDAC1 and 3 (siHDAC1/3) even slightly increased the level of pATM, but did not affect pCHK1 or pSer15 p53. The same was true for cells transfected with HDAC2/3 targeting siRNAs. Loss of these HDACs showed unchanged phosphorylation of DDR kinases and p53 during HU treatment compared to control cells. Interestingly, phosphorylation of ATM, CHK1 and p53 by HU was diminished in cells lacking HDAC1 and 2 (siHDAC1/2). Additional depletion of HDAC3 (siHDAC1/2/3) likewise showed reduced activation of these DDR proteins. However, simultaneous knockout of HDAC3 did not further reduce phosphorylation of ATM, CHK1 or p53 in comparison to HDAC1/2

depleted cells. Unequal amounts of total ATM and CHK1 could not account for their reduced phosphorylation level as their constant expression was verified by respective western blots (Figure 7.15 B). Further detection of PARP protein showed that knockdown of HDAC1/2 and HDAC1/2/3 created a distinct second signal at a lower molecular weight in the context of HU treatment. This signal represents the 89kDa fragment of PARP cleavage and indicates apoptotic cell death. While this fragment was also observed in control cells incubated with HU, it was even more abundant in cells transfected with siHDAC1/2 and siHDAC1/2/3. Again, depletion of HDAC3 showed no additional effect.

Since knockdown of HDAC1/2 and HDAC1/2/3 successfully mimicked the effects of MS-275 on HU-induced DDR signaling and apoptosis, their putative role on PR130 expression was tested next. Simultaneous transfection of siRNAs for HDAC1 and HDAC2 elevated PR130 protein as shown by western blot (Figure 7.16). This induction was independent of HU. Furthermore, the intensity of PR130 signals after HDAC1-3 triple knockdown was the same as for HDAC1/2 depletion.

In summary, simultaneous knockdown of HDAC1 and HDAC2 was sufficient to modulate the cellular response to HU. It increased PR130 protein (Figure 7.16), attenuated phosphorylation of important DDR mediators and finally sensitized cells for apoptotic PARP-1 cleavage (Figure 7.15). Further depletion of HDAC3 showed no additional impact on the examined processes.

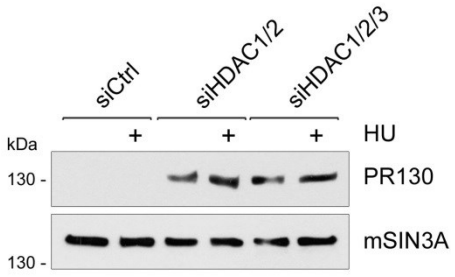

Figure 7.16: Simultaneous knockdown of HDAC1 and HDAC2 increases PR130 protein.

Knockdown of HDAC1/2 and HDAC1/2/3 was performed with respective siRNAs by transient transfection. After 48 h of incubation, 1 mM HU was added for further 24 h. PR130 expression was analyzed by western blot. mSIN3A was used as loading control. The experiment was carried out by Maria Schreiber, former Master student of AG Krämer, FSU Jena.

7.5 PP2A-PR130 as a potential phosphatase of ATM

7.5.1 Elimination of PR130 restores ATM phosphorylation

The experiments described so far revealed, among other things, that MS-275 interferes with replication stress-driven signaling (Figure 7.1) and increases expression of the PP2A regulatory subunit PR130 in HCT116 cells (Figure 7.13). It was also shown that PP2A inhibitors are able to counteract the effect of MS-275 on DDR signaling (Figure 7.12). However, a direct link between the upregulation of a phosphatase subunit and potentially increased dephosphorylation of DDR kinases was still missing.

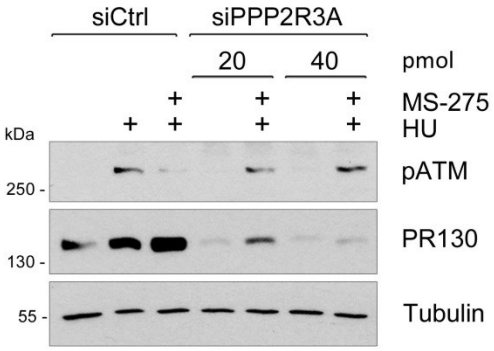

Figure 7.17: Knockdown of PR130 restores ATM phosphorylation.

HCT116 cells were transfected with control siRNA or the indicated amounts of *PPP2R3A*-specific siRNA. After 24 h of incubation, 1 mM HU and 2 µM MS-275 were added and incubation was extended for additional 24 h. Whole cell lysates were used for protein detection by western blot.

For this reason, a specific siRNA for *PPP2R3A* was employed. Different concentrations of the siRNA were used to knock down PR130 protein. As a control, scrambled siRNA was transfected to HCT116 cells and after 24 hours of incubation cells were either left untreated, were exposed to HU or HU+MS-275 for a total of 48 hours. The same time schedule was used for the transfection of siPPP2R3A. Only these cells were solely exposed to the combination treatment of HU and MS-275 or remained untreated. Western blot analysis of PR130 protein showed its induction by MS-275 in control cells (Figure 7.17). Also, transfection with scrambled control siRNA did not affect HU-induced ATM phosphorylation or its loss during co-administration with the HDACi. The specific siRNA for PPP2R3A was able to reduce the basal level of PR130 by at least 90%. Application of the siRNA showed a dose-dependent knockdown of the PP2A subunit. An amount of 40 pmol PPP2R3A siRNA was sufficient to completely block the induction of PR130 by MS-275. More importantly, PPP2R3A siRNA dose-dependently restored ATM phosphorylation in cells exposed to HU and

MS-275. The less PR130 was present, the more ATM molecules were phosphorylated. This fact points to a direct connection of ATM phosphorylation and PR130 protein level.

7.5.2 PR130 physically interacts with ATM

A necessity for the involvement of PR130 in dephosphorylation of ATM is direct interaction of the phosphatase with the targeted kinase. Since regulatory subunits mediate PP2A target specificity [97] PR130 should be present in the complex of phospho-protein and phosphatase. Therefore, pATM antibody was used for co-immunoprecipitation experiments. HCT116 cells were treated with HU and MS-275 for 24 hours to induce PR130. The resulting low level of pATM was restored by further incubation with OA for four hours (see Figure 7.12 B) to provide enough potential interaction partners. Those enzyme-substrate complexes would be very transient. Therefore, they were stabilized prior to lysis by fixation with 1% formaldehyde. This method would also prevent detachment of the proteins during purification of precipitates. As negative controls, normal IgG of pre-immune serum or no antibody were used for immunoprecipitation. Both controls showed no signals for pATM or PR130 in western blot (Figure 7.18). By using pATM antibody, phosphorylated ATM was successfully precipitated from whole cell lysates. Finally, detection for co-immunoprecipitated PR130 resulted in a distinct signal at the same molecular weight as for PR130 in the input control.

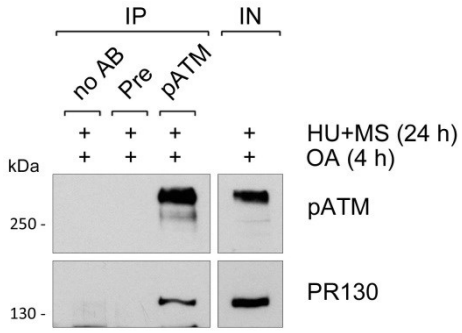

Figure 7.18: Endogenous PR130 physically interacts with phosphorylated ATM *in vivo*.

After 20 h of treatment with 1 mM HU and 2 µM MS-275, Okadaic acid (OA, 25 nM) was added for further 4 h. Co-immunoprecipitations (IP) with anti-pSer1981-ATM antibody, rabbit pre-immune serum (Pre) or no antibody (no AB) were analyzed for ATM phosphorylation (Ser1981) and PR130 protein by western blot with specific antibodies. Right panel shows anti-pATM and anti-PR130 immunoblots of the initial lysate (6% Input, IN).

The experiment showed that PR130 physically interacts with phosphorylated ATM in HCT116 cells. Of note, neither of the proteins was artificially expressed and precipitation was performed with endogenous protein levels.

8 DISCUSSION

The use of anti-metabolites in cancer therapy has become a standard chemotherapeutic approach for a variety of malignancies [197]. Among them are hydroxyurea (HU) and 5-Fluorouracil (5-FU), which are established drugs for leukemia and various solid tumors [154,162]. Both compounds act in a similar manner as they interfere with the *de novo* synthesis of deoxynucleotide triphosphates (dNTPs) – the building blocks of DNA [162,198]. Consequently, a shortage of dNTPs during replication of the genome impedes the complete duplication of chromosomes, which is a prerequisite for cell division. Thus, it provokes activation of the replication checkpoint that functions to stabilize stalled replication forks until the stress is relieved and replication can continue [199]. If the onset of this checkpoint was defective, delayed, or to weak, cellular survival would be endangered. Several *in vitro* studies showed that the desired cytotoxic effect of HU and 5-FU on cancer cells could be increased when histone deacetylase inhibitors (HDACi) were applied simultaneously with the anti-metabolites [156,157,200].

8.1 Inhibition of HDACs interferes with DDR signaling and cell replication

The first line mediator of DNA damage response signaling upon replication stress is the ATR-CHK1 pathway [17,71,201]. Incubation with HU obstructs replication fork progression and in turn creates long stretches of RPA-coated ssDNA, which are the ultimate trigger for activation of ATR [19]. The phosphorylation of its downstream target CHK1 at Ser317 was used as readout for the catalytic activity of ATR in the present study [63] (Figure 7.1 A). Besides activation of CHK1, also ATM and CHK2 phosphorylation were detected in response to HU [9]. These should be considered as secondary downstream effects and this is also illustrated by the increase of pATM levels from six to 24 hours of treatment. The agent 5-FU was used as reference compound and similarly induced DDR signaling in HCT116 cells (Figure 7.1 B). ATM kinase was described as a direct target of active ATR after UV radiation and replication stress [9]. It was shown that ATM is phosphorylated at Ser1981 in an ATR-dependent manner, which in turn promoted ATM activity and CHK2 phosphorylation. Additionally, ATM activation could be a cause of occurring DNA double-strand breaks (DSBs). After prolonged exposure to replication stressors like HU, blocked replication forks progressively collapse and generate fork-associated DSBs that thereupon mediate ATM signaling [36,202]. Resolution of these DSBs by homologous recombination (HR) in return triggers ATR activity [18,203]. Both paths leading

to ATM phosphorylation do not exclude each other and are likely to run in parallel. DSB-associated ATM activation though should be induced at a later time.

Figure 7.1 further shows that even long-time exposure of HCT cells to the HDACi MS-275 does not induce DDR signaling, although HDACi alone can cause damage to DNA [113]. However, MS-275 did affect HU- and 5-FU-induced DNA damage signaling in a way that reduced DDR kinase phosphorylation after long periods of co-treatment with the HDACi (Figure 7.1). Especially the experiment performed with HU showed a clear time-dependent loss of kinase activation in presence of MS-275. Phosphorylation of ATM, CHK1, and CHK2 is still comparable to the HU-induced DDR after six, but diminished after 24 hours. Increased modification by acetylation often alters mRNA or protein stability and can favor stabilization as well as (proteasomal) degradation depending on the modified mRNA/protein [111]. Inhibition of HDACs by MS-275 showed no impact on the protein level of ATM and checkpoint kinases. Consequently, a reduced amount of kinase molecules in the cell as cause for their attenuated phosphorylation after 24 hours of co-treatment with HU and MS-275 can be excluded for the shown experimental setups. The kinase ATM of the DDR cascade is a reported target for acetylation by the HAT TIP60 [41]. The associated deacetylase for ATM is unfortunately unknown until today, although it was shown that the kinase is localized in a complex with catalytically active HDAC1 [204]. So MS-275 would (if at all) increase acetylation of ATM. Sun *et al.* also delineated in their work that acetylation is necessary for activation of ATM. This way, HDAC inhibition would rather increase activity or phosphorylation of ATM, but not account for suppressed pATM levels.

To investigate whether the obtained effects corresponded to a general mechanism, other cell lines were examined for ATM phosphorylation (Figure 7.2). For this, the p53 negative counterpart to HCT116 wild-type cells as well as MEF cells, both harboring and lacking p53 wild-type protein, were chosen. RKO cells were additionally tested as a second cell line originating from human colon carcinoma. In each case, HDAC inhibition by MS-275 reduced the amount of phosphorylated ATM after 24 hours of co-incubation with HU. In RKO cells, the depletion of DDR signaling already occurred after a short period of six hours. Thus, they appear more sensitive to MS-275 compared to HCT116 cells. The results demonstrate that sustained replication stress signaling requires class I HDAC activity and that this seems to be a universal regulatory mechanism (at least for ATM). The MS-275-dependent loss of ATM activity affected murine cells in the same way as human cells and is independent of p53.

The tumor suppressor p53 is an important downstream effector of the DDR and direct substrate for ATM/ATR and CHK1/CHK2 [83]. Treatment with HU triggered phosphorylation at Ser15 by apical and at Ser20 by checkpoint kinases (Figure 7.3 A). Modification of these serine residues in the N-terminal transactivation domain is generally considered to stabilize p53 protein by preventing interaction with the E3 ubiquitin ligase MDM2 (also 'HDM2' in humans) [83]. Consistent with this, the total amount of p53 protein increased after incubation with HU (Figure 7.3 A). The MS-275-dependent reduction in ATM, CHK1, and CHK2 activity also reflected on the p53 phosphorylation status. Phosphorylation at Ser15 and Ser20 of p53 after S phase stress was reduced by the HDACi after 24 hours. Also, total p53 levels were lower if HDAC activity was suppressed. The serine residue 46, in contrast, is substrate to the kinases HIPK2 and DYRK2 [166,167]. Both are activated upon DNA damage and modified p53 Ser46 even stronger in presence of MS-275, which might also indicate increased activity of these kinases (Figure 7.3 A). Densitometric analysis of western blots revealed that the residual accumulation of p53 after long-time exposure to HU+MS-275 cannot only be explained by residual Ser15 phosphorylation (Figure 7.3 B). This is supported by the findings of several studies. They indicate that phosphorylated Ser15 is rather important to stimulate transactivation of target genes whereas Ser20 phosphorylation primarily interferes with HDM2 interaction [87]. Additionally, acetylation of several lysine residues in the C-terminal domain contributes to p53 stabilization. This prevents HDM2-mediated ubiquitination at the same residues [84,205,206]. HDAC1 and SIRT1 are known deacetylases for several of these sites [206,207]. HDAC inhibition would therefore preserve p53 acetylation and increase half-life of p53 protein. This fact also explains slightly increased amounts of the tumor suppressor after single application of MS-275 in HCT116 cells (Figure 7.3).

The amount of activated p53 protein in cells correlated well with p53 target gene expression. When HU was applied, the expression of early response p53 target genes like HDM2 and WIP1 was induced. On the other hand, decreased accumulation of p53 during 24 hours of co-treatment with MS-275 resulted in diminished expression of these proteins (Figure 7.4). As described previously, the cell cycle regulator p21 cannot and was not induced by HU-activated p53 [208] (Figure 7.4). Long-time incubation with MS-275 though promoted p21 expression and this effect was already demonstrated for several HDACi in various cell lines [209,210]. However, additional exposure to HU decreased p21 levels at both indicated time points. The underlying cause is probably the same mechanism that prevents p53-dependent induction of p21 during S phase stress in the first place. Beckermann *et al.*

found that CHK1 activated by the replication checkpoint mediates a blockage of *p21* transcriptional elongation and thereby prevents increase of p21 mRNA [211].

In summary, cells suffering from S phase stress induce proper DDR signaling, whereas simultaneous inhibition of HDAC1-3 activity by MS-275 attenuates DDR kinase phosphorylation and thereby also lowers the HU-triggered increase in p53 protein and target gene expression in a time-delayed manner. A recent publication from Thurn *et al.* demonstrated a diminished phosphorylation of ATM and p53 after combined treatment of cell lines with the DSB-inducing cytostatic Epirubicin and different HDACi [212]. Accordingly, also DDR signaling after DSBs is modified by deacetylases. The group states that HDAC inhibition perturbs ATM expression. They specify that a maximal reduction of 20 to 40% ATM mRNA accounts for an almost complete loss of ATM phosphorylation observed by western blot analysis. This explanation cannot be transferred to the results obtained during this study, since ATM protein levels were not altered by HU and MS-275 treatments (Figure 7.1).

An early marker for the occurrence of DNA damage is phosphorylation of the histone variant H2A.X, commonly referred to as γH2A.X [34,175]. When HCT116 cells were incubated with MS-275 for 24 hours, there were no γH2A.X signals detected (Figure 7.5). Yet, HDACi like TSA, SAHA, or depsipeptide were described to cause DNA damage in malignant cells during single administration [213-215]. It cannot be excluded that MS-275 is also capable to evoke damage in HCT116 cells, but it did not do so in the monitored time frame and with the used concentration. This is also in line with the absence of DDR kinase activation after MS-275 treatment (Figure 7.1). HU triggered H2A.X phosphorylation by activation of ATR kinase and the S phase checkpoint [34] (Figure 7.5). Also phosphorylated ATM could have contributed to the generation of observed γH2A.X foci. They were distributed across the cellular chromatin indicating sites of DNA damage (Figure 7.1 and Figure 7.5 B). Immunofluorescence staining for γH2A.X revealed a variable number of foci in different cells, which could be connected to their respective position within the cell cycle. While healthy cells in G1 should not harbor foci, an increasing amount of γH2A.X in S phase could correlate with the cell´s remaining rate of DNA synthesis or collapsing replication forks. A concurrent exposure of stressed HCT116 cells to MS-275 caused a similar variability in γH2A.X foci. It additionally created cells with a massive amount of the same (Figure 7.5 B). So MS-275 strongly elevated DNA damage induction in individual cells, which could again be correlated to a cell´s position within the cell cycle. Western blot analysis revealed that also the overall amount of H2A.X

phosphorylation after application of HU+MS-275 was higher than in HU-treated cells (Figure 7.5 A). At first sight, this is a contradiction to the MS-275-dependent attenuation of DDR kinase activity since ATM and ATR phosphorylate H2A.X. An intramolecular interplay of phosphorylation and acetylation on H2A.X could be a possible explanation and the histone is indeed targeted by acetylation. However, this modification was reported to be independent of phosphorylation and rather serves to mediate H2A.X ubiquitination [216]. WIP1 was described as phosphatase for γH2A.X and also targets many other substrates of ATM and ATR like p53 [217-219]. The reduced p53-dependent expression of WIP1 in HU+MS-275-treated HCT116 cells could account for increased H2A.X phosphorylation, since dephosphorylation of the histone is hindered (Figure 7.4). It was also shown that CHK1 activation during HU treatment is indispensable to impart repair of occurring DNA damage by HR [201]. HR is the predominantly triggered repair pathway after replication arrest and collapsing forks [203,220,221]. So, reduced checkpoint signaling by CHK1 as a result of HDAC inhibition would consequently restrict the repair capacity of HR and maintain damage (Figure 7.1 and Figure 7.5). The well-defined feature of HDACi to alter gene expression might also negatively affect DNA damage repair pathways. There are several reports about downregulation of repair genes, such as RAD51, by inhibitors of HDACs [113,222]. The protein RAD51 is required to rescue stalled replication forks and for repair by HR and its expression was suppressed by the broad-spectrum HDACi PCI-24781 in HCT116 cells [221,223].

Any repair of the DNA will only be effective if the cell cycle is arrested first. The regular cell cycle profile of HCT116 cells is presented in the lower part of Figure 7.6 A. After 24 hours of incubation with MS-275, the S and G2/M phase are depleted and cells gathered in G1 stage. This effect of HDACi is attributed to their induction of the CDK inhibitor p21 [141] (Figure 7.4). P21 mainly inhibits CDK2 and prevents binding of the cell cycle-regulating kinase to cyclin E and A required for transition into S phase. Consequently, these cells arrest in G1 [224]. In contrast, the compound HU primarily induces the S phase checkpoint as it obstructs replication fork progression. Before discussing the consequences of HU for HCT116 cells, a closer look should be taken on the process of DNA replication itself. Multiple so called ‘origins of replication’ are distributed along the chromosomes and they are initiated in a time-ordered manner (origin firing). Several of them are active at the same time to allow successful DNA duplication within only six hours [225,226]. The replication checkpoint governs the stability of the generated replication forks as well as origin firing during the undisturbed and disturbed cell cycle [227,228]. An essential function of activated CHK1 during replication

checkpoint is to modify CDC25 phosphatases and thereby target them for proteasomal degradation or sequestration in the cytosol [66,67,70] (see also Figure 3.5). These phosphatases are crucial cell cycle regulators and facilitate the binding of cyclins to CDKs by activating dephosphorylation of the latter. If CDK2-cyclinA dimers cannot be established owing to the absence of CDC25A, the cell cycle will arrest in S phase [67]. This is why treatment with HU for 24 hours accumulated cells at the stage of DNA synthesis and the amount of cells in G2 and mitosis is decreased compared to the undisturbed cell cycle (Figure 7.6 A). When the cell cycle progression of HU-treated HCT116 cells was monitored over 12 hours, a considerably decelerated course through S phase was visible (Figure 7.6 B). Most of the cells were located in the early S phase after 16 hours of incubation, but they slowly continued to progress during the next hours. This indicates that the cell cycle arrest was not stable. Indeed, HU reversibly inhibits the enzyme RNR, which is why its catalytic activity might be restored at later time points [229]. It has also been shown in a system with budding yeast that HU only delays fork progression and origin firing, thus does not complete block cell cycle progression [178]. The active ATR-CHK1-mediated replication checkpoint is crucial to stabilize stalled replication forks. It furthermore mediates restart of obstructed forks or repair of collapsed forks by HR [201], which would also encourage transition through the S phase.

After simultaneous administration of HU and MS-275 for 24 hours, cell cycle profiling showed a decreased amount of cells in S phase and a higher percentage in G2/M compared to single HU treatment (Figure 7.6 A). Also, the progression of cells through S phase was accelerated and indicates declining replication checkpoint activity with time (Figure 7.6 B). This correlates well with the phosphorylation status of DDR kinases (Figure 7.1). Inhibition of HDACs did not affect HU-induced DDR signaling after six hours and had just as little effect on S phase arrest after 16 hours. Longer incubation with the HDACi diminished replication stress signaling by ATM, CHK1, and CHK2 and thus abolished cell cycle arrest. The reduced activity of CHK1 in stressed cells would allow new origin firing but also destabilize and restart breakage-prone replication forks. This might subsequently lead to the generation of more severe DSB lesions [185]. Moreover, it would limit repair of these lesions since CHK1 is necessary for induction of HR [201]. That could also contribute to the massive accumulation of γH2A.X in co-treated HCT116 cells (Figure 7.5).

The mediator kinase CHK1 not only critically regulates the intra-S phase checkpoint but also the transition of cells into mitosis at the G2/M checkpoint by targeting CDC25C [67]. A study from Lossaint *et al.* showed that efficient induction of p21 after DSBs was able to

compensate a loss of CHK1 activity and arrested cells in G2 [230]. However, p21 protein was not induced in HU+MS-275-treated cells after 24 hours (Figure 7.4) and hence could not contribute to cell cycle blockage. But how can accelerated completion of DNA replication in presence of MS-275 be explained, considering the depleted synthesis of dNTPs by HU? It is unclear how effective RNR was inhibited by 1 mM HU in HCT116 cells, but most commonly the enzyme is only partially inhibited by HU [177]. So some enzymatic activity remained to supply the pool of dNTPs. Furthermore, it is an acknowledged fact these days that also ribonucleotides can be and are occasionally incorporated in newly synthesized DNA [231,232]. In normal cells, they are far more abundant than deoxyribonucleotides and inhibition of the RNR by HU further increases the imbalance. This would make misincorporation of ribonucleotides even more likely.

How single and combined application of HU and MS-275 affected the induction of (apoptotic) cell death was investigated with western blot and FACS assays (Figure 7.7). Apoptosis was evaluated in western blot with a specific antibody for a caspase 3-dependent PARP cleavage fragment. Incubation of HCT116 cells with MS-275 for 24 hours created no pronounced signal for PARP cleavage. Also Annexin V staining revealed only minor apoptosis with about 8% apoptotic cells after 24 and 48 hours. Long-time exposure for 48 hours though showed 20% of cells in subG1 fraction indicating DNA fragmentation. One explanation would be that, apart from the intrinsic and extrinsic apoptotic pathway, HDACi in general were also shown to eliminate tumor cells via induction of reactive oxygen species (ROS), mitotic cell death, or autophagy [233]. When the lethal capability of HU was quantified, the percentage of apoptotic cells (Annexin V) was a little higher than that of the subG1 fraction. This is because PI staining only reveals the late, but not early apoptotic stage (Figure 7.7 B-C). Cell death of HCT116 cells could be further increased by simultaneous HDAC inhibition. First, western blot analysis indicated higher caspase-dependent turnover of PARP (Figure 7.7 A). Additional FACS analysis confirmed that cytotoxicity after 48 hours was significantly enhanced if HU and MS-275 were applied together instead of alone (Figure 7.7 B-C). These data are in line with earlier publications showing that disturbed HDAC activity contributes to a more efficient induction of apoptosis in HU-stressed cells [156,157]. Moreover, enhanced tumor cell toxicity was also reported for co-administration of 5-FU with MS-275 [200]. It was described above that the HDACi MS-275 disrupts HU-induces cell cycle arrest. This would also impair repair and cause an accumulation of damage. If those cells pre-maturely entered mitosis with damaged DNA, cell death would be certain.

Taken together, inhibition of HDACs reduced checkpoint kinase signaling and consequently disturbed cell cycle checkpoints set on by HU. This was accompanied by increased DNA damage and apoptotic cell death. Replication stress-induced S phase arrest was canceled in presence of MS-275 in a time-dependent fashion and facilitated the progression of cells harboring unrepaired DNA towards mitosis.

8.2 Deactivation of apical kinases during DNA damage conveys the effects of MS-275

The primary ATM-CHK1 and secondary ATM-CHK2 DDR kinase cascades were induced after treatment with HU but were attenuated upon HDAC inhibition by MS-275 (Figure 7.1 and Figure 7.3). Therefore, it was investigated whether impaired activity of only one of these kinases could account for the MS-275-dependent effects on cell signaling and viability. The kinase CHK1 is required for faithful DNA replication during unperturbed S phase and depletion of CHK1 is lethal for every proliferating cell [71,227,234,235]. In the experiments of this study, CHK1 activity was blocked with UCN-01 [186]. Application of the inhibitor increased p53 phosphorylation at Ser15 in HU-treated cells, indicating that ATR and/or ATM activation was elevated (Figure 7.8 A) [75,80]. These data match previous studies, which showed that CHK1 is indispensable to trigger the S phase checkpoint and to avoid replication initiation after occurrence of stalled replication forks [11,236]. So, loss of CHK1 activity must have augmented the formation of DSB after replication inhibition with HU. It additionally must have reduced repair of the damage by HR [201,236]. As a consequence, caspase 3-dependent PARP cleavage was markedly increased in HCT116 cells incubated with UCN-01 (Figure 7.8 A) [237]. In contrast to p53 Ser15, checkpoint kinase-dependent phosphorylation of Ser20 remained unchanged with addition of UCN-01 proving efficient inhibition of CHK1 [81,82]. Despite their partially overlapping substrates, the second checkpoint kinase CHK2 is not crucial for the survival of normal cells [238,239]. This is also demonstrated by the availability of the CHK2-deficient cell line HCT116 CHK2$^{-/-}$. These cells were used to address the role of CHK2 in HU-induced signaling. Wild type and CHK2-negative HCT116 cells show the same accumulation of p53 upon exposure to HU (Figure 7.8 B). Yet, apoptosis was decreased if CHK2 was missing. The data speak against a role of CHK2 as coordinator of replication stress signaling. Hence, increased apoptosis after HU+MS-275 treatment is not reliant on this checkpoint kinase. Reduced CHK1 activity neither completely displays the effects of MS-275 (Figure 7.8 A). UCN-01 clearly accentuated apoptotic death of cells with replicative stress, like MS-275 did.

However, CHK1 inhibition did not cause reduced downstream phosphorylation of p53 after HU and therefore is not the sole mediator for the effects of MS-275.

The apical kinase ATM was selectively inhibited using KU-55933 [188]. This compound was unable to prevent auto-phosphorylation of ATM molecules *in trans*, since HU generated the same amount of Ser1981-phosphorylated ATM irrespective of the inhibitor (Figure 7.9 B). The inhibitory capacity of KU-55933 is demonstrated by the reduction of p53 phosphorylation at Ser15 during simultaneous incubation with HU. This in turn impeded the accumulation of p53 protein. Surprisingly, KU-55933 also diminished HU-induced CHK1 phosphorylation at Ser317. It has been published before that ATM inhibition by KU-55933 reduced phosphorylation of CHK1 following IR or ICRF-193 (an inhibitor of DNA topoisomerase II) [33,230]. KU-55933 did not affect ATR, since work from Liu *et al.* showed that ATR activation is not disturbed in HCT116 cells incubated with 10 µM KU-55933 [22]. This reflects the set-up used in the present study. In contrast to reduced CHK1 phosphorylation, the ATM inhibitor paradoxically increased the amount of Thr68-phosphorylated CHK2 after HU (Figure 7.9 B). This effect of KU-55933 has also been published and the authors state that ATM could be dispensable for phosphorylation of CHK2 at Thr68 [230]. The experiment in Figure 7.9 B furthermore shows that the ATM inhibitor supported induction of apoptosis after replication stress, which is indicated by increased apoptotic PARP cleavage.

Addition of the ATR inhibitor ETP-46464 similarly increased PARP cleavage after 24 hours of HU treatment (Figure 7.9 A). Annexin V staining revealed a highly significant increase in apoptosis after 48 hours, if ATR activity was impaired during replication stress (Figure 7.10 A) [237]. Moreover, ETP-46464 caused massive phosphorylation of ATM in presence of HU (Figure 7.9 A). The induction of cell cycle arrest following perturbations of DNA synthesis is critically dependent on ATR and its downstream kinase CHK1. Both proteins are crucial for cell survival. When replication forks become stalled, ATR signaling mediates fork stabilization and blocks new origin firing [71,185,240]. ETP-46464 suspends this mechanism and leaves S phase uncontrolled, which would result in DSB formation or even chromosomal breakage and explains the high degree of ATM phosphorylation [241] (Figure 7.9 A). In line with this, DNA damage-inducible phosphorylation at Ser46 of p53 was increased when the S phase checkpoint was abrogated by ETP-46464. In contrast, Ser15 of p53 was less phosphorylated in HU-treated HCT116 cells if ATR was inhibited - despite the high levels of activated ATM. This demonstrates the primary role of ATR in p53 phosphorylation after

replication stress. An additional proof for the efficacy of ETP-46464 is the complete loss of CHK1 phosphorylation following HU (Figure 7.9 A). How this loss of CHK1 activity affected cell cycle progression was investigated by PI staining after 18, 22, and 26 hours of treatment. Incubation of HCT116 cells with HU alone activated the replication checkpoint so cells accumulated in early S phase and progressed only very slowly towards G2 (Figure 7.10 B, see also section 8.1 and Figure 7.6 B). Simultaneous inhibition of ATR by ETP-46464 disturbed the onset of both an S phase and G2/M arrest. HCT116 cells instead gathered at the G1/S transition. It is known that the kinase ATR can only be activated in the S and G2 phase of the cell cycle [18,187] which also restricts CHK1-dependent DNA repair via HR to these cell cycle stages [201,242]. If ATR activity is blocked right from the beginning of a HU-induced stress response, only G1 arrest can be initiated. This checkpoint is, unlike the replication and G2 checkpoint, independent of ATR [243,244]. Cells that escape from G1 and re-enter the cell cycle would face an unchecked process of DNA synthesis which is moreover disturbed by HU and lacks DNA repair by HR. As a consequence, these cells would be eliminated. The depiction of subG1 fraction for the treatment of HCT116 cells with HU and ETP-46464 indeed shows an increasing amount of dead cells with time (Figure 7.10 C). After 26 hours of co-incubation, about 12% of cells had died. The effectively established S phase arrest in cells exposed to HU prevented cell death, so the respective proportion of subG1 (3%) is comparable to the control treatments used (1-2%).

The targeted inhibition of the apical kinases ATM and ATR during HU-induced replication stress showed that repression of not only one but rather both kinases conveys the effects of MS-275 on DDR signaling and cell fate. The ATR inhibitor ETP-46464 nicely reproduced the attenuated downstream signaling though CHK1, p53 phosphorylation and accumulation, disturbed cell cycle arrest and also elevated the cytotoxicity of HU in HCT116 cells (Figure 7.9 and 7.10). Nevertheless, it also strongly increased the level of ATM phosphorylation which is why ATM activity has to be counteracted by MS-275 as well.

8.3 HDAC1/2 are important players in the orchestration of DDR signaling

The synthetic benzamide derivate MS-275 was identified as a potent HDAC inhibitor in 1999 and selectively targets HDAC1, HDAC2 and HDAC3 [148,245]. It was therefore tested which of these three HDACs mediate(s) the negative effect of MS-275 on HU-induced DDR signaling. Single HDACs were eliminated by siRNA-mediated knockdown with high efficiency (Figure 7.15 A). Interestingly, application of siRNA against either HDAC1 or HDAC2 (each in

combination with HDAC3) upregulated the endogenous amount of the other HDAC variant. This indicates a mutual compensation of HDAC1 and HDAC2 (Figure 7.15 A) [246]. The combined knockdown of HDAC1/3 and HDAC2/3 still showed a proper induction of the DDR after 24 hours of HU treatment. Phosphorylation of ATM, CHK1, and p53 was induced as in control cells transfected with scrambled siRNA (Figure 7.15 B). That HDAC2 is not necessarily required for ATM activation in response to HU is also demonstrated by the regularly induced phosphorylation of ATM in HCT116 $p53^{-/-}$ cells lacking the deacetylase [247] (Figure 7.2 A). Joint depletion of HDAC1 and HDAC2 in HCT wild-type cells, however, diminished activation of ATM and CHK1 upon exposure to HU whereas the total amount of these proteins was not affected. Furthermore, the p53 residue Ser15 was less phosphorylated and apoptotic PARP cleavage was markedly induced in HU-treated cells depleted of HDAC1 and HDAC2. Hence, a loss of HDAC1/2 protein imitated the effects of their catalytic inhibition by MS-275 (Figure 7.1 and 7.7 A). The additional transfection of siHDAC3 in triple knockdown experiments yielded the same results without additional effects (Figure 7.15 B). This excludes an essential function of HDAC3 in the maintenance of replication stress signaling. Instead, activity of both HDAC1 and HDAC2 is required for the onset of a cellular response after disturbed DNA synthesis. These results are in agreement with a recent publication of Thurn *et al*., which demonstrated that HDAC1 and HDAC2 hold important function in the DDR after DSBs [212].

8.4 PR130 as a new regulator of ATM phosphorylation

What was still missing was a molecular mechanism for the modulation of apical DDR kinases by HDAC1 and HDAC2. Pre-incubation of HCT116 cells with MS-275 was used to gain more insight in its time-dependent regulation of the DDR (Figure 7.11). Simultaneous treatment with HU and MS-275 revealed decreasing phosphorylation of ATM, CHK1, and p53 after more than six hours of exposure. Pre-incubation with MS-275 over-night blunted the induction of HU-induced replication stress signaling via ATR and ATM right from the beginning. These results suggested that MS-275 altered expression of (an) unknown gene(s) which afterward antagonized DDR signaling. A delayed increase in phosphatase activity by MS-275 was a suitable, direct explanation for the observed sensitivity of kinase phosphorylation to HDAC inhibition.

WIP1 acts as an important serine/threonine phosphatase in the DDR and was also reported as a possible negative regulator of ATM and ATR [218,219,248,249]. However, long-time exposure

to HU showed a reduced expression of the p53 target WIP1 in the presence of the HDACi (Figure 7.4). This makes WIP1 an unlikely candidate for ATM/ATR dephosphorylation in cells treated with HU and MS-275. Other researchers also reported additional phosphatases. These might either prevent basal ATM phosphorylation and thereby keep the kinase in an inactive state or facilitate ATM activation and downstream signaling following DNA damage. They include PP1 [250], PP5 [251], and PP2A [252], which are all serine/threonine phosphatases of the PPP family (see also section 3.2). Since PP2A is a key phosphatase in mammalian cells [100], it was investigated whether inhibition of HDACs by MS-275 increased the general catalytic activity of PP2A. The inhibitors Cantharidin and Okadaic acid (OA) were applied to block dephosphorylation by PP2A. Cantharidin increasingly rescued the MS-275-mediated repression of HU-induced DDR signaling with rising concentration (Figure 7.12 A). Phosphorylation of ATM, CHK1, and CHK2 was restored by Cantharidin within four hours, which was reproducible with the PP2A inhibitor Okadaic acid (Figure 7.12). Moreover, Cantharidin accentuated phosphorylation of the mentioned kinases in cells treated with HU alone, but did not do so in untreated HCT116 cells (Figure 7.12 A). These data argue that PP2A rather controls the intensity of DDR kinase activity than their basal phosphorylation. They could further mean that (i) PP2A counteracts phosphorylation of apical as well as checkpoint kinases or (ii) PP2A regulates phosphorylation of apical kinases and restored activity of ATM/ATR by Cantharidin (or OA) in turn increases downstream target phosphorylation. In fact, PP2A was shown to also control phosphorylation of CHK1 (Ser317, Ser345) [253] and CHK2 (Thr68) [254] of the S phase signaling cascade. Albeit many proteins have been described as targets of PP2A, very few publications identified the critical regulatory subunit, which facilitates specificity of the phosphatase for the respective substrate. For example, it was found that the PP2A B'α subunit binds to CHK2 and thereby marks it for dephosphorylation [255].

When the microarray data obtained during this study was searched for genes that were apparently controlled by HDAC activity, one gene encoding for regulatory subunits of PP2A appeared. Expression of the gene *PPP2R3A* yields two protein isoforms called PR72 and PR130 and a third transcript variant that has not been further described [193]. Since PR72 is exclusively found in heart and skeletal muscle tissue, only the level of PR130 was examined in HCT116 cells [101,194]. Quantitative RT-PCR revealed that *PPP2R3A* expression was not affected by HU over 24 hours of incubation (Figure 7.13 A). Also western blot analysis showed comparable signals for PR130 in HU-treated and control samples (Figure 7.13 B). If

MS-275 was applied, mRNA levels of *PPP2R3A* were significantly increased independent of the presence of HU (Figure 7.13 A). More than six hours of HDAC inhibition were necessary to elevate the mRNA amount. Western blot analysis showed that PR130 protein nicely increased with rising concentrations of MS-275 (Figure 7.13 B) and knockdown of HDAC1 and HDAC2 was sufficient to show a strong induction of PR130 (Figure 7.16). These results point to a role of solely HDAC1 and HDAC2 in suppressing expression of the PP2A regulatory subunit PR130 under normal cellular conditions. It is still unclear how both HDACs repress *PPP2R3A*. Either, epigenetic gene regulation by histone deacetylation or direct transcriptional repression is possible. The need to eliminate both, HDAC1 and HDAC2, for the induction of PR130 seems plausible for the latter scenario, because they share overlapping functions and are part of the same transcriptional co-repressor complexes, e.g. mSIN3A [116,123]. Interestingly, the more PR130 was accumulated by MS-275 the stronger HU-induced ATM phosphorylation was lost. This suggested a link between PR130 and ATM activity (Figure 7.13 B).

Until today, PR130 was shown to modulate Wnt signaling [256] and to affect epidermal growth factor (EGF)-mediated signaling by the EGF receptor (EGFR) [257]. PR130 is also associated with the huge scaffolding protein CG-NAP, although the function of the PP2A subunit in this complex remains to be defined [258]. Within cells, PR130 localizes in the cytosol as well as in the nucleus [259-261]. Cellular fractionation of HCT116 cells revealed that the regulatory subunit mainly localized in the cytoplasm and only resided to a small extent in the nucleus (Figure 7.14). MS-275 did not visibly change the balance of this distribution and only elevated the amount of PR130 protein in both compartments. In the same experiment, pATM appeared exclusively in the nucleus. So, if ATM would actually serve as a substrate for PP2A-PR130, just a minor proportion of all PR130 detected in whole cell lysates would be capable to target phosphorylated ATM molecules.

The potential role of PR130 in particular and not PP2A in general in the regulation of ATM Ser1981 phosphorylation was investigated by transfecting PR130-targeted siRNA. The MS-275-induced upregulation of the phosphatase subunit could be efficiently blocked by the specific siRNA. PR130 levels could be reduced to below the basal value in the control sample (Figure 7.17). The elimination of PR130 in HCT116 cells treated with HU and MS-275 had the same effect like inhibition of PP2A as it rescued phosphorylation of ATM (Figure 7.17 and 7.12). To further study the interaction of the phosphatase with its suggested substrate, pSer1981 ATM was precipitated from whole cell lysates of previously fixed cells. To enhance

the usually low amount of phosphorylated ATM molecules after treatment with HU and MS-275 and thereby increase the number of possible PP2A interaction partners, OA was applied (Figure 7.18). Western blots of immunoprecipitates indeed displayed a clear signal for PR130 in pATM-precipitates, which was not the result of unspecific binding of PR130 to sepharose beads ("no AB") or IgG antibody ("Pre"). In addition, the detected signals only reflect complexes that must have existed in the nucleus prior to lysis. Fixation of the cells at the end of the treatment period should have prevented the artefact of an interaction between cytosolic PR130 and nuclear pATM after dissolution of the compartments. Hence, PR130 and pATM can physically interact with each other, whereby both might be part of a larger protein complex.

The results obtained in this study create reasonable evidence that PR130 is part of a phosphatase complex, which mediates deactivation of at least ATM kinase (Figure 7.13 B, 7.17, and 7.18). The degree of ATM dephosphorylation after replication stress is determined by the abundance of PR130, since regulatory subunits are the limiting factor for the assembly of active PP2A holoenzymes [259]. Interestingly, PR130 was identified as a potential target of CHK1 and CHK2 phosphorylation by using *in vitro* kinase assays [262]. This could enable a feedback regulation in cells. Under normal cellular conditions, specifically HDAC1 and HDAC2 mediate transcriptional repression of *PPP2R3A.* Thereby, they facilitate a sustained response of a cell to occurring lesions in the DNA (Figure 7.16 and 7.15 A). Suppression of HDAC1 and HDAC2 activity by MS-275 triggered a delayed expression of PR130 (Figure 7.13). This correlates with the proper activation of replication stress signaling in the beginning as well as its subsequent decline (Figure 7.1 A and 7.11). Perspectively, it would be of great interest to find out how HDAC1 and HDAC2 repress *PPP2R3A*. A regulation via epigenetic modification or modulation of the respective transcription factor(s) is possible.

To draw more general conclusions about the functions of PP2A-PR130 in mediation of the cellular replication stress response, additional experiments need to be carried out. For example, the impact of ectopic PR130 overexpression on HU-induced ATM phosphorylation should be monitored or *in vitro* dephosphorylation assays could be performed. Immunofluorescence studies should reveal whether the phosphatase subunit co-localizes at DNA damage foci with γH2A.X and ATM. It is also of high importance to find out whether ATR phosphorylation is also counteracted by PP2A-PR130 holoenzymes, since ATR is the main DDR kinase activated by stalled replication forks during HU treatment. There is

evidence that also phosphorylated ATR is a substrate of PP2A [263], although ATR activity was induced with IR in the respective experiments. Considering the close relation to ATM [264], it seems conceivable that also ATR is regulated by PR130. Thus, knockdown experiments as shown in Figure 7.17 need to be performed with respect to phosphorylated ATR and DDR downstream signaling. Also co-localization of phosphorylated ATR with PR130 should be tested and an antibody for Thr1989 would be most appropriate [22,23]. Last but not least, the biological roles of PR130 should be addressed in greater detail. One approach would be to compare the cell cycle profiles of HU+MS-275-treated cells with high (siCtrl) and repressed (siPR130) PR130 levels. If the PP2A subunit was involved in the positive control of cell cycle progression, a knockdown of PR130 should restore a sustained HU-mediated cell cycle arrest in S phase. In an opposing approach, cell cycle profiles of HU-treated cells with elevated PR130 by MS-275 and transient overexpression could be compared.

8.5 Summarizing model and conclusions

In summary, this study revealed that HDACs modulate the DNA damage response after replication stress and it also provides a possible mechanistic link for this interference. The following model is proposed:

Replication forks that became stalled during treatment of cancer cells with hydroxyurea trigger a DDR encompassing the apical kinases ATR and ATM, the mediator kinases CHK1 and CHK2, and the effector p53 (Figure 8.1). The cascade-like phosphorylation of these proteins facilitates the cell´s response to inhibited DNA synthesis. It includes p53 target gene expression, activation of cell cycle checkpoints to arrest cells with perturbed S phase, and apoptosis as a last resort to eliminate severely damaged cells. A simultaneous inhibition of HDAC1 and HDAC2 activity in HU-treated cells, however, suppresses DNA damage signaling. The loss of HDAC1/2 activity abolishes the transcriptional repression of the gene *PPP2R3A* which encodes the PP2A B subunit PR130. As a regulatory subunit of PP2A, PR130 specifically mediates substrate specificity for apical DDR kinases. Accumulation of PR130 through MS-275 increases dephosphorylation and therefore deactivation of ATM and ATR. Consequently, the entire DDR downstream signaling pathway is diminished, which also impairs transcriptional activity of p53. All this contributes to insufficient cell cycle checkpoint activation, subsequent reentry of unrepaired cells with impaired DNA synthesis capacity into the cell cycle, replication fork collapse, and accumulation of DNA damage. On the biological level, cells show a higher susceptibility for apoptotic cell death. It was not directly

demonstrated that ATR phosphorylation is counteracted by PP2A-PR130. Yet, experiments with specific kinase inhibitors revealed that deprivation of neither ATR nor ATM activity alone accounts for all effects obtained with MS-275. This is why both apical kinases have to be negatively regulated by the HDAC inhibitor. Furthermore, the amount of phosphorylated CHK1 protein, which is a common readout for catalytically active ATR [63], was decreased by MS-275 and restored with PP2A inhibitors. This supports the idea of MS-275-mediated ATR dephosphorylation by PP2A phosphatase complexes though it cannot be completely ruled out that CHK1 phosphorylation is also modulated by MS-275-induced PP2A activity.

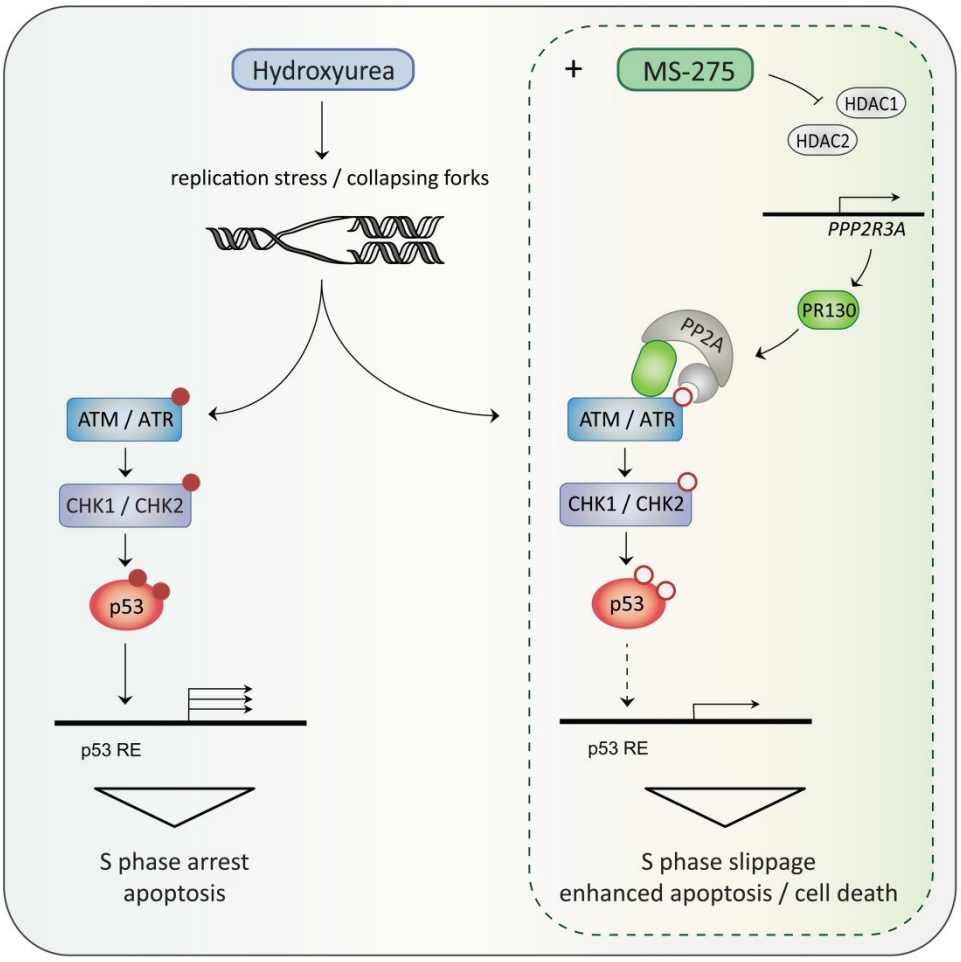

Figure 8.1: Summarizing model for the modulation of the DDR by histone deacetylases 1 and 2.

The image shows a schematic summary of the project´s most important discoveries. Herein, the cell´s condition after hydroxyurea treatment is directly compared to that with additional inhibition of HDAC1 and HDAC2. Further descriptions can be found in the text below. Filled red bullets indicate phosphorylation. Framed red bullets indicate low rates of phosphorylation. p53 RE: p53 response element.

Overexpression of HDAC1 and HDAC2 is found in a variety of tumors and correlates with poor patient prognosis [120,123,124]. According to the results of this study, these cancer entities should show strong repression of PR130 expression and thus little PP2A-PR130 activity. The

phosphatase PP2A is generally considered as tumor suppressor and its functions are often repressed or abolished in solid tumors and leukemia [102]. Experiments in immortalized, non-tumorigenic HEK TER cells also revealed that a loss of PR130 was associated with cell transformation. In this system, the shRNA-mediated suppression of PR130 increased the expression and activity of the proto-oncogene cMyc [265]. The administration of HDAC inhibitors would cancel transcriptional repression and elevate PR130 levels in HDAC1/2 overexpressing tumors and probably also normal cells. Rapidly dividing, cancerous cells are by nature prone to intrinsic replicational stress [187,241]. An exposure to replication inhibitors or genotoxic compounds therefore causes stronger damage in tumorigenic cells. The increase in PR130 protein by simultaneous exposure to HDACi like MS-275 would interfere with the subsequent onset of sustained DDR signaling via ATM/ATR and cell cycle arrest in S or G2 phase. The resulting lack of time for proper repair of damaged DNA would especially trouble tumor cells. Most cancers are moreover p53-deficient, which abrogates induction of the G1 checkpoint [266] – the only remaining cell cycle checkpoint of the interphase if ATR and CHK1 signaling is disturbed [243,244]. All these mechanisms might contribute to the understanding, why HDAC inhibitors are highly tumor cell specific compounds and positively affect the therapeutic outcome in combination regimens with traditional chemotherapeutics.

9 LIST OF REFERENCES

1 Smith J., Tho L.M., Xu N. & Gillespie D.A. (2010) The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Advances in cancer research* **108:** 73-112

2 Shiloh Y. (2003) ATM and related protein kinases: safeguarding genome integrity. *Nature reviews. Cancer* **3:** 155-168

3 Matsuoka S., Ballif B.A., Smogorzewska A. *et al.* (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316:** 1160-1166

4 Linding R., Jensen L.J., Ostheimer G.J. *et al.* (2007) Systematic discovery of in vivo phosphorylation networks. *Cell* **129:** 1415-1426

5 Harper J.W. & Elledge S.J. (2007) The DNA damage response: ten years after. *Mol Cell* **28:** 739-745

6 Dai Y. & Grant S. (2010) New insights into checkpoint kinase 1 in the DNA damage response signaling network. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16:** 376-383

7 Shiloh Y. & Ziv Y. (2013) The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nature reviews. Molecular cell biology* **14:** 197-210

8 Gatei M., Sloper K., Sorensen C. *et al.* (2003) Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *The Journal of biological chemistry* **278:** 14806-14811

9 Stiff T., Walker S.A., Cerosaletti K. *et al.* (2006) ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *The EMBO journal* **25:** 5775-5782

10 d'Adda di Fagagna F. (2008) Living on a break: cellular senescence as a DNA-damage response. *Nature reviews. Cancer* **8:** 512-522

11 Patil M., Pabla N. & Dong Z. (2013) Checkpoint kinase 1 in DNA damage response and cell cycle regulation. *Cellular and molecular life sciences : CMLS* **70:** 4009-4021

12 Durocher D. & Jackson S.P. (2001) DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Current opinion in cell biology* **13:** 225-231

13 Savitsky K., Bar-Shira A., Gilad S. *et al.* (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268:** 1749-1753

14 Alderton G.K., Joenje H., Varon R. *et al.* (2004) Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway. *Human molecular genetics* **13:** 3127-3138

15 Brown E.J. & Baltimore D. (2000) ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes & development* **14:** 397-402

16 Cortez D., Guntuku S., Qin J. & Elledge S.J. (2001) ATR and ATRIP: partners in checkpoint signaling. *Science* **294:** 1713-1716

17 Nyberg K.A., Michelson R.J., Putnam C.W. & Weinert T.A. (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annual review of genetics* **36:** 617-656

18 Jazayeri A., Falck J., Lukas C. *et al.* (2006) ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nature cell biology* **8:** 37-45

19 Zou L. & Elledge S.J. (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300:** 1542-1548

20 Fanning E., Klimovich V. & Nager A.R. (2006) A dynamic model for replication protein A (RPA) function in DNA processing pathways. *Nucleic acids research* **34:** 4126-4137

21 Ball H.L., Ehrhardt M.R., Mordes D.A. *et al.* (2007) Function of a conserved checkpoint recruitment domain in ATRIP proteins. *Molecular and cellular biology* **27:** 3367-3377

22 Liu S., Shiotani B., Lahiri M. *et al.* (2011) ATR autophosphorylation as a molecular switch for checkpoint activation. *Mol Cell* **43:** 192-202

23 Nam E.A., Zhao R., Glick G.G. *et al.* (2011) Thr-1989 phosphorylation is a marker of active ataxia telangiectasia-mutated and Rad3-related (ATR) kinase. *The Journal of biological chemistry* **286:** 28707-28714

24 Bermudez V.P., Lindsey-Boltz L.A., Cesare A.J. *et al.* (2003) Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 1633-1638

25 Parrilla-Castellar E.R., Arlander S.J. & Karnitz L. (2004) Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA repair* **3:** 1009-1014

26 St Onge R.P., Besley B.D., Pelley J.L. & Davey S. (2003) A role for the phosphorylation of hRad9 in checkpoint signaling. *The Journal of biological chemistry* **278:** 26620-26628

27 Delacroix S., Wagner J.M., Kobayashi M., Yamamoto K. & Karnitz L.M. (2007) The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint

signaling via TopBP1. *Genes & development* **21:** 1472-1477

28 Takeishi Y., Ohashi E., Ogawa K. *et al.* (2010) Casein kinase 2-dependent phosphorylation of human Rad9 mediates the interaction between human Rad9-Hus1-Rad1 complex and TopBP1. *Genes to cells : devoted to molecular & cellular mechanisms* **15:** 761-771

29 Kumagai A., Lee J., Yoo H.Y. & Dunphy W.G. (2006) TopBP1 activates the ATR-ATRIP complex. *Cell* **124:** 943-955

30 Lee J., Kumagai A. & Dunphy W.G. (2007) The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. *The Journal of biological chemistry* **282:** 28036-28044

31 Bao S., Tibbetts R.S., Brumbaugh K.M. *et al.* (2001) ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. *Nature* **411:** 969-974

32 Wang X., Zou L., Lu T. *et al.* (2006) Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. *Mol Cell* **23:** 331-341

33 Smits V.A., Reaper P.M. & Jackson S.P. (2006) Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Current biology : CB* **16:** 150-159

34 Ward I.M. & Chen J. (2001) Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *The Journal of biological chemistry* **276:** 47759-47762

35 Cimprich K.A. & Cortez D. (2008) ATR: an essential regulator of genome integrity. *Nature reviews. Molecular cell biology* **9:** 616-627

36 Shiloh Y. (2006) The ATM-mediated DNA-damage response: taking shape. *Trends in biochemical sciences* **31:** 402-410

37 Bakkenist C.J. & Kastan M.B. (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421:** 499-506

38 Lee J.H. & Paull T.T. (2007) Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. *Oncogene* **26:** 7741-7748

39 Kozlov S.V., Graham M.E., Peng C. *et al.* (2006) Involvement of novel autophosphorylation sites in ATM activation. *The EMBO journal* **25:** 3504-3514

40 Kozlov S.V., Graham M.E., Jakob B. *et al.* (2011) Autophosphorylation and ATM activation: additional sites add to the complexity. *The Journal of biological chemistry* **286:** 9107-9119

41 Sun Y., Jiang X., Chen S., Fernandes N. & Price B.D. (2005) A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 13182-13187

42 You Z., Bailis J.M., Johnson S.A., Dilworth S.M. & Hunter T. (2007) Rapid activation of ATM on DNA flanking double-strand breaks. *Nature cell biology* **9:** 1311-1318

43 Soutoglou E. & Misteli T. (2008) Activation of the cellular DNA damage response in the absence of DNA lesions. *Science* **320:** 1507-1510

44 Lee J.H. & Paull T.T. (2005) ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* **308:** 551-554

45 Bhatti S., Kozlov S., Farooqi A.A. *et al.* (2011) ATM protein kinase: the linchpin of cellular defenses to stress. *Cellular and molecular life sciences : CMLS* **68:** 2977-3006

46 Burma S., Chen B.P., Murphy M., Kurimasa A. & Chen D.J. (2001) ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *The Journal of biological chemistry* **276:** 42462-42467

47 Stewart G.S., Wang B., Bignell C.R., Taylor A.M. & Elledge S.J. (2003) MDC1 is a mediator of the mammalian DNA damage checkpoint. *Nature* **421:** 961-966

48 Stucki M., Clapperton J.A., Mohammad D. *et al.* (2005) MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell* **123:** 1213-1226

49 Lukas C., Melander F., Stucki M. *et al.* (2004) Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. *The EMBO journal* **23:** 2674-2683

50 Lou Z., Minter-Dykhouse K., Franco S. *et al.* (2006) MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals. *Mol Cell* **21:** 187-200

51 Lavin M.F. (2008) Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. *Nature reviews. Molecular cell biology* **9:** 759-769

52 Lee J.H., Goodarzi A.A., Jeggo P.A. & Paull T.T. (2010) 53BP1 promotes ATM activity through direct interactions with the MRN complex. *The EMBO journal* **29:** 574-585

53 Gatei M., Jakob B., Chen P. *et al.* (2011) ATM protein-dependent phosphorylation of Rad50 protein regulates DNA repair and cell cycle control. *The Journal of biological chemistry* **286:** 31542-31556

54 Rahal E.A., Henricksen L.A., Li Y. *et al.* (2010) ATM regulates Mre11-dependent DNA end-degradation and microhomology-mediated end joining. *Cell cycle* **9:** 2866-2877

55 Buscemi G., Savio C., Zannini L. *et al.* (2001) Chk2 activation dependence on Nbs1 after DNA damage. *Molecular and cellular biology* **21:** 5214-5222

56 Girard P.M., Riballo E., Begg A.C., Waugh A. & Jeggo P.A. (2002) Nbs1 promotes ATM dependent phosphorylation events including those required for G1/S arrest. *Oncogene* **21:** 4191-4199

57 Matsuoka S., Rotman G., Ogawa A. *et al.* (2000) Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 10389-10394

58 Ahn J.Y., Schwarz J.K., Piwnica-Worms H. & Canman C.E. (2000) Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer research* **60:** 5934-5936

59 Ahn J.Y., Li X., Davis H.L. & Canman C.E. (2002) Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of the Chk2 protein kinase via the forkhead-associated domain. *The Journal of biological chemistry* **277:** 19389-19395

60 Bartek J., Falck J. & Lukas J. (2001) CHK2 kinase--a busy messenger. *Nature reviews. Molecular cell biology* **2:** 877-886

61 Ward I.M., Wu X. & Chen J. (2001) Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *The Journal of biological chemistry* **276:** 47755-47758

62 Lee C.H. & Chung J.H. (2001) The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation. *The Journal of biological chemistry* **276:** 30537-30541

63 Zhao H. & Piwnica-Worms H. (2001) ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Molecular and cellular biology* **21:** 4129-4139

64 Capasso H., Palermo C., Wan S. *et al.* (2002) Phosphorylation activates Chk1 and is required for checkpoint-mediated cell cycle arrest. *Journal of cell science* **115:** 4555-4564

65 Tapia-Alveal C., Calonge T.M. & O'Connell M.J. (2009) Regulation of chk1. *Cell division* **4:** 8

66 Bartek J. & Lukas J. (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer cell* **3:** 421-429

67 Boutros R., Lobjois V. & Ducommun B. (2007) CDC25 phosphatases in cancer cells: key players? Good targets? *Nature reviews. Cancer* **7:** 495-507

68 Shen T. & Huang S. (2012) The role of Cdc25A in the regulation of cell proliferation and apoptosis. *Anti-cancer agents in medicinal chemistry* **12:** 631-639

69 Moore J.D. (2013) In the wrong place at the wrong time: does cyclin mislocalization drive oncogenic transformation? *Nature reviews. Cancer* **13:** 201-208

70 Medema R.H. & Macurek L. (2012) Checkpoint control and cancer. *Oncogene* **31:** 2601-2613

71 Sorensen C.S. & Syljuasen R.G. (2012) Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. *Nucleic acids research* **40:** 477-486

72 Liu P., Barkley L.R., Day T. *et al.* (2006) The Chk1-mediated S-phase checkpoint targets initiation factor Cdc45 via a Cdc25A/Cdk2-independent mechanism. *The Journal of biological chemistry* **281:** 30631-30644

73 Heffernan T.P., Unsal-Kacmaz K., Heinloth A.N. *et al.* (2007) Cdc7-Dbf4 and the human S checkpoint response to UVC. *The Journal of biological chemistry* **282:** 9458-9468

74 Shimada M., Niida H., Zineldeen D.H. *et al.* (2008) Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. *Cell* **132:** 221-232

75 Banin S., Moyal L., Shieh S. *et al.* (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281:** 1674-1677

76 Canman C.E., Lim D.S., Cimprich K.A. *et al.* (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281:** 1677-1679

77 Khanna K.K., Keating K.E., Kozlov S. *et al.* (1998) ATM associates with and phosphorylates p53: mapping the region of interaction. *Nature genetics* **20:** 398-400

78 Haupt Y., Maya R., Kazaz A. & Oren M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387:** 296-299

79 Oren M., Damalas A., Gottlieb T. *et al.* (2002) Regulation of p53: intricate loops and delicate balances. *Biochemical pharmacology* **64:** 865-871

80 Tibbetts R.S., Brumbaugh K.M., Williams J.M. *et al.* (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes & development* **13:** 152-157

81 Shieh S.Y., Ahn J., Tamai K., Taya Y. & Prives C. (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes & development* **14:** 289-300

82 Hirao A., Kong Y.Y., Matsuoka S. *et al.* (2000) DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* **287:** 1824-1827

83 Kruse J.P. & Gu W. (2009) Modes of p53 regulation. *Cell* **137:** 609-622

84 Gu B. & Zhu W.G. (2012) Surf the post-translational modification network of p53 regulation. *International journal of biological sciences* **8:** 672-684

85 Khosravi R., Maya R., Gottlieb T. *et al.* (1999) Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA

damage. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 14973-14977

86 Wade M., Li Y.C. & Wahl G.M. (2013) MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nature reviews. Cancer* **13:** 83-96

87 Olsson A., Manzl C., Strasser A. & Villunger A. (2007) How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? *Cell death and differentiation* **14:** 1561-1575

88 Riley T., Sontag E., Chen P. & Levine A. (2008) Transcriptional control of human p53-regulated genes. *Nature reviews. Molecular cell biology* **9:** 402-412

89 Sullivan K.D., Gallant-Behm C.L., Henry R.E., Fraikin J.L. & Espinosa J.M. (2012) The p53 circuit board. *Biochimica et biophysica acta* **1825:** 229-244

90 Brugarolas J., Moberg K., Boyd S.D. *et al.* (1999) Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 1002-1007

91 Tan T. & Chu G. (2002) p53 Binds and activates the xeroderma pigmentosum DDB2 gene in humans but not mice. *Molecular and cellular biology* **22:** 3247-3254

92 Batchelor E., Loewer A. & Lahav G. (2009) The ups and downs of p53: understanding protein dynamics in single cells. *Nature reviews. Cancer* **9:** 371-377

93 Nakano K. & Vousden K.H. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7:** 683-694

94 Youle R.J. & Strasser A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews. Molecular cell biology* **9:** 47-59

95 Green D.R. & Kroemer G. (2009) Cytoplasmic functions of the tumour suppressor p53. *Nature* **458:** 1127-1130

96 Cohen P. (2001) The role of protein phosphorylation in human health and disease. The Sir Hans Krebs Medal Lecture. *European journal of biochemistry / FEBS* **268:** 5001-5010

97 Moorhead G.B., Trinkle-Mulcahy L. & Ulke-Lemee A. (2007) Emerging roles of nuclear protein phosphatases. *Nature reviews. Molecular cell biology* **8:** 234-244

98 Fellner T., Lackner D.H., Hombauer H. *et al.* (2003) A novel and essential mechanism determining specificity and activity of protein phosphatase 2A (PP2A) in vivo. *Genes & development* **17:** 2138-2150

99 Hombauer H., Weismann D., Mudrak I. *et al.* (2007) Generation of active protein phosphatase 2A is coupled to holoenzyme assembly. *PLoS biology* **5:** e155

100 Seshacharyulu P., Pandey P., Datta K. & Batra S.K. (2013) Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer letters* **335:** 9-18

101 Janssens V. & Goris J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *The Biochemical journal* **353:** 417-439

102 Perrotti D. & Neviani P. (2013) Protein phosphatase 2A: a target for anticancer therapy. *The lancet oncology* **14:** e229-238

103 Eichhorn P.J., Creyghton M.P. & Bernards R. (2009) Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et biophysica acta* **1795:** 1-15

104 Bononi A., Agnoletto C., De Marchi E. *et al.* (2011) Protein kinases and phosphatases in the control of cell fate. *Enzyme research* **2011:** 329098

105 Brautigan D.L. (1995) Flicking the switches: phosphorylation of serine/threonine protein phosphatases. *Seminars in cancer biology* **6:** 211-217

106 Tolstykh T., Lee J., Vafai S. & Stock J.B. (2000) Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits. *The EMBO journal* **19:** 5682-5691

107 Shouse G.P., Nobumori Y., Panowicz M.J. & Liu X. (2011) ATM-mediated phosphorylation activates the tumor-suppressive function of B56gamma-PP2A. *Oncogene* **30:** 3755-3765

108 Muller S. & Kramer O.H. (2010) Inhibitors of HDACs--effective drugs against cancer? *Current cancer drug targets* **10:** 210-228

109 Durrin L.K., Mann R.K., Kayne P.S. & Grunstein M. (1991) Yeast histone H4 N-terminal sequence is required for promoter activation in vivo. *Cell* **65:** 1023-1031

110 Thiagalingam S., Cheng K.H., Lee H.J. *et al.* (2003) Histone deacetylases: unique players in shaping the epigenetic histone code. *Annals of the New York Academy of Sciences* **983:** 84-100

111 Spange S., Wagner T., Heinzel T. & Kramer O.H. (2009) Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *The international journal of biochemistry & cell biology* **41:** 185-198

112 Ocker M. (2010) Deacetylase inhibitors - focus on non-histone targets and effects. *World journal of biological chemistry* **1:** 55-61

113 Ververis K., Hiong A., Karagiannis T.C. & Licciardi P.V. (2013) Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. *Biologics : targets & therapy* **7:** 47-60

114 de Ruijter A.J., van Gennip A.H., Caron H.N., Kemp S. & van Kuilenburg A.B. (2003) Histone

deacetylases (HDACs): characterization of the classical HDAC family. *The Biochemical journal* **370:** 737-749

115 Buck S.W., Gallo C.M. & Smith J.S. (2004) Diversity in the Sir2 family of protein deacetylases. *Journal of leukocyte biology* **75:** 939-950

116 Yang X.J. & Seto E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nature reviews. Molecular cell biology* **9:** 206-218

117 Martin M., Kettmann R. & Dequiedt F. (2007) Class IIa histone deacetylases: regulating the regulators. *Oncogene* **26:** 5450-5467

118 Gregoretti I.V., Lee Y.M. & Goodson H.V. (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *Journal of molecular biology* **338:** 17-31

119 Denu J.M. (2005) The Sir 2 family of protein deacetylases. *Current opinion in chemical biology* **9:** 431-440

120 Bolden J.E., Peart M.J. & Johnstone R.W. (2006) Anticancer activities of histone deacetylase inhibitors. *Nature reviews. Drug discovery* **5:** 769-784

121 Bernstein B.E., Meissner A. & Lander E.S. (2007) The mammalian epigenome. *Cell* **128:** 669-681

122 Wang Z., Zang C., Cui K. *et al.* (2009) Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell* **138:** 1019-1031

123 Spiegel S., Milstien S. & Grant S. (2012) Endogenous modulators and pharmacological inhibitors of histone deacetylases in cancer therapy. *Oncogene* **31:** 537-551

124 Weichert W., Roske A., Niesporek S. *et al.* (2008) Class I histone deacetylase expression has independent prognostic impact in human colorectal cancer: specific role of class I histone deacetylases in vitro and in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14:** 1669-1677

125 Montgomery R.L., Davis C.A., Potthoff M.J. *et al.* (2007) Histone deacetylases 1 and 2 redundantly regulate cardiac morphogenesis, growth, and contractility. *Genes & development* **21:** 1790-1802

126 Montgomery R.L., Hsieh J., Barbosa A.C., Richardson J.A. & Olson E.N. (2009) Histone deacetylases 1 and 2 control the progression of neural precursors to neurons during brain development. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 7876-7881

127 Wilting R.H., Yanover E., Heideman M.R. *et al.* (2010) Overlapping functions of Hdac1 and Hdac2 in cell cycle regulation and haematopoiesis. *The EMBO journal* **29:** 2586-2597

128 Lagger G., O'Carroll D., Rembold M. *et al.* (2002) Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *The EMBO journal* **21:** 2672-2681

129 Huang B.H., Laban M., Leung C.H. *et al.* (2005) Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell death and differentiation* **12:** 395-404

130 Miller K.M., Tjeertes J.V., Coates J. *et al.* (2010) Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining. *Nature structural & molecular biology* **17:** 1144-1151

131 Wen Y.D., Perissi V., Staszewski L.M. *et al.* (2000) The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 7202-7207

132 Guenther M.G., Lane W.S., Fischle W. *et al.* (2000) A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. *Genes & development* **14:** 1048-1057

133 Ishii S., Kurasawa Y., Wong J. & Yu-Lee L.Y. (2008) Histone deacetylase 3 localizes to the mitotic spindle and is required for kinetochore-microtubule attachment. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 4179-4184

134 Eot-Houllier G., Fulcrand G., Watanabe Y., Magnaghi-Jaulin L. & Jaulin C. (2008) Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion. *Genes & development* **22:** 2639-2644

135 Li Y., Kao G.D., Garcia B.A. *et al.* (2006) A novel histone deacetylase pathway regulates mitosis by modulating Aurora B kinase activity. *Genes & development* **20:** 2566-2579

136 Bhaskara S., Chyla B.J., Amann J.M. *et al.* (2008) Deletion of histone deacetylase 3 reveals critical roles in S phase progression and DNA damage control. *Mol Cell* **30:** 61-72

137 Bhaskara S., Knutson S.K., Jiang G. *et al.* (2010) Hdac3 is essential for the maintenance of chromatin structure and genome stability. *Cancer cell* **18:** 436-447

138 Montgomery R.L., Potthoff M.J., Haberland M. *et al.* (2008) Maintenance of cardiac energy metabolism by histone deacetylase 3 in mice. *The Journal of clinical investigation* **118:** 3588-3597

139 Miller T.A., Witter D.J. & Belvedere S. (2003) Histone deacetylase inhibitors. *Journal of medicinal chemistry* **46:** 5097-5116

140 Minucci S. & Pelicci P.G. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nature reviews. Cancer* **6:** 38-51

141 Marks P.A. (2010) Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. *Biochimica et biophysica acta* **1799:** 717-725

142 Burgess A., Ruefli A., Beamish H. *et al.* (2004) Histone deacetylase inhibitors specifically kill nonproliferating tumour cells. *Oncogene* **23:** 6693-6701

143 National-Institutes-of-Health. *clinical trials on HDAC inhibitors*, <http://clinicaltrials.gov/ct2/results?term=HDAC+inhibitor> (03-20-2014).

144 Dokmanovic M. & Marks P.A. (2005) Prospects: histone deacetylase inhibitors. *Journal of cellular biochemistry* **96:** 293-304

145 Ma X., Ezzeldin H.H. & Diasio R.B. (2009) Histone deacetylase inhibitors: current status and overview of recent clinical trials. *Drugs* **69:** 1911-1934

146 Marks P.A. & Breslow R. (2007) Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nature biotechnology* **25:** 84-90

147 Campas-Moya C. (2009) Romidepsin for the treatment of cutaneous T-cell lymphoma. *Drugs of today* **45:** 787-795

148 Bradner J.E., Mak R., Tanguturi S.K. *et al.* (2010) Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 12617-12622

149 National-Institutes-of-Health. *clinical trials on MS-275*, <http://clinicaltrials.gov/ct2/results?term=Entinostat&pg=1 > (03-19-2014).

150 Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685

151 Hehlmann R., Heimpel H., Hasford J. *et al.* (1993) Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. The German CML Study Group. *Blood* **82:** 398-407

152 Harousseau J.L., Martinelli G., Jedrzejczak W.W. *et al.* (2009) A randomized phase 3 study of tipifarnib compared with best supportive care, including hydroxyurea, in the treatment of newly diagnosed acute myeloid leukemia in patients 70 years or older. *Blood* **114:** 1166-1173

153 Vokes E.E., Haraf D.J., Brockstein B.E. & Weichselbaum R.R. (1999) Paclitaxel, 5-fluorouracil, hydroxyurea, and concomitant radiation therapy for poor-prognosis head and neck cancer. *Semin Radiat Oncol* **9:** 70-76

154 Saban N. & Bujak M. (2009) Hydroxyurea and hydroxamic acid derivatives as antitumor drugs. *Cancer Chemother Pharmacol* **64:** 213-221

155 Elford H.L. (1968) Effect of hydroxyurea on ribonucleotide reductase. *Biochem Biophys Res Commun* **33:** 129-135

156 Kramer O.H., Knauer S.K., Zimmermann D., Stauber R.H. & Heinzel T. (2008) Histone deacetylase inhibitors and hydroxyurea modulate the cell cycle and cooperatively induce apoptosis. *Oncogene* **27:** 732-740

157 Stauber R.H., Knauer S.K., Habtemichael N. *et al.* (2012) A combination of a ribonucleotide reductase inhibitor and histone deacetylase inhibitors downregulates EGFR and triggers BIM-dependent apoptosis in head and neck cancer. *Oncotarget* **3:** 31-43

158 Waldman T., Kinzler K.W. & Vogelstein B. (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer research* **55:** 5187-5190

159 Waldman T., Lengauer C., Kinzler K.W. & Vogelstein B. (1996) Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* **381:** 713-716

160 Bunz F., Dutriaux A., Lengauer C. *et al.* (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282:** 1497-1501

161 Grem J.L. (2000) 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Investigational new drugs* **18:** 299-313

162 Longley D.B., Harkin D.P. & Johnston P.G. (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews. Cancer* **3:** 330-338

163 Zhang N., Yin Y., Xu S.J. & Chen W.S. (2008) 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules* **13:** 1551-1569

164 Liu Y. & Kulesz-Martin M. (2001) p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. *Carcinogenesis* **22:** 851-860

165 Lavin M.F. & Gueven N. (2006) The complexity of p53 stabilization and activation. *Cell death and differentiation* **13:** 941-950

166 D'Orazi G., Cecchinelli B., Bruno T. *et al.* (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nature cell biology* **4:** 11-19

167 Taira N., Nihira K., Yamaguchi T., Miki Y. & Yoshida K. (2007) DYRK2 is targeted to the nucleus and controls p53 via Ser46 phosphorylation in the apoptotic response to DNA damage. *Mol Cell* **25:** 725-738

168 Woo G.H., Bak E.J., Nakayama H. & Doi K. (2006) Molecular mechanisms of hydroxyurea(HU)-induced apoptosis in the mouse fetal brain. *Neurotoxicology and teratology* **28:** 125-134

169 Schneider G., Henrich A., Greiner G. *et al.* (2010) Cross talk between stimulated NF-kappaB and the tumor suppressor p53. *Oncogene* **29:** 2795-2806

170 Gartel A.L., Serfas M.S. & Tyner A.L. (1996) p21--negative regulator of the cell cycle. *Proceedings of*

the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine **213:** 138-149

171 Wu X., Bayle J.H., Olson D. & Levine A.J. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes & development* **7:** 1126-1132

172 Lu X., Ma O., Nguyen T.A. *et al.* (2007) The Wip1 Phosphatase acts as a gatekeeper in the p53-Mdm2 autoregulatory loop. *Cancer cell* **12:** 342-354

173 Sowa Y., Orita T., Minamikawa S. *et al.* (1997) Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. *Biochem Biophys Res Commun* **241:** 142-150

174 Rogakou E.P., Pilch D.R., Orr A.H., Ivanova V.S. & Bonner W.M. (1998) DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *The Journal of biological chemistry* **273:** 5858-5868

175 Pilch D.R., Sedelnikova O.A., Redon C. *et al.* (2003) Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **81:** 123-129

176 Errico A. & Costanzo V. (2012) Mechanisms of replication fork protection: a safeguard for genome stability. *Critical reviews in biochemistry and molecular biology* **47:** 222-235

177 Koc A., Wheeler L.J., Mathews C.K. & Merrill G.F. (2004) Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *The Journal of biological chemistry* **279:** 223-230

178 Alvino G.M., Collingwood D., Murphy J.M. *et al.* (2007) Replication in hydroxyurea: it's a matter of time. *Molecular and cellular biology* **27:** 6396-6406

179 Johnson C.A., Forster T.H., Winterford C.M. & Allan D.J. (1992) Hydroxyurea induces apoptosis and regular DNA fragmentation in a Burkitt's lymphoma cell line. *Biochimica et biophysica acta* **1136:** 1-4

180 Gui C.Y., Jiang C., Xie H.Y. & Qian R.L. (1997) The apoptosis of HEL cells induced by hydroxyurea. *Cell research* **7:** 91-97

181 Tewari M., Quan L.T., O'Rourke K. *et al.* (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81:** 801-809

182 Patel T., Gores G.J. & Kaufmann S.H. (1996) The role of proteases during apoptosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **10:** 587-597

183 Kaufmann S.H., Desnoyers S., Ottaviano Y., Davidson N.E. & Poirier G.G. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer research* **53:** 3976-3985

184 Vermes I., Haanen C., Steffens-Nakken H. & Reutelingsperger C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of immunological methods* **184:** 39-51

185 Lopez-Contreras A.J. & Fernandez-Capetillo O. (2010) The ATR barrier to replication-born DNA damage. *DNA repair* **9:** 1249-1255

186 Busby E.C., Leistritz D.F., Abraham R.T., Karnitz L.M. & Sarkaria J.N. (2000) The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. *Cancer research* **60:** 2108-2112

187 Toledo L.I., Murga M., Zur R. *et al.* (2011) A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. *Nature structural & molecular biology* **18:** 721-727

188 Hickson I., Zhao Y., Richardson C.J. *et al.* (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer research* **64:** 9152-9159

189 Qi Y., Schoene N.W., Lartey F.M. & Cheng W.H. (2010) Selenium compounds activate ATM-dependent DNA damage response via the mismatch repair protein hMLH1 in colorectal cancer cells. *The Journal of biological chemistry* **285:** 33010-33017

190 Zakikhani M., Bazile M., Hashemi S. *et al.* (2012) Alterations in cellular energy metabolism associated with the antiproliferative effects of the ATM inhibitor KU-55933 and with metformin. *PloS one* **7:** e49513

191 Honkanen R.E. (1993) Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. *FEBS letters* **330:** 283-286

192 Bialojan C. & Takai A. (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *The Biochemical journal* **256:** 283-290

193 NCBI. *PPP2R3A transcripts*, <http://www.ncbi.nlm.nih.gov/gene?cmd=Retrieve&dopt=full_report&list_uids=5523> (01-17-2014).

194 Hendrix P., Mayer-Jackel R.E., Cron P. *et al.* (1993) Structure and expression of a 72-kDa regulatory subunit of protein phosphatase 2A. Evidence for different size forms produced by alternative splicing. *The Journal of biological chemistry* **268:** 15267-15276

195 Brown K.D., Ziv Y., Sadanandan S.N. *et al.* (1997) The ataxia-telangiectasia gene product, a constitutively expressed nuclear protein that is not up-regulated following genome damage. *Proceedings of the National Academy of Sciences of the United States of America* **94:** 1840-1845

196 Watters D., Khanna K.K., Beamish H. *et al.* (1997) Cellular localisation of the ataxia-telangiectasia (ATM) gene product and discrimination between mutated and normal forms. *Oncogene* **14:** 1911-1921

197 Parker W.B. (2009) Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer. *Chemical reviews* **109:** 2880-2893

198 Szekeres T., Fritzer-Szekeres M. & Elford H. (1997) The enzyme ribonucleotide reductase: target for antitumor and anti-HIV therapy. *Critical reviews in clinical laboratory sciences* **34:** 503-528

199 Branzei D. & Foiani M. (2005) The DNA damage response during DNA replication. *Current opinion in cell biology* **17:** 568-575

200 Flis S., Gnyszka A., Flis K. & Splawinski J. (2010) MS275 enhances cytotoxicity induced by 5-fluorouracil in the colorectal cancer cells. *European journal of pharmacology* **627:** 26-32

201 Sorensen C.S., Hansen L.T., Dziegielewski J. *et al.* (2005) The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. *Nature cell biology* **7:** 195-201

202 Saintigny Y., Delacote F., Vares G. *et al.* (2001) Characterization of homologous recombination induced by replication inhibition in mammalian cells. *The EMBO journal* **20:** 3861-3870

203 Lundin C., Erixon K., Arnaudeau C. *et al.* (2002) Different roles for nonhomologous end joining and homologous recombination following replication arrest in mammalian cells. *Molecular and cellular biology* **22:** 5869-5878

204 Kim G.D., Choi Y.H., Dimtchev A. *et al.* (1999) Sensing of ionizing radiation-induced DNA damage by ATM through interaction with histone deacetylase. *The Journal of biological chemistry* **274:** 31127-31130

205 Tang Y., Zhao W., Chen Y., Zhao Y. & Gu W. (2008) Acetylation is indispensable for p53 activation. *Cell* **133:** 612-626

206 Ito A., Kawaguchi Y., Lai C.H. *et al.* (2002) MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. *The EMBO journal* **21:** 6236-6245

207 Brooks C. & Gu W. (2011) The impact of acetylation and deacetylation on the p53 pathway. *Protein & cell* **2:** 456-462

208 Gottifredi V., Shieh S., Taya Y. & Prives C. (2001) p53 accumulates but is functionally impaired when DNA synthesis is blocked. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 1036-1041

209 Sambucetti L.C., Fischer D.D., Zabludoff S. *et al.* (1999) Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. *The Journal of biological chemistry* **274:** 34940-34947

210 Richon V.M., Sandhoff T.W., Rifkind R.A. & Marks P.A. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 10014-10019

211 Beckerman R., Donner A.J., Mattia M. *et al.* (2009) A role for Chk1 in blocking transcriptional elongation of p21 RNA during the S-phase checkpoint. *Genes & development* **23:** 1364-1377

212 Thurn K.T., Thomas S., Raha P., Qureshi I. & Munster P.N. (2013) Histone deacetylase regulation of ATM-mediated DNA damage signaling. *Molecular cancer therapeutics* **12:** 2078-2087

213 Lee J.H., Choy M.L., Ngo L., Foster S.S. & Marks P.A. (2010) Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 14639-14644

214 Gaymes T.J., Padua R.A., Pla M. *et al.* (2006) Histone deacetylase inhibitors (HDI) cause DNA damage in leukemia cells: a mechanism for leukemia-specific HDI-dependent apoptosis? *Molecular cancer research : MCR* **4:** 563-573

215 Wang H., Zhou W., Zheng Z. *et al.* (2012) The HDAC inhibitor depsipeptide transactivates the p53/p21 pathway by inducing DNA damage. *DNA repair* **11:** 146-156

216 Ikura T., Tashiro S., Kakino A. *et al.* (2007) DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. *Molecular and cellular biology* **27:** 7028-7040

217 Macurek L., Lindqvist A., Voets O. *et al.* (2010) Wip1 phosphatase is associated with chromatin and dephosphorylates gammaH2AX to promote checkpoint inhibition. *Oncogene* **29:** 2281-2291

218 Cha H., Lowe J.M., Li H. *et al.* (2010) Wip1 directly dephosphorylates gamma-H2AX and attenuates the DNA damage response. *Cancer research* **70:** 4112-4122

219 Lu X., Nguyen T.A., Moon S.H. *et al.* (2008) The type 2C phosphatase Wip1: an oncogenic regulator of tumor suppressor and DNA damage response pathways. *Cancer metastasis reviews* **27:** 123-135

220 Arnaudeau C., Tenorio Miranda E., Jenssen D. & Helleday T. (2000) Inhibition of DNA synthesis is a potent mechanism by which cytostatic drugs induce homologous recombination in mammalian cells. *Mutation research* **461:** 221-228

221 Petermann E., Orta M.L., Issaeva N., Schultz N. & Helleday T. (2010) Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol Cell* **37:** 492-502

222 Kachhap S.K., Rosmus N., Collis S.J. *et al.* (2010) Downregulation of homologous recombination DNA repair genes by HDAC inhibition in prostate cancer is mediated through the E2F1 transcription factor. *PloS one* **5:** e11208

223 Adimoolam S., Sirisawad M., Chen J. *et al.* (2007) HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 19482-19487

224 Cazzalini O., Scovassi A.I., Savio M., Stivala L.A. & Prosperi E. (2010) Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutation research* **704:** 12-20

225 Symeonidou I.E., Taraviras S. & Lygerou Z. (2012) Control over DNA replication in time and space. *FEBS letters* **586:** 2803-2812

226 Painter R.B., Drew R.M. & Giauque B.G. (1960) Further studies on deoxyribonucleic acid metabolism in mammalian cell cultures. *Experimental cell research* **21:** 98-105

227 Petermann E. & Caldecott K.W. (2006) Evidence that the ATR/Chk1 pathway maintains normal replication fork progression during unperturbed S phase. *Cell cycle* **5:** 2203-2209

228 Nam E.A. & Cortez D. (2011) ATR signalling: more than meeting at the fork. *The Biochemical journal* **436:** 527-536

229 Bundy R., Marczin N., Chester A.H. & Yacoub M. (1999) Differential regulation of DNA synthesis by nitric oxide and hydroxyurea in vascular smooth muscle cells. *The American journal of physiology* **277:** H1799-1807

230 Lossaint G., Besnard E., Fisher D., Piette J. & Dulic V. (2011) Chk1 is dispensable for G2 arrest in response to sustained DNA damage when the ATM/p53/p21 pathway is functional. *Oncogene* **30:** 4261-4274

231 Dalgaard J.Z. (2012) Causes and consequences of ribonucleotide incorporation into nuclear DNA. *Trends in genetics : TIG* **28:** 592-597

232 Goksenin A.Y., Zahurancik W., LeCompte K.G. *et al.* (2012) Human DNA polymerase epsilon is able to efficiently extend from multiple consecutive ribonucleotides. *The Journal of biological chemistry* **287:** 42675-42684

233 Xu W., Parmigiani R. & Marks P. (2007) Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* **26:** 5541-5552

234 Liu Q., Guntuku S., Cui X.S. *et al.* (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes & development* **14:** 1448-1459

235 Takai H., Tominaga K., Motoyama N. *et al.* (2000) Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes & development* **14:** 1439-1447

236 Syljuasen R.G., Sorensen C.S., Hansen L.T. *et al.* (2005) Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage. *Molecular and cellular biology* **25:** 3553-3562

237 Myers K., Gagou M.E., Zuazua-Villar P., Rodriguez R. & Meuth M. (2009) ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. *PLoS genetics* **5:** e1000324

238 Takai H., Naka K., Okada Y. *et al.* (2002) Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. *The EMBO journal* **21:** 5195-5205

239 Hirao A., Cheung A., Duncan G. *et al.* (2002) Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Molecular and cellular biology* **22:** 6521-6532

240 Casper A.M., Nghiem P., Arlt M.F. & Glover T.W. (2002) ATR regulates fragile site stability. *Cell* **111:** 779-789

241 Toledo L., Altmeyer M., Rask M.-B. *et al.* (2013) ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* **155:** 1088-1103

242 Branzei D. & Foiani M. (2008) Regulation of DNA repair throughout the cell cycle. *Nature reviews. Molecular cell biology* **9:** 297-308

243 Nghiem P., Park P., Kim Y., Vaziri C. & Schreiber S. (2001) ATR inhibition selectively sensitizes G1 checkpoint-deficient cells to lethal premature chromatin condensation. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 9092-9097

244 Deckbar D., Stiff T., Koch B. *et al.* (2010) The limitations of the G1-S checkpoint. *Cancer research* **70:** 4412-4421

245 Saito A., Yamashita T., Mariko Y. *et al.* (1999) A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. *Proceedings of the National Academy of Sciences of the United States of America* **96:** 4592-4597

246 Senese S., Zaragoza K., Minardi S. *et al.* (2007) Role for histone deacetylase 1 in human tumor cell proliferation. *Molecular and cellular biology* **27:** 4784-4795

247 Brandl A., Wagner T., Uhlig K.M. *et al.* (2012) Dynamically regulated sumoylation of HDAC2 controls p53 deacetylation and restricts apoptosis following genotoxic stress. *Journal of molecular cell biology* **4:** 284-293

248 Lu X., Nguyen T.A. & Donehower L.A. (2005) Reversal of the ATM/ATR-mediated DNA damage response by the oncogenic phosphatase PPM1D. *Cell cycle* **4:** 1060-1064

249 Shreeram S., Demidov O.N., Hee W.K. *et al.* (2006) Wip1 phosphatase modulates ATM-dependent signaling pathways. *Mol Cell* **23:** 757-764

250 Peng A., Lewellyn A.L., Schiemann W.P. & Maller J.L. (2010) Repo-man controls a protein phosphatase 1-dependent threshold for DNA damage checkpoint activation. *Current biology : CB* **20:** 387-396

251 Ali A., Zhang J., Bao S. *et al.* (2004) Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. *Genes & development* **18:** 249-254

252 Goodarzi A.A., Jonnalagadda J.C., Douglas P. *et al.* (2004) Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. *The EMBO journal* **23:** 4451-4461

253 Leung-Pineda V., Ryan C.E. & Piwnica-Worms H. (2006) Phosphorylation of Chk1 by ATR is antagonized by a Chk1-regulated protein phosphatase 2A circuit. *Molecular and cellular biology* **26:** 7529-7538

254 Carlessi L., Buscemi G., Fontanella E. & Delia D. (2010) A protein phosphatase feedback mechanism regulates the basal phosphorylation of Chk2 kinase in the absence of DNA damage. *Biochimica et biophysica acta* **1803:** 1213-1223

255 Freeman A.K., Dapic V. & Monteiro A.N. (2010) Negative regulation of CHK2 activity by protein phosphatase 2A is modulated by DNA damage. *Cell cycle* **9:** 736-747

256 Creyghton M.P., Roel G., Eichhorn P.J. *et al.* (2006) PR130 is a modulator of the Wnt-signaling cascade that counters repression of the antagonist Naked cuticle. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 5397-5402

257 Zwaenepoel K., Goris J., Erneux C., Parker P.J. & Janssens V. (2010) Protein phosphatase 2A PR130/B''alpha1 subunit binds to the SH2 domain-containing inositol polyphosphate 5-phosphatase 2 and prevents epidermal growth factor (EGF)-induced EGF receptor degradation sustaining EGF-mediated signaling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24:** 538-547

258 Takahashi M., Shibata H., Shimakawa M. *et al.* (1999) Characterization of a novel giant scaffolding protein, CG-NAP, that anchors multiple signaling enzymes to centrosome and the golgi apparatus. *The Journal of biological chemistry* **274:** 17267-17274

259 Janssens V., Longin S. & Goris J. (2008) PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). *Trends in biochemical sciences* **33:** 113-121

260 Zwaenepoel K., Louis J.V., Goris J. & Janssens V. (2008) Diversity in genomic organisation, developmental regulation and distribution of the murine PR72/B'' subunits of protein phosphatase 2A. *BMC genomics* **9:** 393

261 Elong Edimo W., Derua R., Janssens V. *et al.* (2011) Evidence of SHIP2 Ser132 phosphorylation, its nuclear localization and stability. *The Biochemical journal* **439:** 391-401

262 Kim M.-A., Kim H.-J., Brown A. *et al.* (2007) Identification of novel substrates for human checkpoint kinase Chk1 and Chk2 through genome-wide screening using a consensus Chk phosphorylation motif. *Experimental & molecular medicine* **39:** 205-212

263 Yan Y., Cao P., Greer P. *et al.* (2010) Protein phosphatase 2A has an essential role in the activation of gamma-irradiation-induced G2/M checkpoint response. *Oncogene* **29:** 4317-4329

264 Mordes D.A. & Cortez D. (2008) Activation of ATR and related PIKKs. *Cell cycle* **7:** 2809-2812

265 Sablina A.A., Hector M., Colpaert N. & Hahn W.C. (2010) Identification of PP2A complexes and pathways involved in cell transformation. *Cancer research* **70:** 10474-10484

266 Dixon H. & Norbury C.J. (2002) Therapeutic exploitation of checkpoint defects in cancer cells lacking p53 function. *Cell cycle* **1:** 362-368

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Rauch A.*, Hennig D.*, **Schäfer C.***, Wirth M., Marx C., Schneider G. & Krämer O.H. (2014) Survivin and YM155: How faithful is the liaison? *Biochim Biophys Acta* **1845:** 202-220 (* equal contribution)

Dietrich S., Krämer O.H., Hahn E., **Schäfer C.**, Giese T., Hess M., Tretter T., Rieger M., Hüllein J., Zenz T., Ho A.D., Dreger P. & Luft T. (2012) Leflunomide induces apoptosis in fludarabine-resistant and clinically refractory CLL cells. *Clin Cancer Res* **18:** 417-431

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DECLARATION OF INDEPENDENT ASSIGNMENT - Eigenständigkeitserklärung

Die vorliegende Arbeit habe ich mit den angegebenen Hilfsmitteln selbst angefertigt, keine Textabschnitte Dritter ohne Kennzeichnung übernommen, sowie meine Quellen und Hilfsmittel aufgeführt. Ein Promotionsberater wurde nicht in Anspruch genommen und Dritte bekamen von mir keine Gelder oder geldwerten Leistungen die im Zusammenhang mit der Dissertation stehen.

Die Dissertationsschrift wurde in vorliegender oder ähnlicher Form nicht als Dissertation, Prüfungsarbeit oder anderweitige wissenschaftliche Prüfung bei einer Hochschule eingereicht.

Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität ist mir bekannt.

Jena, den 24. November 2014 _____

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