Investigations into the regulation of histone H2B monoubiquitination

Dissertation

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submitted by
Andrei Shchebet
born in Stolin, Belarus

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Members of the Thesis Committee:

Prof. Dr. Steven A. Johnsen (Reviewer)
Molecular Oncology,
University of Göttingen Medical School, Göttingen

Prof. Dr. med. Heidi Hahn (Reviewer)
Molecular Developmental Genetics,
University of Göttingen Medical School, Göttingen

Prof. Dr. Markus Wahl
Structural Biochemistry,
Freie Universität Berlin

Date of the oral examination: 18th April 2011

Affidavit

I hereby declare that the PhD thesis entitled “Regulation of histone H2B monoubiquitination” has been written independently and with no other sources and aids than quoted.

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Andrei Shchebet
March, 2011

Göttingen

Publications

Pirngruber J, **Shchebet A**, Johnsen SA (2009a) Insights into the function of the human P-TEFb component CDK9 in the regulation of chromatin modifications and co-transcriptional mRNA processing. *Cell Cycle* **8:** 3636-3642

Pirngruber J, **Shchebet A**, Schreiber L, Shema E, Minsky N, Chapman RD, Eick D, Aylon Y, Oren M, Johnsen SA (2009b) CDK9 directs H2B monoubiquitination and controls replication-dependent histone mRNA 3'-end processing. *EMBO Rep* **10:** 894-900

В целях природы обуздания,
В целях рассеять неученья Тьму
Берем картину мироздания — да!
И тупо смотрим, что к чему…

А. и Б. Стругацкие

ABBREVATIONS

53BP1	p53 binding protein 1
APS	Ammonium persulfate
ARF	Alternative reading frame product of CDKN2A locus
ATM	Ataxia telangiectasia mutated
ATP	Adenosin triphosphate
ATR	Ataxia telangiectasia and Rad3 related
ATXN7L3	Spt-Ada-Gcn5-acetyltransferase
BGP	ß-Glycerolphosphate
BRCA1	Breast cancer 1
Brd4	Bromodomain containing 4
Bre1	BREfeldin A sensitivity
BSA	Bovine serum albumin
CB	Cajal body
CCNH	Cyclin H
CCNT1	Cyclin T1
CDK7	Cyclin-Dependent Kinase 7
CDK9	Cyclin-Dependent Kinase 9
cDNA	Complementary DNA
COMPASS	Complex proteins associated with Set1p
CTD	Carboxyterminal Domain
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate

DFC	Dense fibrillar component
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
Dot1L	DOT1-like
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DSIF	(DRB) Sensitivity Inducing Factor
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACT	Facilitates Active Chromatin Transcription
FBS	Fetal Bovine Serum
FC	Fibrillar center
GC	Granular component
GST	Glutathione *S*-transferase
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
H4	Histone 4
Hdm2	Mdm2 p53 binding protein homolog
HEXIM1	Hexamethylene bis-acetamide inducible 1
HMBA	Hexamethylene bisacetamide

HoxA	Homeobox A cluster
HRP	Horseradish peroxidase
HU	Hydroxyurea
IAA	Iodacetamide
JNK	C-Jun N-terminal kinase
MDC1	Mediator of DNA-damage checkpoint 1
MEKK1	Mitogen-activated protein kinase kinase kinase
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
MYBBP1A	MYB binding protein (P160) 1a
MyoD	Myoblast determination protein
NAC	*N*-acetylcysteine
NCL	Nucleolin
NELF	Negative elongation factor
NEM	N-ethylmaleimide
Nopp140	Nucleolar phosphoprotein 140
NOR-90	Nucleolar organizer
NP-40	NonidetTM P40
NPM	Nucleophosmin
PCNA	Proliferating Cell Nuclear Antigen
PIC	Pre-Initiation Complex
PP1α	Protein phosphatase 1alpha
PP2B	Protein phosphatase 2 B
PPRγ	Peroxisome proliferator-activated receptor gamma
P-TEFb	Positive Transcription Elongation Factor beta

RAD18	Radiation sensitivity protein 18
RAD5	Radiation sensitivity protein 5
Rad6	Radiation sensitivity protein 6
RNA	Ribonucleic acid
RNAPII	RNA Polymerase II
RNF20	Ring finger protein 20
RNF40	Ring finger protein 40
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SAPKs	Stress-activated protein kinases
SB203580	4-[5-(4-Fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1***H***-imidazol-4-yl]pyridine
SCP	Small CTD phosphatase
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SP600125	1,9-Pyrazoloanthrone
siRNA	Small interfering RNA
SLBP	Stem loop-binding protein
snoRNP	Small nucleolar ribonucleoprotein
snRNA	Small nuclear ribonucleic acid
snRNP	Small nuclear ribonucleoproteins
SUPT5H	Supressor of Ty Homologue-5
TEMED	Tetramethylethylenediamine
TFIID	Transcription factor II D

TIF-IA	Transcription initiation factor IA
UBE2A	Ubiquitin-conjugating enzyme E2A
UBP10	Ubiquitin protease 10
UBP8	Ubiquitin protease 8
USP22	Ubiquitin specific peptidase 22

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Summary

Transcription-associated chromatin modifications are recognized now as important regulators of gene expression. One of them, the monoubiquitination of lysine 120 of histone H2B (H2Bub1), is associated with actively transcribed genes. It was shown to change the physical properties of the chromatin as well as serve as a recognition mark for regulatory chromatin-binding proteins. The misregulation of H2Bub1-modifying machinery has been observed to be tightly linked with different types of cancer. Many aspects of regulation of H2B monoubiquitination remain unknown.

In the course of this study we presented the data which links H2Bub1 to the modifications of RNA polymerase II, proving that H2Bub1 does not depend on transcription *per se*. We also clarified aspects of the regulation of the H2B ubiquitinating enzymes. A separate interesting question investigated in this study is a rapid and massive loss of H2Bub1 following various cell stresses. Preliminary mechanism and signaling pathways which control this process were discovered. Together, these studies have uncovered important mechanisms controlling H2Bub1 and may serve as a basis for developing more potent strategies to combat cancer.

1 Introduction

1.1 RNA polymerase II-mediated transcription overview

The transcription of most genes is mediated by RNA polymerase II (RNAPII). It performs the cycle of transcription in 4 steps: pre-initiation, initiation, elongation and 3'-end processing/termination (Fig. 1).

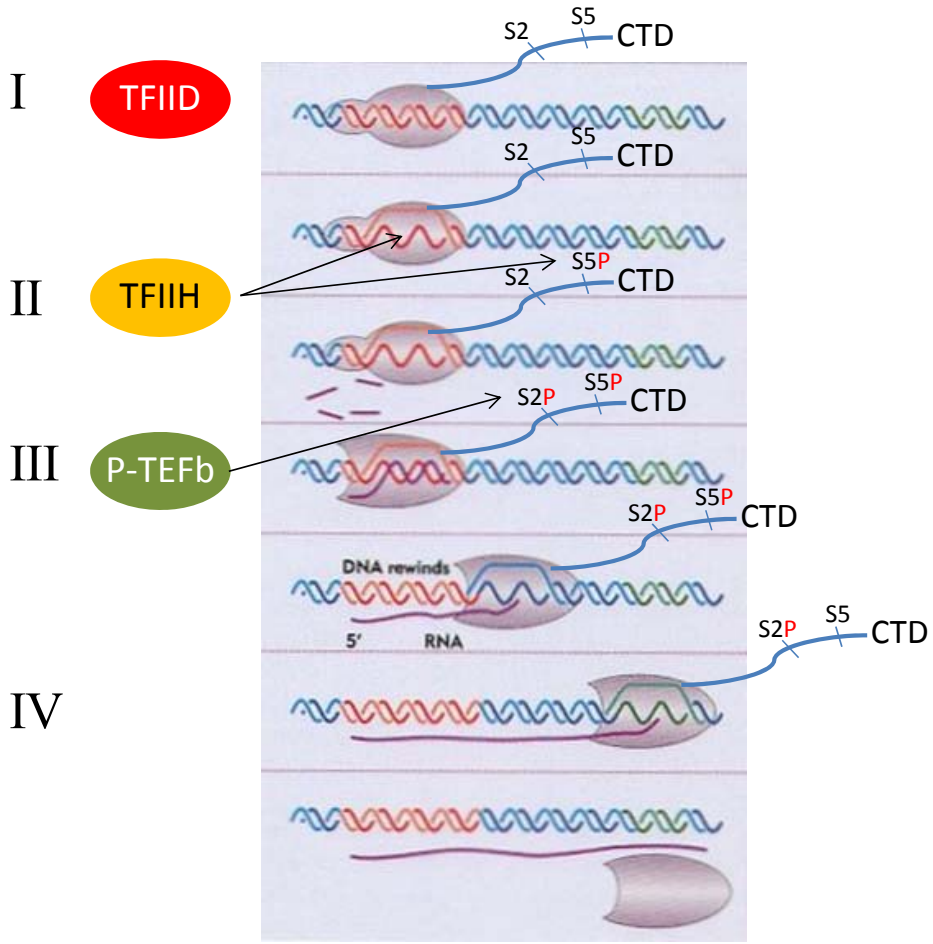

Figure 1. Regulation of different transcription stages. I. Preinitiation. DNA is distorted by the action of TFIID. **II. Initiation.** TFIIH opens the DNA duplex and

phosphorylates serine 5 in RNAPII CTD. The production of abortive transcripts takes place or the polymerase is switched to the paused state. **III. Elongation.** RNAPII is phosphorylated at serine 2 by P-TEFb and starts producing full-length transcript. **IV. 3'-end processing and termination.** The transcript is cleaved by an endonuclease activity and the mRNA is normally polyadenylated (except for histone mRNAs which have stem loop structure). RNAPII CTD is dephosphorylated for the next run and released from DNA. Modified from Lewin's Gene IV.

The pre-initiation step includes the assembly of the Pre-Initiation Complex (PIC) on the promoter. It consists of six general transcription factors and the RNAPII holoenzyme. TFIID is one of these factors. Its subunits create a distortion in the DNA as well as provide proper positioning of the whole complex on the promoter.

The initiation starts when the helicase subunit of TFIIH factor opens the DNA duplex creating a so-called transcription bubble. The kinase component of TFIIH consisting of Cyclin-Dependent Kinase 7, Cyclin H (CDK7/CCNH) and MAT1 phosphorylates serine in the fifth position of RNAPII Carboxyterminal Domain (CTD) repeats. The RNAPII starts producing short abortive transcripts.

For the transition of RNAPII to the next, elongation step, the activity of Positive Transcription Elongation Factor b (P-TEFb) is needed. Its components, Cyclin-Dependent Kinase 9 and Cyclin T1 (CDK9/CCNT1) phosphorylate and inactivate negative elongation factors as well as activate the RNAPII by phosphorylating its second serines in CTD repeats.

At the end of transcription cycle the transcript is terminated and its 3'-end is processed into either poly-A tail or stem loop. The RNAPII is dephosphorylated to be reused in the next round of transcription (Lee & Young, 2000).

1.1.1 Role of CTD modifications in the regulation of the transcription events

The large subunit of RNAPII has a carboxy-terminal domain which consists of 52 heptapeptide repeats (YSPTSPS) in human (Nonet et al, 1987). CDK9 can phosphorylate serine in second position of each repeat. This phosphorylation marks the onset of productive elongation of transcription (Kim et al, 2002). P-TEFb remains associated with RNAPII as it goes along the gene. Subsequently the number of phosphorylated serines in RNA polymerase CTD

increases while the enzyme moves toward the 3'-end of the gene (Komarnitsky et al, 2000). At the end of the gene the phosphorylation declines and RNAPII gets rapidly dephosphorylated to be reused in another cycle of transcription (Cho et al, 2001).

Serines in the fifth position of CTD repeats are phosphorylated by the TFIIH components CDK7 and Cyclin H during the initiation of transcription (Giglia-Mari et al, 2004; Komarnitsky et al, 2000). Unlike p-Ser2 the phosphorylation of Ser5 mostly happens near the 5'-end of the gene (Komarnitsky et al, 2000). Selective phosphorylations of serines in the positions 2 and 5 were the first well-studied elements of a regulatory paradigm later called a "CTD code" (Buratowski, 2003). More recently, Ser7 was also shown to be phosphorylated (Chapman et al, 2007; Egloff et al, 2007). Tyr1 and Thr4 can also be potential phosphorylation sites. Two conserved proline residues (Pro3 and Pro6) can be also modified by peptidyl-prolyl isomerases (Shaw, 2007) thus increasing the complexity and possible number of combinations of modifications. The variety of modification makes the RNAPII CTD a universal docking site for the proteins involved in different stages of transcription (Fig. 2).

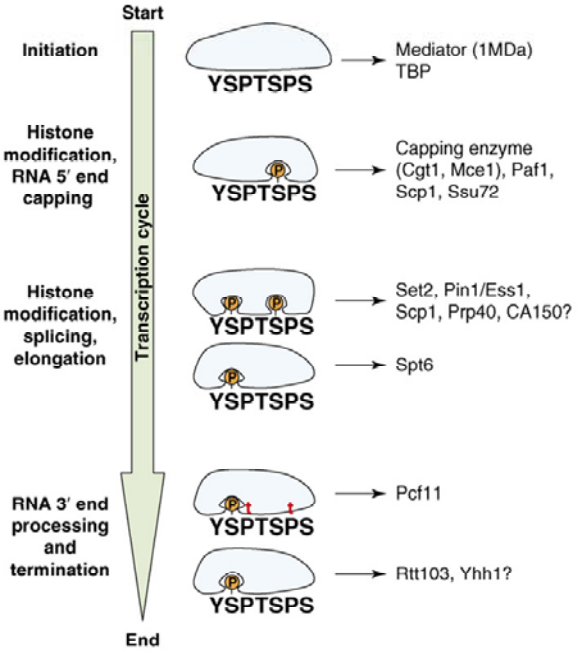

Figure 2. The carboxyl-terminal domain of RNAPII is subjected to various posttranslational modifications and serves as a docking site for many transcription-related factors. The stages of transcription and co-transcriptioonal processes are listed on the left. Yellow circles and red "t"s represent phosphorylations and proline isomerisations, respectively. CTD-binding factors are shown as light blue shapes. The proteins listed on the right were all shown to directly bind the RNAPII CTD. Taken from (Egloff & Murphy, 2008).

In brief, the unphosphorylated RNAPII binds TBP and Mediator complex in the beginning of transcription cycle (Myers et al, 1998; Usheva et al, 1992).The phosphorylation of serine 5 was shown to promote the binding of RNA capping enzymes (Phatnani & Greenleaf, 2006) since the cap is required for further mRNA survival. As the serine 2 gets phosphorylated and the polymerase goes into the elongation stage the phosphorylation of serine 5 declines which can be explained by the recruitment of CTD phosphatases to the bi-phosphorylated CTD (Zhang et al, 2006). The elongation factor hSUPT6H binds only Ser2-phosphorylated polymerase (Yoh et al, 2007).

Apart from elongation in yeast p-Ser2 has been also shown to play an important role in 3'-end processing. The Pcf11, a component of yeast cleavage/polyadenylation factor CFIA, is specifically binding the Ser-2 phosphorylated CDT of RNAPII (Licatalosi et al, 2002).

1.2 P-TEFb complex

1.2.1 Regulation of P-TEFb activity

The P-TEFb complex plays an important role in the regulation of transcription and transcription-coupled processes such as mRNA processing and chromatin modifications. In this way P-TEFb plays an important role in controlling gene transcription under many conditions and can influence such diverse processes as cell differentiation and DNA damage repair.

In its active form the complex consists of a kinase component CDK9 and cyclin T1 (CCNT1), and less often T2 or K (Fu et al, 1999; Peng et al, 1998). Unlike most other cyclins, CCNT1 is not subjected to a cell cycle-dependent regulation and its amount remains constant throughout the cell cycle (Grana & Reddy, 1995). However, the activity of P-TEFb complex is tightly regulated.

CDK9-CCNT1 heterodimers are kept in an inactive state in a complex with the HEXIM1 protein and 7SK snRNA (Nguyen et al, 2001; Yang et al, 2001; Yik

et al, 2003). About half of P-TEFb complexes can be sequestered in this way (Nguyen et al, 2001).

Like other cyclin-dependent kinases, CDK9 has a conserved threonine residue in its T-loop domain. The phosphorylation of this residue changes the conformation of the active center to allow for the interaction with a substrate (Russo et al, 1996). Thr186 in CDK9 can be dephosphorylated by several phosphatases such as PP1α or PP2B (Chen et al, 2008). This dephosphorylation happens upon UV-irradiation or hexamethylene bisacetamide (HMBA)-induced stress. The activity of these phosphatases is regulated by the Ca2+-calmodulin pathway. The dephosphorylated form of CDK9 is released from the HEXIM/7SK complex (Chen et al, 2008). Upon release CDK9 can be bound by Brd4 which can specifically recognize acetylated histones (Wu & Chiang, 2007) and brings the CDK9-CCNT1 complex to the transcription initiation site where it remains inactive until the start of productive elongation(Jang et al, 2005; Yang et al, 2005).

1.2.2 P-TEFb substrates

One of the first described targets of P-TEFb was RNA polymerase II CTD whose function and regulation were described above (Marshall et al, 1996). RNAPII is not the only target of CDK9 in the transcription elongation complex. Negative elongation factor, NELF, is known to inhibit the transcription elongation by binding RNAPII together with another inhibitor, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) Sensitivity Inducing Factor DSIF (Wada et al, 1998a; Yamaguchi et al, 1999). P-TEFb phosphorylates the NELF-E subunit which leads to its release from RNAPII binding (Fujinaga et al, 2004). Supressor of Ty Homologue-5 (SUPT5H), a component of DSIF, is also phosphorylated by P-TEFb (Kim & Sharp, 2001; Wada et al, 1998b). The phosphorylation is localized in CTR1, a repeat domain containing several repeats of PSPSPASY sequence analogous to RNAPII CTD (Ivanov et al, 2000). In contrast to released NELF, the phosphorylated DSIF stays in the complex with RNAPII becoming now a positive factor of elongation (Yamada et al, 2006).

Apart from the regulation of transcription elongation P-TEFb was also shown to directly control transcription factor activity. For example, the transcription factor MyoD, a key regulator of myoblasts differentiation (Davis et al, 1987), is phosphorylated by the CDK9/CCNT2 complex. This phosphorylation

increases MyoD activity and promotes the muscular differentiation (Simone et al, 2002).

Peroxisome proliferator-activated receptor gamma (PPARγ) is another P-TEFb-regulated transcription factor. Upon CDK9-mediated phosphorylation PPARγ is activated which promotes adipocyte differentiation (Iankova et al, 2006).

1.3 H2B ubiquitination.

1.3.1 Overview of chromatin structure

Chromatin is a complex of DNA, histones and other proteins that form chromosome. The basic unit of the chromatin is the nucleosome. The typical nucleosome is a heterooctamer consisting of two histone H3-H4 dimers which form a stable tetramer as well as two flanking histones H2A-H2B dimers. Standard nucleosome consisting of canonical histones is wrapped by 147 base pairs of DNA (Davey et al, 2002; Luger et al, 1997).

While the core domains of histones perform solely structural function, the N- and C-terminal tails are exposed outside the nucleosome and often subjected to a wide range of posttranslational modifications including acetylation and ubiquitination of lysines, methylation of lysines and arginines, and phosphorylation of serines and threonines (Turner, 2007) (Fig. 3).

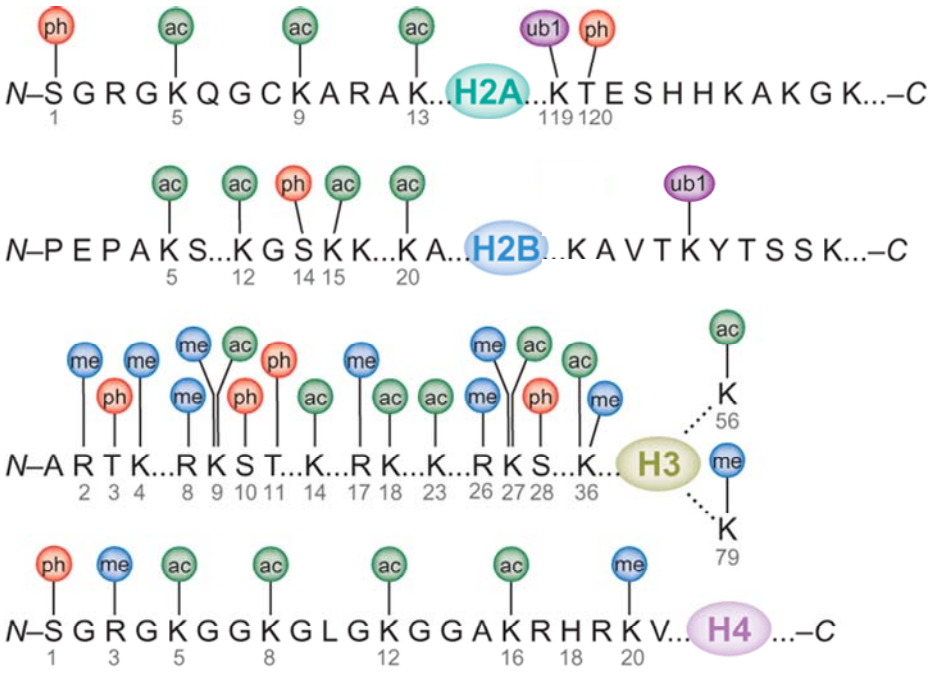

Figure 3. Variety of histone modifications. The core parts of histones are represented as colored ovals. Tails are shown as sequences of aminoacids. Modifications: me-methylation, ph-phosphorylation, ac-acetylation, ub1-monoubiquitination. Taken from: (Bhaumik et al, 2007).

This wide variety of modifications and their relatively uniform distribution in the same regions of many genes (Fig. 4) gave rise to the concept of histone code (Strahl & Allis, 2000). This hypothesis suggests a combinatorial effect of histones modifications on the recruitment of chromatin-interacting factors with specific biological functions. In general, there are marks mostly associated with inactive chromatin, such as hypoacetylation, H3K9me3 and H3K27me3 while the hyperacetylation, H3K4me3 and H3K36me3 are found mostly on transcribed genes (Ruthenburg et al, 2007).

The precise location of histone modifications in different regions of a gene is achieved by close integration of chromatin-modifying enzymes with the transcription apparatus (Fig. 4). A good example of such regulation is trimethylation of histone H3K4. It is performed by Set1p methyl transferase which is recruited to the serine 5-phosphorylated RNAPII at the 5'-end of a gene (Liu et

al, 2005; Ng et al, 2003; Santos-Rosa et al, 2002). Similarly, H3K36me3 is controlled by Set2p which is associated with a dually Ser2/Ser5-phosphorylated elongating RNAPII and is mostly detected in the middle and 3'-end regions of active genes (Carrozza et al, 2005; Keogh et al, 2005).

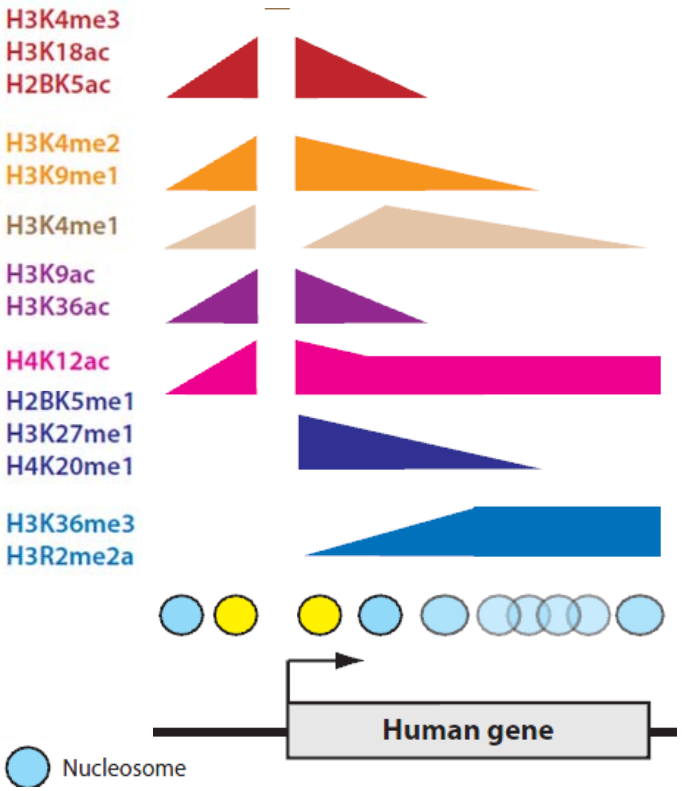

Figure 4. Chromatin map of an active human gene. Modified from (Rando & Chang, 2009).

Apart from modifications of canonical histones the epigenetic background can be altered by the incorporation of histone variants. Histones H2A and H3 have physiologically important variants while H2B and H4 are mostly invariant (Malik & Henikoff, 2003).

The H2A.Z variant in mammals is predominantly localized in the enhancer elements, insulator regions and around the transcription a start site (Barski et al, 2007; Schones et al, 2008)

H2A.Bbd lacks 13 of 14 acetylatable lysines, the ubiquitinatable C-terminal tail and and the acidic region which contacts H4 (Doyen et al, 2006). As a result the H2A.Bbd-containing nucleosomes bind only 118 DNA residues and are significantly less stable (Bao et al, 2004).

In contrast macroH2A contains large non-histone c-terminal tail and is much bigger than canonical H2A (Pehrson & Fried, 1992). Due to the increased stability of macroH2A-containing nucleosomes they are mostly found in transcriptionally inactive regions such as the inactivated X-chromosome and methylated CpG islands (Choo et al, 2006; Costanzi & Pehrson, 1998).

The highly transcribed regions of chromatin are also marked by the the H3.3 variant. Being only 5 amino acids different from canonical H3 it nevertheless makes nucleosomes extremely unstable (Jin & Felsenfeld, 2007). In contrast, H3.1 variant is also found in significant proportion in silenced chromatin regions (Hake & Allis, 2006).

The H2A.X variant constitutes about 10% of total H2A in human cells. It is phosphorylated in the region of double strand DNA break by DNA damage response kinases like ATM, ATR and DNA-PK (Burma et al, 2001; Stiff et al, 2004)4-6. The size of phosphorylated region around the break can reach a megabase (Rogakou et al, 1999). The loss of H2A.X causes impaired formation of DNA damage repair loci with reduced accumulation of basic repair factors like MDC1, BRCA1 and 53BP1 while the initial recruitment of this factors still takes place (Celeste et al, 2003; Paull et al, 2000; Ward et al, 2003).

1.3.2 H2B monoubiquitination

Monoubiquitination of Lysine in the C-terminal tail of histone H2B is conserved from yeast (K123) to human (K120) (Robzyk et al, 2000; Thorne et al, 1987). Like all other ubiquitination reactions this requires the activity of E1, E2 and E3 ubiquitinating enzymes which subsequently transfer the activated ubiquitin molecule to the target protein (Pickart, 2001). The E2 ubiquitin conjugating enzyme for H2B in yeast is called Rad6 (Robzyk et al, 2000). In human this function is assigned to the Rad6 homolog the UBE2A protein (Kim et al, 2009). Bre1 serves as an E3 ubiquitin ligase of H2B in the yeast *Saccharomyces cereviseae* (Wood et al, 2003). In human Bre1 has two homologs, Bre1A and Bre1B, also refered to as RNF20 and RNF40. A heterodimeric complex of the E3

ring-finger ubiquitin ligases RNF20 and RNF40 is responsible for the final step of ubiquitin transfer to the H2B, however, *in vitro* only RNF20 enzymatic activity is needed for the reaction (Kim et al, 2005; Zhu et al, 2005).

Since monoubiquitination of H2B does not lead to protein degradation the ubiquitin moiety has to be removed in order to suppress the signaling when necessary. In yeast two ubiquitin proteases were described, Ubp8 and Ubp10. Ubp8 is a component of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (Daniel et al, 2004). In humans the ortholog of Ubp8, USP22 was found (Zhang et al, 2008). As in yeast, USP22 requires the interaction with other SAGA complex subunits CG13379 and ATXN7L3 for its deubiquitination activity (Henry et al, 2003; Zhao et al, 2008). Interestingly, unlike yeast, in which H2A is not ubiquitinated, USP22 deubiquitinated not only H2B, but also H2A (Zhao et al, 2008)

Unlike Upb8 the ubiquitin protease Ubp10 is not associated with the active fraction of the genome. Instead it interacts with the silencing protein Sir4 and plays a role in silencing telomeric regions as well as rRNA genes (Emre et al, 2005). No human orthologs of Ubp10 were described so far.

1.3.3 Function of H2B ubiquitination

The attachment of a bulky ubiquitin to H2B histone was shown to significantly modify the properties of chromatin in vitro. The compaction of the chromatin into 30 nm fiber was severely impared when ubiquitinated H2B was incorporated into nucleosomes. Besides, such chromatin was shown to be more available for the Dot1L methyltransferase (Fig. 5) (Fierz et al, 2011).

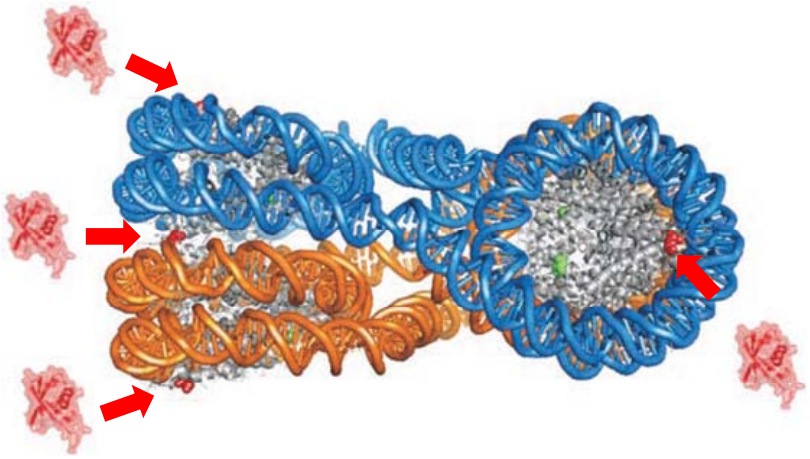

Figure 5. H2B ubiquitination in the context of 30-nm fiber. Tetranucleosome unit extracted from the fiber and rotated by 38.1°. Red arrows indicate the sites of ubiquitin (pink) attachment. Modified from (Fierz et al, 2011)

In yeast, monoubiquitinated H2B has been generally associated with the promoters and coding regions of actively transcribed genes (Henry et al, 2003; Kao et al, 2004; Xiao et al, 2005). Yeast strains which have a K123R mutation in H2B demonstrated strong transcriptional defects. H2B ubiquitination was shown to be important for the recruitment of COMPASS methyltransferase complex to the chromatin. This way of epigenetic regulation was called trans-tail histone modifications (Fischle et al, 2003). Di- and trimethylation of lysines 4 and 79 on histone H3 were significantly impaired in strains lacking H2Bub1 (Lee et al, 2007). The absence of H2Bub1 caused the overall reduction of nucleosome occupancy in the transcribed regions of the genes. The initiation of transcription from intragenic cryptic transcription sites was also detected. This way be explained by the fact that H2Bub1 was found to be in a close functional interaction with the FACT (Facilitates Active Chromatin Transcription) complex (Fleming et al, 2008; Pavri et al, 2006). FACT, being a H2A/H2B chaperone is involved in the disassembly and reassembly of nucleosomes during the transcription (Fig. 6) (Belotserkovskaya et al, 2003).

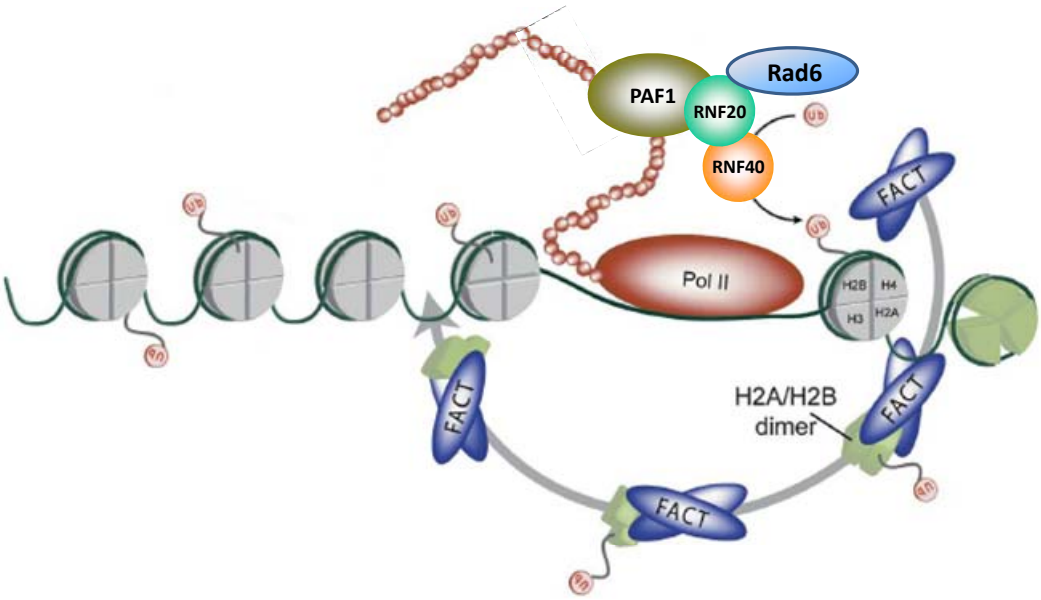

Figure 6. Dynamics and role of H2B monoubiquitination in transcription elongation. Modified from (Laribee et al, 2007)

Quite recently the mechanistic explanation was given for H2Bub1-mediated chromatin alterations. It was shown that despite disrupting the 30-nm fiber structure H2Bub1 strongly increases the stability of individual nucleosomes. This, in fact, stabilized the binding of the FACT subunit Spt16 to the nucleosome in front of RNAPII and insures the proper chromatin reconstitution in the wake of RNA polymerase (Chandrasekharan et al, 2009).

In human cells the role of H2Bub1 in supporting the level of histone methylation was also shown. The overexpression of RNF20 led to the accumulation of H3K4me1, H3K4me3 and H3K79me3 (Zhu et al, 2005). The depletion of RNF20 and RNF40 with siRNA resulted in a significant decrease of the same histone methylations. The presence of RNF20/40 was shown to be essential for the expression of genes from HoxA cluster. Most of the genes were upregulated and downregulated by the overexpression and siRNA-mediated depletion of H2B E3 ubiquitin ligases, respectively. Moreover, chromatin immunoprecipitation showed that HoxA genes, which where regulated by H2Bub1, had significant changes of histone H3 methylations upon RNF20/40 overexpression or knockdown. Altogether that provided first robust model of gene

expression regulation by H2Bub1 in human cells. Supporting evidence for a positive role of H2Bub1 in transcription came from further studies in which the retinoic acid-induced transcription of retinoic acid receptor-alpha gene was shown to depend upon RNF20 and 40 *in vivo* and H2Bub1 *in vitro* (Pavri et al, 2006).

One of the components of H2B ubiquitinating complex, RNF20 has been found to be a putative tumor suppressor. Surprisingly, it has a repressive influence on the induction of EGF-responsive genes (Shema et al, 2008). Moreover, its depletion promotes cell migration and its promoter is hypermethylated in many tumors.

Not only the expression of genes but also the processing of transcripts is regulated by H2Bub1 in human. The 3'-ends of the replication dependent histone genes are normally processed into stem loop structure by stem loop-binding protein SLBP and U7 small nuclear ribonuclear protein (snRNP) (Marzluff et al, 2008). Recently it has been shown that upon the depletion of RNF20/40 RNAPII with higher probability skips the site of 3'-end processing and synthesizes a longer polyadenylated transcript (Pirngruber et al, 2009b).

In the sites of DNA brakes the chromatin undergoes different modifications. Recent reports show an accumulation of H2Bub1 on the damaged chromatin as well as the interaction of H2B ubiquitinating enzymes with DNA repair machinery (Moyal et al, 2011; Nakamura et al, 2011). In accordance with the above mentioned *in vitro* data H2Bub1 accumulation might play an important role in decompacting the chromatin for the double-stranded DNA break repair.

1.4 Rad6

1.4.1 Rad6 in yeast

Rad6 was one of the first yeast proteins identified as a regulator of H2B ubiquitination. This E2 ubiquitin conjugating enzyme was shown to ubiquitinate H2B in *vivo* and *in vitro* (Robzyk et al, 2000). Later it was demonstrated that the *in vivo* activity of Rad6 is not detected if Bre1, an E3 RING-finger ubiquitin ligase was not present (Wood et al, 2003).

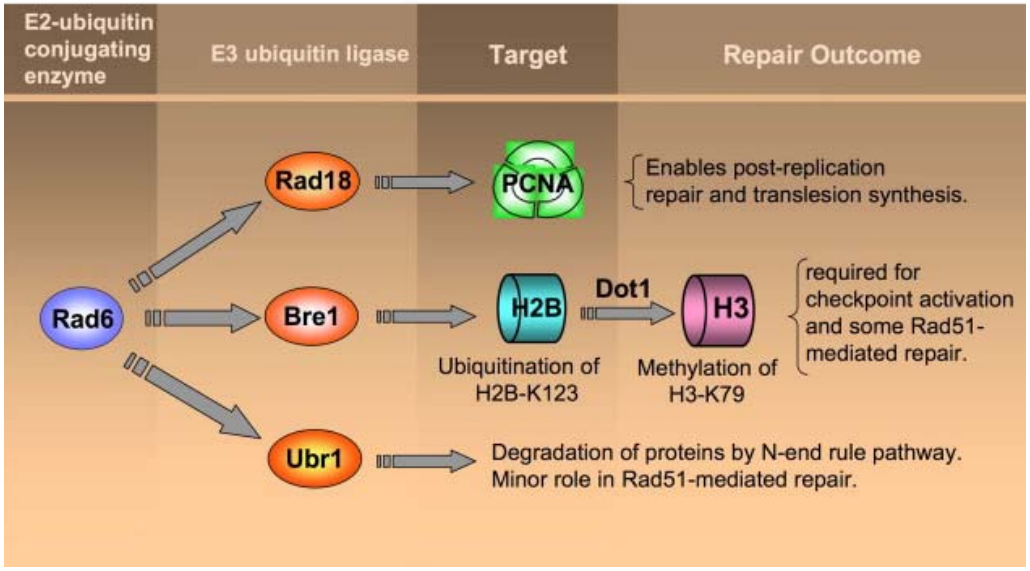

Figure 7. Multiple functions of Rad6 in yeast. Taken from (Game & Chernikova, 2009)

Like most E2 enzymes, Rad6 is involved in the ubiquitination of several substrates (Fig. 7). Proliferating Cell Nuclear Antigen (PCNA) has been shown to be ubiquitinated at lysine 164 by Rad6 in a complex with the E3 ubiquitin ligase Rad18. In a complex with another E3 ubiquitin ligase, Rad5, Rad6 can convert the monoubiquitination of PCNA into a K63-linked poliubiquitin chain (Hoege et al, 2002). This ubiquitination mark promotes the interaction of DNA polymerase eta with the DNA clamp PCNA to activate the translesion DNA synthesis pathway in both yeast and human (Haracska et al, 2001; Haracska et al, 2004; Kannouche et al, 2004). The N-end rule pathway of protein degradation also makes use of Rad6 where it serves as an E2 enzyme for E3 ubiquitin ligase Ubr1 (Dohmen et al, 1991)

1.4.2 UBE2A (hRAD6)

The closest homolog of yeast Rad6 in human are called UBE2A (Fig. 8) and UBE2B. However, UBE2A appears to be the predominantly expressed form. In resemblance to its yeast homolog it is involved in the ubiquitination of histone H2B in a complex with E3 ubiquitin ligases RNF20/RNF40 (Kim et al, 2009). The

ubiquitination of human PCNA is also mediated by UBE2A in association with E3 ligase hRAD18 (Watanabe et al, 2004).

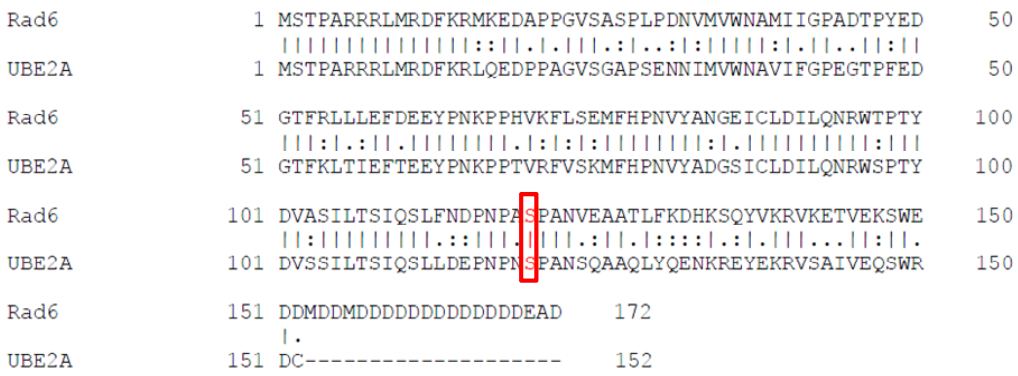

Figure 8. Alignment of amino acid sequences of yeast Rad6 and human UBE2A. Conserved phosphorylated serine residue is marked in red. (Done by EMBOSS alignment tool.)

1.4.3 Regulation of Rad6 activity

The Bur1/Bur2 complex is essential for yeast growth and consists of the cdc28-related kinase Bur1 and cyclin T family member Bur2 (Yao et al, 2000). It has been shown to phosphorylate Rad6 at serine 120 in yeast and this phosphorylation is necessary for the ability of Rad6 to ubiquitinate H2B (Wood et al, 2005).

In human cells UBE2A can also be phosphorylated at conserved serine 120 by CDKs 1 and 2 (Sarcevic et al, 2002). In vivo the phosphorylation of UBE2A increases during the G2/M transition. The mutation of serine 120 into alanine abolishes UBE2A activity

1.5 Loss of H2B ubiquitination

H2Bub1 is a dynamic chromatin modification. A severe decrease of H2B ubiquitination was shown upon treating cells with different stress-inducing factors such as proteosome inhibitors, heat shock, sodium arsenite, cadmium chloride, doxorubicin, Actinomycin D or DRB (Davie & Murphy, 1990; Mimnaugh et al, 1997; Minsky et al, 2008). That suggests the existence of a universal mechanism of

chromatin modification in response to different stresses. As an explanation of the observed effect the depletion of free ubiquitin or the inhibition of transcription were proposed (Davie & Murphy, 1994).

1.5.1 Nucleoli and stress conditions

1.5.1.1 Nucleoli overview

Nucleoli are subnuclear organelles where the production of small and large subunits of ribosomes takes place. Therefore their function must be tightly regulated in different growth conditions (Lempiainen & Shore, 2009). Three distinct processes occur in the nucleoli: pre-rRNA transcription, processing, and ribosomal RNP assembly. Accordingly, three compartments are distinguished in nucleolar microstructure. The fibrillar center (FC) hosts the transcription, dense fibrillar component (DFC) is a place of rRNA processing while the assembly of ribosomal subunits happens in the granular component (GC) (Boisvert et al, 2007) .

1.5.1.2 Nucleolar reaction to stress

In stress conditions the structure of nucleoli undergoes different changes. One of them is called nucleolar segregation and is caused by UV irradiation, topoisomerase inhibitors or actinomycin D. During this process the FC and GC are condensed and separated while the nucleolar cap is formed from nucleolar and nuclear proteins around the nucleolar remnant (Al-Baker et al, 2005; Shav-Tal et al, 2005).

The nucleolar proteome contains about 4500 different proteins while only 30% of them are directly involved in ribosomal biosynthesis (Ahmad et al, 2009). The rest are involved in DNA replication and repair, apoptosis control, RNP biogenesis and cell cycle regulation. The studies involving quantitative mass spectrometry and fluorescence microscopy of more than 1000 nucleolar proteins revealed a massive translocation of factors involved in nucleolar stress, DNA damage pathway and oxidative stress response upon treatment with the topoisomerase I inhibitor camptothecin. This translocation has been shown to be quite rapid so that some components changed their localization already in two min after adding the drug (Cohen et al, 2008).

A typical mechanism of cell cycle regulation by nucleolar signaling involves the regulation of p53 level. In normally growing cells p53 protein levels

are kept low due to its constant ubiquitination by the E3 ubiquitin ligase HDM2 which marks p53 for proteosomal degradation (Kruse & Gu, 2009). The activity of HDM2 can be inhibited by $p14^{ARF}$, a protein with nucleolar localization (Sherr, 2001). The ARF protein is itself very unstable. It is also ubiquitinated by the E3 ligase UlF and subsequently degraded. Another important nucleolar component, nucleophosmin (NPM) has been shown to abrogate ARF degradation thus inhibiting HDM2 activity and stabilizing p53 to block the progression of cell cycle progression (Chen et al, 2010).

1.5.2 Cajal bodies and nucleoli

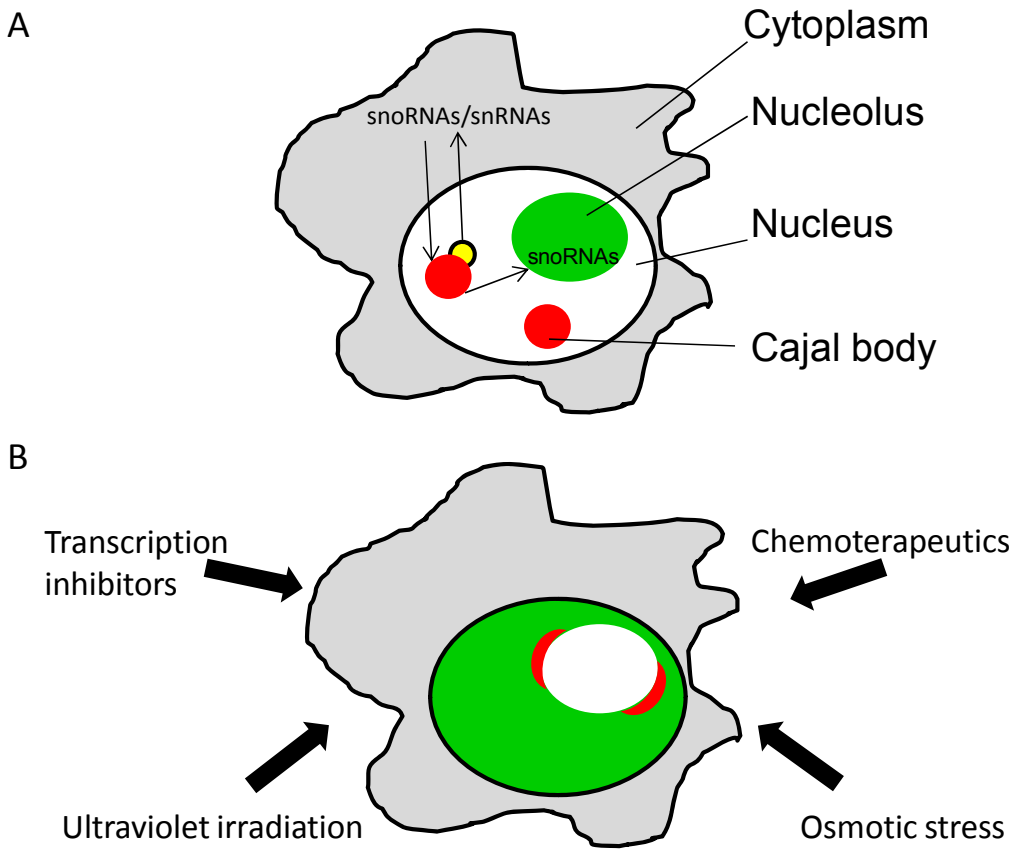

Figure 9. Nucleoli and Cajal bodies. A. Functional interaction between CBs and nucleoli. SnRNAs are transcribed from their locus (yellow) adjacent to CBs (red) (Frey & Matera, 2001) and then are transported to the cytoplasm (grey) for splicing. After re-import to the nucleus snRNAs undergo final maturation in CBs and migrate to the nucleolus (green) as a

part of snRNP complexes which are involved in rRNA processing. **B.** Upon different stress conditions nucleolar components (nucleophosmin, green) are released to the nucleoplasm whereas CB components (p80-coilin, red) migrate to the periphery of the nucleolus.

Cajal bodies (CBs) are distinct nuclear structures which function in the processing of different types of RNAs such as snRNAs, snoRNAs and histone mRNAs (Kiss et al, 2006; Matera et al, 2007; Nizami et al, 2010). One of the main components of CBs, p80-coilin is used as a CB marker and plays an important role in snRNP biogenesis and splicing (Cioce & Lamond, 2005; Strzelecka et al, 2010).

CBs are linked to nucleoli both physically and functionally. In the nuclei of neurons these organelles are closely associated (Lafarga et al, 1986). The snoRNPs which undergo maturation in CBs are later transported to the nucleoli to serve for the rRNAs processing (Fig. 9A). Moreover, a constant shuttling of proteins like Nopp140 between the nucleoli and CB makes the connection between these compartments even more obvious. Nopp140 is a snoRNP chaperone and it probably acts as a transporter of snoRNPs to the nucleoli (Isaac et al, 1998). In micronucleated cells coilin was shown to be co-localized with nucleolar components such as fibrillarin, NOR-90 and RNA polymerase I. The functional role of such a protein localization shift remains unclear (Silva et al, 2004) .

1.5.3 Cajal bodies under stress

CBs undergo different transformations upon the exposure of cells to different stress conditions. Starvation decreases the number of CBs (Andrade et al, 1993), UV-C irradiation, osmotic stress and heat shock cause redistribution of coilin to nucleoplasmic microfoci as well as formation of so called nucleolar caps which also form upon actinomycin D treatment (Cioce et al, 2006; Handwerger et al, 2002; Shav-Tal et al, 2005). In HeLa cells, the overexpression of a phosphorylation site-mimicking mutant coilin (S202D) resulted in nucleolar localization of this protein together with splicing snRNAs, suggesting the importance of S202 phosphorylation for the localization of CB components (Lyon et al, 1997).

1.5.4 Nucleoli and JNK signaling

C-Jun N-terminal kinase JNK belongs to the stress-activated protein kinase (SAPK) family and plays an important role in the cellular reaction to stress factors, regulating the choice between survival and apoptosis (Chen et al, 1996). The

activity of JNK is increased upon exposure to UV-irradiation, alkylating agents, actinomycin D, hyperosmotic shock and oxidative damage (Martindale & Holbrook, 2002; Song & Lee, 2007; Yoon & Kim, 2004). JNK activity is regulated by phosphorylation of threonine and tyrosine residues in the T*PY* motif by SEK1/MKK4 (Derijard et al, 1995). JNK inhibits the function of nucleoli by phosphorylating the key rRNA transcription factor TIF-IA (Bodem et al, 2000; Mayer et al, 2005). The inhibition of RNA polymerase I activity itself results in the development of the so-called "nucleolar stress response" which can cause p53 stabilization by ARF, L5 and L11 (Dai & Lu, 2004; Lohrum et al, 2003) release as described previously.

2. Materials

2.1 Equipment

Agarose gel chamber	Harnischmacher Labortechnik, Kassel
Balance	Sartorius AG, Göttingen
Bandelin Sonoplus Sonicator	Bandelin electr. GmbH & Co. KG, Berlin
Biological Safety Cabinet "Hera Safe"	Thermo Fisher Scientific, Waltham, USA
Bioruptor	Diagenode SA, Liège, Belgium
Centrifuge (Megafuge 1.OR)	Thermo Fisher Scientific, Waltham, USA
Centrifuge 4 °C (5417R)	Eppendorf AG, Hamburg
C1000TM Thermal Cycler	Bio-Rad Laboratories GmbH, München
CFX96TM Optical Reaction Module	Bio-Rad Laboratories GmbH, München
Confocal microscope LSM510 META	Carl Zeiss GmbH
Counting chamber (Neubauer)	Brand GmbH & Co. KG, Wertheim
5100 Cryo 1 °C Freezing Container	Thermo Fisher Scientific
Electrophoresis & Electrotransfer Unit	GE Healthcare Europe GmbH, München
Freezer -20 °C	Liebherr GmbH, Biberach
Freezer -80 °C "Hera freeze"	Thermo Fisher Scientific, Waltham, USA
Gel Imager "Gel iX imager"	Intas Science Imaging GmbH, Göttingen
Incubator (bacteria)	Memmert GmbH & Co. KG, Schwabach
Incubator (bacteria culture)	Infors AG, Bottmingen
Incubator (cell culture) "Hera cell 150"	Thermo Fisher Scientific, Waltham, USA
Inverse Microscope "Axiovert 40 CFL"	Carl Zeiss MicroImaging GmbH, Göttingen
Magnet stirrer "MR3001"	Heidolph GmbH & Co. KG, Schwabach

Microscope “Axiovert 40 C“	Carl Zeiss MicroImaging GmbH, Göttingen
Microwave	Clatronic International GmbH, Kempen
Nano Drop® ND-1000 Spectrophotometer	Peqlab Biotechnology GmbH, Erlangen
Pestle	Sartorius AG, Göttingen
pH meter	inoLab® WTW GmbH, Weilheim
Phosphoimager TYPHOON 9400	Amersham Biosciences
Pipette Aid® portable XP	Drummond Scientific Co., Broomall, USA
Pipettes “Research” Series	Eppendorf AG, Hamburg
Power supply “Power Pack P25T”	Biometra GmbH, Göttingen
Refrigerator	Liebherr GmbH, Biberach
Repeat Pipette	Eppendorf AG, Hamburg
Scanner (CanoScan 8600F)	Canon GmbH, Krefeld
Shaker “Rocky”	Schütt Labortechnik GmbH, Göttingen
Table centrifuge (GMC-060)	LMS Co., Ltd., Tokyo, Japan
Test tube rotator	Schütt Labortechnik GmbH, Göttingen
Ultrapure Water System “Aquintus”	membraPure GmbH, Bodenheim
Vacuum pump	Integra Bioscienc. AG, Zizers, Switzerland
Vortex mixer	Scientific Industries, Inc., Bohemia, USA
Water bath “TW 20”	JULABO Labortechnik GmbH, Seelbach
X-Ray Cassettes	Rego X-ray GmbH, Augsburg

2.2 Consumables

Cellstar 6- and 12-well cell culture plate	Greiner Bio-One GmbH, Frickenhausen
Cellstar PP-tube 15 and 50 ml	Greiner Bio-One GmbH, Frickenhausen
Cellstar tissue culture dish 100×20 mm	Greiner Bio-One GmbH, Frickenhausen
Cellstar tissue culture dish 145×20 mm	Greiner Bio-One GmbH, Frickenhausen
Cell scraper (16 cm)	Sarstedt AG & Co., Nümbrecht

Cryo TubeTM Vial (1.8 ml)	Thermo Fisher Scientific, Waltham, USA
Falcon® assay plate, 96 well	VWR Int., LLC, West Chester, USA
Gel blotting paper (Whatman paper)	Sartorius AG, Göttingen
Glass coverslips (18 mm)	Gebr. Rettberg GmbH, Göttingen
HybondTM-PVDF Transfer Membrane	GE Healthcare Europe GmbH, München
Microtube 1.5 ml	Sarstedt AG & Co., Nümbrecht
Microtube 1.5 ml, conical	VWR International GmbH, Darmstadt
Microtube 2 ml	Sarstedt AG & Co., Nümbrecht
96 Multiply® PCR plate white	Sarstedt AG & Co., Nümbrecht
96-well Multiplate® PCR plate white (low)	Bio-Rad Laboratories GmbH, München
Parafilm® "M"	Pechiney Plastic Packaging, Chicago, USA
Petri dish 92×16 mm	Sarstedt AG & Co., Nümbrecht
Pipette tips	Greiner Bio-One GmbH, Frickenhausen
Pipette filter tips	Sarstedt AG & Co., Nümbrecht
Protan® Nitrocellulose transfer membrane	Whatman GmbH, Dassel
X-ray films "Super RX"	Fujifilm Corp., Tokyo, Japan

2.3 Chemicals

2.3.1 General chemicals

Acetic acid	Carl Roth GmbH & Co. KG, Karlsruhe
Adefodur WB developing concentrate	Adefo-Chemie GmbH, Dietzenbach
Adefodur WB fixing concentrate	Adefo-Chemie GmbH, Dietzenbach
Adenosin triphosphate (ATP)	Fermentas GmbH, St. Leon-Rot
Agarose	Carl Roth GmbH & Co. KG, Karlsruhe
Albumin Fraction V (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium sulfate (NH4)2SO4	Carl Roth GmbH & Co. KG, Karlsruhe
Aprotinin	Carl Roth GmbH & Co. KG, Karlsruhe
Bromophenol blue	Sigma-Aldrich Co., St. Louis, USA

Calcium Chloride (CaCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Chelex	Bio-Rad Laboratories GmbH, München
Chloramphenicol	Serva Electrophoresis GmbH, Heidelberg
Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Diethylpyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe
Dimethyl sulfoxide (DMSO)	AppliChem GmbH, Darmstadt
Dithiothreitol (DTT)	Carl Roth GmbH & Co. KG, Karlsruhe
DMEM	GIBCO®, Invitrogen GmbH, Darmstadt
dNTPs	Promega GmbH, Mannheim
Doxorubicin	Enzo Life Sciences GmbH, Lörrach
Ethanol absolute	Th. Geyer GmbH & Co. KG, Renningen
Ethidium bromide	Carl Roth GmbH & Co. KG, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe
Fetal Bovine Serum (FBS)	Thermo Scientific HyClone, Logan, USA
Formaldehyde	Sigma-Aldrich Co., St. Louis, USA
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe
ß-Glycerolphosphate (BGP)	Sigma-Aldrich Co., St. Louis, USA
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe
GlycoBlue	Applied Biosystems/Ambion, Austin, USA
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe
Kanamycin	AppliChem GmbH, Darmstadt
Leupeptin	Carl Roth GmbH & Co. KG, Karlsruhe
Magnesium chloride (MgCl2)	Carl Roth GmbH & Co. KG, Karlsruhe
Methanol	M. Baker B.V., Deventer, Netherlands
MG-132	Biomol GmbH, Hamburg
Monopotassium phosphate (KH2PO4)	Carl Roth GmbH & Co. KG, Karlsruhe
N-ethylmaleimide (NEM)	Sigma-Aldrich Co., St. Louis, USA
NonidetTM P40 (NP-40)	Sigma-Aldrich Co., St. Louis, USA
Opti-MEM	GIBCO®, Invitrogen GmbH, Darmstadt
PBS tablets	GIBCO®, Invitrogen GmbH, Darmstadt
Pefabloc SC Protease Inhibitor	Carl Roth GmbH & Co. KG, Karlsruhe
Penicillin-Streptomycin solution	Sigma-Aldrich Co., St. Louis, USA
Peptone	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium acetate (KOAc)	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium chloride (KCl)	AppliChem GmbH, Darmstadt

Potassium dihydrogen phosphate (KH2PO4)	Carl Roth GmbH & Co. KG, Karlsruhe
Propidium iodide solution	Sigma-Aldrich Co., St. Louis, USA
Protein A SepharoseTM CL-4B	GE Healthcare, Uppsala, Sweden
RNase inhibitor	New England Biolabs, Frankfurt am Main
RNAiMAX	Invitrogen GmbH, Karlsruhe
Roti®-Phenol	QIAGEN Sciences
Rotiphorese® Gel 30	Carl Roth GmbH & Co. KG, Karlsruhe
Rotipuran® Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Rotipuran® Isoamylalcohol	Carl Roth GmbH & Co. KG, Karlsruhe
Salmon sperm DNA	Stratagene, La Jolla, USA
SepharoseTM CL-4B	GE Healthcare, Uppsala, Sweden
Skim milk powder	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium deoxycholate	AppliChem GmbH, Darmstadt
Sodium dodecylsulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
di-Sodium hydrogen phosphate dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium pyruvate (Na-Pyr)	GIBCO®, Invitrogen GmbH, Darmstadt
SYBR Green	Roche Diagnostics GmbH, Mannheim
TEMED	Carl Roth GmbH & Co. KG, Karlsruhe
α,α-Trehalose Dihydrate	USB Corporation, Cleveland, USA
Tris	Carl Roth GmbH & Co. KG, Karlsruhe
Triton X-100	AppliChem GmbH, Darmstadt
TRIzol® Reagent	Invitrogen GmbH, Karlsruhe
Trypsin-EDTA (0.05%)	GIBCO®, Invitrogen GmbH, Darmstadt
Tween-20	AppliChem GmbH, Darmstadt
Yeast Extract	USB Corporation, Cleveland, USA

2.3.2 Inhibitors

Actinomycin D	Sigma-Aldrich
alpha-amanitin	Sigma-Aldrich
Caffeine	Sigma-Aldrich
Camptothecin	Sigma-Aldrich
Cholerae toxin	EMD Chemicals

Cisplatin	Zytostatika
Cyclosporine A	Sigma-Aldrich
Doxorubicin	Enzo Life Sciences
DRB	Sigma-Aldrich
Etoposide	Sigma-Aldrich
Hydroxyurea	Sigma-Aldrich
Iodacetamide	Sigma-Aldrich
KM05283	Thermo Fisher Sc. Int.
MG132	Sigma-Aldrich
NAC	Affymetrix
NiCl2	Sigma-Aldrich
Nutlin-3A	Sigma-Aldrich
Ocadaic acid	Tocris
Olaparib	Santa Cruz
Rapamycin	Calbiochem
SB 203580	Biomol
Sorbitol	USB Corporation
SP600125	Sigma-Aldrich
Trolox	Sigma-Aldrich

2.4 Kits and reagents

LipofectamineTM 2000	Invitrogen GmbH, Karlsruhe
LipofectamineTM RNAiMAX	Invitrogen GmbH, Karlsruhe
PureYieldTM Plasmid Midiprep	Promega GmbH, Mannheim
QIAprep® Spin Miniprep Kit	Qiagen GmbH, Hilden
SuperSignal® West Dura	Thermo Fisher Scientific, Waltham, USA
SuperSignal® West Femto Maximum	Thermo Fisher Scientific, Waltham, USA

2.5 Nucleic acids

2.5.1 Plasmids

Name	Source
pCDNA3.1hygro(+)	Invitrogen

pCDNA3.1hygro(+)-HA-hCDK9	this study
pGEX-6P-1	GE Healthcare
pGEX-6P-1-hUBE2AA-WT	this study
pGEX-6P-1-hUBE2AA-S120A	this study
pCS2	(Knockaert et al, 2006)
pCS2-Flag-SCP1	(Knockaert et al, 2006)
pCS2-Flag-SCP2	(Knockaert et al, 2006)
pCS2-Flag-SCP3	(Knockaert et al, 2006)
pSTC-TK-WTRpb1-EGFP	D. Eick
pSTC-TK-Rpb1-dCTD-EGFP	D. Eick
pSTC-TK-Rpb1-1-3+S2A48+52	D. Eick
pSTC-TK-Rpb1-1-3+S5A48+52	D. Eick
pHA-JNK1	R. Janknecht
pHA-MEKKc	R. Janknecht

2.5.2 Oligonucleotides

2.5.2.1 siRNAs

Gene	Cat. Num	Sense sequence	Source
CDK9	S2834	UGA GAU UUG UCG AAC CAA Att	Ambion
CCNT	S2541	CGACCCAGACAAUAGACUAtt	Ambion
NCL	SI00300923	GCUAUGGAGACUACACCAGtt	Qiagen
NPM	SI00300979	GAAUUGCUUCCGGAUGACUtt	Qiagen
UBE2A	S14567	UUCAUAUUCCCGUUUGUUCtc	Ambion
RNF20	S32088	GGUCCGCAAGGAGUAUGAAtt	Ambion
RNF40	S18962	GUACUACAGUUCAAGAACAtt	Ambion
USP22	S23566	GGAGAGAAGUUUUCAACUUtt	Ambion

2.5.2.2 Primers

Experiment	Name	5'-3' sequence	Source
CDK9 cloning	HA-hCDK9-For	GCTGACGGATCCGCCA CCATGTACCCATACGA TGTTCCAGATTACGCT GAATTCATGGCAAAGC AGTACGACTC GGTGGAGTGCCC	This study
CDK9 cloning	hCDK9-Rev	GTCAGCCTCGAGTCAG AA GACGCGCTCAAACTCC	This study

		GTCTG	
Nucleolin knockdown	NCL50F	TGGCTCCTCCTCCAAA GGAGGT	This study
Nucleolin knockdown	NCL235R	TGGCTGGTGTGGCAAC TGCA	This study
Nucleophosmin knockdown	NPM250F	CAGCCAACGGTTTCCC TTGGG	This study
Nucleophosmin knockdown	NPM375R	TGACTCTGCATCTTCC TCCACAGC	Gomes et al, 2006
p21 ChIP	p21 TSS F	GGGGCGGTTGTATATC AGG	Gomes et al, 2006
p21 ChIP	p21 TSS R	GGCTCCACAAGGAACT GACT	Gomes et al, 2006
p21 ChIP	p21 TR for	CCAGGGCCTTCCTTGT ATCTCT	Gomes et al, 2006
p21 ChIP	p21 TR rev	ACATCCCCAGCCGGTT CT	Gomes et al, 2006
p21 RT-PCR	p21 +8000 for_1	CTCCAGGTGGCTCTGA GGT	Gomes et al, 2006
p21 RT-PCR	p21 +8000 rev_1	GCCCTTCTTCTTGTGTG TCC	Gomes et al, 2006
UBE2A cloning	hRad6A HA Hind3 For	GCTGACAAGCTTGCCA CCATGTACCCATACGA TGTTCCAGATTACGCT CAATTGATGTCCACCC CGGCTCGGCGGCGCCT CATG	This study
UBE2A cloning	hRad6A XhoI Rev	GTCAGCCTCGAGTCAA CAATCACGCCAGCTTT GTTC	This study
UBE2A mutagenesis	hRad6A S120A C-For	CAATCCCAATGCTCCA GCAAACAGCCAGGCT GCTC	This study
UBE2A mutagenesis	hRad6A S120A N-Rev	GTTTGCTGGAGCATTG GGATTGGGTTCATCCA AC	This study
USP22 cloning	hUSP22For	GCTGACGGATCCATGG TGTCCCGGCCAGAGCC CG	This study
USP22 cloning	hUSP22Rev	GTCAGCGCGGCCGCCT ACT	This study

		CGTATTCCAGGAACTG TTTG	

2.6 Proteins

2.6.1 Protein molecular weight markers

PageRulerTM Prestained Protein Ladder Fermentas GmbH, St. Leon-Rot

2.6.2 Enzymes

c-Jun N-terminal kinase 1	ProQinase GmbH, Freiburg
Cyclin Dependent Kinase 9	Cell Signaling
Phusion® High-Fidelity DNA Polymerase	New England Biolabs, Frankfurt am Main
Proteinase K	Invitrogen GmbH, Karlsruhe
Restriction enzymes	New England Biolabs, Frankfurt am Main
Reverse Transcriptase (M-MuLV)	New England Biolabs, Frankfurt am Main
RNase A	Qiagen GmbH, Hilden
T4 DNA Ligase	New England Biolabs, Frankfurt am Main
Taq DNA Polymerase	Prime Tech, Minsk, Belarus

2.6.3 Antibodies

2.6.3.1 Primary antibodies

Name	Clone	Cat. Number	WB	IP	IF	Source
β-Actin	-	ab6276-100	1:10000	-	-	Abcam
CCNT1	H-245	sc-10750	1:1000	1 µg	-	Santa Cruz
CDK9	C-20	sc-484	1:1000	1 µg	-	Santa Cruz
Coilin	Pdelta	sc-56298	1:1000	-	1:50	Santa Cruz
FLAG	M2	F1804	1:10000	4 µg	-	Sigma
H2B	-	07-371	1:3000	-	-	Upstate
H2Bub1	56	05-1312	1:5000	-	1:500	Millipore
H2Bub1	-	-	-	75 µl	-	Minsky et al, 2008
HSC70	B-6	sc-7298	1:25000	-	-	Santa Cruz
mouse	-	12-371	-	1 µg	-	Upstate

IgG						
NPM	-	32-5200	1:1000	-	1:100	Invitrogen
PCNA	PC10	ab29100	1:1000	-	-	Abcam
p-JNK	-	46685	1:1000	-	1:100	Cell Signaling
p-UBE2A	5A10	-	1:5	-	-	D. Eick/ E. Kremmer
p-Ser2	H5	MMS-129R	-	2 µl	-	Covance
p-Ser2	3E10	-	1:10	-	-	Dirk Eick (Chapman et al, 2007)
p-Ser5	H14	MMS-134R	1:10000	1 µl	-	Covance
UBE2A	-	A300-282A	1:3000	-	-	Bethyl
RNAPII	N-20	sc-899	-	1 µg	-	Santa Cruz
RNF20	-	NB100-2242	1:2000	-	-	Novus Biologicals
RNF40	-	ab26082	1:1000	-	-	Abcam
USP22	2391	-	1:1000	-	-	(Zhao et al, 2008)
γ-H2A.X	-	05-636	1:1000	-	-	Millipore

2.6.3.2 Secondary antibodies

Name	**Cat. Number**	**WB**	**IP**	**IF**	**Source**
Goat Anti-Mouse IgM	M 8644	-	3 µg	-	Sigma
Donkey Anti-Mouse IgG-HRP	715-036-150	1:10000	-	-	Jackson ImmunoResearch
Donkey Anti-Mouse IgM-HRP	115-035-044	1:3000	-	-	Jackson ImmunoResearch
Donkey Anti-Rabbit IgG-HRP	711-036-152	1:10000	-	-	Jackson ImmunoResearch
Goat Anti-Rat IgG + IgM-HRP	112-035-068	1:10000	-	-	Jackson ImmunoResearch
Donkey Anti-Mouse IgG-Alexa 488	A21202			1:500	Invitrogen

2.7 Cells

2.7.1 Bacterial cells

Name	Source
BL21 (Ril DE3)	Invitrogen
DH10B	M. Dobbelstein, GZMB, Goettingen

2.7.2 Eucaryotic cells

Name	**Source**
H1299	M. Dobbelstein, GZMB, Goettingen
HCT116 WT	M. Dobbelstein, GZMB, Goettingen
HEK293	M. Dobbelstein, GZMB, Goettingen
U2-OS	M. Dobbelstein, GZMB, Goettingen

2.8 Buffers and solutions

RIPA buffer

PBS 1X

NP40 1%

Na-deoxycholate 0.5%

SDS 0.1%

10X TBS-T

Tris 0.1M

NaCl 1.5M

Tween-20 0.5%

H_2O to 1l

Western salts 10X

Tris 0.25M

Glycin 0.86M

SDS 0.7mM

H_2O to 1l

PBS-T 10X (pH 7,4)

NaCl 0,73M

KCl 0.027M

NaH_2PO_4*7H_2O 14.3 mM

KH_2PO_4 14.7 mM

Tween 20 1%

H_2O to 4l

Frackelton buffer

Tris (pH 7.1) 5mM

NaCl 25 mM

Triton X-100 0.5%

DTT 0.2%

Protease and phosphatase inhibitors 1X

PCR-Mix 10X

Tris-HCl (pH 8.8) 750 mM

$(NH_4)_2SO_4$ 200 mM

Tween-20 0.1%

RT-PCR Master Mix

PCR-Mix 1X

$MgCl_2$ 3mM

SYBR Green 1:80000

dNTPs 0.2 mM

Taq-polymerase 20U/ml

Triton X-100 0.25%

Trehalose 300 mM

Pagano buffer

Tris-HCl (pH 7.4) 20 mM

DTT 2 mM

EDTA 0.25 mM

Deubiquitination buffer

Tris-HCl (pH 7.4) 60 mM

Glycerol 20%

$MgCl_2$ 10 mM

Blocking solution

PBST 1X

Milk 5%

Cell culture freezing medium

DMEM

FBS 50%

DMSO 8%

ChIP IP buffer

NaCl 150 mM

EDTA 5 mM

Tris (pH 8) 50 mM

NP-40 0.5%

Triton X-100 1%

Protease and phosphatase inhibitors

Pefabloc 1 mM

Aprotinin/Leupeptin 1 ng/μ l

BGP 10 mM

NEM 1 mM

DMEM cell culture "normal" medium

Phenol red-free, high-glucose DMEM

FBS 10%

Penicillin 100 U/ml

Streptomycin 100 μg/ml

Sodium pyruvate 1 mM

6× Lämmli buffer

Tris (pH 6.8) 0.35 M

Glycerol 30%

SDS 10%

DTT 9.3%

Bromphenol blue 0.02%

LB Agar

LB medium 1X

Agar 1.5%

LB medium

Peptone 1%

Yeast extract 0.5%

NaCl 86 mM

SDS separating gel (X%)

Acrylamide X%

Tris-HCl (pH 8.8) 375 mM

SDS 0.1%

APS 0.1%

TEMED 0.04%

SDS stacking gel (5%)

Acrylamide 5%

Tris-HCl (pH 6.8) 125.5 mM

SDS 0.1%

APS 0.1%

TEMED 0.1%

TAE buffer (50×)

Tris 2 M

Acetic acid 1 M

EDTA 0.1 M

Transfer buffer

10× Western salts 10%

Methanol 15%

2YT medium

Peptone 1.6%

Yeast extract 1%

NaCl 86 mM

2.9 Software

Primer designing tool	NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/)
Confocal pictures processing	LSM viewer, ZEISS

3. Methods

3.1 Cell culture

Culturing cells

H1299 (human non-small cell lung carcinoma), HEK293 (human embryonic kidney) and U2-OS (human osteosarcoma) cells were cultured in phenol red-free high-glucose Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100

µg/ml streptomycin and 1 mM sodium pyruvate at 37 C under 5% CO2 atmosphere. HCT116 (human colorectal carcinoma) cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate in the same conditions.

Plasmid DNA transfection in 6-well plate

2.4 µg of plasmid DNA were diluted in 200 µl of Opti-MEM. In a separate tube 8 µl of Lipofectamine 2000 were diluted in 200 µl of Opti-MEM. After 5 min DNA- and Lipofectamine 2000-containing solutions were mixed and incubated for 20 min at room temperature. After the incubation transfecting mixture was added to a well with adherent 80%-confluent cells cowered with 2 ml of DMEM-FBS. After 4 h of transfection cells were washed with PBS and covered with fresh DMEM-FBS-P/S.

siRNA transfection protocol in 6-well plate

30 pmol siRNA and 5 µl RNAiMAX were diluted in 500 µl OptiMEM. Components were incubated at room temperature for 20 min and mixed with 2.5 ml antibiotic-free normal growth medium containing 2.0-3.0 x 10^5 cells. The medium was replaced with a fresh one after 24 h. Cells were harvested in 48 h after transfection.

Immunofluorescence

Cells were grown on glass cover slips on a 12 well plate. After washing with PBS cells were fixed for 20 min with 4% formaldehyde and permeabilized for 10 min with 0.1% TritonX-100. Further cells were blocked with Blocking Solution (10% FCS in PBS) for 10 min and treated with primary antibodies in Blocking Solution for 1-12 h. Secondary antibodies were applied in the dark for 30 min together with DAPI stain. Cover slips were removed from the plate and mounted on a glass slide for the microscopy.

3.2 Molecular biology

Molecular cloning

The expression constructs used in this study were created by amplifying the gene from cDNA using specific primers and Phugene polymerase. The product of PCR and appropriate vector molecule were digested with Fermantas restriction enzymes, gel-purified and ligated with T4 DNA ligase from Fermentas. The mutagenesis was performed by amplifying the plasmid with the gene using the primers, containing the mutation. The product of PCR was treated with DpnI to degrade the template DNA and transformed into E.coli DH10B cells.

TRIZOL RNA isolation from 6-well plates

Cells were washed twice with ice-cold PBS. 500 µl Trizol reagent were added to each well. Cells were scraped and transferred to 1.5 ml tube. 100 µl of chloroform were added followed by vortexing for 15 s. After 20 min of centrifugation at maximum speed, the aqueous phase was taken and washed with 100 µl of chloroform again. The resultant solution was mixed with equal volume of isopropanol and kept at -20C for 2 h. The mixture was centrifuged for 20 min at full speed, the pellet was washed once with 500 µl of 70% ethanol and dried. Dry RNA was dissolved in 40 µl of DEPC water and its concentration was measured with a NanoDrop.

cDNA synthesis by reverse transcription

1 microgram of total RNA was mixed with 2ul 15uM random nonamer, dT23VN or combined primers and 4 µl of 2.5mM dNTPs and heated at 70 C for 5 min. After cooling on ice 4 µl of mastermix were added, containing 10X reaction buffer, 10 units of RNAse inhibitors and 25 units of M-MuLV Reverse Transcriptase. The reaction was incubated at 42 C for 1 hour and stopped by heating to 95 C for 5 min.

Real-time PCR

14 µl of RT-PCR Master Mix were mixed with primers (final concentration 0.3 µM), 1 µl of ChIP-DNA or cDNA and water to final volume 25 µl. The PCR was performed according to the following program:

2 min – 95 C

15 sec – 95 C

1 min – 60 C x40

Melting curve 55-95 C to ensure primer specificity

Chromatin Immunoprecipitation

ChIP analysis was performed essentially as described (Pirngruber *et al.*, 2009). In brief, growing medium was removed from plates and cells were crosslinked by adding 1.42% formaldehyde diluted in PBS. After 15 min of incubation at room temperature the crosslink was quenched by adding glycine to a final concentration of 125 mM. 5 min later cells were washed twice with PBS and scraped in 1 ml of ChIP buffer. All further manipulations were performed on ice or at 4 C. Scraped cells were cleared from the buffer by centrifugation (12000g x 10 min) and washed again with 1 ml of ChIP buffer. After second centrifugation the pellet was resuspended in 300 µl of ChIP buffer and sonicated by Bioruptor (3 x 10 min) at "H" setting with 10 s breaks between 10 s pulses. The soluble chromatin was cleared from the debris by centrifugation (12000g x 10 min) and pre-cleaned with 100 µl of 50% slurry sepharose beads for 1 hour. After the pre-clean the chromatin was centrifuged, aliquoted, frozen in liquid nitrogen and stored at -80 C. For the analysis 50 µl of chromatin were diluted up to 0.5 ml with ChIP buffer and incubated overnight with indicated amounts of antibodies. Immunobound complexes were precipitated by adding 30 µl of 50% slurry Protein-A or Protein-G sepharose and incubation for another 2 h. After the incubation samples were centrifuged (2000g x 2 min). The beads were washed with ChIP buffer 6 times and the crosslink was reversed by adding 10% slurry Chelex with subsequent heating to 95 C for 10 min. The proteins in the sample were eliminated by adding 40 µg of Proteinase K and incubating for 30 min at 55 C. The samples were centrifuged (12000g x 1 min) and the supernatant was analysed by RT-PCR. The inputs were prepared by precipitating the DNA from 50 µl of undiluted chromatin by standard ethanol precipitation.

Kommentar [SJ1]: Might want to do this with your reference program.

3.3 Protein biochemistry

In vitro deubiquitination

Cells were washed with PBS and scraped in Pagano buffer. After mixing cells were sonicated with tip sonicator for 15 s and centrifuged at full speed to remove the cell debris. 1 volume of target supernatant was mixed with 1 volume of effector supernatant and 2 volumes of Deubiquitination buffer. The reaction was performed with shaking at 37 C for 15 min and stopped by adding 6X Laemmli buffer and heating to 95 C for 5 min. The products of reaction was analyzed by SDS-PAGE Western blot.

Protein co-immunoprecipitation

After washing with PBS cells were scraped in 1 ml Frackelton buffer. Cells were vortexed for 15 s and tumbled for 45 min at 4 C. Cell lysate was cleared by centrifugation and the supernatant was pre-cleaned by adding 50 µl of sepharose beads for 1 hour. The pre-cleaned supernatant was incubated with 1-3 µg of antibodies for 2 h and 30 µl of protein A or G coupled sepharose beads for 1 hour at 4 C. Beads were washed 4 times with Frackelton buffer and heated at 95 C with 70 µl of 2X Laemmli buffer. The supernatant was analyzed by SDS-PAGE and Western blot.

GST protein purification

BL21 E. coli strain containing a plasmid coding for an appropriate gene under GST tag was grown in 2X YT medium to the OD_{600} 300, then induced with 1 mM IPTG and incubated overnight at 16 C. The bacteria were harvested by centrifugation and lysed in a GST Lysis Buffer containing 0.2 mg/ml Lysozyme. Cells were lysed by sonication (5 pulses 30 s each, full power). The solution was cleared by centrifugation and incubated with glutathione-sepharose beads for 2 h at 4 C. After triple washing with GST Lysis Buffer protein was eluted with 25 mM glutathione in Lysis Buffer for 1 hour at 4 C. Eluted protein was analyzed by SDS-PAGE and measured by Coomasie staining in comparison to the serial dilution of BSA, then stored at -80 C.

In vitro kinase assay

150 ng of recombinant kinase were diluted in 20 µl of Kinase buffer and incubated for 30 min at 30 C to block all possible auto-phosphorylation sites with

non-radioactive phosphates. After that 500 ng of target protein and 50uCi of $\gamma[^{32}P]$-ATP in kinase buffer were added to the final volume of 30 µl. The reaction was performed for 20 min at 30 C and stopped by adding 6X Laemmli buffer and heating up to 95 C for 5 min. Later it was separated by SDS-PAGE, the gel was dried and analyzed by phosphoimager to detect the phosphorylated proteins.

SDS-PAGE

Protein extracts were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Adherent cells were lysed with RIPA buffer with Pefabloc (1 mM), Aprotinin/Leupeptin (1 ng/ul), betaglycerol phosphate (10 mM) and N-ethylenmaleimid (1 mM). The extracts were then sonicated for 15 s with Bandelin Sonoplus tip sonicator to shear the genomic DNA. The extracts were mixed with Laemmli buffer and heated at 95 C for 5 min. Proteins were separated in 8% or 15% acrylomide gels in SDS running buffer at 25 mA.

Western blot analysis

Separated proteins were transferred to nitrocellulose membranes by Western blot procedure (Towbin *et al*., 1979). The transfer was performed at 100V for 1 hour using Transfer buffer. The membrane was blocked in Blocking solution (PBS-T with 5% dry milk) and incubated overnight with primary antibodies diluted in Blocking solution. On the next day the membrane was washed 3 times with PBS-T and incubated for 1 hour with secondary HRP-coupled antibodies in Blocking solution. After the incubation the membrane was washed with PBS-T 3 times and developed using the enhanced chemoluminiscence solution and X-ray films.

4. Results

In this study different mechanisms of regulation of H2B monoubiquitination were examined.

First, the role of P-TEFb complex in maintaining the level of H2Bub1 was described. P-TEFb was found to be responsible not only for gene-specific but also for a global level of H2B ubiquitination.

Second, the role of UBE2A regulation by CDK9 through site-specific phosphorylation has been demonstrated. The ubiquitination of PCNA, another target of UBE2A, was also shown to be dependent upon P-TEFb activity.

The last part of this study describes strong and rapid loss of H2B monoubiquitination caused by various stress-inducing factors, such as chemotherapeutics, μltraviolet radiation, osmotic shock, etc. The signaling and deubiquitinating enzymes responsible for this global epigenetic shift were identified.

4.1 CDK9 positively regulates global and gene-specific levels of H2B monoubiquitination.

4.1.1 CDK9 activity is necessary for supporting global level of H2Bub1.

Since phosphorylation of Ser2 of RNAPII CTD and H2Bub1 have the same pattern of accumulation on a gene, we hypothesized that the activity of the P-TEFb complex including CDK9 and CCNT1 is important for H2B monoubiquitination. H1299 cells treated with different inhibitors of CDK9 demonstrated a loss of H2Bub1 (Fig. 10A). The overexpression of the recombinant CDK9 led to the increased p-Ser2 and H2Bub1, whereas the knockdown of CDK9 by a specific siRNA caused a strong reduction of p-Ser2 as well as H2Bub1 (Fig. 10B,C). RNAPII carboxy-terminal domain (CTD) phosphatases SCP1, SCP2 and SCP3 were found to have high specificity towards Ser2 in comparison to Ser5 (Fig. S1). The overexpression of these phosphatases reduced both the phosphorylation of Ser2 of RNAPII CTD and monoubiquitination of H2B (Fig. 10D).

To address the question whether Ser2 of RNAPII CTD is really the target of CDK9 which regulates H2Bub1 the following experiment was performed. Recombinant large RNAPII subunits resistant to α-amanitin and having either wild type or mutated CTD were overexpressed in HEK293 cells. The cells were treated with α-amanitin for 48 h to block all the transcription by endogenous RNAPII. The result of the experiment clearly shows that cells which had no CTD or CTD with S2A mutations had a significantly lower level of global H2B monoubiquitination whereas cells with wild type CTD or S5A mutations maintain a similar levels of H2Bub1 (Fig. 10E).

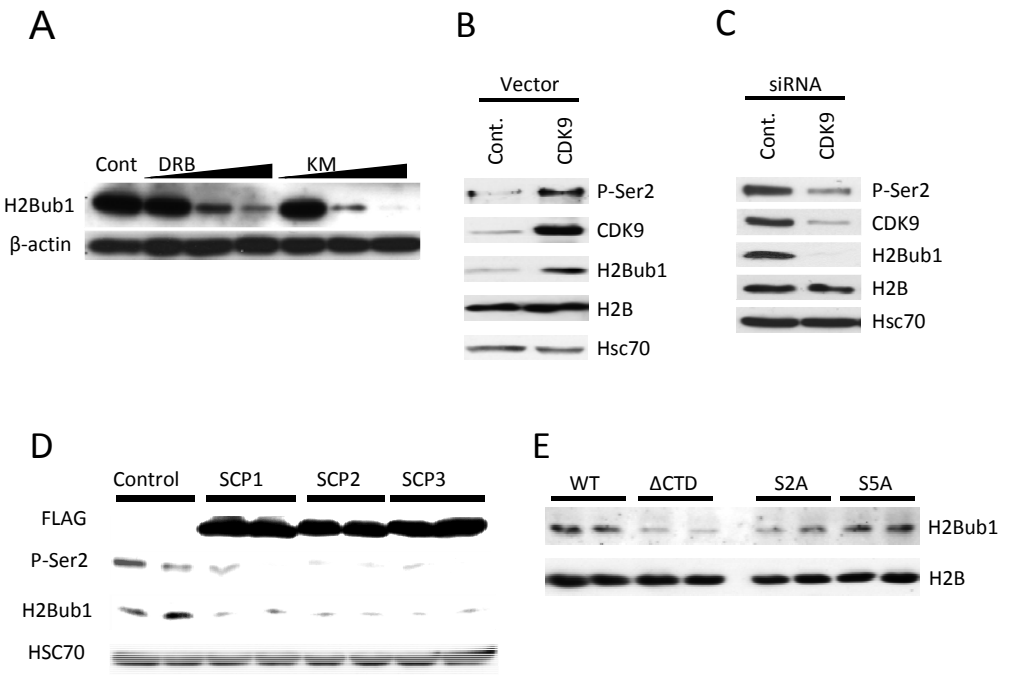

Figure 10. CDK9 activity and RNAPII CTD Ser2 phosphorylation regulate H2B ubiquitination. A. H1299 cells were treated with 10, 50 and 100 μM of CDK9 inhibitors 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) and 8-(methylthio)-4,5-dihydrothieno[3',4':5,6]benzoisoxazole-6-carboamide (KM05283) for 4 h and analyzed by Western blot. **B**. H1299 cells were transfected with an empty vector or recombinant hCDK9 expression vector. Following 48 h of expression cell extracts were analyzed by Western blot. **C**. H1299 cells were transfected with control or anti-CDK9 siRNAs for 48 h and analyzed by Western blot. **D**. H1299 cells were transfected with plasmids encoding for FLAG-tagged CTD phosphatases SCP1, SCP2 and SCP3. 72 h after transfection protein extracts were analyzed by

Western blot with specified antibodies. **E**. HEK293 cells were transfected with plasmids encoding α-amanitin resistant large RNAPII subunit with different mutations in the CTD. After 24 h of expression cells were grown in medium containing 10 mM α-amanitin for another 48 h. Protein extracts were analyzed by Western blot.

4.1.2 CDK9, not transcription *per se* is required for maintaining H2Bub1 on an actively transcribed gene.

In an attempt to test whether CDK9 activity plays a positive role in the maintaining H2B ubiquitination on a single gene chromatin immunoprecipitation analyses were performed using the p21 gene as a model system. The p21 gene is unique in that it could be transcribed in the absence of active P-TEFb complex (Gomes et al, 2006). The gene was induced by two different chemicals. First, Nutlin-3A, is a selective inhibitor of HDM2, the E3 ubiquitin ligase that targets p53 for proteosomal degradation. Following Nutlin-3A treatment the polyubiquitination and subsequent degradation of p53 is blocked which leads to its rapid accumulation and induction of p53-regulated genes, such as p21 (Vassilev et al, 2004). DRB (5, 6-dichloro-b-D-ribofuranosyl benzimidazole) can also induce transcription of some p53 target genes while paradoxically inhibiting CDK9 activity.

Due to the different nature of the two drugs the dynamics of p21 induction are different as well. A time-course of the two drugs was performed to estimate an appropriate time point for the experiment. As seen from the graph in 8 h after the treatment both drugs actively induced p21 expression (Fig. 11A).

Both Nutlin-3A and DRB increased the occupancy of RNAPII to the transcribed region (Fig. 11B, C), whereas only upon Nutlin-3A treatment did the CTD of RNAPII remain phosphorylated at Ser2 since DRB prevents this phosphorylation by inhibiting CDK9 (Fig. 11D). In accordance with our model H2B ubiquitination is detected only following Nutlin-3A-induced gene induction while the transcription of p21 in the absence of CDK9 activity does not lead to the accumulation of this transcription-related chromatin mark (Fig. 11E).

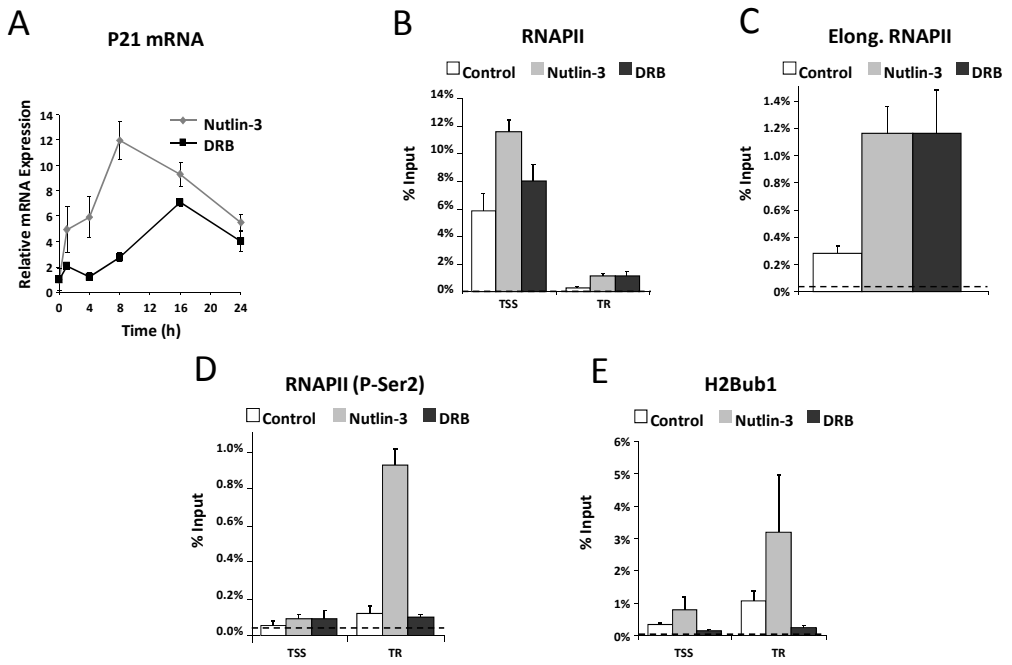

Figure 11. H2Bub1 depends on CDK9 activity but not on transcription *per se*. A. Nutlin-3A and DRB induce p21 expression. U2OS cells were treated with 8 μM of Nutlin-3A and 50 μM of DRB for 4, 8, 16 and 24 h. RNA was extracted and reverse transcribed. The cDNA was analyzed by real-time PCR and results were normalized to mitochondrial 16S rRNA. **B-E**. H2Bub1 increases on the p21 gene only in the presence of Ser2-phosphorylated RNAPII. U2OS cells were treated with Nutlin-3A and DRB for 8 h and the chromatin was immunoprecipitated using the antibodies against total RNAPII **(B,C)**, pSer2 RNAPII **(D)** and H2Bub1 **(E)**. Chromatin was analyzed by real-time PCR, the data was normalized to input DNA and expressed as percent of recovery, mean values + standard deviation, n=3. Dotted lines represent the experimental background. TSS - transcription start site, TR – transcribed region.

4.2 CDK9 regulates UBE2A by site-specific phosphorylation

4.2.1 CDK9 and CCNT1 interact with UBE2A

UBE2A (hRad6A) is an E2 ubiquitin conjugating enzyme involved in the ubiquitination of histone H2B and DNA clamp PCNA. In yeast Rad6A is activated

by phosphorylation of serine 120. The kinase responsible for this phosphorylation, called Bur1 (Wood et al, 2005) is homologous to human CDK9. Furthermore, the human homolog UBE2A was also shown to be phosphorylated at the same position by other cyclin-dependent kinase members (Sarcevic et al, 2002). We hypothesized that human UBE2A function may also be regulated by CDK9-mediated site-specific phosphorylation.

In order to test whether CDK9 and UBE2A interact in human cells we performed coimmunoprecipitation studies between the kinase and its putative substrate. We were able to successfully demonstrate the binding of endogenous UBE2A with CDK9 and Cyclin T1 (Fig. 12A) as well as CDK9 with UBE2A (Fig. 12B).

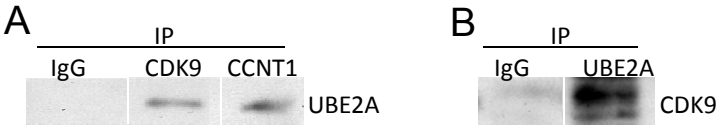

Figure 12. UBE2A interacts with CDK9 and CCNT1. H1299 cells were harvested and coimmunoprecipitation was performed using the antibodies against endogenous CDK9, Cyclin T1 **(A)** and UBE2A **(B)** as well as unspecific IgGs. The presence of coimmunoprecipitated proteins was detected by Western blot with specific antibodies.

4.2.2 CDK9 phosphorylates UBE2A *in vitro* and *in vivo*

Besides the binding we also wanted to demonstrate site-specific kinase activity of CDK9 towards UBE2A. For that wild type UBE2A as well as S120A mutant were purified from *E. coli* as GST-fusion proteins and phosphorylated by CDK9 *in vitro* using γ-[^{32}P]-ATP. Serine 120 is homologous to the yeast Rad6 phosphorylation site (also S120) of human UBE2A. By mutating it to alanine we expected to abolish the CDK9-mediated phosphorylation.

While GST alone and the S120A mutant of UBE2A were only mildly phosphorylated (Fig. 13A), wild-type GST-UBE2A was significantly phosphorylated by P-TEFb *in vitro.* After quantifying the intensity of bands and normalizing them to the molecular weights of proteins the specificity of P-TEFb for S120 became clear, since the S120A mutant was phosphorylated at a level similar to GST alone (Fig. 13B).

In collaboration with Dirk Eick and Elisabeth Kremmer (Helmholtz Center for Environmental Health, Center for Integrated Protein Science, Munich) we generated a mouse monoclonal antibody that specifically recognizes S120-phosphorylated form of UBE2A. Importantly, the signal of this antibody in Western blot is strongly decreased following the knockdown of either CDK9 or UBE2A suggesting that the phosphorylation of UBE2A *in vivo* depends on CDK9 activity (Fig. 13C).

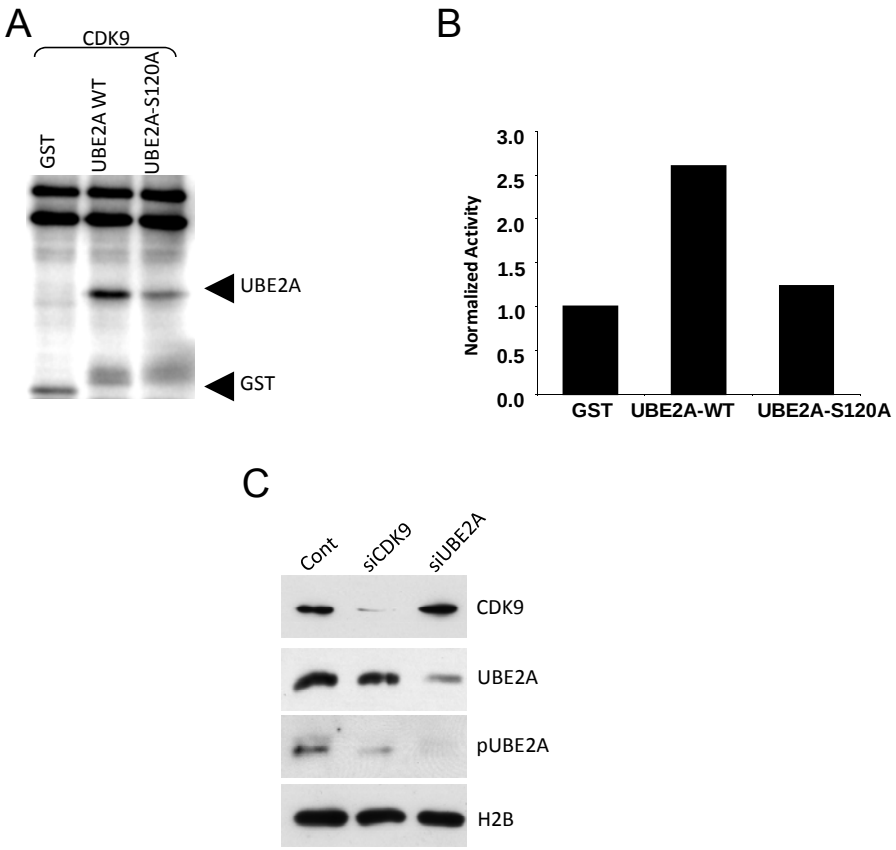

Figure 13. CDK9 phosphorylates UBE2A *in vitro* and *in vivo*. A. GST-UBE2A fusions were phosphorylated by CDK9 in the presence of γ-[^{32}P]-ATP. Arrows indicate specific bands obtained with phosphoimager. High molecular weight bands represent the products of auto-phosphorylation of kinase complex components. **B.** Quantification of band intensity was performed using TYPHOON Scanner Control 3.0 software. Results were normalized to the protein size and presented as fold relative to GST background. **C.** HCT116 cells were transfected

with siRNAs against CDK9 or UBE2A. Protein extracts were analyzed by Western blot 48 h after transfection.

4.2.3 CDK9 is necessary for both H2B and PCNA monoubiquitination

As is typical for most E2 ubiquitin conjugating enzymes, UBE2A supplies the ubiquitin for several E3 ubiquitin ligase complexes. In this case RAD18 and RNF20/40 which ubiquitinate PCNA and H2B, respectively, both utilize UBE2A for substrate ubiquitination (Kim et al, 2009; Watanabe et al, 2004). Assuming that CDK9 might regulate UBE2A function we hypothesized both ubiquitinations may be affected by changing the level of CDK9. Indeed, the reduction of PCNA and H2B monoubiquitinations in UVC-treated CDK9-depleted cells was comparable to the effect of knocking down UBE2A alone (Fig. 14A).

Cyclin T1 is the major cyclin component of P-TEFb and is known to be necessary for its activity (Peng et al, 1998). As predicted by our model the depletion of CCNT1 also led to the reduction of H2B monoubiquitination as well as PCNA monoubiquitination following the treatment by UVC and hydroxyurea (Fig. 14B).

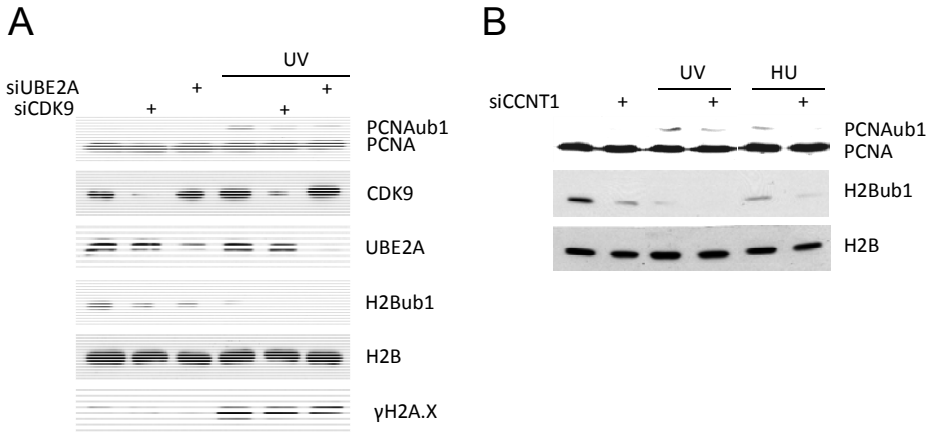

Figure 14. CDK9 regulates PCNA monoubiquitination. A. HCT116 cells were transfected with siRNAs against CDK9 or UBE2A. After 48 h 80J/m^2 UVC was applied and cells were harvested 3 h later. **B**. HCT116 cells were transfected with a control siRNA or an siRNAs against Cyclin T1. 80J/m2 UVC and 3mM of hydroxyurea were applied 3 and 20 h before harvesting protein extract.

4.3. H2B is rapidly deubiquitinated following various stress conditions

4.3.1. Different stress factors rapidly reduce H2B monoubiquitination

As shown in the previous chapter treatment with µltraviolet light drastically reduced the levels of H2Bub1 (Fig. 14A). In the course of this study we observed a severe reduction of histone H2B monoubiquitination caused by a wide palette of factors, such as DNA-damaging chemotherapeutic drugs (doxorubicin, camptothecin, etoposide, cisplatin, hydroxyurea), proteasome inhibitors (MG132), heat shock, osmotic stress and nucleolar function disruption (Fig. 14B, 15A, S2). Surprisingly we were able to rescue this effect by pre-treating the cells with caffeine, which is widely used to inhibit the activity of PI3-kinase-related protein kinases following DNA damage (Fig. 15B).

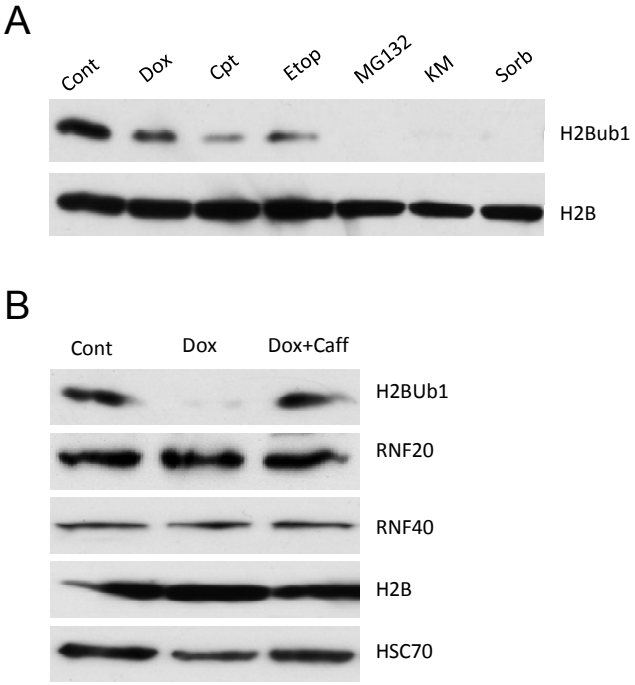

Figure 15. Various factors downregulate the H2B ubiquitination. A. H1299 cells were treated for 1 h with 7.5 µM doxorubicin, 10 µM camptothecin, 100 µM etoposide, 20 µM MG132, 30 µM KM05283 and 0.5 M sorbitol. Protein extracts were analyzed by Western blot.

B. H1299 cells were pretreated with 4 mM of caffeine for 1 h, then treated with 7.5 μM doxorubicin for 3.5 h. Protein extracts were analyzed by Western blot.

The chemotherapeutic drugs used in this study cause the formation of single- or double-strand DNA breaks. Ultraviolet light, heat shock and osmotic stress can also act as DNA-damaging factors. Caffeine which is able to rescue the H2Bub1 is a well known inhibitor of DNA damage response PI3-kinases. Therefore, we further tested whether DNA damage *per se* causes a loss of H2B ubiquitination. Surprisingly, cells irradiated with 10 Gy of γ-radiation showed no significant decrease of H2Bub1 (Fig. 16A). Moreover, in a concentration course of the DNA-damaging drug doxorubicin we can clearly see that the cells develop a strong DNA-damage response (as indicated by the level of γH2AX) at concentrations which do not affect H2Bub1 (Fig. 16B). We therefore analyzed several signaling pathways which are known to be activated by cell stress and potentially lie downstream of the effects of caffeine to determine if these are involved in the stress-induced loss of H2Bub1. As seen from Table 1 only a few of these could rescue the post-stress H2Bub1 levels.

Target	**Rationale**	**Inhibition**	**Effect**
ATM	inhibited by caffeine (Sarkaria et al, 1999)	KU55933, siRNA	-
ATR	inhibited by caffeine (Sarkaria et al, 1999)	siRNA	-
DNA-PK	inhibited by caffeine (Block et al, 2004)	siRNA	-
PI3Ks	Include ATM, ATR and DNA-PK	Wortmannin	-
JNK	Stress-activated (Ip & Davis, 1998)	SP600125	+
p38	Stress-activated (Roux & Blenis, 2004)	SB203580	-
PP1, 2A	CDK9 phosphatases (Chen et al, 2008)	Okadaic acid	-
PP2B	CDK9 phosphatases (Chen et al, 2008)	Cyclosporin A	-
PARP	Activated by stresses (Nicoletti & Stella, 2003)	Olaparib	-
mTOR	stress-activated (Reiling & Sabatini, 2006)	Rapamycin	-
G-protein	regulates cAMP level, as caffeine does	Cholera toxin	-
ROS	Produced by various stresses	NAC/Trolox	-
DUBs	remove ubiquitin tags	NiCl2/IAA	+

Table 1. Signaling pathways analyzed in the course of the study.

Most of these drugs used require transport mechanisms and subsequent steps in order to get inside the cell and cause the damage. Thus in order to analyze the dynamics of H2Bub1 reduction we treated cells with ultraviolet radiation and followed the effects of treatment on global H2Bub1 levels. From the results of a

time course treatment it is clear that most of H2Bub1 loss happens within 15 min after the treatment indicating that H2B ubiquitination and deubiquitination are very fast and highly dynamic processes (Fig. 16C).

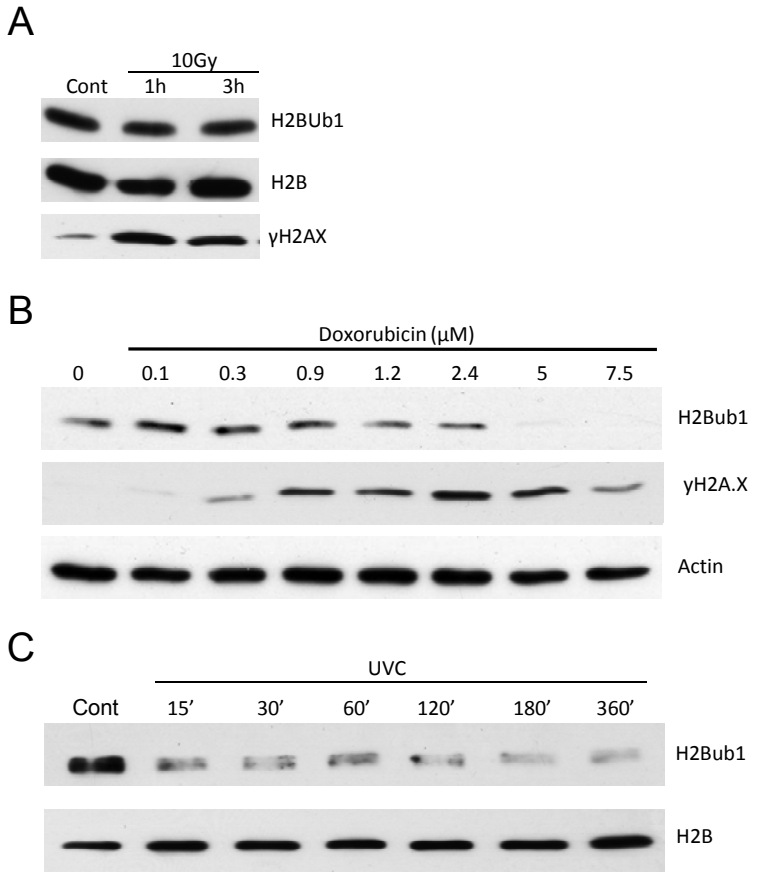

Figure 16.The reduction of H2B ubiquitination happens rapidly but is not caused by DNA damage *per se*. A. H1299 cells were irradiated with 10 Gy γ-irradiation 1 and 3 h before harvesting for Western blot analysis. **B.** H1299 cells were treated with the indicated concentrations of doxorubicin for 3.5 h, proteins were analyzed by Western blot. **C.** H1299 cells were treated with UVC (80 J/m^2) and harvested for Western blot after indicated times.

4.3.2. Nucleolar function is necessary for maintaining H2B ubiquitination

Nucleoli are known to quickly respond to different stress factors by exchanging their components with the nucleoplasm (Boulon et al, 2010). Some consider nucleoli to be one of the most important stress sensors in the cell (Mayer

et al, 2005; Olson, 2004). We hypothesized that this effect could be involved in the regulation of H2Bub1 upon stress. At low concentrations Actinomycin D is a potent RNAPI inhibitor and can rapidly disrupt the function of nucleoli by blocking ribosomal RNA transcription (Perry & Kelley, 1968). Concentration course studies showed that the decrease of H2Bub1, stress-induced release of the nucleolar protein nucleophosmin (NPM) and activation of the stress-responsive kinase JNK happen in the same range of concentrations (Fig. 17A, B, S3).

Nucleophosmin and nucleolin are two important components of nucleoli and are essential for proper rRNA processing and ribosomal subunits biogenesis (Ginisty et al, 1998; Lindstrom, 2011). Nucleophosmin is a chaperone and one of its functions is retaining the ARF protein in the nucleolus (Colombo et al, 2005). Upon stress-induced nucleoplasmic translocation NPM releases ARF to the nucleus where it can trigger p53-signaling in previously described manner. The knockdown of nucleophosmin and nucleolin led to the reduction of H2B ubiquitination indicating that ribosomal function is needed for H2Bub1 maintanence. Since the cells used in the experiment were p53-negative it is unlikely that the observed effects depend on the p53 pathway. (Fig. 17C, D).

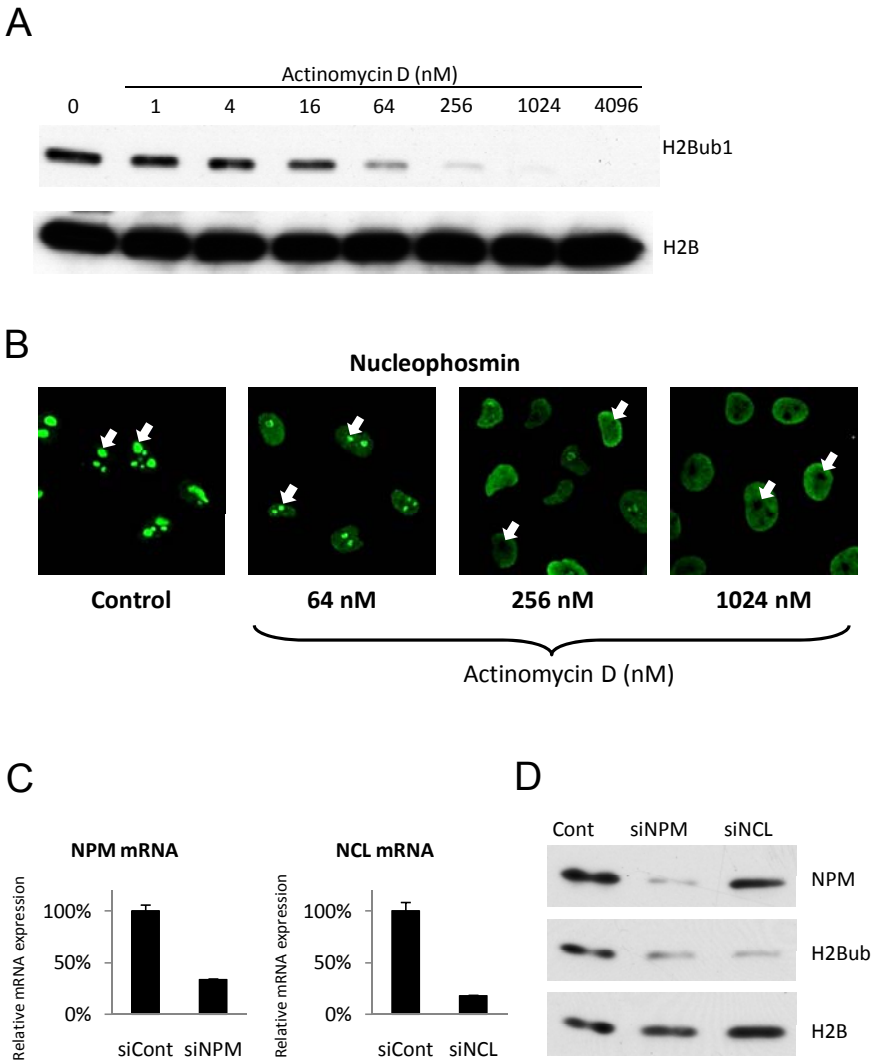

Figure 17. Disruption of nucleolar function causes a loss of histone H2B monoubiquitination. A. H1299 cells were treated with the indicated concentrations of actinomycin D for 3.5 h. Protein extracts were subjected to Western blot analysis. **B.** H1299 cells were treated with the indicated concentrations of actinomycin D for 3.5 h and immunostained with antibodies against nucleophosmin. White arrows indicate individual nucleoli with or without nucleophosmin. **C.** H1299 cell were transfected with siRNAs against nucleophosmin and nucleolin. After 48 h after transfection total RNA was harvested, reverse transcribed and analyzed by RT-PCR. The results were normalized to 36B4 expression and presented as percent

of expression. n=3, error bars represent standard deviation **D**. H1299 cell were transfected with siRNAs against nucleophosmin and nucleolin. In 48 h after transfection cells were harvested and protein extracts were analyzed by Western blot.

Nucleoli tightly interact with specific subnuclear structures involved in snRNA maturation referred to as Cajal bodies (Kiss et al, 2006). This interaction is mediated by protein migration. We observed a massive migration of one of the main Cajal body structural proteins, Coilin, to the periphery of the nucleoli following the induction of stress response by the same factors that induce the loss of H2Bub1. Interestingly, same as H2B ubiquitination, proper localization of Coilin can be rescued by caffeine treatment (Fig. 18).

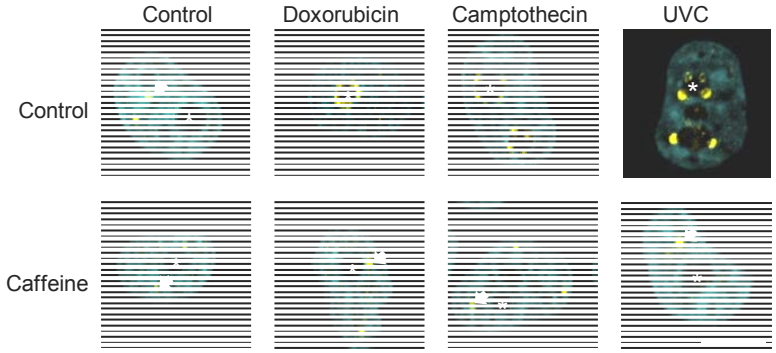

Figure 18. Disruption of Cajal bodies structure upon stress can be rescued by caffeine. H1299 cells were pretreated with 4 mM caffeine for 30 min and treated with 7.5 μM doxorubicin, 10 μM camptothecin and 50 J/m^2 UVC. After 3.5 h cells were fixed and stained with antibodies against Coilin (yellow) and with DAPI (cyan). Stars represent nucleoli, arrows show intact Cajal bodies. Scale bar is 10 μm.

4.3.3. JNK activity regulates the level of H2B ubiquitination

Activation of JNK is a hallmark of various stress inducers. Therefore, we used a specific JNK inhibitor SP600125 to determine whether increased JNK activation might be essential for the stress-induced loss of H2Bub1. Indeed, pre-treatment with the JNK inhibitor partially blocked the ability of doxorubicin or actinomycin D to decrease global H2Bub1 levels (Fig. 19A, S4).

Further support for the role of JNK in this process was provided by overexpressing it together with constitutively active form of its activating kinase

MEKK1. As seen from the results of Western blot and immunofluorescence staining, the overexpression of activated JNK leads to the reduction of H2B ubiquitination (Fig. 19B, C). Furthermore, the effect of overexpression can be rescued by the treatment with the JNK inhibitor (Fig. 19D).

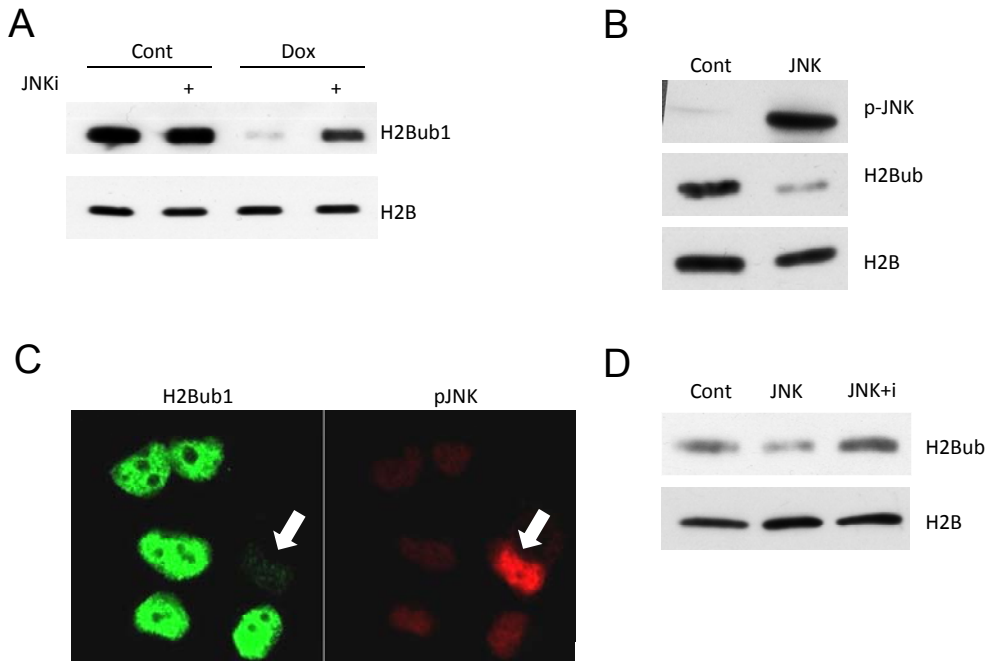

Figure 19. JNK activity regulates the level of H2B ubiquitination. A. H1299 cells were pretreated with 20 μM of JNK inhibitor SP600125 for 30 min before 3.5 h treatment with 7.5 μM of doxorubicin. Proteins were analyzed by Western blot. **B, C.** H1299 cells were transfected with control vector or plasmids encoding human JNK1 and its activator MEKK1. After 24 h cells were **(B)** harvested for Western blot or **(C)** stained with antibodies against H2Bub1 (green) and phospho-JNK (red). White arrows indicate the nucleus of the cell with overexpressed activated JNK. **D.** H1299 cells were transfected as in B and 24 h later treated with 20 μM of SP600125 for 3 h before harvesting for Western blot.

4.3.4. H2Bub1 levels are reduced due to the activation of a cellular deubiquitinating enzyme

We questioned whether the activity of deubiquitinating enzymes (DUBs) is responsible for the rapid removal of H2B ubiquitination following cellular stress.

Two wide range DUBs inhibitors, $NiCl_2$ and iodacetamide (IAA), significantly rescued doxorubicin or actinomycin D induced decrease in H2Bub1 (Fig. 20A).

However, based on the data it is not clear whether the effects of $NiCl_2$ and IAA are due to the basal level of DUB activity (i.e. the loss of H2Bub1 is primarily caused by decreased ubiquitination) or whether a specific DUB activity is increased. In order to clarify this question we performed an in vitro deubiquitination experiment (Fig. 20B). In brief, protein extracts from normal cells (substrate) were mixed with extracts from cells where RNF20 and RNF40 were depleated with siRNA to minimize the level of H2Bub1 (effector). The reaction buffer was added to the mixed extracts and they were incubated at 37 C. After mixing the substrate extract with the control effector extract we observed no change of H2Bub1 meaning that neither additional ubiquitinated H2B nor any appreciable DUB activity were present in the control effector extract. Note that the level of total H2B doubled at the same time due to additional histones present in the effector extracts. When the doxorubicin-treated cells were used for the effector extract the amount of H2B ubiquitination dropped below the basal level indicating that an increased deubiquitinating activity was present in treated cells (Fig. 20C).

In order to prove that the loss of ubiquitination caused by the presence of the doxorubicin-induced effector extract was provided by DUBs we performed another in vitro deubiquitination experiment with DUB inhibitors. The result clearly shows all three DUB inhibitors were able to successfully rescue the level of H2B ubiquitination (Fig. 20D).

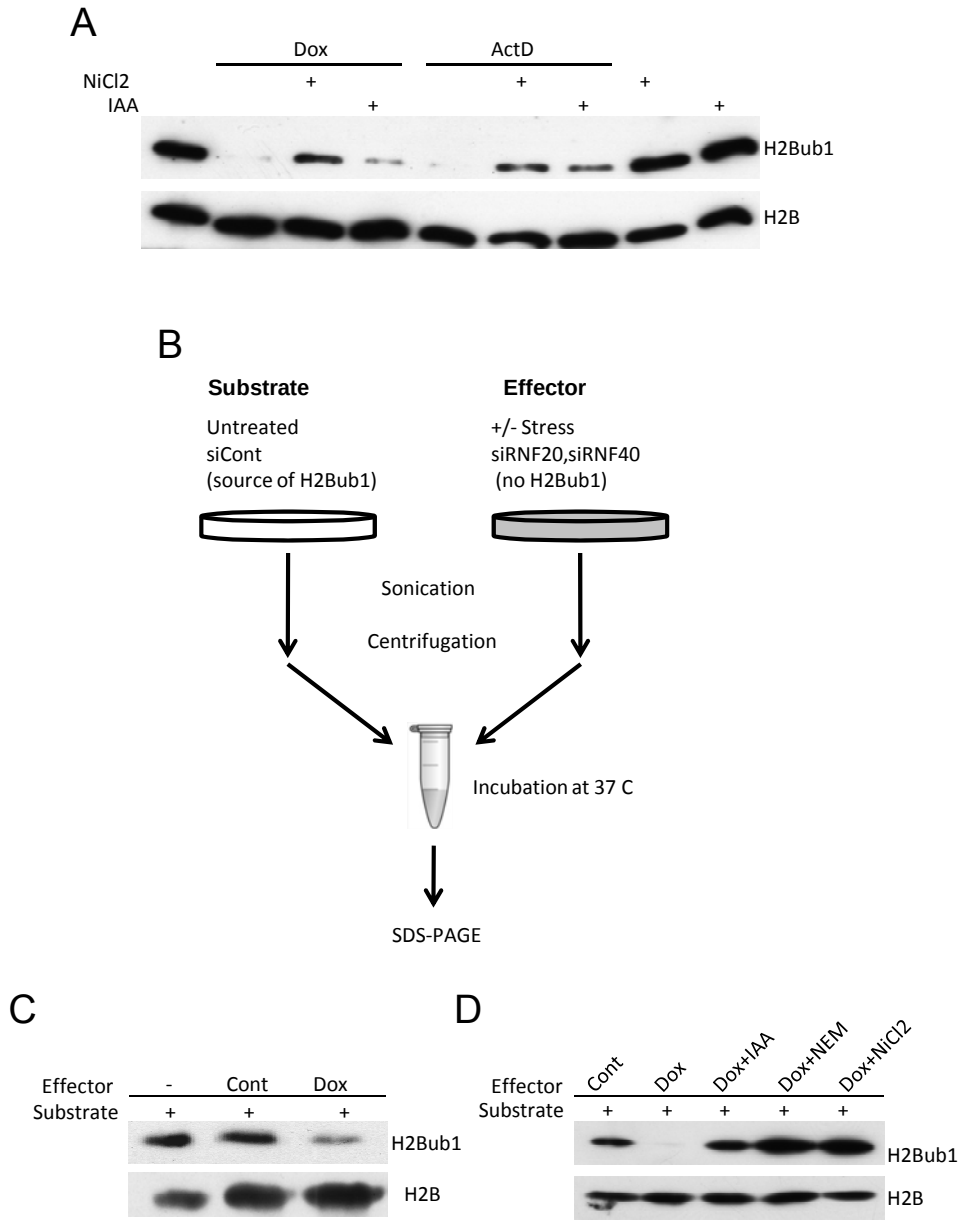

Figure 20. An H2B deubiquitinating activity is increased during cellular stress. A. H1299 cells were pretreated with 1 mM of $NiCl_2$ and 1 μM of iodacetamide for 30 min before treatment with 3.75 μM doxorubicin or 256 nM actinomycin D for 3.5 h. Protein extracts were analysed by Western blot. **B.** Scheme of the in vitro deubiquitination experiment. For details see

"Materials and methods". **C.** H1299 cells were transfected with control or RNF20/40 siRNA and treated with 7.5 μM doxorubicin for 3.5 h prior to harvesting and performing in vitro deubiquitination assay. **D.** Cells were treated same way as in **C**, but DUB inhibitors (IAA 1 μM, NEM 1mM, $NiCl_2$ 1mM) were added to the reaction mix.

4.3.5 USP22 is needed for the stress-induced JNK-regulated deubiquitination

USP22 has been shown to deubiquitinate nucleosomes containing monoubiquitinated H2B *in vitro*. We hypothesized that its activity could be crucial for the stress-induced deubiquitination as well. As shown in Fig. 21A and supplementary Fig. S5 siRNA-mediated knockdown of USP22 rescues the H2B ubiquitination after treatment with actinomycin D or the DNA-damaging drug cisplatin.

Knowing the role of USP22 in H2B deubiquitination and the importance of JNK activity for this process we suggested the possibility that JNK may directly regulate USP22 activity through phosphorylation. Therefore we performed *in vitro* kinase assays using GST-tagged *E. coli*-purified USP22 as a substrate for recombinant JNK in the presence of γ-[^{32}P]-ATP. We could observe specific phosphorylation of USP22 by JNK while phosphorylation of GST alone was nearly undetectable (Fig. 21B).

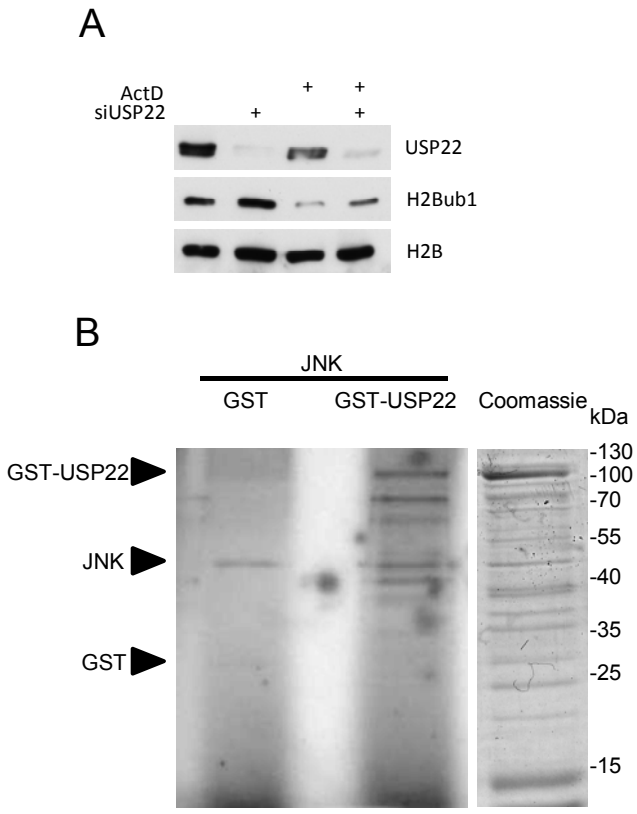

Figure 21. USP22 regulates the level of H2Bub1 and can be phosphorylated by JNK. A. H1299 cells were transfected with siRNAs against USP22. After 48 h cells were treated with 50 nM of actinomycin D for 3.5 h. Extracts were analyzed by western blot. **B.** GST-USP22 fusion protein was phosphorylated in vitro by JNK in presence of γ-[^{32}P]-ATP. The products of the reaction were separated by SDS-PAGE and analysed with phosphoimager. Black arrows indicate phosphorylated full-size GST-USP22, autophosphorylated JNK and GST. Other bands are phosphorylated degradation products of GST-USP22.

Supplemental material

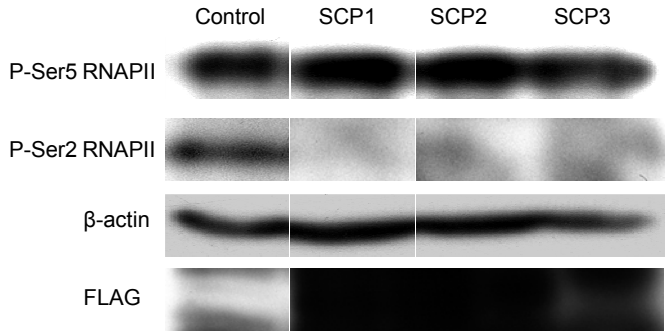

Figure S1. SCP1, SCP2 and SCP3 dephosphorylate Ser2 but not Ser5 of RNAPII CTD. H1299 cells were transfected with plasmids coding for SCP1, SCP2 and SCP3. After 48 h cells were harvested for and analyzed by Western blot using the indicated antibodies.

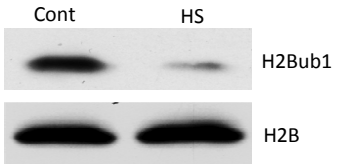

Figure S2. Heat shock reduces H2B ubiquitination. H1299 cells were heat-shocked by incubation at 42 C for 1 h. Protein extracts were analyzed by Western blot.

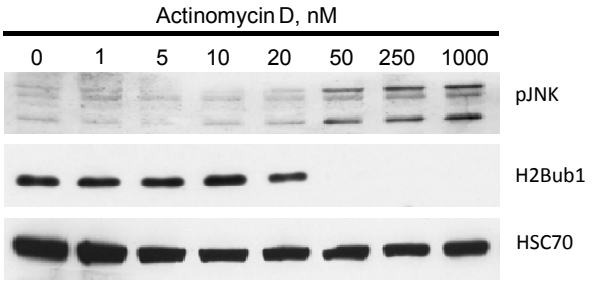

Figure S3. Activation of JNK correlates with the reduction of H2Bub1. H1299 cells were treated with indicated concentrations of actinomycin D for 12 h. Proteins were analyzed by Western blot using the indicated antibodies. These data were kindly provided by Theresa Gorsler.

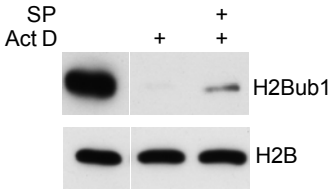

Figure S4. JNK inhibitor rescues H2Bub1 level after actinomycin D treatment. H1299 cells were pretreated with 20 μM of JNK inhibitor SP600125 for 1 h and then treated with 256 nM of actinomycin D for 3.5 h. Protein extracts were harvested and analyzed by Western blot.

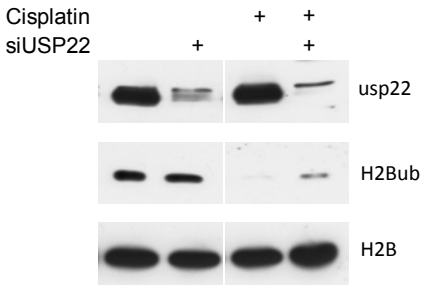

Figure S5. USP22 is needed for cisplatin-mediated reduction of H2Bub1. H1299 cells were transfected with siRNA against USP22. After 48 h cells were treated with 50 μM cisplatin for 12 h. Protein extracts were analyzed by Western blot using the indicated antibodies. These data were kindly provided by Theresa Gorsler.

5 Discussion

Gene transcription is a tightly regulated process. It is controlled at all stages from initiation complex formation to the processing of the 3'-end of the transcript. One of the important regulatory steps is a decision to switch the paused RNA polymerase complex to the elongation mode. The importance of pausing the transcription for the gene regulation became more obvious in recent years. The paused polymerase can wait for the induction for quite a long time providing the cell the possibility to rapidly activate transcription. In yeast two thirds of genes with paused RNA polymerase appeared to belong to the groups encoding the factors of cellular metabolism reprogramming (Radonjic et al, 2005). Besides that, pausing also provides the chance for improperly assembled transcription complex to fall off. In humans about 30% of all genes were shown to have a significant amount of RNA polymerased paused in the proximal promoter region while not not producing any detectable amount of RNA (Guenther et al, 2007). The release of RNAPII from pausing is primarily regulated by the Positive Transcription Elongation Factor-b (P-TEFb) (Yamada et al, 2006). The posttranslational modification of histones plays an important regulatory role in coordination of transcription events. Monoubiquitination of lysine 120 of histone H2B (H2Bub1) is an elongation epigenetic mark of actively transcribed genes (Minsky et al, 2008). Despite the high occupancy of H2Bub1 in the transcriptionaly active fraction of the genome relatively little is known about its regulation during the transcription cycle as well as the dynamics of this mark in different physiological conditions.

5.1 P-TEFb as a key regulator of transcription elongation and co-transcriptional histone modification

P-TEFb is known to regulate the elongation step of transcription. Its kinase component CDK9 phosphorylates multiple target proteins. The phosphorylation of the NELF-E subunit of the Negative Elongation Factor leads to its release from the paused RNA polymerase complex (Fujinaga et al, 2004). Another negative elongation factor, DSIF, stays associated with RNAPII and is converted to a positive elongation factor upon CDK9-mediated phosphorylation (Yamada et al, 2006). The RNA polymerase II itself can also be phosphorylated by CDK9 at second serine residues of its carboxy-terminal domain repeats. This not only

changes the charge and conformation of the CTD but also provides a binding surface for RNAPII associated factors (Buratowski, 2003; Egloff & Murphy, 2008).

Based on a similar distribution pattern of H2Bub1 and P-TEFb-mediated RNAPII CTD Ser2 phosphorylation in human cells (Gomes et al, 2006; Komarnitsky et al, 2000; Minsky et al, 2008) we hypothesized that P-TEFb activity might be essential for the accumulation of H2Bub1. Indeed we were able to show that both inhibition and knockdown of the P-TEFb kinase component CDK9 resulted in a strong decrease of H2Bub1 levels while the overexpression of CDK9 had an opposite effect on H2Bub1. We were also able to convincingly show that Ser2 in RNAPII CTD is at least one of the CDK9 targets whose phosphorylation is needed for maintaining H2B ubiquitination. Although H2Bub1 was thought to be a co-transcriptional histone mark we were able to prove that it's only observed on a gene, which is being transcribed by CDK9-phosphorylated RNAPII. It is not detected in the presence of non-phosphorylated RNAPII in cases when the transcription happens in the absence of active P-TEFb. The recent discovery of a new role of Ww domain-containing Adaptor with Coiled-coil (WAC) protein provided a solid support for our model (Zhang & Yu, 2011). WAC was shown to be a physical link between phospho-RNAPII and H2Bub1. It can simultaneously bind phosphorylated RNAPII CTD and heterodimeric complex of E3 ubiquitin ligases RNF20/40 which ubiquitinates histone H2B.

The P-TEFb complex has been shown to regulate not only RNA polymerase dynamics but also the co-transcriptional modifications of chromatin. The histone methyl transferase SETD2 is recruited to the CDK9-phosphorylated RNAPII CTD (Egloff & Murphy, 2008). This leads to the increase of H3K36 trimethylation in the transcribed region of the gene. In addition, H2B monoubiquitination can itself regulate the deposition of tri-methyl marks on H3K4 and H3K79 by a trans tail regulation mechanism while its own regulation remains elusive in many aspects (Kim et al, 2005; Zhu et al, 2005).

Other targets of CDK9 appeared to be less important for maintaining H2Bub1 level. The knockdown of NELF-E affected neither H2Bub1 nor H3 methylations dependent on it. The knockdown of the DSIF component SUPT5H also did not change the level of H2Bub1 while the amount of H3K36me3 was

significantly reduced, proving that H3K36me3 is regulated by additional factors besides RNAPII phosphorylation. Both H2Bub1 and H3K36me3 are predominantly localized in the transcribed part of the gene, however different regulation suggests different roles. Indeed, unlike ubiquitously distributed H2Bub1 H3K36me3 is highly enriched on exons (Kolasinska-Zwierz et al, 2009). This localization was found to be connected to pre-mRNA splicing (Luco et al, 2011). It also should be mentioned that some published data is contradictory to our observations. The siRNA-mediated knockdown of SUPT5H was shown to strongly decrease the level of H2Bub1 in human cells (Chen et al, 2009). Additional experiments involving another siRNAs and probably the rescue experiment are needed to clarify the situation. In accordance with previously shown data that H2Bub1 regulates H3K4me3 and H3K79me3 we were able to demonstrate a strong downregulation of all these marks following the CDK9 knockdown (Pirngruber et al, 2009a).

Despite playing a significant role in the coordination of other chromatin marks H2Bub1 may also play an important role in transcription by itself. As was recently observed the ubiquitination of H2B disrupts the structure of the chromatin at the level of the 30 nm fiber (Fierz et al, 2011). This structural change could promote the transcription by opening it for RNAPII. However, previous reports suggest otherwise. The rate of *in vitro* transcription did not depend on the presence of ubiquitin tags on histones (Kim et al, 2009). The most probable role of H2Bub1 in this case is regulation of other histone modifications which might be more important for the transcription rate. At the same time other researchers reported direct stimulating effect of H2Bub1 on *in vitro* transcribing RNAPII. A model was proposed where H2Bub1 acts as a mark for the FACT complex to remove the H2A/H2B dimer from the nucleosome thus promoting the movement of RNAPII through the chromatin (Pavri et al, 2006). In general, this provides a novel mechanism of transcriptional regulation by P-TEFb through H2Bub1.

5.2 P-TEFb and genome integrity

Despite having a significant role in the regulation of transcription, P-TEFb or at least its kinase component CDK9, is clearly involved in maintaining genome integrity. CDK9 was recently isolated in a screen as a HU-sensitivity factor (Yu et al, 2010). Cells lacking CDK9 were less able to recover from HU-induced cell cycle arrest. Cyclin K was shown to be necessary as the CDK9 partner for this

activity. The accumulation of γH2A.X in cells depleted of CDK9 was also observed suggesting the inability of cells to repair spontaneous DNA breaks. The interaction of CDK9 with DNA damage-related proteins ATR, ATRIP and Ku70 also provides good evidence for the role of CDK9 in genome integrity maintenance (Yu et al, 2010). However, based on these studies the exact mechanism of CDK9-mediated control of DNA damage repair remains unknown.

The yeast CDK9 homolog Bur1 may also be important for DNA damage repair. It specifically phosphorylates and activates Rad6, an E2 ubiquitin ligase for PCNA. PCNA is a DNA clamp and in its ubiquitinated state recruits DNA polymerase eta for the translesion synthesis (Haracska et al, 2001). It is also worth mentioning that the putative phosphorylation site in the human Rad6 homolog UBE2A likely plays an important role in the regulation of its function. Many other members of the UBE family have single or double negatively charged aminoacids in the site where UBE2A has a phosphorylatable serine (Fig. 22). It appears that a strong negative charge in this region is essential for protein function.

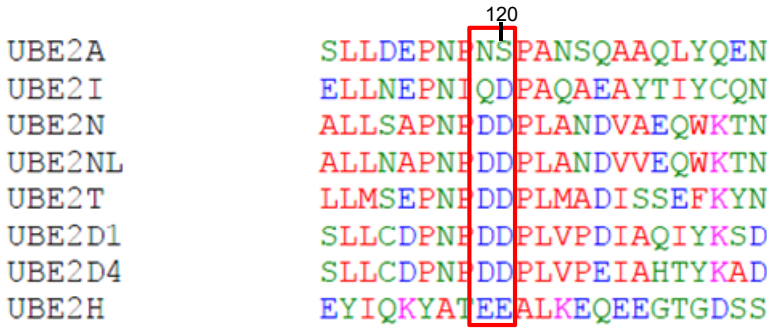

Figure 22. Analysis of UBE-family members. Serine is substituted to one or two negatively charged amino acids in multiple homologues of UBE2A. (Done by ClustalW software).

We hypothesized a positive role for CDK9 in the regulation of the human Rad6 homolog UBE2A. Indeed CDK9 and cyclin T1 can bind a UBE2A and phosphorylate it in vitro at the conserved serine 120. Moreover, the antibody raised against a UBE2A phosphopeptide showed a decreased signal after siRNA-mediated CDK9 depletion. Importantly, the knockdown of CDK9 also reduced the ubiquitination of PCNA and H2B which both depend on Rad6 activity.

The ubiquitination of PCNA is an important component in the Fanconi anemia network which is essential for the repair of interstrand DNA crosslinks (Geng et al, 2010). Previously the increased sensitivity of Fanconi anemia cells to DNA-crosslinking drugs like mitomycin C (MMC) was demonstrated (Geng et al, 2010). Moreover, the combinatorial treatment with flavopiridol and MMC significantly increased the efficiency of apoptosis induction in breast cancer cells (Schwartz et al, 1997). Since CDK9 is strongly inhibited by flavopiridol we now provide a potential mechanistic model for the observed effects which may provide a rational basis for testing more specific CDK9 inhibitors in combination with interstrand crosslinkers.

Very recently the importance of H2B ubiquitination in DNA damage repair was shown (Moyal et al, 2011; Nakamura et al, 2011). Although details are still to be clarified it is already obvious that H2Bub1 in the sites of DNA damage is absolutely crucial for proper repair process. The cumulative effect of DNA break-inducing treatments and potent CDK9 inhibitor flavopiridol on promoting apoptosis in cancer cells has been known for a long time. Flavopiridol was able to induce apoptosis in colon carcinoma HCT116 cells treated with low concentrations of topoisomerase I inhibitor camptothecin (Motwani et al, 2001). Ovarian carcinoma cells exhibited increased radiosensitivity upon flavopiridol treatment (Raju et al, 2003). The deletion of the tumor suppressor p53 and overexpression of the antiapoptotic protein Bcl-2 could not impair the ability of flavopiridol to increase the effect of ionizing radiation (Hara et al, 2008). The radiosensitivity of xenographic mammary carcinoma, ovarian carcinoma, lymphoma and glioma in mice was improved by flavopiridol as well (Mason et al, 2004; Newcomb et al, 2004). Different mechanisms of flavopiridol action were proposed starting from the cell cycle regulation to the modulation of PARP activity. Although some authors mentioned CDK9 as one of the relevant flavopiridol targets no clear connection to the chromatin modifications has been made so far.

Taken together the radiosensitizing activity of CDK9 inhibitor flavopiridol, H2Bub1-dependent dsDNA breaks repair and our data connecting CDK9 and H2Bub1 provide a completely new, distinct mechanism of P-TEFb-mediated genome integrity control.

5.3 Stress-induced reduction of H2B ubiquitination

5.3.1 H2Bub1 is strongly reduced by various treatments

In the course of this study we have demonstrated an interesting phenomenon partly described previously. The ubiquitination of histone H2B is rapidly and strongly reduced upon various treatments such as chemotherapeutic drugs (doxorubicin, etoposide, camptothecin, cisplatin), μltraviolet irradiation, osmotic stress (sorbitol) and heat shock. Quite surprisingly ionizing irradiation did not affect H2Bub1 levels although causing significant DNA damage. Remarkably, the chemotherapeutic DNA-damaging drug doxorubicin decreased the H2Bub1 only at concentrations at least 5 fold higher than needed for a strong DNA damage-response induction. This finding looks, however, less surprising if taken into the context of recent research reporting that H2B ubiquitination is rather induced at the sites of DNA damage and needed for the proper repair (Moyal et al, 2011; Nakamura et al, 2011). This suggests the existence of another pathway regulating H2Bub1 under unfavorable conditions.

Previous studies also reported strong reduction of H2B ubiquitination following the treatment with proteosome inhibitors, heat shock and inhibitors of transcription (Davie & Murphy, 1990). The model proposed by authors included the depletion of free monomeric ubiquitin due to the insufficient turnover of polyubiquitinated proteins in the cell. This explanation apparently does not fit the picture we observe since most treatments we apply do not cause the accumulation of polyubiquitinated proteins. Moreover, the reduction of H2Bub1 upon treatment can be rescued by pre-treatment with caffeine. Altogether this data suggests an actively regulated multiple factor-induced pathway of H2Bub1 reduction.

The physiological role of this massive change of chromatin status is not clear yet. Yeast cells are known to rapidly shut off the transcription of almost all genes within the first 10 min after strong stress like osmotic stress (Miller et al, 2011). The new wave of gene expression which starts after 15 min is already corrected in accordance with new conditions. We can speculate that similar reprogramming takes place in human cells as well. H2Bub1, being a transcription-specific chromatin mark, would be expected to be rapidly removed.

Another possible explanation of the observed effects may utilize the novel finding that H2Bub1 keeps the chromatin uncompacted. The dosages of treatments

needed to induce a quick loss of H2Bub1 are usually very high and are very likely to induce apoptosis. As we know, one of the apoptotic events is an ATP-dependent chromatin condensation (Kass et al, 1996). Thus, the rapid loss of H2B ubiquitination we observe might be one of early apoptotic events.

5.3.2 JNK and nucleolar signaling are involved in reduction of H2Bub1

Nucleoli are known to rapidly respond to a variety of stresses and, what is important for us, their function is tightly controlled by c-Jun N-terminal kinase (JNK) signaling. JNK has been shown to phosphorylate and inactivate TIF-IA, a specific RNAPI transcription factor. It in turn leads to the failed ribosomal biosynthesis and nucleoplasmic translocation of nucleolar proteins like NPM, L5, L11, ARF, etc. (Mayer et al, 2005).

Further analysis of stress-induced H2Bub1 changes revealed a strong dependence of this chromatin modification on the activity of JNK. JNK is known to be activated by various environmental stresses (Minden & Karin, 1997). Pretreatment of cells with a specific JNK inhibitor partially rescued the effects of both UV and doxorubicin on H2Bub1. Importantly, both these treatments increase JNK activity. In opposite, the overexpression of activated JNK led to the reduction of the global H2Bub1 level. At the moment JNK seems to be the only known kinase whose activity downregulates H2B ubiquitination.

Thus, the induction of nucleolar stress via a direct mechanism (e.g. actinomycin D) or indirect mechanisms involving JNK or rRNA processing (i.e. NPM or NCL knockdown) suggest an intimate connection between nucleolar integrity and the maintenance of H2Bub1. It was recently shown that MYBBP1A and MKI67IP proteins are released from nucleoli upon blocking their function by knocking down TIF-IA (Kuroda et al, 2011). The described function of these two proteins is acetylation of p53 in order to stabilize it and induce a p53-response which leads to either cell cycle arrest or apoptosis. However, all our experiments with H2Bub1-reducing treatments were done in the H1299 cell line, which is p53-negative. The recent data obtained in our lab shows that MYBBP1A and MKI67IP are also RNF40 binding proteins. We also cannot exclude the possibility that MYBBP1A and MKI67IP not only interact with RNF40 but also inhibit it by acetylation. Nopp140, which is also normally localized in nucleoli, was also shown

to interact with RNF40 in our experiments. We thus propose an inhibition H2B ubiquitination by stress-released nucleolar components.

5.3.3 Stress and DUB activity

According to our data the loss of H2B ubiquitination happens very rapidly which is difficult to explain with inhibition of RNF40 alone. We hypothesized the activation of certain de-ubiquitinating enzymes (DUBs) as a mechanism of quick H2B deubiquitination. We were indeed able to demonstrate the increase of a deubiquitinating activity in cells treated with doxorubicin. This activity was completely abolished by adding DUB inhibitors such as IAA, NEM or $NiCl_2$ proving that it came from one of ubiquitin-specific proteases (USPs). Further analysis allowed us to conclude that USP22 was the enzyme which was at least partially responsible for the stress-induced deubiquitination. USP22 was already shown to deubiquitinate H2B in vitro (Zhao et al, 2008). Normally it is found as a component of Spt-Ada-Gcn5-Acetyltransferase (SAGA) complex and is involved in the deubiquitination of histone H2A and H2B. USP22 was also identified as a component of a so-called “death from cancer” signature, a specific gene expression profile containing several genes whose expression is associated with poor clinical prognosis of cancer patients (Glinsky et al, 2005). We also showed in vitro phosphorylation activity of JNK on USP22. Thus, this phosphorylation may potentially modify the affinity of the USP22 zinc-finger domain to certain interaction partners thereby changing its activity and specificity. For example, the USP22 deubiquitinating module (containing ATXN7L3 and ENY2) may be released from the core SAGA complex and function in a SAGA-independent manner. Consistent with this hypothesis, no effect of the core SAGA component GCN5 was observed on the stress-induced loss of H2Bub1 (data not shown). The knockdown of USP22 gave partial rescue of H2Bub1 level upon several stresses. It is quite possible that several USPs such as USP27 perform this function though.

The above given information provides interesting possibilities for improving the efficiency of X-ray therapy of tumors. As we know from previous research the successful repair of double strand DNA breaks requires the deposition of ubiquitinated H2B into the region of damaged chromatin repair (Moyal et al,

2011; Nakamura et al, 2011). It is quite easy to imagine the impairment of the DNA repair process in case we were able to abolish the H2Bub1 in tumor tissues.

5.4 The role of H2Bub1 in cancer biology

The role of H2Bub1 in tumors biology is another interesting aspect. As we mentioned before the proteins involved in H2Bub1 level regulation are often mis-expressed in cancers. It's not easy now to explain the details of this regulation, but most probably H2Bub1 would be reduced in tumors. In support of this, a CpG island in the promoter of the RNF20 gene was shown to be highly methylated in human breast tumors in comparison to normal tissues (Shema et al, 2008). Moreover, changes in the expression or activity of USP22, RNF40 or UBE2A could also potentially be altered during tumorogenesis.

One possible outcome of such a reduction might be an increase of metastatic activity. It is known that diffusion-based hypoxia in tumors promotes the spreading of metastasis (Chaudary & Hill, 2007). At the same time the activity of H2B E3 ubiquitin ligase RNF20 has been shown to reduce the migration potential of cancer cells (Shema et al, 2008). We can hypothesize that tumor cells which manage to inactivate H2B ubiquitinating system would gain an advantage of high motility in hypoxic conditions. Alternatively, hypoxic stress itself, like the other investigated stresses, might lead to a loss of ubiquitination, thereby increasing the cell's propensity.

Another possible role of H2Bub1 loss in tumor biology came out of the experiment in which we observed a strong reduction of γH2AX upon RNF40 knockdown (data not shown). Some cancer cell lines with a high growth rate demonstrate quite a significant level of γH2AX in the absence of any DNA damage. This could be a result of so-called replication stress, which is caused by disruption of DNA replication forks during the S-phase of cell cycle. The cells with low levels of H2Bub1 might not recognize such events properly and keep growing at a higher rate meanwhile accumulating chromosomal aberrations, which is typical for cancer cells.

To shed some light on these and other problems further careful and extensive study of this process is required for the development of solid knowledge

of stress-induced H2B deubiquitination and the role of H2B monoubiquitination during tumorogenesis and metastasis.

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Curriculum Vitae

Personal Information

Name Andrei Shchebet

Address Petrikirchstr. 12-45

37077 Göttingen

Date of birth August 04, 1984 in Stolin, Belarus

University

since 10/2007 PhD thesis

"Regulation of histone H2B monoubiquitination"

University of Göttingen, Department of Molecular Oncology

09/2006 – 09/2007 Graduate studies

International Max Planck Research School for Molecular Biology,

University of Göttingen, Germany.

09/2001 – 06/2006 Diploma studies

"Construction of the In Vivo Expression Technology (IVET) system for host-induced genes search in Erwinia carotovora."

Belarusian State University, Minsk, Belarus

Education

09/1997 – 05/2001 High school #2, Glubokoe, Belarus.