The role of H2B monoubiquitination in cellular differentiation

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Affidavit

I hereby declare that the PhD thesis entitled “The role of H2B monoubiquitination in cellular differentiation” has been written independently and with no other sources and aids than quoted.

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List of Publications

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“What are you working on?” I asked.

“As with all science — the happiness of man.”

Arkadi and Boris Strugatsky

Table of Contents

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Abstract

Histones, subjected to post-translational modifications, are important regulators of the cellular processes. One of these modifications is monoubiquitination of histone H2B (H2Bub1). H2Bub1 is associated with the actively transcribed genes. Moreover, H2Bub1 is required for the proper DNA repair and was recently reported to be lost during tumor progression.

The levels of H2Bub1 in the cell are tightly regulated. In mammals, ubiquitination is mediated by the E3 ubiquitin ligase RNF20/40. Another important upstream regulator of H2Bub1 is the CDK9 enzyme that promotes transcriptional elongation. It, together with an adaptor protein WAC, facilitates the RNF20/40 recruitment to the chromatin.

Differentiation of the cell is a process that results in cellular specialization and acquiring a physiological function. It is accompanied by the significant changes in gene expression and in histone modification patterns.

This project aimed to understand the role of H2Bub1 in cellular differentiation. Investigating human mesenchymal stem cells (hMSCs) it was observed that the H2Bub1 levels increase during differentiation into osteoblasts and adipocytes. Depletion of the H2Bub1 regulators RNF40, WAC and CDK9 resulted in inhibition of the hMSC differentiation suggesting that H2Bub1 is required for the correct progression of this process. Mechanistically, H2Bub1 was shown to participate in the activation of the “bivalent” genes that carry activatory and inhibitory histone marks. H2Bub1 deposition is required for removal of the repressive H3K27me3 from the differentiation-dependent genes.

Taken together, these observations for the first time demonstrate the involvement of H2Bub1 in cellular differentiation. The proposed model suggests that H2Bub1 executes its function via promoting the resolution of bivalency on the differentiation-specific genes. These results give additional insights into H2Bub1 function during transcription of the certain subsets of genes. The obtained knowledge increases our understanding of the transcriptional regulation, carcinogenesis and stem cell biology.

Abbreviations

ATXN7L3	Ataxin 7-like 3
BGP	ß-Glycerolphosphate
BGLAP	bone gamma-carboxyglutamate (gla) protein
BGS	Bovine Growth Serum
BRD4	Bromodomain containing 4
Bre1	BREfeldin A sensitivity
BSA	Bovine serum albumin
°C	degree Celsius / centrigrade
CDK7	Cyclin-Dependent Kinase 7
CDK9	Cyclin-Dependent Kinase 9
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP followed by high-throughput sequencing
COMPASS	Complex proteins associated with Set1p
CTD	Carboxyterminal Domain
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
DOT1L	Dot1-like
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DSIF	DRB Sensitivity Inducing Factor
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E1 enzyme	ubiquitin-activating enzyme
E2 enzyme	ubiquitin-conjugating enzyme
E3 enzyme	ubiquitin-ligase
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia = for example

EGF	Epidermal growth factor
ENY2	Enhancer of yellow 2 homolog
EtOH	Ethanol
Facs	Fluorescence-Activated Cell Sorting
FACT	Facilitates Active Chromatin Transcription
GCN5	Histone asetyltransferase, general control of amino acid synthesis protein 5
H2A	Histone 2A
H2Aub1	monoubiquitinated histone 2A
H2B	Histone 2B
H2Bub1	monoubiquitinated histone 2B
H3	Histone 3
H3K4me3	Histone 3 trimethylated at position lysine 4
H3K27me3	Histone 3 trimethylated at position lysine 27
H3K36me3	Histone 3 trimethylated at position lysine 36
H3K79me3	Histone 3 trimethylated at position lysine 79
H4	Histone 4
hFOBs	Human fetal osteoblasts
hMSCs	Human Mesenchymal Stem Cells
hnRNPK	Heterogeneous Nuclear Ribonucleoprotein K
IAA	Iodacetamide
IBMX	Isobutyl-methyl-xanthine
IgG	Immunoglobulin G
kDa	kilo Dalton
m	milli (10^{-3})
µ	micro (10^{-6})
MEM	Minimum Essential Media
MLL	Myeloid/lymphoid or mixed-lineage leukemia
n	nano (10^{-9})
NELF	Negative elongation factor
NEM	N-ethylmaleimide
NP-40	Nonidet P40
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with Tween-20

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDK4	Pyruvate Dehydrogenase Kinase, isozyme 4
pH	Measurement of acidity or alkalinity of a solution
PI	Propidium Iodide
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
P-TEFb	Positive Transcription Elongation Factor beta
Rad6	Radiation sensitivity protein 6
RASD1	RAS, dexamethasone-induced 1
RING	Really Interesting New Gene
RNA	Ribonucleic acid
RNAPII	RNA Polymerase II
RNF20	Ring finger protein 20
RNF40	Ring finger protein 40
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
RXR	Retinoid X Receptor
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SUPT5H	Supressor of Ty Homologue-5
TEMED	Tetramethylethylenediamine
TFF1	Trefoil factor 1
Tris	Tris(hydroxymethyl)aminomethane
UBE2A	Ubiquitin-conjugating Enzyme E2A
UBE2B	Ubiquitin-conjugating Enzyme E2B
USP22	Ubiquitin Specific Peptidase 22
VDR	Vitamin D Receptor
WAC	WW domain containing adaptor with coiled-coi
WB	Western blot

List of Figures

1 Introduction

1.1 Introduction to histone modifications

1.1.1 Chromatin organization and histone modifications

The genomic DNA of living cells is a long and highly charged polymer. In diploid human genome there are approximately 6 billion base pairs of DNA that, assuming that each base pair is 0.34 nm long, form about 2 meters of double-stranded DNA (Annunziato, 2008). Therefore genomic DNA undergoes strong compaction processes mediated by proteins that coil or fold DNA resulting in higher levels of compaction (Alberts, 2002). Association of DNA with nuclear proteins forms chromatin.

DNA within the cell can exist in different compaction states which are essential for its vitality (Lewin, 1999). Very compact states, like segregation into chromosomes, are required for correct cell division and distribution of genetic information. More relaxed states are important during most of the cellular life cycle since they allow proteins to access DNA for performing various biological tasks, e.g. transcription, DNA replication or repair. Another important feature associated with DNA compaction is compartmentalization of the nucleus which regulates accessibility of the genetic information and reading it at the right time. To transform into the more compacted state DNA interacts with different nuclear proteins forming a complex structure called chromatin (Alberts, 2002). In general, cellular chromatin exists in two states: heterochromatin, which represents highly compacted and transcriptionally silent regions, and euchromatin, that is less condensed and transcriptionally active (reviewed in Tamaru, 2010; Kwon and Workman, 2011).

The first level of DNA compaction is the interaction with a protein complex called nucleosome. Nucleosome is composed of 8 protein subunits (two H2A-H2B dimers and two H3-H4 dimers) and of DNA helix of 147bp wrapped around a histone octamer (McGhee, 1980; Luger, 1997). Another histone protein, histone H1, does not participate in the nucleosome formation, but serves as a linker connecting nucleosomes to form higher levels of chromatin compaction (Allan, 1981; Bates, 1981).

Histones are globular basic proteins which consist of a core domain and C- and N-terminal tails. While core domains are required for the nucleosome formation, histone tails remain at the surface of nucleosome which makes them accessible for enzymes and allows them to

undergo different posttranslational modifications (reviewed in Jenuwein and Allis, 2001; Rando, 2012; Portela and Esteller, 2010). The most studied among them are methylation of lysine and arginine residues, phosphorylation of serine and threonine and acetylation of lysine (Fig. 1). More rare modifications include O-glycosylation, ADP-ribosylation, carbonylation, formylation, crotonylation and citrinullation. Sometimes an entire protein (in case of ubiquitination or sumoylation) can be added as a modification (reviewed in Jenuwein and Allis, 2001; Rando, 2012; Portela and Esteller, 2010; Johnsen, 2012). Due to posttranslational modifications the function of histones is not limited to scaffolding the chromatin structure and extends to the regulation of the DNA-assosiated processes like replication, repair, spatial arrangement and compartmentalization of the genome and transcriptional regulation.

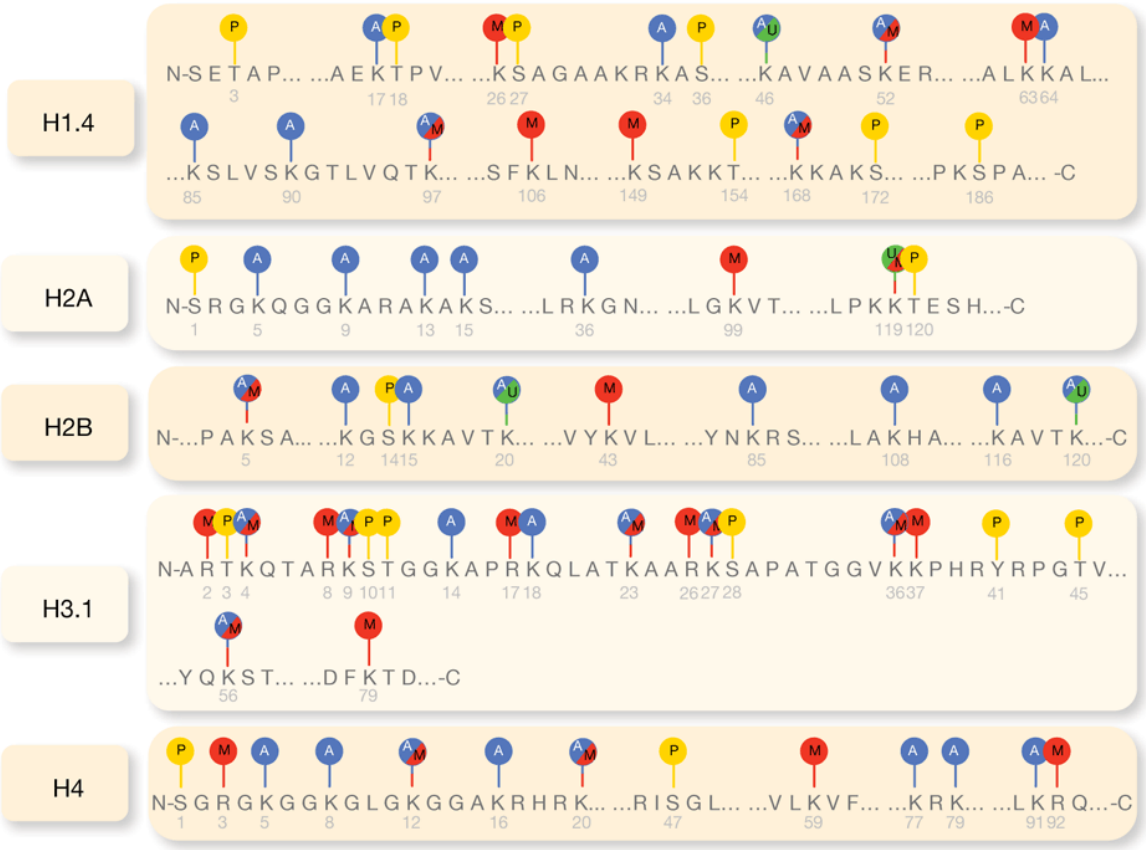

Fig. 1. Main posttranslational histone modifications (from Portela and Esteller, 2010). Acetylation is represented in blue, methylation – in red, phosphorylation – in yellow and ubiquitination – in green. The number under each amino acid indicates its position in the sequence for the human cells.

1.1.2 “Histone code”

The protein-coding genes consist of different compartments required for their proper transcription. At the 5’end they carry a promoter region which is a site for the binding of the transcription factors. The transcription start site (TSS) is located near promoter and indicates a position from which the RNAPII starts the transcript production. (Lewin, 1999). It was observed that specific histone modifications are often found at the same position on the gene. For example, H3K4 di- (me2) and trimethylation (me3) is mostly located around promoter regions, while H3K36me3 is found over the middle and 3’end of the transcribed regions (reviewed in Rando and Chang, 2009). Moreover, some modifications are associated primarily with repressed genes, e.g. H3K27me3, while others, e.g. H3K79me3, – with activated. These findings led to the hypothesis of the “histone code” which suggests that type and position as well as number of histone modifications or their combinations that are present on the gene defines its transcriptional status (reviewed in Jenuwein and Allis, 2001; Strahl and Allis, 2000). This function is mediated either by changing physical of chemical properties of the chromatin fiber or by recruitment of defined chromatin-modifying complexes that activate or repress transcription. In other words, histone modifications appear to regulate the transcriptional state of the gene. In addition to transcription, posttranslational modifications of histones are involved in DNA damage response, telomere silencing, dosage compensation or histone exchange (reviewed in Jenuwein and Allis, 2001; Strahl and Allis, 2000).

1.2. Monoubiquitination of histone H2B

Monoubiquitination of histone H2B (H2Bub1) is a posttranslational modification that occurs at the C-terminus of histone H2B (West, 1980). It is conserved from yeasts to mammals, although the numbering of the ubiquitinated lysine residue might vary across the species (K123 for yeasts and K120 for mammals) (Sun, 2002; West, 1980). H2Bub1 is not followed by polyubiquitination and H2B degradation being rather a signaling modification.

H2Bub1 is synthesized in classical ubiquitination reaction. First of all, an E1 ubiquitin-activating enzyme transforms ubiquitin into an active form in ATP-dependent manner. After that E2 ubiquitin-conjugating enzyme together with E3-ligase transfer ubiquitin onto a lysine of the target protein. This reaction does not require ATP. For H2Bub1 the E2 and E3 enzymes are known. The E2 enzyme in yeast is called Rad6 (Robzyk, 2000; Hwang, 2003; Kao, 2004) and its human orthologs are known as UBE2A and UBE2B (Kim, 2005; Kim, 2009).

Interestingly, Rad6 is also involved in DNA damage signaling via PCNA ubiquitination in yeast (Hoege, 2002) and, as it was shown recently, in human (Shchebet, 2012). This suggests a common regulation and a possible interplay between H2B and PCNA monoubiquitination during DNA repair.

In yeast the E3 ligase for H2Bub1 is Bre1 (Wood, 2003; Hwang, 2003). It is important to underline that Bre1 is a RING-domain ubiquitin ligase which means that it does not possess intrinsic enzymatic activity but serves to position the E2 enzyme relative to its target protein. Thus, the enzymatic function is executed by RAD6 but only in the presence of Bre1 which was also confirmed in vivo (Wood, 2003; Hwang, 2003). In human there are two orthologs of Bre1 called RNF20 (BRE1A) and RNF40 (BRE1B) (Kim, 2005; Kim, 2009). To perform their biological function these proteins form an obligatory heterodimer in vivo (Karpiuk, 2012; Fuchs, 2012; Pirngruber, 2009; Kari, 2011; Chernikova, 2012). Interestingly, both RNF20 and RNF40 possess an intact RING-finger domain, however only RING-domain of RNF20 participates in the ubiquitination of H2B in vitro (Kim, 2009).

1.3 Regulation of H2Bub1 levels

1.3.1 H2Bub1 is regulated by the CDK9-WAC-RNF20/40 axis

1.3.1.1 CDK9 and regulation of transcription

RNAPII in eukaryotes transcribes protein-coding genes (reviewed in Alberts, 2002). It is a large protein-RNA complex consisting of catalytic core and carboxy-terminal domain (CTD). CTD is an amino acid chain formed by evolutionary conserved repeating heptapeptides Tyr-Ser-Pro-Thr-Ser-Pro-Ser ($Y_1S_2P_3T_4S_5P_6S_7$) (Corden, 1985; Corden, 1990); however the length of CTD is species-dependent (Egloff and Murphy, 2008). Serines of CTD in position 2 and 5 and, as it was shown recently, in position 7 can undergo phosphorylation which has an important regulatory role in transcription. Ser5 phosphorylation is mediated by CDK7, a component of general transcription factor TFIIH (Komarnitsky, 2000). This modification occurs near 5'end of the gene and is required for proper mRNA capping (Cho, 1998). Pohosphorylation of Ser2 is accomplished by Positive Transcription Elongation Factor β (P-TEFb). It consists of the Cyclin-Dependent kinase 9 (CDK9) and cyclin T or Cyclin K (Fu, 1999; Peng, 1998a; Peng, 1998b) and is required for releasing RNAPII from promoter

proximal pausing (Marshall, 1996), discussed in the next paragraph. Phosphorylation of Ser7 is important for the snRNA transcription and is not required for protein-coding genes (Egloff, 2007).

The generation of the mRNA transcripts includes three steps: initiation, elongation and termination. Before initiation general transcription factors build a complex at the promoter and recruit RNAPII (reviewed in Cooper, 2000). After RNAPII recruitment some of the transcription factors leave the promoter (promoter clearence) allowing the elongation to start. At this point the CTD of RNAPII is phosphorylated at Ser5, but not at Ser2. Before the start of productive elongation RNAPII is paused after transcribing first 20-50 bases (Bentley, 2005). This process is called promoter-proximal pausing and is required as an additional regulatory step of transcription (Gilmour and Lis, 1986). Pausing is mediated by two factors – DRB Sensitivity-Inducing Factor (DSIF) and Negative Elongation Factor (NELF). The release of RNAPII into productive elongation is dependent on the P-TEFb. CDK9 of the P-TEFb complex phosphorylates the CTD of RNAPII at Ser2 allowing for processive elongation (Marshall, 1996). It also phosphrylates SUPT5H subunit of DSIF leading to its conversion into positive elongation factor (Peterlin and Price, 2006; Yamada, 2006). Finally, P-TEFb stimulates release of RNAPII from the NELF complex by phosphorylation of NELF-E subunit (Fujinaga, 2004; Peterlin and Price, 2006). All described phosphorylation events mediated by CDK9 as a part of P-TEFb complex result in repression of negative regulators and promotion of effective elongation by RNAPII.

1.3.2.2 CDK9 also regulates H2Bub1 deposition

Levels of H2Bub in the cell are tightly regulated. The deposition of H2Bub1 is tightly connected to active transcription. One of the main regulators of H2Bub1 is CDK9 and its orthologs. In metazoans, CDK9 interacts with Cyclin T or Cyclin K form P-TEFb (Fu, 1999; Peng, 1998a; Peng, 1998b). As it was mentioned above, P-TEFb complex phosphorylates the CTD of RNAPII, as well as DSIF and NELF (Marshall, 1996; Peterlin and Price, 2006). It was recently shown that H2Bub1 levels are regulated by CDK9. Upon CDK9 inhibition or depletion H2Bub1 is globally decreased while CDK9 overexpression leads to increase in H2Bub1 levels (Pirngruber, 2009; reviewed in Johnsen, 2012). Moreover, H2Bub1 deposition requires presence of Ser2 phosphorylation, because S2A CTD mutant also leads to the loss of H2Bub1 (Pirngruber, 2009).

Until recently there was no mechanistic explanation of CDK9 action on RNF20/40 recruitment due to their different recruitment sites. The explanation of such cooperation came with an identification of WW domain-containing adaptor with coiled-coil protein (WAC) as an interaction partner of RNF20/40 complex (Zhang, 2011b). WAC interacts with phosphorylated Ser2, generated by CDK9, and at the same time recruits RNF20/40 complex to the chromatin (Fig. 2) leading to H2Bub1 deposition.

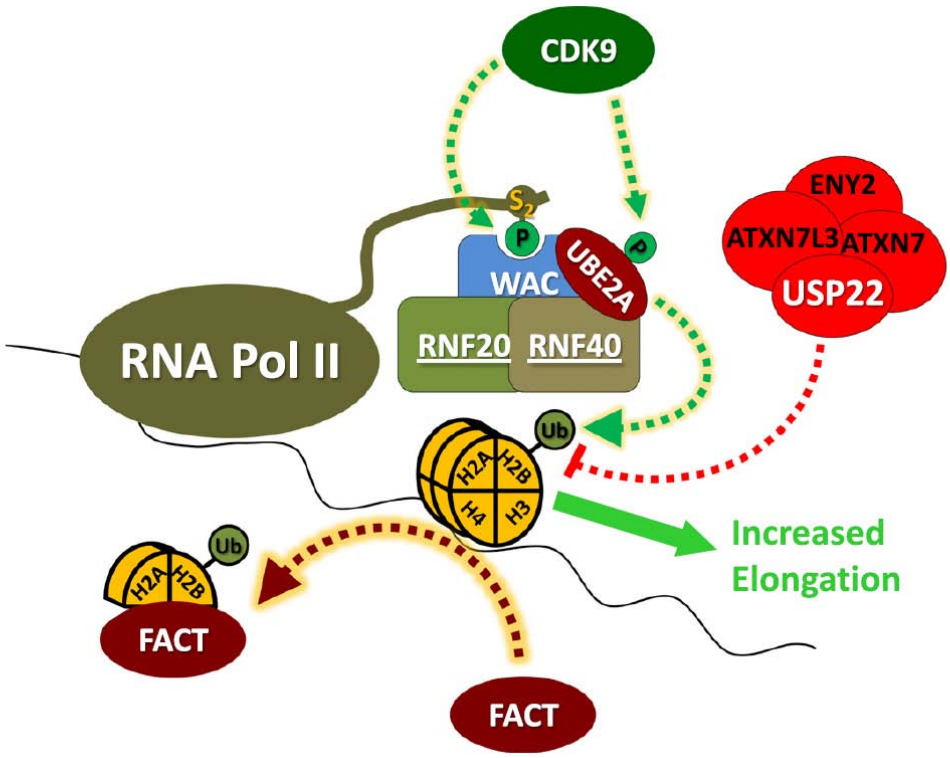

Fig 2. H2Bub1 regulation via the CDK9-WAC-RNF20/40 axis (from Johnsen, 2012). CDK9 phosphorylates Ser2 of RNAPII CTD. An adaptor protein WAC binds to P-ser2 and promotes recruitment of RNF20/40 and UBE2A to the activated gene. UBE2A is phosphorylated by CDK9 and becomes activated. RNF20/40 and UBE2A ubiquitinated H2B leading to promotion of elongation. Ubiquitination is then removed either by active deubiquitination via USP22 or by histone exchange via FACT complex.

Other organisms, e.g. budding yeast, have certain differences in controlling H2Bub1 in comparison to human. First of all, there are two orthologs of CDK9 in S. cerevisiae – Bur1 and Ctk1 (reviewed in Wood and Shilatifard, 2006). While Ctk1 performs RNAPII phosphorylation at Ser2 of the CTD it does not have an ability to facilitate H2Bub1 deposition (Krogan, 2003; Xiao, 2003). Moreover, presence of H2Bub1 blocks Ctk1 recruitment and Ser2 phosphorylation suggesting that H2Bub1 deposition is rather P-Ser5 then P-Ser2-

dependent. H2Bub1 regulation in S. cerevisiae is mediated by Bur1 and does not depend on the Ser2 phosphorylation of RNAPII (Wood, 2005; Laribee, 2005). Moreover, Bur1 phosphorylates Rad6, E2 enzyme for H2Bub1 leading to its activation (Wood, 2005). In correspondence to this data human CDK9 was recently shown to phosphorylate human Rad6 ortholog UBE2A in vitro and in vivo (Shchebet, 2012).

In S. pombe function of CDK9 ortholog spCdk9 is even more special. It phosphorylates elongation factor Spt5 which is a part of DSIF complex (Pei, 2003). Spt5 phosphorylation by spCdk9 stimulates H2Bub1 accumulation and this function is not dependent on FACT complex in contrary to S. cerevisiae and human (Sanso, 2012). Moreover, H2Bub1 regulates Spt5 phosphorylation via a positive feedback loop: H2Bub1-enriched chromatin facilitates spCDK9 recruitment (Sanso, 2012).

Taken together, described data suggest that, despite of H2Bub1 being conserved among species, its regulation evolved differently in different species.

1.3.2 H2Bub1 deubiquitination

H2Bub1 levels are regulated not only by addition, but also by active removal of this modification from the chromatin. In yeast it is mediated by two de-ubiquitinating enzymes Ubp8 (Henry, 2003; Daniel, 2004) and Ubp10 (Emre, 2005; Gardner, 2005). Ubp8 acts as a part of Spt-Ada-Gcn5-Acetylating complex (SAGA) which plays a role of transcriptional coactivator (reviewed in Daniel and Grant, 2007), while Ubp10 is associated with non-transcribed regions and plays a role in the telomere silencing (Emre, 2005). In humans no Ubp10 ortholog was described up to date, but a homolog of Ubp8, USP22, was discovered (Zhao, 2008; Zhang, 2008). It is also a part of human SAGA complex (reviewed in Rodríguez-Navarro, 2009).

In yeasts SAGA complex consists of 21 subunits, most of them are essential and evolutionary conserved (reviewed in Rodríguez-Navarro, 2009). All the subunits unite in two enzymatic modules – acetylating and deubiquitinating. The main compomnent of acetylating complex is GCN5 – a bromodomain-containing protein that mediates acetylation of H3 (Grant, 1997). The deubiquitination module of SAGA is composed of Sgf11, Sus1 and Ubp8 (Köhler, 2006) with corresponding human orthologs ATXN7L3, ENY2 and USP22. Interaction between the DUB module and other components of SAGA is mediated by Sgf73 (human – ATXN7) which removal leads to a release of the DUB module from the SAGA complex (Lee, 2009).

Deubiquitination of H2Bub1 in human is predominantly dependent on the SAGA complex and its disruption via ATXN7L3 knockdown leads to great increase in H2Bub1 (Lang, 2011). However, the role of USP22 as a main DUB for H2B is not well established. It was shown that USP22 deubiquitinates H2Bub1 on the interferon-regulated gene IRF1 (Chipumuro, 2012) and is essential for estrogen (Zhang, 2008) and androgen-dependent (Zhao, 2008) transcription. But depletion of USP22 results in only mild decrease in global H2Bub1 (Zhao, 2008; Zhang, 2008, Chipumuro, 2012). Due to these facts a model of alosteric regulation of USP22 by SAGA was proposed were enzymatic activity of this enzyme depends on interactions with other components of the complex (reviewed in Rodríguez-Navarro, 2009). However, there is a possibility that other ubiquitin hydrolases perform deubiquitination of H2Bub1 in addition to USP22. For example, enzyme USP27X shares structural homology with USP22 and was shown to interact with it (Sowa, 2009) suggesting it as another DUB for H2Bub1.

On the other hand, USP22 might act independently of SAGA. An indirect evidence for this comes from the USP22 role in cancer (Zhang, 2011c; Liu, 2011). It was observed that USP22 overexpression in tumors correlates with poor clinical prognosis (Glinsky, 2006). Since no other SAGA components were reported as potential oncogenes this effect of USP22 might be SAGA-independent.

1.4. Cellular functions of the H2Bub1

1.4.1 The role of H2Bub1 in the chromatin compaction

The role that H2Bub1 plays in the cell is largely connected to its structure. While most of the histone modifications take place at the N-terminus it is has been suggested that a bulky ubiquitin is added to the C-terminus of H2B which is located close to the interface of two adjacent nucleosomes (Fig. 3) (Fierz, 2011). This positioning can potentially interfere with the formation of higher order chromatin compaction. However, a deeper structural analysis is required to confirm this statement.

The presence of H2Bub1 in the nucleosome may also disrupt higher order chromatin structures making it more accessible to chromatin-modifying enzymes like DOT1L (Fierz, 2011). Moreover, the decompaction of the chromatin seems to be dependent on the chemical

nature of ubiquitin since the addition of a similar, but distinct chemical moiety with different surface charges did not mimic the action of H2Bub1 (Fierz, 2011).

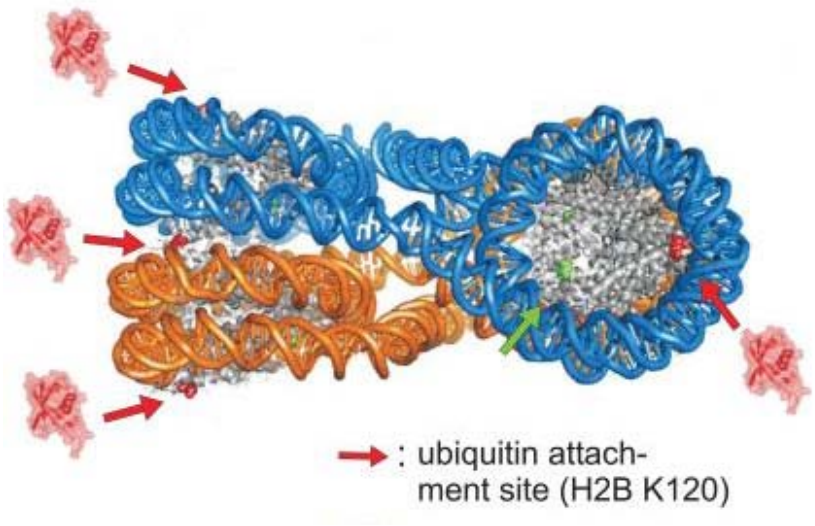

Fig. 3. Positioning of H2Bub1 within chromatin (modified from Fierz, 2011**).** Red arrows indicate attachment of ubiquitin residues.

1.4.2 The fuctions of H2Bub1 in yeast

Structural modeling suggests that H2Bub1 can be involved in transcriptional regulation since it requires opening of the chromatin (Fierz, 2011). Indeed, in yeast H2Bub1 is mostly associated with actively transcribed genes and is excluded from heterochromatin (Kao, 2004). Yeast strains bearing a mutation of lysine 123 to arginine (K123R) which prevents monoubiquitination of H2B have been instrumental in deciphering the function of H2Bub1 in vivo. In vitro H2Bub1 promotes transcript elongation (Kim, 2009), which is also supported by the in vivo finding that Rad6 together with Bre1 is recruited to elongating RNA Polymerase II (RNAPII) to deposit H2Bub1 (Wood, 2003; Hwang, 2003). Rad6 recruitment requires yeast Paf1 complex and is dependent upon the phosphorylation of the RNAPII CTD (Xiao, 2005). Interestingly, the repression of H2Bub1 deubiquitination inhibits transcription in yeast (Henry, 2003) suggesting that H2Bub1 has to be dynamically regulated. In human cells H2Bub1 is also found on actively transcribed regions with the increased levels downstream of the transcription start site (Minsky, 2008). Although generally associated with active transcription, H2Bub1 may also inhibit expression of certain genes (Shema, 2008).

Another way by which H2Bub1 facilitates transcription is interplay with other histone modifications. As it is established for yeast, H2Bub1 is a pre-requisite for H3K4me3 deposition (Dover, 2002). Trimethylation of H3K4 is performed by Set1/COMPASS complex in S. cerevisiae (Briggs, 2001; Nagy, 2002) and associates with actively transcribed genes (Briggs, 2001). H2Bub1 serves as recognition signal for Set1/COMPASS recruitment since Rad6 deletion mutant drastically decreases H3K4me3 levels (Dover, 2002). Another confirmation of H3K4me3 dependancy on H2Bub1 comes from S. cerevisiae K123R strain that exhibits no detectable H3K4me3 (Sun, 2002). H3K4me3 associates with H2Bub1 both on selected active genes (Sun, 2002) and genome-wide (Dover, 2002). Interestingly, depletion of H3K4me3 does not decrease H2Bub1 levels (Sun, 2002) suggesting the unidirectional cross-talk.

Another mark of actively transcribed genes, H3K79 trimethylation, deposited by the Dot1 lysine methyltransferase, is also dependent on H2Bub1 in yeast (Briggs, 2002; Ng, 2002) agreeing with the previously mentioned H2Bub1-dependent facilitation of DOT1L (the human Dot1 ortholog) function in vitro. H2B K123 mutant displayed loss of H3K79me3, however this loss was not mediated by H3K4me3 depletion since it was not affected in Set1 mutant (Briggs, 2002). Similarly to H3K4me3, depletion of H3K79me3 did not result in decrease of H2Bub1 levels (Briggs, 2002) indicating that H2Bub1 is an upstream regulator of H3K4me3 and H3K79me3.

One more possible function of H2B is connected with the nuclear transport. Translation of mRNA templates requires their transport to the cytoplasm via nuclear pore complex (NPC) (Iglesias, 2008). Recently the novel role in nuclear transport was demonstrated for SAGA complex in S. cerevisiae (Rodriguez-Navarro, 2004): Suz1 subunit of SAGA was shown to interact with the Transcription-Export Complex 2 (TREX2) required for mRNA export (Pascual-Garcia, 2009). Since the DUB enzyme for H2Bub1 is a part of SAGA (Henry, 2003), it is possible that H2Bub1 also participates in the nuclear transport of mRNA transcripts.

Recruitment of H2Bub1 machinery to the chromatin in S. cerevisiae is mediated via the Paf1 complex (Shi, 1996) that was shown to be associated with RNAPII as well as with histone methyltransferases (Li, 2002; Wood, 2003). In its turn Paf1 interacts with FACT histone chaperone complex (Krogan, 2002) which facilitates elongation by displacing H2A/H2B dimer from the core nucleosomes (Belotserkovskaya, 2003) and allows RNAPII to pass

through nucleosomal barrier (Kireeva, 2002). Summarizing, FACT associated with RNAPII recruits Rad6/Bre1 via interaction with Paf1.

1.4.3 H2Bub1 in higher eukaryotes

There are several similarities and differences in H2Bub1 functions between S. cerevisiae and higher eukaryotes. In human chromatin H2Bub1 is also associated with transcribed regions of active genes (Minsky, 2008; Shema, 2008). However, a knockdown of either RNF20 or RNF40 that form an E3 ligase complex for H2Bub1 results in a rather moderate effect on gene expression despite of the substantial decrease in H2Bub1 levels (Shema, 2008; Prenzel, 2011). Moreover, H2Bub1 is also required for repression of certain genes (Shema, 2008), suggesting that in mammals this modification plays a more complex role in cellular processes than in budding yeast.

Similarly to S. cerevisiae H2Bub1 in higher eucariots cooperates with FACT (Pavri, 2006; Prenzel, 2011) and PAF (Pavri, 2006) complexes to facilitate gene expression. In human, H2Bub1 is also required for H3K4me3 deposition, however there are six orthologs of COMPASS complex which could be grouped in three subfamilies: Set1/COMPASS, MLL1/2 hCOMPASS-like and MLL3/4 hCOMPASS-like (reviewed in Shilatifard, 2012). Downregulation of H2Bub1 also interferes with H3K4me3. Removal of Bre1 analog in *D.melanogaster* results in decrease of both H3K4me3 and H3K79me3 (Wood, 2003; Mohan, 2010). H2Bub1 requirement for H3K4me3 deposition was also confirmed for human cells (Kim, 2009).

Furthermore, H2Bub1 also regulates gene expression by interfering with 3'end mRNA processing. As it was recently shown (Pirngruber, 2009) H2Bub1 is required for correct stem loop-dependent processing of histone genes. Upon RNF40 depletion the stem loop site is likely to be skipped by polymerase resulting in production of longer polyadenylated transcripts.

The conncetion between nuclear transport and H2Bub1, described earlier for yeast, may also be possible for higher eucariots. SAGA requirement for mRNA transport was demonstrated in D. melanogaster where e(y)2 (human – ENY2) interacts with A Homolog of TREX (AMEX) complex, which is an ortholog of TREX2 (Kurshakova, 2007) that was mentioned earlier.

Apart from its transcription-associated functions H2Bub1 also participates in other cellular processes including DNA damage signaling (Moyal, 2011; Nakamura, 2011; Kari, 2011;

Chernikova, 2012). In human cells double strand break (DSB) generation leads to ATM-dependent RNF20/40 recruitment to the DSB-site. It is proposed that H2Bub1 deposition at DSB sites is required for chromatin unwinding followed by the recruitment of the repair machinery.

1.4.4 H2Bub1 and cancer

During last few years it was shown that H2Bub1 is lost during the carcinogenesis (Schema, 2008; Prenzel, 2011; Chernikova, 2012; Urasaki, 2012). First indications that H2Bub1 could be involved in the regulation of this process as a tumor-suppressor came from the observations that RNF20 promoter is hypermethylated in breast cancer (Shema, 2008). Recently RNF20 levels were shown to be downregulated in seminomas (Chernikova, 2012). It was proposed that downregulation of RNF20 results in increased frequencies of chromosomal aberrations due to the loss of H2Bub1 (Chernikova, 2012). RNF20 depletion also increases migratory potential of the cells and thereby facilitates metastasis (Shema, 2008). Later that was also demonstrated for the knockdown of RNF40 together with its ability to induce estrogen-independent growth and proliferation of breast cancer cells (Prenzel, 2011). Finally, direct studies on breast cancer samples demonstrated nearly complete loss of H2Bub1 in malignant tissues while adjacent non-transformed cells possessed substantial amount of this modification (Prenzel, 2011). Going along with this data USP22 was reported to be upregulated and correlated with a poor prognosis in colon (Liu 2011, Liu 2010) and breast (Zhang, 2011c) cancer. Taken together these observations suggest that the loss of H2Bub1 correlates with the increased cancer progression. This modification play a role in modulating proliferation and migration of the cells (Shema, 2008; Prenzel, 2011), alteration of gene expression and chromosomal instability (Chernikova, 2012).

1.5 Histone modifications in cellular differentiation

Cellular differentiation is a process of committing a particular cellular fate. It is driven by activation of lineage-specific genes and silencing of genes required for other lineages (reviewed in Dillon, 2012). Embryonic stem cells (ES cells) have the biggest differentiation potential – they can differentiate into all types of somatic cells, while the differentiated cells are committed to a certain lineage and are difficult to be trans-differentiated into another cell

type. Since differentiation results in changes in transcription histone modifications as one of the main transcription regulators directly influence this process.

One of the ways is priming of lineage-specific genes in stem cells. Priming is an addition of certain properties to the gene that will make it easier to activate upon a signal (reviewed in Dillon, 2012). An example of positive priming can be observed in B-cell differentiation. Differentiation-dependent enhancer loci, important for B-cell maturation, are marked with activating modification H3K4me2 in pre-B-cells as well as in ES cells (Liber, 2010). They are not active in ES cells due to repression by Foxd3, but upon differentiation signal repression is removed and the enhancer becomes fully functional. Another type of priming is establishing of bivalent domains which will be explained in the next chapter.

In more differentiated precursors, priming is less spread and replaced by classical signal-coupled deposition of histone modifications. Switching on the differentiation-dependent genes requires activating histone marks as acetylation of H3 and H4, H3K4me3 and H3K79me3 (Gan, 2006). For example, GCN5, a histone acetyl-transferase (HAT) subunit of SAGA complex, in required for cardiomyocyte differentiation of rat MSCs (Li, 2010); another HAT called MOZ is essential for hematopoetic stem cell development (Perez-Campo, 2009). Histone deacetylating enzymes (HDACs) also play a role in differentiation, e.g., knockout of HDAC1 and HDAC2 results in abnormal neuronal differentiation (Montgomery, 2009). SETD3 which deposits H3K4me3 and H3K36me3 is required for transcription of muscle specific genes such as myogenin and creatine kinase (Eom, 2011).

On the other side, repressive histone modifications prevent transcription of genes from other lineages. One of the most important players in this regard is the Polycomb Repressive Complex-2 (PRC2). It binds to gene promoters and deposits H3K27me3 (Sparmann, 2006). PRC2 has several components: Suppressor of Zeste-12 (SUZ12), Embryonic Ectoderm Development (EED), and Enhancer of Zeste Homolog 2 (EZH2). SUZ12 and EED are required for stabilization of the complex while EZH2 possesses methyl-transferase activity (Pasini, 2004). PRC2 is required for intestine epithelium (Benoit, 2012), cardiomyocyte (He, 2012), myoblast (Stojic, 2011) differentiation and lineage commitment of hematopoetic precursors (Mochizuki-Kashio, 2011).

Summing up, the main function of histone modifications during differentiation is stabilizing activation or silencing of certain subsets of genes and preserving this transcription pattern across cellular generations. Ultimately that leads to establishing of cell lineage "memory".

1.5.1 Gene bivalency

To understand the phenomenon of gene or chromatin bivalency it is important to know the chromatin organization of ES cells, where it was discovered (Bernstein, 2006; Azazura, 2006). Undifferentiated ES cells have less heterochromatin (Mattout, 2010) and express more transcription factors and remodeling proteins than differentiated cells (Efroni, 2008). Chromatin of ES cells has a so-called "open" structure: it is transcriptionally permissive and possesses transcription-associated histone modifications (H3K9me3, H3K4me3 and H3K36me3) (Mattout, 2010, Efroni, 2008). At the same time the transcription of the lineage-specific genes is very low.

The chromatin immunoprecipitation (ChIP) coupled with DNA hybridization on a microarray (ChIP-Chip) and ChIP sequencing (ChIP-seq) studies revealed that certain areas of genome in human and mouse ES cells are enriched with functionally opposite histone modifications (Azuara, 2006; Bernstein, 2006; Pan, 2007; Zhao, 2007). These structures were called bivalent. They are usually located near the TSS and consist of regions enriched with H3K27me3 (a repressive mark) and H3K4me3 (an activating mark). Genes carrying bivalent domains usually belong to development or differentiation-regulated transcription factors (Bernstein, 2006). H3K4me3 is deposited by proteins of Trithorax group (Ingham, 1983; reviewed in Ingham, 1998; Schuettengruber, 2011) and composition of the complex varies between different species and cell lineages. Trimethylation of H3K27 is executed by PRC2 as mentioned before.

Most of the bivalent marks are resolved during differentiation and the transcriptional state of the gene depends on the mark that remained (Bernstein, 2006; Mikkelsen, 2007; Pan, 2007) (Fig.4). If differentiation signal leads to gene induction, H3K4me3 mark remains on the gene followed by RNAPII recruitment, while H3K27me3 is removed. In Drosophila this function is performed by UTX demethylase (Smith, 2008) which has two orthologs in human – UTX and JMJD3 (Agger, 2007; De Santa, 2007). In case of gene repression the PRC2-deposited mark H3K27me3 is preserved on the chromatin leading to recruitment of other silencing proteins and H3K4me3 is removed. Several enzymes were described to perform this reaction in

mammalians, among them are KDM5B (JARID1B) (Frescas, 2007) and KDM2B (JHDM1B) (Christensen, 2007). Finally, in certain progenitor cells bivalent state of the gene can be preserved across generations until appropriate differentiation signal.

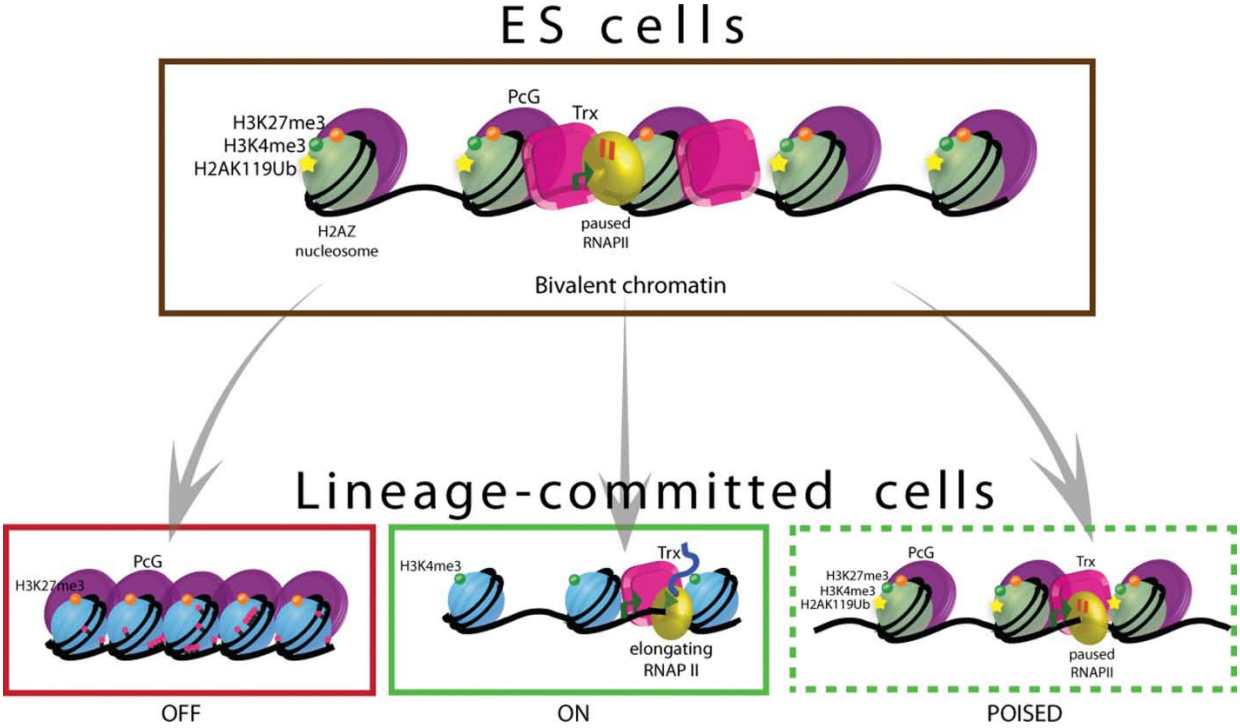

Fig. 4. Resolution of gene bivalency (from Sha, 2011). Many genes in ES cells exist in poised state characterized by presence of paused RNAPII and functionally opposite histone marks – H3K4me3 and H3K27me3 (upper panel). Chromatin possessing these features was called bivalent. Upon differentiation bivalent state can be resolved in different ways (lower panels). (1) The gene loses activating marks (H3K4me3) and becomes repressed ("OFF" state). (2) Gene becomes activated. In this case RNAPII is released and repressive H3K27me3 is removed ("ON" state). (3) Gene remains poised until later differentiation stages ("POISED" state) which is characteristic for many progenitor cells. PcG – PRC2 complex that deposits H3K27me3; Trx – Trithorax complex that performs trimethylation of H3K4; RNAPII – RNA polymerase II

Bivalent chromatin is evolutionarily conserved and can be found in mammals as well as in Zebrafish (Lindeman, 2010). Apart from ES cells bivalent domains can be also found, although in lesser extent, in neural progenitors, mesenchymal (Mikkelsen, 2007) and hematopoietic (Cui, 2009) stem cells. As in ES cells, bivalent marks are also resolved upon differentiation in these systems.

Concluding, the presence of conserved bivalent domains suggests the importance of these marks for regulation of developmental and differentiation-activated genes. However, the questions about establishing and maintaining bivalency as well as signaling that leads to its resolution remains opened.

1.6 Human MSCs as a differentiation system

hMSCs are fibroblast-like cells which were firstly obtained from the bone marrow and isolated due to their ability to grow in the adherent conditions (Luria, 1971; Kassem, 1993). These cells do not originate from the hematopoietic lineage and are negative for hematopoietic markers CD34, CD45 and CD14 (Pittenger, 1999). Although hMSCs are "classically" isolated from the bone marrow they can also be found in other tissues like peripheral blood (Kuznetsov, 2001), adipose tissue (Gronthos, 2001a), dental pulp (Gronthos, 2001b; Otaki, 2007). Their main characteristic is a capacity to differentiate into multiple mesodermal cell types, like osteoblasts (Kassem, 1993), adipocytes (Justesen, 2002), and chondrocytes (Johnstone, 1998).

Several transcription factors are implicated in hMSC differentiation (reviewed in Abdallah and Kassem, 2012). One of the adipocyte regulators is a transcription factor Peroxisome Proliferator–Activated Receptor γ (PPARG) (Rosen, 1999). PPARG is a nuclear receptor whose in vivo ligand has not been defined; however it is activated upon binding of thiazolidinediones, insulin-sensitizing drugs (Lehmann, 1995). Early in adipogenesis PPARG dimerizes with the Retinoid X Receptor (RXR) to activate transcription of CCAAT/Enhancer-Binding Protein β (C/EBPβ) and C/EBPδ (Chawla, 1994) followed by C/EBPα activation and establishment of a differentiated phenotype. C/EBPα also elevates PPARG expression via a positive feedback loop (Wu, 1999).

During osteoblast differentiation Runt-Related Transcription Factor 2 (RUNX2) plays an essential role in MSC commitment (reviewed in Komori, 2010). RUNX2 induces transcription of another master transcription factor Osterix (SP7) that coordinates bone mineralization (Nakashima, 2002). RUNX2 also activates expression of bone matrix proteins like collagen type II (COL2A1) (Aubin and Triffitt 2002), osteopontin (SPP1) and bone sialoprotein 2 (IBSP) (Aubin and Triffitt, 2002) in immature osteoblasts and osteocalcin (BGLAP) in mature osteoblasts (Nakashima, 2002). Although RUNX2 directs hMSCs into osteoblast lineage its expression keeps osteoblasts in immature state and has to be shut down to complete the differentiation (reviewed in Komori, 2010).

Another important compound for osteoblast differentiation is 1,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin D_3 or calcitriol), the activated form of vitamin D (Piek, 2010; Kroeze, 2011; Erben, 1997). All the intracellular actions of calcitriol are mediated by Vitamin D

Receptor (VDR). In the absence of ligand VDR resides on the chromatin VDR responsive elements (VDREs), but only upon ligand binding it gains an ability to activate or suppress gene expression (Haussler, 2008; Pike and Meyer, 2010; Pike, 2010). Except of calcitriol binding VDR activation also requires dimerization with RXR which is similar to PPARG. VDR controls the expression of the genes related to mineral homeostasis (DeLuca, 1998; Plum and DeLuca, 2010); VDR knockout mice develop osteomalacia (bone softening) and lack bone mass (Amling, 1999). Interestingly, VDR expression is regulated by Osterix binding (Zhang, 2011a) suggesting a complex crosstalk between regulators of osteoblast differentiation. Other factors like TAZ (Hong, 2005) and ΔFosB also promote osteogenesis (Sabatakos, 2000).

Wnt signaling, both canonical and non-canonical, is also involved in determination of lineage for hMSCs (reviewed in Abdallah and Kassem, 2012). Canonical Wnt-β-catening pathway promotes osteoblast differentiation of hMSCs via an increase in RUNX2 expression. At the same time it inhibits adipocyte differentiation by downregulation of C/EBPα and PPARγ and upregulation of RUNX2 and SP7 (Kang, 2007). Non-canonical Wnt signaling also promotes osteoblast differentiation (Taipaleenmäki, 2011). Moreover, hMSCs themselves regulate their differentiation via paracrine secretion of growth factors like Dlk1/FA1 (Abdallah, 2004) Dlk1/FA1 is a member of EGF-like protein family (Laborda, 2000) which was shown to repress osteoblast as well as adipocyte differentiation of hMSC and to maintain undifferentiated state of these cells (Abdallah, 2004).

1.6.1 Application of hMSCs in medicine and biology

hMSCs are potential targets for regenerative medicine which is based on transplantation of biologically competent cells or tissues to treat degenerative or age-related diseases.

hMSCs possess a well-characterized ability to suppress immune responses from cells of the innate and adaptive immunity via cytokine production (Chen, 2006). These properties also make them attractive for treatment of transplantation-related and autoimmune diseases (Ciccocioppo, 2011; Mannon, 2011; Le Blanc, 2004; Zanone, 2010; Kocher, 2001). One more feature of hMSCs is an ability to migrate to damaged tissues (Chen, 2006). Due to this feature together with their immuno-suppressive activity, hMSCs were successfully used for the treatment of myocardial infarction, spinal cord injury, bone injury, damaged kidney and diabetes (Reagan and Kaplan, 2011).

Only a limited number of molecular biology techniques can be applied to hMSCs due to their relatively short lifespan - up to 40 population doublings (Stenderup, 2003). The solution to this problem was proposed by the generation of hMSCs overexpressing reverse transcriptase (hTERT) (Simonsen, 2002). These cells have an increased life span while maintaining the ability to differentiate (Abdallah, 2005).

Another aspect of interest in hMSCs is their contribution to the development of osteoporosis. Osteoporosis is a bone disease resulting in decreased mineral density and increased risk of fractures. One of the reasons of osteoporosis development is decreased osteoblast formation in the bone possibly due to decreased differentiation of progenitors (reviewed in Gimble, 2006). As it was shown for hMSCs commitment to one lineage inhibits differentiation to other cell types (Beresford, 1992; Falconi, 2007; Gimble, 1995). PPARG selectively promotes adipocyte differentiation of hMSC which results in accumulation of fat cells and reduced number of osteoblasts in the bone marrow (Wang, 2012). However more studies are required to describe a mechanism of hMSCs lineage commitment as well as to interfere with it.

1.7 Aim of the project

H2Bub1 is implicated in various cellular processes including gene expression, DNA damage repair and nucleosomal exchange. However, there is almost nothing known about H2Bub1 involvement in differentiation and lineage commitment. Therefore, the aim of this study was to examine, whether H2Bub1 in required for cellular differentiation. To answer this question H2Bub1 levels were monitored in hMSCs during their differentiation into osteoblasts and adipocytes. Moreover, to study the effect of H2Bub1 removal on differentiation main regulators of this modification, like RNF40 and CDK9, were depleted in hMSCs and the influence of this depletion on the gene expression was examined. Finally, the mechanistic effect of H2Bub1 removal was studied by monitoring the distribution of other histone marks, H3K4me3 and H3K27me3 on the adipocyte-specific genes.

2 MATERIALS

2.1 Technical 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 MicroImaging GmbH, Göttingen
Counting chamber (Neubauer)	Brand GmbH & Co. KG, Wertheim
5100 Cryo 1 °C Freezing Container	Thermo Fisher Scientific, Waltham, USA
MiniVE (mini vertical electrophoresis unit)	GE Healthcare Europe GmbH, München
Freezer -20 °C	Liebherr GmbH, Biberach
Flow cytometry system Guava EasyCyte Plus	Millipore, Billerica, USA
Freezer -80 °C “Hera freeze”	Thermo Fisher Scientific, Waltham, USA
Gel Imager “Gel iX imager”	Intas Science Imaging GmbH, Göttingen
Incubator (cell culture) “Hera cell 150“	Thermo Fisher Scientific, Waltham, USA
Magnet stirrer “MR3001”	Heidolph GmbH & Co. KG, Schwabach
Microscope Axio Scope A1	Carl Zeiss MicroImaging GmbH, Göttingen
Microscope “Axiovert 40 C“	Carl Zeiss MicroImaging GmbH, Göttingen
Microwave	Clatronic International GmbH, Kempen

Nano Drop® ND-1000 Spectrophotometer	Peqlab Biotechnology GmbH, Erlangen
OptiMax X-ray Processor	Typon Medical, Krauchthal
Pestle	Sartorius AG, Göttingen
pH meter	inoLab® WTW GmbH, Weilheim
Pipette Aid® portable XP	Drummond Scientific Co., Broomall, USA
Pipettes “Research” Series	Eppendorf AG, Hamburg
Power supply “Power Pack P25T”	Biometra GmbH, Göttingen
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
Thermomixer comfort	Eppendorf AG, Hamburg
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 Consumable materials

Cellstar 6-, 12-, 24-well cell culture plates	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
Cellstar tubes, 15ml and 50 ml	Greiner Bio-One GmbH, Frickenhausen
Cell scraper (16 cm, 25 cm)	Sarstedt AG & Co., Nümbrecht

Cryo TubeTM Vial (1.8 ml)	Thermo Fisher Scientific, Waltham, USA
Gel blotting paper (Whatman paper)	Sartorius AG, Göttingen
Glass coverslips (18 mm)	Gebr. Rettberg GmbH, Göttingen
Microtube 0,5 ml, 1.5 ml, 2ml	Sarstedt AG & Co., Nümbrecht
Microtube 1.5 ml, conical	VWR International GmbH, Darmstadt
96 Multiply® PCR plate white	Sarstedt AG & Co., Nümbrecht
96-well Multiplate® PCR plate white (low)	Bio-Rad Laboratories GmbH, München
NORM-JECT Syringes of different volume	Henke Sass Wolf GmbH, Tuttlingen
FrameStar® 96 Skirted qRT-PCR plates	4titude Ltd., Wotton, UK
Parafilm® "M"	Pechiney Plastic Packaging, Chicago, USA
Pipette tips	Greiner Bio-One GmbH, Frickenhausen
Pipette filter tips	Sarstedt AG & Co., Nümbrecht
Pro-Bind 96-Well Plates for flow cytometry	BD Biosciences, Franklin Lakes, USA
Protan® Nitrocellulose transfer membrane	Whatman GmbH, Dassel
Syringe filter, CA-membrane, 0,20 µm	Sartorius AG, Göttingen
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
Acetone ROTISOLV	Carl Roth GmbH & Co. KG, Karlsruhe
Adefodur WB developing concentrate	Adefo-Chemie GmbH, Dietzenbach
Adefodur WB fixing concentrate	Adefo-Chemie GmbH, Dietzenbach
Agarose	Biozym Scientific GmbH, Hessisch Oldendorf
Albumin Fraction V (BSA)	Carl Roth GmbH & Co. KG, Karlsruhe

Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium sulfate $(NH_4)_2SO_4$	Carl Roth GmbH & Co. KG, Karlsruhe
Antibiotic-Antimycotic	Life Technologies, Carlsbad, USA
Aprotinin	Carl Roth GmbH & Co. KG, Karlsruhe
Bovine Growth Serum (BGS)	Thermo Scientific HyClone, Logan, USA
Bromophenol blue	Sigma-Aldrich Co., St. Louis, USA
Calcium Chloride ($CaCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
Chelex 100 (Chelating Ion Exchange Resin)	Bio-Rad Laboratories GmbH, München
Chloroform	Carl Roth GmbH & Co. KG, Karlsruhe
Ciprofloxacin	Sigma-Aldrich Co., St. Louis, USA
Co-precipitant Pink	Bioline, Luckenwalde
Diethylpyrocarbonate (DEPC)	Carl Roth GmbH & Co. KG, Karlsruhe
Dimethyl sulfoxide (DMSO)	AppliChem GmbH, Darmstadt
Dithiothreitol (DTT)	Carl Roth GmbH & Co. KG, Karlsruhe
DMEM, no Phenol Red	Life Technologies, Carlsbad, USA
DMEM/F-12, no Phenol Red	Life Technologies, Carlsbad, USA
dNTPs	Prime Tech, Minsk, Belarus
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
Formaldehyde	Sigma-Aldrich Co., St. Louis, USA
Glycerol	Carl Roth GmbH & Co. KG, Karlsruhe
ß-Glycerolphosphate disodium salt hydrate (BGP)	Sigma-Aldrich Co., St. Louis, USA
Glycine	Carl Roth GmbH & Co. KG, Karlsruhe
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG, Karlsruhe

HygroGold (Hygromycin B)	InvivoGen, San Diego, USA
Iodoacetamide	AppliChem GmbH, Darmstadt
Isopropanol	Carl Roth GmbH & Co. KG, Karlsruhe
Leupeptin	Carl Roth GmbH & Co. KG, Karlsruhe
Lithium chloride (LiCl) solution, 8M	Sigma-Aldrich Co., St. Louis, USA
Magnesium chloride ($MgCl_2$)	Carl Roth GmbH & Co. KG, Karlsruhe
MEM, no Glutamine, No Phenol Red	Life Technologies, Carlsbad, USA
Methanol	Carl Roth GmbH & Co. KG, Karlsruhe
M-MuLV Reverse Transcriptase Reaction Buffer	New England Biolabs, Frankfurt am Main
N-ethylmaleimide (NEM)	Sigma-Aldrich Co., St. Louis, USA
Nickel chloride	Sigma-Aldrich Co., St. Louis, USA
Nile Red	Sigma-Aldrich Co., St. Louis, USA
NonidetTM P40 (NP-40)	Sigma-Aldrich Co., St. Louis, USA
Oil Red O	Sigma-Aldrich Co., St. Louis, USA
Opti-MEM	Life Technologies, Carlsbad, USA
PBS tablets	Life Technologies, Carlsbad, USA
Pefabloc SC Protease Inhibitor	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium chloride (KCl)	AppliChem GmbH, Darmstadt
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth GmbH & Co. KG, Karlsruhe
Propidium iodide solution	Sigma-Aldrich Co., St. Louis, USA
Protein A Sepharose CL-4B	GE Healthcare, Uppsala, Sweden
Protein G Sepharose 4 Fast Flow	GE Healthcare, Uppsala, Sweden
RNase inhibitor	New England Biolabs, Frankfurt am Main
RNAiMAX	Invitrogen GmbH, Karlsruhe

Rotiphorese® Gel 30	Carl Roth GmbH & Co. KG, Karlsruhe
Salmon sperm DNA	Invitrogen GmbH, Karlsruhe
Sepharose CL-4B	GE Healthcare, Uppsala, Sweden
Skim milk powder	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium acetate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium azide	AppliChem GmbH, Darmstadt
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium citrate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium deoxycholate	AppliChem GmbH, Darmstadt
Sodium dodecylsulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium fluorid (NaF)	AppliChem GmbH, Darmstadt
di-Sodium hydrogen phosphate dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. KG, Karlsruhe
SYBR Green I	Roche Diagnostics GmbH, Mannheim
TEMED	Carl Roth GmbH & Co. KG, Karlsruhe
α,α-Trehalose (+)	AppliChem GmbH, Darmstadt
Tris	Carl Roth GmbH & Co. KG, Karlsruhe
Triton X-100	AppliChem GmbH, Darmstadt
Trypsin-EDTA (0.05%)	Life Technologies, Carlsbad, USA
Tween-20	AppliChem GmbH, Darmstadt
QIAzol Lysis Reagent	QIAGEN, Hilden

2.3.2 Differentiation chemicals

Ascobic acid	Sigma-Aldrich Co., St. Louis, USA
Calcitriol (1α,25-dihydroxy Vitamin D_3)	Cayman chemicals, Ann Arbor, USA

Dexamethasone	Sigma-Aldrich Co., St. Louis, USA
ß-Glycerolphosphate (BGP)	Sigma-Aldrich Co., St. Louis, USA
Insulin	Sigma-Aldrich Co., St. Louis, USA
Isobuthylmetylxantine (IBMX)	Sigma-Aldrich Co., St. Louis, USA
Troglitazone	Sigma-Aldrich Co., St. Louis, USA

2.3.3 Kits and reagents

Alkaline phosphatase leukocyte kit	Sigma-Aldrich Co., St. Louis, USA
FITC Mouse Anti- BrdU Set	BD Biosciences, Franklin Lakes, USA
Immobilon Western Chemiluminescent HRP Substrate	Millipore, Billerica, USA
Lipofectamine RNAiMAX	Invitrogen GmbH, Karlsruhe
PageRulerTM Prestained Protein Ladder	Fermentas GmbH, St. Leon-Rot
Salmon Sperm DNA	Invitrogen GmbH, Karlsruhe
SuperSignal West Femto Maximum	Thermo Fisher Scientific, Waltham, USA

2.3.4 Nucleic acids

2.3.4.1 Custom genes

Name	**Sequence (5' – 3')**	**Source**
Synthetic H2B	GGATCCGCCACCATGGACTACAAGGACGATGATGACA AGGGCATGCCCGAGCCCGCAAAAAGTGCACCAGCAC CAAAAAAAGGAAGTAAGAAAGCAGTAACAAAAGCTC AAAAGAAAGATGGGAAAAAAAGGAAAAGGTCAAGG AAAGAATCATATAGTGTTTATGTCTATAAAGTCCTCA AACAAGTGCATCCGGATACGGGGATAAGCAGTAAGG CGATGGGTATAATGAATAGTTTTGTGAATGATATATTT GAAAGGATAGCTGGGGAAGCCAGTAGGTTAGCCCACT ATAATAAAAGGAGTACGATAACGAGTCGTGAAATTCA AACTGCGGTCAGGCTCTTATTACCAGGTGAACTCGCG AAACATGCAGTCAGTGAAGGGACGAAAGCAGTGACG AAGTATACGTCGAGTAAGTAGCTCGAG	Eurogentec, Seraing, Belgium

2.3.4.2 Plasmids

Name	Source
pcDNA5/TO	Invitrogen GmbH, Karlsruhe
pcDNA5/TO-Flag-H2B	This study
pcDNA5/TO-K120R	This study

2.3.4.3 siRNA Oligonucleotides

Target Gene	siRNA target sequence	Source	Cat.No.
Negative control #1 siRNA	-	Ambion	4457287
siGENOME Non-targeting siRNA pool # 1	-	Dharmacon	D-001206-13
HIST1H2BC	GCAGUGACCAAAGCGCAGAtt	Ambion	s15862
HIST1H2BG	CGUCUGGCCCACUACAACAtt	Ambion	s194890
HIST1H2BO	GCAAAGAGAGUUACUCUAUtt	Ambion	
RNF20 siGenome (#1)	CCAAUGAAAUCAAGUCUAA	Dharmacon	D-007027-01
RNF20 si Genome (#2)	UAAGGAAACUCCAGAAUAU	Dharmacon	D-007027-02
RNF20 si Genome (#3)	GCAAAUGUCCCAAGUGUAA	Dharmacon	D-007027-03
RNF20 si Genome (#4)	AGAAGAAGCUACAUGAUUU	Dharmacon	D-007027-04
RNF40	UGAGGACAUGCAGGAACAGAA	Ambion	s18960
RNF40 siGenome (# 1)	GAGAUGCGCCACCUGAUUAUU	Dharmacon	D-006913-01
RNF40 siGenome (# 2)	GAUGCCAACUUUAAGCUAAUU	Dharmacon	D-006913-02
RNF40 siGenome (# 3)	GAUCAAGGCCAACCAGAUUUU	Dharmacon	D-006913-03
RNF40 siGenome (# 4)	CAACGAGUCUCUGCAAGUGUU	Dharmacon	D-006913-04
CDK9	UGAGAUUUGUCGAACCAAAtt	Ambion	S2834
WAC siGenome (# 1)	CAACAUAACGUCUCUGAUU	Dharmacon	D-013325-01

WAC siGenome (# 2)	UAAGCACACCUCAAACUAA	Dharmacon	D-013325-02
WAC siGenome (# 3)	GAGACAAACCCGUAUCACA	Dharmacon	D-013325-03
WAC siGenome (# 4)	CGAUCCACGUGUUCAUUAA	Dharmacon	D-013325-04

2.3.4.4 Cloning primers

Name	**Sequence (5' – 3')**	**Reference**
Flag-H2B Flag For	CTGAGCGGATCCGCCACCATGGACTACAAGG ACGATGATGACAAGGGCATGCCCGAGCCCGC AAAAAGTGCAC	This study
Flag-H2B Rev	CAGGCTCTTATTACCAGGTGAACTCGCGAAA CATGCAGTCAGTGAAGGGAC	This study
K120R-H2B Rev	GCTCAGCTCGAGCTACTTACTCGACGTATAC CTCGTCACTGCTTTCGTCCCTTCACTGACTGC ATGTTTCGCG	This study

2.3.4.5 Reverse Transcription primers

Random nonamer	Metabion International AG, Munich

2.3.4.6 qPCR primers

All Primers were synthesized by Metabion International AG (Munich, Germany) Primers generated for this study were designed using a primer designing tool (www.ncbi.nlm.nih.gov/tools/primer-blast/).

Name	**Sequence (5' – 3')**	**Reference**
ALPL F	TGGGCCAAGGACGCTGGGAA	Karpiuk, 2012
ALPL R	AAGGCCTCAGGGGGCATCTCG	Karpiuk, 2012
BGLAP F	GCCCTCACACTCCTCGCCCT	Karpiuk, 2012
BGLAP R	CGGGTAGGGGACTGGGGCTC	Karpiuk, 2012
CDK9 F	AGAGGGTTTCCATGGGGTAG	Karpiuk, 2012
CDK9 R	TCAGCCCGAGAATAGGATTG	Karpiuk, 2012
G6PD F	CGACGAAGCGCAGACAGCGTCA	Karpiuk, 2012

G6PD R	CAGCCACATAGGAGTTGCGGGC	Karpiuk, 2012
HIST1H2BA F	CAGGTCCATCCGGACACTGGCA	This study
HIST1H2BA R	CAAACGTGATGCCTCGCTCGCT	This study
HIST1H2BB F	CCCGACACCGGCATCTCATCCA	This study
HIST1H2BB R	CCTTAGTGCCCTCGGACACAGCA	This study
HIST1H2BC F	AGAAGGCAGTGACCAAAGCGCAG	This study
HIST1H2BC R	GCCCATGGCCTTGGAAGAGATGC	This study
HIST1H2BD F	ACGATGCCTGAACCTACCAA	This study
HIST1H2BD R	AGCCTTAGTCACCGCCTTCT	This study
HIST1H2BE F	GTGACCAAGGCGCAGAAGAAGGAC	This study
HIST1H2BE R	TTTAGAGGAGATGCCGGTGTCGGG	This study
HIST1H2BF F	ACCGGCATCTCATCCAAGGCCA	This study
HIST1H2BF R	TGACACGGCGTGCTTAGCCAG	This study
HIST1H2BG F	AGAAGCGCAAGCGCAGTCGT	This study
HIST1H2BG R	TAGTGGGCCAGACGGGAAGCC	This study
HIST1H2BH F	GCGTAAACGCAGCCGCAAGG	This study
HIST1H2BH R	GCCAGTTCCCCAGGCAGCAG	This study
HIST1H2BI F	GGGAGATCCAAACGGCTGTGCG	This study
HIST1H2BI R	GAGCCTTTGGGTCGTTAGCGCTTT	This study
HIST1H2BJ F	GCCAGCGAAGTCTGCTCCCG	This study
HIST1H2BJ R	CTCTCCTTGCGGCTGCGCTT	This study
HIST1H2BK F	TGCTGCTCGTCTCAGGCTCGT	This study
HIST1H2BK R	CTCTCCTTGCGGCTGCGCTT	This study
HIST1H2BL F	CCAAGAAGGCGGTGACCAAGGC	This study
HIST1H2BL R	AGAAGAGATGCCGGTGTCGGGG	This study
HIST1H2BM F	GGCCGTGCGCCTACTGCTAC	This study

HIST1H2BM R	GGTGTGGGTCACGGCGGAAC	This study
HIST1H2BN F	CAAAGTCCGCTCCTGCCCCG	This study
HIST1H2BN R	TGACCGAACGTTCCGCGGTG	This study
HIST1H2BO F	TTCACTCTCCTCCGCCATGCCC	This study
HIST1H2BO R	CTCTTTGCGGCTGCGCTTGC	This study
HIST2H2BE F	CCTGGTGGCTCCTTGGGTCTGT	This study
HIST2H2BE R	TATCCACAGGAGGCCCCATCGC	This study
HIST2H2BF F	CCTCCACCCCACCACCCCTC	This study
HIST2H2BF R	ATGGACTCGGGAACCGCCGA	This study
HIST3H2BB F	TCTTCGAGCGCATCGCCAGC	This study
HIST3H2BB R	CAGGACGCCGAGGAACGCC	This study
HNRNPK F	ATCCGCCCCTGAACGCCCAT	Karpiuk, 2012
HNRNPK R	ACATACCGCTCGGGGCCACT	Karpiuk, 2012
LPL F	TCAGCCGGCTCATCAGTCGGT	Karpiuk, 2012
LPL R	AGAGTCAGCACGAGCAGGGCT	Karpiuk, 2012
PDK4 F	TTCACTCCGCGGCACCCTCA	Karpiuk, 2012
PDK4 R	TCGGAGCAGAGCCTGGTTCCG	Karpiuk, 2012
PPARG F	ACCTCCGGGCCCTGGCAAAA	Karpiuk, 2012
PPARG R	TGCTCTGCTCCTGCAGGGGG	Karpiuk, 2012
RASD1 F	CAAGACGGCCATCGTGTCGCG	Karpiuk, 2012
RASD1 R	GCTGCACCTCCTCGAAGGAGTCG	Karpiuk, 2012
RNF20 F	TGGCCAAGCAGGAAGAAG	Karpiuk, 2012
RNF20 R	ACGCTCTGACATGAGCTTGA	Karpiuk, 2012
RNF40 F	AGTACAAGGCGCGGTTGA	Prenzel, 2011
RNF40 R	GAAGCAGAAAACGTGGAAGC	Prenzel, 2011
RPLP F	GATTGGCTACCCAACTGTTG	Fritah, 2005

RPLP R	CAGGGGCAGCAGCCACAAA	Fritah, 2005
WAC F	AGTGGGTTTGCCAGTGGAATGGAAGA	Karpiuk, 2012
WAC R	ACAGTGCTTGGGGTAGCAGTTGGA	Karpiuk, 2012

2.3.4.7 ChIP primers

Name	**Sequence**	**Reference**
HIST1H2AC F	AAAAGCGGCCATGTTTTACA	Pirngruber, 2009
HIST1H2AC R	AAAAATCACCAAAACCAGCG	Pirngruber, 2009
GAPDH +1061F	CCGGGAGAAGCTGAGTCATG	Shema, 2008
GAPDH +1111R	TTTGCGGTGGAAATGTCCTT	Shema, 2008
PDK4-BV+468F	GCGTCGAGGCTCCAGGGCT	Karpiuk, 2012
PDK4-BV+570R	GCCCAAGCTGGGTCCTAGGGTT	Karpiuk, 2012
PDK4 +3831F	CTCGGATGCTGATGAACCAGCACAGTAAG	Karpiuk, 2012
PDK4 +3963R	AGTACTATCACTGAGAATGTGACCCGCTGAT	Karpiuk, 2012
PPARG-BV+655F	AGCCGCTCCGGGGGAACTT	Karpiuk, 2012
PPARG-BV+850R	ACAGGGCCTGGCCAGCTACAA	Karpiuk, 2012
PPARG +91888F	GGCCCACCAACTTTGGGATCAGC	Karpiuk, 2012
PPARG +91922R	GAGTGGGAGTGGTCTTCCATTACGGAG	Karpiuk, 2012
RASD1-BV +768R	GATCTGCTGCCTGAGCCGCTG	Karpiuk, 2012
RASD1-BV +666F	CGGCCACCCTCACCTTCTCCT	Karpiuk, 2012

TFF1 +6KB F	CAGGCTTCTCCCTTGATGAAT	Pirngruber and Johnsen, 2010
TFF1 +6KB R	ACACCCACCTTCCACAACAC	Pirngruber and Johnsen, 2010

2.3.5 Proteins

2.3.5.1 Enzymes

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-Polymerase	Primetech, Minsk, Belarus

2.3.5.2 Antibodies

2.3.5.2.1 Primary antibodies

Dilutions of antibodies used for ChIP or WB are indicated in corresponding columns.

Target protein	ChIP	WB	Clone	Cat. No.	Source
CDK9		1:1000	C-20	sc-484	Santa Cruz
H2B		1:3000		07-371	Millipore
H2Bub1		1:100	7B4		(Prenzel et al., 2011)
H2Bub1	2 µg		56	05-1312	Millipore
H2Bub1	2 µl		D11	5546	Cell Signaling
H3K4me3	2 µg			MAb-003-050	Diagenode

H3K27me3	2 µg			pAb-069-050	Diagenode
IgG	2 µg			ab46540-1	Abcam
P-Ser2 RNAPII		1:10	3E10		(Chapman et al., 2007)
RNF20		1:1000		R8904	Sigma
RNF40		1:1000	KA7-27	R9029	Sigma

2.3.5.2.2 Secondary antibodies

Name	Dilution (WB)	Cat. No.	Source
Goat anti-mouse IgG-HRP	1:5000	sc-2005	Santa Cruz
Goat anti-rabbit IgG-HRP	1:5000	sc-2004	Santa Cruz
Goat Anti-Rat IgG + IgM-HRP	1:10000	112-035-068	Jackson ImmunoResearch

2.4 Cells

2.4.1 Bacterial cells

Name	Source
E. coli XL-1 Blue	Prof. A. Wodarz, Göttingen University

2.4.2 Human cells

Name	Source
H1299	Prof. M. Dobbelstein, Göttingen University
HEK293	Prof. M. Dobbelstein, Göttingen University

Human fetal osteoblasts (hFOB1.17)	Prof. T. Spelsberg, Mayo Clinic, USA
Human mesencymal stem cells (hMSC)	Prof. M. Kassem, Odense University Hospital, Denmark
U2OS with stable TetR overexpression	Prof. T. Spelsberg, Mayo Clinic, USA

2.5 Buffers and media

Ascorbic acid stock solution (1000×): 0.2 M ascorbic asid in water

Blocking solution: 1× PBS-T, 5% (w/v) milk

Calcitriol stock solution (1000×): 10 µM calcitriol in 100% DMSO

Cell culture freezing medium: MEM, 50% (v/v) FBS, 8% DMSO

Chelex (10%): 10% (w/v) Chelex in ddH_2O

ChIP crosslinking buffer: 1% formaldehyde in PBS

Gomes Lysis Buffer (ChIP): 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 50 mM Tris-HCl (pH 8), 20 mM EDTA, 20 mM NaF, 1x inhibitor cocktail.

Gomes Wash Buffer (ChIP): 100 mM Tris-HCl (pH 8.5), 500 mM LiCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 20 mM EDTA, 20 mM NaF, 1x inhibitor cocktail.

Dexamethasone stock solution (1000×): 100 µM dexamethasone in 100% EtOH

DMEM cell culture medium: phenol red-free, high-glucose DMEM, 10% BGS, 1× Penicillin/Streptomycin

DMEM/F-12 cell culture medium: phenol red-free, high-glucose DMEM/F-12, 10% BGS, 1× Penicillin/Streptomycin

β-glycerol phosphate (BGP) stock solution (100×): 1 M BGP in water

IBMX stock solution (100×): 0.45 M isobutyl-methyl-xanthine in 100% EtOH

Inhibitor cocktail (1×): 1 mM Pefabloc, 1 ng/µl Aprotinin/Leupeptin, 10 mM BGP, 1 mM NEM, 10 µM iodoacetamide and 1 mM nickel chloride

MEM cell culture "normal" medium: phenol red-free, high-glucose MEM,
10% BGS, 1× Antibiotic-Antimycotic solution, 1µg/ml ciprofloxacin

MEM cell culture "adipocyte" medium: phenol red-free, high-glucose MEM,
15% BGS, 1× Antibiotic-Antimycotic solution, 1µg/ml ciprofloxacin, 10 nM dexamethasone, 0.45 mM isobutyl-methyl-xanthine, 2 µM insulin, 10 µM Troglitazone

MEM cell culture "osteoblast" medium: phenol red-free, high-glucose MEM, 10% BGS, 1× Antibiotic-Antimycotic solution, 1µg/ml ciprofloxacin, 10 nM dexamethasone, 10 mM β-glycerol phosphate (BGP), 0.2 mM ascorbic acid, 10 nM calcitriol

Nelson Lysis Buffer (ChIP): 150 mM NaCl, 20 mM EDTA (pH 8), 50 mM Tris (pH 7.5), 0.5% (v/v) NP-40, 1% (v/v) Triton X-100, 20 mM NaF, 1x inhibitor cocktail.

DEPC water: 0.1% (v/v) DEPC in ddH_2O

Lämmli buffer (6×): 0.35 M Tris (pH 6.8), 30% (v/v) glycerol, 10% (w/v) SDS, 9.3% (w/v) DTT, 0.02% (w/v) bromphenol blue

PBS (1×): 137 mM NaCl, 2.68 mM KCl, 4.29 mM $Na_2HPO_4 \times 2H_2O$, 1.47 mM KH_2PO_4, pH 7.4

PBS++: 1× PBS, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$

PBS for cell culture: 1 PBS tablet per 500 ml ddH_2O

PBS-T: PBS including 0.1% (w/v) Tween-20

qPCR buffer: 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween-20, 3 mM $MgCl_2$, 200 µM dNTPs, 0.5 U/reaction *Taq* DNA Polymerase, 0.25% (v/v) Triton X-100, 1:80,000 SYBR Green I, 300 mM α,α-Trehalose, 300 nM Primers

RIPA buffer: 1× PBS, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxychelate, 0.1% (w/v) SDS,

SDS separating gel (8%, 10%, 12%, 15%): 8%, 10%, 12% or 15% (v/v) acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.04% (v/v) TEMED

SDS stacking gel (5%): 5% (v/v) acrylamide, 125.5 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED

TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH to 8.0

Transfer buffer: 10% (v/v) 10× Western salts, 15% (v/v) Methanol

Tris-glycine electrophoresis buffer: 25 mM Tris, 200 mM Glycine, 0.1% (w/v) SDS

Troglitazone stock solution (1000×): 10 mM Troglitazone in 100% DMSO

10× Western salts: 1.92 M Glycine, 250 mM Tris-HCl (pH 8.3), 0.02% (w/v) SDS

2YT medium: 16 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl

2.6 Software

Name	Sourse
CytoSoft 5.3	Millipore, Billerica, USA
Image J	http://rsbweb.nih.gov/ij/
AxioVision Software	Carl Zeiss MicroImaging GmbH, Göttingen
Limma (Linear Models for Microarray Data) package	http://bioconductor.org/
Primer designing tool NCBI/Primer-BLAST	www.ncbi.nlm.nih.gov/tools/primer-blast/
R statistical sofware	http://www.r-project.org/

3 METHODS

3.1 Cell culture

3.1.1 Culturing cells

H1299, HEL293 and U2OS cells were cultured in high glucose, phenol red free DMEM supplemented with 10% Bovine Growth Serum (BGS) and 1× Penicillin/Streptomycin at 37°C with 5% CO_2.

hFOB1.17 cells were routinely cultured in high glucose, phenol red free DMEM/F-12 supplemented with 10% Bovine Growth Serum (BGS) and 1× Penicillin/Streptomycin at the permissive temperature 33°C with 5% CO_2 supply. For induction of the differentiation to osteoblasts cells were cultured in the same medium but at 39°C (restrictive temperature) with 5% CO_2.

hMSCs were cultured in low glucose Minimum Essential Media (MEM) without glutamine and Phenol Red supplemented with 10% BGS, 1× Antibiotic-Antimycotic and 1µg/ml ciprofloxacin solution at 37°C with 5% CO_2. To induce adipocytic differentiation cells were cultured in presence of 15% BGS, 10 nM dexamethasone, 0.45 mM isobutyl-methyl-xanthine, 2 µM insulin, 10 µM Troglitazone, 1× Antibiotic-Antimycotic solution and 1µg/ml ciprofloxacin. For osteoblast differentiation the media contained 10% BGS, 10 nM dexamethasone, 10 mM β-glycerol phosphate (BGP), 0.2 mM ascorbic acid, 10 nM calcitriol, 1× Antibiotic-Antimycotic solution and 1µ/ml ciprofloxacin.

3.1.2 Plasmid DNA transfection

For transfections in 6-well format 2.4 µg of plasmid DNA were diluted in 200 µl of OptiMEM media. Separately, 8 µl of Lipofectamine^{TM} 2000 were diluted in 200 µl of OptiMEM. Both solutions were incubated for 5 min at RT followed by mixing and additional incubation for 20 min at RT. After that mix was added to the cells grown in media without antibiotics. Finally, after 4 h of transfection cells were washed with PBS and antibiotic-containing media was added.

3.1.3 Stable transfection with plasmid DNA

To create a stable cell line, H1299 or U2OS Tet-R cells were transfected with linearized plasmid DNA as described in 3.1.2. After the transfection cells were grown for 4 h in media

without antibiotics and for 2 d in media with antibiotics. Then cells were trypsinized, diluted, transferred on 15 cm plates and grown in selective media containing Hygromycin (200 µg/ml). For the induction of the trasgene expression cells were grown in the presence of 1µg/ml doxycycline.

3.1.4 Reverse transfection with siRNAs

siRNA transfections were performed with Lipofectamine™ RNAiMAX according to manufacturer's instructions. For transfections in 6-well plate format for each well 30 pmol of corresponding siRNAs were mixed with 500 µl of OptiMEM media. Then 5 µl of Lipofectamine™ RNAiMAX were added and the mix was incubated for 20 min at RT. Cells were washed twice with PBS, trypsinized and counted. 200,000 – 250,000 cells per well were used for transfections in 6-well format. Cells were transferred to the wells, diluted to the volume of 1.5 ml with MEM culturing media without Antibiotic-Antimycotic solution and mixed with siRNA master mix. After 24 h of transfection media was exchanged to MEM culturing media with Antibiotic-Antimycotic solution or corresponding differentiation media. For transfections in 10 cm format reagent amounts and cell number were scaled up four times.

3.1.5 Cell cycle analysis by BrdU and PI staining (flow cytometry)

Prior to fixation cells were grown in presence of BrdU for 1 h. After that cells were trypsinized, centrifuged for 7 min at 2200g (+4°C), washed with ice-cold PBS and resuspended in 500 µl of ice-cold PBS. Then cells were fixed by step-wise addition of 1.5 ml of ice-cold EtOH (500 µl per step) and incubated overnight at +4°C.

On the next day cells were centrifuged for 10 min at 2400g (+4°C) followed by removal of supernatant and rehydration by PBS addition. After that cells were centrifuged again, incubated with 2M HCl for 30 min at 37°C and washed with PBS-T. Then cells were incubated with FITC-BrdU antibody in PBS-T according to manufacturer's instructions. After washing with PBS pellets were resuspended in 500 µl of RNAse A solution and kept on ice. Before measurement 200 µl of cell suspension were stained with 6 µl of propidium iodide solution.

Cell cycle profiling was performed on Guava EasyCyte flow cytometry system using CytoSoft 5.3 software. Cells were filtered by size. Cell cycle stages were assigned by following principle:

PI positive, BrdU negative – G1 phase

PI positive, BrdU positive – S phase

PI positive with twice stronger intensity, BrdU positive – G2 phase

3.2 Chemical staining

3.2.1 Alkaline phosphatase staining

Staining for alkaline phosphatase activity was used to assess osteoblast differentiation efficiency. Staining was performed with alkaline phosphatase kit for leukocytes according to manufacturer's instructions. All steps were performed at RT. In brief, cells were washed with PBS and fixed for 30 s with Citrate fixing solution containing (for 98 ml): 66 ml acetone, 25 ml Citrate solution (Sigma-Aldrich Co., St. Louis, USA), 8 ml 37% formaldehyde. After fixation cells were washed three times with DI H_2O and incubated for 15 min with diazonium salt followed by rinsing with DI H_2O and drying. Diazonium salt preparation: 1 ml of FRV-Alkaline solution was mixed with 1 ml of sodium nitrate solution and incubated for 2 min. Then 45 ml of DI H_2O and 1 ml of Naphtol AS-BI Alkaline solution were added to the mix. Diazonium salt was prepared freshly before each staining. Pictures of the stained plates were taken under light microscope using 10x magnification.

3.2.2 Oil Red O staining

To examine adipocyte differentiation efficiency we visualized lipid drops with Oil Red O staining. All steps were performed at RT. Preparation of Oil Red O working solution: 3 parts of Oil Red O stock solution (300 mg/ml of Oil Red O powder in 99% isopropanol) were mixed with 2 parts of DI H_2O and incubated for 10 min followed by filtration. Cells were washed with PBS, fixed with 10% formaldehyde for 30 min and incubated with 60% isopropanol for 5 min. after that cells were stained with Oil Red O working solution for 5 min followed by rinsing with DI water and drying. Pictures of the stained plates were taken under light microscope using 10x magnification.

3.2.3 Quantification of staining

Chemical staining was quantified by Dr. Magali Hennion. Each picture used for quantification contained 500-1000 cells. The intensity and area of stained material was quantified by Image J software using the Threshold_Color plugin (http://www.dentistry.bham.ac.uk/landinig/software/software.html). For Oil Red O staining, the threshold was defined in RGB

color space (typically in the range of: R:150-255; G:0-140; B:0-140) and the staining-positive area of each picture was measured. For alkaline phosphatase staining, the threshold was defined in CIE Lab color space for each experiment (typically : L*:5-255; a*:125-255; b*:0-255) and the positive area of each picture was measured.

3.3 Molecular biology

3.3.1 Molecular cloning

H2B and K120R constructs used in this study were created by amplification of synthetic H2B sequence using primers listed in 2.3.4.4 and Phusion DNA polymerase. Obtained products and pcDNA5/TO vector were digested by restriction enzymes followed by ligation with T4 DNA ligase and clone selection on Ampicillin agar plates. For expansion constructs were transformed into E. coli XL1-Blue strain followed by culturing in Ampicillin-containing 2YT medium.

3.3.2 RNA isolation

RNA isolation was performed with QIAzol® reagent according to manufacturer's instructions. Cells were washed with PBS, lysed by addition of 1ml of QIAzol® reagent to each well (6-well format) and collected into 1.5 ml tubes. After addition of 200 µl of chloroform samples were vortexed and centrifuged at 10,000g for 20 min (4°C). After that the aqueous phase was collected and chloroform extraction was performed a second time followed by overnight isopropanol precipitation at -20°C. After that samples were centrifuged at maximal speed for 20 min (4°C), pellets were washed twice with 70% ethanol, dried on vacuum concentrator and re-dissolved in 50 µl of DEPC water. RNA concentration was measured using a NanoDrop.

3.3.3 cDNA synthesis

For DNA synthesis 1 µg of total RNA was mixed with 2 µl of 15 µM random primers and 4 µl of 2.5 mM dNTP mix and incubated 5 min at 70°C. After that 4 µl of reverse transcription master mix containing 2 µl 10× reaction Buffer, 10 units of RNAse Inhibitor, 25 units of Reverse Transcriptase and 1.625 µl of DEPC water were added to each sample. cDNA synthesis was performed at 42°C for 1 h followed by enzyme inactivation for 5 min at 95°C. Finally, samples were brought to 50 µl volume by DEPC water.

3.3.4 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed in final volume of 25 µl. For each reaction 1 µl of cDNA or ChIP DNA was mixed with 8.5 µl of ddH_2O, 1.5 µl of 5 µM primer mix and 14 µl of qRT-PCR mix containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 3 mM $MgCl_2$, 200 µM dNTPs, 20 U/ml Taq Polymerase, 0.25% Triton X-100, 1:80,000 SYBR Green I and 300 mM Trehalose.

The PCR reaction was performed using two-step protocol:

95 °C	2 min		
95 °C	15 s	}	40 cycles
60 °C	1 min		

The PCR reaction was followed by a melting curve recording from 70 °C to 95 °C with the plate read every 0.5 °C. All qRT-PCR samples were normalized to an internal reference gene (*HNRNPK*) and displayed relative to the control non-differentiated sample. Statistical analysis was done with ANOVA test.

3.3.5 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (Karpiuk et al., 2012). Briefly, cells were crosslinked by 1% formaldehyde in PBS for 1 min. The reaction was quenched by addition of 1 ml 1.25M glycine for 5 min. After that cell were washed with ice-cold PBS and scraped in Nelson Lysis Buffer with inhibitor cocktail. Obtained nuclei were centrifuged at 12,000g for 1 min (4°C), washed with Nelson lysis buffer and resuspended in 300 µl of Gomes Lysis buffer in the presence of 1% w/v SDS. Sonication was performed with Bioruptor at high power settings three times 10 min with 10s pulses followed by 10s pause. After that samples were pre-cleared with 100 µl of Sepharose 4B (50% slurry in Gomes Lysis Buffer) for 1h at 4°C, diluted, aliquoted, snap frozen in liquid nitrogen and stored at -80 C until further use.

One aliquot was taken for each antibody. Chromatin was diluted to 1 ml with Gomes Lysis Buffer without SDS and with inhibitor cocktail and an appropriate amount of antibody was added (see Materials section). Samples were incubated with the antibodies overnight at 4°C, then 30 µl of Protein-G Sepharose (50% slurry in Gomes Lysis Buffer with inhibitor cocktail) were added and incubated for another 2 h at 4°C. After centrifugation (2000g, 2 min, 4°C) beads were washed three times with Gomes Lysis Buffer with inhibitor cocktail and without

SDS, three times with Gomes Wash Buffer with inhibitor cocktail and twice with TE-buffer. Reverse crosslinking was performed by adding 100 µl of 10% (w/v) Chelex 100 slurry and incubating at 95 °C for 10 min. After that 2 µl of Proteinase K (20 µg/µl) were added to each sample, followed by the incubation at 55°C for 30 min. Proteinase K was inactivated by heating the samples to 95 °C for 10 min. After that samples were centrifuged at 12,000g for 1 min at 4°C and the supernatant containing DNA was used for quantitative real-time PCR. The background binding was determined by performing a ChIP with a non-specific IgG antibody. ChIP inputs preparation: 5 µl (10% relative to ChIPs) of chromatin extracts were precipitated by adding 100% EtOH and 1 µl of Pink Precipitant (5 mg/ml) and incubating overnight at -20°C. The pellets were washed twice with 70% EtOH, dried and processed with Chelex addition as described above for ChIP samples. ChIP samples were normalized to input DNA samples, and displayed as "% of input". Statistical analysis was done with ANOVA test.

3.3.6 Microarray

Total RNA for microarray experiments was isolated as described in 3.3.1 and Illumina whole-genome gene expression analysis using a human HT-12 v4 beadchip was performed by the Vancouver Prostate Centre Laboratory for Advanced Genome Analysis, Vancouver, Canada.
Gene expression data was analyzed by Frank Krammer, WG Statistical Bioinformatics, University of Goettingen, using log2 transformation and quantile normalization of expression levels (Bolstad et al., 2003). Background correction was applied according to the manufacturer's advice. In order to determine significant differences of expression levels between the different groups a moderated Student's *t*-test was computed on a gene-by-gene basis using the empirical Bayesian statistics in the 'limma' package (Smyth, 2004). P-values were adjusted for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) to avoid a high number of false positives and to stay below a false discovery rate of 5%. All analyses were performed using the free statistical software R (version 2.12.2) (RDev, 2011).

3.4 Protein biochemistry

3.4.1 SDS-PAGE

Protein samples were separated via sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Cells were washed with PBS and lysed in RIPA buffer containing inhibitor cocktail (1 mM Pefabloc, 1 ng/µl Aprotinin/Leupeptin, 10 mM BGP and 1 mM NEM). To separate chromatin-bound proteins chromatin was sheared by sonication for 15 s at 5% power using a Bandelin Sonoplus sonicator. After that protein samples were boiled in Laemmli Buffer for 5 min and subjected to SDS-PAGE. Stacking and resolving gels used in this study are described in Materials section. Proteins were separated in SDS running buffer at 25 mA.

3.4.2 Western blot analysis

After separation by SDS-PAGE proteins of interest were detected by Western blot analysis (Towbin *et al.*, 1979) using specific antibodies. Proteins were transferred on the nitrocellulose membrane at 100 V for 1 h. After that the membrane was washed in blocking solution (5% milk in PBS-T) for 30 min and incubated with primary antibodies in blocking solution overnight at 4°C. Subsequently, membranes were washed three times in PBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies in blocking solution for 1 h at RT. After that membranes were again washed three times with PBS-T and HRP signals were detected by X-ray films using enhanced chemoluminescence solution.

4 Results

In this study the role of monoubiquitinated H2B (H2Bub1) on the differentiation of human mesenchymal stem cells (hMSCs) was examined. As stem cells hMSCs have a high clinical potential because they can be obtained from the adult donor and undergo differentiation into various cell types. On the other side, these cells also participate in pathology development, for example during osteoporosis progression. Taken together these data show the importance of studying the regulation of hMSC differentiation and understanding the signaling that drives this regulation.

4.1 Establishing a "knockdown-overexpression" system for H2B

Most of the knowledge about H2Bub1 function comes from yeast studies utilizing mutant strains with mutation of K123 in H2B (Sun, 2002). Unfortunately, this approach cannot be utilized in mammal systems due to the high number of H2B genes (Marzluff, 2002). In the present study it was decided to use a "knockdown-overexpression" system to overcome this problem. In this system endogenous H2B is depleted with siRNAs and at the same time synthetic H2B, introduced via stable plasmid transfection, is overexpressed. By changing the codons of the H2B sequence it is possible to make the overexpressed protein resistant to siRNAs that target H2B. Exchange of amino acids that undergo post-translational modifications, e.g. lysine 120 that is subjected to monoubiquitination, allows to investigate their functions in more detail.

4.1.1. Overexpression of Flag-H2B

The H2B overexpression construct was based on pcDNA5/TO plasmid (Fig.5A). To differentiate from endogenous H2B (endo-H2B) a Flag-tag was added to N-terminus of the overexpressed protein. Two constructs were created: H2B with wild-type protein sequence (Flag-H2B) and mutant H2B that cannot undergo ubiquitination (K120R). To test the constructs H1299 cells were transiently transfected with the corresponding plasmids and Flag-H2B expression was examined by Western blot (Fig. 5B). Both constructs were properly expressed, however the amount of H2B expressed from the transgene was far lower than the amount of endogenous protein. Examination of H2Bub1 levels showed that Flag-H2B was successfully ubiquitinated, although in a far lower amount than endogenous H2B, whereas the K120R mutant was not ubiquitinated (Fig. 5C). Confocal immunofluorescence staining of

overexpressed proteins demonstrated that both of them correctly localize to the nucleus (Fig. 5D), suggesting that Flag-H2B and K120R proteins are incorporated into chromatin.

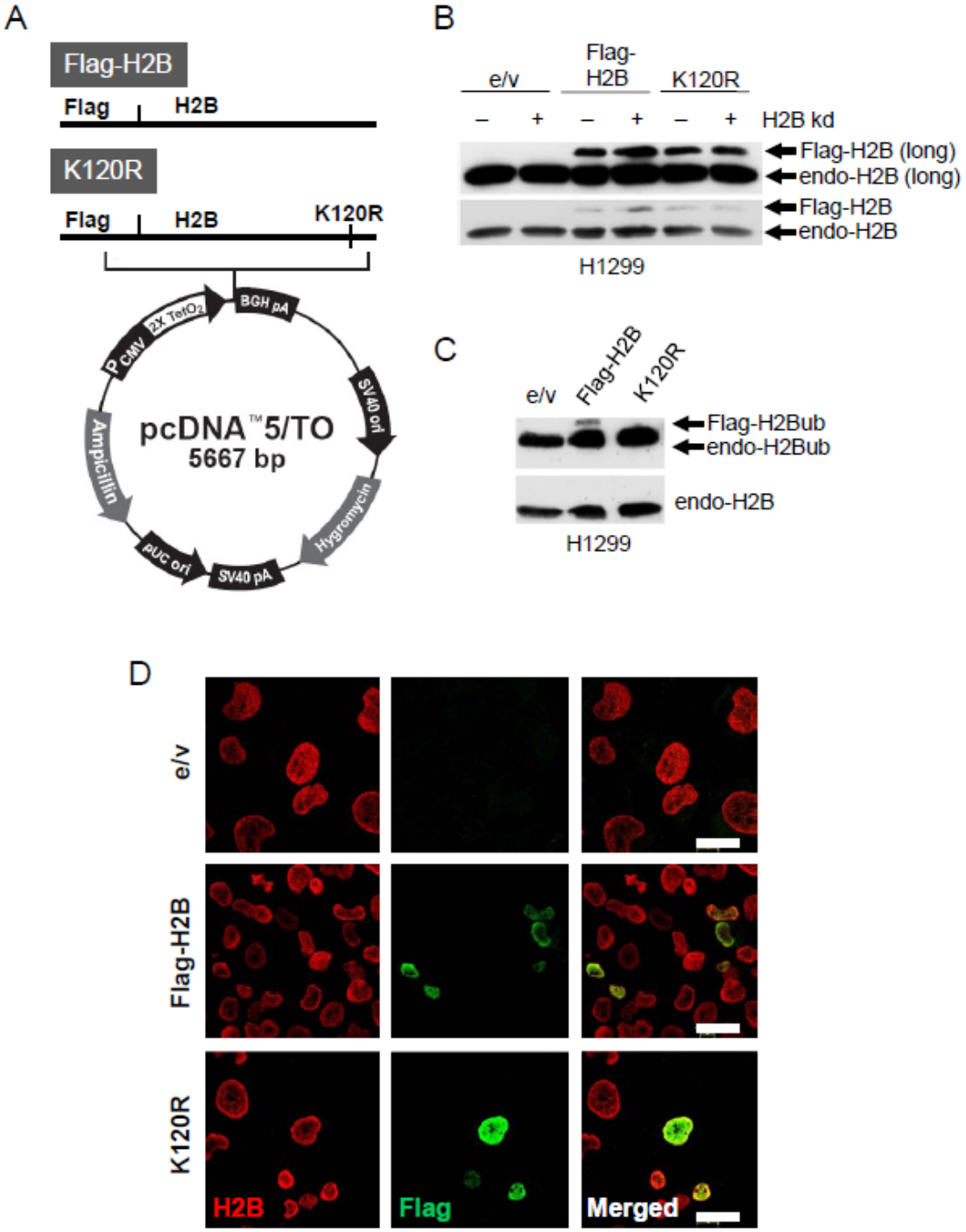

Fig. 5. H2B constructs are correctly expressed in H1299 cells.
(A) Scheme of Flag-H2B and K120R overexpressing constructs.
(B) H1299 cells were transiently transfected with Flag-H2B or K120R expressing constructs for 24h followed by H2B depletion for 48h. Protein lysates were collected and analyzed by Western blot using H2B antibodies. Different exposures of the same film are shown.
(C) Transient transfection of H1299 cells with H2B constructs was performed for 24h. Samples were analysed by Western blot with antibodies to H2Bub1 and H2B as a loading control.
(D) H1299 were transfected as in (C) and visualized by immunofluorescence using antibodies to H2B or Flag-tag. Scale bar corresponds to 20 µm.

4.1.2 H2B depletion leads to cell cycle arrest

The next step in creating a “knockdown-overexpression” for H2B was establishing an H2B knockdown in human cells. Due to nucleotide sequence dissimilarity between H2B genes it was not possible to target all of them with a low number of siRNAs, thus we focused on targeting individual H2B transcripts with siRNA. To establish efficient H2B knockdown, expression of different histone genes was examined in H1299 cells (Fig. 6A). It was observed that the amount of mRNA transcripts can vary up to s thousand fold between different H2B genes. The highest levels of transcription were observed for HIST1H2BK, HIST1H2BC, HIST1H2BD, HIST1H2BJ, HIST1H2BN, HIST1H2BE, HIST1H2BG and HIST1H2BO. Unfortunately, the commercially available siRNAs did not show the efficient targeting for most of the transcripts, so taking into account the expression levels and feasibility of targeting, the following genes were chosen for knockdown: HIST1H2BC, HIST1H2BG and HIST1H2BO. The sequences of chosen siRNAs also allowed targeting of other H2B genes. Transfection of H1299 cells with individual siRNA demonstrated a significant decrease in the expression of corresponding H2B genes for each of siRNAs (Fig. 6B) as well as for siRNA mix (Fig. 6C).

Examining the effect of knockdowns on global H2B level with Western blot is difficult due to huge amounts of produced protein. To overcome this problem the effect on cellular processes upon H2B depletion was examined. H1299 cells were transfected with siRNA mix containing siRNAs to HIST1H2BC, HIST1H2BG and HIST1H2BO for 48h. After that the cell cycle profile was monitored by flow cytometry using propidium iodide (PI) and Bromodeoxyuridine (BrdU) staining (Fig. 6D). Upon H2B knockdown cells synchronized in G1/S phase agreeing with the idea that H2B is synthesized at the end of G1 phase and without it cell is not able to proceed to S-phase. To test whether G1-arrest is a specific response of H1299 cells or it is a more general effect H2B knockdown was also performed in HEK293 cells (Fig. 6E), which demonstrated a similar and even more pronounced increase in the G1/early S fraction. Described observations suggest that knocking down of several H2B genes is sufficient to affect cellular metabolism and cause a G1-arrest or block S-phase entry.

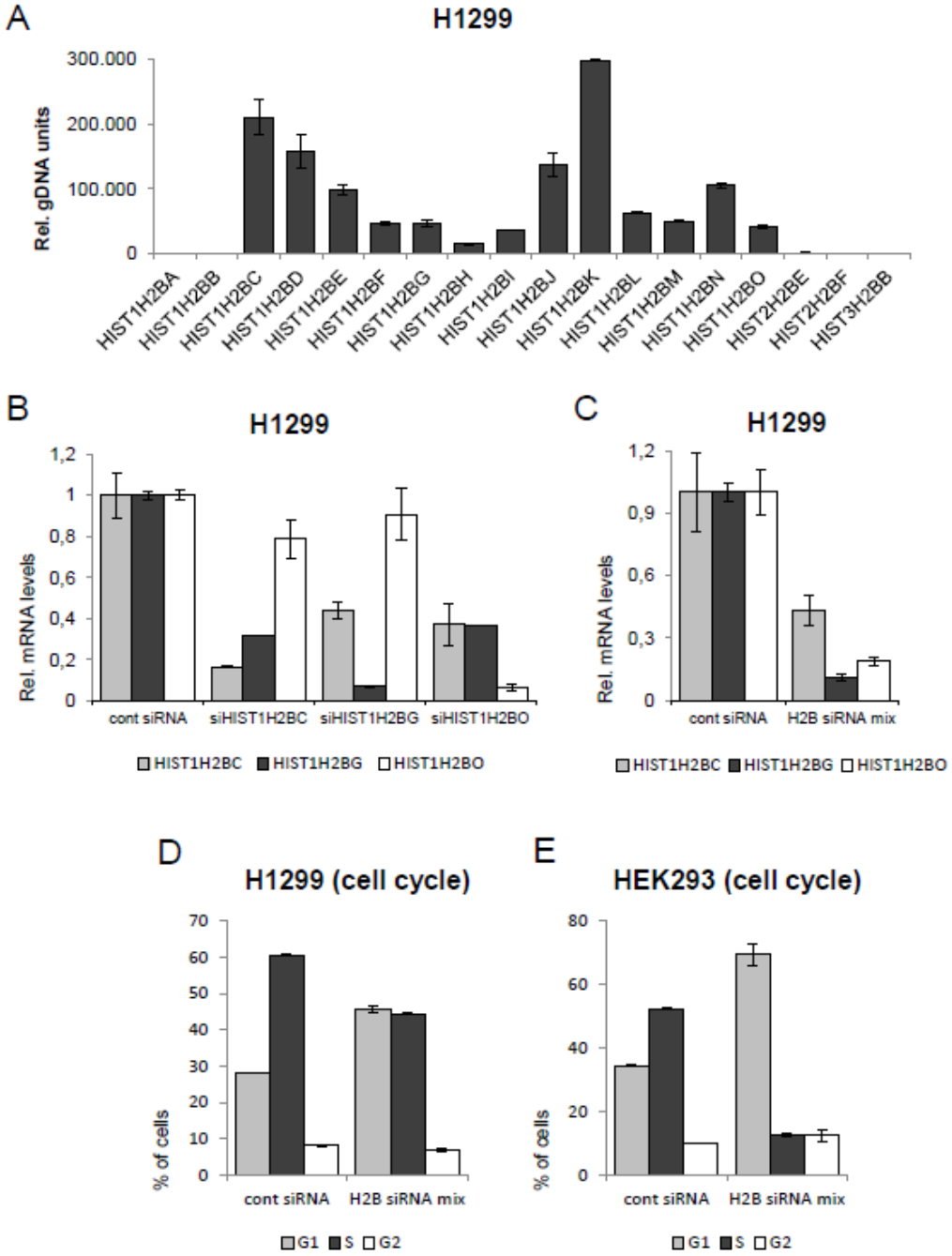

Fig. 6. H2B knockdown inhibits cell cycle progression.
(A) Expression of H2B genes in H1299 cells. RNA was isolated from H1299 cells followed by cDNA synthesis. Expression of H2B was analysed by qRT-PCR. Number of copies was calculated based on C(t) values of genomic DNA with known copy number and indicated as "Rel. gDNA units". Mean ±SD, n = 3.
(B, C) H1299 cells were transfected with control siRNA, individual siRNAs against HIST1H2BC, HIST1H2BG and HIST1H2BO (B) or a mix of them (C) for 48h. After that RNA was isolated followed by cDNA synthesis. Gene expression was examined by qRT-PCR and normalized to RPLP0 expression (indicated as "Rel. mRNA levels"). Mean ±SD, n = 3.
(D) H1299 cells were transfected with siRNA mix as in (C). After 48h of knockdown cells were incubated with BrdU for 1h followed by fixation, incubation with FITC-coupled anti-BrdU antibody and staining with PI. Cell cycle distribution was measured by flow cytometry. Mean ±SD, n = 3.
(E) HEK293 cells were transfected and analysed as in (D). Cell cycle distribution was measured by flow cytometry. Mean ±SD, n = 3.

4.1.3 “Knockdown - overexpression” approach is not applicable for human cells

After checking H2B overexpression and knockdown their combination was tested as an approach to study H2Bub1 function. H1299 were transiently transfected with Flag-H2B for 24h or K120 followed by H2B depletion for 48h (Fig 5B). Unfortunately, no significant depletion of endogenous H2B was observed. To examine if replacement of endo-H2B with Flag-H2B increases with longer overexpression or knockdowns H1299 were stably transfected with Flag-H2B or K120R (Fig. 7A). Two clones for each construct were analyzed but no significant increase in overexpressed H2B was observed. Depletion of endogenous H2B also did not enhance replacement of endo-H2B with transgene overexpression.

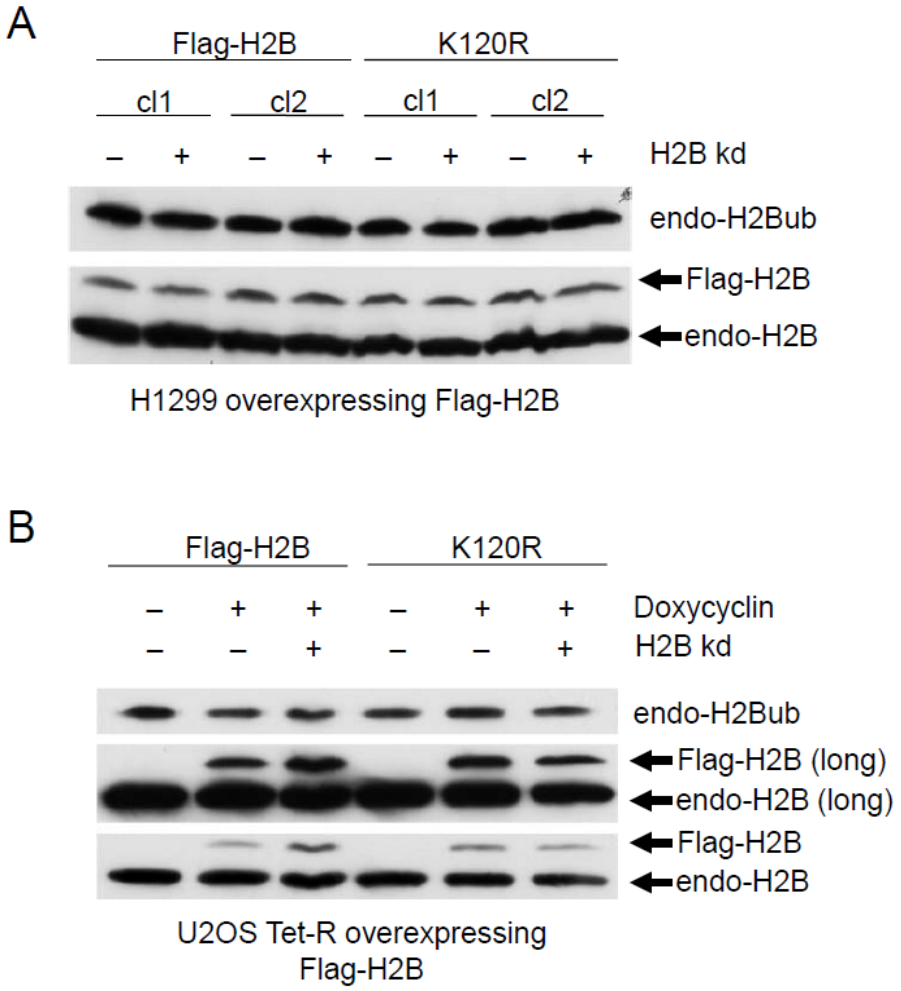

Fig.7. Optimizing Flag-H2B overexpression.
(A) H1299 cells were stably transfected with Flag-H2B or K120R construct. Clones were selected using Hygromycin-containing medium. Cells were transfected with mix of siRNAs against HIST1H2BC, HIST1H2BG and HIST1H2BO or control siRNA for 48h. Protein lysates were analyzed by Western blot with antibodies for H2Bub1 and H2B.
(B) U2OS cells expressing the Tet-R were stably transfected with Flag-H2B or K120R constructs and grown on Hygromycin. Cells were pre-treated with doxycyclin for 24h followed by H2B knock down as in (A) for 48h. Proteins were collected and analysed by Western blot using antibodies to H2Bub and H2B.

The next step was creation of stable cell lines with inducible Flag-H2B expression were created using U2OS cells expressing Tet-R (Monroe, 2003) since the inducible expression helps to avoid negative selection. To replace endo-H2B with overexpressed Flag-H2B or K120R U2OS Tet-R cells were treated with doxycyclin for 24h to induce transgene expression followed by H2B knockdown for 48h (Fig.7B). Unfortunately, this approach did not lead to increased expression of synthetic proteins and no effect of H2B knockdown was observed.

Analyzing all used approaches it can be concluded that overexpressed synthetic H2B is not able to replace endogenous protein. Increasing the overexpression to the cellular levels was technically possible neither with transient nor with stable construct expression. Knocking down some of H2B genes did not increase ratio of synthetic H2B to total H2B either. Summing up, H2Bub1 function is difficult to decipher by knockdown-overexpression system in mammalian cells.

4.2 Cellular differentiation is accompanied by elevated H2Bub1 levels

4.2.1 Levels of H2B monoubiquitination increase during differentiation of hMSCs

To assess the function of H2B monoubiquitination it was decided to examine the role of its ubiquitin ligases within a physiological cellular context. The main way to dissect H2Bub1 function in these conditions is depletion of one of the ubiquitinating enzymes - RNF20 or RNF40. Due to the fact that H2Bub1 involvement in transcription and DNA damage response is established, this project focused on H2Bub1 functions in other cellular processes. One of them is cellular differentiation, which was already shown to involve changes in other histone modifications (Asp, 2011; Yasui, 2011; Lindeman, 2010; Carrozza, 2003), but with no systematic data about H2Bub1 involvement.

In order to investigate the changes in H2Bub1 a differentiation system based on hMSCs was established. hMSCs immortalized with the expression of telomerase were obtained from Prof. M. Kassem, Odense University Hospital, Denmark. These cells originate from the bone marrow and can differntiate in the laboratory conditions into various cell types including osteoblasts, adipocytes and chondrocytes (Simonsen, 2002). Due to the long time required for chondrocyte differentiation it was not chosen for this study and only osteoblast and adipocyte differentiations were examined.

To differentiate hMSCs into osteoblasts they were treated with a set of chemicals including calcitriol (vitamin D, nuclear receptor ligand), dexamethasone (glucocorticoid receptor agonist), ascorbate (vitamin C, required for collagen synthesis) and β-glycerophosphate (required for mineralization of collagen matrix). As a readout for differentiation efficiency cells were chemically stained for Alkaline phosphatase (AP) activity. Alkaline phosphatase is a known marker of osteoblast differentiation and is required for mineralization. AP expressing cells can convert certain chemicals into colored products and become violet (Fig.8A). After 5 days of osteoblast differentiation hMSCs were stained for ALPL activity which confirmed its expression in osteoblasts but not in control cells (Fig. 8A).

After that, to confirm the differentiation of hMSCs the expression of osteoblast-specific genes was studied by qRT-PCR. After 5 days of differentiation hMSCs showed greatly elevated levels of ALPL and osteocalcin (BGLAP), a bone structural protein, comparing to undifferentiated cells (Fig.8B). Taken together these data demonstrate that hMSCs can be successfully differentiated into osteoblasts.

Finally, H2Bub1 levels increased during osteoblast differentiation (Fig. 8C) consistently with marker gene expression, which gives the first indication that H2Bub1 might play a role during differentiation progression.

To check whether the H2Bub1 increase is specific for osteoblasts adipocyte differentiation of hMSCs was also tested. To promote adipocyte differentiation hMSCs were treated with dexamethasone, insulin (inhibitor of lipolysis), isobutylmethylxantine (enhancer of cAMP signalling) and Troglitazone (agonist of PPARγ receptor). To monitor the differentiation cells were stained with Oil Red O which specifically stains accumulated lipid drops in red. After 5 days of treatment hMSCs successfully accumulated lipid drops (Fig. 9A). This finding was confirmed by staining cells with Nile Red – a red fluorescent dye that also marks lipid drops (Fig. 9B). Moreover, the expression of adipocyte-specific genes during differentiation was examined with qRT-PCR (Fig. 9C). Expression of the major adipocyte regulator Peroxisome proliferator-activated receptor gamma (*PPARG*) was highly upregulated after 5 days of differentiation, as well as expression of lipoprotein lipase (*LPL*) that produces lipoproteins.

After confirmation of efficient adipocyte differentiation H2Bub1 levels were examined in hMSCs (Fig. 9D). H2Bub1 was elevated in adipocytes similarly as in osteoblasts suggesting

that the observed increase is not lineage specific and might play a general role in differentiation.

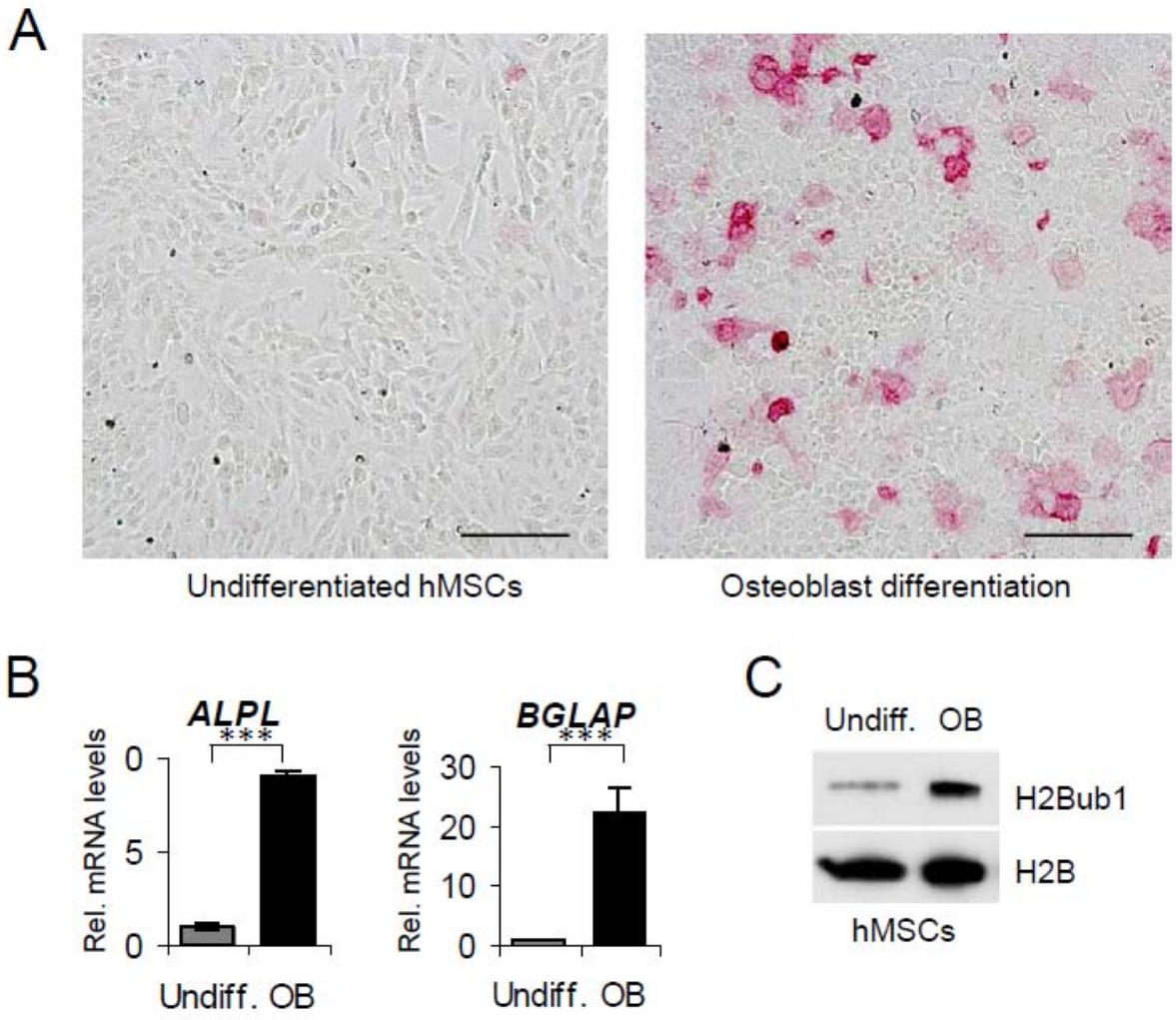

Fig. 8. Differentiation of hMSCs into osteoblasts is accompanied by an increase in H2Bub1 levels.
(A) hMSCs were differentiated in osteoblast media for 5 days, followed by fixation and staining for ALPL activity. Scale bar corresponds to 200 μm.
(B) hMSCs were differentiated as in (A) followed by RNA isolation and cDNA synthesis. Gene expression was examined by qRT-PCR and normalized to hnRNPK expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts. Mean ±SD, n = 3.
(C) Cells were differentiated as in (A) and analyzed by Western blot for H2Bub1 levels. H2B serves as a loading control.

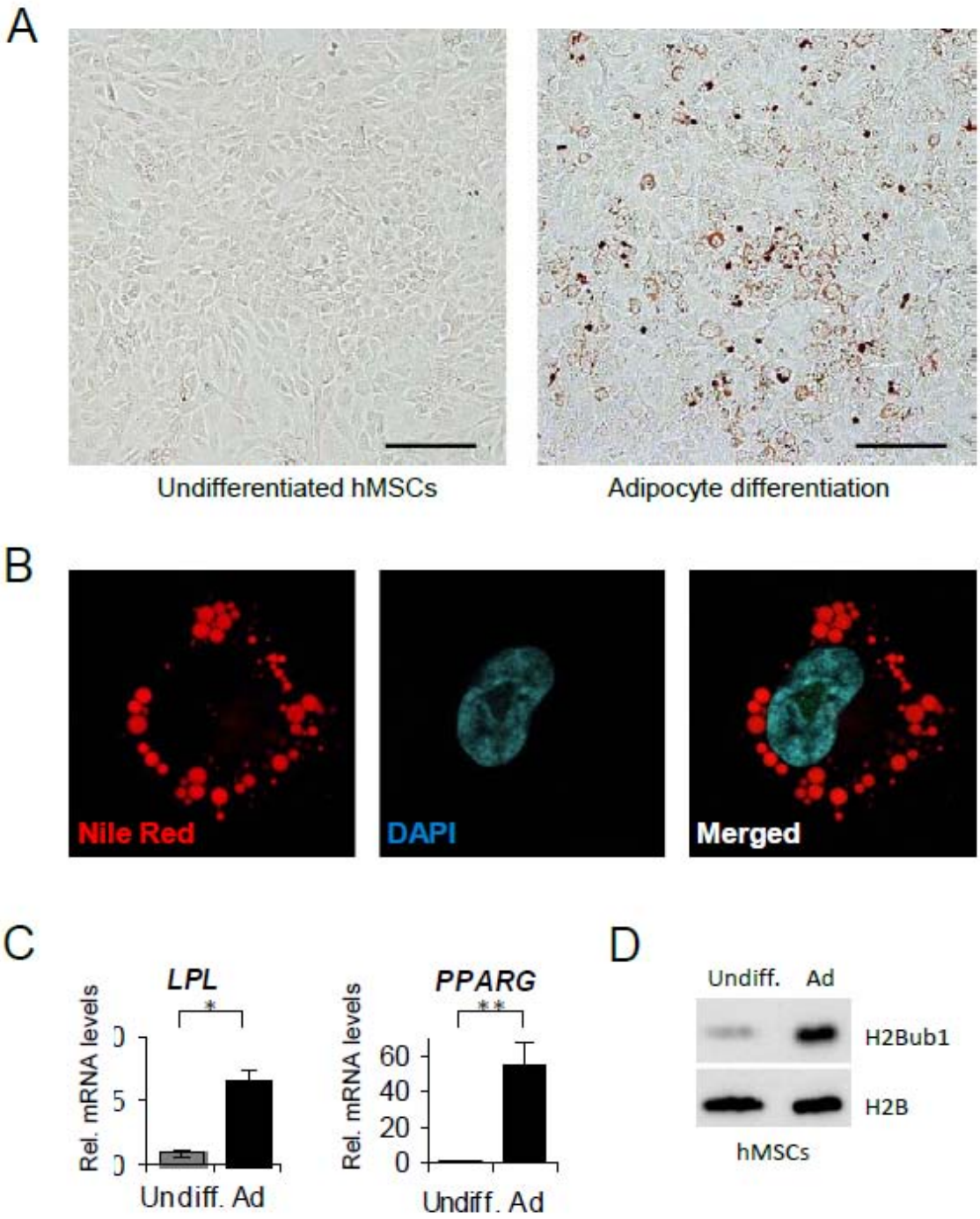

Fig. 9. H2Bub1 levels also increase during adipocyte differentiation of hMSCs.
(A) hMSCs were differentiated into adipocytes for 5 days, fixed and stained with Oil Red O for lipid droplet visualisation. Scale bar corresponds to 200 μm.
(B) hMSCs were differentiated as in (A) and stained with fluorescent dye Nile Red for lipid drops visualization. Cellular nuclei were stained with DAPI.
(C) Cells were differentiated as in (A), RNA was isolated and cDNA was analysed by qRT-PCR. Gene expression was normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, Ad – adipocytes. Mean ±SD, n = 3.
(D) hMSCs were differentiated as in (A) followed by Western blot for H2Bub1 levels. H2B serves as a loading control.

4.2.2 H2Bub1 is also elevated during hFOB differentiation into osteoblasts

To further investigate how general the increase of H2Bub1 during differentiation is it was decided to establish differentiation system with another cell line. The human fetal osteoblast cell line hFOB1.17 was chosen due to their ability to differentiate into mature osteoblasts. hFOB1.17 (obtained from Thomas Spelsberg, Mayo Clinic, USA) express a temperature-sensitive T-antigen that maintains them in a non-differentiated proliferative state at 33ºC. Growing cells under higher temperature (39°C) inactivates the T-antigen and promotes osteoblast differentiation.

Firstly, hFOB1.17 cells were examined with qRT-PCR for differentiation efficiency. After 7d of differentiation the expression of *ALPL* and *BGLAP* was significantly increased (Fig. 10A) demonstrating osteoblast commitment of FOB1.14. H2Bub1 levels were increased upon differentiation (Fig.10B) as observed in hMSCs. These observations suggest that H2Bub1 increase during differentiation is not cell type-specific but is a rather general feature of differentiation.

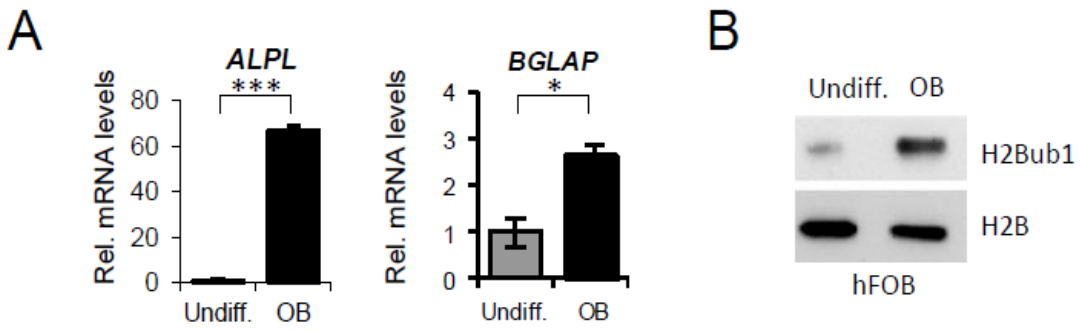

Fig. 10. hFOBs accumulate H2Bub1 during differentiation similarly to hMSCs.
(A) hFOB1.17 cells were differentiated for 7 days. cDNA was analyzed by qRT-PCR. Gene expression was normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts. Mean ±SD, n = 3.
(B) hFOB1.17 were differentiated as in (A) and examined for H2Bub1 levels by Western blot. H2B serves as a loading control.

These results correlate with recently published observations that the increase in H2Bub1 during differentiation is conserved among tissues and species (Fuchs, 2012; Karpiuk, 2012). It was demonstrated that differentiation of the mouse stem cells as well as maturation of mouse oligodendrocytes and neurons were also accompanied with the increase in H2Bub1.

4.3 Inhibition of signaling that leads to H2Bub1 accumulation results in decreased differentiation of hMSCs

4.3.1 RNF40 knock down inhibits differentiation of hMSCs

In order to understand the role of H2Bub1 increase during hMSCs differentiation the next step was to examine the effect of depletion of this modification on differentiation. Unfortunately, due to the difficulty encountered in replacing endogenous human H2B it was not possible to generate an H2Bub1 mutant in human cells. However, knockdown of RNF40 which is an E3 ubiquitin ligase for H2B effectively depletes H2Bub1 levels in the cell and helps to overcome this problem.

To deplete H2Bub1 hMSCs were transfected with RNF40 siRNA 24h before addition of differentiation media. After 3 days of RNF40 knock down and, correspondingly, after 2 days of either osteoblast or adipocyte differentiation, hMSCs showed a significant decrease in RNF40 protein levels (Fig. 11A) as well as mRNA levels, examined by qRT-PCR (Fig. 11B). RNF40 depletion in differentiated and control cells resulted in decreased H2Bub1 levels comparing to the cells transfected with control siRNA (Fig.11A). The impact of H2Bub1 depletion on differentiation was examined with chemical staining. After 5 days of differentiation control hMSCs demonstrated elevated AP-activity or lipid droplet accumulation and those effects were greatly decreased upon RNF40 depletion (Fig. 12A, B). Quantification analysis of stainings showed a significant decrease in the amount of differentiated cells upon RNF40 knockdown (Fig. 12C, D) suggesting that H2Bub1 is required for differentiation and its accumulation requires RNF40 activity.

To confirm this finding on the transcriptional level the effect of RNF40 knockdown on gene expression was examined by qRT-PCR analysis. Upon RNF40 depletion the expression of osteoblast-specific genes *ALPL*, *BGLAP* and *G6PD* was significantly decreased compared to differentiated cells without RNF40 knockdown (Fig. 13A). The same effect was observed for adipocyte marker genes *PDK4*, *PPARG* and *RASD1* (Fig. 13B) strengthening the data obtained from the staining studies. However, it has to be mentioned that some of the known regulators of differentiation did not change the expression upon RNF40 knockdown. For example, expression of osteoblast transcription factor *RUNX2* was not affected by RNF40 knockdown (Fig. 13C) as well as adipocyte transcription factor *CEBPB*. These observations suggest that RNF40 knockdown differentially influences gene expression.

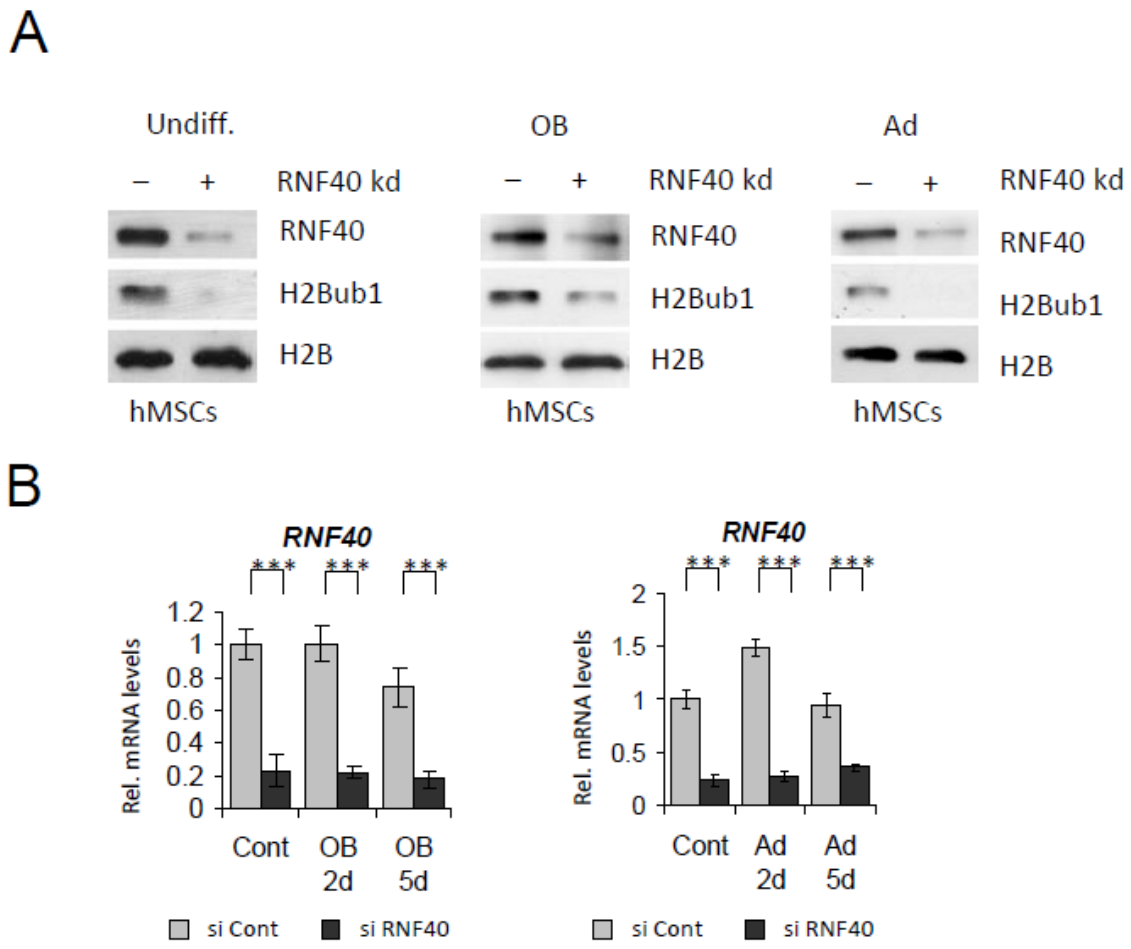

Fig. 11. Knockdown of RNF40 decreases H2Bub1 levels in hMSCs.
(A) hMSCs were transfected with siRNAs to RNF40 or control siRNA for 24h and differentiated into osteoblasts (OB), adipocytes (Ad) or remained undifferentiated (Undiff.) for 48h. Protein lysates were analyzed by Western blot with antibodies to RNF40, H2Bub1and H2B as a loading control.
(B) hMSCs were transfected with siRNAs to RNF40 or control siRNA for 24h followed by osteoblast or adipocyte differentiation for 2d or 5d. RNA isolation was followed by cDNA synthesis and gene expression analyis by qRT-PCR. Gene expression was normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Cont – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean ±SD, n = 3.

4.3.2 RNF20 depletion inhibits differentiation of hMSCs similarly to RNF40 knockdown

H2B monoubiqitination is performed by the RNF20/RNF40 heterodimer. Depletion of one of its components effectively decreases amount of H2Bub1. In this study RNF40 knockdown inhibits differentiation of hMSCs which suggests differentiation to be dependent on H2Bub1 levels. However, there is a possibility that RNF40 also acts independently of RNF20 and has several substrates. Thus its depletion leads to decreased ubiqitination of another protein which in its turn inhibits differentiation making the process H2Bub1-independent. To rule out this hypothesis the effect of RNF20 depletion was examined in differentiating hMSCs.

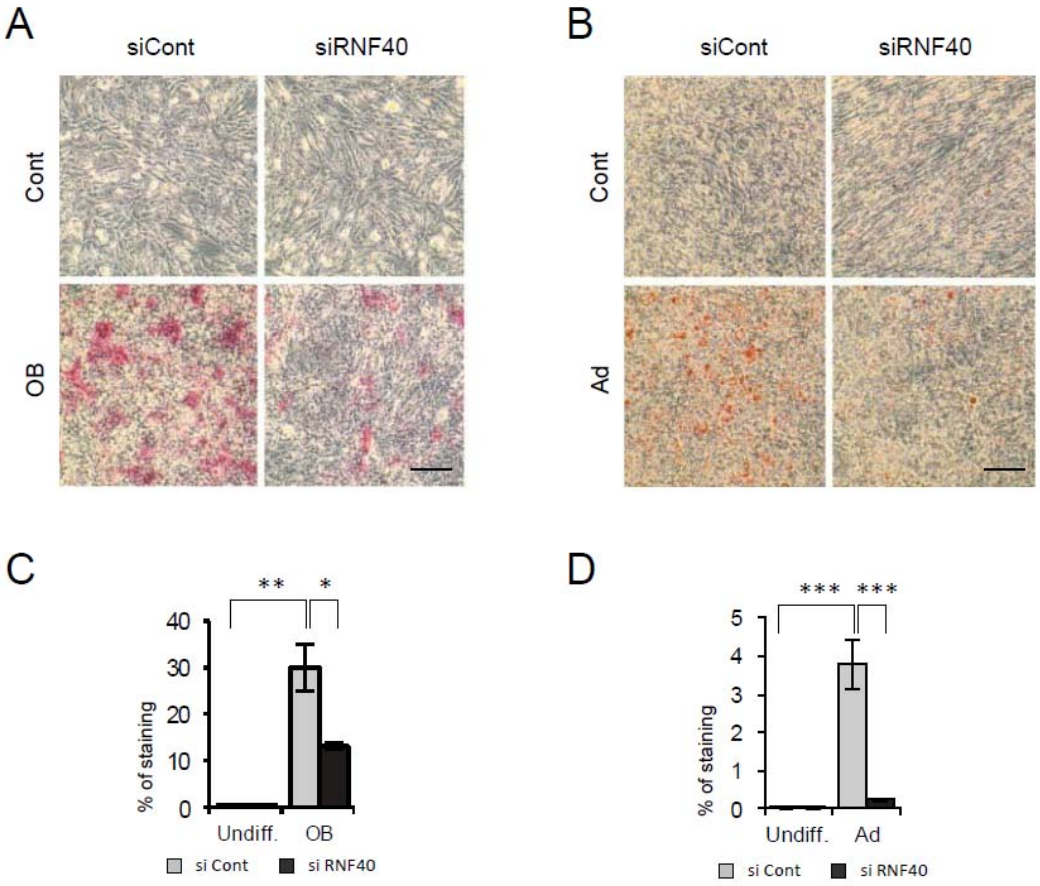

Fig. 12. RNF40 depletion results in decreased differentiation of hMSCs.
(A, B) Knockdown of RNF40 was performed in hMSCs for 24h followed by osteoblast (A) or adipocyte (B) differentiation for 5d. After that cells were fixed and stained for ALPL activity (A) or lipid drop accumulation (B). Scale bar corresponds to 200 μm.
(C, D) Quantification of ALPL staining (C) from Fig. 5A and lipid drop staining (D) from Fig. 5B. Quantification was done my measuring the area of stained regions with ImageJ software. Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean ±SD, n = 3.

Knockdown of RNF20 in both osteoblasts and adipocytes resulted in a strong decrease of H2Bub1 levels (Fig. 14A, B) confirming that RNF20 is an essential component of the H2B E3-ligase complex. Gene expression studies confirmed a significant decrease in RNF20 mRNA levels (Fig. 14C, D) which resulted in downregulation of osteoblast-and adipocyte-specific genes compared to differentiated hMSCs transfected with control siRNA (Fig. 14C, D). These observations demonstrate that RNF40 or RNF20 has a similar effect on the differentiation of hMSCs and suggests that the monoubiquitination of H2B is required for the proper differentiation progression. However, that does not completely exclude the possibility that RNF20 and RNF40 ubiquitinate an another target together and this target promotes differentiation independently of H2Bub1.

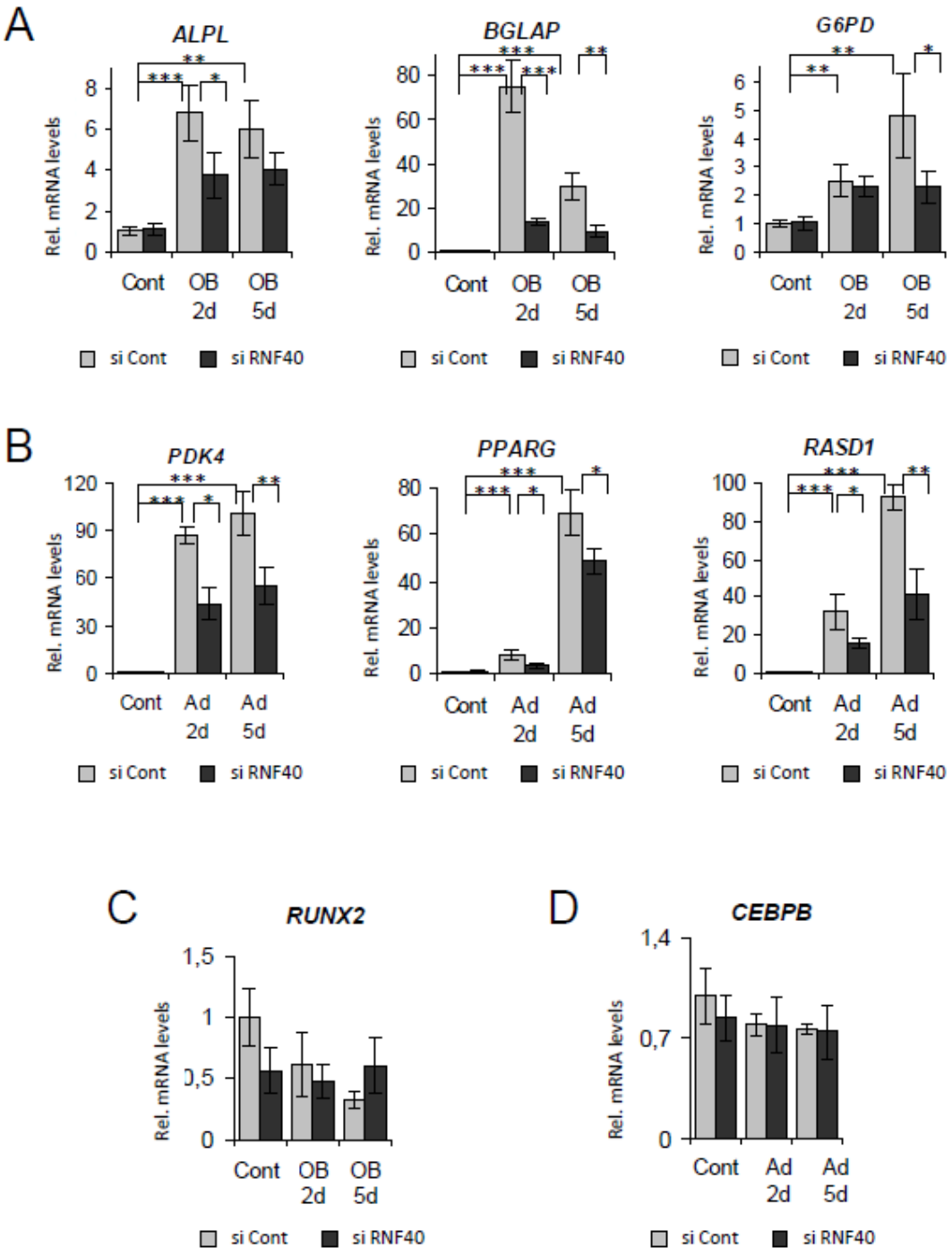

Fig. 13. Expression of differentiation-dependent genes is downregulated upon RNF40 knock down.

(A) hMSC were transfected with siRNAs to RNF40 or control siRNA for 24h and differentiated into osteoblasts for 48h. Marker gene expression was analyzed by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Cont – undifferentiated hMSCs, OB – osteoblasts. Mean ±SD, n = 3.

(B) hMSCs were transfected as in (A) and differentiated into adipocytes (Ad) for 48h. Adipocyte specific gene expression was analyzed as in (A) Cont – undifferentiated hMSCs, Ad – adipocytes. Mean ±SD, n = 3.

(C, D) hMSCs were transfected as in (A) and differentiated to osteoblasts (C) or adipocytes (D) for 48h. Gene expression analysis was performed as in (A). Cont – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean ±SD, n = 3.

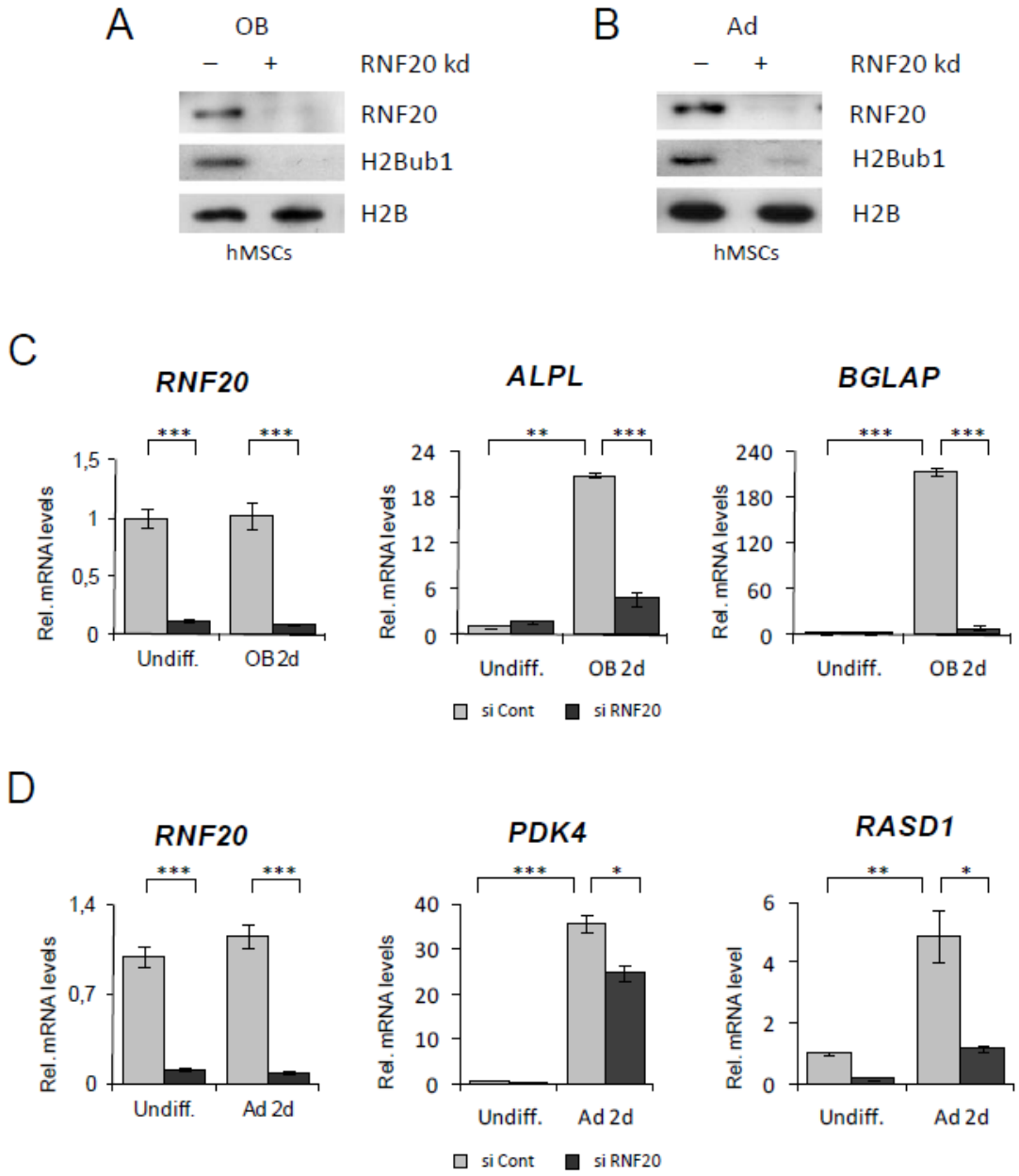

Fig. 14. RNF20 depletion inhibits differentiation similarly to RNF40 knockdown.
(A, B) RNF20 knockdown was performed in hMSCs for 24h followed by osteoblast (A) or adipocyte (B) differentiation for 2d. Protein samples were obtained and analyzed by Western blot with antibodies to RNF20, H2Bub1 and H2B as a loading control.
(C, D) hMSCs were transfected and differentiated as in (A). Gene expression was studied by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean ±SD, n = 3.

4.3.3 Depletion of RNF40 results in the transcriptome-wide changes in differentiating hMSCs

As it was mentioned previously it is proposed that H2Bub1 is required for transcriptional elongation and its depletion can result in altered gene expression. According to this, the

inhibitory effect of RNF40 knockdown on hMSCs differentiation could be driven by a general effect on transcription rather than by gene-specific effects. To differentiate between the described possibilities, the transcriptional profile of the hMSCs was investigated by microarray analysis. To conduct the studies hMSCs were differentiated into osteoblasts or adipocytes in the presence or absence of RNF40 knockdown. Cells were differentiated for 2d and 5d to examine the expression of early and late-induced differentiation genes. mRNA levels were normalized to gene expression in undifferentiated hMSCs transfected with control siRNAs.

First of all, the effect of RNF40 knockdown on undifferentiated cells was examined. Interestingly, microarray data showed that only 5 genes were downregulated more than 50% (-1.5 fold) upon RNF40 depletion (Table 1, Fig. 15A) indicating that the maintenance of the basal hMSC transcriptional profile occurs in an RNF40-independent manner. These results help to rule out the hypothesis of general transcription failure upon RNF40 knockdown confirming that its influence is differentiation specific.

Table 1. Transcriptome-wide changes during hMSC differentiation.
Gene expression was normalized to expression profile of undifferentiated hMSCs transfected with control siRNAs. Cut-off: upregulated genes – +1.5 fold above control expression, downregulated genes – - 1.5 fold of control expression. q-values < 0.05.

	Upregulated >+1.5 fold	Downregulated <-1.5 fold
RNF40 kd to Undiff.	**6**	**5**
OB 2d to Undiff.	**105**	**67**
OB 5d to Undiff.	**747**	**194**
Ad 2d to Undiff.	**457**	**122**
Ad 5d to Undiff.	**803**	**237**

Both osteoblast and adipocyte differentiation resulted in increased transcription of hundreds of genes with the largest number of genes upregulated at 5 days of differentiation (Table 1). Heat maps composed from these data also confirm massive transcriptional changes during differentiation (Fig. 15A).

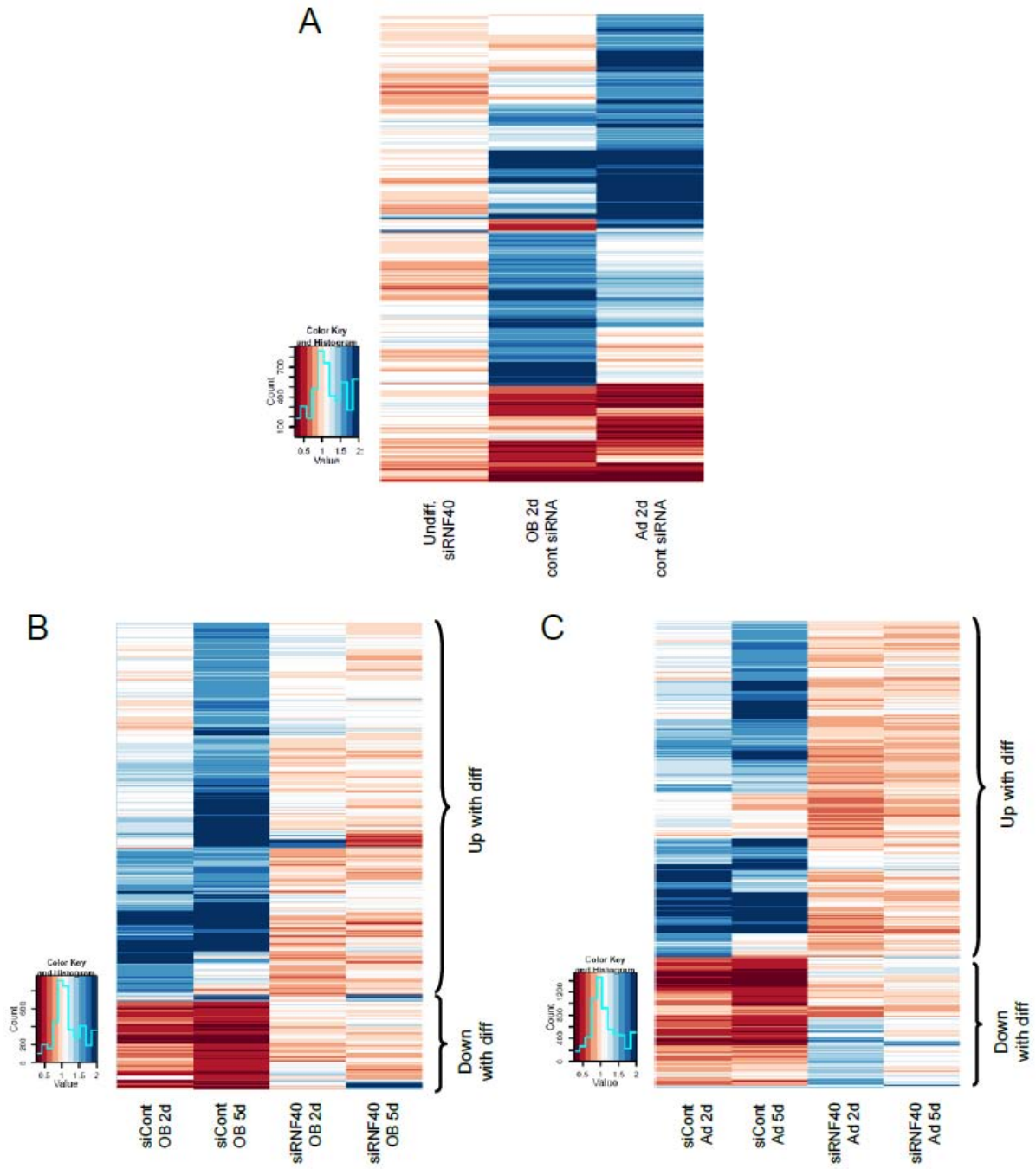

Fig. 15. The decrease in differentiation-induced gene expression upon RNF40 knock down is transcriptome-wide.

(A) hMSCs were transfected with siRNAs to RNF40 or control siRNAs and differentiated into osteoblasts or adipocytes for 2 days. mRNA was harvested and used for microarray studies. Expression values were normalized to the expression of the corresponding gene in undifferentiated hMSCs transfected with control siRNA. Heat maps were composed of statistically significant (q-values < 0.05) up- (blue) or down- (red) regulated genes with the cut-off of –1,5 and +1,5 fold change respectively. Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean values, n = 3.

(B, C) Cells were transfected as in (A) and differentiated into osteoblasts (B) or adipocytes (C) for 2 days or 5 days. mRNA was used for microarray studies. Expression values were normalized to the expression of the corresponding gene in undifferentiated hMSCs transfected with control siRNA. Heat maps were composed of statistically significant (q-values < 0.05) up- (blue) or down- (red) regulated

genes with the cut-off of –1,5 and +1,5 fold change respectively. Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean values, n = 3.

Next, the effect of RNF40 depletion on differentiation-induced transcription was examined (Fig. 15B, C). Heat-maps demonstrate that upregulated genes (blue) in both differentiations become repressed upon RNF40 knockdown. This effect is observed for early as well as late differentiation-induced genes. Moreover, genes that are repressed during differentiation (red) lose their repression upon RNF40 knockdown suggesting that RNF40 depletion reverses the action of differentiation signal independent of whether the individual gene is activated or repressed.

Concluding from the described results, RNF40 knockdown (which leads to H2Bub depletion) causes transcriptome-wide changes in differentiating cells while having almost no effect on undifferentiated hMSCs. Upon RNF40 depletion, differentiation-activated genes become inhibited and inhibited genes increase their transcription suggesting that absence of H2Bub1 reverses the effect of the differentiation signal. Moreover, RNF40 knockdown does not lead to a switch in differentiation but is rather equally suppresses both osteoblast and adipocyte commitment. All these observations suggest that H2Bub1 is essential for activation of differentiation-driven transcription in hMSCs.

4.3.4 CDK9 together with RNF40 regulates H2Bub1 accumulation during hMSC differentiation

In the knockdown experiments described above we observed that the increase in H2Bub1 requires RNF20/RNF40 activity. However, it remains unknown which upstream signals lead to the accumulation of H2Bub1. Previously it was shown (Pirngruber, 2009) that H2Bub1 can be regulated by CDK9, a kinase required for RNAPII phosphorylation at Ser2 that allows elongation of the transcript (Marshall, 1996). It was observed that upon CDK9 depletion levels of H2Bub1 also decrease. Correspondingly, CDK9 overexpression leads to H2Bub1 accumulation. Taking into account these observations it was decided to examine if CDK9 has the same effect on H2Bub1 in differentiation conditions.

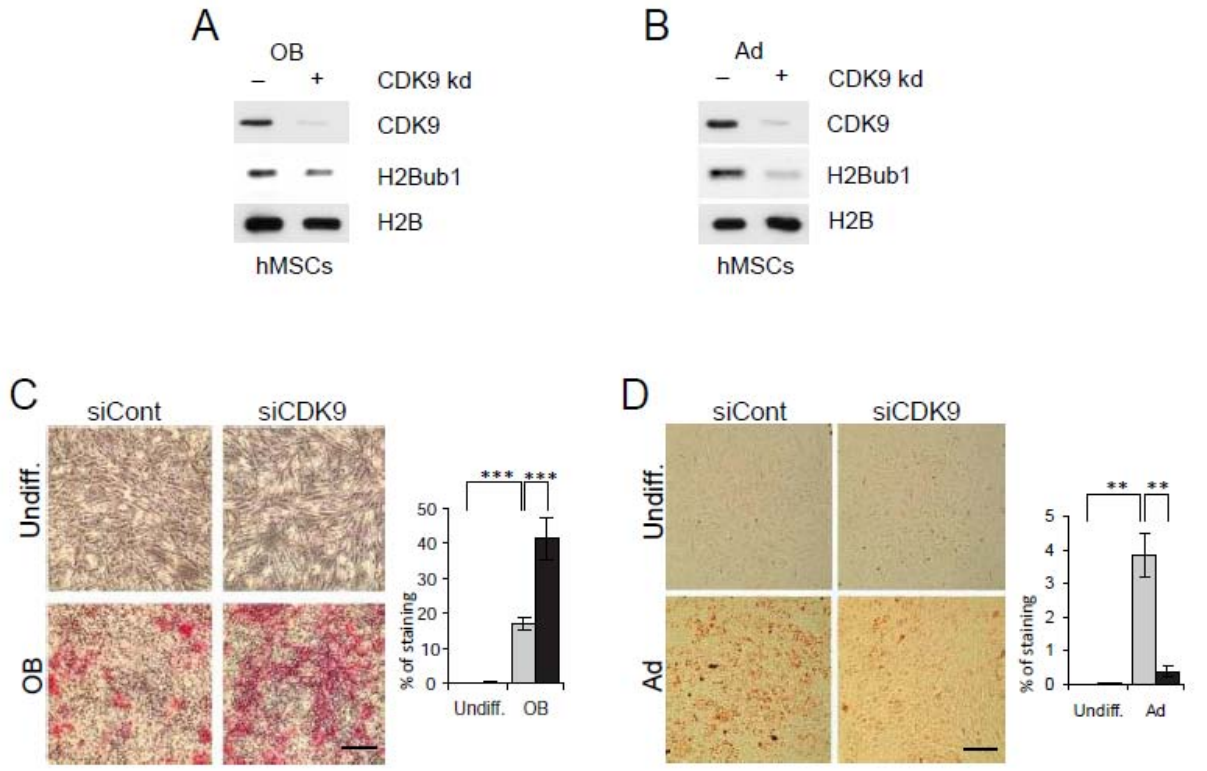

Fig. 16. CDK9 depletion results in decreased H2Bub1 levels in differentiated hMSCs.
(A, B) hMSCs were transfected with siRNAs to CDK9 or control siRNAs for 24h followed by osteoblast (A) or adipocyte (B) differentiation for 2d. Protein lysates were analyzed by Western blot for CDK9, H2Bub1 and H2B as a loading control.
(C, D) hMSCs were transfected as in (A) and differentiated to osteoblasts (C) or adipocytes (D) for 5d. Next, cells were fixed and stained for AP activity (C) or lipid drop accumulation (D). Scale bar corresponds to 200 μm. Quantification of AP staining (C) and lipid drop staining (D) was done by measuring the area of stained regions with ImageJ software. Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean ±SD, n = 3.

To answer this question CDK9 knockdown was performed in hMSCs followed by adipocyte or osteoblast differentiation. Western blot analysis showed that CDK9 depletion decreases H2Bub1 levels in both osteoblasts and adipocytes (Fig. 16A, B) concluding that H2Bub1 dependence on CDK9 is a general effect which can be observed in various cell lines. The next step was to investigate the role of CDK9 in differentiation using chemical staining of hMSCs differentiated for 5 following CDK9 knockdown (Fig. 16C, D). Oil Red O staining of adipocytes (Fig. 16D) showed a significant decrease in lipid droplet number in differentiated hMSCs with CDK9 knockdown compared to differentiated hMSCs transfected with control siRNA. This data is consistent with previous observations upon RNF40 knockdown and suggests that CDK9 together with RNF40 regulates H2Bub1 accumulation. However, AP

staining showed the opposite results. Upon CDK9 depletion hMSCs induced to differentiate to osteoblasts exhibited higher AP activity in comparison to control differentiated cells. The observed inconsistency with adipocyte differentiation may potentially be explained by AP regulation. This enzyme is not osteoblast-specific, it is also expressed in kidney and liver and can be activated by various stimuli. Moreover, *ALPL* expression is an established marker of ES cells. If CDK9 knockdown prevents hMSC differentiation it may bring them to an even more undifferentiated state resulting in the expression of stem cell markers like *ALPL*. To confirm this hypothesis it is important to investigate the expression of classical stem cell markers like NANOG or OCT3/4 upon CDK9 depletion in the cells grown in osteoblast differentiating media.

To further investigate the effect of CDK9 depletion the expression of differentiation markers was examined by qRT-PCR. Gene expression studies showed a significant decrease in CDK9 mRNA level upon knockdown in both osteoblasts and adipocytes (Fig. 17A, B). Osteoblasts with CDK9 knockdown showed increased levels of *ALPL* expression (Fig. 17A) confirming the results of AP staining. In contrast, the expression of the other osteoblast markers, *BGLAP* and *G6PD*, was greatly decreased in osteoblasts upon CDK9 depletion suggesting that loss of CDK9 has rather inhibitory then activating effect on osteoblast differentiation. All studied adipocyte genes (*PDK4*, *PPARG*, *RASD1*) decreased their expression upon CDK9 depletion compared to control differentiated cells (Fig, 17B). These observations confirm the data obtained with chemical staining that CDK9 knockdown suppresses adipocyte differentiation. On the other hand this effect can also be explained by the requirement of CDK9 as an elongation-promoting enzyme for RNF20 or RNF40 transcription. However qRT-PCR studies showed no significant difference in RNF40 expression in either of differentiations upon CDK9 knockdown and a slightly elevated expression of RNF20 in osteoblasts compared to control differentiated cells (Fig. 17C, D). Those results indicate that CDK9 participates in differentiation as a regulator of H2Bub1 accumulation. Moreover, it supports the previously described model of CDK9 regulating H2Bub1 deposition possibly by performing recruitment of the RNF20/40 complex.

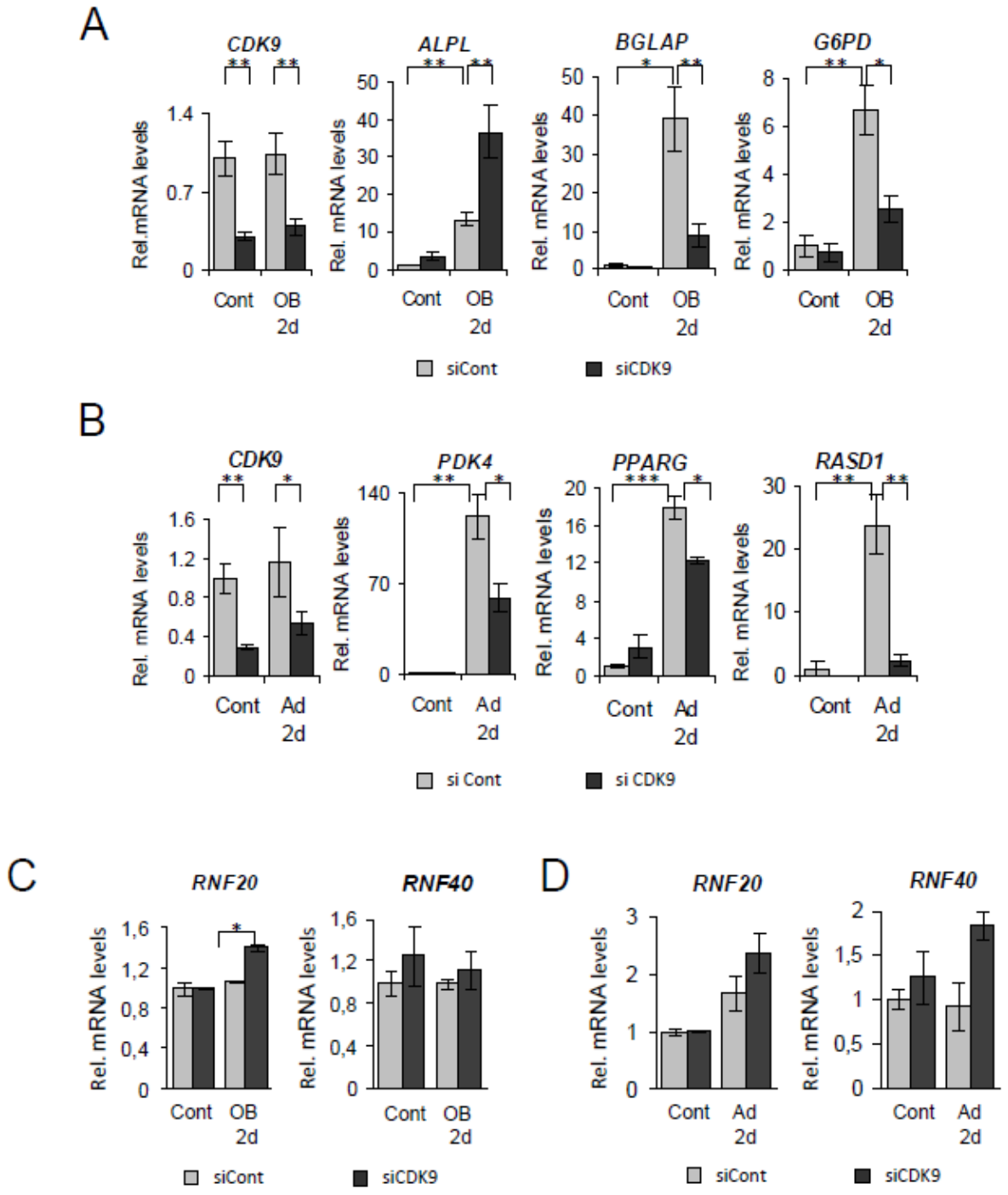

Fig. 17. Knockdown of CDK9 results in lower expression of differentiation markers.
(A, C) hMSCs were transfected with siRNA to CDK9 or control siRNA for 24h followed by osteoblast differentiation for 2d. Gene expression was analyzed by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts, Mean ±SD, n = 3.
(B, D) hMSCs were transfected as in (A) followed by adipocyte differentiation for 2d. Gene expression was studied by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, Ad - adipocytes. Mean ±SD, n = 3.

4.3.5 WAC mediates crosstalk between CDK9 and RNF40

Despite the fact that CDK9 and RNF40 participate in the regulation of H2Bub1 levels the mechanism of their interaction remains unclear. The main difficulty in modeling such a mechanism is the differences in CDK9 and RNF40 location. CDK9 is recruited to the poised RNA polymerase II and travels with it along the gene, when RNF20/40 complex is directly recruited to the histones downstream of TSS. The recently described WW domain containing adaptor with coiled-coil (WAC) is likely to be a "missing link" in the CDK9 and RNF20/RNF40 interaction. This protein interacts with Ser2P on the CTD of RNAPII, which accumulates due to CDK9 activity and, at the same time, it is able to recruit RNF20/40. Thus, due to the described features, WAC can be a regulator of differentiation-dependent H2Bub1 accumulation.

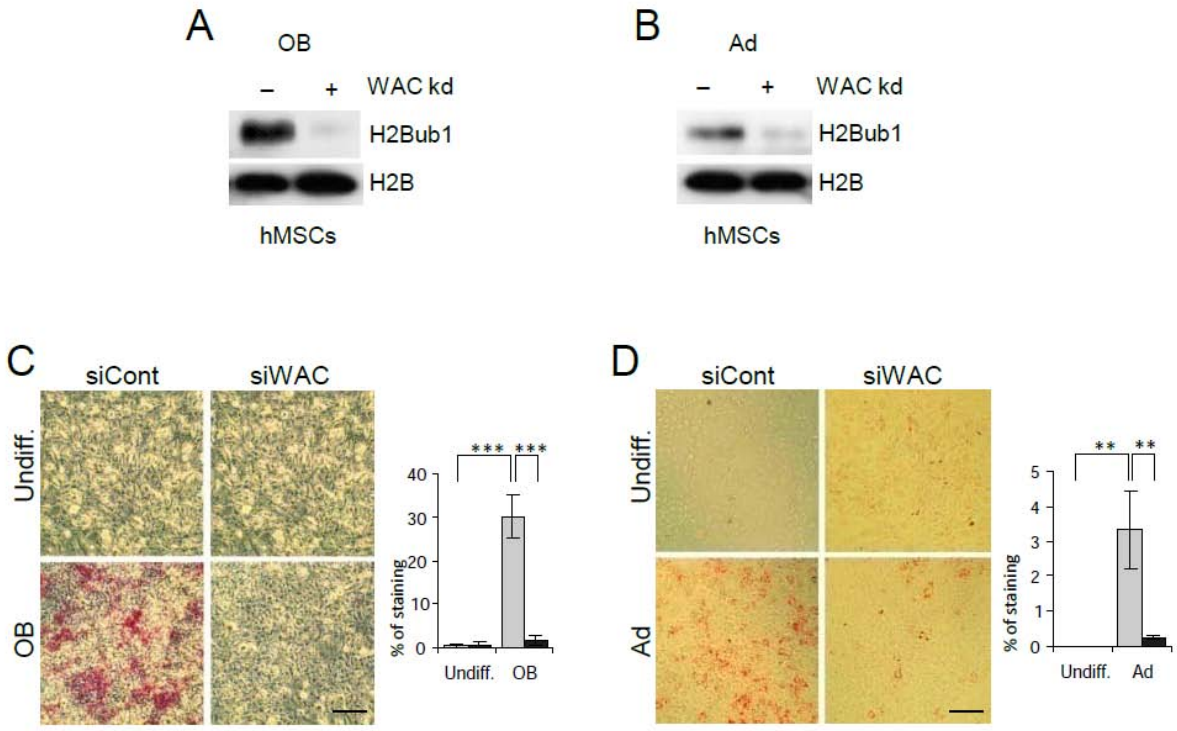

Fig. 18. WAC knockdown inhibits differentiation of hMSCs.
(A, B) Knockdown of WAC was performed in hMSCs for 24h. After that cells were differentiated to osteoblasts (A) or adipocytes (B) for 48h. Protein lysates were obtained and analyzed by Western blot for H2Bub1 and H2B as a loading control.
(C, D) hMSCs were transfected as in (A) followed by 5 days of differentiation into osteoblasts (C) or adipocytes (D). After that cells were fixed and stained for AP activity (C) or lipid drop accumulation (D). Scale bar corresponds to 200 μm. Quantification of AP staining (C) and lipid drop staining (D) was done my measuring the area of stained regions with ImageJ software. Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad - adipocytes. Mean ±SD, n = 3.

To test this hypothesis hMSCs were depleted of WAC by siRNAs followed by differentiation induction. First of all, protein lysates were examined by Western blot. In the case of both osteoblast and adipocyte differentiation WAC depletion resulted in a great decrease in H2Bub1 levels in comparison to differentiated cells transfected with control siRNA (Fig. 18A, B). Unfortunately, it was not possible to check protein levels of WAC upon the knockdown due to the absence of specific commercially available antibodies. However, WAC mRNA levels examined by qRT-PCR showed an efficient knockdown in both differentiations (Fig. 19A, B).

Chemical staining showed less AP activity and less lipid drop accumulation in WAC-depleted MSCs compared to control differentiated cells (Fig. 18C, D) Quantification of the staining confirmed visual observations (Fig. 18C, D). Interestingly, WAC knockdown did not result in elevated AP activity upon osteoblast differentiation making this effect CDK9 depletion-specific. Differentiation rates observed after WAC depletion were similar to those observed after RNF40 knockdown suggesting WAC as an upstream regulator of H2Bub1 accumulation.

To further validate this hypothesis, expression of the differentiation-dependent genes was studied upon WAC depletion. Confirming the data from the stainings, WAC knockdown resulted in downregulation of all studied osteoblast and adipocyte differentiation-dependent genes (Fig.19A, B). To rule out that WAC influences differentiation due the regulation of *RNF20*, *RNF40* or *CDK9* transcription the mRNA levels for these genes were also examined by qRT-PCR (Fig. 19C, D). There was no significant difference in *RNF20* expression upon WAC knockdown neither in adipocytes nor in osteoblasts, a slight decrease in *CDK9* expression (0.3 fold) in adipocytes and a slight increase in *RNF40* expression (0.2 fold) in osteoblasts. However, these changes cannot results in massive inhibition of differentiation-dependent transcription suggesting that WAC directly participates in H2Bub1 regulation.

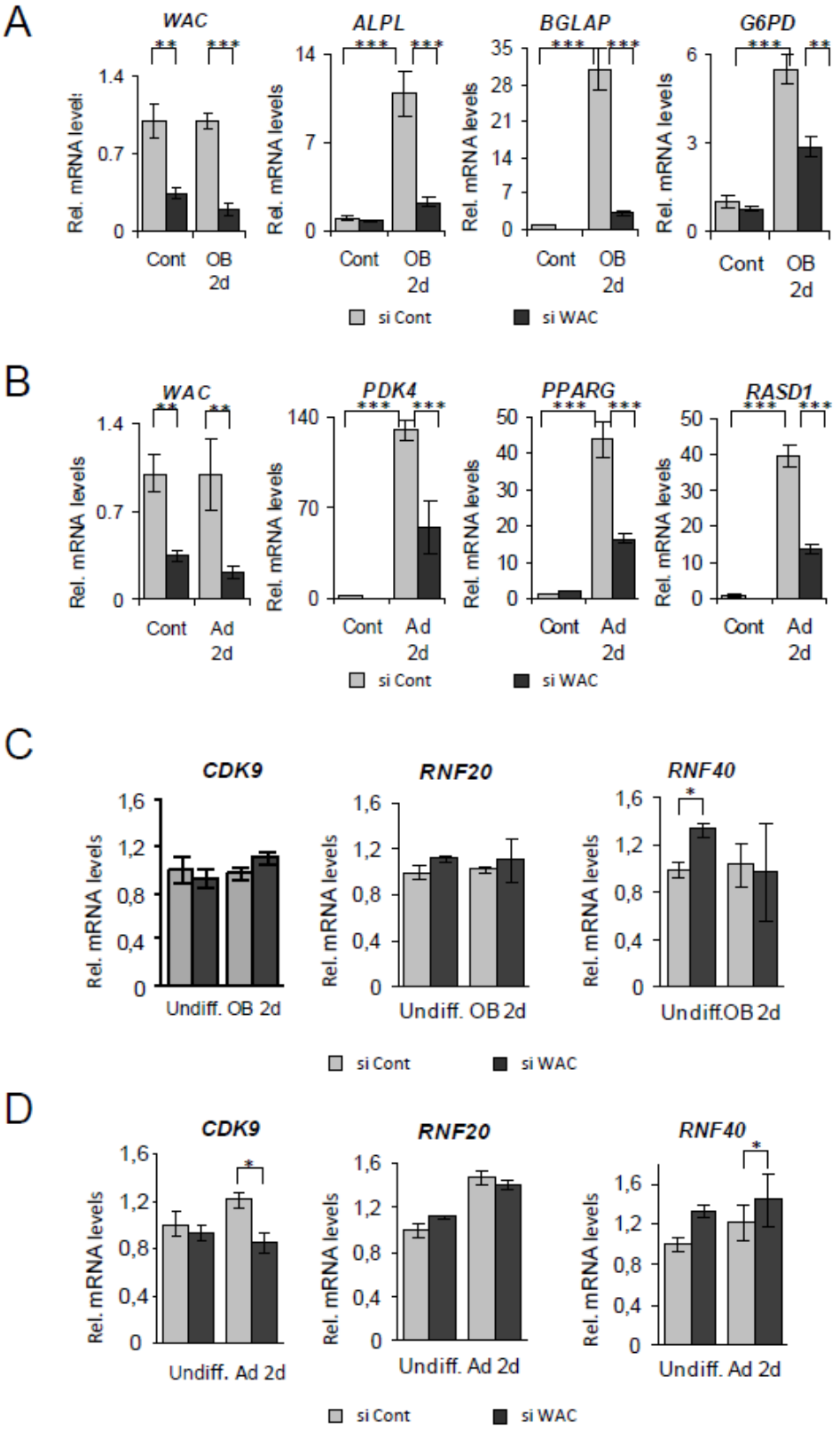

Fig. 19. WAC depletion specifically results in decreased differentiation-specific transcription.
(A, C) hMSCs were transfected with siRNAs to WAC for 24h and differentiated into osteoblasts for 2 days. mRNA levels were analyzed by qRT-PCR. Gene expression was normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts, Mean ±SD, n = 3.
(B, D) hMSCs were transfected as in (A) followed by differentiation into adipocytes for 2 days. Gene expression was analyzed by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, Ad – adipocytes, Mean ±SD, n = 3.

These results strengthen the hypothesis that WAC is required for differentiation-dependent H2Bub1 accumulation. Concluding, H2Bub1 increase during differentiation is mediated by RNF20/RNF40 and is regulated by CDK9 with the help of WAC adaptor protein. Depletion of one of the described players results in a block of H2Bub1 accumulation and a failure in differentiation.

4.4 H2Bub1 executes its function via regulating differentiation-induced changes in other histone modifications

After gaining insight into the upstream regulation of H2Bub1 the next step was an investigation of the downstream targets of this histone modification. As it is known from previous findings, H2Bub1 plays a role in controlling other histone marks like H3K4me3 or H3K79me3. So the next step of the project was to describe the downstream targets of H2Bub1 during differentiation.

4.4.1 Adipocyte-specific genes carry bivalent histone modifications

To understand the interplay between H2Bub1 and other histone modifications it is essential to study which histone modifications are characteristic for differentiation-driven genes. Adipocytic differentiation of hMSCs and adipocyte gene markers were chosen for this study due to the well characterization of this pathway as well as the availability of high-resolution ChIP-seq data for adipose nuclei.

Epigenome Roadmap Resource (http://www.roadmapepigenomics.org/) was used to investigate epigenomic patterns of adipocyte-specific genes *PDK4*, *PPARγ* and *RASD1* used in this study. Two sets of ChIP-seq data were compared – from ES cells where these genes are inactive and from adipose tissue where these genes are transcribed. In adipose tissue, as expected, all three genes carried high levels of H3K4me3 indicating the potential to be actively transcribed (Fig. 20). In ES cells *PDK4*, *PPARG* and *RASD1* were not transcribed and marked by H3K27me3 indicating Polycomb-dependent repression. But surprisingly H3K4me3 was present in ES cells together with H3K27me3 indicating that adipocyte-specific genes in undifferentiated cells carry both activatory and inhibitory histone marks. This state is referred as “histone bivalency” and is characteristic for pluripotent stem cells and some types of multipotent cells. Upon differentiation, one of the marks disappears (“resolution of

bivalency") leading to activation or to silencing of the gene. In case of *PDK4*, *PPARG* and *RASD1*, upon differentiation, the inhibitory mark H3K27me3 will be removed resulting in transcriptional activation.

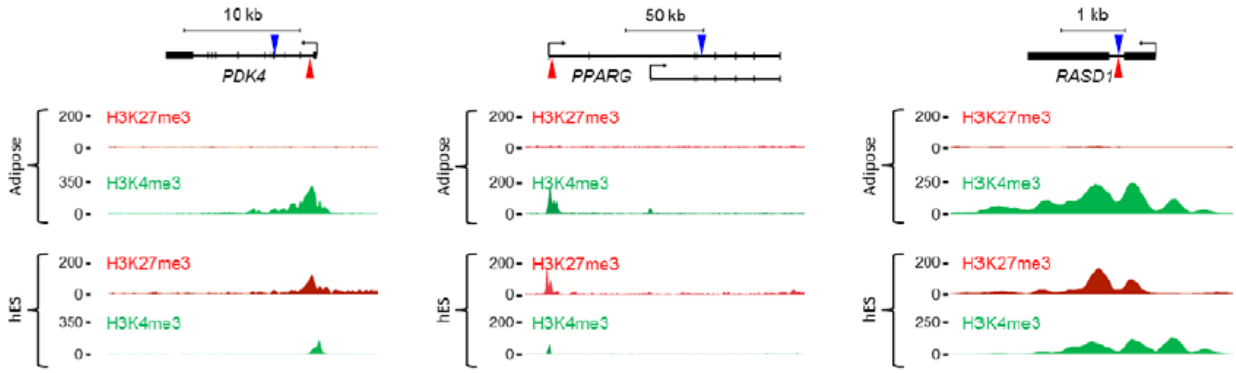

Fig. 20. Adipocyte-specific genes are bivalent. ChIP-seq profiles of H3K4me3 (green) and H3K27me3 (red) on adipocyte-specific genes. Data was obtained from the Roadmap Epigenomics Project (http://www.roadmapepigenomics.org/). Red arrows on the gene schemes (top) indicate primer positions for bivalent sites used in Fig. 15, blue arrows – for H2Bub1 sites.

To investigate the effect of H2Bub1 of bivalency and its resolution hMSCs were differentiated into adipocytes in presence of RNF40 knockdown. First of all, H2Bub1 occupancy in the presence or absence of RNF40 knockdown was examined on adipocyte-specific genes by ChIP (Fig. 21A). First of all, H2Bub1 was detected on all adipocyte genes even in the absence of differentiation signal followed by a significant increase during differentiation. Upon RNF40 depletion H2Bub1 occupancy was significantly reduced on all adipocyte-specific genes as well as on the *GAPDH* gene that was chosen as a positive control for an active gene. Correspondingly, no H2Bub1 above background level was detected on the silent *TFF1* gene.

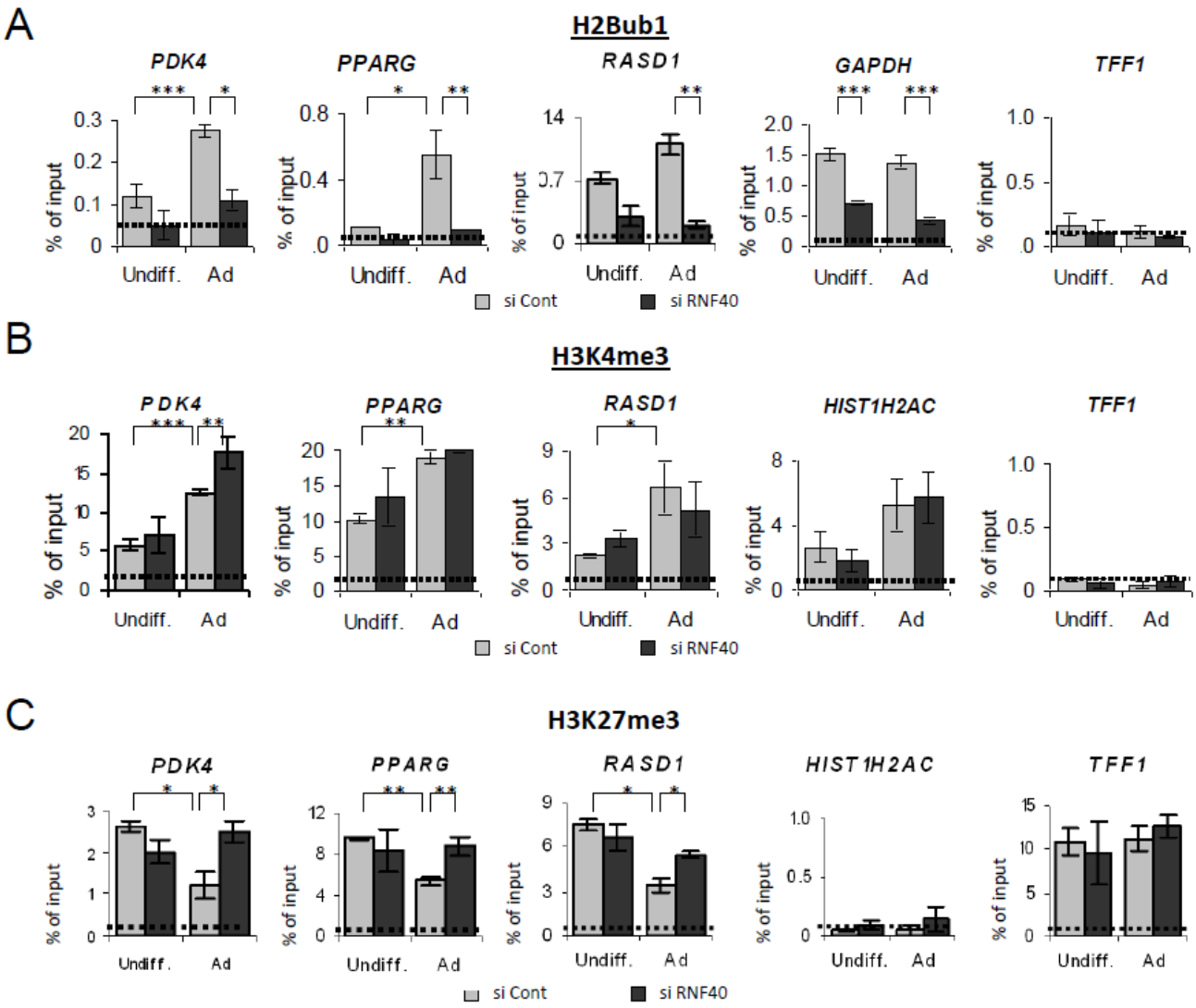

Fig. 21. H2Bub1 is required for correct bivalency resolution during adipocyte differentiation in hMSCs.
(A) hMSCs were transfected with siRNAs to RNF40 for 24h and differentiated into adipocytes for 5 days. Chromatin extracts were analyzed by ChIP for presence of H2Bub1. IgG antibody was used to determine background binding (indicated as dotted line). H2Bub1 occupancy was normalized to input (indicated as "% of input"). Undiff. – undifferentiated hMSCs, Ad – adipocytes, Mean ±SD, n = 3.
(B, C) Cells were transfected and differentiated as in (A). Chromatin extracts were analyzed for H3K4me3 (B) or H3K27me3 (C) occupancy by ChIP. IgG antibody was used to determine background binding (indicated as dotted line). H3K4me3 and H3K27me3 occupancy was normalized to input (indicated as "% of input"). Undiff. – undifferentiated hMSCs, Ad – adipocytes, Mean ±SD, n = 3.

After verifying the efficiency of H2Bub1 reduction the levels of bivalent marks were examined. H3K4me3 increased on all adipocyte genes in differentiated cells compared to control hMSCs, confirming the activation of transcription (Fig. 21B). Surprisingly, there was no significant difference in H3K4me3 occupancy in the presence or absence of RNF40 knockdown despite the established connection between H2Bub1 and H3K4me3 in budding yeast (Sun, 2002; Dover, 2002). No change in H3K4me3 was detected on the active *HIST1H2AC* gene suggesting that this feature is not restricted to adipocyte-specific genes.

Examination of H3K27me3 levels revealed a decrease of this modification upon differentiation (Fig. 21C) confirming the resolution of bivalency. However, upon RNF40 knockdown H3K27me3 occupancy was not reduced and remained at the same levels as in control cells. To check whether that was a specific effect, H3K27me3 occupancy was examined on the *HIST1H2AC* gene, but no increase upon RNF40 depletion was detected. Examination of the repressed gene *TFF1* also demonstrated no H3K27me3 accumulation upon H2Bub1 depletion. These results suggest that depletion of RNF40 results in prevention of H3K27me3 removal and resolution of bivalency.

In summary, H2Bub1 accumulation is required for regulating the levels of other histone modifications. An increase in H2Bub1 results in removal of the repressive H3K27me3 modification of adipocyte-specific genes and the activation of their transcription. A loss of H2Bub1 prevents the resolution of bivalency and differentiation-dependent genes remain transcriptionally inactive. This mechanism represents a new level of regulation of hMSC differentiation.

4.5 The SAGA complex is also required for hMSC differentiation

Knowing that ubiquitination of H2B is required for hMSC differentiation it is logical to hypothesize that deubiquitinating enzymes (DUBs) also play a role in this process. In mammalian cells the DUB module for H2Bub1 is incorporated into the large SAGA complex that also exhibits histone acetylation. Despite the several H2Bub1 DUBs reported, depletion of none of them does not lead to a substantial decrease in H2Bub1 levels (Zhao, 2008; Zhang, 2008, Chipumuro, 2012). However, knockdowns of core SAGA components like ATXN7L3 led to a significant increase in H2Bub1 (Lang, 2011). To study the importance of deubiquitination in hMSC differentiation the effect of ATXN7L3 depletion was analyzed in this system.

4.5.1 Depletion of SAGA component ATXN7L3 leads to H2Bub1 accumulation

To test whether SAGA depletion leads to increased H2Bub1 levels hMSCs were transfected with siRNA to ATXN7L3 for 24h followed by osteoblast or adipocyte differentiation for 5d. Western blot analysis confirmed an increase in H2Bub1 levels upon ATXN7L3 depletion in differentiated as well as in control cells (Fig. 22A). To check whether H2Bub1 is also

increased on differentiation-dependent genes its occupancy in adipocyte-differentiated hMSCs was examined by ChIP with corresponding antibodies (Fig. 22B). All three adipocyte-specific genes, *PDK4*, *RASD1* and *PPARG*, demonstrated increased H2Bub1 occupancy on their transcribed regions upon ATXN7L3 depletion. Interestingly, *GAPDH*, used as a differentiation-independent gene, also possessed high levels of H2Bub1 after ATXN7L3 knockdown suggesting that inactivation of SAGA leads to genome-wide H2Bub1 accumulation.

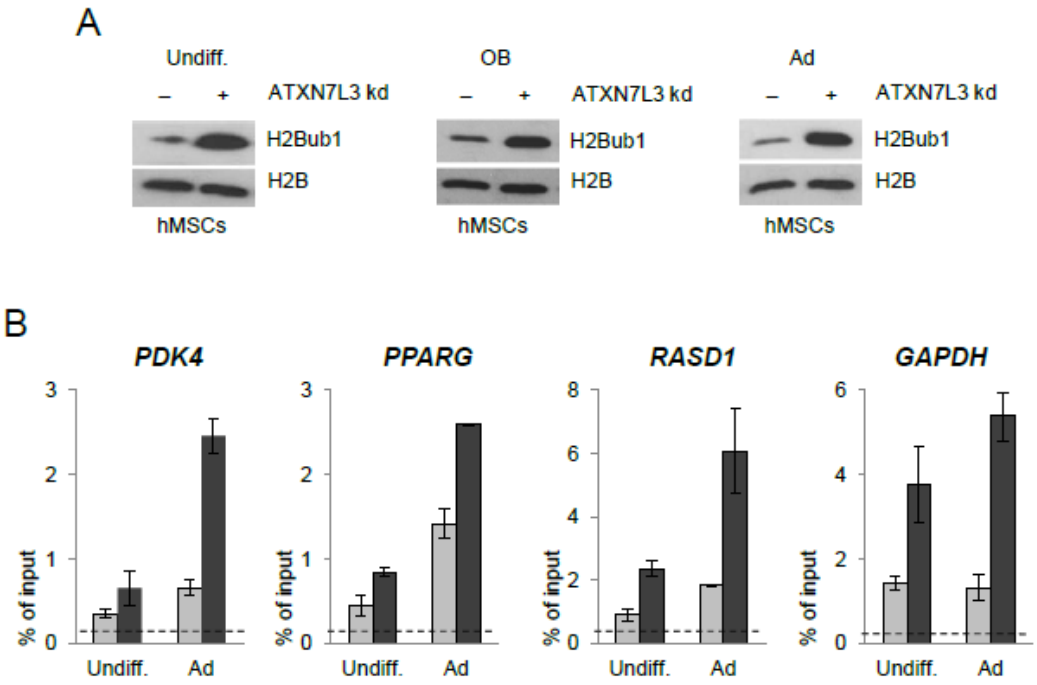

Fig. 22. H2Bub1 levels increase upon ATXN7L3 knockdown in hMSCs.
(A) hMSCs were transfected with ATXN7L3 or control siRNAs for 24h followed by adipocyte or osteoblast differentiation for 5d. Protein lysates were analyzed by Western blot for H2Bub1 and H2B as a loading control. Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad – adipocytes.
(B) hMSCs were transfected as in (A) and differentiated into adipocytes for 2d. chromatin extracts were analyzed by ChIP for H2Bub1 occupancy. IgG antibody was used to determine background binding (indicated as dotted line). H2Bub1 occupancy was normalized to input (indicated as "% of input"). Undiff. – undifferentiated hMSCs, Ad – adipocytes, Mean ±SD, n = 3.

4.5.2 ATXN7L3 knockdown inhibits differentiation of hMSCs

As it was shown earlier, H2Bub1 depletion decreases differentiation of hMSCs. Based on the accumulation of H2Bub1 due to ATXN7L3 it was hypothesized that increased or accelerated differentiation would occur. To test this hypothesis hMSCs were transfected with ATXN7L3 siRNA, differentiated for 5 days and subjected to chemical staining for AP activity and lipid droplet accumulation. Surprisingly, ATXN7L3 depletion inhibited osteoblast and adipocyte

differentiation compared to differentiated cells transfected with control siRNAs (Fig 23A, B). This observation suggests that a balance of H2B monoubiquitination and deubiquitination is essential for induction of differentiation-specific genes.

To further test this model differentiation-driven gene expression after ATXN7L3 knockdown was examined in hMSCs. Cells were treated with siRNAs to ATXN7L3 or control siRNAs followed by differentiation and gene expression analysis by qRT-PCR. As a result of significant ATXN7L3 depletion (Fig. 24A, B; first graphs) osteoblast- and adipocyte-specific genes were induced to a lesser extent than in differentiated cells transfected with control siRNAs (Fig. 24A, B). This data confirms observations obtained from the chemical staining experiments. To exclude the downregulation of SAGA components an expression of another SAGA protein, *ENY2*, was examined and appeared to be unchanged upon ATXN7L3 depletion. This suggests that ATXN7L3 depletion inhibits differentiation-driven genes.

The results suggest that disruption of the SAGA complex via ATXN7L3 depletion leads to H2Bub1 accumulation. However, this accumulation does not promote hMSC differentiation, but rather inhibits it. This leads to the hypothesis that differentiation of hMSCs is not dependent on elevated H2Bub1 per se, but requires a precise balance of ubiquitination and deubiquitination. The exact mechanisms that sustain this balance require further investigations.

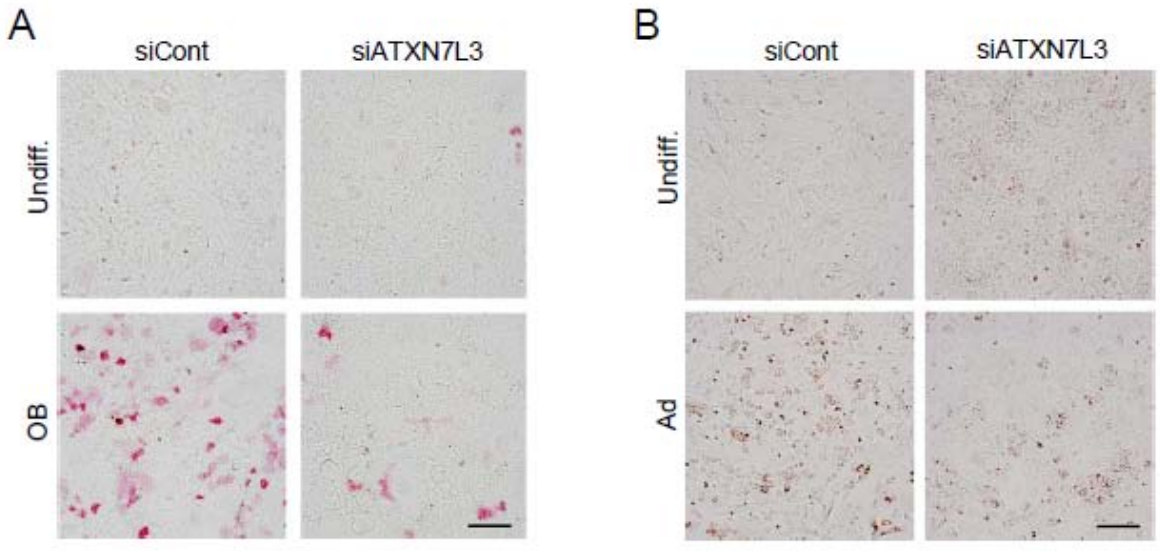

Fig. 23. ATXN7L3 depletion leads to decreased hMSCs differentiation.
(A, B) Transfection with ATXN7L3 siRNAs or control siRNA was performed in hMSCs for 24h followed by differentation into osteoblasts (A) or adipocytes (B) for 5d. After that cells were stained for ALPL activity (A) or lipid drop accumulation (B). Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad – adipocytes.

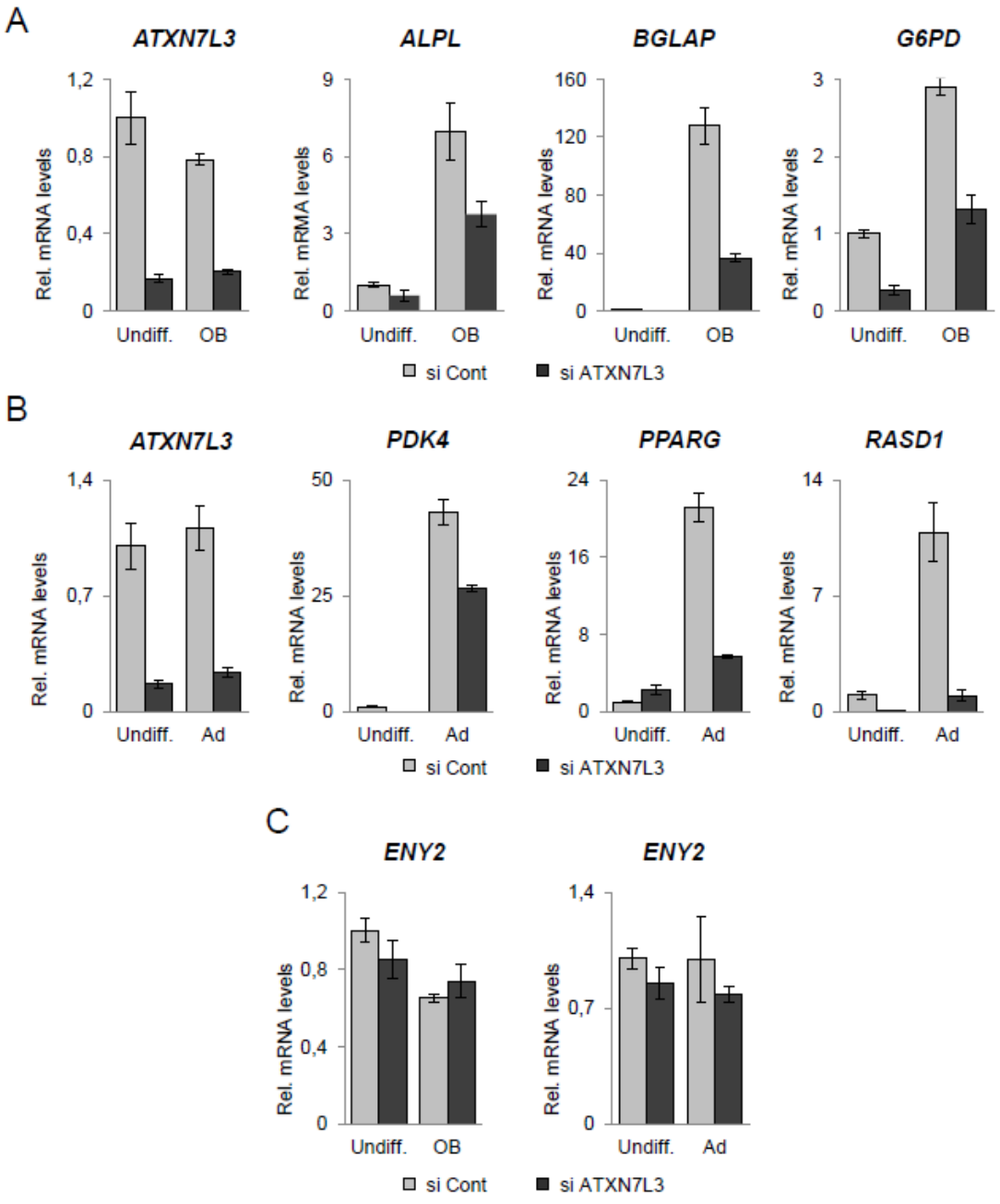

Fig. 24. Differentiation-dependent genes are downregulated upon ATXN7L3 knockdown.
(A, B) hMSCs were transfected with siRNA to ATXN7L3 or control siRNAs and differentiated into osteoblasts (A) or adipocytes (B) for 48h. Gene expression was analysed by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad – adipocytes. Mean ±SD, n = 3.
(C) hMSC were transfected and differentiated as in (A) and into osteoblasts or adipocytes for 48h. Gene expression was analysed by qRT-PCR and normalized to *HNRNPK* expression (indicated as "Rel. mRNA levels"). Undiff. – undifferentiated hMSCs, OB – osteoblasts, Ad – adipocytes. Mean ±SD, n = 3.

4.6 Summary

Summarizing this study it was shown that the “knockdown-overexpression” system for H2B is not suitable to use due to the technical difficulties. Next, it was demonstrated that H2Bub1 elevation is a characteristic feature of the cellular differentiation. Moreover, the H2Bub1 depletion, as well as depletion of its upstream regulators, was shown to inhibit differentiation progression. It was also described that H2Bub1 executes its differentiation-promoting effect by interfering with the other histone modifications like H3K27me3. Finally, it was proposed that depletion of the SAGA component ATXN7L3 also inhibits differentiation suggesting the importance of dynamic ubiquitination-deubiquitation. Taken together this data for the first time demonstrates that H2Bub1 is involved in cellular differentiation and is essential for differentiation of hMSCs.

5 Discussion

Gene expression is tightly regulated not only by availability of transcription factors, but also by the epigenetic factors that control accessibility of the chromatin. Among them, post-translational histone modifications play an important role in many cellular processes via facilitating or inhibiting gene activation. In this study it was demonstrated that one of the histone modifications, monoubiquitination of histone H2B, is essential for differentiation of hMSCs. Using adipocyte differentiation the following model for H2Bub1 function was proposed (Fig. 25). In the absence of a stimulus adipocyte-specific genes are not transcribed and carry bivalent histone modifications – activating H3K4me3 and inhibitory H3K27me3 (Fig. 25 I). Poised RNAPII might be present on the gene but due to the absence of Ser2 phosphorylation no transcriptional elongation occurs. Upon induction of differentiation, CDK9 is recruited to the gene probably via interactions with a tissue-spesific transcription factor (Fig. 25 II). CDK9 phosphorylates Ser-2 of C-terminal domain of RNAPII that leads to WAC and subsequent RNF20/RNF40 recruitment (Fig. 25 III). Monoubiquitination of H2B deposited by RNF20/40 stimulates the removal of inhibitory H3K27me3 followed by efficient transcription progression and production of the complete mRNA transcript (Fig. 25 IV).

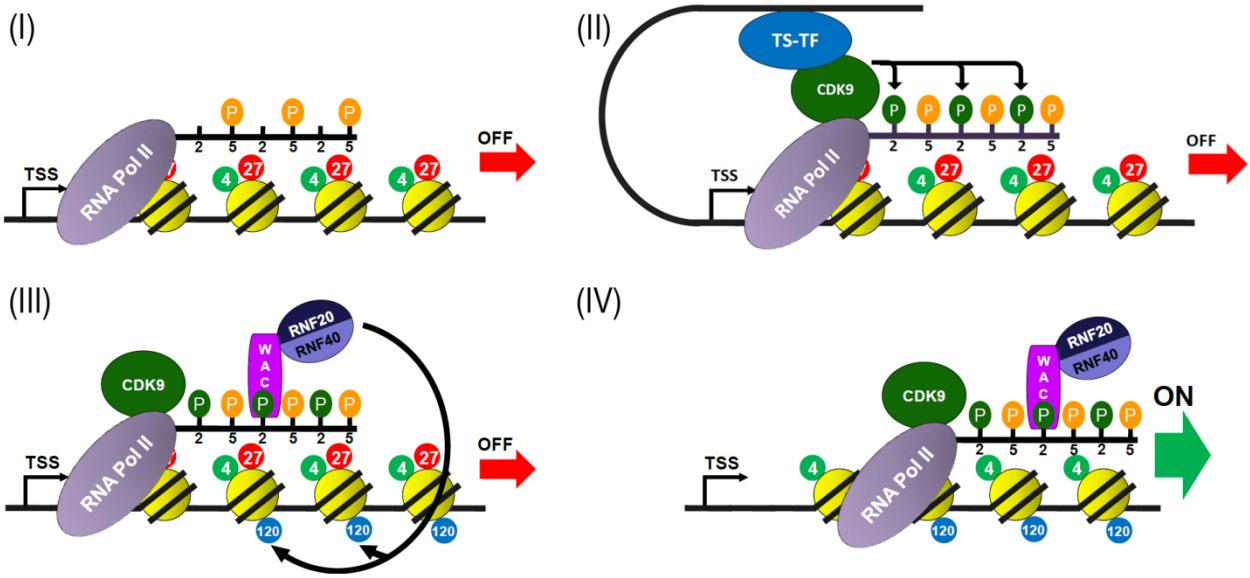

Fig. 25. A suggested model for the H2Bub1 involvement in the resolution of bivalency (from Karpiuk, 2012)**.** (I) RNA Pol II is poised near the promoter, both bivalent marks (H3K4me3 and H3K27me3) are present. (II) Recruitment of CDK9 via a transcription factor leads to phosphorylation of Ser2 in the C-terminal domain of RNA Pol II. (III) Interaction of WAC adaptor protein with P-Ser2 leads to RNF20/RNF40 recruitment and subsequent H2B monoubiquitination. (IV) H2Bub1 stimulates demethylation of H3K27me3 which permits the productive elongation. Abbreviations: RNA Pol II – RNA polymerase II, TS-TF – tissue-specific transcription factor.

5.1 Mechanistic insights into the resolution of bivalency

5.1.1 H2A monoubiquitination and bivalency

Embryonic stem cells and, to a lesser extent, other somatic stem cells possess a more complex chromatin structure than differentiated cells. Some of their genes are occupied with activators and repressors at the same time; this chromatin state was named "bivalent" (Azuara, 2006; Bernstein, 2006; Pan, 2007; Zhao, 2007). Most common bivalency is co-occupancy of the gene with the transcription activating mark H3K4me3 and inhibiting mark H3K27me3 (Azuara et al., 2006; Bernstein et al., 2006). Trimethylation of H3K4 is deposited by members of the Thrithorax family of histone methyltransferases (Shilatifard, 2011; Schuettengruber, 2011). Di- and trimethylation of H3K27 is performed by Polycomb Repressive Complex 2 (PRC2). For many genes that are silenced by PRC2, H3K27me3 deposition leads to the further recruitment of PRC1 (reviewed in Richly, 2011). In mammalian cells this complex contains two ring finger proteins, RING1A and RING1B, that mediate the monoubiquitination of H2A at lysine 119 (Wang, 2004; Cao, 2005). In *D. melanogaster* this modification is present on about 10% of H2A which makes it one of the most abundant histone modifications (Levinger, 1982). PRC1 is believed to be required for further stable silencing of the gene and facilitate additional compaction (Eskeland, 2010; Francis, 2004; Stock, 2007). Interestingly, PRC1 co-localizes with PRC2 at bivalent domains taking part in repression of developmental genes (Azuara, 2006) probably through enhancing RNAPII pausing on the promoter (Stock, 2007).

Loss of RING1B or A results in a genome-wide loss of H2Aub1 and de-repression of bivalent genes (Endoh, 2008; Stock 2007). Due to the fact that hMSCs possess bivalent domains on adipocyte-specific genes it is probable that PRC1 also regulates their silencing by H2A monoubiquitination (H2Aub1). Thus, it would be important to monitor H2Aub1 levels during differentiation progression as well as H2Aub1 occupancy on the bivalent genes. Since PRC1 is required for maintaining the pluripotency state (Stock, 2007), depletion of its components will likely lead to early activation of bivalent genes and enhanced adipocyte differentiation. However, it is possible that PRC2 depletion will not mimic the effect of H2K27me3 removal since PRC2 and PRC1 do not always act together (Asp, 2011). For example, PRC2 and PRC1 depletion during myogenic differentiation resulted in opposite effects where PRC2 knockdown promoted differentiation and PRC1 inhibited it (Asp, 2011).

Another open question is whether RNF20/RNF40 complex regulates H2A monoubiquitination and whether its depletion results in alterations of H2Aub1 levels. One possible way of H2Aub1 and H2Bub1 is mutual exclusion. We still know very little about the interplay between different histone modifications on the same nucleosome and which sets of modifications that can exist together. Since addition of a bulky ubiquitin moiety interferes a lot with the chromatin structure (Fierz, 2011) it is possible that two ubiqitinations cannot take place at one nucleosome. H2Bub1 may prevent monoubiquitination of H2A thus maintaining an active transcription. In this case RNF20 or RNF40 depletion may result in stable levels of H2Aub1 thereby preventing differentiation-activated gene expression. On the other hand, H2Aub1 may regulate genes that are required only during the first and middle stages of differentiation and have to be silenced in the mature somatic cells. In this case the RNF20/40 complex may recruit PRC1 upon a certain signal for shutting down the activated genes. According to this hypothesis depletion of RNF20 or RNF40 might result in decreased H2A monoubiquitination. In this case hMSCs would successfully pass through the early stages of differentiation but would fail to reach the terminally differentiated state.

5.1.2 The role of histone demethylases in differentiation

The resolution of bivalency in differentiating hMSCs requires the removal of the repressive mark H3K27me3. This removal is most likely an active process which requires the presence of a demethylating enzyme (Agger, 2007; De Santa, 2007). Up to date two H3K27me3 demethylating enzymes have been reported – UTX (KDM6A) and JMJD3 (KDM6B) (Agger, 2007; De Santa, 2007). Both of them possess a Fe(II)-containing jumonji (JmjC) domain that mediates their catalylic activity (Tsukada, 2006) and are able to specifically remove tri- and dimethylation of H3K27 (Hong, 2007). Although there is no direct evidence that JMJD3 or UTX are responsible for the resolution of bivalency, an indirect proof of this comes from their requirement for HOX gene activation during differentiation (Agger, 2007). JMJD3 is also required for murine osteoclast maturation where it resolves bivalency on one of the master transcription factors NFATc1 (Yasui, 2011). UTX executes an opposite biological role: it is required for re-establishing of pluripotency in iPSCs and is involved in germ cell reprogramming (Mansour, 2012). However, it was also reported to be involved in myoblast differentiation (Seenundun, 2010). Since there is no comparative studies of JMJD3 and UTX expression in hMSCs both of these enzymes should be considered in future studies related to the resolution of bivalency in hMSCs.

Due to a relatively recent discovery of UTX and JMJD3 the mechanism of their recruitment to the activated genes is not well understood. Studies on the INK4A-ARF locus in mouse MEFs suggest that JMJD3 can be recruited upon activation of ERK kinase via interaction with transcription factor AP-1 (Agger, 2009). On the other side, data obtained from breast cancer studies proposes an interaction between JMJD3 and ERα nuclear receptor (Svotelis, 2011). In this case JMJD3 is recruited via the AF1 domain of ERα to the Estrogen-responsive elements (EREs) of the BCL2 gene and suggested to interact with RNAPII via chromatin looping. The second model is particularly interesting in regard to hMSC differentiation since PPARγ also has an AF1 domain that targets it to PPARγ responsive elements (PPREs) (reviewed in Daynes, 2002) and can potentially recruit JMJD3 to bivalent PPARG-activated adipocyte genes. In this case depletion of JMJD3 would result in the preservation of the bivalency and the absence of transcription. Interestingly, JMJD3 expression can also be activated by VDR in colon cancer cells suggesting a potential involvement in osteoblast differentiation (Pereira, 2011). Since hMSCs can differentiate into both adipocyte and osteoblast lineages it is an ideal system to compare JMJD3 involvement in two different lineage commitment pathways.

As it was demonstrated in the current study, the removal of H3K27me3 is abolished upon H2Bub1 depletion. Several models can explain this observation. (1) A demethylating enzyme for H3K27me3 is recruited directly or via a mediator protein to H2Bub1. However, according to this theory H2Bub1 occupancy should overlap with H3K27me3 which is usually not the case since H2Bub1 is found preferentially in transcribed regions while H3K27me3 is situated primarily near transcriptional start sites. Nonetheless, demethylases may not be recruited directly to H2Bub1, but to one of its upstream regulators such as the RNF20/40 complex. (2) The presence of H2Bub1 changes the chromatin accessibility which may allow a demethylase to interact with H3K27me3. The addition of a ubiquitin moiety to H2B “opens” chromatin making it more accessible to other factors. In this case no direct interaction between the demethylase and H2Bub1 or the ubiquitination machinery would be required. In the case of H2Bub1 depletion the chromatin would remain “closed” and bivalency would not be resolved.

5.1.3 Involvement of H3K79me3 in the resolution of bivalency

Another modification directly dependent on H2Bub1 is the trymethylation of H3K79 (H3K79me3) (Briggs, 2002; Ng, 2002). This modification has diverse functions: it is involved in telomere silencing in yeast (Ng, 2002) and at the same time is required for transcrptional elongation (Wood, 2003; Lee, 2007). H3K79 di- and trimethylation is catalysed by Dot1 in

yeast or by the corresponding human ortholog DOT1L (Feng, 2002; Lacoste, 2002; Ng, 2002a; van Leeuwen, 2002). Structural studies on yeast nucleosomes demonstrated that H3K79 and H2BK123 lie in close proximity suggesting a crosstalk between H2Bub1 and H3K79me3 (Luger, 1997). Indeed, Bre1 mutation as well as H2B K123 mutation prevented H3K79me3 deposition (Sun, 2002; Ng, 2002). The observed effect is unidirectional since Dot1 mutation did not influence H2Bub1 levels (Sun, 2002).

Several mechanisms connecting H2Bub1 and H3K79me3 were proposed (reviewed in Nguyen, 2011). A direct interaction of Dot1 and H2Bub1 was suggested since Dot1 has a lysine-rich patch that can interact with ubiquitin (Oh, 2010). Moreover, chemically synthesized H2Bub1 can promote Dot1 activity without the addition of other factors (McGinty, 2008). An indirect interaction was also demonstrated wherein H2Bub1 facilitates the recruitment of the 19S proteasome Rpt4 and Rpt6 ATPase subunits which facilitate H3K79 trimethylation (Ezhkova, 2004). Finally, as discussed above for H3K27 demethylation, monoubiquitination of H2B appears to bring chromatin into a more “opened” conformation (Fierz, 2011) thereby making it accessible to Dot1.

H3K79me3 is enriched within the transcribed regions of active genes (Pokholok, 2005). This connection and the dependence of H3K79me3 on H2Bub1 make it a potential player in differentiation and the resolution of bivalency. Although H3K79me3 levels were not monitored in this study it can be expected that they also increase on genes regulated upon differentiation concomitant with the induction of transcription and increase in H2Bub1. The important question that has to be answered is if H3K79me3 deposition takes place before or after removal of H3K27me3 from the bivalent genes. If it happens before, H3K79me3 can be a pre-requisite for the resolution of bivalency. The initial idea of H3K79me3 involvement in hMSC differentiation can be obtained from ChIP studies on bivalent genes upon DOT1L knockdown. Since it was reported that DOT1L knockdown promotes formation of induced pluripotent stem cells (iPS) (Onder, 2012), DOT1L depletion in hMSCs may result in the inhibition of differentiation and in the acquiring of more “stem cell-like” phenotype by hMSCs.

5.2 CDK9 recruitment to chromatin

CDK9 is an important regulator of the H2B monoubiquitination in mammalian cells since its interaction with RNAPII is required for RNF20/40 recruitment (Pirngruber, 2009; Karpiuk,

2012). CDK9 together with cyclin T forms P-TEFb that is required for phosphorylation of Ser2 withinthe RNAP II CTD. Apart from this CDK9 also phosphorylates DSIF and NELF which is also required for the release from the pausing (Fujinaga, 2004; Peterlin and Price, 2006; Yamada, 2006; Core, 2008).

Despite several proposed models it is still not known how P-TEFb is recruited to chromatin. Budding yeast is a good model to study different aspects of P-TEFb function since they have two homologs of P-TEFb – Ctk1 and Bur1. Ctk1 mediates CTD phosphorylation at Ser2 and a Bur1 is required for H2Bub1 and H3K4me3 (Wood, 2006; reviewed in Jones, 2008). Ctk1 phosphorylates CTD which facilitates transcription and in parallel stimulates recruitment of Bur1. Bur1 executes several functions: it prosphorylates Rad6 and activates it; it transforms DSIF into the positive elongation factor by phosphorylating its subunit Spt5; finally, it facilitates Bre1 recruitment and H2Bub1 deposition. H2Bub1 stimulates H3K4 trimethylation by SET1/COMPASS complex (Wood, 2006). Interestingly, H2Bub1 presence inhibits Ctk1 recruitment, so H2B has to undergo deubiquitination (Wyce, 2007). This reaction is performed by Ubp8 assosiated with the SAGA complex (Baker, 2007). It was also shown that Ctk1 preferentially binds to non-ubiquitinated H2A:H2B dimers obtained from cell extracts (Wyce, 2007) which leads to a conclusion that Ctk1 recruitment to chromatin requires the deubiquitination of H2B.

The mammalian SAGA complex also contains a DUB module. Since H2B deubiquitination is required for the P-TEFb recruitment in budding yeast, similar mechanism might be true for human P-TEFb. If this is the case, the interference with the H2Bub1 turnover may result in decreased P-TEFb recruitment even to the genes with high H2Bub1 levels. An indirect confirmation for this suggestion comes from the data of the present study where accumulation of H2Bub1 induced by ATXN7L3 knockdown led to decreased transcription of differentiation-dependent genes. If P-TEFb indeed interacts with non-ubiquitinated H2B then an excess of H2Bub1, generated due to ATXN7L3 knockdown, would be predicted to prevent CDK9 recruitment to the chromatin and result in observed transcription inhibition.

5.2.1 BRD4-mediated CDK9 recruitment

BRD4 is a mammalian bromodomain protein that interacts with cyclin T1 and recruits P-TEFb to chromatin (Jang, 2005; Yang, 2005). BRD4 binds to highly acetylated H3 and H4 histones (Wu, 2007) and recruits P-TEFb to activated genes. It was also shown that it interacts

with the mediator complex and can recruit RNAPII to chromatin (Wu, 2007). BRD4 is a possible link between differentiation signals and CDK9 recruitment to activated genes in a differentiation context. In human cells the treatment with serum stimulates H3S10 phosphorylation by MSK1/2 (Macdonald, 2005; Soloaga, 2003). That facilitates binding of the adaptor protein 14-3-3 which recruits the histone acetyltransferase MOF (Zippo, 2009). MOF deposits H4K16 acetylation which is recognized by BRD4 (Zippo, 2009). Thus, BRD4 could "sense" extracellular signals and recruit CDK9 to the genes that have to be activated.

5.2.2 CDK9 and bivalent chromatin

Can CDK9 influence the resolution of bivalency? This question is difficult to answer since there is no data about interaction between P-TEFb and UTX or JMJD3. However, it is known that JMJD3 knockdown decreases efficient elongation of the transcript (Chen, 2012). Moreover, JMJD3 interacts with the elongation factor Spt6 (Chen, 2012) that was shown to interact with P-TEFb and P-Ser2 of CTD (Yoh, 2007). The current study suggests that CDK9 recruitment precedes the resolution of bivalency since H3K27me3 removal requires H2Bub1, which in turn requires CDK9 activity deposition as a pre-requisite. From myoblast differentiation it is known that UTX travels with elongating RNA Pol II (Seenundun, 2010) which makes its interaction with CDK9 or Spt6 theoretically possible. CDK9 and this demethylase could be recruited together with the same transcription factor, e.g. E2F or AP-1, or independently via a coordinated action of several transcription factors, which is the case in some differentiation pathways (Aziz, 2010). Another option is the recruitment of JMJD3 or UTX with Spt6 (Fig. 26A). Since the demethylase apparently cannot perform its action without the monoubiquitination of H2B it is possible that this enzyme constantly resides on the bivalent genes and becomes active upon CDK9 recruitment (Fig. 26B).

A more special case of CDK9 involvement in the resolution of bivalency is via nuclear receptors (Fig. 26C). An interaction of CDK9 with a nuclear receptor upon ligand binding might stimulate H2B monoubiquitination which in turn will lead to the recruitment of a histone demethylase to the activated genes. This possibility is strengthened by the requirement of H2B ubiquitinating machinery for estrogen- (Prenzel, 2011) and androgen- (Jaaskelainen, 2012) activated transcription. Despite the apparent prevalence of this kind of regulation it is unlikely to be universal since many of differentiation pathways are not nuclear receptor-driven.

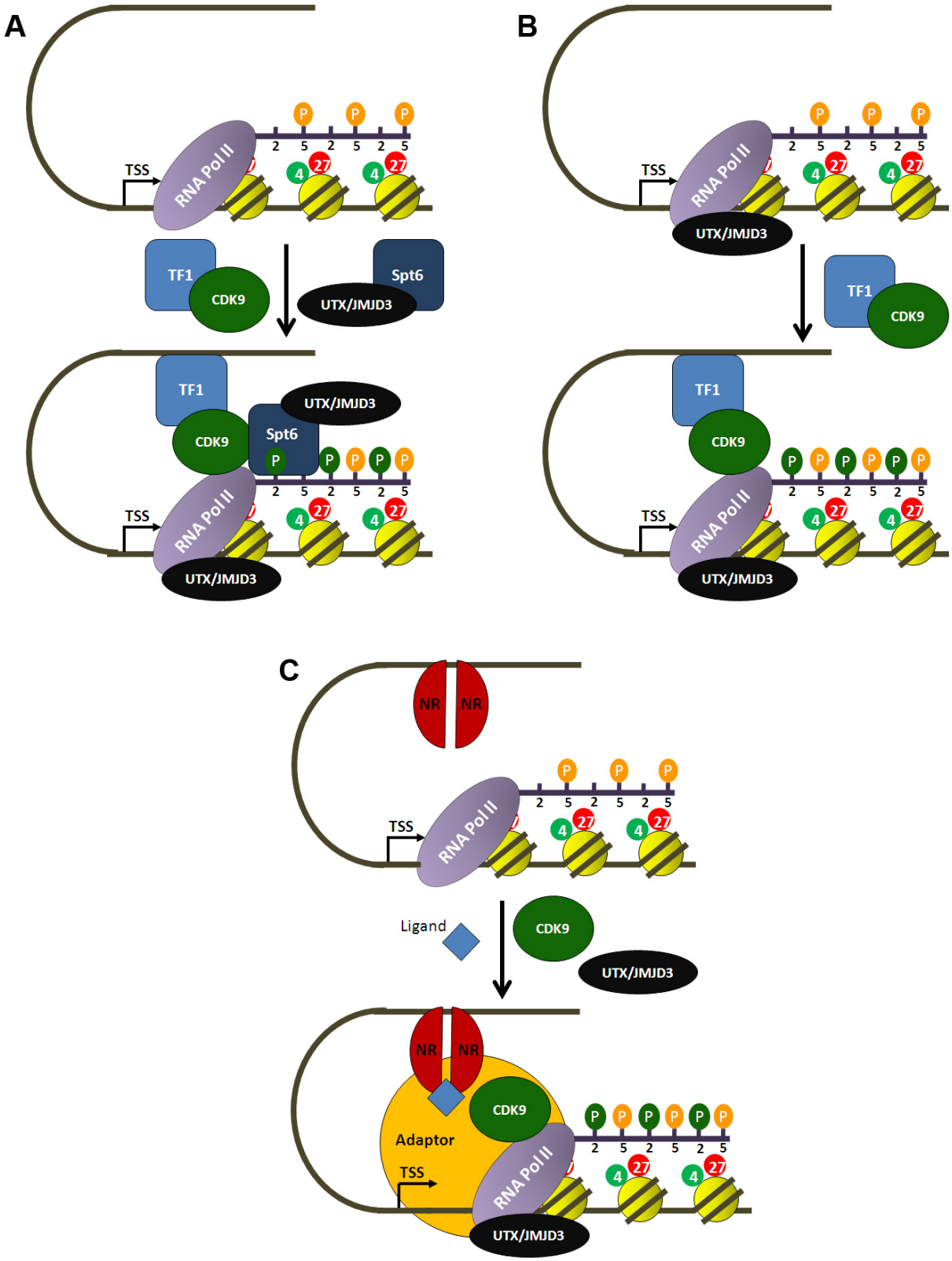

Fig. 26. Possible mechanisms of CDK9-dependent resolution of bivalency. (A) Independent recruitment of CDK9 and JMJD3 or UTX by transcription factors. (B) Demethylase is constantly bound to chromatin and becomes activated upon CDK9 recruitment. (C) CDK9 and demethylase recruitment is nuclear-receptor dependent. Upon ligand binding a bigger complex containing CDK9, JMJD3 or UTX, nuclear receptor and adaptor proteins is formed. Abbreviations: NR – nuclear receptor, P – phosphorylation, TF – transcription factor, TSS – transcription start site.

5.3 The role of H2Bub1 in transcription

5.3.1 How essential is H2Bub1 for transcription?

One of the most interesting observations was that despite the requirement of H2Bub1 for differentiation of hMSCs, upon RNF40 knockdown and H2Bub1 depletion, the cells remained completely viable and able to divide. This raises a question whether H2Bub1 is a compulsory pre-requisite for transcription. As it was shown in this study knockdown of RNF40 in undifferentiated hMSCs changed the expression of a very small number of genes. The same effect was observed for RNF20 knockdown (Shema, 2008; Fuchs, 2012). Thus it appears that, although H2Bub1 is universally associated with active gene transcription, many genes can apparently bypass a need for it. Then when is this modification required? Recalling once again the microarray data with RNF40 knockdown it can be concluded that almost all differentiation-activated genes remained inactive in the absence of H2Bub1. This leads to the idea that H2Bub1 is essential for the rapid and strong transcription activation as it happens during differentiation. Due to its ability to loosen chromatin structure H2Bub1 may make differentiation-activated genes more accessible to the transcription machinery and facilitate transcription elongation.

5.3.2 Genome-wide distribution of H2Bub1

As it was demonstrated in this study, differentiation of hMSCs leads to an elevation in global H2Bub1 levels. ChIP studies showed that H2Bub1 occupancy increases on differentiation-specific genes, however, it remains unknown whether these genes are the only ones affected. Despite the large number of differentiation-regulated genes most of the genome does not appear to require this signal. An accumulation of H2bub1 on the differentiation- regulated genes would likely not account for the observed increase of the H2Bub1 levels. Although it is established that H2Bub1 is associated with the transcribed regions of active genes (Minsky, 2008), it is possible that it also accompanies transcription of micro-RNAs (miRNAs) or long non-coding RNAs (lnRNAs) that are known to participate in differentiation (Crippa, 2012; Dong, 2012; Sartipy, 2009; van Leeuwen, 2010). ChIP-seq studies investigating the genome-wide occupancy of H2Bub1 might shed light on this question.

5.3.3 Requirement of RNF20/40 for differentiation

A high number of H2B genes in mammalian cells extremely complicates a creation of a cell line with mutated H2B that cannot be ubiquitinated. Thus, the main way to investigate H2Bub1 functions is depletion of its E3-ligase components RNF20 or RNF40. This kind of approach raises a concern whether H2B is the only substrate of the RNF20/40 complex. Although it is frequently assumed that RNF20/40 ubiquitinate only H2B, the protein Ebp1 was reported as another possible target of RNF20 (Liu, 2009). Interestingly, this ErbB3-assosiated protein is a tumor suppressor and its ubiquitinated form is absent in cancer tissues (Liu, 2009) similarly to H2Bub1 (Prenzel, 2011). It is unknown if CDK9 or WAC can regulate ubiquitination of the other RNF20/40 targets, but this finding leaves room for speculations that RNF40 regulates differentiation of hMSCs via ubiquitination of additional substrates, and H2Bub1 may be a secondary product of its activity. One more substrate was reported for the rat ortholog of RNF20 and 40 called Staring (Chin, 2002). It was also shown to ubiquitinate Syntaxin 1 targeting it for degradation (Chin, 2002). Another fact that suggests the existence of multiple RNF20/40 substrates is the composition of the complex itself. Most metazoans utilize a heterodimeric complex for H2B monoubiquitination while budding yeast and Drosophila have only one Bre1/RNF20/RNF40 ortholog. Moreover, both RNF20 and RNF40 have RING finger domains required for ubiquitination, but only the RING finger of RNF20 is required for transcription-coupled H2B monoubiquitination in vitro (Kim, 2009). Evolutionary preservation of the RNF40 RING finger domain might indicate its requirement for other ubiquitination reactions, maybe even as a part of another complex.

5.4 H2Bub1 as a differentiation regulator

5.4.1 H2Bub1 - a link between differentiation and carcinogenesis

A cell that undergoes malignant transformation acquires and loses a certain set of features. It becomes immortal and gains an ability to divide without external stimuli and, on the other side, it reduces or loses lineage-specific protein production and it no longer performs its physiological function. This transformation has certain similarities with turning back into progenitor cells, thus this process is essentially a “de-differentiation”. Looking at carcinogenesis as the opposite process to lineage commitment raises a possibility that

H2Bub1, as a differentiation-associated modification, can also perform tumor-supressor functions.

So does H2Bub1 accumulation negatively correlate with malignancy? The first indirect answer comes from the studies on non-transformed MCF10A breast epithelial cells (Shema, 2008) where depletion of RNF20 resulted in a significant increase in cellular migration and proliferation. Later these findings were also confirmed for RNF40 (Prenzel, 2011). These findings support that the RNF20/40 complex has a tumor-suppressor function probably through its regulation of H2Bub1. On the other hand, the H2Bub1 deubiquitinating enzyme USP22 was identified as one of the "stem cell signature" genes whose overexpression correlates with poor cancer prognosis (Glinsky, 2006; Liu 2011, Liu 2010). Finally, it was shown that H2Bub1 is not detectable in malignant and metastatic breast cancer tissues while being abundant in differentiatednormal breast tissue and benign tumors (Prenzel, 2011). Taken together, these observations suggest that H2Bub1 is an important marker of differentiated, non-transformed tissue and reduction of its levels can possibly be used as a diagnostic tool.

Considering H2Bub1 as a potential tumor suppressor allows utilizing its regulators as potential therapeutic targets. For example, inhibitors of deubiquitinating enzymes might be used for patients with USP22-overexpressing tumors. On the other side, usage of specific CDK9 inhibitors, like flavopiridol, in H2Bub1-low tumors should be controlled since it can lead to even greater decrease of H2Bub1 and heavier malignant transformation.

Despite the fact that H2Bub1 downregulation during malignancy agrees with "de-differentiation" model, the functional importance of H2Bub1 absence in malignant tissues remains unknown. One model might be that the decrease of H2Bub1 levels is a cause or consequence of shutting down the lineage-specific genes. An active suppression of H2Bub1 accumulation is also possible, since several tumors overexpress USP22 or hypermethylate the RNF20 promoter to downregulate its expression (Glinsky, 2006; Shema, 2008). To understand which advantages H2Bub1 depletion might bring, the H2Bub1, RNF20/40, USP22 occupancies and their correlation to global gene expression in malignant and normal cells should be analyzed.

5.4.2 H2Bub1 in regenerative medicine and stem cell biology

During the last decades a lot of research has been dedicated to regenerative medicine as an approach to treat degenerative diseases connected with aging or differentiation failures. Its main idea is replacing of the non-functional tissues or even organs with ones grown in laboratory conditions. This approach allows avoiding problems with histocompatibility and also does not create ethical complications since it is based on obtaining cells from the same patient to which they will be transplanted. The main caveat lies in the restricted differentiation potential of somatic cells and can be partially overcome by two major ways: (1) using less differentiated precursors that can be found in the adult organism or (2) de-differentiating somatic cells to a pluripotency state via genetic and/or chemical manipulations.

The first approach utilizes stem cells available from adult body, e.g. hematopoietic, adipose tissue- or bone marrow-derived mesenchymal stem cells (hMSCs). MSCs are especially attractive in this regard due to their ability to suppress immune response which makes them easier to transplant. These cells were successfully transplanted in trials for Crohne's disease (Ciccocioppo, 2011; Mannon, 2011), steroid refractory acute graft-versus-host disease (GvHD) (Le Blanc, 2004), type I diabetes mellitus (DM) (Zanone, 2010), acute myocardial infarction (AMI) (Kocher et al., 2001) and chronic obstructive pulmonary disease (COPD) (D'Agostino, 2010) treatment. Understanding the signaling that allows hMSCs to differentiate into so different tissues and its regulation will increase the efficiency of treatment and expand the list of possible applications. For example, sensitization of hMSCs towards differentiation signaling prior to transplantation might increase their therapeutic efficacy. In the current study it was suggested that H2Bub1 is a general feature of hMSC differentiation independent of the specific signal. Thus pre-treatment with USP22 siRNAs or inhibitors could be used of faster H2Bub1 accumulation and earlier induction of lineage-specific genes.

Another area of regeneration therapy is the generation of iPS cells based on transfecting the somatic cells with pluripotency inducing factors and culturing them in special conditions (reviewed in Shafa, 2010). Most often a combination of four pluripotency factors is used: OCT4, SOX2, KLF4 and c-MYC (Yamanaka, 2008) or OCT4, SOX2, NANOG and LIN28 (Yu, 2007). Due to the low efficiency of iPS cell formation much effort was put into enhancing the process with additional transformations or chemical treatment. In this regard H2Bub1 as a differentiation-associated modification may be inhibited to potentially increase the de-differentiation of the cells. Its downregulation may prevent expression of lineage-

specific genes and decrease spontaneous differentiation of iPS cells. Since RING-ligases are difficult to target with chemical inhibitors, the upstream regulators (e.g. CDK9) of H2Bub1 can be used for this purpose. Another interesting target is the BRD4 protein that recruits CDK9 to transcribed genes because several specific inhibitors of its interaction with chromatin were described recently (reviewed in Dai, 2004). Another fact that makes CDK9 an interesting target for iPS cell generation is its regulation of AP expression observed in this study. CDK9 depletion led to increased production of AP that, apart from of being a bone-associated protein, is also a well established stem cell marker (MacGregor, 1995; Shamblott, 1998; reviewed in Saito, 2004). This may indicate that upon CDK9 knockdown hMSCs acquired a more "stem cell-like" phenotype. If this observation will be confirmed with elevated expression of other stem cell markers, down-regulation of CDK9 or its upstream regulators might significantly improve iPS cell generation.

In summary, this study reveals that H2Bub1 regulates differentiation of hMSCs. Its presence is required for the resolution of bivalency on differentiation-regulated genes possibly via affecting the recruitment of a demethylating enzyme for H3K27me3. The proposed mechanisms of H2Bub1 involvement of activating poised genes might be utilized in other cell types as well as other organisms (Karpiuk, 2012). A better understanding of H2Bub1's role in transcription will help to determine whether this histone modification may serve as a target for cancer treatment and a tool for regenerative medicine.

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- Faculty of Biology, International Max Plank Research School, MSc/PhD program in Molecular Biology

2004-2008 Kyiv National Taras Shevchenko University

- Faculty of Biology, specialization: biochemistry
- Bachelor Diploma with overall score 5.0 (out of 5 maximum)
- Bachelor Thesis – "Glutathione impact on apoptotic protein content in parietal cells under the chronic atrophic gastritis in rats"

Laboratory experience

PhD student, group of Prof. Dr. S. Johnsen

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Lab rotations

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Project: Characterization of spinal cord lesions by immunohistochemical methods

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Jan 2009 – Feb 2009 Department of Molecular Oncology, University of Göttingen
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Part-time assistant in biochemical laboratory
May 2006 – June 2008 Department of Biochemistry, Taras Shevchenko University, Kyiv

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Awards

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Publications

Karpiuk O. et al. "The Histone H2B Monoubiquitination Regulatory Pathway is Required for Differentiation of Multipotent Stem Cells". Mol Cell. 2012 Jun 8;46(5):705-13.

Shchebet A., **Karpiuk O.**, Kremmer E., Eick D., Johnsen SA. Phosphorylation by cyclin-dependent kinase-9 controls ubiquitin-conjugating enzyme-2A function. Cell Cycle. 2012 Jun 1;11(11): 2122-7.

Competences

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- work with laboratory animals and cell cultures;
- molecular biology methods;
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