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MASTERARBEIT

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„Consequences of SUMO E2 Deregulation on Chromatin“

Verfasser

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For my family

1 Abstract

SUMOylation (Small Ubiquitin-related MOdifier) is a post-translational protein modification that can alter the sub-cellular localization, the activity or the interaction partners of proteins. Thus, this reversible modification has been associated with many important cellular processes and the number of newly identified SUMO substrates is steadily increasing. Several chromatin modifying enzymes are modified by SUMO – many of which are responsible to set and to maintain repressive Histone and DNA methylation characteristic for silent heterochromatin. The essential tri-enzymatic SUMO cascade comprises a single E1, a single E2 and several E3 enzymes. The E2 enzyme Ubc9 was found to be deregulated in cancer and is associated with metastasis, tumor cell invasion and resistance to chemotherapy. This study aims to investigate the consequences of Ubc9 deregulation on chromatin in NIH 3T3 mouse fibroblasts. Therefore, the Histone lysine methylation at H3K9 and H4K20, which are characteristic for pericentromeric heterochromatic regions, were investigated. Thereby, Ubc9-siRNA treatments of cells lead to growth arrest and an increase in H4K20 methylation. In contrast to that, no change was observed for the other prominent repressive histone mark, the H3K9 tri-methylation.

2 Introduction

2.1 Principle of SUMOylation

SUMOylation is an essential posttranslational modification related to protein ubiquitinylation. Although SUMO and ubiquitin share a similar structural fold their sequence homology is only 18%[1]. Ubiquitinylation often functions as signal for degradation whereas SUMOylation is involved in diverse cellular functions as transcription, chromatin structure, DNA repair, signaling and stability of target proteins [2, 3, 4, 5]. This is due to the change of this posttranslational modification in binding affinities with proteins, DNA or RNA[6, 7]. Thereby SUMO can even compete with ubquitin for the lysine residue and thus contributes to target stability[8]. SUMOylation is highly conserved throughout evolution, but was not found in eubacteria and archea. Eukaryotes have varying numbers of SUMO paralogues ranging from one protein in yeast to four SUMO variants in mammals[9].

SUMO is an 11kDa protein and its conjugation to substrates (sumoylation) is performed by an energy dependent hierarchical tri- enzyme cascade (Figure 1). In the first ATP consuming reaction SUMO gets activated and forms a thioester bond with the E1 activating enzyme which is the heterodimer between SAE1 and SAE2 (Aos1 and Uba2 in yeast)[10, 11]. Subsequently, SUMO is transferred from the E1 to the E2 conjugating enzyme Ubc9 again resulting in a thioester bond[12, 13]. Ubc9 can either directly transfer SUMO to the substrate or - more commonly – needs the help of an E3 ligating enzyme for efficient substrate modification[14, 15]. This results in an isopeptide bond between the C-terminal glycine of SUMO and the ε-amino group of the target lysine[16] . Such lysines are often part of the SUMO consensus motif ψKXE/D with Ψ as a hydrophobic amino acid and X as any residue[17, 18].

SUMO can also regulate proteins functions in a non-covalent manner by binding to a specific SUMO interactions motif (SIM) in a substrate. Such motifs are short hydrophobic regions in a β-sheet structure often flanked by acidic amino acids which interact with the β-sheet in SUMO[19, 20, 21]. SUMOylation is a reversible process and different SUMO specific proteases are identified. The most prominent representatives are the six members of the Senp family (Senp family

members 1-3 and 5-7)[22, 23] but very recently two novel classes of SUMO proteases were described, DeSI-1 and Uspl1[24]. SUMO proteases can carry out different functions: isopeptide cleavage leading to dissociation of SUMO from its substrate, SUMO processing for maturation of the SUMO precursor or SUMO chain editing (Figure 3). Also preferences for either SUMO1 or SUMO 2/3 are described for specific Senp family members[25, 26, 27, 28].

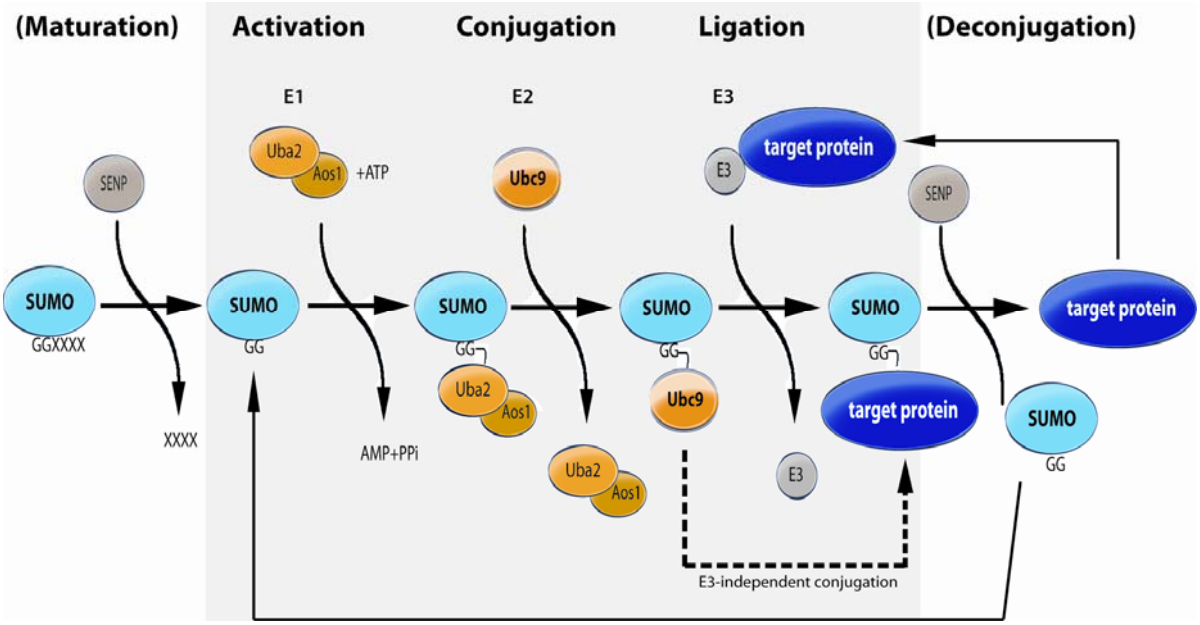

Figure 1: SUMO conjugation to its substrate: After removal of SUMO C-terminal residues by SUMOproteases SUMO is activated by the E1 heterodimer Aos1- Uba2 by the expense of ATP and forms a thioester bond with the E1 enzyme. SUMO is then transferred to the E2 enzyme Ubc9 again forming a thioester bond. Finally, the C-terminal glycine forms an isopeptide bond with a lysine in the target protein, which is normally assisted by an E3. However, there are targets which are SUMOylated directly by Ubc9. SUMOylation is reversible and demodification is performed by SUMOspecific proteases. (Adaped from *Meulmeeser and Melchior* [9])

2.2 Ubc9 deregulation

SUMOylation is mainly regulated at the level of E3 ligases and isopeptidases since these enzymes ensure substrate specificity. Little is known about regulation at the level of the sole E2 enzyme which is essential for SUMO conjugation. Regulation at this step of the enzyme cascade is expected to have severe consequences on all downstream events, the E3 ligases and substrates.

Ubc9 is highly conserved and its deletion is lethal in most organism from baker´s yeast to mice[29, 30]. However, deletion of fission yeast homologue, hus5, is viable

although these cells are severely impaired in growth with defects in mitosis resulting in chromosome missegregation[31, 32]. Ubc9-depleted Caenorhabditits elegans displays developmental defects comparable to mutations in developmental regulators, as for example in Hox genes[33]. Also in Drosophila melanogaster the semushi (semi is the Drosophila SUMO-E2) mutant strain suffers from defects in the arterior-posterior patterning during development[34]. In zebrafish, expression of a dominant negative mutant causes widespread apoptosis whereas anti-sense mediated Ubc9 knock down showed defects in later proliferating tissues such as brain and eyes. More detailed analyses indicated that Ubc9 depletion results in a G2/M arrest of the cell cycle and the accumulation of polyploid cells [35]. Conditional Ubc9 depletion in DT40 chicken cells demonstrated chromosomal loss and apoptosis and a subpopulation of multinucleated cells. In contrast to other studies in those cells there a G2/M arrest was not detected[36].

In mice Ubc9 is essential and Ubc9 deficient mouse embryos die early in development after the blastocyte stage. Interestingly, such blastocytes are viable for up to two days before undergoing apoptosis. Analysis of these cells indicated severe chromosomal defects and major changes in the nuclear architecture of the inner cell mass[30].

Interestingly, not only the downregulation of Ubc9 has severe consequences on cell homeostasis also its upregulation by ectopic expression promotes cell invasion and metastasis. In line, Ubc9 upregulation correlates with different human malignancies[37]. In colon adenocarcinoma and prostate intraepithelial neoplasia Ubc9 was found to be elevated compared to normal tissue and Ubc9 expression increased with progressed disease stages. Luminal and non-luminal breast cancer tissue also significantly differed in their levels of Ubc9 expression with upregulation in luminal breast cancer tissue. Moreover, hormone (estrogen and/or progesterone) receptor-positive breast cancer tissues also showed higher Ubc9 levels than hormone receptor-negative tumors[38]. Furthermore, stable expression of a Ubc9 wt or Ubc9 double mutant in breast cancer cells yielded to higher tissue invasion as a control vector and accordingly breast cancer cells treated with Ubc9-siRNA did not show tissue invasion[39]. In agreement, clinical

responses of breast cancer patients to neoadjuvant chemotherapy were compromised by higher Ubc9 expressing tumors[40] .

However, these data clearly demonstrate that Ubc9 deregulation has severe consequences on cell homeostasis: Ubc9 upregulation appears to promote cancer progression whereas its downregulation results in severe chromosomal defects indicating an important role for Ubc9 in the regulation of chromatin structure and function.

2.3 Chromatin and its regulation

Chromatin describes the complex organization of DNA with proteins which achieves the dynamic regulation between DNA compaction and accessibility to regulate chromatin functions. In metaphase chromosomes are highly condensed, whereas in interphase chromatin can be divided in decondensed euchromatin and highly condensed heterochromatin[41].

The basic unit of chromatin is the nucleosome in which 146bp of DNA are wrapped around a protein octamer. Such octamers consist of each two of the core histones H2A, H2B, H3 and H4[41, 42]. However, the length of linker regions between the nucleosomes varies between species and cell type. Consistently, the histones associated with linker regions such as histone H1, are also less conserved as core histones and regulate nucleosome spacing and compaction[43].

Changes in the highly organized chromatin structure are introduced by various mechanisms such as DNA methylation, posttranslational histone modifications, chromatin remodelling enzymes which allow the exchange of core histones with their variants[42].

DNA methylation at cytosines next to guanines (CpGs) is a covalent DNA modification in which a methyl group is added at the 5´position on the pyrimidine ring[44]. The functional consequences of DNA methylation on chromatin include transcriptional gene silencing, genome stability, chromatin compaction, suppression of homologous recombination between repeats and X chromosome inactivation[45]. Two classes of enzymes responsible for DNA methylation are described: the maintenance methyltransferase Dnmt1, and the de novo methyltransferases Dnmt3a and Dnmt3b[46]. DNA methylation together with histone methylation leads to gene repression and forms condensed heterochromatin for transient (facultative heterochromatin) or stable gene

silencing (constitutive heterochromatin)[47]. Besides DNA methylation, histone modifications are key players in regulating chromatin structure and function[48].

The small histones consist of core domains involved in octamer formation and unstructured N-terminal tails which are particularly rich in lysine and arginine residues[42]. These tails protrude out of the histone complex making them easily accessible for diverse post-translational modifications. Such modifications change the charge of the highly basic histones which alters DNA accessibility and protein binding affinities to the nucleosome. A large number of enzymes are described which regulate addition and removal of methyl groups on lysines and arginines, phosphorylation on serines and threonines, and acetylation or ubiquitination on lysines, as just to mention the best understood and most studied examples[42, 48]. The consequences of these modifications can be diverse as methylation can either be a repressive or an activating mark, depending on the position of the modified residue and whether the residue is mono-, di- or trimethylated[48]. In contrast, phosphorylation and acetylation are rather enriched at actively transcribed genes in open chromatin[49]. The term 'histone code' refers to the combination of all the post-translational modifications that are interpreted by specific proteins determining the chromatin status[42]. The highly dynamic chromatin state and the transition from closed to open chromatin is achieved by the activity of a variety of such multiprotein chromatin regulatory complexes[50]. One important class are the ATP-dependent chromatin remodelling complexes with functions in increasing DNA accessibility, nucleosome sliding and dissociation of histones from DNA, a dynamic process required for exchange of histone variants[51].

Histone variants are described for different core histones and mark specific regions on chromatin. Prominent examples are the histone H3 variant CENP-A which is associated with silent centromeric regions or H3.3 often found in actively transcribed genes (Figure 2)[42, 48, 49]. Most variations are known for H2A including H2A.X involved in DNA repair, H2A.Z in transcription regulation and macroH2A present on the inactive X chromosome of female mammals[42,48].

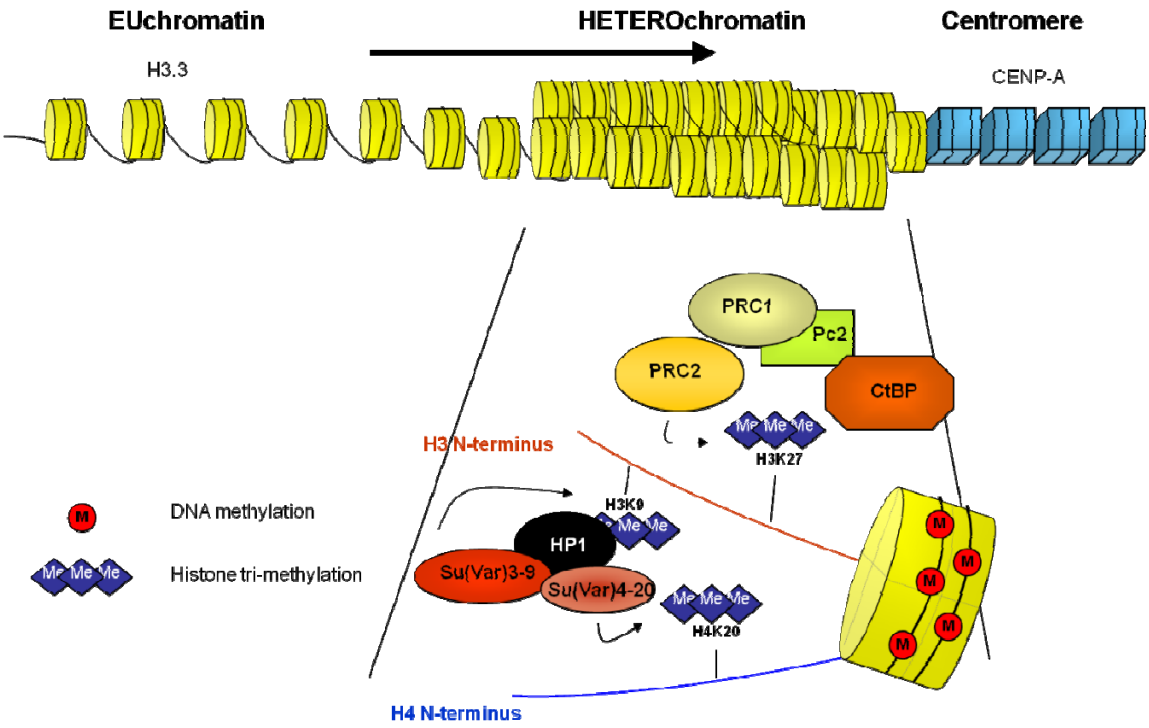

Figure 2: Proteins that establish and maintain heterochromatin: Histone composition changes with chromatin condensation: H3.3 is found in open, transcribed regions whereas CENP-A is only found in the centromere of the chromosome. Heterochromatin is characterized by H3K9me3, H3K27me3 and H4K20me3 which are recognized by specific protein complexes. Su(Var)3-9 sets H3K9me3. HP1 binds to H3K9me3 and is in a complex with Su(Var)4-20 for setting of H4K20me3. PRC2 sets H3K27me3 which attracts further repressive complexes (eg.: PRC1-Pc2) and corepressors (eg.: CtBP) DNA methylation at Cytosines in CpG context aids in gene repression as a signal for further repressive complexes (e.g.: MDB-1, not indicated). Arrows indicate the enzymes setting the respective tri-methylation.[42]

The methylation of histones is achieved by enzymes being specific for the target and/or even for their mono-, di and tri-methylation at a distinct lysine[52, 53, 54]. Thereby, H3K9me3, H4K20me3 and H3K27me3 were found to localize to pericentric constitutive heterochromatin. Several enzymes and co-enzymes establishing and maintaining condensed and silent chromatin were associated with sumoylation[55, 56, 57].

2.4 Sumoylation and chromatin

Although sumoylation is involved in most biological processes it has distinguished functions in the nucleus including intracellular transport, cell cycle progression, DNA repair, transcriptional control and chromatin regulation[2,3,4]. Consistently, components of the general transcription machinery[58], several transcription factors

(e.g. CBP, Smad4, p53,...)[8, 59, 60] and their regulators[61, 62] are modulated via sumoylation. Moreover, also several key regulators of chromatin function are substrates for sumoylation. This includes all DNA methylating enzymes Dnmt1, Dnmt3a and Dnmt3b[63, 64]. Also in regard of histone modifications sumoylation has a major role. All core histones and some variants are modified with SUMO and several enzymes controlling histone acetylation (e.g. HDACs) and methylation (e.g. LSD1) are substrates of sumoylation or affected by sumoylation of interaction partners[65, 66, 67, 68, 69, 70, 71].

Moreover, enzymes of the SUMO machinery were linked to chromatin regulation as a drosophila PIAS E3 ligase mutant was identified as a Su(var)2-10 (suppressor of position effect variegation 2-10), suggesting that PIAS has an important role in heterochromatin formation. Su(var)2-10 locus was reported to stabilize chromosome structure and function[72]. Furthermore, Pc2, a member of polycomb proteins which are chromatin factors maintaining developmental gene silencing, is described to have SUMO E3 ligase functions[73].

Taken together, several studies identified sumoylation as major regulator of many chromatin associated proteins and this modification is often found connected to gene silencing and repressed transcription activity. Interestingly, a recent study investigated the occupancy of SUMO1 on chromatin and identified it enriched upstream of the transcription start sites of many active housekeeping genes correlating with the transcription active mark H3K4me2[74]. This finding suggests that we are just at the very beginning in understanding the function of SUMO at chromatin.

2.5 Aim of the project

Sumoylation is an essential posttranslational modification. Deregulation of Ubc9, one key enzyme for sumoylation has severe consequences: Ubc9 deficiency in mice or baker´s yeast is lethal and results in cell cycle arrest and severe chromosomal defects and major changes in the nuclear architecture. This is consistent with a large number of SUMO substrates involved in chromatin regulation. Furthermore, Ubc9 upregulation was found in different types of cancer and ectopic expression of Ubc9 promotes cell invasion and metastasis.

In the proposed thesis I aimed to analyse the consequences of Ubc9 deregulation on heterochromatin formation. Ubc9 deregulation was aimed to be studied by both overexpression as it is found in various cancer cells but also by its downregulation. For investigating Ubc9 overexpression a stable, Ubc9 inducible, NIH3T3 cell line was established. Downregulation was studied by siRNA-mediated Ubc9 knock down. Finally, cells were investigated for changes in specific heterochromatin associated histone marks by immunoblotting and immunofluorescence.

3 Results

Ubc9 is a key enzyme for sumoylation. Different studies indicate that its deregulation has severe consequences on cell homeostasis. Whereas downregulation is associated with severe chromosomal aberrations its upregulation was found in different types of cancer. To study Ubc9 deregulation requires the availability of good anti-Ubc9 antibodies.

3.1 Purification and Characterization of Ubc9 Antibodies

To obtain Ubc9 specific antibodies a goat was immunized with recombinant human Ubc9 protein. To reduce unspecific background staining and increase antibody specificity, affinity purification of the goat serum was performed. This requires the purification of recombinant Ubc9 from bacteria and subsequent coupling to cyanbromide-activated sepharose. Ubc9 specific antibodies were enriched on this Ubc9 coupled sepharose and were then eluted.

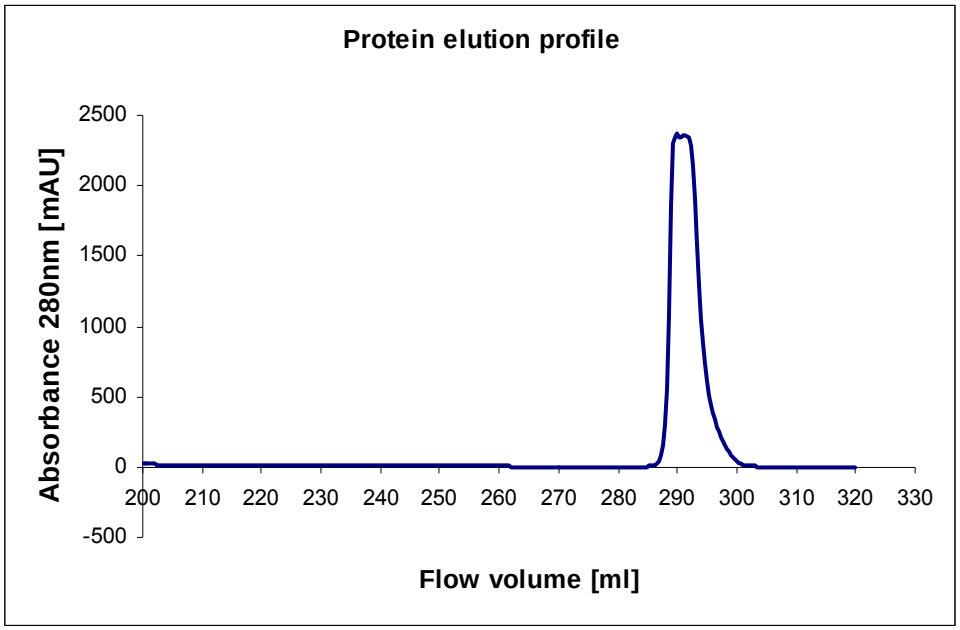

Figure 3: Protein density profile of Ubc9 elution after size exclusion chromatography on an ÄKTA FPLC system: Ubc9 eluted highly concentrated between 289ml and 294ml as one peak. The x-axis represents the eluted volume in ml and the y-axis the protein concentration in mAU (milli absorbance unit) at 280nm.

3.1.1 Purification of recombinant Ubc9

Ubc9 has the size of 18 kDa and was expressed as untagged protein from the pET23a plasmid driven by the IPTG inducible T7 promoter. The plasmid was transformed into the bacteria strain BL21 gold and induction was with 1 mM IPTG for 4 hours at 37°C. Lyses was done by a freeze/thaw cycle followed by sonication on ice. Because of its positive charge, Ubc9 was first enriched by ion exchange chromatography on SP sepharose, a strong cation exchange matrix. Upon high salt elution, the protein was concentrated and further purified by a HiLoad S200 size exclusion chromatography on a FPLC Äkta system. Figure 3 shows Ubc9 elution in one clean peak monitored by a protein density profile measured at 280nm in milli Abosrbance units (mAU).

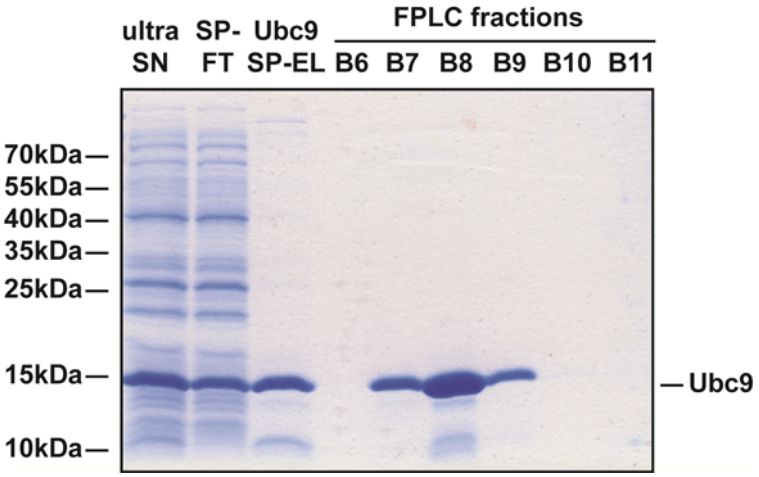

Figure 4: Steps of Ubc9 purification. Samples were separated by 12.5% SDS-PAGE and protein was stained with Coommassie brilliant blue. Lane 1 "ultra SN" shows an aliquote of the crude bacterial lysate after ultra centrifugation. Subsequent Ubc9 was enriched by ion exchange chromatography on SP-beads. "bead FT" or "SP FT" indicates the flow through after enrichment on the beads whereas lane 3 demonstrates an aliquote after elution from the beads "Ubc9 SP-EL". The eluted protein was finally separated by size exclusion chromatography and protein containing and adjacent fractions (FPLC fractions) are shown.

In the next step all peak fractions containing high protein amounts and adjacent fractions were evaluated by separation on a 12.5% SDS PAGE followed by staining of proteins with Coomassie blue. As demonstrated in Figure 4 fractions B7 (A_{280nm} measurement: 285.20-289.70ml), B8 (A_{280nm} measurement: 290.05-294.90ml), B9 (A_{280nm} measurement: 295.25-299.75ml) contained Ubc9 enriched to near homogeneity compared to the starting material (ultra SN). In addition critical steps of the purification protocols are shown: Ubc9 elution from SP-sepharose (Ubc9 SP-EL) and the flow through after SP beads incubation (SP

FT). Of note, the presence of Ubc9 in the SP-FT sample indicates that the binding to SP-sepharose was incomplete.

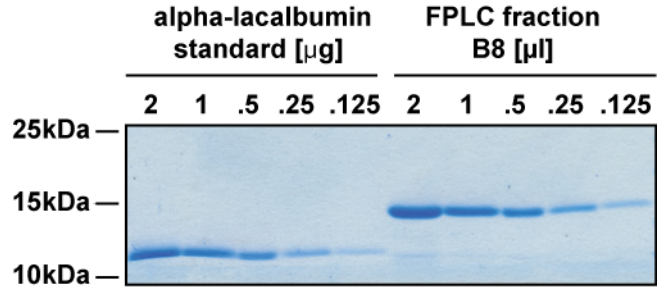

Figure 5: Concentration determination of purified recombinant Ubc9 with the reference protein α-Lactalbumin. A serial dilution of Ubc9 was compared to defined concentrations of α-Lactalbumin as indicated. Samples were separated on 12,5% SDS PAGE and protein was stained with Coomassie blue. Ubc9 concentration was estimated as 3 µg/µl.

1.1.2. Coupling of recombinant Ubc9 to cyanbromide-activated sepharose

1mg of the recombinant Ubc9 was coupled to cyanbromide-activated sepharose which finally served as the matrix for antibody purification. Binding of Ubc9 to the beads was verified by investigating aliquots of beads before ("beads before") and after Ubc9 binding ("beads after") as well as testing the flow through after binding ("flow through"). Samples were analyzed by separation on SDS PAGE followed by protein staining with Coomassie brilliant blue. As demonstrated in Figure 6, recombinant Ubc9 was saturated on beads since the flow through fraction contained low Ubc9 levels.

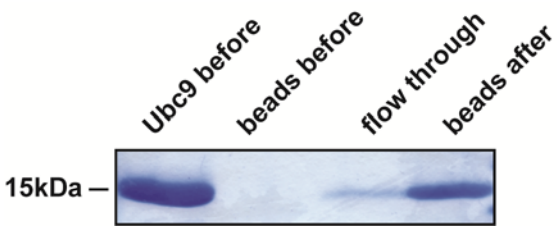

Figure 6: Coupling of recombinant Ubc9 to cyanbromide-activated sepharose. Ubc9 binding was followed by analysis of beads ("beads before") and after Ubc9 binding ("beads after"). Ubc9 depletion was analysed before (Ubc9 before) and after binding (flow through) to the sepharose. Samples were separated by 12.5% SDS PAGE and protein was stained with Coomassie brilliant blue.

3.1.2 Affinity purification of anti-Ubc9 specific antibodies

Affinity purification is performed to obtain antigen specific antibodies with high reduction in unspecific background binding. Such antibodies are favored for immunoblotting and immunofluorescence analysis. Therefore, the serum from a goat immunized with human Ubc9 as antigen, was incubated with the recombinant Ubc9 bound to sepharose.

Elution was done with acidic acid in 1ml fractions, which were immediately neutralized and tested for their protein content by spotting small aliquots on a nitrocellulose membrane. Protein containing fractions were stained with Ponceau S (data not shown), pooled, concentrated to a volume of approximately 500µl and buffer exchanged. Aliquotes were stored for further usage at -20°C.

3.1.3 Specificity and sensitivity of affinity purified anti-Ubc9 antibodies in immunoblot analysis

To determine the specificity and sensitivity of the newly purified antibodies different dilutions were tested on crude cell lysates from NIH3T3 mouse fibroblasts: 12µg of these protein extracts were separated on a 12.5% SDS PAGE and immunoblotted with indicated concentrations of the affinity purified anti-Ubc9 antibodies. As shown in Figure 7, antibody dilutions up to 1:4000 resulted in detection of a single band at approximately 18 kDa, the expected size for Ubc9. Also longer exposures exceeding 10 minutes did not indicate any significant background staining (data not shown)

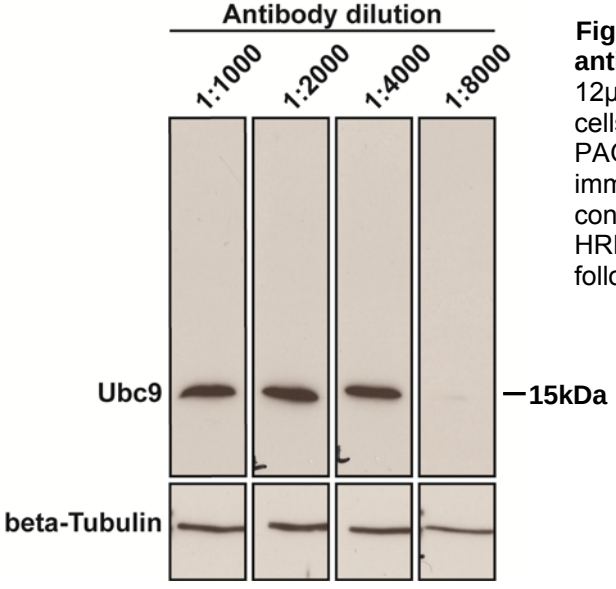

Figure 7: Affinity purified anti-Ubc9 antibodies in immunoblot analysis: 12µg crude cell lysates of NIH 3T3 cells were separated on 12.5% SDS PAGE. Ubc9 was detected by immunoblotting with indicated antibody concentrations. Detection was with HRP-labelled secondary antibodies followed by chemiluminescence.

In summary, the new affinity purified Ubc9 antibodies worked nicely in the detection of endogenous Ubc9 in cell extracts.

3.1.4 Specificity and sensitivity of affinity purified anti-Ubc9 antibodies in immunofluorescence analysis

Since the affinity purified antibodies specifically recognized Ubc9 in immunoblot analysis the next question was whether they can also be used for immunofluorescence analysis.

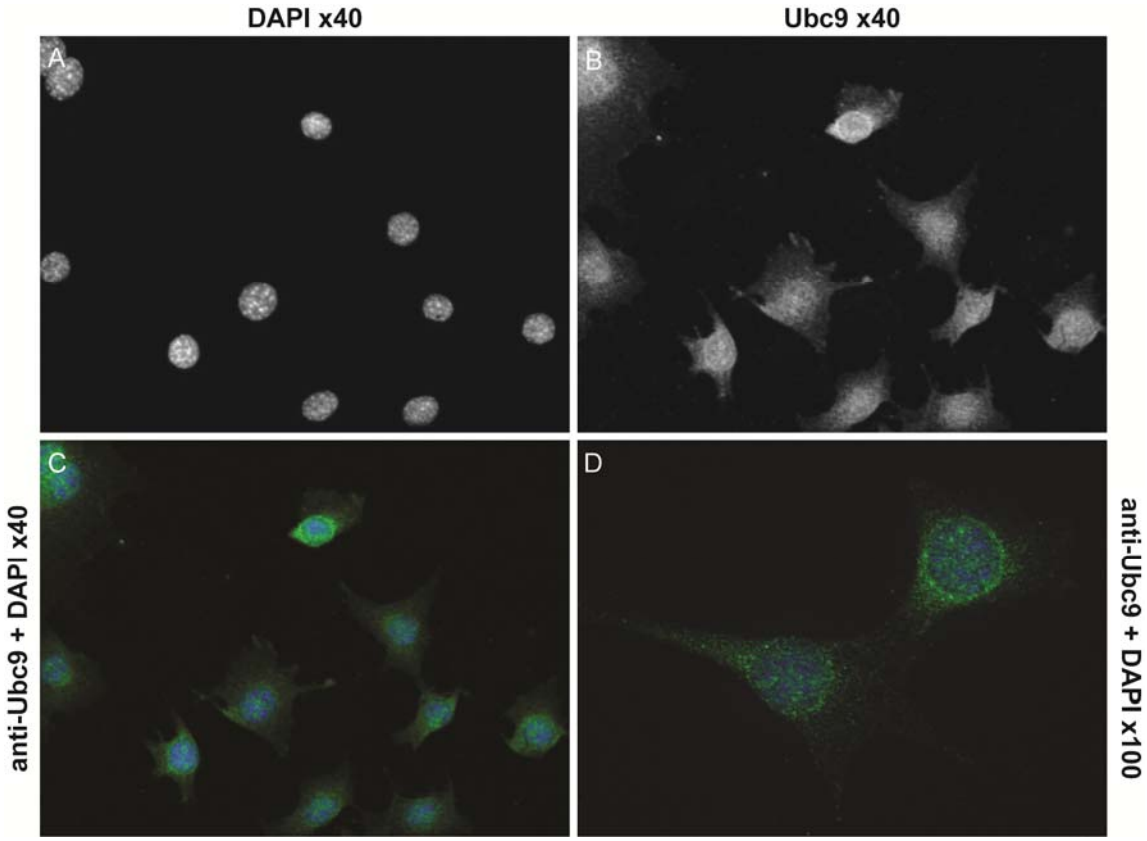

Figure 8: Affinity purified anti-Ubc9 antibodies in immunofluorescence analysis. NIH 3T3 were fixed with 2% Formaldehyde/PBS and stained with anti-Ubc9 antibodies (1:100). Detection was accomplished with an Alexa Fluor®-488-anti-goat secondary antibody. DNA was stained with DAPI. Pictures were taken with Zeiss Axiovert microscope. Indicated magnification of 40x and 100x.

NIH 3T3 fibroblasts were fixed with 2% Formaldehyde in PBS. As for immunoblotting also for immunofluorescence different primary antibody dilutions were tested in various experiments including 1:100, 1:200, 1:400, 1:500, 1:800,

1:1000 and 1:1600. As secondary antibody an Alexa Fluor® 488-anti-goat-fluorophore-coupled donkey-antibody in a 1:1000 dilution was used. Antibody dilutions lower than 1:100 showed very weak and diffuse staining without any distinct sub-cellular localization. Since this staining was not obtained with the secondary antibody alone it is suggested as background staining from the primary antibody. Also at the highest antibody concentration (1:100) the staining was very weak and nearly at the detection limit for the Zeiss Axiovert camera used.

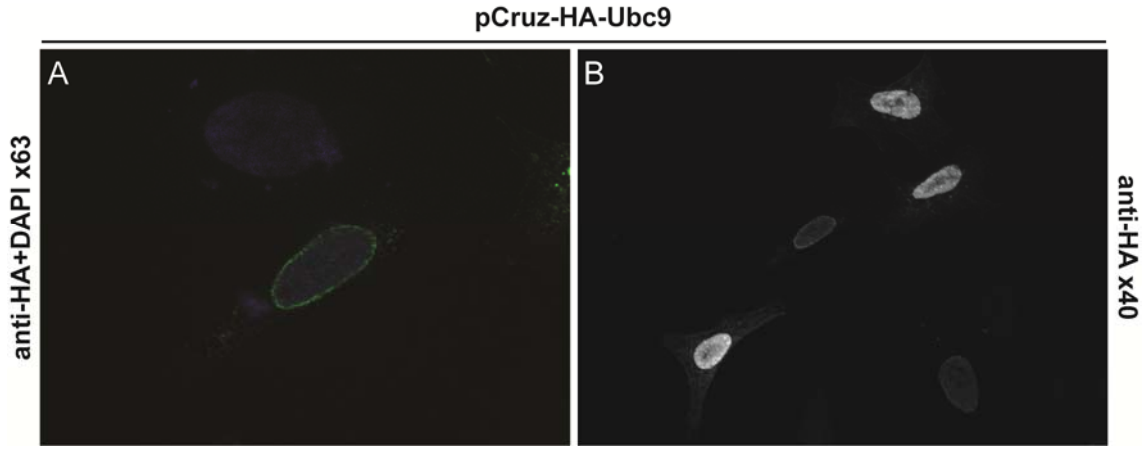

Figure 9: Transient transfection of Hela cells with pCruz HA-Ubc9. Hela were fixed with 2% Formaldehyde/PBS and stained with anti HA monoclonal antibody (1:1000) 36h after transfection. Detection accomplished with an Alexa Fluor®-488-anti-mouse secondary antibody. DNA was stained with DAPI. Pictures were taken with Zeiss Axiovert microscope. Indicated magnifications 63x (A merge: anti-HA+DAPI) and 40x (B:anti-HA)

At the highest magnification of the device (Figure 8 D) Ubc9 showed a faint staining in the cytoplasm with a slight enrichment in the nucleus and at the nuclear envelope. In earlier studies in HS68 human diploid fibroblasts Ubc9 levels were much more pronounced in the nucleus compared to the cytoplasm[75]. This suggests that either Ubc9 expression differs between mice and human fibroblasts or that Ubc9 is expressed at levels below the detection limit for the newly purified antibody. To distinguish between these scenarios a HA (human influenza hemiglutinin) tagged version of Ubc9 was transiently expressed and immunofluorescent staining was performed with a monoclonal anti-HA antibody recognizing the tag. Whereas this antibody showed a distinct staining as

presented in Figure 9, the staining with the purified anti-Ubc9 antibodies again was too weak for taking comparative pictures. This initial immunofluorescence analysis of the affinity purified anti-Ubc9 antibodies did not lead to conclusive results and more conditions (fixing, higher antibody concentrations) will need to be tested.

3.2 Ubc9 overexpression in NIH 3T3 mouse fibroblasts

The generation of an inducible stable Ubc9 overexpression cell line was the central part of this research project. Creating such a system aimed to establish a model for high Ubc9 expression, as it was often found in cancer tissues of various origins. Concomitant with elevated Ubc9 expression, tumor cells acquired crucial properties for tumor survival, as for instance increased metastasis and evading chemotherapy. Thereby, the inducible Ubc9 overexpression aimed to study whether the elevated Ubc9 level is a driver for tumor formation or rather a secondary hit in progressed cancer stages. To answer that question, the Tet-On® 3G expression system was used. That system was reported to have a sensitive responsiveness to the expression-activating tetracycline-derivate Doxycycline (Dox) with only low baseline expression levels. At that time it was the newest version of Tet systems which is also applicable for related mice studies planned in the future. There the addition of Dox to the drinking water would induce transgene expression.

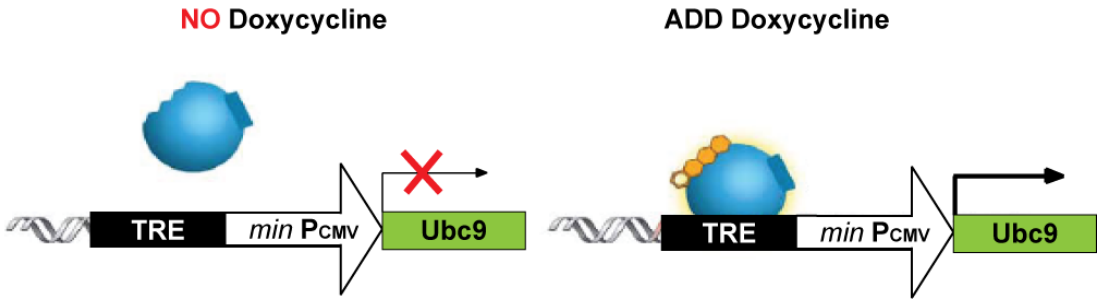

Figure 10: Tet-on 3G Gene Induction: The transactivator protein (blue) undergoes a conformational change in the presence of Doxycycline which causes its binding to the Tet responsive element (black; TRE) consisting out of multiple copies of the signal sequence for the Tet protein. This in turn induces the transcription of Ubc9 downstream of a minimal Cytomegalovirus promoter (white).

In the Tet-On® 3G system a stable cell line needed to be generated, which constitutively expressed a transactivator protein (TetA). In a second transfection the expression plasmid was introduced containing Ubc9 downstream of the TetA-responsive promoter. In the presence of Dox, TetA underwent a conformational change and could only then bind to repetitive sequences (TRE) upstream of the responsive promoter. By that, the minimal CMV promoter was activated and transcription of Ubc9 was induced (Figure 12).

3.2.1 Generation of stable inducible cell lines using the Tet transactivator system

3.2.1.1 Tet Transactivator expression analysis by immunoblotting

The prerequisite for inducible Ubc9 overexpression was the generation of a cell line expressing TetA. After transfection of the pCMV-TetA plasmid cells were selected for resistance to G418. That was due to the co-expression of the antibiotic resistance marker *neo*^{r} on the transfected plasmid. G418 is also named Geneticin® and inhibits the 80S ribosomal subunit essential for the protein translation of eukaryotic cells. Expression of the *neo*^{r} gene protects the cells from the antibiotic action of G418. [76] That *neo*^{r} gene is placed downstream of an SV40 early promoter on the pCMV-Tet3G plasmid (Clontech). Seven clones were isolated and tested for TetA protein expression after induction with 1µg/ml Dox for 48h and immunoblotting of 20µg total cell lysates.

Wild type NIH 3T3 cells were included to monitor any physiological deviation caused by the applied concentration of Dox in the growth medium and as negative control for TetA expression. To test the impact of Dox on TetA expression lysates from induced as well as un-induced TetA cells were compared. Cells denoted clone 7 was the only clone out of the seven resistant clones that produced a high level of the transactivator protein (Figure 11).

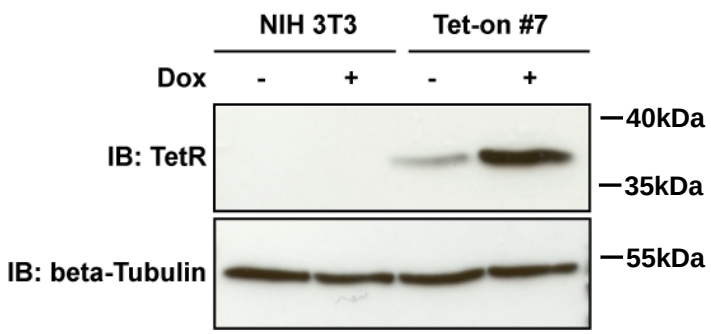

Figure 11: Dox-inducible expression of the transactivator protein. NIH 3T3 cells transfected for TetA protein expression after selection by G418. 20µg of total cell lysate were separated on 12.5% SDS PAGE. Ubc9 was detected by immunoblotting with 1:500 commercial monoclonal mouse TetR antibody Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. Cells were incubated with or without 1µg/ml Doxycycline (Dox) for 48h. Beta-Tubulin as loading control confirms similar protein amounts.

3.2.1.2 Functional dual luciferase reporter gene assay

A dual reporter gene assay was used to assess the capability of the isolated TetA clones to induce gene expression measured with the Firefly Luciferase reporter gene. The screening of a reporter gene like Luciferase was easier to measure than the TetA expression and much more sensitive than immunoblotting. Moreover, it allowed the more accurate quantification of target gene induction than determining the ability to express Ubc9. The chemical Luciferase reaction produces light in a linear intensity over a wide range[77]. Due to its absence in the mouse genome, Luciferase does not produce any background noise from endogenous expression. Additionally, the co-transfection of a constitutive expression plasmid containing the Renilla Luciferase gene normalized the experiment in terms of varying transfection efficiency, protein concentration and any pipetting errors. That was because chemical structures of firefly (*Photinus pyralis*) and Renilla Luciferase (*Renilla reniformis*) molecules are different which results in different substrate requirements. So, it was possible to measure them sequentially.

The readout of the Luciferase activity confirmed the initial screen of the clones by the immunoblot giving the by far highest Luciferase expression for the new TetA clone 7 cells (Figure 12, red bar). A previously established Tet-on cell line was included in that experiment, because they failed to produce detectable protein amounts when used as positive control in the immunoblot before. Its potential to induce Luciferase expression after multiple cell passages (green bar) was also

lower than for the early passage of the actual TetA cells. Their Luciferase gene expression represents less than 10% of the new clone 7 (87.6 versus 931.5 fold induction). Besides that, only clone 2 showed some rather low induced expression of Luciferase (70.6 fold induction) whereas all other clones did not exceed a 10-fold induction relative to the negative control.

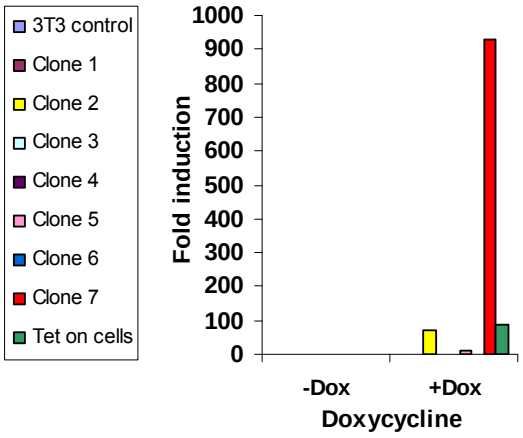

Figure 12: Induction of the Luciferase reporter gene. Seven NIH 3T3 clones stably transfected with pCMV-TetA for the constitutive transactivator expression were transiently transfected with a pTre3G-Luciferase plasmid and assayed for induction of Luciferase. The cells were induced with 3µg/ml Doxycycline (Dox) for 24h and then analysed for Luciferase activity which is proportional to induced gene expression. Clone 7 performed best compared to all other clones. Former established NIH 3T3 Tet-on cells were used as positive control.

Moreover, concentrations above 1µg/ml Doxycycline did not elevate the induced expression level of Luciferase. Higher Dox concentrations thereby rather decreased than increase the fold induction of Luciferase (not shown).
The evaluation of TetA protein expression by immunoblotting and the level of Luciferase reporter gene induction qualified clone 7 for the generation of a stable Ubc9 inducible cell line.

3.2.1.3 Transient Ubc9 overexpression

To test the potential to induce Ubc9 cloned into the pTre3G-Ubc9 expression plasmid it was transiently expressed in the established stabe TetA expressing cell line. Cells were transfected with pTre3G-Ubc9 in medium containing or not containing Dox which was added together with the Fugene transfection reagent for 48h hours. Cells were lysed in SDS buffer and 10µg of total cell lysates from Dox-induced and non-induced cells were compared by immunoblotting after separation on SDS-PAGE. As shown in Figure 13, in the presence of 1µg/ml Dox for 48h Ubc9 expression was nicely induced compared to the non-induced endogenous Ubc9 levels (Figure 13). Of note, the Dox dependent induction of

Ubc9 is even higher since only approximately half of the cells were transfected as monitored by eGFP expression (not shown). That implies that the Ubc9 levels per cell are even higher as it appears in immunoblotting. In summary this results clearly show that Ubc9 expression can be nicely induced upon Dox induction in the stable TetA expressing cells.

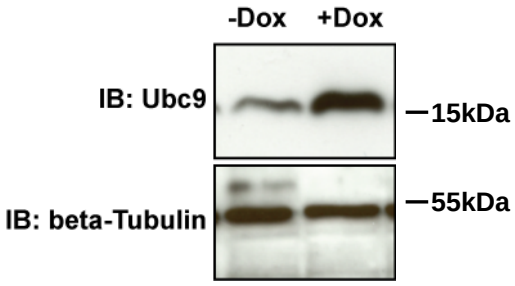

Figure 13: Transient transfection of NIH 3T3 TetA cells with pTre3G-Ubc9. NIH 3T3 TetA cells were transiently transfected with the pTre3G-Ubc9 plasmid and expression was induced with 1µg/ml Doxycyline for 48h. 10µg crude SDS-cell lysates were separated on 12.5% SDS PAGE. Ubc9 was detected by immunoblotting with Ubc9 antibodies (1:1000). Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. Beta-Tubulin confirms that similar protein amounts were loaded.

3.2.2 Establishing a stable Ubc9 inducible cell line

Since transient expression only targets approximately 50% of the cells and the levels of expression differ from cell to cell, the establishment of a homogeneous stable Ubc9 inducible cell line was conceived. Therefore, NIH 3T3 TetA cells were co-transfected with the pTre3G-Ubc9 and a linear Hygromycin restance marker required for selection of transfected cells. Hygromycin B is an aminocyclitol antibiotic inhibiting 70S and 80S large ribosomal subunits and is produced by the gram positive bacterium *Streptomyces hygroscopicus*. [78] The Hygromycin B phosphotransferase found in the same organism confers resistance to that antibiotic[79]. 30 hours after transfection, the cells were challenged with 150µg/ml Hygromycin B in the growth medium for approximately two weeks. While the untransfected cells are supposed to die during that time, cells which express both transfected plasmids are expected to form large resistant colonies. These colonies were individually isolated and separated in 24 well plates were they grew to at least 60% confluence. 20 clones were obtained after this procedure and subsequently analyzed by a genotyping PCR.

The PCR was designed to evaluate each clone for the integration of the pTre3G-sUbc9 expression plasmid into its genome. This step was necessary to exclude

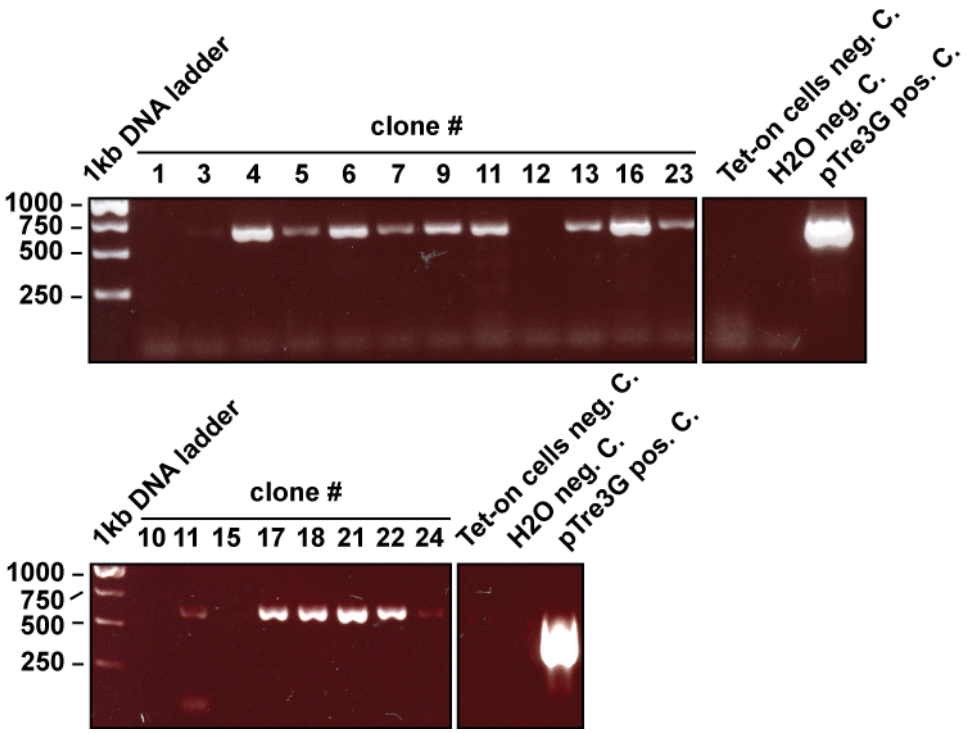

Figure 14: Genotyping PCR of stable NIH 3T3 TetA-pTre3G-Ubc9 clones. The genomic DNA of NIH 3T3 TetA cells transfected with pTre3G-Ubc9 plasmid and resistant to Hygromycin B was genotyped. Thereby, the primers enclosed the pTre3G promoter sequence at the 5'-end and the Ubc9 3' end. 10µl of the PCR reaction were separated on 2% agarose gels and detection with UV-light and gel red. Negative clones were excluded from further analysis. The negative controls were TetA cells which were not transfected, the H_2O control contained water instead of DNA. pTre3G was the positive control which contained 10ng of pTre3G-Ubc9 expression plasmid.

false positive clones which integrated only the linear Hygromycin resistance gene, but not the circular plasmid for conditional Ubc9 expression. For PCR analysis the genomic DNA of each cell line was extracted and 500ng/µl or less was amplified with primers enclosing the 5' promoter sequence of the pTre3G plasmid and the Ubc9 3' end to distinguish the endogenous from the exogenous Ubc9.

As shown in Figure 14 indeed 16 out of 20 clones integrated the pTre3G-Ubc9 as detected by PCR amplification and subsequent agarose gel electrophoresis (Figure 14). In the next step all positive pTre3G-Ubc9 positive clones were further tested for their ability to inducibly express Ubc9 after induction with Dox. The cell lines with the strongest signals in the PCR reaction were the most promising candidates for inducible Ubc9 expression.

3.2.3 Inducible Ubc9 expression in stable NIH 3T3 TetA-pTre3G-Ubc9 cell lines

After genotyping, only clones which had integrated the Ubc9 expression plasmid into their genome were tested for inducible Ubc9 overexpression. The biological function of Ubc9 was expected to be completely retained since an untagged human Ubc9 sequence was cloned into the pTre3G plasmid. The human and the murine Ubc9 genes share 93% sequence homology when aligned in a BLAST, whereas the amino acid sequences are 100% identical. In order to evaluate the cell lines for their Ubc9 protein expression levels, all NIH 3T3 pTre3G clones were induced with 1 to 3µg/ml Dox for 24 to 96 hours. After that treatment cells were lysed in SDS buffer and separated on SDS PAGE. Analysis was by

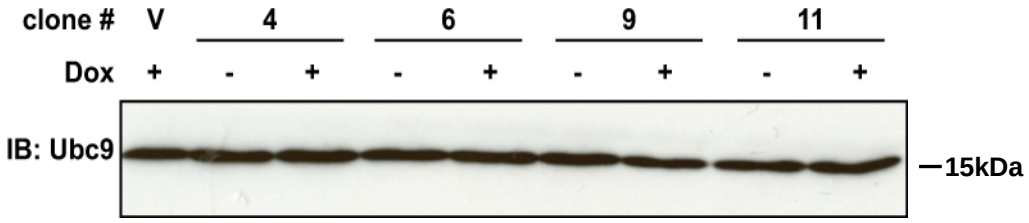

Figure 15: Inducible Ubc9 expression in NIH 3T3 TetA clones. Stable clones #4, 6, 9 and 11, positive for pTre3G-Ubc9 in geneotyping were treated as indicated with 1µg/ml Doxycycline (Dox) for 48 hours. were tested for induced Ubc9 expression. 10µg crude SDS. cell lysates were separated on 12.5% SDS PAGE. Ubc9 was detected by immunoblotting with Ubc9 antibodies (1:1000). Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. V: cells stably transfected with the pTre3G backbone was used as negative control

immunoblotting with anti-Ubc9 anitbodies. Figure 15 is representative for all clones which had been positive for the pTre3G-Ubc9 in PCR genotyping. In the example presented, clone 4, clone 6, clone 9 and clone 11 were evaluated for their Ubc9 overexpression after induction with 1µg/ml Dox for 48h. The cell lysates were compared to an equally induced stable empty vector control clone (V) which was transfected with the pTre3G backbone.

Disappointingly, not a single of the pTre3G–Ubc9 positive clones showed increased Ubc9 levels after Dox induction at any tested condition. In summary, although being resistant to Hygromycin as well as having the Ubc9 expression plasmid integrated into the genome, none of the clones was inducible for Ubc9 overexpression. This data clearly indicate that the tet- inducible system is not

suitable to obtain inducible Ubc9-overexpressing cells and other strategies have to be established.

3.3 Ubc9 Knock Down

The approach to transiently knock down Ubc9 was an alternative attempt to deregulate Ubc9 and to investigate changes in heterochromatin. Due to the fact that SUMOylation was found to be a modification of enzymes responsible for repressive chromatin modifications the knock down of Ubc9 was hypothesized to result in changes of heterochromatin[57, 66, 105].

3.3.1 Individual siRNA sequences differentially reduce Ubc9

In that experiment, the fundamental set up for the Ubc9 knock down was evaluated by immunoblotting (Figure 16). NIH 3T3 cells were separately transfected with three different siRNAs targeting different exons and a control pool of non targeting RNAs (scrambled control, Sc). All three individual siRNAs reduced endogenous Ubc9 levels, although at different levels. The best result was obtained with siRNA1. However, the pool of all three specific Ubc9 siRNAs was even a bit more efficient. Ubc9 reduction was already observed after 24h (not shown) but the best reduction was obtained after 48h.

In summary, treatment of a pool of Ubc9 targeting siRNAs for 48 hours was established as standard method for all further knock down experiments.

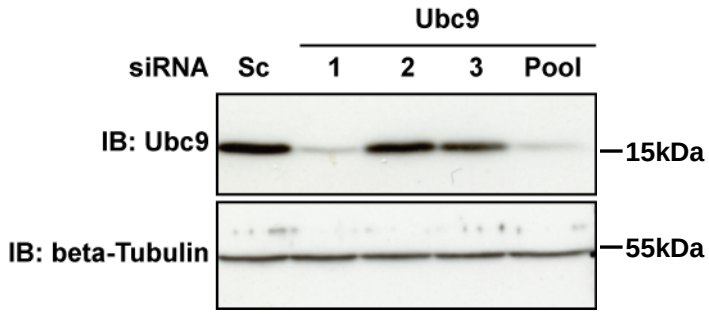

Figure 16: Ubc9 knock down in NIH 3T3 cells with 3 siRNAs. Cells were transfected with three individual Ubc9 targeting siRNA sequences for 48h. Each sequence was compared to the pool consisting of all three siRNAs of equal concentration. Immunoblotting of 10µg crude cell lysates of NIH 3T3 cells were separated on 12.5% SDS PAGE. Ubc9 was detected by immunoblotting with Ubc9 antibodies (1:1000). Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. siRNA: small interfering RNA, Sc: Scrambled control, pool of non-targeting siRNAs

3.3.2 Ubc9 is knocked down for 72h

The next question asked was when Ubc9 will reappear after the RNAi knock down. Determination of the time frame of Ubc9 depletion was crucial for further analysis to investigate changes in the Histone methylation. Thus, the Ubc9 knock down was investigated in a time course for five days.10µg protein samples of total cell lysates were generated after 24h, 48h, 72h, 96h and 120h. In parallel, the cells were monitored in the light microscope for any differences in morphology and growth.

Immunoblotting analysis indicated that up to 72 hours, Ubc9 was not detectable and slowly appeared after 96 hours (Figure 17). Analysis in light microscopy indicated that cells treated with Ubc9 siRNAs showed an increase in rounded and dead cells starting from day two onwards compared to the normal growing cells treated with scrambled siRNAs. This finding is in line with earlier studies reporting that Ubc9 is essential for mammalian cell growth[37].

In summary, a siRNA mediated Ubc9 knock down was successfully established was with a pool of three siRNAs in NIH3T3 cells. So, this method was then further used to study the consequences of Ubc9 deregulation on heterochromatin formation.

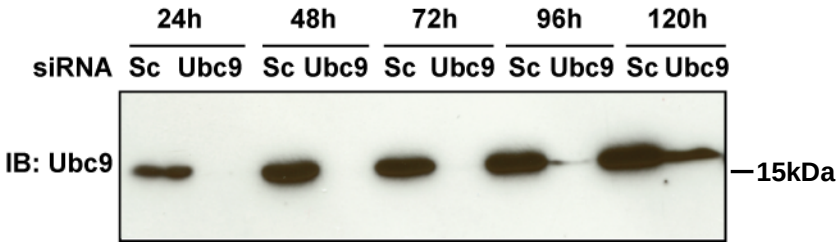

Figure 17: Time course of Ubc9 knock down. 10µg. crude cell lysates of NIH 3T3 cells were separated on 12.5% SDS PAGE. Ubc9 was detected by immunoblotting with Ubc9 antibodies (1:1000). Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. siRNA: small interfering RNA, Sc: Scrambled control

3.3.3 Histone Methylation changes in Ubc9 Knock down cells

Different studies indicated that the sumoylation machinery is implicated in the formation of heterochromatin-like structures in euchromatic loci[64, 66]. The feature of such facultative heterochromatin is characterized by low levels of activating histone marks like histone acetylation and histone H3K4 methylation but high

levels of repressing marks including DNA methylation and H3K27 methylation, H3K9 di-and trimethylation and H3K20 mono-, di- and trimethylation[113]. The aim of this work was to analyse if changes in heterochromatin specific histone marks can be detected after Ubc9 knock down.

3.3.3.1 Preparative histone acid extraction

To test whether histone methylation is affected in Ubc9 knock down cells, first global changes of various histone methylations by immunoblotting of purified histones were investigated. Because of their net positive charge histones can be purified by acid extraction of cell nuclei. Determination of the histone concentration was done by visual comparison of different dilutions of purified histone extracts to defined concentrations of alpha-lactalbumin separated on SDS PAGE and stained with coomassie brilliant blue (Figure 18). That assay allowed not only the quantification of the individual histone concentrations, but also provided information of the quality of the histone preparation.

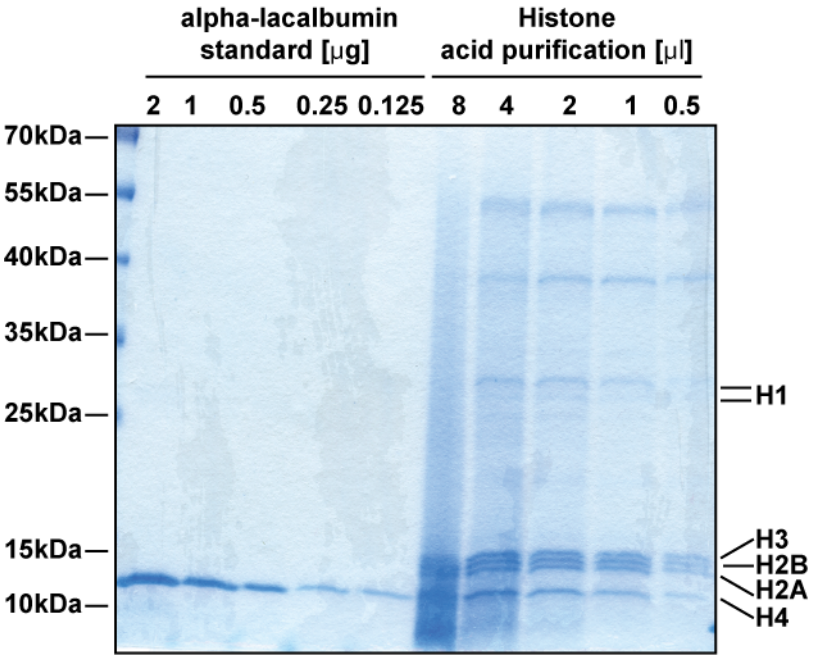

Figure 18: Concentration determination and purity test of Histone acid extracts. Histones were purified from NIH 3T3 cells treated with Ubc9 siRNAs for 48h Ubc9 siRNA knock down cells by extraction with hydrochloric acid and compared to a defined concentration of α-lacalbumin standards by SDS PAGE. Detection was by staining with Coomassie brilliant blue. H: histone

After establishing that preparative method global changes of the histone methylation in Ubc9 knock down cells could be subsequently studied by immunoblotting.

3.3.3.2 Analysis of histone methylation of NIH 3T3 cells upon Ubc9 knock down

To gain insights into the consequences of Ubc9 down regulation on heterochromatin formation, NIH 3T3 cells were transfected with either Ubc9 targeting or scrambled siRNAs for 48 hours. Analysis was done with a variety of antibodies detecting specific repressive histone methylation marks[80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90], as shown in Figure 19. To confirm that equal concentrations of histones were loaded to the gel lanes, an antibody recognizing the histone H3 core was used. Under conditions where Ubc9 was significantly reduced no difference could be detected for mono-, di- and trimethylation of the histone H3 at lysine 9 and lysine 27 (H3K9, H3K27). Monomethylation of histone H4 at lysine 20 seemed slightly enhanced in Ubc9 knock down. In contrast, the enhancement of histone H4 di- and tri-methylation at lysine 20 (H4K20me2, H4K20me3) in histones extracted from Ubc9 knock down cells was much more pronounced.

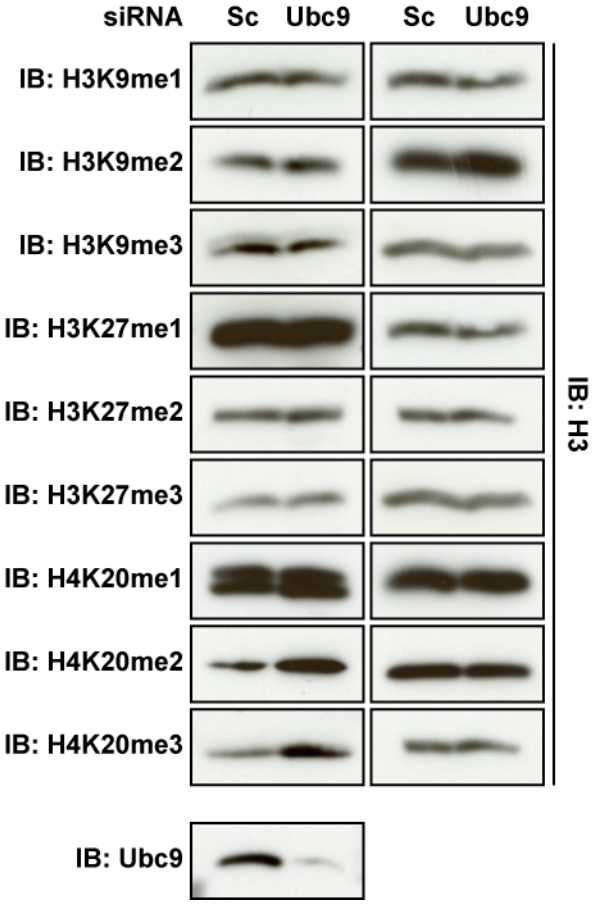

Figure 19: Immunoblot analysis of histone lysine methylation: Equal concentrations of histone acid extracts from NIH 3T3 cells transfected with Ubc9 or scrambled (Sc) siRNAs were separated on SDS PAGE. Immunoblotting was with indicated antibody (1:1000). Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. Knock down was controlled by immunoblotting with anti-Ubc9 antibodies.

Together studying different repressive heterochromatin marks after Ubc9 knock down indicated increased levels of H4K20 mono-, di- and trimethylation whereas all different stages of H3K9 and H3K27 methylation did not change.

3.3.3.3 Analysis of pericentric heterochromatin changes upon Ubc9 knock down in NIH 3T3 cells

Another method to study general changes in heterochromatin is by immunofluorescence analysis. Pericentric heterochromatin, a major repressive chromatin mark can be visualized as DAPI-dense foci in interphase nuclei of NIH3T3 cells. Different repressive chromatin marks are enriched at pericentric heterochromatin including DNA methylation, H3K9m3 and H4K20m3.

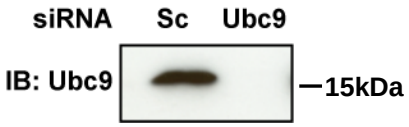

Figure 20: Ubc9 knock down in microscopy chamber wells. Cells were transfected with Ubc9 or scrambled (Sc) siRNA pool for 48h. Each Immunoblotting of 10µg crude cell lysates of NIH 3T3 cells were separated on SDS PAGE. Ubc9 was detected by immunoblotting Ubc9 antibodies (1:1000). Detection was with HRP-labelled secondary antibodies followed by chemiluminescence. siRNA: small interfering RNA

Since downregulation of Ubc9 indicated unchanged H3K9m3 levels but increased H4K20m3 levels in immunoblot analysis these studies were continued with the analysis of pericentric heterochromatin in immunofluorescence stainings. Therefore, NIH 3T3 cells were transfected for 48 hours in microscopy chamber slides with either the pool of siRNAs specific for the Ubc9 knock down or with scrambled siRNAs, as control. The transfections were performed in quartets to confirm on one hand the efficiency of the knock down and on the other hand to analyze the cells for the two prominent pericentric heterochromatin marks H3K9m3 and H4K20m3 in immunofluorescence stainings. The knock down of Ubc9 in the microscopy chambers was verified by separating whole protein from curde cell lysates on a 12.5% SDS PAGE gel and immunoblotting was with Ubc9 antibodies (Figure 20). For immunofluorescence analysis cells were fixed with 2% Formaldehyde and stained with antibodies raised in rabbits specific for the H3K9me3- or the H4K20me3-antigens. Detection was done with a fluorescent labelled Alexa Fluor®-488-anti-rabbit antibody. In the first wash after the

secondary antibody DAPI was added to the washing buffer to stain the DNA. This allowed the detection of highly condensed DNA enriched in foci which indicate pericentric heterochromatin. Slides were finally mounted with DAKO mounting medium.

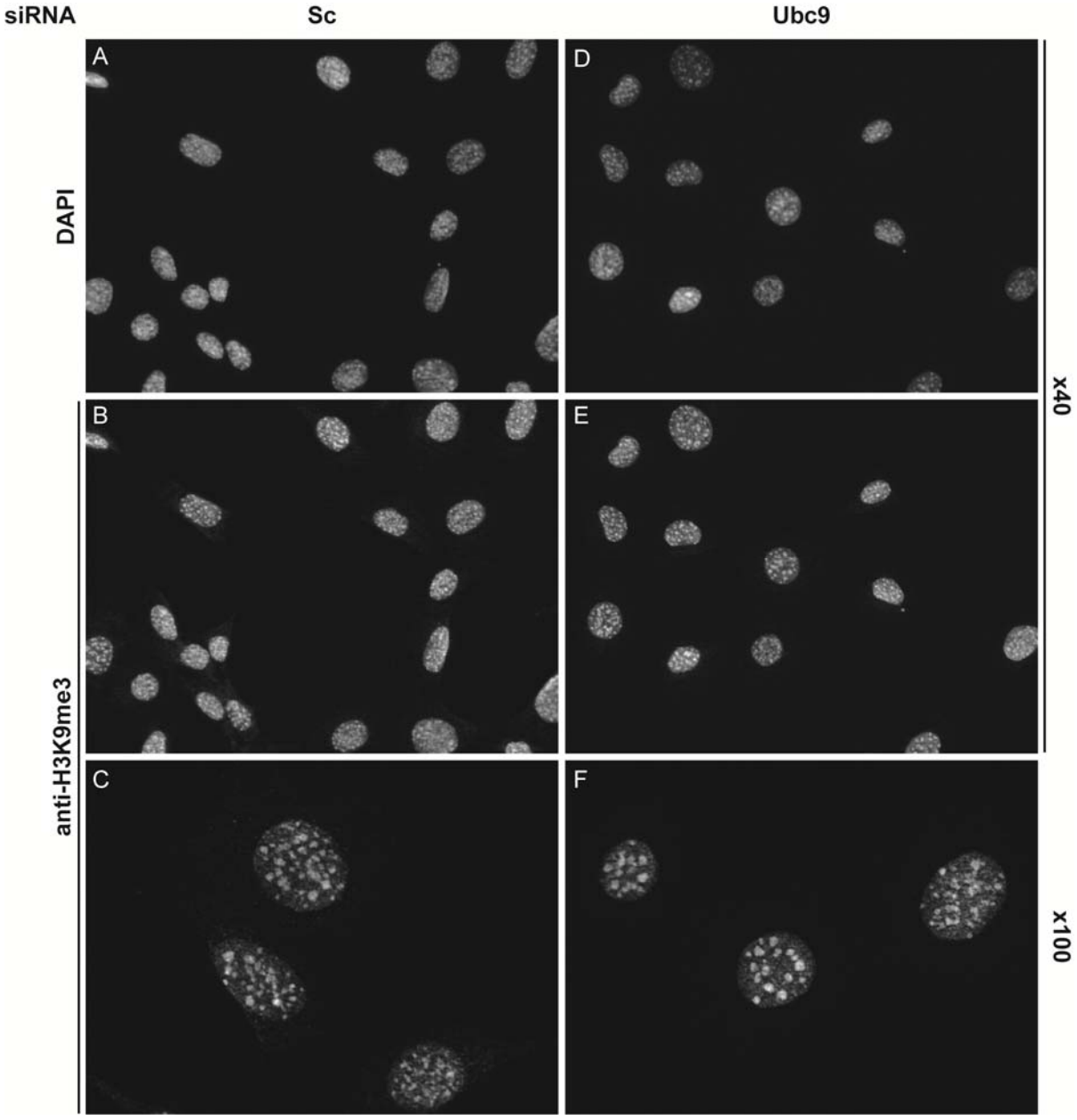

Figure 21: Immunofluorescence staining of H3K9me3 in 48h NIH 3T3 cells upon Ubc9 knock down: Cells were fixed with 2% Formaldehyde/PBS and stained with anti H3K9me3 antibodies (1:1000). Detection was accomplished with an Alexa Fluor®-488-anti-rabbit secondary antibody. DNA was stained with DAPI. Pictures were taken with Zeiss Axiovert Apotome microscope at magnification of 40x and 100x, as indicated.A: DAPI of scrambled control (Sc), B,C: H3K9me3 staining of scrambled control in 40x (B) and 100x magnifications (C), D: DAPI of Ubc9 knock down, E,F: H3K9me3 Ubc9 knock down in 40x (E) and 100x (F) magnifications.

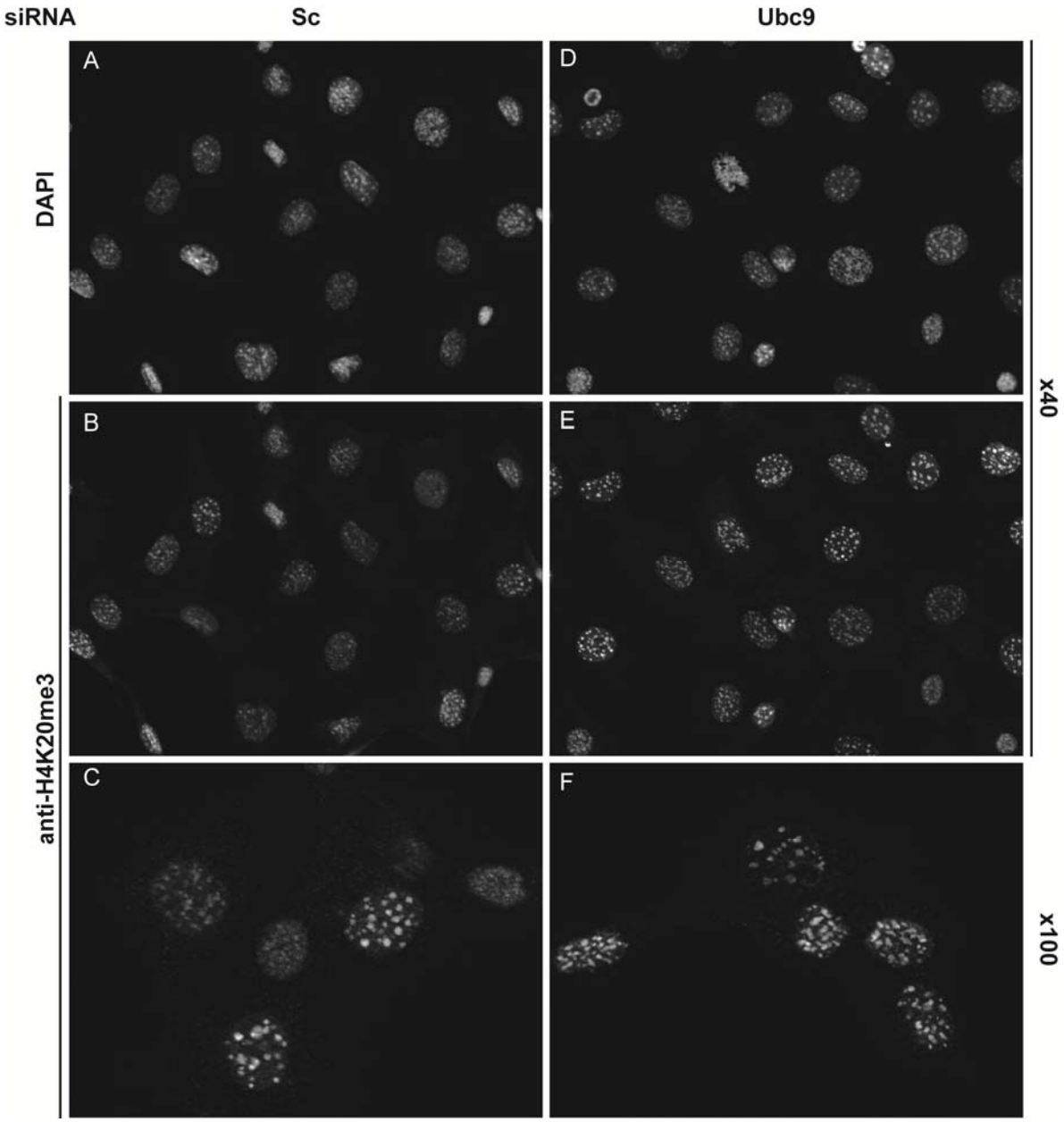

Figure 22: Immunofluorescence staining of H4K20me3 in 48h NIH 3T3 cells upon Ubc9 knock down cells: Cells were fixed with 2% Formaldehyde/PBS and stained with anti H4K20me3 antibodies (1:1000). Detection was accomplished with an Alexa Fluor®-488-anti-rabbit secondary antibody. DNA was stained with DAPI. Pictures were taken with Zeiss Axiovert Apotome microscope at magnification of 40x and 100x, as indicated.A: DAPI of scrambled control (Sc), B,C: H4K20me3 staining of scrambled control in 40x (B) and 100x magnifications (C), D: DAPI of Ubc9 knock down, E,F: H4K20me3 Ubc9 knock down in 40x (E) and 100x (F) magnifications.

Images were taken in 40-times and 100-times magnifications with the Zeiss Axiovert Apotome Imagining microscope. Equal exposure times were applied to normalize for the intensity of the stainings. The Apotome device mimicked a

confocal scanning of the sample by applying a static as well as a moving optical grid. Thus, the software eliminated the scattered light which was not in the focal plane.

In agreement with the immunoblot analysis also the immunofluorescence staining of H3K9me3 did not reveal any significant difference in heterochromatin formation in cells upon Ubc9 knock down compared to the control cells (Figure 21). The foci that are indicative for densely packed heterochromatin remained unaltered in their number and intensity when Ubc9 was knocked down.

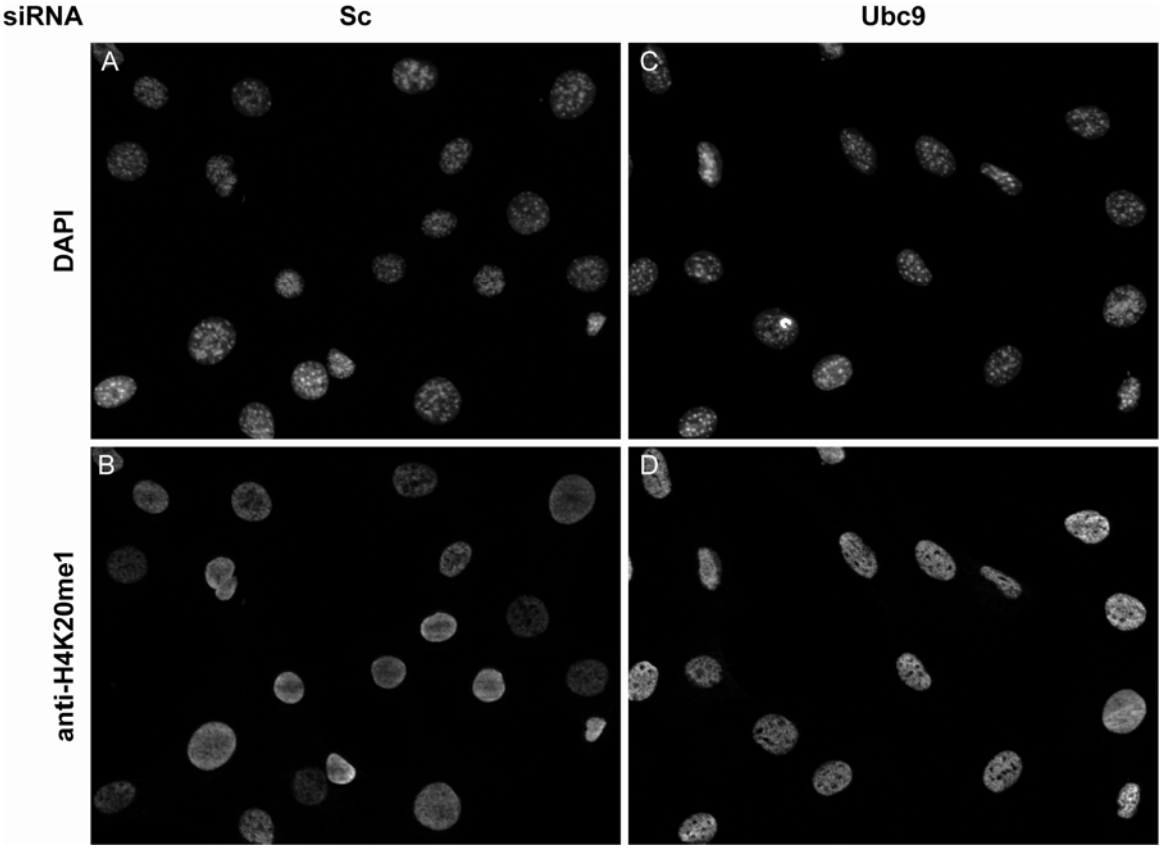

Figure 23: Immunofluorescence staining of H4K20me1 in 48h NIH 3T3 cells upon Ubc9 knock down: Cells were fixed with 2% Formaldehyde/PBS and stained with anti H4K20me1 antibodies (1:1000). Detection was accomplished with an Alexa Fluor®-488-anti-rabbit secondary antibody. DNA was stained with DAPI. Pictures were taken with Zeiss Axiovert Apotome microscope at magnification of 40x. A: DAPI of scrambled control (Sc), B: H4K20me1 staining of scrambled control, C: DAPI of Ubc9 knock down, D: H4K20me1 Ubc9 knock down in 40x

Histone H4K20me3 was significantly increased upon Ubc9 knock down in immunoblot analysis which may result in changes in pericentric heterochromatin.

Therefore also H4K20me3 staining was performed as described above for H3K9me3. As shown in Figure 22, although the number and shape of heterochromatic foci did not change significantly in the cells upon Ubc9 knock down. It appears that more cells are showing higher intensity in H4K20me3 staining in the cells upon Ubc9 knock down compared to the control cells.

Ubc9 depletion results in a G2/M arrest of the cell cycle[109]. H4K20me1 is a highly dynamic chromatin mark which significantly changes during the different cell cycle phases. It appears in S-phase and is highly enrichment in G2/M and at meiotic chromosomes [91, 92]. Therefore, I also investigated H4K20me1 in NIH3T3 cells upon Ubc9 knock down. As it is demonstrated in Figure 21 the control cells show a very heterogeneous population of H4K20me1 positive cells whereas in the Ubc9 knock down all cells showed a strong enrichment in H4K20me1 staining confirming a Ubc9 depletion induced G/2M arrest.

In conclusion, immunofluorescence analysis of Ubc9 depleted cells confirmed a significant enrichment in H3K9me3 modification but did not indicate a difference in DAPI dense foci number or foci shape. Also the cell cycle regulated modification, H4K20me1 was highly enriched, that Ubc9 depletion induced a cell cycle arrest at G2/M (Figure 22, Figure 23).

4 Discussion

Sumoylation is an essential posttranslational modification involved in most cellular pathways. SUMO is covalently linked to its substrates via a hierarchical enzymatic cascade involving one E1 activating, one E2 conjugating and several substrate specific E3 ligating enzymes. Although regulation is mainly performed by E3 ligases different studies point to an important role for the sole SUMO E2 enzyme Ubc9[93, 94]. Its upregulation promotes cell invasion and metastasis and is found in different types of cancers whereas its depletion is lethal in mice and results in severe chromosomal defects and major changes in the nuclear architecture[95, 96, 97, 98, 99, 100, 101, 102, 103].

In line, sumoylation was demonstrated to be implicated in the formation of heterochromatin, highly condensed chromosomal regions characterized by low levels of transcriptional activating histone marks (e.g. acetylation and histone H3K4 methylation) but high levels of repressing marks (e.g. DNA methylation and H3K27 methylation, H3K9 di-and trimethylation and H4K20 mono-, di- and trimethylation)[104, 105, 106, 107]. The aim of this work was to analyze if deregulation of Ubc9, either by overexpression or by down regulation, results in overall changes of such heterochromatin specific histone marks.

To study Ubc9 overexpression a Dox inducible system in NIH3T3 cells was established. Although several clones with the inserted plasmid were obtained, none of them showed inducible Ubc9 expression.

Analysis of Ubc9 down regulation mediated by a pool of three siRNAs resulted in significantly reduced Ubc9 levels in NIH3T3 cells. This system then allowed the analysis of diverse repressive heterochromatin marks by immunoblot and immunofluorescence studies. Initial immunoblot analysis did not indicate severe changes in mono-, di- and trimethylation of H3K9 and H3K27. Interestingly, an increase in all stages (mono-, di- and tri) of H4K20 methylation was obtained. The most severe difference was observed with H4K20me3 which was further investigated in immunofluorescence staining. Also with this approach, the increase in H4K20me3 was detected upon Ubc9 knock down but only the

intensity of foci representative for heterochromatin was enhanced rather than a change in shape or number.

Ubc9 knock down results in a G2/M cell cycle arrest and H4K20me1 fluctuates during the cell cycle with high enrichment in G2/M and at mitotic chromosomes[108]. Therefore, also this particular histone mark in immunoflourescence staining was investigated. Indeed, H4K20me1 is enriched in all cells upon Ubc9 knock down confirming a G2/M arrest. This finding raises the question whether Ubc9 down regulation is directly involved in H4K20me1 enrichment or whether it is just the consequence of the induced cell cycle arrest. Support for a direct role for Ubc9 in regulating H4K20me1 comes from a recent study demonstrating PR-Set7, the key enzyme for setting H4K20me1, as SUMO substrate[109].

The identification of increased H4K20 methylation upon Ubc9 down regulation strengthens the crosstalk between sumoylation and chromatin. The different stages of H4K20 methylation are involved in several biological pathways including transcription, DNA repair and heterochromatin formation[110]. These are all pathways in which sumoylation is also known to play a major regulatory function[111, 112, 113]. Therefore, it will be of key importance to further dissect the link between the regulation of the SUMO system and their consequences on chromatin.

5 Acknowledgements

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Thanks are also given to Dr. Thomas Jenuwein for providing the Histone methylation antibodies which made that study possible.

6 Materials and Methods

6.1 Amplification of plasmid DNA

All the plasmids used in this study had been already cloned and were taken from frozen stocks. These plasmids had been sequenced and used before by other scientists. Thus, in an initial working step personal plasmid stocks were produced. That was done by transformation of bacteria followed by purification of the plasmid DNA with a kit.

6.1.1 Heat shock transformation

For all Midipreps, competent (processed by the technical assistant, S Koidl) E.Coli DH5α cells were used. Therefore, 100µl of bacteria were thawed on ice, ~1.5µg of the respective plasmid was added, mixed by pipetting and incubated on ice for 30 min. After a heat shock at 42°C (Eppendorf Thermomixer Comfort) for 45s, bacteria were incubated on ice for 3 min. Next, 900µl of LB-medium (5g/l yeast extract, 5g/l NaCl, 10g/l bacto-tryptone, pH7, room temperature) were added, and the mix was transferred to 14ml bacterial growth tubes (Falcon) and incubated at 37°C with 170-200rpm shaking (New Brunswick Scientific Innova® 44) for 1h.

In the meantime, a petri dish with agar containing the appropriate antibiotic (Ampicillin: 100µg/ml, or Kanamycin: 50µg/ml) (for the corresponding resistance gene) on the plasmid were prewarmed at 37°C. Finally, bacteria were spun down at 4000rpm (~1500rcf) for 5 min and the supernatant was discarded. The pellet was resuspended in the remaining volumeand plated on the agar plate, distributed using a glass spreader and then incubated at 37°C over night.

The next day, a single colony was picked using an inoculation needle or a sterile pipette tip and transferred to a bacterial growth tube containing 5ml LB containing the same type and concentration of antibiotic. That starter culture was incubated at 37°C with shaking for 4 to 8h. After that, depending on the OD600 density of the culture, an aliquot or the whole starter culture was scaled up in 100ml of the same selective medium in a 300ml or larger Erlenmeyer flask.

For the pCruz plasmids, usually giving low yields, Chloramphenicol was added to the culture the next day for approximately 4h to block translation and thus to enhance plasmid synthesis.

6.1.2 Plasmid purification

For the large scale purification of plasmid-DNA, the PureLink™ Hipure Plasmid Filter Midiprep kit (Invitrogen) was used. The procedure was done according to the manufacturers' instructions with the following amendments: The cells were harvested for 15min by centrifugation (Eppendorf Centrifuge 5810R, rotor: A-4-81) instead of 10min, the optional washing step of the column filter cartridge was performed to increase the yield, the plasmid-DNA was precipitated using a 50ml instead of a 15ml Falcon tube, and centrifugation after isopropanol precipitation as well as after ethanol washing was done at 4000rpm (3113rcf - maximum speed) for 45-60min and 15min instead of $15x10^3$g for 30min or 10min, respectively.
The pellet was resuspended in 50-100µl TE-buffer provided in the kit and concentration was determined using the Nanodrop 1000 (Thermo Scientific). Subsequently, plasmids were stored at -20°C and always thawed and kept on ice prior to use.

6.2 Cell culture of NIH 3T3, NIH 3T3 Tet-On and NIH 3T3 Tet-off

6.2.1 Maintenance

The NIH 3T3 cell line (a generous gift from Thomas Jenuwein) was routinely maintained in 10 centimetre dishes (BD Falcon) at 37°C/5% CO_2 (Heracell 240i, Thermo Scientific) in complete medium: Dulbecco's Modified Eagles Medium (DMEM; Sigma Aldrich) supplemented with 10% FBS (Fetal bovine Serum) gold (PAA), 1x Glutamax (Gibco, 100x stock), 1xPenecillin/Streptomycin (Sigma Aldrich, 100x stock) and 1x Non-essential Amino acids (Sigma Aldrich, 100x stock). Microscopic evaluation of cells was done on a regular basis in 5x and/or 10x magnification (Zeiss, Axiovert 40 CFL) and cells were split in a ratio of maximum 1:8 every two to four days. Thereby they were exclusively handled in the laminar flow hood (Heraeus Herasafe K12, Thermo Scientific), where the medium was aspirated (Vacuu Hand Control, vacuubrand; house aeration),

cultures gently washed once with sterile prewarmed 1xPBS (PAA), chemically detached with 1ml of pre-warmed (Memmert UNB 400 heating cabinet) 1x Trypsin-EDTA (Sigma Aldrich, stock 10x) in 1xPBS for three to five minutes at 37°C/5% CO_2, which was then inactivated with 2ml of complete medium. After re-suspension of cells with the 1ml Pipette (Gilson, filter tips: greiner bio one), they were transferred to a 15ml Falcon tube and spun down at 300g (Heraeus Megafuge 16, Thermo Scientific, rotor: Thermo Scientific 75003629) for 5min. The pellet was resuspended in complete medium and the sufficient volume was added to a new 10cm dish containing five to eight millilitres (ml) of fresh, pre-warmed, complete medium.

The same procedure was applied to the NIH 3T3 Tet-On and the NIH 3T3 Tet-off cell lines except for the fact that the medium additionally contained 100µg/ml G418 (G418 disulfate biochemica, AppliChem, stock 1g lyophilized, diluted to working concentration of 10mg/ml in DMEM).

For freezing of cells, trypsinized cells were spun down again after passaging as described, to be then re-suspended in FBS containing 10% DMSO (Sigma). Thereof, 1ml aliquots were put into cryovials (Greiner-bio one) which were transferred to freezing containers (Nalgene) containing Isopropanol (Sigma Aldrich) and stored at -80°C (Sanyo MDF-U73V) for at least 24h. After that, they were shifted to -150°C (Sanyo MDF-1155) for long term storage. Cells were thawed rapidly in the 37°C waterbath (Stuart SBS40 or GFL 1008) and then immediately pelleted as mentioned above and re-suspended in complete medium. After plating them, the cells were given time to recover for at least two days.

6.2.2 Generation of stable cell lines

The Tet-on3G system for the inducible Ubc9 expression demanded a double-stable cell line. In a first transfection and selection a cell line was generated that stably expressed the transactivator protein as confirmed by immunoblot. The evaluation whether the expressed TetA level can induce a target gene, a Luciferase expressing plasmid was transiently transfected and expression was induced. The luminescent readout was directly proportional to gene induction. In a second step the best TetA cell line was used for the transfection with the

expression plasmid containing the TetA responsive promoter upstream of the gene of interest. After the selection, the resistant clones were screened by genotyping PCR and by immunoblotting of induced versus non-induced cell lysates.

6.2.2.1 Stable cell lines for Tet-On 3G transactivator protein expression

For the generation of stable cell lines expressing the transactivator (TetA), $1x10^5$ NIH 3T3 cells were seeded per well of a 6-well-plate (Corning Incorporation Costar) one day prior to transfection to yield a culture of approximately 60% confluency. The transfection was performed using the Polyfect reagent (Qiagen). The plasmid containing the gene for the TetA was the commercial pCMV-Tet3G plasmid from the Tet-On 3G inducible expression system (Clontech). For the transfection, the suggested optimized protocol of Polyfect was followed in terms of reagent to DNA ratio. So, for one well of a 6-well-plate, 1.5µg of plasmid well mixed by vortexing with 100µl of DMEM, followed by addition of 10µl of Polyfect were added. Again, the tube was mixed well by vortexing, spun down for 3s to avoid any drops at the sides or on the lid and then incubated for 30min at room temperature to allow complex formation. In the meantime, complete medium was aspirated, cells were gently washed with 1xPBS and 2.5ml of transfection medium was added, which did not contain Penicillin/Streptomycin. The culture was incubated at 37°C/5% CO_2 for 4h and subsequently the medium was aspirated and exchanged for complete growth medium. The transfected cells were then incubated until reaching 80-90% confluence (24h) and then passaged into a 10cm dish. After another 24h, the selective medium was added containing the sufficient concentration of 500µg/ml G418, which beforehand was assessed to kill non-resistant cells.

After densely packed colonies had formed they were isolated with cloning cylinders (polymer: Corning; glass: ZITT-THOMA GmbH Laborbedarf und Glasbläserei, Freiburg). Therefore, the position of the selected colonies was marked with a waterproof pen on the bottom of the dish under the light microscope, medium was aspirated, the bottom edge of a cloning cylinder was dipped into high vacuum grease (Corning) and placed as such to enclose and seal the area where the colony was situated. Thereby, it was crucial that neither

any of the grease infiltrated the enclosed area of the cylinder where the colony was located nor that there was an insufficient amount of grease which would cause a leaking of any of the 100µl 1xTrypsin-EDTA which was added immediately after placement of the cylinder. After that procedure had been conducted with all clones to be grown, the plate was incubated at 37°C/5% CO_2 for 4min. Then, 200µl of complete medium w/o antibiotics were added and the liquid was resuspended within the cylinder. The suspension was put into a well of a 12-well-plate containing 1ml of complete medium w/o antibiotics which was swirled gently after adding the cell suspension. Selective medium was added after 24h and when those cultures reached 70-80% confluence the cells were passaged into 6-well-plates and consequently after expansion finally into 10cm dishes. From there, back up freezes were done applying the procedure described in 6.2.1. The screening for the Tet-On 3G transactivator protein was performed in terms of protein expression and its potential to induce transgene expression (6.3.1).

6.2.2.2 Stable cell lines for inducible expression of wild type Ubc9

The TetA cells were then used to generate double positive cell lines constitutively expressing the transactivator as well as conditionally the Ubc9 transgene downstream of the TetR-responsive promoter. Again, Polyfect was used with the same protocol as described before for the transactivator except for some amendments.

Before that transfection was carried out, the concentration of Hygromycin B was assessed in non-transfected NIH 3T3-TetA cells. Thereby, the required lethal dose of that antibiotic was found to kill all non-resistant cells. The transfection was again performed in 6-well plates, a concentration of 2.5µg of pTre3G-Ubc9 plasmid was used, diluted with 150µl of DMEM and mixed with 15µl Polyfect. As there was no resistance gene present on the pTre3G-Ubc9 plasmid a linear selection marker had to be cotransfected in a concentration of 50ng per 1µg of plasmid DNA. The Hygromycin resistance gene was governed by a SV40 promoter and flanked by a SV40 polyadenylation signal at the 3' end. The transfection procedure was conducted equally as already described above. The

cloning of Ubc9 into the multiple cloning site of the pTre3G plasmid was done by the former lab member Yvonne Steuernagel.
For the selection of colonies, the 6-well was split in four 10cm dishes 24h post transfection, after a recovery time of 48h 150µg/ml Hygromycin B (stock 50mg/ml, Clontech) was added to the medium. After colony formation for about 12 days, many resistant large colonies had been formed, whereas a control culture dish without the linear Hygromycin resistancy marker suffered from 90% or more cell death. Subsequently, colonies were picked applying the same procedure as described before (6.2.2.1) and grown up in wells of a 24 well plate (Costar). After being split for the first time, one third of the cells was passaged on in another 24well and the other two thirds were spun down again and genotyped (6.4.1)

6.2.3 Knock down of Ubc9 Transient knock down of Ubc9 with siRNA

Seeding of NIH 3T3 cells was performed in accordingly selected plates one day prior to transfection to obtain 30-40% confluence at transfection. For the transfection, a pool of three siRNAs (Thermo Scientific) was used routinely. Sequences were chosen as such that all of them were specifically targeting different regions of the longest of three murine transcript variants of Ubc9 identified at NCBI Nucleotide database entry NM_001177609.1. The sequences were the following:
siRNA 1: CACUGGCAUAUUCAGUCUUUU
siRNA 2: CCAGAAAGGUGAAAGACUGUU
siRNA 3: CUGUGAUCUUAGGGCUUACUU
Each siRNA was re-suspended to 40µM diltions in nuclease-free water, aliquoted and stored in several working stocks at -20°C. Major stocks were preserved at -80°C. For the transfection, 1.5µl of each siRNA and 5µl of Lipofectamine RNAi max (Invitrogen) were used per well of a 6-well plate. As control, there was always 4.5µl of a non-targeting scrambled siRNA-pool of the same concentration tested in a separate culture well. The transfection reagent as well as the siRNAs were diluted in total volumes of 250µl of OptiMEM (Gibco), mixed by pipetting up and down at least 20 times, spun down briefly, pooled, mixed again and incubated for 30min at room temperature. In the meantime, the complete growth

medium was aspirated, cultures were washed with 1xPBS, and 2.5ml of complete medium w/o antibiotics (refer to 6.2.2.1) was added. Then the transfection mix was added slowly, plates were swirled and incubated for the indicated time points, normally for 48h. The ratio between the transfection mix and the medium was always kept constant irrespective of the medium volume used.

6.2.4 Transient overexpression of wild type Ubc9

6.2.4.1 Testing the pTre3G-gene of interest expression in NIH 3T3 Tet-On cells

Overexpression of Ubc9 was done transiently in NIH 3T3 TetA clone 7 cells. Therefore the Fugene reagent (Promega) was used in a ratio of 4µl of Fugene per 1µg of DNA for 48h which had been found to yield the best transfection efficiency in NIH 3T3 cells[114]. The protocol was calculated using the Fugene HD database, which aimed to give an optimal protocol in terms of cell seeding number, amount of DNA and the volume of Fugene reagent[115]. However, multiple steps of the protocol were modified. The cell numbers recommended by the database were found to lead to very confluent cultures after 48h, so they were lowered as following. For 6-well plates, the protocol database recommended to seed $2.5x10^5$ cells per well, but only $1.5\text{-}2x10^5$ cells were seeded, for the histone extraction, only $1x10^6$ instead of $3x10^6$ cells per 10cm dish were seeded. As the medium suggestions were also rather high only the halves of the proposed media volumes were taken per culture dish or well. Hence, for the calculation of the DNA and the Fugene reagent the number of wells was reduced by half to keep the ratio of medium to DNA-Fugene-complexes constant. The DNA was diluted in OptiMEM to a concentration of 0.020µg/µl, the indicated Fugene reagent was added, the solution was mixed by resuspending cautiously 30 times, spun down briefly and then incubated for complex formation at room temperature for 30min. Then, complete medium w/o antibiotics was added to the transfection mix, cells were washed with 1xPBS, and the transfection medium containing the pTre3G-Ubc9 plasmid was added to the cells. The medium containing the transfection complexes was then left in the culture vessel until analysis, as the Fugene reagent does not demand a medium exchange.

In order to have a rough estimation of the transfection efficiency, a pCruz-eGFP construct was transfected in a separate well using the same conditions. This well

was then evaluated for GFP expression before the protein lysates were prepared from the other cells. The pCruz-eGFP was provided by the lab technician Stefanie Koidl.

6.3 Characterization of stable clones

The previous section explained how the cells were treated. After that, clones which had grown up and had managed to survive the stringent antibiotic selection processes, their ability to stably express the protein of interest was evaluated by different means. Thereby, according to the protein analyzed, different approaches were applied.

6.3.1 Tet-On 3G transactivator screening in stable clones

First, the TetA expression level was evaluated by immunoblotting. Second, a functional reporter gene assay was run to test the efficiency of the TetA to induce the reporter gene.

6.3.1.1 Tet-On 3G transactivator protein expression screen by immunoblotting

As an initial screen, the protein expression of the Tet transactivator in the absence and the presence of Doxycycline were analysed. Each of the seven clones that were picked and grown up was seeded in 6cm cell culture dishes (BD Falcon) at a density of approximately $2x10^5$ cells. 2.5ml of Induction medium [DMEM (Sigma Aldrich) supplemented with 10% Tetracycline-free FBS (Clontech), 1x Glutamax (Gibco, 100x stock), 1xPenecillin/Streptomycin (Sigma Aldrich, 100x stock) and 1x Non-essential Amino acids (Sigma Aldrich, 100x stock) was added per well. That medium either contained or did not contain 1µg/ml Doxycycline. The seeded cell density was sufficient to yield a 60-70% confluence after 48h. As negative control, non-transfected NIH 3T3 cells were also seeded in the same media either devoid of or containing 1µg/ml Doxycycline. As positive control a previously identified transactivator protein expressing NIH 3T3 cell line passage 20 was re-evaluated.
48h after the seeding and the therewith accompanied induction, whole cell lysates were prepared (6.5.1) and 20µg of total protein were applied to a 12.5% SDS PAGE gel. For SDS PAGE and the Western blot procedures refer to 6.5.2-6.5.3.1.

The primary antibody was a monoclonal TetR mouse antibody (Clontech) stored at -20°C and thawed and kept on ice throughout its usage. For the immunodetection the antibody was diluted 1:500 in 5% (w/v) milk in PBST (0.05% (v/v) 20% Tween-20) and incubated at room temperature with slow agitation for 2h. The antibody was suited to detect both the transactivator as well as the transrepressor.

The secondary antibody against mouse (Sigma) was diluted 1:2500 in 5ml of 50g/l non fat dry milk powder in PBST (0.05% (v/v) 20% Tween-20). Further details for the immunoblot detection are specified in 6.5.3.1.

6.3.1.2 Functional Dual Luciferase Assay

To find out whether the transactivator is able to bind to the Tet-resposive promoter and to activate target gene transcription, a dual Luciferase assay was carried out. The candidate NIH 3T3 TetA clone cultures were seeded at a density of $8x10^4$ cells per well of a 12 well plate one day before transfection. The next morning, cells were transiently co-transfected with 1µg per well of a commercial pTre3G-Luciferase plasmid (Clontech) and 20ng of pLG4.70-synthetic Renilla Luciferase gene plasmid (Promega) using 10µl of Polyfect reagent, which were added to 100µl DMEM. The detailed transfection procedure is delineated in 6.2.2.1. All clones were transfected in triplets for the uninduced, as well as for the induced condition.

The medium containing the transfection complexes was exchanged for induction medium either being devoid of or containing 1µg/ml or 3µg/ml Doxycycline and incubated for 24h.

For the detection of Luciferase activity the Dual-Luciferase® Assay Reporter System (Promega) was used in a modified protocol. The cells were lysed by adding 100µl of ice cold 1x cell lysis buffer (stock 5x) from the kit diluted with mQH_2O, slowly agitating the plate for 5min, scraping the cell slurry and after transferring it to a Eppendorf tube spinning it down at 4°C and maximum speed for 20min. The supernatant was transferred to a new tube and stored on ice. If the protocol was not continued the same day lysates were frozen at -20°C for short term or at -80°C for long term storage.

Table 1: Luciferase dual measurement

Injector I
50µl LARII reagent injected
2s delay
10s measurement Firefly Luciferase
Injector II
50µl Stop & Glo®-Reagent-Renilla substrate injected
2s delay
10s measurement Renilla Luciferase

The protocol was continued by applying 20µl of each lysate in triplicates to a white, flat bottom 96-well plate (Nunc) which was kept on ice. The Dual luciferase assay was further conducted according to the manufacturers' instructions with the following amendment: the Luciferase Assay Reagent II (LARII) and the Stop & Glo® Buffer were diluted 1:1 with mQH_2O. Then, the samples were measured in a plate reader (Berthold) by injecting first the Firefly substrate contained in the LARII buffer and then the quencher with the Renilla substrate contained in the Stop & Glo® buffer by a sequential program (

Table 1). By dividing the Firefly readout through the Renilla readout the samples were normalized, by dividing each value through the measured value of the negative control the measurement was normalized against the blank. In multiple experiments means, standard deviations and student t-test were used to evaluate the significance of the data in Microsoft EXCEL.

6.4 Characterization of inducible Ubc9 overexpression cell clones

6.4.1 Genotyping PCR

PCR was used as an initial screen in order to rule out false positive stable clones, which were stably expressing the Hygromycin resistance gene, even though having not integrated the pTre3G plasmid into their genome. Rather than testing all clones for protein expression genotyping considerably reduced the number of clones which had to be scaled up and maintained.

Therefore, clone cultures originating from Hygromycin-resistant colonies were expanded in wells of a 24-well plate and split at the ratio of 1:3 when they had reached a minimum confluence of 60%. One third was passaged into a new 24-well plate and the other two thirds were genotyped as following.

6.4.1.1 DNA Isolation

For the DNA isolation, a crude extraction protocol was used. Two thirds of the cell mass of the clones in suspension from a 24 well plate were spun down at the same conditions applied for the cell culture. After the supernatant had been aspirated 50µl of lysis buffer [1xPCR buffer (Quiagen), 200µl/ml TritonX, 100µg/ml Proteinase K (Thermo Scientific - Fermentas), mQH_2O] were added to the pellet, resuspended and transferred to an Eppendorf tube. Then, the digestion was done at 55°C in a waterbath for 4h. After that, the proteinase K was inactivated by heating the sample at 65°C for 30min. Then, the lysate was spun down at maximum speed for 10min and the supernatant was transferred to a fresh tube. 100µl of isopropanol was added, briefly mixed by resuspending and incubated at room temperature for 5min. Furthermore the samples were spun down at maximum speed for 15min, the supernatant was removed, 70% EtOH was added and tubes were tabbed, spun down for 5min again, pellets were dried by inverting the tubes as long as pellets became transparent (approximately 10min). After that, 30µl of TE buffer preheated to 60°C were added and pellets were resuspended therein. Each sample was measured in the Nanodrop and if the DNA concentration was above 500ng/µl the concentration was adjusted to that threshold.

6.4.1.2 PCR program

After the DNA concentrations had been approximated to 500ng/µl with nuclease-free H_2O, 2µl of each sample were pipetted into an emtpy vial of a PCR tube-strip (Thermo Scientific). All master mix reagents were thawed, briefly vortexed and kept on ice throughout their use. The master mix was prepared according to Table 2**: Master mix per sample**. As negative control the DNA of NIH 3T3 Tet-on cells was used; the master mix control was 2µl of nuclease free H_2O as template instead of sample DNA whereas the positive control was 10ng of pTre3G-wt-

Ubc9 plasmid DNA. The forward primer was designed to bind to the vector within the promoter region and the reverse primer was homologous to the 3'end of the inserted, transgenic hUbc9 gene (

Table **3**).

Table 2: Master mix per sample

Reagent	Volume [µl]
Nuclease-free H_2O (Quiagen)	17
pTre3G forward primer (Eurofins MWG Operon)	0.1
Ubc9 reverse primer (Eurofins MWG Operon)	0.1
dNTPs	0.5
Crimson LongAmp® Taq Polymerase buffer (New England Biolabs Inc.)	5
Crimson LongAmp® Taq Polymerase (New England Biolabs Inc.)	0.25

Table 3: Primer sequences for genotyping

Primer	Sequence 5' -> 3'
pTre3Gforward primer	GTGAACCGTCAGATCGCCTGGAGC
hUbc9 reverse primer	TACATTGGATCCTTATGAGGGCGCAAACTTCTTGGC

Table 4: PCR cycle program

Step	Temp [°C]	Time
1	94	5min
2	94	20s
3	55	30s
4	65	40s
5	Go to step 2 30-times	
6	65	7min
7	8	for ever

The PCR tube strips were closed, drops sticking to the walls of the tubes were spun down by a short centrifugation step and tubes were put into the thermo cycler (DNA Engine Peltier Thermal Cycler, Biorad) pre-heated to 94°C. The genotyping program was then run in 30 cycles (cycle steps 2-5 in

Table 4: PCR cycle program).

1% (w/v) Agarose gel solution in TAE buffer was prepared by weighing 4g of Agarose (Lonza), adding 400ml of 1xTAE buffer (diluted from 50x stock solution: 2M Tris, 5.7% (v/v) acetic acid, 0.05M EDTA pH 8.0, pH adjusted with HCl to pH 7.7) and boiling it in the microwave

until the Agarose dissolved completely and 1:10000 dilution of Gel red (Biotium) were added. Air bubbles were avoided during polymerization at room temperature.

After polymerization, the gel was submerged in 1xTAE buffer. 5µl of 1kb DNA ladder and 10µl of the amplified samples and controls were loaded next to each other. The gels were run at constant voltage (Electrophoresis power pack EPS 601, GE Healthcare) depending on the gel size between 100V and 200V for 25min to 45min.

Pictures were taken in the UV transilluminator after the gel had been positioned directly onto the illuminator surface and focus and exposure time had been adjusted by previewing the saturation of the signal.

6.5 Western Blot

The immunoblot is the most frequently applied technique to analyze the expression level of specific proteins. First, the content of the TetA and Ubc9 in cell lysates was determined using specific monoclonal and polyclonal antibodies, respectively. Second, the Histone extracts from Ubc9 knock down cells were evaluated. Moreover, the coomassie staining of SDS PAGE gels was used to determine concentrations and the purity of proteins.

6.5.1 Protein preparation of whole cell lysates

For the analysis of whole cell protein lysates from cultivated cells, they were washed once with cold 1xPBS. Then, 95°C-heated 2x SDS-Laemmli buffer (100mM Tris pH 6.8, 4% SDS, 20% Glycerin, dH_2O) was added directly to the cells and distributed by rapidly swirling the plate. After that a cell scraper (1.8cm blade, Costar) was used to collect the cell suspension and transfer it to an Eppendorf tube. Then, the tubes were sonicated (Diagenode Bioruptor) for 5min at cycles of 15s high level pulse and a 15s intermission. That was done to break up all cellular compartments and to shear the DNA in order to get a homogeneous sample. After sonication, the cell lysates were heated for 1min and then spun down briefly.

In order to load comparable protein amounts in the SDS PAGE later on, a protein estimation was performed. Therefore, 10µl of each lysate were pipetted in a clear

flat bottom 96-well plate (Nunc) in duplicates. Then, the reagents of the BCA kit (Thermo Scientific) were mixed in 1:50 ratio and 200µl were added to each well. After incubation at 37°C for 30min (Thermo Scientific Heraeus Function line), the plate was cooled down to room temperature again and the absorbance at 562nm was measured. Initially, equations of multiple standard curves were calculated by assaying a BSA standard (contained in the kit). The sample concentration was afterwards determined in the same way by the comparison to a mean standard equation. After adding the adequate sample volumes sufficient for the required protein content, the volumes were equalized using 2x SDS-Laemmli buffer supplemented with 0.2% (w/v) Bromophenol blue and 200mM freshly added DTT.

6.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl-sulfate polyacrylamide-gel-electrophoresis was used either for preparative reasons in the immunoblot protocol. Alternatively, instead of preparative gels, also analytical gels were used, which were then coomassie stained.

6.5.2.1 Preparation of denaturing polyacrylamide gels

Table 5: Formulae of SDS PAGE separation gels of different percentages

Reagent	Gel percentage		
	4% (staking)	12.5%	20%
mQH_2O	42.9ml	25.5ml	7.9ml
2M Tris pH 8.8	14ml	14ml	14ml
30% Acrylamide:Bis 30:0.8 (Roth)	11.7ml	29.2ml	46.7ml
20% SDS	350µl	350µl	350µl
Mix well			
10% APS	700µl	700µl	700µl
TEMED	70µl	70µl	70µl

In general, two different gel types were used. For separation of Ubc9, 12.5% Polyacrylamide gels were used cast in a 10 gel stack: alumina support plates, glass plates (both 10cmx8cm, GE Healthcare) and spacers (GE Healthcare) were cleaned with 70% Ethanol and then with mQH_2O with fibre-free tissue paper (Kimberly-Clark professional) to remove any gel residues. Self-designed casting trays were cleaned as well and components were assembled as following: a

transparent plastic foil at the very bottom of the tray, a alumina plate, spacers at the side edges and a glass plate on top, then a alumina plate again and so on. This was done 10 times, after the last glass plate again a plastic foil was placed and the casting tray was closed tightly with screw clamps. 12.5% gel solution was mixed according to the formula (Table 5: Formulae of SDS PAGE separation gels of different percentages) under the fume hood using a magnetic stirrer, finally 10% APS and TEMED were added, mixed well and then the solution was poured in the middle of the casting tray until an appropriate height for the separation gel was reached. The tray was positioned in a diagonal angle to ensure equal levels for the separation gel, put back into a horizontal position and then every gel was covered with isopropanol and let polymerize for 1h. Residual isopropanol was removed by inversion of the cast tray, rinsing the gel with 0.5M Tris pH 6.8 and drying remaining liquid in the corners with chromatography paper.

The stacking gel was a 4% polyacrylamide gel by default: 22.7ml mQH_2O, 3ml 0.5M Tris pH 6.8, 4ml 30:0.8 Acrylamide:Bis, 150µl 20% SDS (w/v), mixed in the fume hood on a magnetic stirrer, 150µl 10% APS and 30µl TEMED were added, mixed and poured on top of the separation gel. Immediately, 12 slot-gel combs were rapidly inserted into every gel and again it was given 1h for polymerization.

After that, the casting tray was cautiously opened, the gel stack was taken out by pulling the plastic sheet behind the bottom gel, gels were laid on plastic foil which had been topped with H_2O-wettened paper towels, and the gel stack was wrapped therein and stored at 4°C until use.

The separation of histones required a higher resolution and thus gradient gels were the formulation of choice. To pour gradient gels, a more elaborate procedure was demanded. The alumina supports as well as the glass plates were larger (10cmx12cm, GE Healthcare). The same was true in regard of the size of the casting tray and moreover it had an inlet opening at the rear bottom. The separation gel 5% as well as 20% gel solutions (Table 5) were prepared separately in 50ml Falcon tubes. The assembled casting tray as described before was connected via a clamped tube to the outlet of the mixing unit resting on a magnetic stirring plate and consisting of two by a cylinders being interconnected. In the first outlet cylinder contained a stirring bar and was directly connected to the casting tray. That first cylinder was filled first with the 5% gel. Then, the clamp

blocking the interconnecting tube was loosened and the 5% was let into tube until entering the second cylinder to remove any air out of the system. Immediately, the clamp was closed again, the 20% gel solution was poured into the second cylinder, the 5% gel was again leveled to an equal amount, then the interconnection was opened first followed by opening the tube connecting the mixing unit and the cast tray. The 20% gel entered the 5% cylinder through the connecting tube; the solutions were mixed by the stirring bar and entered the casting tray filling it from the bottom. When the desired height of the separation gel was reached, the clamp of the tube connecting the casting tray with the mixing cylinders was closed, the single gel solutions were again leveled, covered with isopropanol and let polymerize for 1h.

For the stacking gel the formula stated in Table 5: Formulae of SDS PAGE separation gels of different percentages and the same procedure as described for the separation gel were conducted. After pouring the gel into the casting tray, the gel combs were inserted.

6.5.2.2 Resolution of proteins by SDS PAGE

Depending on the sample and its protein concentration, sample volumes between 10µl and 40µl were applied to a lane of the gel. In the outermost left lane 4µl of a pre-stained protein standard ladder (Fermentas) and outermost right 8µl of an unstained protein standard (Fermentas) ladder were loaded by default. Every sample contained 2x Laemmli buffer containing 0.02% (w/v) bromophenol blue and supplemented with 250mM fresh DTT. All gels were run in SDS PAGE chambers (ELPHO “V100”, GE Healthcare) filled with running buffer (25mM Tris, 192mM Glycine, 0.05% SDS (w/v), in mQH_2O) at 25mA constant current (Electrophoresis power pack EPS 601, GE Healthcare) per gel as long as the bromophenol blue front started to elute at the bottom or before the 10kDa band of the pre-stained protein standard ladder reached the very bottom.

6.5.3 Semi-dry Western Blot

The specific detection of a particular protein was only achieved after the transfer of all proteins from the gel to a nitrocellulose membrane. Therefore, a cut piece of Whatman paper (GB005, Whatman) topped with the membrane (Protan®

Nitrocellulose Transfer membrane, Whatman) was soaked and activated in transfer Buffer [25mM Tris, 193mM Glycine (from a 10x stock solution), 20% (v/v) Methanol and 1.8ml of 20% (w/v) SDS added freshly, mQH_2O up to 1l]. The gel spacers and the glass plate were removed with a razor blade and the stacking gel was cut off and discarded. In contrast, the separation gel was placed on top of the activated membrane, and another Whatman paper was placed on top of the gel and soaked in the Transfer Buffer as well. Then that sandwich was taken out of the buffer, pressed in between two layers of paper towel. Finally, it was placed onto the blotting apparatus (Peqlab) in the same orientation as it was assembled. The sandwich was rolled two times to remove any air bubbles; any excessive buffer on the electrode was dried with paper towels, the lid of the blotter was placed on top and screws were equally tightened. The blotting apparatus was connected to the power supply (Power Pac® HC Power Supply 305300, BioRad) which was set to 100mA per small membrane for 1h. When there was only one sandwich in the blotter, the current was increased from 150mA to 250mA for 1h. Otherwise the voltage for the transfer would have been to low resulting in incomplete blotting. For the larger gradient gels the applied conditions were at least 200mA for 1h.

After the blotting procedure was finished, the blotted membranes were either left in the blotting apparatus over night or stained with 0.5% (w/v) Ponceau S (Sigma Aldrich) solution (in 1% acetic acid) for approximately 5min and then destained using 1% (v/v) acetic acid or dH_2O. The unstained protein ladder bands were indicated using a pen, edges were cut and the membranes were scanned (Epson 500 Series). This was done to evaluate the correct transfer of proteins and to evaluate any imbalances in the protein contents of the individual sample lanes. Furthermore, the staining was removed in PBS Tween-20 (Tween concentration range from 0.05%-0.2% (v/v) - depending on the primary antibody used) and then blocked in 5% (w/v) non-fat dried milk (Milk powder blotting grade, Roth) in PBST, either at room temperature with agitation for 1h or at 4°C with agitation over night.

6.5.3.1 Antibody detection

The membranes were incubated with primary antibodies diluted depending on the antibody and the sample, in 5% (w/v) milk in PBST at room temperature with slow agitation for 1-2h. After that the membrane was once rinsed with PBST and then washed at least 3 times with PBST with faster, orbital shaking for 10min each time.

The secondary antibody was applied according to the species the primary antibody was raised in. However, all of them were from Jackson Immuno Research, coupled to Horse Reddish Peroxidase (HRP) and diluted in 5% (w/v) milk PBST 1:2500 to 1:5000 depending on the primary antibody. The incubation of the secondary antibodies was always carried out at room temperature with slow agitation for 1h.

Table 6: Antibodies for immunoblotting

Antibody	Diluton(s)	Species	Source
Ubc9	1:1000	rabbit	homemade
Ubc9	1:1000-1:4000	goat	homemade
TetR	1:500	mouse	Clontech
H3	1:3000	rabbit	Upstate
H3K9me1	1:1000	rabbit	Thomas Jenuwein
H3K9me2	1:1000	rabbit	Thomas Jenuwein
H3K9me3	1:1000	rabbit	Thomas Jenuwein
H3K27me1	1:1000	rabbit	Thomas Jenuwein
H3K27me2	1:1000	rabbit	Thomas Jenuwein
H3K27me3	1:1000	rabbit	Thomas Jenuwein
H4K20me1	1:1000	rabbit	Thomas Jenuwein
H4K20me2	1:1000	rabbit	Thomas Jenuwein
H4K20me3	1:1000	rabbit	Thomas Jenuwein
beta-tubulin	1:2500	rabbit	Santa Cruz
rabbit-HRP	1:5000		Jackson Immuno Research
goat-HRP	1:5000		Jackson Immuno Research
mouse-HRP	1:5000		Jackson Immuno Research

After the secondary antibody incubation the membrane was again washed for three times applying the same conditions as before. In the meantime, the ECL reagent (Thermo Scientific) which contains the substrate for the horseradish peroxidase was prepared. Therefore, the two reagents contained in the kit were mixed 1:1. When Super-ECL was used, the two reagents of that kit were mixed 1:1 and then diluted in ECL or PBS to a final concentration from 10 to 50% (v/v).The washing solution was drained from the membrane by gravity and then the membrane was placed horizontally. The ECL reagent was slowly applied to the surface of the membrane until it was covered completely. Then it was incubated for 1.5min and then the membrane was rinsed with the reagent for another 3min. After that the membrane was put in between of two plastic sheets fixed in a light tight developing cassette. The membrane was exposed to an x-ray film (GE Healthcare) in the dark room for different time spans ranging from 1s to 10min. After that the film was developed in the Kodak M35 B x-ray film developer.

6.6 Histone modification analysis

The techniques for the evaluation of histone methylation were a histone acid extraction from Ubc9 knock down cells, followed by immunoblotting and further the immunofluorescent staining of Histones in knock down cells.

6.6.1 Histone acid extraction of 3T3 Ubc9 knock down

The extraction was done according to the adapted Histone acid extraction protocol provided by Abcam[116]. For analysis, Ubc9 knock down cells were prepared as detailed in 6.2.3 and immunoblot was done as described in 6.5.1. Two dishes were prepared for each sample, one for the histone acid extraction and one to follow the Ubc9 expression pattern from total cell lysates. For the preparation of histone acid extracts, each plate was washed twice with ice cold 1xPBS. Then 1ml of Triton extraction buffer was added (TEB: 0.5% Triton-X 100 (v/v), 2mM phenylmethylsulfonyl fluoride, 0.02% (v/v) NaAz in 1xPBS) and plates were put on ice on the shaker to enable cell lysis for 10min. After that, the lysate was scraped off the cell culture dishes and transferred to Eppendorf tubes. The nuclei were spun down at 6400g and 4°C for 11min and the supernatant was removed. Then, 0.5ml of TEB was added and tubes were flicked to wash the

pellets. Next, the tubes were spun down again applying the conditions as before. The supernatant was removed and the pellets were resuspended in 250µl of 0.2N hydrochloric acid (Merck, prepared from 37% (v/v) stock) and histones were extracted by end over end shaking at 4°C over night. The next day, the debris was centrifuged at the same conditions as mentioned above and the supernatant was transferred to a fresh tube which was stored on ice.

The purity and the concentration of the extracted histones were determined on coomassie stained SDS gradient gels by comparison to a α-lactalbumin standard. Therefore, all extracts were loaded onto an SDS gel applying different volumes and comparing them to the α-lactalbumin standard of known concentration. The staining procedure of gels was started by fixing them in 40% (v/v) Ethanol in 10% (v/v) acetic acid shaking at room temperature for 10min. Then, the Coomassie staining solution was added and either left shaking at room temperature for 3h or for a quick-stain it was heated in the microwave for 30s. Then, it was given time to cool down for 1min followed by heating it for another 15s. Then, the gels were left on the shaker for another 10min to an hour and after that destained. The destaining solution was 1% (v/v) acetic acid. For destaining, the solution was exchanged every 2-4 hours. Destaining was ultimately either done over night or also with a quick microwave protocol by heating the gels in destaining solution for 30s. Then, it was shaken at room temperature for 30min, exchanging the destaining solution and let therein until concentration evaluation on the white light transilluminator or drying it.

For the gel drying procedure, gels were rinsed with distilled water, put on a wet Chromatography paper (Munktell), covered with a transparent plastic foil and laid on the vacuum heating plate (Biometra). Then, the vacuum pump (Eppendorf concentrator 5301) was switched on. A plastic cover enclosed the gels by the formed vacuum and then 60°C were applied for 2h.

Extracts were always as pure as figures shown by *Shechter et. al* and that publication was used to determine the single Histone bands and their concentrations. [117]

6.7 Ubc9 Antibody Affinity Purification and Characterization

6.7.1 Expression and purification of recombinant human Ubc9

The purification of recombinant, untagged human Ubc9 was performed for the serum affinity chromatography purification of Ubc9-specific goat antibodies, which required Ubc9.

BL21 gold bacteria, a competent protein expression strain, were heat shock transformed with a pET23a-hUbc9 plasmid construct according to the procedure described before (6.1.1). The plating on one Petri dish was done only for back up purposes in that case. Instead of growing resistant colonies the culture was directly grown up in 5ml LB medium containing 100µg/ml Ampicillin for 8h. After that, the whole culture was scaled up to 100ml LB medium containing 100µg/ml Amp and grown at 37°C with 200rpm shaking over night. The next morning, that culture was scaled up to 4l adding 25ml of the overnight culture to 1l of LB/Amp medium in a 2l Erlenmeyer flask and incubating the culture again at 37°C, 170rpm shaking (New Brunswick Scientific innova 40) until it reached an OD_{600} of 0.65 (Eppendorf Biophotometer plus). A sample of the non-induced culture was taken. Then, 1mM of IPTG was added to each liter of culture to induce the protein expression and incubated at 37°C for 4h. Then, the culture was harvested by spinning them in 6 250ml centrifuge buckets at 10^4g for 20min, 4°C (Sorvall RC 6 plus, rotor: PTI F10S-6x500y), the supernatants were discarded. Having the pellets from multiple spins enriched at one side of the containers they were then resuspended in a total volume of 45ml ice cold lysis buffer (25mM Na_3HPO_4, 50mM NaCl, 0.1mM PMSF, 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin). The protease inhibitors and DTT were added freshly just before resuspending the pellets. After that, the homogeneous lysates were divided in three 50ml Falcon tubes containing equal volumes, snap frozen in liquid nitrogen and stored at -80°C over night.

The next day the lysates were thawed on ice and then sonicated on ice 3 times at 90% intensity for 10s each time with 20s breaks in between. Then, the lysate was filled into ultracentrifuge tubes, which were balanced and spun at 10^5g, 4°C for 1h.

For the ion exchange chromatography, 5ml of SP Sepharose beads (GE Healthcare) were packed into a column and equilibrated with 10 column volumes (50ml) of lysis buffer. The supernatant of the ultracentrifugation was collected and passed through the beads for 3 times, each time washing the column with 5 column volumes (25ml) of lysis buffer in between. To elute the bound protein from the ion exchange resin 10 times 2ml of elution buffer (25mM Na_3HPO_4, 300mM NaCl 1mM DTT, 1µg/ml Aprotinin, 1µg/ml Leupeptin/Pepstatin) were added and 2ml fractions were collected. Then, the fractions were tested for their protein content by applying 1µl of each fraction to a strip of nitrocellulose membrane and staining that with 0.5% (w/v) Ponceau S followed by destaining with 1% (v/v) acetic acid. The protein containing elution fractions were pooled and concentrated using protein concentrators (Vivaspin, Santorius) with a molecular weight cut off of 10kDa at 1500g (maximum speed) as long as a volume of just below 5ml was obtained.

Then, the concentrated sample was injected to an ÄKTA tm purifier 10 FPLC system with UC 900 (GE Healthcare) consisting out of pHIC-900, UV-900, P-900, Frac-150 and connected to the Hiload 26/60 S200 Superdex prep grade size exclusion column which had been equilibrated before. For that the system and the column were rinsed with 318ml of degased (sonication: 2x8min 90% continuous with 10min break) transport buffer (100mM NaCl, 20mM Tris-HCl pH8.0) at a maximum (alarm) pressure of 0.65MPa (0.5MPa column pressure + ~0.15MPa system pressure) and a flow rate of 2ml/min.

For running of the sample the same conditions were applied and 36 fractions of 5ml were collected starting from 200ml after eluting the column void volume of approximately 318ml.

10µl of the fractions containing the highest protein content including the proximate fractions were diluted with 40µl of 2xLaemmli containing 200mM DTT, were run on an SDS-PAGE gel and coomassie stained by the described methods (6.5.2, 6.6.1). One fraction contained the highest protein content, whereas the previous and the subsequent ones also contained some protein. So, the highly concentrated fraction was divided in 1ml aliquots whereas the less concentrated fractions were pooled and split in 2ml aliquots. Small aliquots were taken for concentration determination on a SDS-PAGE gel by the previously described

method (6.5.2, 6.6.1). Then, the aliquots were snap frozen in liquid nitrogen and stored at -80°C.

6.7.2 Affinity chromatography of crude Ubc9 goat serum

The first step was to dialyse 1mg of purified recombinant Ubc9 into the adequate carbonate buffer [0.2M $NaHCO_3$ (Merck), 0.2M Na_2CO_3 (Riedel del Haën) in the ratio 10:1, pH 8.8] for the affinity chromatography. A dialysis cassette with a membrane cut off of 10kDa was equilibrated in ice cold carbonate buffer with slow agitation at 4°C. Then, 1mg of Ubc9 in transport buffer was cautiously injected into the cassette using a 1ml syringe, the cassette was put into at least 500ml of carbonate buffer and dialysed for at 4°C for approximately 4h. After that, the carbonate buffer was exchanged and dialysis was continued at 4°C, slow agitation over night. The next morning, again the buffer was exchanged and dialysis was continued for another 2h applying the same conditions.

Cyanogen bromide-activated Sepharose® (Sigma Aldrich) beads stored at 4°C were equilibrated to room temperature, 800mg were weighed and swollen in 10ml of 1mM HCl (37% (v/v) stock, Merck) for 10min with slow agitation (neoLab® shaker DRS-12) in a 15ml falcon tube, spun down at 500g for 5min to settle, supernatant was decanted and beads were resuspended in 10ml of carbonate buffer for washing. This was repeated two times. The 3rd time, a 40µl beads sample was taken with a cut pipette tip before adding the dialysed recombinant Ubc9 protein and incubating the beads with the protein at room temperature for 1h with slow agitation and then at 4°C with agitation over night. The next day, the beads with the bound protein were spun down, supernatant was kept on ice and a sample was taken up in an Eppendorf tube. Then, the beads were blocked with 100mM Ethanolamine (Acros Organics) for 1h at room temperature with slow agitation to block all unoccupied coupling sites of the beads followed by 3 washes with 10 column volumes 500mM NaCl in 1xPBS.

For the purification of antibodies, 9ml of crude 3rd bleed serum from an immunized goat from day 88 was thawed on ice and 3 aliquots of 12ml were generated after adjusting the overall volume to 36ml with ice cold 500mM NaCl in 1xPBS. That was done to run several purification batches. In order to remove any insoluble material, the serum was ultracentrifuged at 10^5g at 4°C for 1h and the

supernatant was added to the beads. That was mixed carefully into the 15ml Falcon tube containing the beads and incubated at 4°C with slow agitation over night to bind the antibodies to the beads.
The following day, the slurry was spun down, the supernatant was kept as further flow through sample, and the beads were resuspended in 10 column volumes of 500mM NaCl in 1xPBS and subjected to a chromatography column. After draining of washing solution, the column was washed again with 10 column volumes ice cold 500mM NaCl in 1xPBS. 1ml fractions were collected by adding 5ml of elution buffer (200mM acetic acid pH 2.7, 500mM NaCl) for two times in 1.5ml Eppendorf tubes, which contained 50µl of Tris-HCl pH8.0 to buffer the antibodies back to their native pH. Immediately after 5 fractions had been collected, the tubes were closed and inversed several times for mixing. For the rapid evaluation of the elution profile, 1µl of each fraction was spotted on a piece of nitrocellulose membrane and stained with 0.5% Ponceau S solution followed by destaining with 1% acetic acid. The fractions which contained protein were pooled, mixed, a sample was taken and then they were concentrated to approximately 500µl using protein concentrators with a MWCO of 10kDa (Vivaspin, Santorius). Finally the concentrated antibodies were buffer exchanged into 50% Glycerol (AppliChem) in 1xPBS using 2.5ml PD-10 disposable size exclusion columns (GE Healthcare) according to the manual and stored at -20°C.
12.5% SDS-PAGE gels with the drawn samples were run, the antibodies in reducing as well as non-reducing buffer, Coomassie-stained and dried according to described procedures (6.5.2, 6.6.1)

6.7.3 Characterization of affinity purified antibodies

6.7.3.1 Specificity and sensitivity assessment by immunoblot

The probing of 12µg Ubc9 knock down versus scrambled control cell lysates with the purified Ubc9 antibodies by immunoblot determined the specificity and the sensitivity of the antibodies.
The knock down of Ubc9 was done applying the same ratio of siRNA to RNAimax reagent, as described in 6.2.3. One day before the knock down, $1x10^6$ cells were seeded per 10cm dish. For the transfection, the triple amount of the transfection

mix and medium of a 6well (9ml) was appropriated to overcome the already observed and rather inefficient knock down of Ubc9 in 10cm dishes.
The SDS-PAGE and the Western Blot were carried out as described (6.5). The purified goat-antibody dilutions tested were 1:1000, 1:2000, 1:4000 and 1:8000 in 5% (w/v) non-fat dried milk/PBS 0.2% (v/v) Tween-20 and incubated at room temperature with agitation for 1.5h. The secondary goat-HRP antibody was used as described for all secondary antibodies in 6.5.3.1.
Detection was done with ECL as well as with 20% Super-ECL to be able to visualize any additional nonspecific signal.

6.7.3.2 Qualification for immunofluorescence

In order to observe a gradually increasing expression of Ubc9, cells were fixed with 2% (v/v) Formaldehyde after 12h, 24h and 36h. After fixing the cells were overlayed with 1xPBS and stored at 4°C as long as the cultures from the last time point were fixed. Then, the immunofluorescence protocol described in 6.8 was conducted with all slides in parallel.
For each time point one well was stained with a 1:1000 HA antibodies, one well was stained with 1:500 purified Ubc9-goat antibodies and a third well was stained with a 1:500 purified Ubc9-goat antibodies which were challenged with excess of recombinant Ubc9 protein prior to their use. For the challenge of antibodies, 3µl of the goat antibody were added to 10µl of the same recombinant Ubc9 protein and incubated at 22°C with 350rpm in the heating block (Eppendorf Thermomixer comfort) for 30min. Then, the required 1.5ml of blocking solution was added and mixed cautiously. The fourth well of each chamber was a secondary antibody control containing both antibodies used in a 1:1000 dilution. The secondary antibodies were both donkey-Alexa-488 (Sigma), one against mouse for the detection of HA, the other one against goat. Pictures were taken using the Zeiss Axioimager Apotome 390 (Xenon lamp power supply ebx75, Apotome power supply PC 167) with 6D acquisition mode in the Axiovision 4.1 imaging software.

6.8 Immunofluorescence

6.8.1 Testing of histone modifications in Ubc9 knock down

$1x10^4$ NIH 3T3 cells per well of microscopy slide chambers (Labtek) were seeded the day before transfection. The transfection for the knock down of Ubc9 was carried out as detailed in 6.2.3. 500µl of medium per well of the slide were used, so the siRNA-Lipofectamine complexes were mixed to the transfection medium in a 15ml falcon tube applying the same ratio as indicated before. The cells were then grown for the indicated time points and after that medium was aspirated, cells were washed once with ice-cold 1xPBS, fixed with 2% (v/v) formaldehyde (Thermo Scientific) in 1xPBS at room temperature for 10min, washed for 10min, permeabilized with 0.1% TritonX-100 for 5min and then washed two times with 1xPBS for 5min. After that, cells were washed with washing solution [0.25% (w/v) BSA (Sigma Aldrich), 0.1% (v/v) Tween in 1xPBS] for 5min. Then the blocking solution (washing solution supplemented with 10% donkey serum and 2.25% (w/v) BSA) was added at room temperature for 30min. Furthermore, the primary antibody was added at the indicated dilution in blocking solution and cultures were kept overnight at 4°C.

The next day, the primary antibody was incubated at room temperature for another 30min, and then washed with washing solution three times for 10min, the AlexaFluor® 488 secondary antibody (Sigma) was added fitting the species of the primary antibody, and slides were incubated in the dark for 1h. After that, again 4 washes in washing solution at room temperature for 10min were performed. During the second wash, DAPI was added to the washing solution in a 1:1000 dilution. Finally, wells were removed, one drop of mounting medium (DAKO) was applied to each preparation and cover slips (Roth) were slowly placed onto the stained cells starting from one side and using forceps. Then, the slides were let dry for approximately two hours in the dark at room temperature and in the following stored in the dark at 4°C. Images were taken using the Zeiss Axiovert 390 with or without the Apotome.

Two microscopy slides treated exactly the same as the other ones in terms of cell number seeded were used as a Western blot control. So cells from 1 well that had been transfected with the scrambled control as well as one culture treated for

the Ubc9 knock down were lysed in 30µl of 2x SDS-Laemmli buffer without bromophenol blue, 10µl of 2xSDS-Laemli buffer containing 0.2% (w/v) bromophenol blue and 100mM DTT were added, samples were boiled for 5min at 95°C, spun down and separated on a 12.5% SDS gel and then blotted. For details refer to section 6.5.

7 Appendix

7.1 List of Abbreviations

Abbreviation	Full name
APS	Ammonium Per-Sulfate
AMP	Adenosine Mono-Phosphate
Amp	Ampicillin
ATP	Adenosine Tri-Phosphosphate
ATPase	An enzyme, that converts ATP to ADP or AMP by hydrolysis
CAF1	Chromatin Activating Factor 1
CO_2	Carbon Dioxide
CpG	Cystein paired to a Guanin in the same DNA strand
DNA	Di-Nucleotide-Deoxy-Ribonucleic Acid
Dnmt1	De Novo Methyl Transferase 1
Dnmt3a	De Novo Methyl Transferase 3a
Dnmt3b	De Novo Methyl Transferase 3b
dNTPs	Mix of Deoxy-Adenosine Tri-phosphate (dATP), Deoxy-Guanosine Tri-phosphate (dGTP), Deoxy-Cytidine-Tri-phosphate (dCTP), Deoxy-Thymidine Tri-phosphate (dTTP)
Dox	Doxycycline
DTT	Dithiothreitol
E. Coli	Escherichia Coli
EDTA	Ethylene-Diamine-Tetra-acetic Acid
EtOH	Ethanol, ethyl-alcohol
FBS	Fetal Bovine Serum
FPLC	Fast Performance Liquid Chromatography
g	Gravity, 9.81m/s
GFP	Green Fluorescent Protein
G418	Geneticin
h	hours
H1	Histone 1
H2a	Histone 2a
H2b	Histone 2b
H_2O	Water
H3	Histone 3
H3.3	Histone 3.3

H3K9	Lysine 9 of Histone 3
H3K9me1	Mono-Methylation of Lysine 9 in Histone 3
H3K9me2	Di-Methylation of Lysine 9 in Histone 3
H3K9me3	Tri-Methylation of Lysine 9 in Histone 3
H3K27	Lysine 27 of Histone 3
H3K27me1	Mono-Methylation of Lysine 27 in Histone 3
H3K27me2	Di-Methylation of Lysine 27 in Histone 3
H3K27me3	Tri-Methylation of Lysine 9 in Histone 3
H4	Histone 4
H4K20	Lysine 20 of Histone 4
H4K20me1	Mono-Methylation of Lysine 20 in Histone 4
H4K20me2	Di-Methylation of Lysine 20 in Histone 4
H4K20me3	Tri-Methylation of Lysine 20 in Histone 4
HA	Human influenza hemagglutinin
HA-Ubc9	Human influenza hemagglutinin-Ubc9
Hox genes	Homeobox genes
HP1	Heterochromatin protein 1
HRP	Horse Reddish Peroxidase
ICM	Inner Cell Mass
kDa	Kilo Dalton
kb	Kilobases
LARII	Luciferase Activating Reagent II
LB medium	lysogeny broth medium
mA	Milli-Ampere
MDB1	Methyl-DNA-binding protein 1
MEF	Mouse Embryonic Fibroblasts
min	Minute(s)
mg	Milligram(s)
ml	Millilitre(s)
mM	Milli-Mol(es)
MPa	Mega-Pascal
mQ H_2O	Milli-Q-water, deionized water filtered through 0.22µm (Millipore)
mRNA	Messanger RNA
MWCO	Molecular weight cut off
µg	Microgram(s)
µl	Microlitre(s)

µM	Micro Mol(es)
NaAz	Sodium azide
ng	Nanogram(s)
nm	Nanometer
NRB	Non-Reducing Buffer
Pa	Pascal
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PML	Progressive Multifocal Leukoencephalopathy
PMSF	Phenyl-Methyl-Sulfonyl Fluoride
PRC1, PRC2	Polycomb Repressive Complex 1, 2
RB	Reducing Buffer
rcf	Relative centrifugal force
RNA	Ribonucleic Acid
RNAi	RNA interfance
rpm	Revolutions per Minute
rRNA	Ribosomal RNA
S	Sedimentation Coeffecient
s	Second(s)
Sc	Scrambled Control, mix of non-targeting siRNAs
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis
SIM	SUMO Interaction Motif
siRNA	Small interfering RNA
SP Sepharose	Sulfopropyl Sepharose (Chemically modified beaded Agarose)
SUMO	Small Ubiquitin-related modifier
TAF12	Transcription Activating Factor 12
TAE	Tris-acetic acid-EDTA
TE buffer	Tris-EDTA buffer
TEB buffer	Triton Extraction Buffer
TEMED	Tetra-Methyl-Ethylene-Diamine
TetA	Transactivator protein in the Tet-on3G system
TRE	Tet-Responsive Element
V	Volts
°C	Degree Celsius

7.2 Zusammenfassung

SUMOylierung (SUMO= Small Ubiquitin-related MOdifier) ist eine post-translationale Proteinmodifikation, welche die zellinterne Lokalisation, die Aktivität oder die Interaktionspartner eines Proteins verändern kann. Diese reversible Modifikation wurde mit vielen wichtigen zellulären Abläufen in Verbindung gebracht und Zahl ihrer Substrate steigt stets an. Einige Enzyme, die Chromatin modifizieren, werden auch von SUMO modifiziert. Viele dieser Enzyme sind für die Etablierung und Aufrechterhaltung von repressiver Histon- und DNA-Methylierung verantwortlich, die charakteristisch für inaktives Heterochromatin sind. Die essentielle SUMO-Kaskade besteht aus drei Enzymen: einem einzigen E1-, einem einzigen E2- und einigen E3-Enzymen. Bei Krebserkrankungen wurde das E2-Enzym dereguliert vorgefunden und mit der Bildung von Metastasen, der Verbreitung von Tumorzellen in Geweben und mit Chemotherapeutika-Resistenzen assoziiert.

In dieser Arbeit werden die Auswirkungen der Deregulierung von Ubc9 in der NIH 3T3 Maus-Fibroblasten Zelllinie geprüft. Hierfür wurden die für an das Centromer angrenzendes Heterochromatin bestimmenden Histon Lysin Methylierungen H3K9 und K4K20 untersucht. Die dabei angewendete Ubc9-siRNA-Behandlung der Zellen führte zu einem Wachstumsstopp und einem Anstieg der H4K20 Methylierung. Im Gegensatz dazu wurde keine Veränderung in der zweiten für diesen Abschnitt bekannten reprimierenden Histonmodifikation, der Tri-Methylierung von H3K9, gefunden.

7.3 Curriculum Vitae CHRISTIAN FALTUS

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03/2011-03/2012	Master thesis at the Max Planck Institute of Immunobiology and Epigenetics – Supervisor: Andrea Pichler in cooperation with Thomas Jenuwein, Topic: "Role of SUMO E2 enzyme Ubc9 deregulation in epigenetics"
10 -11/2010	Study Internship at the Max F. Perutz Laboratories – Supervisor: Johannes Nimpf, Topic: "Investigation of substrates for ApoER2 and VLDLR in the Reelin Signalling Pathway in primary embryonic rat neurons"
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