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Studies of Histone Modification Systems in *Schizosacchromyces pombe*

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To my beloved parents,
Eleanor and Esnoraldo

”Hjärtat har motiv som förståndet inte känner.”

Blaise Pascal

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ABSTRACT

The genetic information in every cell is carried in the DNA. In eukaryotes the DNA is wrapped twice around proteins called histones forming the fundamental repeating unit called the nucleosome, in fibers known as chromatin. Nucleosomes are folded into higher order structures forming the chromosomes. The chromatin can be more or less compacted, controlling the accessibility of the DNA to ensure correct gene expression. Histones have long N-terminal tails, which can be covalently modified by different modifying enzymes. Different types of histone modifications are linked to the different functional properties of chromatin. In this thesis we have studied three histone modification systems in the model organism *Schizosaccharomyces pombe* (fission yeast).

In paper I, the Rpb7 subunit of the RNA Pol II polymerase function is analyzed in cells with a missense mutation in the *rpb7* gene (*rpb7-G150D*) within an RNA binding motif. Chromatin Immunoprecipitation showed decreased levels of methylation of histone H3 at lysine 9 (H3K9me2), heterochromatin protein Swi6 and cohesin Rad21, which is the cause of lagging chromosomes and defects in chromosome segregation in *rpb7-G150D*. Analysis of centromeric forward and reverse pre-siRNA transcripts showed accumulation in the *dcr1*Δ mutant. In *rpb7-G150D* the reverse transcripts decreased to 25% of wild type levels. In the double mutant *dcr1*Δ*/rpb7-G150D* the reverse transcript levels remained low indicating that Rpb7 functions upstream of Dcr1 in the RNAi pathway that maintains the H3K9me2 in heterochromatin. More specifically, we show that Rpb7 is required for initiation of pre-siRNA transcription in this pathway.

In paper II, we show that the amino-oxidase family demethylases Lsd1 and Lsd2 from fission yeast are able to demethylate H3K9 but not H3K4 *in vitro*. Microarray studies showed increased H3K9me2 in 8,2% of the genes both in promoter and coding regions in the *lsd1*Δ knock-out and 3,8% of the genes also showed increased H3K4me2 in promoter regions. These genes also tend to become upregulated in *lsd1*Δ. Conversely, the down-regulated genes in *lsd1*Δ showed increased levels of H3K9me2, consistent with the notion that H3K9me2 is needed for gene repression and silencing. Our data also suggest that Lsd1 and the HDAC Clr6 cooperate to repression of genes. Thus, Lsd1 seems to have demethylase activity at both H3K4 and K9 *in vivo* but *in vitro* only K9me activity was detected, suggesting that additional factors are directing Lsd1 specificity *in vivo*.

In paper III, we found that Pst3, one of the three Sin3 homologs in fission yeast, is localized to the entire nuclear space, including the nucleolar core. This is in contrast to the other two homologs Pst1 and Pst2 which are not present in the nucleolus. The deletion of the *pst3+* gene affects genome stability, and causes sporulation defects, altered nucleolar structure and chomosome mis-segregation. Our data indicate that genome stability requires an established heterochromatic environment at rDNA repeats maintained by the Clr6 HDAC and Pst3. Pst3 co-purifies with two different Clr6 multi-protein complexes, suggesting that Clr6 HDAC complexes are dynamic. We show that Pst3 is associated with rDNA chromatin and is involved in rDNA silencing. Interestingly, Pst3 is specifically required for repression of endogenous Pol II mediated non-coding RNA transcripts within the rDNA spacer region.

LIST OF PUBLICATIONS

I. Djupedal I., Portoso M., Spåhr H., **Bonilla C**., Gustafsson C. M., Allshire R. C., Ekwall K. RNA Pol II subunit Rpb7 promotes centromeric transcription and RNAi-directed chromatin silencing. Genes Dev. 2005 Oct 1;19(19):2301-6.

II. Opel M., Lando D., **Bonilla C**., Trewick S. C., Boukaba A., Walfridsson J., Cauwood J., Werler P.J., Carr A. M., Kouzarides T., Murzina N. V., Allshire R. C., Ekwall K., Laue E. D. Genome-wide studies of histone demethylation catalysed by the fission yeast homologues of mammalian LSD1. PLoS One. 2007 Apr 18;2(4):e386.

III. **Bonilla C**.*, Silverstein R. A.*, Walfridsson J., Durand-Dubief M, Ekwall K. SIN3 homolog Pst3 is required for rDNA structure and function in S*chizosaccharomyces pombe*. Manuscript.

* These authors contributed equally to this work.

OTHER PUBLICATIONS

I. Durand-Dubief M, Sinha I, Fagerström-Billai F, **Bonilla C**, Wright A, Grunstein M, Ekwall K. Specific functions for the fission yeast Sirtuins Hst2 and Hst4 in gene regulation and retrotransposon silencing. EMBO J. 2007 May 16;26(10):2477-88.

II. Dunleavy E. M., Pidoux A. L., Monet M., **Bonilla C**., Richardson W., Hamilton G. L., Ekwall K., McLaughlin P. J., Allshire R. C. A NASP (N1/N2)-related protein, Sim3, binds CENP-A and is required for its deposition at fission yeast centromeres. Mol Cell. 2007 Dec 28;28(6):1029-44.

III. Sinha I., Buchanan L., Rönnerblad M., **Bonilla C**., Durand-Dubief M., Shevchenko A., Grunstein M., Stewart F. and Ekwall K. Genome wide mapping of histone modifications and mass spectrometry reveal an H4 acetylation bias in coding regions and a role for H3K36 methylation at gene promoters in fission yeast. Epigenomics in press.

CONTENTS

LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl Coenzyme A
ActD	Actinomycin D
AOL	Amine oxidase-like
bp	Base pair
ChIP	Chromatin Immunoprecipitation
Cnt	Centromere central core
DBP	DNA-binding protein
DMA	Diadipimidate
DNA	Deoxyribonucleic acid
ds	Double-stranded
ETS	External transcribed spacers
FACS	Fluorescence-activated cell sorting
FAD	flavin adenine dinucleotide
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IF	Immunofluorescence Microscopy
Imr	Innermost repeats (heterochromatin flanking the cnt)
ITS	Internal transcribed spacers
JmjC	Jumonji C family
KAT	Lysine acetyltransferase
KDM	Lysine demethylase
LSD1	Lysine Specific demethylase 1
NAD	Nicotineamide adenine dinucleotide
NTS	Non Transcribed Spacer in the rDNA region
Otr	Outermost repeats at centromeres
PCR	Polymerase chain reaction
PEV	position effect variegation
Pol II	RNA Polymerase II
Pst	*pombe* Sin three
rDNA	Ribosomal DNA
RITS	RNA-Inducing Transcriptional Silencing Complex
RT-PCR	Real-time PCR
RNA	Ribonucleic acid
RNAi	RNA interference
Rpb7	RNA polymerase II subunit, 7:th largest
siRNAs	small-interfering RNAs
Sin3	Switch independent 3
Swi6	Heterochromatin protein 1 homolog in *S. pombe*

1 INTRODUCTION TO CHROMATIN

The genetic information is carried in the DNA. The DNA is long and needs to be folded to fit into the nucleus but still be accessible for transcription, repair and replication. The DNA is wrapped around proteins called histones. Histones have long amino-terminal tails, which can be covalently modified by different modifying enzymes such as lysine acetyltransferases (KATs) that add an acetyl group to the lysine residues. The tails can be enzymatic modified by different histone modifying enzymes such as: histone deacetylases (HDACs), lysine methyltransferases (KMT) and lysine demethylases (KDMs), but other residues can also be modified by additional enzymes.

Together, DNA and histones form the fundamental repeat unit, called nucleosome, in fibers known as chromatin. Nucleosomes are folded into higher order structures forming the chromosomes. The chromatin can be more or less compacted, thereby controlling the accessibility of the DNA to ensure correct gene expression. Compacted chromatin is often associated by non-expressed genes and called heterochromatin whereas active and expressed genes are often accompanied with less dense chromatin and is known as euchromatin. Different types of histone lysine modifications are linked to the different functional properties of chromatin.

Euchromatin is typically marked by acetylation of histones in combination with di- or tri-methylation of histone H3 on lysine 4 (H3K4me2/3) and mono-methylation of histone H3 on lysine 36 (H3K36me). In contrast, heterochromatin is accompanied by hypoacetylation in combination with di- or tri-methylation of histone 3 on lysine 9 (H3K9me2/3). Heterochromatin is important for telomere and centromere function but is also involved in suppression of homologous recombination and thereby represses transposition of selfish DNA.

In this thesis three different types of histone modification systems have been studied. The first system is involved in centromere function where the methylation of H3K9 is important for proper centromere and chromosome function. We found that the RNA polymerase II subunit Rpb7 is needed for centromeric transcription and RNAi-directed chromatin silencing. This was shown by studying altered heterochromatin marks, such as H3K9 methylation, the heterochromatin protein Swi6 and cohesin Rad21 in the r*pb7* mutant.

The second histone modification system studied is the demethylation of methylated H3K4 and H3K9. Methylation is important to keep the genes at the right expression level. In paper II, we found that the homolog of the human demethylase LSD1^{KDM1} in fission yeast, had demethylase activity on both H3K4 and K9 *in vivo* but *in vitro* only K9me activity was detected, suggesting that additional factors direct Lsd1 specificity *in vivo*. Our data also suggest that Lsd1 and the HDAC Clr6 cooperate to silence genes.

The third modification system studied is the involvement of the corepressor Pst3 (*pombe* Sin three), found in complex with the HDAC Clr6, in maintaining genome stability. Pst3 is one of three Sin3 homologs in fission yeast. Sin3 links DNA binding proteins to chromatin modifying enzymes, most often HDACs, and is involved in the set up of chromatin environments important for proper gene regulation and genome stability. Sin3 is a conserved protein and is present from yeast to humans. We found that Pst3 is localized to the entire nuclear space, including the nucleolus, where it seems to have an important function. The nucleolus is where the ribosomal RNA (rRNA) genes are transcribed. The deletion of the *pst3+* gene causes genome instability and increased ploidy. Other phenotypes of the *pst3Δ* mutation are sporulation defects, altered nucleolar structure and chomosome mis-segregation.

1.1 THE NUCLEOSOME

The nucleosome contains a histone octamer with two of each core histone: H2A, H2B, H3 and H4. In this structure histone H2A dimerizes with H2B (H2A/H2B) and H3 and H4 forms a tetramer (H3/H4)2 (Luger, Mader et al. 1997; Luger and Hansen 2005). Nucleosomes are assembled in a two-step process; first a tetramer of H3/H4 is deposited onto the DNA, after which the two heterodimers of H2A/H2B are added (Kleinschmidt, Seiter et al. 1990).

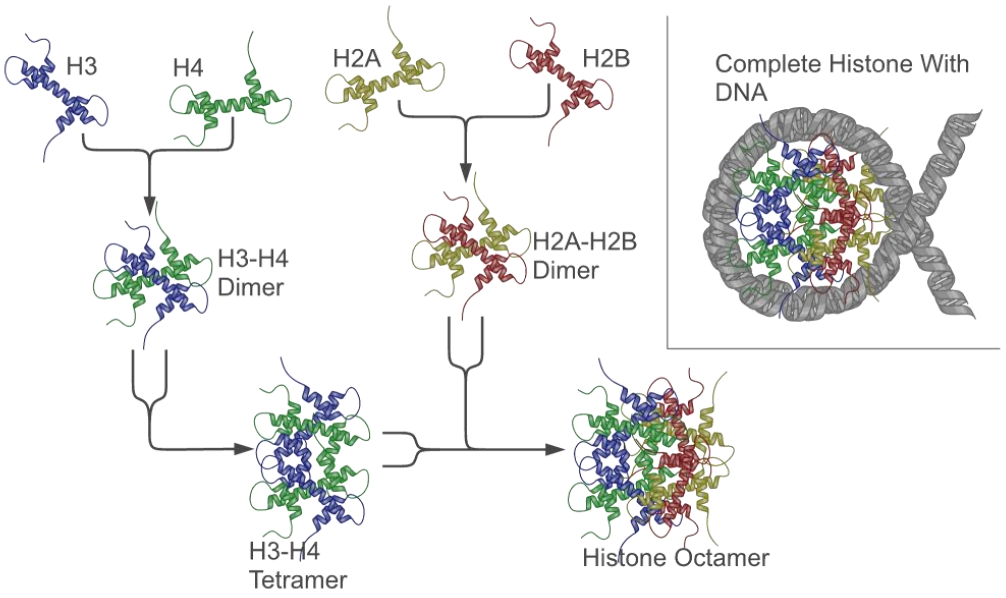


Figure 1. *Nucleosome composition. The nucleosome consist of 147 bp of DNA wrapped twice around the histone octamer complex with 2 copies of each core histone (H2A, H2B, H3 and H4). Each histone has tails than can be enzymatically modified by different chromatin remodeling enzymes. Reprinted with permission from Wheler R., Zephyris, 2005.*

Nucleosome positioning regulates the accessibility of the DNA (Luger, Mader et al. 1997). This is important for gene regulation since the nucleosome can block access of the DNA to transcription factors or, alternatively, help transcription by binding of additional factors (Park and Luger 2006). Chromatin remodelers are ATP-dependent enzymes involved in nucleosome positioning and organization, regulating the accessibility of the DNA (Jiang and Pugh 2009). The structure of the nucleosome can be altered by modifications of the histones (Wang, Wang et al. 2004) and by incorporation of histone variants (Chakravarthy, Gundimella et al. 2005). This can lead to changes in the charge of the nucleosomes leading to conformational changes of the chromatin, with local effects at single gene level but also global on large parts of the genome or entire chromosomes (Grewal and Moazed 2003).

1.1.1 Histone Variants

In addition to the canonical histones, there are histone variants with small (H2B.1, H3.3) or large (H2A.Z, H2A.Bbd, CENP-A) differences in their amino acid sequence. Histone variants are usually involved in special biological processes and in formation of specialized chromatin structures (Segal and Widom 2009). Histone variants

incorporated into nucleosomes might alter bonds and interactions with the DNA. One of the most studied variants is H2A.Z (Htz1 in yeast), which is conserved in eukaryotes. The crystal structure of a nucleosome with H2A.Z incorporated revealed a destabilization of the interaction between the H2A.Z-H2B-dimer and the tetramer of H3-H4. Thus, H2A.Z is involved in nucleosome stability (Suto, Clarkson et al. 2000). H2A.Z is found at gene promoters and seems to be involved in poising genes for active transcription. It is likely that nucleosomes containing H2A.Z are disassembled faster than regular nucleosomes and thereby exposing transcription factor binding sites in the promoter region (Cairns 2009). Possibly due to different experimental approaches, H2A.Z has been reported to be associated both with transcriptionally active and repressed gene promoters in different model organisms (Campos and Reinberg 2009). In fission yeast H2A.Z and heterochromatin factors, i.e. the KMT Clr4 and the RNAi-component Ago1 are involved in the suppression of antisense transcription (Zofall, Fischer et al. 2009). Another conserved histone variant, the H3 variant, CENP-A (called Cnp1 in *S. pombe*) replaces H3 at the centromeres. It is required for the special chromatin structure and alters the interaction of the nucleosome with the DNA so that the kinetochore, *i.e.* the structure that is responsible for chromosome segregation in mitosis, can form (Black, Foltz et al. 2004). Thus, histone variants can both change the chromatin structure to affect gene expression and contribute to anchoring a non-coding chromosome structure such as the kinetochore.

1.2 EUCHROMATIN & HETEROCHROMATIN

Chromatin exists in two forms, euchromatin and heterochromatin. Active regions of the genome are known as euchromatin while silenced or inactive regions are known as heterochromatin. Heterochromatin is more compact and may be facultative or constitutive. Facultative heterochromatin refers to chromosomal regions that sometimes can be modified, for example by histone modifications, to form heterochromatin. Constitutive heterochromatin is defined chromosomal regions that always have a high enrichment of heterochromatin and often contains DNA repeat elements. The heterochromatin protects DNA repeats from homologous recombination (Grewal and Klar 1997) and thereby represses transposition of selfish DNA.

At pericentromeric and subtelomeric regions, heterochromatin has a structural role. Heterochromatin can also affect transcription, as in X chromosome inactivation (Sidhu *et al.*, 2008) where the X-linked gene codes for an untranslated RNA that coats the

inactive X chromosome, which then gets histone modifications typical of heterochromatin (H3K9me2/3, H4K20me1). Heterochromatin can propagate along chromosomes, as in position effect variegation (PEV), where heterochromatin can intrude into what normally would be euchromatin and repress neighboring genes. The limitation of the spreading of the heterochromatin is very important since heterochromatin at the wrong place may silence genes that need to be expressed and can lead to a dangerous situation for the cell.

1.3 EPIGENETICS

Epigenetics is the study of heritable changes in gene expression that occur without alteration of the DNA sequence of the genome. I think of this as the DNA working as a template and epigenetics being the interpretation of the template depending on the situation at the moment. Epigenetic modifications seem to be stable but can be affected by physiological and pathological conditions as well as by the environment (Aguilera, Fernandez et al.). Environmental factors may cause epigenetic changes sometimes leading to adaptation. For example, plants are immobile and need to adapt to different environmental factors such as snow, cold, dry- or toxic soil. Plants have evolved different epigenetic ways to encounter different problems.

Epigenetic changes have been linked to aging, where DNA methylation was shown to decrease with age in mice (Bjornsson, Sigurdsson et al. 2008), rats (Vanyushin, Nemirovsky et al. 1973) and humans (Wilson, Smith et al. 1987). It has also been shown that increased levels of trimethylated histone H4 on lysine 20 (H4K20me3) is associated with aging processes in liver and kidney tissue in rats (Sarg, Koutzamani et al. 2002). Thus, the epigenome accumulates changes over time and these changes can depend on genotype, the environment and hitherto undetermined factors.

It is not know exactly how the environment affects the epigenome but it is thought that dietary intake of methyl donors for DNA methyltransferases and/or KMT and KAT activators/inhibitors and HDAC inhibitors can affect the epigenetic state of the genome. Garlic compounds (DADS) (Myzak and Dashwood 2006) and green tea component (EGCG) (Waladkhani and Clemens 1998) seem to inhibit the function of HDACs and therefore seem to be involved in prevention of age-related diseases by influencing epigenetic factors. The mechanisms involved in epigenetics are among others, DNA methylation, covalent histone modifications and non-coding RNA.

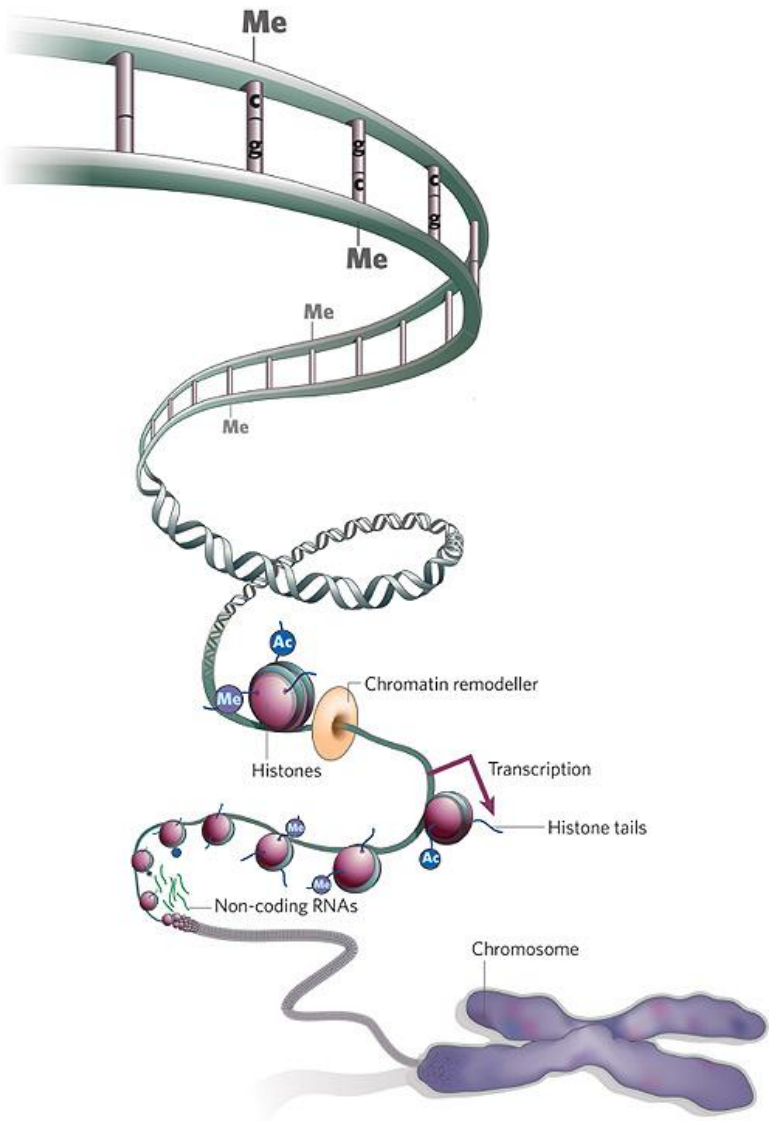


Figure 2. *Epigenetic mechanisms include DNA methylation, histone modification, alternation of nucleosome structure by histone variants and chromatin remodeling enzymes and non-coding RNAs. Reprinted with permission from Jones et al., Macmillian PublishersLtd: Nature, 2008 (2008).*

1.3.1 DNA methylation

DNA methylation mainly occurs in cytosines that precede guanines (CpG) to yield 5-methylcytosine (5-meC). The methylated Cytosine 5 in CpG dinucleotides can form a binding site for methyl-binding proteins, which induce transcriptional repression by recruiting repressors such as HDACs and KMTs. Repression by DNA methylation is essential during development (Morgan, Santos et al. 2005), X chromosome inactivation in females (Hellman and Chess 2007) and genomic imprinting (Tremblay, Saam et al.

1995). Long-term gene silencing is generally accompanied by DNA methylation (Hoffman and Hu 2006; Klose and Bird 2006). However, this epigenetic mark is not found in fission yeast.

1.3.2 Histone modifications

The core histones have a globular structure except for the amino (N) terminal tails protruding from the nucleosome working as a signaling platform. The histones are positively charged, which make the interaction with the negatively charged DNA easier. The histone tails have a regulatory function through the tail modifications. These can affect the chromatin structure by making it more or less packed and can by this mechanism regulate gene expression. The tails can recruit other factors that might have an effect on structure or transcription. Histone modifications can either function alone or with other histone modifications and one or more histone modification is often required for a specific function (Xu, Bai et al. 2009).

Over 60 different types of modified residues have been detected on histones and at least eight types of modifications have been identified (Kouzarides 2007). The histone N-terminal tails can be post-transcriptionally modified by addition or removal of different chemical groups by a variety of enzymes leading to acetylation, methylation, ubiquitation, phosphorylation, demethylation and deacetylation. Other histone modifications that are known are: sumoylation, biotinylation, ADP-ribosylation, proline isomerization and deimination but the information on these types of modifications is limited.

Lysine acetylation is associated with active gene transcription while lysine methylation is associated with both activation (H3K4, H3K36 and H3K79) and repression (H3K9, H3K27 and H3K20) of transcription depending on which of the histone tail residues are modified (Kouzarides 2007).

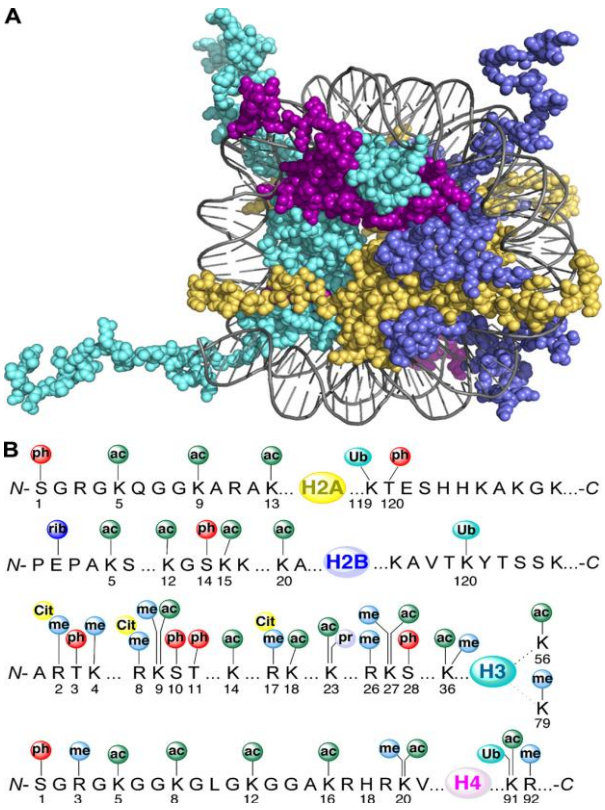


Figure 3. *Histones and the histone modifications.* ***A****, a single nucleosome structure with histone tails protruding from the core histones. H2A is represented by the yellow color, H2B in blue, H3 in turquoise and H4 in purple.* ***B****, schematic representation of histone tails and their modifications. The histone modifications are indicated as: ac, for acetylation; Cit, citrullination; me, methylation; ph, phosphorylation; pr, propionylation; rib, ADP-ribosylation; and Ub, ubiquitination. Reprinted with permission from Chatterjee and Muir, J Biol Chem, 2010 (Chatterjee and Muir).*

1.3.3 Histone modifying enzymes

The DNA molecule is long and needs to be correctly packed so that it fits in to the nucleus of the cell. At the same time the right factors need access to the DNA for processes such as gene transcription, DNA replication, recombination and repair at the proper time. Therefore regulation of the chromatin structure is of key importance in eukaryotes. To this end, several different histone modifying enzymes and nucleosome remodeling enzymes (SNF2 family of ATP helicases) use their activity to modulate the chromatin structure. Regarding the histone modifying enzymes they have been identified for acetylation, deacetylation, methylation, demethylation, phosphorylation, sumoylation, ADP-ribosylation, deimination and proline isomerization.

1.3.3.1 Lysine acetyltransferases (KATs)

Histone acetylation is the addition of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the ε-amino group of a lysine (K) residue on histone tails, which are carried out by lysine acetyltransferases (KATs). The histone tails are normally positively charged since they contain amine groups on their lysines- and arginines residues. This positive charge makes it easier to bind to the negatively charged phosphate groups on the DNA. Acetylation neutralizes the charge of the histones by transferring a negative charge to the N-terminal tail, decreasing the interaction of the negatively charged DNA with the histone tail. The change of the charge causes loosing of the DNA around the nucleosomes thereby allowing transcription (Roth, Denu et al. 2001).

During the discovery of histone acetyltranferases (HATs), these were divided in two group according to their location in the cell; A-HATs for the nuclear ones and B-HATs for the cytoplasmic ones. A-HATs are located in the nucleus and are involved in gene transcription-associated acetylation. B-HATs are involved in the assembly and transport of newly synthesized histones from the cytoplasm to the nucleus. HATs are divided in three families depending on their structural properties: Gcn5-related *N*-acetyltransferases (GNAT), MYST (for MOZ, Ybf/Sas3, Sas2 and Tip60) and p300/CBP (Roth, Denu et al. 2001). HATs are found in multi-subunit complexes such as the yeast SAGA complex (Spt-Ada-Gcn5 acetyltransferase) and SLIK (SAGA-like) which preferentially acetylates H3 and H2B and deubiquinate H2B (Pray-Grant, Daniel et al. 2005). HATs interact with sequence-specific transcription factors which target the HATs to specific promoters where histones or transcription factors can be modified and thereby affect gene regulation (Legube and Trouche 2003).

1.3.3.2 Histone deacetylases (HDACs)

Deacetylation is the removal of an acetyl group from the histone tails by enzymes called histone deacetylases **(**HDACs). Histone deacetylation is coupled to transcriptional repression since the removal of acetyl groups increases the charge attraction between histones and DNA, leading to tighter chromatin packing. In addition histone acetylation can also alter the affinity for chromatin binding proteins and thereby regulate transcription.

HDACs are evolutionarily conserved and they are phylogenetically divided into four families with HDAC activity. Class I contains human HDACs 1, 2, 3 and 8 and HDAC Clr6 and Hos2 in fission yeast (de Ruijter, van Gennip et al. 2003; Ekwall 2005). Class I are homologs to yeast Rpd3 and class II members resembles Hda1. Class II is subdivided in class IIa and IIb. Human HDACs 4, 5, 7 and 9 belongs to class IIa and HDACs 6 and 10 belong to class IIb. In fission yeast it is only Clr3 that belongs to this group. Class III HDACs (Sirtuins) are homologs to the *Saccharomyces cerevisiae* gene silent information regulator (Sir2) with nicotineamide adenine dinucleotide (NAD) dependent activity. There are seven Sirtuins (SIRT1-7) in mammals involved in many cellular processes such as cell cycle control, DNA repair and ribosomal DNA (rDNA) transcription. In *S. pombe* Sir2, Hst2 and Hst4 belong to the Sirtuins. Class IV is represented by human HDAC 11 which is related to both Rpd3 and Hda1 and is therefore grouped in another class, but little is known about its biological function (Yang and Seto 2008).

The class I HDACs are expressed in most cells but the class II HDACs have more tissue specific expression, suggesting that they might be involved in cellular differentiation and development (de Ruijter, van Gennip et al. 2003). In fission yeast, Clr3 and Clr6 localize to the nucleus while Hda1 localizes to the cytoplasm (Bjerling, Silverstein et al. 2002).

HDACs are as HATs found in multi-subunit complexes. In fission yeast Clr6 exist in at least two complexes. Clr6 forms two different complexes with the Sin3 homologs, Pst1 and Pst2. Sin3 binds HDACs and/or modifying enzymes to assemble silencing complexes that can target the chromatin. Complex I contain Pst1 and is essential, needed for repression of gene promoters and suppression of reverse transcripts from the centromere. Complex II contains Pst2, is not essential and deacetylates transcribed euchromatic regions and centromere cores. Complex II is implicated in protective functions of the genome where mutations of the components showed sensitivity of the DNA to genotoxic agents but also enhanced antisense transcripts that are processed by the exosome (Nicolas, Yamada et al. 2007). Complex II is also involved in repression of forward centromeric transcripts. HDACs and specially the proteins involved in different HDAC complexes are important for the total outcome of the gene expression.

1.3.3.3 Lysine methyltransferases (KMTs)

Histone methylation is very important for many cellular processes such as X chromosome inactivation, genomic imprinting, transcription and DNA repair. Histones can be methylated on either lysine (K) residues or arginine (R) residues. Here, only lysine methylation will be discussed. More than 20 KMTs have been identified with diverse functions (Yang, Lamb et al. 2009). KATs have a conserved catalytical motif known as the SET (Su(var)3-9, Enhancer of zeste E(z) and Trithorax) domain and can subject lysine residues to be mono-, di- or tri-methylated by addition of methyl group(s).

Lysine methylation can result in either transcriptional activation or repression depending on the methylated residues. As already mentioned, transcriptional activation is usually accompanied by methylated H3K4, H3K36 and H3K79 (Schubeler, MacAlpine et al. 2004; Martin and Zhang 2005). In contrast, methylated H3K9, H4K20 and H3K27 are associated with transcriptional repression (Cao, Wang et al. 2002; Reinberg, Chuikov et al. 2004; Schotta, Lachner et al. 2004).

1.3.3.4 Lysine demethylases (KDMs)

Histone lysine methylation was until 2004 assumed to be irreversible (Shi, Lan et al. 2004). The removal of methyl groups on histone lysines is catalyzed by the enzymes lysine demethylases (KDMs). Two groups of KDMs have been identified, including the amino-oxidase family and Jumonji C (JmjC) domain family proteins.

Lysine specific demethylase 1 ($LSD1^{KDM1}$) was shown in 2004 by Shi and colleagues, to be a H3K4 (H3K4me1/2) specific demethylase but could not demethylate tri-methylated H3K4 (H3K4me3). This is because $LSD1^{KDM1}$ belongs to the amine oxidase family and contains a C-terminal amine oxidase-like (AOL) domain homologous to flavin adenine dinucleotide (FAD)-dependent oxidases. The α-carbon bond of the substrate is cleaved by the amine oxidase to form an imine intermediate. The imine is then hydrolyzed to form an aldehyde and amine via a non-enzymatic process. $LSD1^{KDM1}$ depends on the cofactor FAD to be able to catalyze the oxidation reaction (Tian and Fang 2007). Protonated nitrogen is required for $LSD1^{KDM1}$ demethylase as a substrate for the reaction.

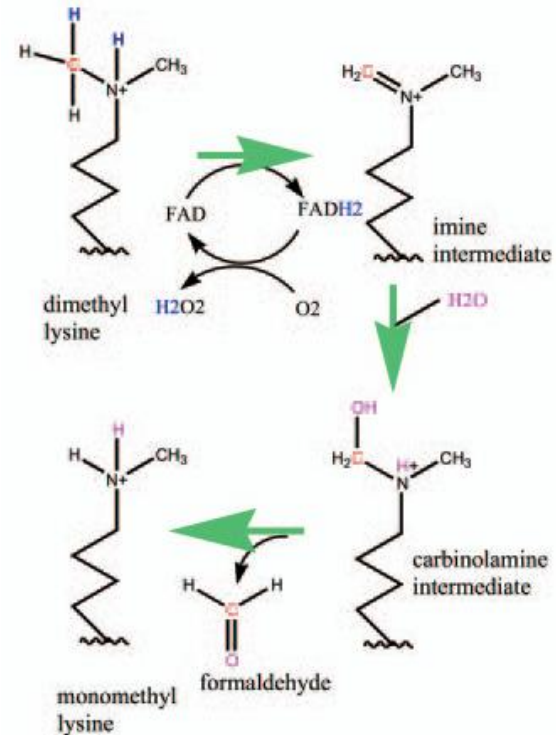


Figure 4. *Mechanism of lysine demethylase LSD1^{KDM1}. LSD1 transfers two hydrogen atoms from methylated H3K4 to FAD and forms a imine intermediate. The imine intermediate is then hydrolyzed to result in an unstable carbinolamine intermediate followed by formaldehyde release. Reprinted with permission from Anand and Marmorstein, J Biol Chem, 2007 (Anand and Marmorstein 2007).*

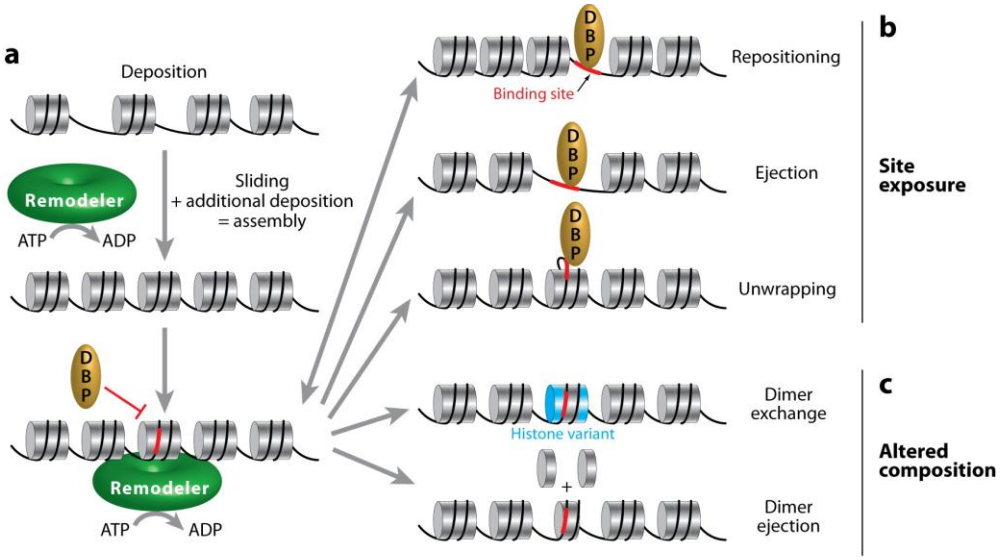
LSD1^{KDM1} is found in complex with hHDAC 1 or 2. LSD1^{KDM1} and HDACs may regulate gene repression by first deacetylation and then removal of the methyl group from H3K4 (Tian and Fang 2007). The function of LSD1^{KDM1} depends on the proteins interacting with it.

There are three JmjC domain subfamilies, JHDM1, JHDM2 and JHDM3 that have histone demethylase activity. JmjC domain protein families depends on Fe(II) and α-ketoglutarate as cofactors to hydroxylaze the substrate and does not require protonated nitrogen and can therefore demethylate tri-methylated residues, exept JHDM1, that is incapable to demethylate tri-metylated histones (Tian and Fang 2007).

Specific histone methylation sites are usually accompanied by other histone modifications, such as acetylation, showing that the combination of histone modifications might contribute to the final biological outcome.

1.3.3.5 Chromatin Remodeling Enzymes

Chromatin remodeling enzymes are ATP-dependent and use ATP hydrolysis to be able to catalyze the alteration of chromatin packing, by moving, ejecting or restructuring the nucleosome, resulting in modification of nucleosome positioning or accessibility of the nucleosomal DNA (Clapier and Cairns 2009). Chromatin remodeling enzymes are needed for different processes in the cell, such as following replication when nucleosomes are deposited and need to be properly spaced, during DNA repair and recombination when nucleosomes are removed, slided and restored and during transcription when nucleosomes can be released by either ejecting or chaperoning the histone octamer around the advancing polymerase.



Clapier CR, Cairns BR. 2009.
Annu. Rev. Biochem. 78:273–304

Figure 5. *Outcomes of chromatin remodeling. Chromatin remodeling enzymes (green) can move already deposited histone octamers making room for additional deposition. (a). the mechanism used by chromatin remodeling enzymes. (b) site exposure, in which a site (red) for a DNA-binding protein (DBP), initially hidden by the histone octamer, becomes accessible by nucleosomal sliding, or nucleosomal ejection, or unwrapping. (c) altered composition, in which the nucleosome content is modified by dimer replacement or through dimer ejection. Reprinted with permission from Clapier and Cairns, Annu. Rev. Biochem., 2009 (Clapier and Cairns 2009).*

Chromatin remodeling complexes are divided into distinct families based on their helicase domains. The families are SWI/SNF, ISWI, CHD and INO80. All chromatin remodelers share five basic properties: 1) affinity for the nucleosome, beyond the

DNA; 2) domains that recognize covalent histone modifications; 3) a similar DNA-dependent ATPase domain, needed to break DNA-histone contacts; 4) domains and/or proteins that regulate the ATPase domain; and 5) domains and/or proteins that interacts with other chromatin or transcription factors.

The SWI/SNF (switching defective/sucrose nonfermenting) family consists of multi-subunit complexes and function by sliding and ejecting nucleosomes but do not have any involvement in chromatin assembly. The ISWI (imitation switch) family contains 2 to 4 subunits. The ISWI ATP-dependent chromatin assembly and remodeling factor (ACF) and chromatin accessibility complex (CHRAC) establish nucleosome position (Jiang and Pugh 2009) and transcriptional repression by optimizing nucleosome spacing (Clapier and Cairns 2009). Another ISWI complex, nucleosome remodeling factor (NURF) can randomize spacing of nucleosome, involved in RNA pol II activation. In *Drosophila melanogaster* (fruit fly) ISWI complexes have also been shown to be required for maintaining higherorder structure of male X chromosome (Deuring, Fanti et al. 2000).

The CHD (chromodomain helicase DNA binding) remodeling complexes are both involved in activation of transcription by sliding or ejecting nucleosomes and repression by for example Mi-2/NURD (nucleosome remodeling and deacetylase) which contains histone deacetylases (HDAC 1/2) and methyl CpG-binding domain (MBD) proteins. The range of the CHD family can be caused by the chromodomain variety. The INO80 (inositol requiring 80) family contains more than 10 subunits and are involved in promoting transcription activation and DNA repair. INO80 complexes have also been shown to be involved in telomere regulation, chromosome segregation and DNA replication during cell division (Ho and Crabtree). Chromatin remodeling enzymes share some properties but have distinct targets that require specialization of the different enzymes.

1.4 RNA INTERFERENCE (RNAi)

At centromeres in fission yeast, heterochromatin formation requires the RNA interference (RNAi) pathway. The centromeres contain a central core (*cnt*) and flanking heterochromatin domains, the inner (*imr*) and the outer (*otr*) repeats, all important for proper centromere and chromosome function. During cell division the kinetochore is attached to chromatin in the *cnt* region and it is required for the

connection of the centromere to the spindle microtubules. The flanking heterochromatin in the *otr* region is important for sister chromatid cohesion (Ekwall 2004). Heterochromatin is established in centromeres by deacetylation of H3 and H4 tails by Clr3, Clr6 and Sir2 HDACs allowing for methylation of H3K9 by the methyltransferase *SuVar(3-9)* Clr4 in *S. pombe* (Rea *et al*., 2000). This mark is a special binding site for the Heterochromatin protein (HP1) homolog, Swi6, which binds to the methylated H3K9 via a chromodomain. Swi6 mediates binding of cohesion to centromeric heterochromatin.

The formation of heterochromatin requires RNAi, which is initiated by long double-stranded RNA (dsRNA) precursors from the flanking repeats. These are generated by RNA polymerase II (pol II) transcription requiring the pol II Rpb7 subunit for transcription initiation (paper I). The RNAi pathway is initiated by the ribonuclease III protein, Dcr1, which cleaves long dsRNA or folded RNA into small dsRNA fragments of around 23 nucleotides in length, called small-interfering RNAs (siRNAs). These siRNAs are incorporated into the RNA-induced transcriptional silencing (RITS) complex. A member of the PAZ/Piwi family, Argonaute, is involved in this gene silencing complex. The complex is guided to complementary nascent RNA at centromeres, which recruits the Clr4-Rik1-Cul4 (CLRC) complex that includes the KMT Clr4 and generates H3K9 methylation in this region (Bayne, White et al.), coupling RNAi to chromatin modification.

2 MATERIAL & METHODS

2.1 *Schizosaccharomyces pombe* as MODEL ORGANISM

Schizosaccharomyces pombe (*S. pombe*), also known as fission yeast, was first isolated from African brew. Pombe means alcoholic beverage in Swahili. *S. pombe* is a unicellular fungus with a genome of 13.8 Mbp and containing 4,824 protein coding genes. The genome consists of three chromosomes where chromosome I is the largest at 5.7 Mbp, chromosome II is 4.6 Mbp and the smallest is chromosome III with 3.5 Mbp. Chromosome III contains 100-120 repeats in tandem of the 5.8S, 18S and 25S ribosomal RNA genes (Wood, Gwilliam et al. 2002) arrayed in two clusters at each end. In eukaryotes, each rRNA gene consists of a promoter, internal and external transcribed spacers (ITS/ETS), rRNA coding sequences (5.8S, 18S and 28S) and an external non-transcribed spacer (NTS). *S. pombe* has three centromeres where centromere I is 35 kb, centromere II is 65 kb and centromere III is 110 kb long. The centromeres of *S. pombe* are larger than in budding yeast and resemble the centromeres of metazoa.

S. pombe is widely used by scientists since it is relatively easy to manipulate genetically, easy and fast to grow and relatively inexpensive. Also, many genes are found in *S. pombe* with high similarly to human disease genes including cancer (Wood, Gwilliam et al. 2002). At least 23 *S. pombe* genes are related to other diseases such as cardiac-, renal-, metabolic- and neurological diseases.

2.2 CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) is widely used for studies of protein-DNA interactions. This method can be used for investigation of occupancy for a protein of interest, localization of histone variants, transcription factors or for studies of different chromatin modifications. For ChIP of yeast cells, cultures are grown to logarithmic-phase and centrifuged to get a cell pellet, which is washed and then treated with formaldehyde to fix the proteins to the DNA. The cells are then lysed, usually using a bead-beater machine to disrupt the tough cell walls of yeast cells. The cell extract is sonicated to shear the DNA to smaller fragments to an average size of around 500 bp. Immunoprecipitation is made by incubation with an antibody targeted to the protein of

interest to distinguish DNA bound by the specific protein. Protein-A beads are added to the extract for isolation of the bound DNA to the specific antibody. The immunoprecipitated material is then washed several times before reversing the formaldehyde crosslinking by heat and SDS. Protein is removed by adding proteases to the immunoprecipitate to achieve the DNA bound to the protein of interest. Analysis of the specific immunoprecipitated DNA regions can be made by quantitative PCR amplification. The immunoprecipitated DNA can also be used to make genome-wide binding maps by amplification, labeling and hybridization of the DNA to microarrays (ChIP on chip).

The ChIP technique has a big advantage compared to *in vitro* techniques since the occupancy of a specific protein can be investigated *in vivo* and at a particular time. There are some aspects to consider for this technique, for example the distance of the protein of interest from the DNA. For proteins with a large distance from the DNA, Di-Methyl Adipimidate (DMA) can be added and the incubation with both or only formaldehyde can be extended. The antibodies used for immunoprecipitation need to be highly specific against the epitope in a free solution in a mix with other proteins to reduce the background. Washing of the protein-A beads is also crucial for keeping the background levels low. The size of the sonicated DNA can be a problem for the tiling arrays and limits the resolution of the arrays. A disadvantage of ChIP on chip is the expense of microarrays, which limits the use of this technique.

2.3 EXPRESSION ANALYSIS

Gene activity is reflected by transcript levels. To be able to measure transcript levels, first the RNA is extracted from cells. The cells in logarithmic phase are pelleted, washed and treated with hot acid phenol for subsequent extraction of the RNA. The method gives high yield of RNA but includes many steps. Disadvantages are that the samples might have traces of phenol and chloroform, which may inhibit enzymatic reactions. Also, degradation of the RNA might occur if too many cells to phenol/chloroform are added, so fast and careful proceeding of the samples is needed in order to not introduce any nucleases.

There are different methods to detect mRNA levels in a cell. In this thesis some of the methods used are: RT-PCR, Northern analysis, Transcription Run On analysis (TRO), 3´-and 5´-RACE and genome-wide expression profiling. Strand specific labeling of

RNA combined with high-resolution ‘tiling’ arrays have made it possible to detail study both sense and antisense transcripts from different organisms. In this type of analysis it has been a concern that antisense transcripts could be artifacts from the reverse transcription (Johnson, Edwards et al. 2005). This because the enzyme used for generation of cDNA has a secondary activity as a DNA-dependent DNA polymerase. The enzyme has the ability to generate spurious transcripts by priming to hairpin loops at the 3´-or 5´-ends or by priming to RNA fragments formed by degradation of RNA template or by priming to primers from the first-strand synthesis. In 2007, Perocchi *et al*., validated the use of reverse transcriptase in the cDNA reaction for microarray use. They show that use of Actinomycin D (ActD) during reverse transcription inhibits ability of the reverse transcriptase to create antisense artifacts (Perocchi, Xu et al. 2007). Therefore we used ActD in paper III to reduce the antisense artifacts (see below).

2.4 MICROARRAY ANALYSIS

Different DNA microarray platforms have been used for the studies in this thesis. In papers I and II Eurogentec ‘spotted’ microarrays with 500 bp cDNA probes for each gene of 99% of the annotated genes in *S. pombe* were used (Xue, Haas et al. 2004). cDNA samples were labeled with different fluorescent dyes and two cDNA samples were hybridized to the same microarray glass slide (two-channel array). After hybridization the slide was washed, dried and scanned. It is crucial to have good quality of the target sample, hybridization and washing of the microarray slide to obtain good results.

In paper III both Eurogentec spotted microarrays and Affymetrix GeneChip® *S. pombe* ‘tiling’ 1.0FR microarrays were used. The Affymetrix tiling arrays have 20 bp resolution where 25 bp are tiled and 5 bp are overlapping from the adjacent probes. The array contains more than 1.2 million probes covering the whole *S. pombe* genome, including both strands of DNA. The Affymetrix tiling arrays are highly reproducible, have bigger coverage and higher resolution than the spotted arrays (Eurogentec).

Normalization of the microarray data is crucial for the interpretation of thousands or millions of data points. Therefore adjustments by normalizing the data are done to reduce the technical variance and to enhance the biological differences in the samples. Normalization was done with the software GeneSpring (Agilent) for the Eurogentec

microarrays and for the tiling microarrays the Affymetrix Tiling analyzing software (TAS) and the Integrated Genome Browser (IGB) were used.

2.5 IMMUNOFLUORESCENCE MICROSCOPY

Immunofluorescence microscopy (IF) is a technique that is used to find the cellular location of a protein *in vivo* and to get a hint of the proteins function by knowing where it is located. IF is used to show different proteins or antigens in fixed cells or tissues. Here, antibodies labeled with fluorophores, a fluorescent dye, are used to visualize the target antigen in the sample. The fluorophore is excited by absorption of high energy light and emits lower energy light which can be visualized as fluorescence in a fluorescence microscope. IF can be done in two ways, either direct or indirect. In paper III, indirect IF was used. Here, the cells are cross-linked with formaldehyde and treated with partial enzyme digestion to digest the cell wall. To reduce non-specific antibody interactions the cells are blocked with Bovine serum albumin or milk proteins. A primary unlabeled antibody is added to the fixed cells and is allowed to react with a specific epitope (from the protein of interest). A second, fluorophore-labeled antibody is added to allow recognition of the first antibody. Immunolabeled cells are studied in a fluorescent microscope, which irradiates the samples with wavelengths that excite the fluorophores.

By using different secondary antibodies with different fluorophores, different antigens can be localized in the same sample. This method is very sensitive and allows for great selection. As always, when using antibodies, the specificity of the antibodies can be a problem. Reduction of the fluorescence emission can be a cause of intensity reduction. This means fading of the fluorescence, caused by photobleaching, which is irreversible when the fluorescent dye in the excited state interacts with oxygen before emission of the light or that the properties of the fluorophore are changed and can therefore not emit light.

3 RESULTS & DISCUSSION

3.1 AIMS

The aim of this thesis was to investigate different histone modification systems in fission yeast. First, the general features and specific role of the Rpb7, a RNA polymerase II subunit was studied. The aim in paper I was to investigate whether the centromeric *dg-dh* transcripts (pre-siRNA) were transcribed by RNA pol II and where needed to establish and maintain high H3K9me2 levels in centromeric heterochromatin. Then, the homologs of the human lysine specific demethylase ($LSD1^{KDM1}$), Lsd1 (Swm1) and Lsd2 (Swm2) in fission yeast were studied, to investigate the specificity of these enzymes *in vivo* and *in vitro*. The third histone modification system studied was the characterization of one specific Sin3 homolog, named Pst3. Sin3 acts as a targeting factor for HDAC enzymes and the aim of this study was to investigate the specific cellular role of the Pst3 protein.

3.2 PAPER I

A temperature sensitive mutant *csp3* (centromere: suppressor of position effect) was isolated in a screen for *trans*-acting mutants involved in centromeric silencing (Ekwall, Cranston et al. 1999). The Rpb7 subunit of RNA polymerase II (pol II) function is analyzed in a mutant with a mis-sense mutation in the *rpb7* gene (*rpb7-G150D*) within an RNA binding motif. Purification of pol II from wild type and *rpb7-G150D* cells showed identical composition of the subunits indicating that the phenotype observed in *rpb7-G150D* is not caused by altered composition of the mutant pol II. ChIP analysis showed decreased levels of H3K9me2, heterochromatin Swi6 and cohesion Rad21, which is the cause of lagging chromosomes and defects in chromosome segregation defects in *rpb7-G150D*. It was important to investigate if *rpb7-G150D* was involved in the same pathway as *dcr1*. Strand specific RT-PCR and Northern analysis showed accumulation of centromeric forward and reverse *dg-dh* transcripts in *dcr1*Δ mutant. In *rpb7-G150D* the reverse transcripts decreased to 25% of wild type levels. In the double mutant *dcr1*Δ*/rpb7-G150D* the reverse transcripts levels remained low indicating that Rpb7 functions upstream of Dcr1 in the RNAi pathway and is required for initiation of pre-siRNA transcription. 3´-RACE of

centromeric *dg-dh* repeats (pre-siRNA) showed that reverse transcripts are polyadenylated and are therefore transcribed by RNA polymerase II. 5´-RACE was conducted in order to map the 5´-end of the pre-siRNA. The region upstream of the transcription start site was cloned upstream of a reporter gene, lacZ, and β-galactosidase assay was done to measure the activity of the enzyme. The results showed that the *rpb7-G150D* mutant had defects in initiation of transcription of the reverse promoter. Hence, we show that Rpb7 is needed for initiation of transcription of pre-siRNA and that these are transcribed by RNA pol II and are required for H3K9me2 in centromeric heterochromatin, important for chromosome function.

3.3 PAPER II

Histone methylation is involved in many important cellular processes and the discovery of the enzymes that demethylate histones opened a new world of thinking. Here, we investigate the Lysine Specific Demethylase 1 ($LSD1^{KDM1}$) homologs Lsd1 and Lsd2. Human LSD1 is a flavine-dependent amine oxidase that catalyzes the removal of mono- and dimethylated Lysine 4 or 9 on histone H3. LSD1 can serve as a platform for stabilization of associated corepressor (HDAC) complexes on chromatin (Shi, Lan et al. 2004).

According to previous results in *S. pombe* we could purify Lsd1 and Lsd2 with each other and with two PHD containing proteins (Nicolas, Lee et al. 2006). In our assay the purified Lsd1 and Lsd2 were not co-purified with any HDAC, as in mammals, (Shi, Lan et al. 2004) or with the chromatin remodeler factor Hrp1. This inconsistancy might have been caused by the different protein tags used in the studies.

We show that purified Lsd1 and Lsd2 from fission yeast could only de-methylate H3K9 *in vitro*. The *lsd2*Δ knockout is lethal so the phenotypic analysis could only be performed for *lsd1*Δ. The gene expression analysis shows that Lsd1 is involved in the repression of weakly expressed genes. ChIP-chip showed an increase of H3K9me2 in *lsd1*Δ in 8.2% of the genes both in the promoter regions (IGR) and in coding regions (ORF). In addition we found that 3.8% of the genes in *lsd1*Δ had an increase of H3K4me2 in IGR's. Upregulated genes in *lsd1*Δ displayed increased H3K4me2 in the ORF regions. Down-regulated genes in *lsd1*Δ showed increased levels of H3K9me2 in ORFs, consistent with the notion that H3K9me2 is needed for gene repression and silencing. Our data also suggest that Lsd1 and the HDAC Clr6

cooperate to repress the chromatin. Thus, Lsd1 has demethylase activity on both H3K4 and K9 *in vivo* but *in vitro* only K9me Lsd1 activity was detected, suggesting that additional factors are directing Lsd1 specificity *in vivo*.

3.4 PAPER III

Sin3 (switch independent 3) was first discovered in genetic screens for factors involved in regulation of mating type switching in *S. cerevisiae* (budding yeast), as well as transcriptional regulation (Nasmyth, Stillman et al. 1987; Sternberg, Stern et al. 1987). Sin3 is conserved from yeast to humans and forms a platform to coordinate DNA binding proteins with chromatin modifying enzymes, most often HDACs, to establish chromatin environments important for proper gene regulation and genome stability. The Sin3 homolog, Pst3, in fission yeast was studied in this paper. The *pst3* gene was knocked out by homologous recombination and the phenotype from the mutation shows that the DNA is unevenly divided during cell division.

We found that Pst3 is the only one of the three Sin3 homologs in fission yeast that is localized to the entire nuclear space, including the nucleolus, where it seems to have an important function. The nucleolus is the site for ribosome formation. The rRNA genes are transcribed forming long pre-ribosomal RNA, which is then processed and forms the 5.8S, 18S and 28S rRNA. Each rRNA gene is made of a transcribed sequence and a non-transcribed spacer (NTS) in which all sequences needed for RNA Pol I transcription are situated. The deletion of *pst3* causes genome instability, characterized by sporulation defects, altered nucleolar structure and chomosome mis-segregation, indicating a special function for Pst3 in the nucleolus.

Fluorescence-activated cell sorting analysis showed that the *pst3*Δ mutant had much higher DNA content compared to wild type and this suggests that genome duplications might have occurred. Purified Pst3 is associated with members of two different Clr6 multi-protein complexes suggesting that Clr6 HDAC complexes are dynamic and play a role at rDNA being targeted by Pst3 to establish a heterochromatic environment required for genome stability. Since *pst3*Δ have a compromised genome, the *pst3* gene promoter was replaced with the thiamine repressible *nmt-41* promoter where the *pst3* gene is repressed when thiamine is added.

ChIP on CHIP experiments showed that Pst3 is associated with rDNA chromatin and spotting assays showed that Pst3 is involved in rDNA silencing. We used microarray

analysis to study the rDNA region and we found, in accordance with previous reports (Perocchi, Xu et al. 2007) that the spurious artifact antisense RNA was reduced with use of ActD. Interestingly, this analysis revealed that Pst3 is also required for repression of endogenous Pol II mediated non-coding RNA transcripts within the rDNA spacer region (NTS). In budding yeast the transcription of non-coding RNA leads to rDNA recombination defects and arrangement of the repeats (Li, Mueller et al. 2006). Hence, Pst3 seems to have an important role in the function of the nucleolus, transcription regulation and is probably involved in heterochromatin formation.

4 CONCLUSION & FUTURE PERSPECTIVES

The condensation of DNA to fit into a cell provides different obstacles to processes such as replication, transcription or DNA repair. Therefore the folding of the chromatin is very important so that when needed, the DNA can be accessed. The structure of the chromatin changes dynamically, allowing for local decondensation and remodeling that facilitates the access of the DNA. Extensive work has been performed in the field of chromatin research searching for the role of histone modifications in gene regulation and other cellular processes. Over past few years, significant progress has been made into the identification of enzymes involved in histone modification, such Lsd1 (paper II) shown to be a lysine specific demethylase both for H3K4me2 and H3K9me2 *in vivo* but only H3K9me2 activity *in vitro*, demonstrating that methylation is reversible.

Histone modifications have been shown to be important for different cellular processes such as proper chromosome segregation. In paper I we show that Rpb7 is needed for proper pre-siRNA transcription initiation required for H3K9me2 in centromeric heterochromatin, important for chromosome function. Another histone modification needed for accurate centromere function is deacetylation of histones, where the HDACs Clr6, Clr3 and Sir2 are important for the formation of heterochromatin (Ekwall 2004). The HDAC Clr6 is found in complex with corepressors such as Sin3. We have characterized the Sin3 homolog, Pst3, in paper III. We have preliminary indications that the three Psts in *S. pombe* have developed specific functions in different cellular processes since investigation of Pst1 showed that Pst1 and Clr6 are required for the hypoacetylation of centromeres and mutation in *pst1* showed a centromeric sister-chromatid cohesion defect (Silverstein, Richardson et al. 2003). Mutation in *pst2* caused defects in chromosome segregation, resolution and/or

condensation during mitosis (Nakayama, Xiao et al. 2003). Mutations in both *pst1* and *pst2* do not cause any effect in silencing of rDNA. Recently, the rDNA silencing in budding yeast was reported to be the natural way to repress synthesis of Pol II-dependent ncRNA from the NTS that can be dangerous for the cell by altering histone modifications and induction of hyperrecombination. When Pol II activity is increased more ncRNAs are produced resulting in increased histone acetylation allowing for more transcription (Cesarini, Mariotti et al.). The limiting factor is the transcription of the 35S pre-rRNA by Pol I because when the Pol I is maximal then the acetylation of Pol II promoters goes down, thereby repressing the production of ncRNAs.

Still there are many questions about histone modifications that need to be further investigated, among these are for example, is there any universal histone "code"? Which combinations form the code and what is the outcome? How are siRNAs involved in chromatin modification? How is the rDNA affected by histone modifications in the r-*pst3* mutant? Regulation of histone modification and chromatin has been an extensive field of research that has provided a lot of surprises and will probably continue to surprise us in the future.

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