Localization and Function of Histone Methylation at active Genes in *Drosophila*

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List of Abbreviations

ASF1	Histone chaperone
ATP	Adenosine Triphosphate
CAF1	Histone chaperone
CTD	RNA Polymerase II C-terminal Domain
dHypb	*Drosophila* homologue of yeast Set2
DOT1	yeast Histone Methyltransferase specific for H3K79
dMes-4	*Drosophila* homologue of worm Maternal-effect-sterile Histone Methyltransferase specific for H3K36
FACT	Nucleosome remodeling complex
Gal4	Transcriptional activator specific for Galactosidase 4
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HMT	Histone Methyltransferase
ISWI	ATPase-dependent nucleosome remodeling enzyme
NAP1	Histone chaperone
nm	nano meter
ORF	Open Reading Frame
PIC	Pre-Initiation Complex
Pol II	RNA Polymerase II
RSC	Nucleosome remodeling complex
Set1	yeast Histone Methyltransferase specific for H3K4
Set2	yeast Histone Methyltransferase specific for H3K36
TFIIA, B, D, H	general transcription factors for RNA Polymerase II
TBP	TATA-box binding protein

Table of Content

1. SUMMARY

In the eukaryotic nucleus, DNA is bound by an octamer of four core histones forming the fundamental repeating unit of chromatin, called the nucleosome. Presenting a barrier to virtually all DNA-templated events, nucleosomal packaging is subject to dynamic alterations.

Nucleosomal histone modifications have emerged as a major determinant of chromatin structure and gene expression. Genome-wide and local profiling of chromatin structure in *Drosophila* cells reveals a complex landscape of histone methylation marks along the body of active genes. Methylation of lysine 4 and lysine 79 of histone H3 coincide at promoters and gradually decrease towards the 3' end. Conversely, H3 lysine 36 methylation states show very different distribution patterns. Dimethylation of H3K36 peaks downstream of promoter-proximal K4 methylation, whereas trimethylation accumulates towards the 3' end of genes. These topographic differences do not reflect deposition-coupled targeting by histone variant H3.3 but instead argue for discrete regulation and function of active methylation marks during transcription elongation.

Indeed, H3K36 di- and trimethylation states rely on two distinct HMTs and display opposite effects on H4K16 acetylation at autosomal genes. This crosstalk is reminiscent of K36me3-dependent deacetylase recruitment in budding yeast, yet it is more intricate as dimethylation appears to signal for increased H4K16 acetylation. Apart from its autosomal function, H3K36me3 has a separate role to enhance H4K16 acetylation at the dosage-compensated X chromosome in male *Drosophila* cells. This additional function most likely involves MSL complex recruitment to dosage compensated genes.

Together, our results reveal a complex pattern of histone methylation marks at active genes, which may enable dynamic chromatin changes during transcription elongation in higher eukaryotes. Furthermore, the context-dependent readout of H3K36me3 implies that methylation marks act as general signaling platforms, which impart their specificity by recruiting effector proteins to characteristic landmarks along the transcription unit.

2. INTRODUCTION

Eukaryotic cells have to condense their DNA into the confines of a cell nucleus and yet provide accessibility for proteins to regulate gene transcription, replication and repair. Such balancing act requires a dynamic and yet highly structured organization of eukaryotic genomes. Inevitably, such organization will have a major effect on DNA readout as various regions can be more or less condensed. At the same time, it provides an opportunity for the differential expression of cell-type specific programs from identical sequence information. The following section will give an introduction to the organization of eukaryotic DNA in the cell nucleus and highlights aspects of its dynamic regulation in particular with respect to gene expression.

2.1. The nucleosome – fundamental subunit of chromatin

In the eukaryotic nucleus, DNA is coated with an equal mass of proteins to form a hierarchical structure called chromatin (Felsenfeld & Groudine, 2003). Histones, which are the principle protein components of the nucleo-protein complex, are among the most highly conserved proteins encoded in eukaryotic genomes (Sullivan & Landsman, 2003). The near perfect conservation of its basic subunits suggests that the fundamental structure of chromatin is common to all eukaryotes. In general, histones consist of a highly-structured, globular histone-fold domain, an unstructured highly basic N-terminal tail and a short basic C-terminal tail. Four canonical core histones are expressed in eukaryotes at an equimolar ratio: H2A, H2B, H3 and H4. These histones readily oligomerize such that histones H3 and H4 form hetero-tetramers and H2A and H2B form hetero-dimers (Kornberg, 1974; Oudet et al, 1975).

The first level of chromatin organization is the formation of the nucleosome “core particle” which comprises of 146 bp of DNA wrapped around a histone octamer (Kornberg, 1974). Formation of the core particle initiates with binding of 121 bp of DNA to a $(H3/H4)_2$ tetramer followed by binding of H2A/H2B dimers to either side. Upon assembly, DNA is wound in 1 ¾ superhelical turns around the nucleosome, which results in a compaction of approximately seven fold (Figure 1A). The modular assembly exhibited by the nucleosome argues that dimers of H2A/H2B can be removed while interaction between the DNA and the $(H3/H4)_2$ tetramer is maintained (Akey & Luger,

2003). Transient displacement of H2A/H2B dimers allows previously masked DNA sequence to be exposed and enhances nucleosomal rearrangments. In addition, it also creates an opportunity for histone variant incorporation, which appears to play an important role in specifying localization along the chromosome or altering of the nucleosome integrity (Jin et al, 2005). Particular aspects of histone exchange will be again addressed in another section below.

High resolution crystallography of the core particle revealed that DNA contacts are limited to the globular histone-folds and the phosphodiester backbones on the inner surface of the superhelix allowing the N-terminal histone tails to extend outwards from the core particle (Luger et al, 1997). This is in accordance with the fact that histone octamers have the capacity to package virtually any DNA independent of the underlying sequence. It also opens the possibility for internucleosomal histone-tail interaction and targeting of covalent modifications to histone tail residues. In addition to the core histones, metazoan chromatin also contains linker histones (such as histone H1) which are not related in sequence to the core histones, but also contain a globular domain flanked by N- and C-terminal tails (Thomas, 1999). Binding of linker histones to core nucleosomes protects an additional sequence of ~20 bp (167 bp in total) from nuclease digestion suggesting that linker histones associate with linker DNA at the entry/exit point of nucleosome core particles at a ratio of one histone per nucleosome. Although only the globular domain is essential for binding to nucleosomes, the tail domains are believed to be important for their role in chromatin folding (Ramakrishnan, 1997).

2.2. Higher-order chromatin structure

Each nucleosome “core particle” is connected to its neighbor via a stretch of “linker” DNA that varies in length between 10 – 60 bp. This polynucleosomal array, also referred to as “beads on a string” form of chromatin, is the basic functional unit of chromatin (Olins & Olins, 1974; Oudet et al, 1975). Under physiological salt conditions, such nucleosomal arrays, with a diameter of 10 nm, can condense even further forming a compact 30 nm fiber with a DNA compaction of approximately 50-fold (Widom, 1998). Little is known about the actual nature of the 30 nm fiber, but

the well-characterized structure of the nucleosome core makes predictions on how higher-order chromatin compaction could be mediated from the nucleosomal array. For instance, amino acids on the surface of the nucleosome core define a contoured landscape of distinctive charge distribution which could promote nucleosome-nucleosome interactions (Luger et al, 1997). Histone tails protruding from the nucleosomes are essential for array folding (Dorigo et al, 2003; Shogren-Knaak et al, 2006) and could promote these interactions by contacting adjacent nucleosomes or influencing the configuration of the linker DNA. Accordingly, covalent modifications of histone tail residues may promote or disrupt contacts important for fiber formation and thereby modulate the accessibility of chromatin. Although higher-order folding is an intrinsic property of the nucleosomal array, binding of linker histones stabilizes both intramolecular folding and fiber-fiber interaction. This notion is reinforced by the observation that addition of linker histone to heterogeneously condensed nucleosomal arrays produces homogeneous, fully compacted 30 nm fibers *in vitro* (Carruthers et al, 1998). Importantly, upon removal of histone tails nucleosome array folding is impaired even in the presence of the linker histone, implying that H1 serves mainly to stabilize an intrinsic tail-mediated condensation. In fact, linker histones are dispensable for the folding of chromatin even into the highest levels of compaction and yeast histone H1 deletion mutants are viable (Widom, 1998; Woodcock et al, 2006). These results suggest that linker histones may be involved in stably locking down regions of chromatin into a condensed state in order to facilitate formation of even higher-order structures.

The isolation of native chromatin has presented a major obstacle for the characterization of the actual structure of condensed chromatin. Several competing models have been proposed involving distinct assumptions of linker DNA conformation and position of linker histones (Widom, 1998). To date, no single model fits the existing empirical data, with contradictory evidence supporting one or the other model. In the solenoid model, an array of six to eight consecutive nucleosomes is arranged in a helix such that successive nucleosomes are adjacent to each other (Finch & Klug, 1976; Widom & Klug, 1985). The linker DNA and histone H1 face the inside of the

coil promoting the interaction between histone tails to mediate stability. The solenoid model has been challenged by recent biochemical and EM data supporting the alternative model of a zigzag conformation of the 30 nm fiber (Dorigo et al, 2004; Schalch et al, 2005). This model is based on a zigzag arrangement of straight linker DNA connecting nucleosomes of two adjacent supercoil stacks. Despite fundamental differences between the two models, limitations in studying the irregular structure of native chromatin prevented differentiation between them unequivocally. At the same time, the differences of the available data could imply that *in vivo* chromatin does not exist as a regular structure but rather as a mixture of different conformation fibers (van Holde & Zlatanova, 1995).

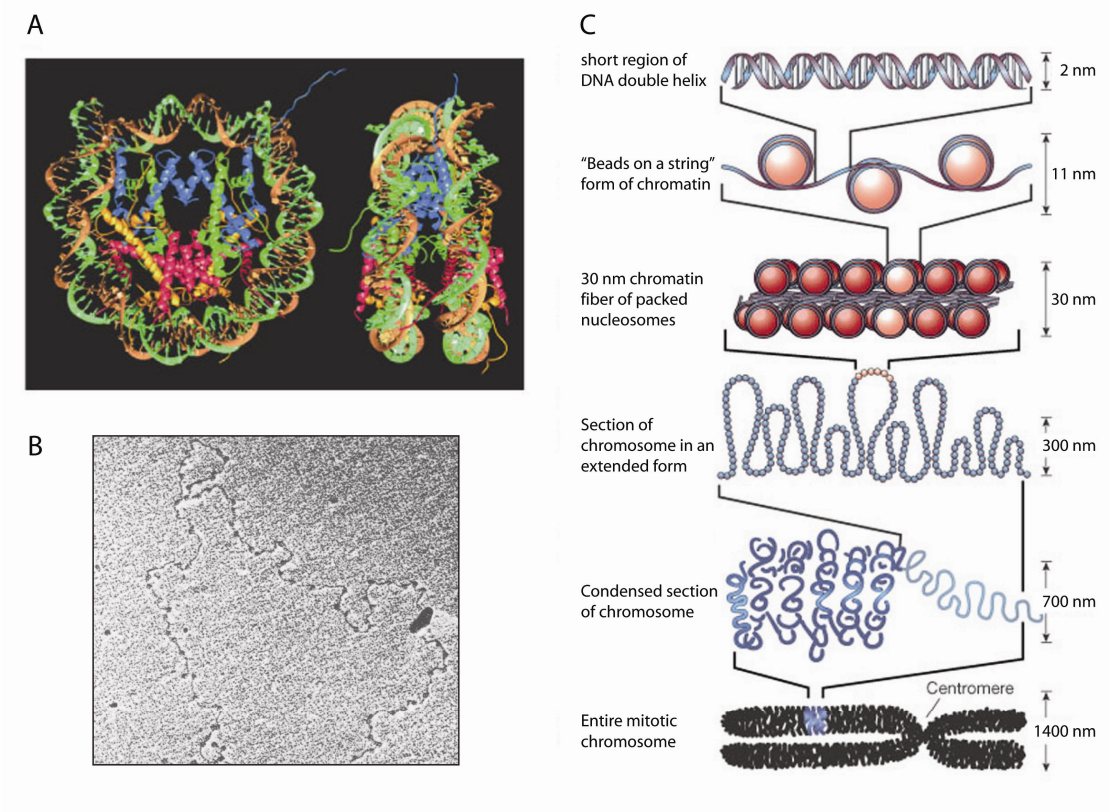


Figure 1: Packaging of DNA. A) X-ray structure of the nucleosome core particle at a resolution of 2.8Å (Luger et al, 1997). B) Electron micrograph of linear arrays of chromatin in "Beads on a string" conformation isolated rat thymus. C) The organization of DNA within the chromatin structure. (Felsenfeld & Groudine, 2003).

Despite lack of insight into the precise structure of condensed chromatin, estimations of the physiological salt concentrations would predict not only condensation of nucleosomal arrays, but also higher-order chromatin folding beyond 30 nm through substantial fiber-fiber interactions (Schwarz & Hansen, 1994). Based on these assumptions, it is reasonable to believe that *in vivo* gene expression occurs primarily in the context of chromatin that exists in a highly ordered state (Belmont et al, 1999). Compaction beyond the 30 nm fiber can be appreciated in electron microscopy studies of mitotic and interphase chromosomes. Electron micrographs of interphase chromosomes reveal a nuclear organization of condensed chromatin regions called heterochromatin and more open chromatin called euchromatin. Euchromatic regions are more sensitive to nuclease digestion and represent sites “poised” for gene transcription, although not necessarily active. In contrast, heterochromatin remains compact throughout interphase rendering the sequestered DNA inaccessible for biochemical processes such as gene transcription. Biophysical studies on chromatin fragments isolated from interphase nuclei revealed a mixture of fibers of increasing thicknesses, indicating that arrays of nucleosomes form a hierarchy of higher-order structures that can range between 60 to 300 nm in diameter (van Holde & Zlatanova, 1995). According to the chromonema model, the highly condensed chromosome structure arises from three helical folding levels of chromatin fibers. Fibers of 60 to 80 nm in width are coiled into fibers of 100 to 130 nm, which are further coiled to the 200 to 300 nm structure of metaphase chromatids.

2.3. Chromatin dynamics and transcription

As highly folded chromatin fibers are inaccessible to the cellular machinery, chromatin structure must be actively remodeled to let DNA-dependent processes to occur. Indeed, it has been shown that even compact chromatin is highly dynamic undergoing different structural rearrangements to facilitate accessibility of DNA (Cheutin et al, 2003). Several mechanisms have been implicated in modulating DNA accessibility by acting at different levels of chromatin structure including transient dissociation of nucleosomal DNA (DNA breathing), nucleosome sliding and remodeling, posttranslation modification of

histones and histone variant exchange (Workman, 2006; Workman & Kingston, 1998). Some of these processes contribute in a combinatorial manner to the structural changes that are necessary to modify access to the DNA template. In the following, I will explore the dynamic nature of chromatin structure with emphasis on the mechanisms involved in transcription regulation. I will commence with introducing some of the basic processes of transcriptional initiation and elongation (Figure 2).

2.3.1. Steps in transcription initiation and elongation

Binding of sequence-specific activators at enhancers and upstream elements of the core promoter trigger transcription initiation. This initial step is followed by recruitment of co-activators (such as chromatin-remodeling enzymes, and the Mediator) which make DNA elements more accessible and facilitate the binding of general transcription factors (GTFs, (Thomas & Chiang, 2006). Next, RNA polymerase II is assembled at the core promoter following sequential binding of TFIID, TFIIA and TFIIB to form the preinitiation complex (PIC). For most genes, this first stage of transcription initiation is rate-limiting and most susceptible to effective regulation. TFIIH-dependent melting of the DNA and positioning of the single strand into the open PIC allows initiation of RNA synthesis. Pol II is released from the PIC as a consequence of TFIIH phosphorylation of the carboxy-terminal domain (CTD), which is believed to destabilize tethering to the PIC. At this stage, GTFs dissociate from the complex while polymerase itself begins transcribing. Productive elongation coincides with additional CTD phosphorylation which controls the binding of factors important for polymerase passage and RNA processing (Buratowski, 2003).

It is evident that packaging of DNA into nucleosomes can interfere with many of the processes necessary for gene transcription to occur. Indeed, activator binding (Lorch et al, 1987) and efficient elongation (Izban & Luse, 1991) are significantly reduced on DNA template assembled into nucleosomal arrays, *in vitro*. Thus, chromatin assures the repression of undesirable transcription by default and requires the contribution of positive remodeling mechanisms to facilitate the activation of transcription. Some of these mechanisms are discussed below.

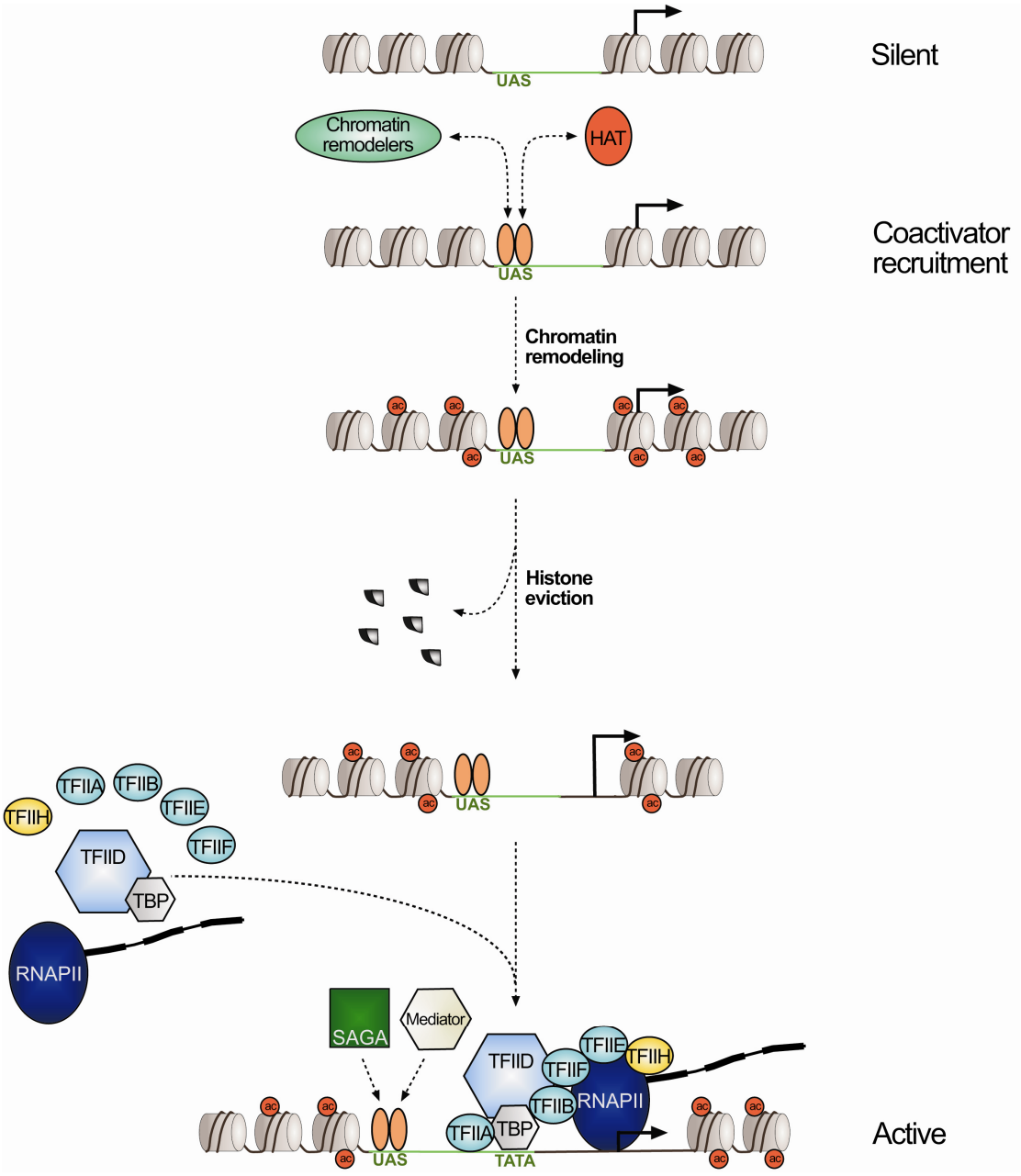


Figure 2: Regulatory steps during transcription initiation at an idealized gene promoter. At a silent promoter, positioned nucleosomes flank a nucleosome- free region over the promoter. Sequence-specific binding of transcription factors is followed by recruitment of coactivators, which leads to acetylation and remodeling of promoter-proximal nucleosomes. As a consequence, nucleosomes are displaced which exposes the entire gene promoter and allows subsequent formation of the PIC. (adapted from (Li et al, 2007a)

2.3.2. ATP-dependent nucleosome remodeling

Eukaryotic genomes display a well-conserved nucleosome positioning pattern, which reflects differential binding affinities between histone octamers and the sequestered DNA sequence (Segal et al, 2006; Yuan et al, 2005). Although some sequences are readily accessible, being either on the nucleosome surface or in linker regions, most are buried inside the nucleosome. Consequently, as binding of sequence-specific factors is at the start of most biochemical reactions with the chromatin substrate, interaction of these factors with their target elements requires a partial unraveling of the repressive chromatin structure.

Chromatin remodeling complexes use the energy of ATP hydrolysis to alter canonical histone-DNA interactions within a nucleosome (Flaus & Owen-Hughes, 2004; Smith & Peterson, 2005). Usually, the accessibility of nucleosomal DNA is increased during this process, such that DNA-binding proteins can productively interact with previously occluded sequences. *In vitro* it has been demonstrated that perturbation of nucleosome stability results either in sliding of nucleosomes along the DNA *in cis*, or nucleosome removal to an acceptor DNA *in trans* (Lorch et al, 1999). In addition, remodeling enzymes have been shown to generate di-nucleosomal particles from mono-nucleosomes and to catalyze replacement of canonical histones with histone variants (Kusch et al, 2004; Mizuguchi et al, 2004). Although the exact biochemical mechanisms as to how the remodeling complexes affect different outcomes is still debated, it is clear that ATP-dependent remodeling results in altered DNA accessibility.

All remodeling enzymes share the same catalytic ATPase domain, which assigns them to the Swi2/Snf2 superfamily of helicases (SFII). Phylogenetic analysis of sequence features outside of the common ATPase domain allows further division into several subfamilies which are conserved in all eukaryotes (Becker & Horz, 2002). The following section will list some of the main subfamilies of ATP-dependent chromatin remodeling enzymes and examples of their roles *in vivo* and their characterized reactions *in vitro*.

2.3.2.1. ISWI containing complexes

ISWI (imitation Swi/Snf) protein has been shown to exist in all eukaryotes constituting a predominant subgroup of the SNF2 ATPase superfamily. *Drosophila* ISWI is the catalytic subunit of three remodeling complexes: ACF (ATP-utilizing chromatin assembly and remodeling factor), CHRAC (chromatin accessibility complex) and NURF (nucleosome remodeling factor) (Ito et al, 1997; Mizuguchi et al, 1997; Varga-Weisz et al, 1997). All three complexes can induce nucleosomal sliding *in vitro*, yet while ACF and CHRAC catalyze arrays of regularly spaced nucleosomes related to compaction, the NURF complex facilitates the exact opposite reaction by disrupting nucleosome regularity. A hydrophilic patch (aa 17-19) in the N-terminal tail of histone H4, which interacts with nucleosomal DNA, appears to be is important for ISWI-mediated nucleosomal sliding since tail deletion or mutation of these residues abolishes its remodeling activity.

In vivo, ISWI is required for large-scale maintenance of chromosome structure. Null mutation in *Drosophila* ISWI results in larval lethality and decondensation of the male X chromosome (Deuring et al, 2000). Interestingly, the male X is globally hyperacetylated at the acetic patch of histone H4 (H4K16ac) which is critical for *Drosophila* male dosage compensation (Akhtar & Becker, 2000). In ISWI mutants, blocking of H4K16 acetylation rescues the chromatin structure of the male X (Corona et al, 2002) suggesting that acetylation of this residue interferes with ISWI-mediated compaction at the male X chromosome.

In addition to the role in maintenance of the overall structure of entire chromosomes, mutations in non-catalytic subunits of ISWI-containing complexes revealed specific functions necessary for appropriate expression of individual genes (Badenhorst et al, 2002; Langst & Becker, 2001).

2.3.2.2. CHD-type remodeling complexes

CHD-type (chromodomain helicase and DNA-binding protein) nucleosome remodeling enzymes have been identified in most eukaryotic organisms and are characterized by the presence of a pair of chromodomains. Phylogenetic analysis of additional sequence features co-lineates the family into the following subgroups: Chd1, Chd2, Chd3/4 and

Chd5. The Chd1 subgroup is associated with active transcription as it is confined to interband regions and puffs on *Drosophila* polytene chromosomes and co-localizes extensively with active forms of RNA polymerase II (Srinivasan et al, 2005). In agreement, yeast Chd1 has been shown to interact with a subunit of the transcription elongation factor FACT, which facilitates transcription through nucleosomes by destabilizing one H2A-H2B dimer (Kelley et al, 1999; Krogan et al, 2002). Further, mammalian Chd1 is part of the SAGA complex (Pray-Grant et al, 2005) which is recruited to active promoters through specific interaction of its chromodomain with methylated histone tails (H3K4me). *In vitro*, Chd1 generates regularly-spaced nucleosome arrays and has been shown to support NAP1-mediated chromatin assembly (Lusser et al, 2005). Taken together, these observations point towards a role of Chd1 in promoting permissive chromatin structure required for the process of transcription.

Unlike Chd1, Chd3/4 (Mi-2) proteins harbor additional PHD (plant-homeo-domain) zinc fingers in their N-termini. Mi-2 was shown to reside in nucleosome remodeling histone deacetylase (NuRD) complexes in various species (Tong et al, 1998; Wade et al, 1998; Xue et al, 1998; Zhang et al, 1998). Despite differences in the precise subunit composition of NuRD complexes purified, all contain a Mi-2 ATPase, a histone deacetylase core made of histone deacetylases 1 and 2 (HDAC1 and HDAC2) and the histone H4-binding proteins Retinoblastoma-associated p46 and p48 (RbAp46 and RbAp48). Identification of a complex that combines activities for covalent histone modification and ATP-dependent remodeling suggests that unlike other remodelers, NuRD may utilize the energy of ATP hydrolysis to render N-terminal histone tails accessible for modification. *in vivo* NuRD specificity could involve targeting of the MBD (Methyl-cytosine binding protein) subunit to methylated CpG di-nucleotides, which correlate with repressive chromatin structure. Through MBD binding, NuRD is targeted to bind, remodel and deacetylate nucleosomes containing methylated DNA and thus may be involved in establishing a repressive chromatin environment. However, DNA methylation does not exist in *Drosophila* and staining of Mi-2 on polytene chromosomes reveals extensive co-localization with active forms of RNA polymerase II, which are difficult to reconcile with the notion of repressive

NuRD function in chromatin. A model of dynamic chromatin structure may be one possible explanation to reconcile Mi-2's repressive nature with its presence at sites of gene transcriptions. Gannon and colleagues propose that histone deacetylases act on chromatin while it is being transcribed (Metivier et al, 2003). Clearly, NuRD targeting must not affect promoter hyperacetylation, which is required for efficient transcript initiation. Instead, recent work by Gozani et al. suggests that Mi-2 could be targeted through interaction of its PHD domain with methylated H3K36 (Shi et al, 2006), a mark that is characteristic for the 3' end of active genes. Since histone acetylation in coding regions facilitates passage of RNA polymerase (Carey et al, 2006), recruitment of NuRD might be involved in reestablishing a compact chromatin structure to prevent transcription from intragenic sequences (Carrozza et al, 2005b; Joshi & Struhl, 2005; Keogh et al, 2005). Similar to ISWI-containing complexes, functions of Mi-2-containing complexes might be diverse and specificity is likely to be mediated by interactions with NuRD complex subunits.

2.3.2.3. SWI/SNF-type remodeling complexes

The yeast SWI/SNF (mating type switching/sucrose non fermenting) complex was originally discovered for its ability to promote Gal4 activator binding to nucleosomal DNA in an ATP-dependent reaction (Cote et al, 1994). Similarly, human Swi/Snf complex facilitates Gal4 and TBP binding at promoter regions (Imbalzano et al, 1994; Kwon et al, 1994) supporting the concept that nucleosome remodeling unravels the chromatin substrate for transcriptional activation. Recruitment of SWI/SNF to certain sites is dependent on binding of its bromodomain (acetyl-group binding domain) to lysines modified by HATs such as SAGA. Accordingly, acetylation of histone tails stimulates SWI/SNF containing RSC complex in facilitating polymerase elongation on nucleosomal substrate (Carey et al, 2006).

Moreover, recent evidence points towards SWI/SNF being involved in eviction of nucleosomes *in trans* from the yeast PHO5 promoter *in vivo* (Boeger et al, 2003; Reinke & Horz, 2003), and both biochemical and genetic evidence support cooperation between the *Drosophila* SWI/SNF complex and the histone H3/H4 chaperone ASF1 (Moshkin et al, 2002).

Depletion of nucleosomes is not limited to PHO5 as genome-wide analysis revealed low nucleosomal occupancy at many active promoters in yeast (Bernstein et al, 2004; Lee et al, 2004). In addition, transiting RNA polymerases displace nucleosomes, leading to variation in nucleosomal occupancy over transcribed regions (Kristjuhan & Svejstrup, 2004; Schwabish & Struhl, 2004). The loss of histone is at least partially compensated by replacement with histone variants.

2.3.3. Replacement histones

In vivo, the chromatin fiber is a heterogeneous nucleoprotein complex, which contains several types of histone variants in addition to the canonical ones. Histone variants can be very similar in amino acid sequence and thus are mainly distinguished from canonical histones by the fact that they are expressed outside of S-phase and are incorporated into chromatin in a DNA replication-independent manner (reviewed in (Malik & Henikoff, 2003). In some cases, their chromosomal deposition is highly localized or imparts distinct biophysical characteristics on the nucleosome. For instance, variant incorporation might alter nucleosome stability and thus affect folding of the chromatin fiber, or it might introduce additional surface residues that are available for modifications or interaction with cellular proteins. Altogether, histone variants are believed to have specialized functions in regulating chromatin structure and dynamics. With the exception of histone H4, variants have been identified for all major histones. In the context of transcription-coupled changes of chromatin structure, I will concentrate on some variants of histones H2A and H3 and their modes of deposition as these are best characterized.

2.3.3.1. Histone H2A variants

Among the core histones, H2A has the largest number of variants, including H2A.Z, MacroH2A, H2A-Bbd, H2AvD, and H2A.X (reviewed (Malik & Henikoff, 2003). Some H2A variants, like H2A.Z, are conserved through evolution, while others such as MacroH2A and H2A-Bbd are restricted to vertebrates or mammals. H2A variants are distinguished from the major H2A

histones by length and sequence divergence in the C-terminal tail, as well as their genomic distributions.

In different species, deposition H2A.Z has been linked to diverse chromatin functions. For example, the H2A.Z orthologue Htz1 localizes to repressed/basal RNA polymerase II promoters (Millar et al, 2006) and transcribed sub-telomeric regions (Meneghini et al, 2003; Shia et al, 2006). These patterns suggest involvement in transcriptional activation or repression and telomeric silencing and do not allow a firm conclusion for the conserved role of the variant in eukaryotic chromatin. Thus, it remains to be determined whether different patterns of posttranslational modifications help to reconcile the diversity in functional readout and chromosomal localization.

Whereas the mechanism by which H2A.Z affects chromatin remains uncertain, much has been learned about how it is deposited into chromatin. In yeast, ATP-dependent chromatin remodeling factor Swr1 forms a complex with Htz1/H2B dimers and is required for their deposition into chromatin (Kobor et al, 2004; Krogan et al, 2003a; Mizuguchi et al, 2004). Swr1-mediated deposition of Htz1 at active chromatin regions could involved Swr1 subunit Bdf1 which can interact with acetylated histones via its two bromodomains and has been shown to associate with the TFIID complex (Matangkasombut et al, 2000). In addition, Htz1 incorporation depends on Yaf9, a common component of both Swr1 and NuA4 histone acetyltransferase complexes (Zhang et al, 2004), further supports an involvement of acetylation in specific targeting. Since histone acetylation correlates with active transcription the question remains if acetylation is cause or consequence of H2A.Z targeting. While these results provide a handle for understanding H2A.Z deposition at transcriptionally active chromatin, there are no indices for the mechanism of incorporation at inert regions.

2.3.3.2. Histone H3 variants

Similar to histone H2A, canonical histone H3 has diverse replication-independent variants with specialized functions (Malik & Henikoff, 2003). Variant H3.3 is highly similar to canonical H3 differing at only four amino acid positions. While the structure of H3.3-containing nucleosomes is virtually identical to the canonical one, the difference in primary sequence has a

marked effect on the H3.3 assembly pathway. Replacing three of four amino acids in the canonical sequence with the variant amino acids results in deposition of histone H3 in a replication-independent manner throughout the cell-cycle similar to H3.3 (Ahmad & Henikoff, 2002). This suggests that these minute differences between H3 and H3.3 determine a pathway for replication-independent assembly that is distinct from the replication-coupled assembly of the major H3 histone. In addition, whereas the N-terminal H3 tail is required for replication-coupled incorporation, it is dispensable for replication-independent deposition. This indicates a possible requirement for histone tail modification prior to histone assembly behind the replication fork.

Purification of canonical H3 and variant H3.3 from distinct chaperone complexes is in agreement with the existence of two alternative assembly pathways. Canonical H3 was isolated from the CAF1 histone chaperone complex that is known to interact with proteins present at replication foci such as proliferating cell nuclear antigen (PCNA) (Loyola & Almouzni, 2004) while H3.3 copurified with the replication-independent histone chaperone HIRA (Tagami et al, 2004). In line with H3.3 deposition independent of DNA replication is evidence that the variant is the dominant H3-subtype in non-dividing differentiated vertebrate cells (Pina & Suau, 1987; Urban & Zweidler, 1983) and becomes incorporated into decondensing male pronuclei prior to DNA replication (Loppin et al, 2005).

In *Drosophila* H3.3 displacement and deposition is associated with transcription as cytological studies using epitope-tagged H3.3 found assembly localized to highly induced heat shock genes as well as active, but not inactive rDNA genes (Ahmad & Henikoff, 2002; Schwartz & Ahmad, 2005). Based on these observations it has been suggested that H3.3 is specifically incorporated into chromatin to serve as an epigenetic mark at sites of active gene transcription. This is supported by observations that the variant is enriched in posttranslational modifications associated with transcription while H3 is enriched in modifications linked to gene silencing (McKittrick et al, 2004). However, very recent work extended the spectrum of replication-independent H3 deposition showing that in addition to replacement at promoters and transcribed coding regions, H3.3 incorporation also compensates for rapid histone turnover at boundary elements and regulatory

regions such as distal enhancers (Dion et al, 2007; Mito et al, 2007). Rather than being an epigenetic or structural mark of transcriptionally active genes these results argue that incorporation of H3.3 is not more than a mere consequence of its availability during interphase.

Clearly, the cues and mechanism for H3.3 incorporation at specific sites remain to be determined with particular focus on posttranslational modifications of the variant compared to canonical H3 before and after introduction into chromatin.

2.3.4. Posttranslational histone modifications

Histones are subject to a variety of posttranslational modifications including acetylation, phosphorylation, methylation, and ubiquitination (Figure 3). Many of these modifications have been known to exist for more than three decades (Allfrey et al, 1964) and their chromosomal distribution suggested a high degree of specificity (Grunstein, 1997; Turner et al, 1992). However, although correlations between modifications and specific transcriptional states were observed, their functional relevance for chromatin structure and readout has only been realized recently with the discovery of enzymes that catalyze them. The first histone acetyltransferase (HAT) was isolated from the macronucleus of Tetrahymena and subsequently found to be homologous to the yeast transcriptional coactivator Gcn5 (Brownell et al, 1996). At the same time, the first histone deacetylase (HDAC) enzyme was identified and found to be related to the yeast transcriptional co-repressor protein Rpd3 (Taunton et al, 1996). These discoveries initiated a shift in the perception of chromatin from being a passive structural scaffold to playing an elementary role in the regulation of DNA-templated processes. The characterization of additional enzymes that for example catalyze acetylation, deacetylation, methylation and phosphorylation (Chen et al, 1999; Kleff et al, 1995; Parthun et al, 1996; Rea et al, 2000) provided a handle to experimentally test the significance of different modifications in processes of transcription, DNA repair, and replication. More rapid, however, is the pace at which new modification are being identified with the help of mass spectrometry and specific antibody detection creating an impressive catalogue of posttranslational modifications

that particularly concentrate along the N-terminal tails of histones (Kouzarides, 2007).

In general, three conceptual models have been proposed for the mechanism by which posttranslational modifications affect the chromatin template and related processes such as gene transcription or repression. Chemical modification of histone residues may alter physical properties of the chromatin structure. Alternatively, modifications could occlude binding of factors to the chromatin template or conversely create binding sites for chromatin interacting proteins. Although numerous types of posttranslational histone modifications have been described, I will limit my discussion to lysine acetylation and methylation, which have been widely studied on a genomic scale.

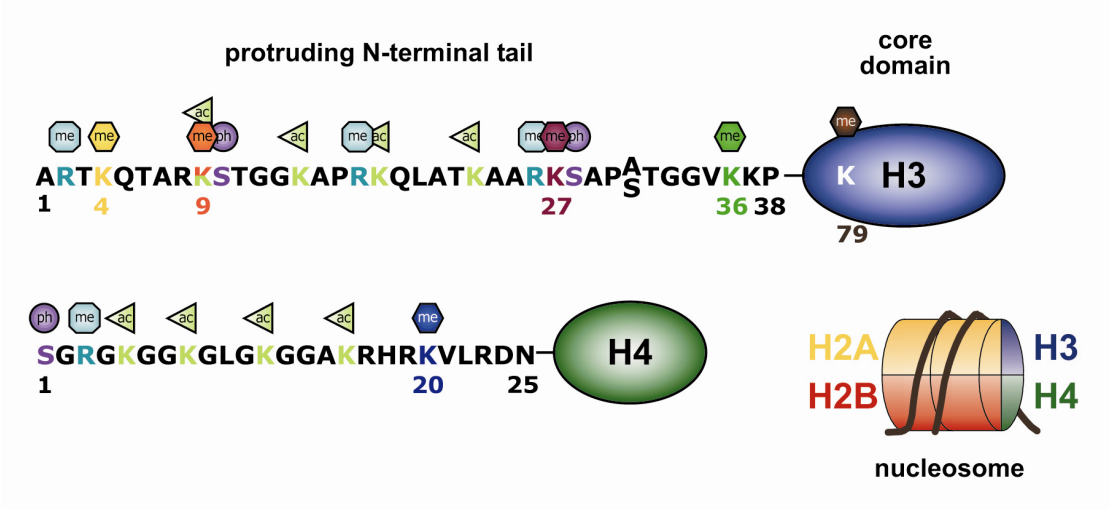


Figure 3: Posttranslational modifications along N-terminal tails of histones H3 and H4. Indicated are sites of lysine (K – color-coded) and arginine (R – turquoise) methylation, lysine acetylation (green) and serine (S – violet) phosphorylation. Only the mono-methylated states are presented. H3K9 can either be methylated or acetylated. (adapted from (Peters & Schubeler, 2005)

2.3.4.1. Acetylation

Acetylation occurs at multiple lysine residues of histones H3, H4 and H2B. In general, this modification is associated with enhanced chromatin accessibility and transcriptional activity (Hebbes et al, 1994), while

transcriptionally silenced regions of the genome show very low levels of histone acetylation (Braunstein et al, 1993). As discussed above there are several theories that have been put forward to reconcile the effects of histone acetylation on chromatin structure. The first mechanism considers that acetylation induces structural changes to chromatin by affecting the nucleosomal net charge. Based on the assumption that nucleosomes present a barrier for transcription, acetylation of positively charged lysine residues would neutralize and reduce interactions between highly basic histones or histone tails and negatively charged DNA rendering more access to DNA-binding sites (Vettese-Dadey et al, 1996). Indeed, protein binding to DNA is increased in hyperacetylated chromatin *in vitro* (Anderson et al, 2001; Lee et al, 1993) and results in destabilization of nucleosomes (Wolffe & Hayes, 1999). The nucleosome crystal structure revealed interactions between the H4 tail and an acidic patch of the H2A/H2B dimer of an adjacent nucleosome (Luger et al, 1997) which could enhance formation of higher-order chromatin. Along the lines of the charge neutralization model, it is also conceivable that acetylation of N-terminal lysines of histone H4, in particular H4K16ac, would interfere with the internucleosomal interactions resulting in decompaction of nucleosomal arrays (Shogren-Knaak et al, 2006).

Alternatively, histone acetylation could create a signal for recruitment of regulatory proteins to the chromatin template. As such, acetylated lysines are specifically recognized and bound by conserved protein modules called bromodomains which are commonly found in many chromatin-associated proteins (Dhalluin et al, 1999). These include for example components of HAT complexes, such as Gcn5 and CBP/p300, members of the TFIID complex such as Taf1 and Bdf1 or Rsc4, which is a component of the RSC nucleosome remodeling complex. Note, that unlike the charge neutralization models, the bromodomain-recruitment model implies that acetylation of a single lysine residue is relevant, while cumulative hyperacetylation would not contribute further to recruitment. Therefore, it is possible that acetylation of specific lysine residues have a dual functions, as recently suggested from *in vitro* studies of H4K16 acetylation (Shogren-Knaak et al, 2006). Nevertheless, the majority of lysine acetylation is likely to play a nonspecific cumulative role in the regulation of chromatin-templated processes (Dion et al, 2005).

Identifications of enzymes responsible for the turnover histone acetylation further facilitated our understanding of this modification. Being highly dynamic and reversible (Waterborg & Matthews, 1983), levels of acetylation are balanced by opposing activities of HATs and enzymes that remove acetyl groups, termed histone deacetylases (HDACs). HATs and HDACs alike exhibit broad activity and rarely target just individual sites. Moreover, several enzymes even catabolize acetylation on a number of non-histone substrates (Glozak et al, 2005; Kouzarides, 2000). Nevertheless, despite individual promiscuity, HATs and HDACs achieve specificity in combination with complex subunits that influence their recruitment. This is exemplified by differential targeting of the Rpd3 deacetylase in the context of two separate complexes. While the large complex (Rpd3L) represses transcription activation through interaction with multiple DNA-binding proteins at specific promoters (Carrozza et al, 2005a; Yang & Seto, 2003), the small Rpd3 complex is globally targeted to open reading frames (ORFs) through binding of a histone methylation mark in order to suppress spurious polymerase inititation (Carrozza et al, 2005b; Joshi & Struhl, 2005).

2.3.4.2. Methylation

Methylation of histones can occur at lysine and arginine residues, most of which reside in the N-terminal tails of histones H3 and H4. Lysines can be mono- (me1), di- (me2) or trimethylated (me3), whereas arginines can be either mono- or dimethylated (symmetric or asymmetric). Unlike acetylation, methyl-groups do not neutralize the residue charge, but instead have been proposed to serve as marks for the recruitment of proteins to the chromatin template. Indeed, recent studies identified at least three different protein motifs; chromodomain (Bannister et al, 2001; Lachner et al, 2001; Pray-Grant et al, 2005), tudor domain (Huyen et al, 2004) and PHD domain (Shi et al, 2006; Wysocka et al, 2006); that are able to bind to methylated residues. These proteins carry with them enzymatic activities, such as remodeling ATPases. As a result, histone methylation can mediate either positive or negative effects on chromatin structure and gene transcription depending on the position of the modified residue within the histone (Peters & Schubeler, 2005). Further complexity is added by different methylation states of the same

residue and the possibility that adjacent modifications might influence each others binding affinities (reviewed by (Kouzarides, 2007)).

On this basis, it was proposed that different combinations of histone modifications encode a complex language that translates into unique cellular responses (Jenuwein & Allis, 2001; Strahl & Allis, 2000; Turner, 2000). However, while the "histone code" would predict various combinations of posttranslational modifications to be linked to diverse chromatin-templated processes, recent global localization studies in yeast and flies indicated a rather simple binary relationship between modifications and transcriptional state (Liu et al, 2005; Pokholok et al, 2005; Schubeler et al, 2004). Therefore, an alternative scenario would be that multiple histone modifications simply combine redundantly to ensure robustness of chromatin regulation (Schreiber & Bernstein, 2002). In the future, more correlative analysis of histone modifications on a genome-wide scale will be required to conclusively address the complexity of chromatin modifications. For the purpose of this introduction to histone methylation, I will limit myself to the discussion of individual methylated residues in the context of gene expression.

At least 24 sites of lysine and arginine methylation have been identified, yet to date only few of them have been well characterized. A major obstacle in studying the function of individual histone methylation marks is the lack of information regarding the catalytic enzymes. Three distinct protein families have been described to catalyze site-specific histone methylation. The PRMT1 family mediates arginine methylation, whereas the SET-domain containing family and the non-SET-domain proteins DOT1/DOT1L target lysine residues (reviewed by (Zhang & Reinberg, 2001). Global chromatin-immunoprecipitation (ChIP) analyses revealed that active genes are methylated at lysine 4, lysine 36 and lysine 79 of histone H3, suggesting a role for these modifications in transcription (Mikkelsen et al, 2007; Pokholok et al, 2005; Rao et al, 2005; Schubeler et al, 2004). In fact in *S. cerevisae*, the enzymes responsible for the former two modifications, Set1 and Set2, have been shown to physically associate with transcribing RNA polymerase II resulting in histone methylation in the coding region (Krogan et al, 2003b; Ng et al, 2003b).

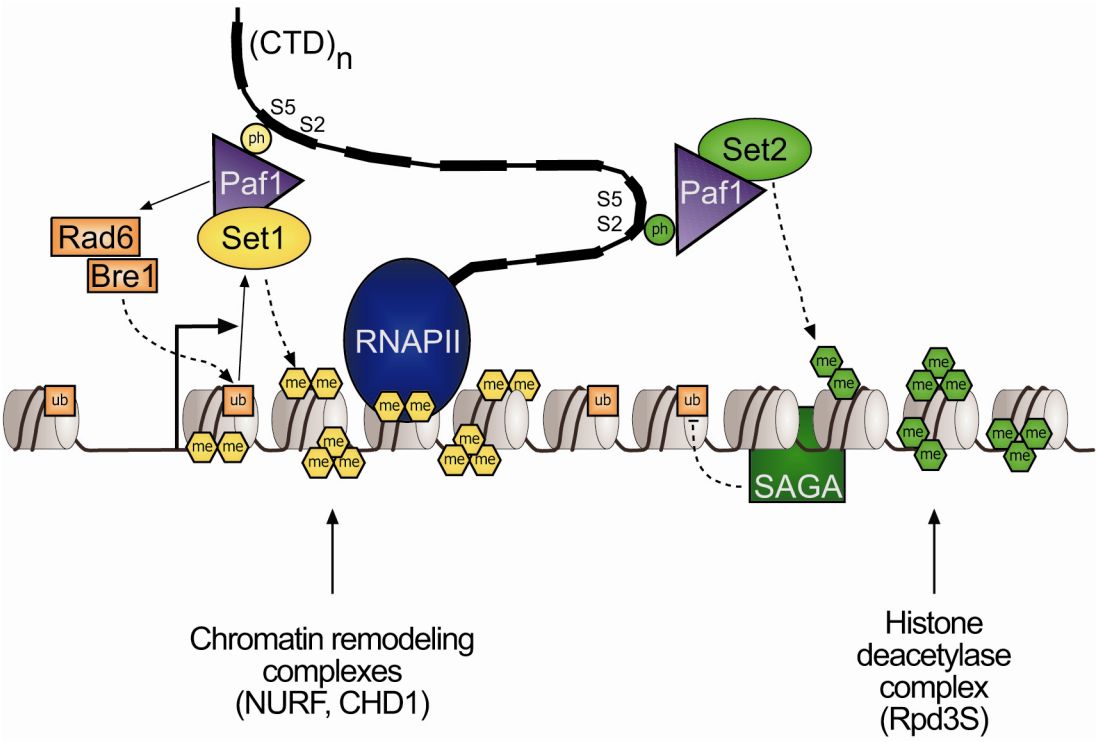


Figure 4: Regulation of chromatin structure during transcriptional elongation. The chromatin landscape during elongation is determined by the factors associated with different phosphorylated forms of RNA polymerase II. The PAF elongation complex serves as a platform to facilitate binding of H3K4 HMT Set1 and Rad6/Bre1 to Ser5-phosphorylated CTD, which results in H2B ubiquitylation and accumulation of di- and trimethylation of H3K4 at the 5' end of the gene. Ubp8, a component of the SAGA complex, mediates H2B deubiquitylation followed by recruitment of Set2 and methylation of H3K36 at the 3' end of through interaction with Ser2 phosporylated CTD. (adapted from (Peters & Schubeler, 2005))

Initial targeting of the H3K4 HMT Set1 complex to 5' end of the ORF requires Rad6-dependent H2B123 monoubiquitination (H2B123ub1) and coincides with phosphorylation of serine 5 of the CTD of RNA polymerase II. The PAF elongation complex, which controls most serine 5 CTD binding regulators, is dispensable for Set1-dependent H3K4 monomethylation but facilitates further conversion into di- and eventually trimethylation (Ng et al, 2003a; Shahbazian et al, 2005; Wood et al, 2003). This results in a distribution of H3K4me where trimethylation peaks at the 5' end of the ORF and di- and monomethylation gradually decrease towards the 3' end (Pokholok et al, 2005). Remarkably similar is the regulation of H3K4

methylation in metazoa (Wysocka et al, 2005) highlighting the importance of this modification in transcription. It is conceivable that H3K4me3 provides a critical signal for the recruitment of chromatin modifiers to the beginning of the transcription unit. This is supported by recent reports of chromatin-remodeling factors (Pray-Grant et al, 2005; Wysocka et al, 2006) and histone-modification complexes (Dou et al, 2005; Taverna et al, 2006) specifically recognizing H3K4me3.

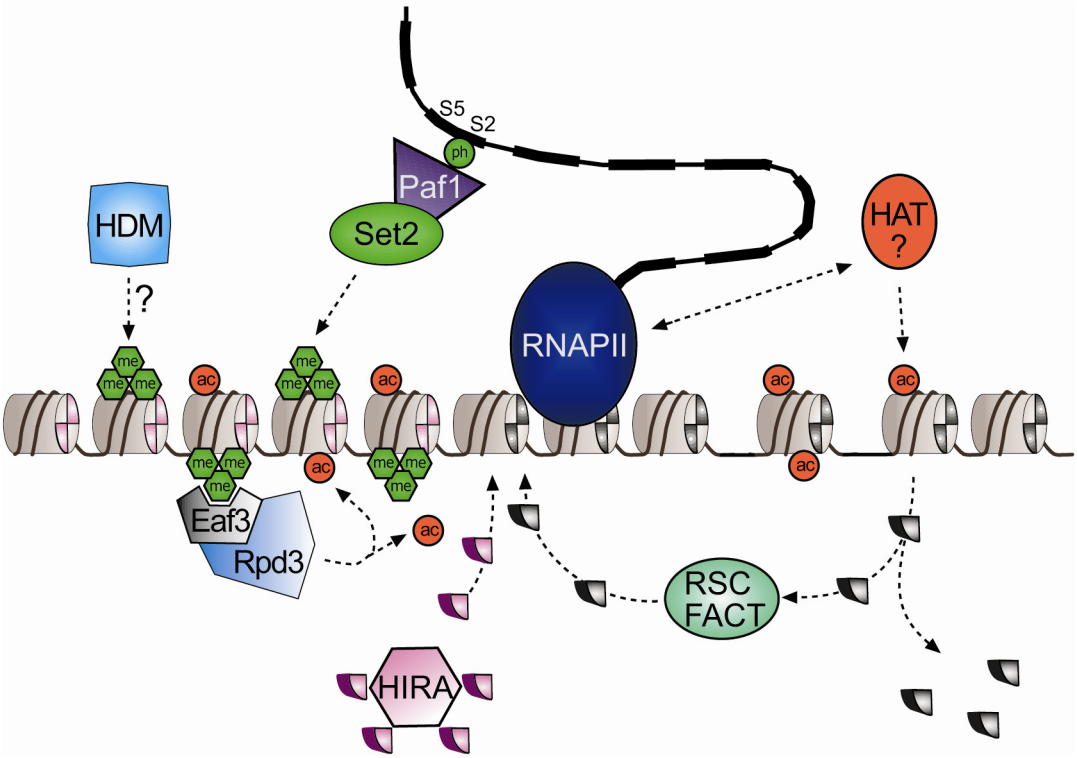


Figure 5: Dynamic regulation of chromatin structure during transcription elongation. The positive effects of promoter-proximal acetylation diminish towards the 3' end of the genes. To ensure efficient elongation through the chromatin template at promoter-distal regions, HATs acetylate of nucleosomes in front of the transcribing polymerase. In addition, remodeling enzymes catalyse displacement of histones and mobilize nucleosomes. Subsequently, histones are reassembled behind Pol II involving the concerted action of histone chaperones. Loss of canonical H3 histones (grey) might be compensated by HIRA-dependent incorporation of H3.3 variant histones (violet). Hyperactylated nucleosomes, that have not been displaced from the DNA, are methylated by Set2 HMT at H3K36. As a result, methylation is recognized by chromodomain of Eaf3, which in turn recruits the Rpd3S deacetylase complex to remove acetyl-groups and reestablish a compact chromatin state. H3K36 methylation is eventually eliminated by activity of histone demethylases when the gene is shut off. (adapted from (Li et al, 2007a))

Methylation of histone H3 lysine 36 (H3K36me) also marks active genes but unlike lysine 4 methylation, it accumulates in the 3' end (Bannister et al, 2005; Barski et al, 2007; Kizer et al, 2005; Mikkelsen et al, 2007; Pokholok et al, 2005; Rao et al, 2005). In *S. cerevisiae*, targeting to transcribed regions entails association of H3K36-specific HMT Set2 with the elongating RNA polymerase II (Kizer et al, 2005; Krogan et al, 2003b; Li et al, 2003; Xiao et al, 2003). This recruitment is further enhanced through interactions with components of the PAF complex (Krogan et al, 2003b) and removal of H2B monoubiquitylation (Henry et al, 2003). Recent progress in yeast advanced our understanding of the role of lysine 36 methylation in transcriptional elongation. Efficient polymerase elongation through chromatin is facilitated by acetylation of nucleosomes and chromatin remodeling activities. Yet, compaction must be reestablish after polymerase passage to prevent aberrant transcription from cryptic internal start sites (Kaplan et al, 2003). In yeast, H3K36me signals for recruitment of the Rpd3S HDAC complex to the body of active genes (Carrozza et al, 2005b; Joshi & Struhl, 2005; Keogh et al, 2005). Recognition of the K36 methyl mark by Rpd3S relies on cooperative binding of chromo and PHD domains of its subunits Eaf3 and Rco1, respectively (Li et al, 2007b). The deacetylase activity removes transcription-coupled histone acetylation which otherwise would unmask cryptic promoters (Carrozza et al, 2005b; Joshi & Struhl, 2005; Keogh et al, 2005). Thus, H3K36 methylation has been proposed to be involved in maintenance of repressive chromatin structure.

Comparably little is known about the function of H3K79 methylation. The responsible enzyme, DOT1, is the only histone methyltransferase identified that lacks the catalytic SET domain. The enzyme was originally discovered in a screen for genes that interfere with telomeric silencing in *S. cerevisae* (Singer et al, 1998). In yeast, deletion or overexpression of DOT1 disrupts silencing at telomeres and HM loci, and overexpression interferes with silencing at rDNA arrays (Ng et al, 2002; van Leeuwen et al, 2002). Remarkably, H3K79 methylation is very abundant at transcribed regions while being absent from silent chromatin. Given that disruption of DOT1 or mutation of H3K79 increase the interaction of silencing proteins Sir2 and Sir3 with euchromatin, it was proposed that K79me functions to prevent promiscuous

binding of silencing proteins to euchromatin thereby enhancing specific targeting to silent chromatin (van Leeuwen et al, 2002). In mammals, H3K79me signals for binding of tudor domain protein 53BP1 at sites of DNA damage (Huyen et al, 2004). While this indicates an additional function of lysine 79 methylation in DNA repair, there is no evidence that 53BP1 is involved in regulation of gene transcription.

The recent discovery of enzymes responsible for demethylation indicated that similar to acetylation, histone methylation is subject to dynamic regulation. Since the breakthrough discovery of the first histone demethylase (HDMs) LSD1 (Shi et al, 2004), many more enzymes have been identified which on the basis of their catalytic domain are separated into two distinct classes (Shi & Whetstine, 2007). While LSD1-domain-containing HDMs can only catalyze removal of mono- and dimethyl groups (Shi et al, 2004), JmjC enzymes have the capacity to reverse trimethylation as well. Moreover, HDMs can act on several residues *in vivo* arguing that specificity is most likely imparted by interaction with cofactors. This ability to selectively target distinct residues and methylation states implies a tight regulation of histone methylation and furthermore supports a model of methylation state specific functions.

2.4. Open Questions

Research over the last decade greatly improved our understanding of how eukaryotic cells modify chromatin to regulate gene expression. On the one hand, nucleosome remodeling complexes utilize the energy of ATP hydrolysis to mobilize, dissemble or exchange nucleosomal histones so that DNA can be accessed for short periods. On the other hand, chemical modifications of histones can directly affect the physical properties of the chromatin fiber or provide signals for the binding of specific proteins. Moreover, it is becoming evident that these types of mechanisms do not operate independently, but that histone modifications and chromatin remodeling enzymes crosstalk in multiple ways. Yet, we are still only beginning to understand the function of histone modification patterns and the recruitment of specific modifying enzymes to reorganize the chromatin structure. Does colocalization of various histone modifications reflect redundant function to ensure robust chromatin regulation? Or is there greater complexity involving modifications, which have not yet been subject to global analysis? What are the responsible enzymes and what are the mechanisms involved in directing their activity to certain genomic loci? How dynamic are euchromatic histone modifications at active genes? Are they simply part of the transcriptional signaling pathway or do they provide a memory of previously active chromatin state?

During the course of my PhD, I have tried to address some of these questions by identifying location and function of chromatin marks at active genes using *Drosophila* as a model.

2.5. Scope of the thesis

At the start of my thesis work in 2003, genome-wide ChIP-chip analysis had revealed a simple relationship between gene activity and the presence of euchromatic histone modifications in higher eukaryotes. Several marks including acetylation at histones H3 and H4 and methylation at lysine 4 and lysine 79 of histone H3 were found to be enriched and coincided at active genes, while being absent from inactive genes (Schubeler et al, 2004). This study had two important implications. On one hand, the observations suggested a common mode for the targeting of these histone modifications that is tightly coupled to polymerase activity. On the other hand, the binary pattern challenged the existing hypothesis of the combinatorial nature of histone modifications (Jenuwein & Allis, 2001; Strahl & Allis, 2000; Turner, 2000). One caveat of this analysis, however, was the spatial limitation to a single probe per gene. This restriction did not allow discerning potential differences in the distributions throughout transcribed regions, which could argue for a greater complexity in the regulation of histone modifications.

Addressing this question, my thesis was aimed to determine the patterns of histone modification at a higher resolution along the body of active genes. This entailed chromatin-immunoprecipitations (ChIP) of several euchromatic marks in *Drosophila* cell lines and quantification of their enrichments along individual active and inactive genes using real-time PCR analysis. Furthermore, to gain a better understanding of the regulation of individual active modifications, we intended to identify the responsible enzymes and study their role in transcription and chromatin structure using an RNA interference approach.

3. RESULTS AND DISCUSSION

3.1. Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active histone modifications show a promoter-proximal bias

Christiane Wirbelauer, Oliver Bell, and Dirk Schübeler

3.1.1. Summary

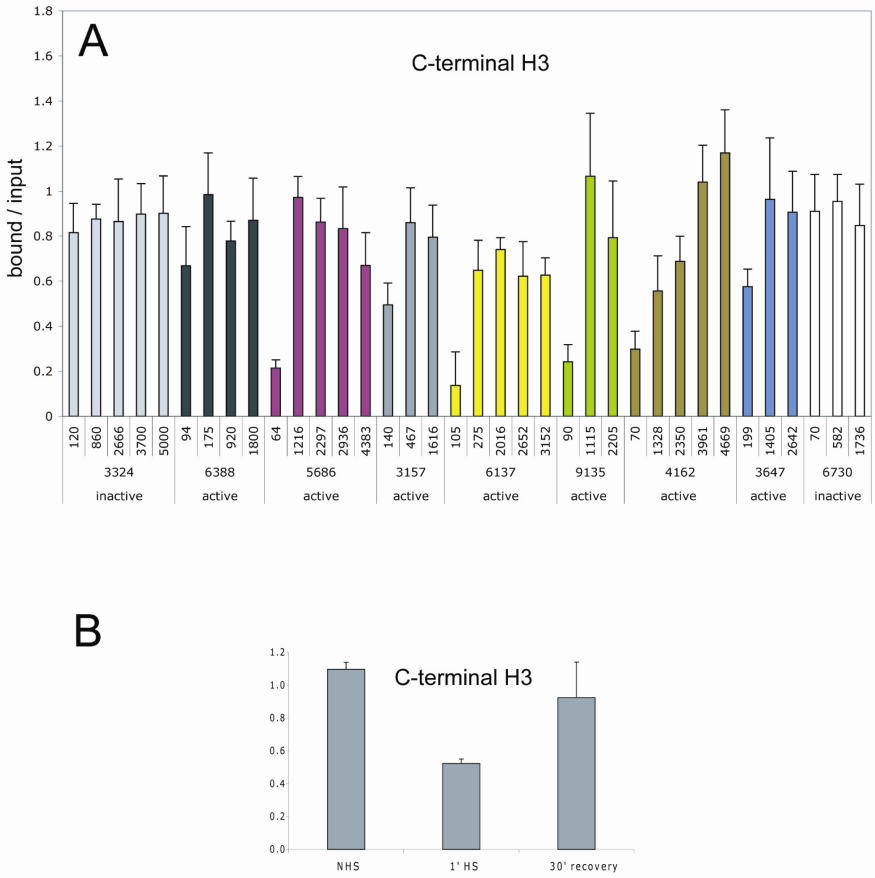
The expression and deposition of canonical histone H3 is tightly coupled to DNA replication during S-phase. In contrast, chromatin incorporation of the variant H3.3 occurs throughout the cell cycle and seems to be linked to high levels of transcription (Ahmad & Henikoff, 2002; Schwartz & Ahmad, 2005). This observation and the fact that H3.3 is highly decorated with active histone modifications (McKittrick et al, 2004) led to the proposal that variant deposition might serve to target these modifications to active genes. Indeed, a role for H3.3 to predetermine chromatin state would be consistent with the all-or-nothing pattern of euchromatic modifications observed by genome-wide profiling (Schubeler et al, 2004).

To address these issues, we decided to study the pattern and dynamics of histone H3.3 incorporation at individual genes in *Drosophila* Kc cells and compare it to the localization of euchromatic histone modifications. If H3.3 predetermines active chromatin state, then the distribution of the modifications should closely resemble the pattern of H3.3 deposition. Using chromatin-immunoprecipitation and quantitative real-time PCR, we found that euchromatic histone modifications are preferentially enriched at the 5' end of active genes and gradually decrease towards the 3' end. Similar analysis of H3.3 deposition revealed a uniform distribution pattern that was clearly distinct from euchromatic histone marks and instead reflected the abundance of transcribing RNA polymerase at active genes. The link between variant deposition and polymerase activity was further supported by our measurements of H3.3 occupancy during a time course of gene induction and subsequent shut-down. Initiation and elongation of RNA polymerase upon induction of the heat shock gene led to a marked displacement of canonical and variant H3 histones. Remarkably, after transcriptional shut-down, ChIP measurements showed a selective deposition of H3.3 variant even compensating for the loss of canonical histones throughout the transcribed region.

These results suggested a role for H3.3 incorporation in compensating transcription-coupled histone eviction at active genes. Evidently, this function is not compatible with the 5' bias distribution of euchromatic histone modifications and thus strongly suggests independent modes of targeting.

3.1.2. Published Manuscript

3.1.3. Supplementary Data



Supplemental Figure 1: Nucleosomal abundance determined by H3 C-terminal IP: Chromatin-IP was performed with an antibody recognizing the C-terminus of H3 and H3.3. Resulting enrichments were normalized to an intergenic sequence. The efficiency of the IP was validated by analyzing a nucleosome-free mitochondrial control sequence, which could not be detected in the bound fraction. Shown are the averages of three independent experiments. A: Nucleosomal abundance throughout all 9 tested genes. The resulting C-terminal values are used to normalize enrichments shown in Figure 2 of the main text. B: Nucleosomal abundance during and after heatshock. The C-terminal antibody was used to determine nucleosomal abundance at 673bp of the HSP70 gene during a heatshock experiment as shown in Figure 4 of the main text. Similar to the results with the epitope tagged histone the C-terminal antibody detects a temporary reduced nucleosomal abundance during heatshock, which recovers after 30 minutes.

Wirbelauer et al.: SUPPLEMENTAL Figure 1

Supplementary Figure 1

Supplemental Table 1: Primers and antibodies used in Wirbelauer et al.

1. PRIMERS:

1.1 Cloning of H3 constructs:

pIB-H3:
forward primer (5'-3'): ACCATGGCTCGTACCAAGC
reverse primer (5'-3'): AGCACGCTCGCCGCGAATGCG

pIB-H33:
forward primer (5'-3'): ACCATGGCACGTACCAAGCAAACAGC
reverse primer (5'-3'): GGCCCGCTCGCCACGGATGCGTC

1.2. Primer set for ChIP analysis:

Gene Annotation	Middle position of amplicon Relative to start site in bp (size of amplicon in bp)	forward primer	reverse primer
Pkg21D (CG3324)	120 (141)	GTTTGAGGACCGAACATCGT	CCATTCTGCCAGTCTGCTGC
	860 (156)	TAC TTG AGC CCC CGT CCA CT	CGC AGT CGC AAG TCT CGA A
	2666 (121)	CCTCTGGACGAACTTCTGCT	TAGGATTGCTGCATGGACTG
	3700 (139)	TGC AAC TCA CAA CCC TGA TCC	CGG CTG CTA TGC TCT TGG AG
CG6388	94 (148)	ATCCAAATGGAAGTGGACGA	ACGATCTCCGCATTACGTTC
	175 (127)	CCTGTCCAGGAATTCAATCG	CATCCTCCTGTTCCTTGACC
	920 (106)	TGCAAGCTGTCGATGAGCAA	TACCAGCCGTGGGATTTGG
	1800 (137)	ACAAAGCGTCGGAGCAACAG	GGGACACAGAAGCAGTCGTCA
chico (CG5686)	64 (192)	CGG GAT GGC AAC TTC GTA CT	ACG TCT AGC TGC CCG TAT GC
	1216 (177)	ACG AGG AGA CGA GCA CTT CG	CGA ATC CAC CGT CTC TGG AG
	2297 (166)	TCG GCA GAG AGA GAT GCG AT	GGC ATG GTG AAC GTT GGA CT
	2936 (149)	GCTTCAACTGTGCCACGTAA	GATTCGGATTCTGTCCCGTA
	4383 (105)	GTCCACAAGTGCTCCACTCC	TGCTTTTCCAAATTGGTTGC

CG3157	140 (76)	CGACTAATTCGACCTTAAACG	TTCACTTGGCATTGTGGTGT
	467 (100)	AAAGATTGTGCCTGGAGCAT	TCCGCCTGGTAGAAGAACAC
	1616 (130)	CGCGTGAGGACTACATGCAGT	GCATGGCACGCATTTCTTCT
aubergine (CG6137)	105 (97)	CACTGAACGGCATTTGTGACG	GTCCACGAGCAATTACAGG
	275 (91)	ATTCGCAGGGTCAGAAGTCG	GGACCCACTTGAGCATCACC
	2016 (157)	ACGGGAATGACGGACGCTAT	GTTCGATGCGACGATCTGGA
	2652 (101)	CTTTCCGCTACTTCTCCACCG	CTCCTGATACGACCTCAGGGC
	3152 (86)	AACCATACGAGTCCCCGCTG	CTGCTGAAGGCGCACGATTA
CG9135	90 (180)	AATGTTGGGATTACTCAAATGG	TTTTCGCCGTTCACACTGGG
	1115 (160)	GCCGAGCAGAGAGATCCAGA	GGACTCGTCGCTGAACTCGT
	2205 (123)	ATCGACTCCAAGAAGCGCGT	CACCCCGACCCTGAGTATCG
lace (CG4162)	70 (120)	GCCACACCATTCCAATTAGA	AAACCGTTCCAGAATCGC
	1328 (129)	AAGCCTCGCAGAATGCGATT	TTCTTTGGCAGCCCAGACG
	2350 (100)	CGATTAAGCTGACGTTCAAC	GCTGGCCAAAGGATCCG
	3961 (113)	GGG TCC ATA GTC CGT TTG CC	AAC TCC TCG TCC ACG GGA AC
	4669 (113)	GGTTCGCACCTTGACGACTC	TCTCGTCTATCGCCTCCAGG
shuttle craft (CG3647)	199 (185)	CGTCCAGTTGGCCGTAGAAA	CCCAGGAAAAGCTCCAGACA
	1405 (149)	GGGAGGACCGGTACGAAAGA	TCGTGTCCAAATCGTCAACG
	2642 (155)	CAAAATGCCTGTACCGGCTG	TTGGGACATGGAGGGCACT
CG6730	70 (140)	CCAACCCTGAATCGATTCGC	CCATTGTTTGCGACTATTATCC
	582 (101)	TTTTCTTGGCGATGGACTTC	CGAAGTTTTCAAGGCATTTG
	1736 (149)	GGCAGAAATTCGCCAACCTT	TCTTGGCTTGAGGCCAACATT
Hsp70	+682 (73)	ATA TCT GGG CGA GAG CAT CAC A	GTA GCC TGG CGC TGG GAG TC
intergenic g	(200)	CGAAGCCTCAAAACGAGTTC	ACTCAACGCTTGGCTTCACT

2. ANTIBODIES:

Primary:
- mouse monoclonal anti-V5 (Invitrogen)
- rabbit αH3K4me2 (Upstate Biotechnology)
- rabbit αH3K4me3 (gift from Bryan Turner described in Schübeler et al., 2004)
- rabbit αH3K9me2 (Upstate Biotechnology)
- rabbit αH3K79me2 (Upstate Biotechnology)
- rabbit αH3AC (Upstate Biotechnology)
- rabbit αH3 C-terrminus (Abcam)
- mouse monoclonal αRNA Polymerase II (8WG16, Santa Cruz Biotechnology)
- mouse monoclonal αRNAPII CTD-Ser5 (H14) (BAbCO Covance)

Secondary:
- FITC-conjugated AffiniPure Donkey α-mouse IgG (H+L) (Jackson ImmunoResearch)
- FITC-conjugated AffiniPure Donkey α-rabbit IgG (H+L) (Jackson ImmunoResearch)
- sheep α-mouse IgG-peroxidase linked, whole antibody (Amersham Biosciences)
- donkey α-rabbit IgG-peroxidase linked, whole antibody (Amersham Biosciences)

3.2. Localized H3K36 methylation states define histone H4K16 acetylation during transcriptional elongation in *Drosophila*

Oliver Bell, Christiane Wirbelauer, Marc Hild, Annette N.D. Scharf, Michaela Schwaiger, David M. MacAlpine, Frederic Zilberman, Fred van Leeuwen, Stephen P. Bell, Axel Imhof, Dan Garza, Antoine H. F. M. Peters and Dirk Schübeler

3.2.1. Summary

Our analysis of histone H3.3 occupancy at heat shock genes indicated that chromatin structure is dynamically remodeled during the course of transcription. Nucleosomes are being displaced in the wake of traversing RNA polymerases and become rapidly reassembled after polymerase clearance. This inverse relationship indicates that the presence of nucleosomes intrinsically suppresses transcriptional activity. Indeed, transcription initiation requires histone eviction (Boeger et al, 2003; Reinke & Horz, 2003) and coincides with nucleosomal depletion in the promoter regions of active genes in *S. cerevisae* (Lee et al, 2004). Nucleosomes are also dissembled to facilitate polymerase passage downstream of promoters (Schwabish & Struhl, 2004). However, reassembly of compact chromatin at the body of transcribed genes is critical as failure to do so results in aberrant transcription initiation from internal start sites (Kaplan et al, 2003; Schwabish & Struhl, 2006). Interestingly, similar phenotypes of spurious intragenic transcription were also observed in yeast SET2 mutants (Carrozza et al, 2005b; Joshi & Struhl, 2005; Keogh et al, 2005) suggesting a role for H3K36 methylation in maintenance of chromatin structure at transcribed regions. In budding yeast, methylation of lysine 36 resides in the 3' end of active genes where it signals for removal of transcription-coupled hyperacetylation of histones through recruitment of the Rpd3 deacetylase complex.

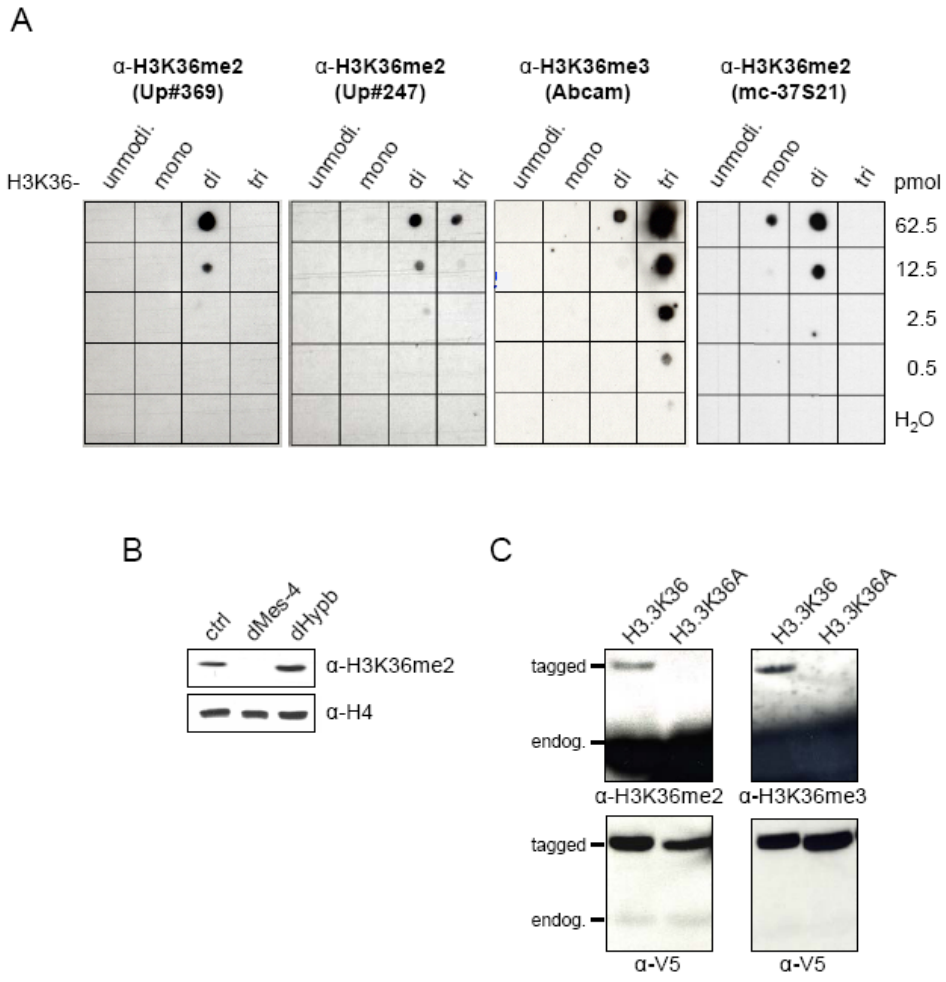
To investigate the function of this modification in a higher eukaryote, we characterized the distribution and regulation of H3K36 di- and trimethylation in *Drosophila melanogaster* using chromatin-immunoprecipitation and RNA interference. Local and global profiling of K36 methylation states revealed distinct localization patterns throughout the body of transcribed genes. Dimethylation peaked towards the 5' end, yet downstream of promoter-proximal H3K4 methylation, whereas trimethylation accumulated in the 3' end. This differential targeting at active genes reflects binding of two separate enzymes, which display distinct specificities *in vivo*. dHypb mediates trimethylation while dMes-4 is required for both methylation states of lysine 36. Reduction of trimethylation by dHypb knockdown is lethal in *Drosophila* larvae, exposes K36me2 and coincides with hyperacetylation of H4 lysine 16 at 3' ends. Acetylation of lysine 16 has been shown to prevent

the formation of higher-order chromatin folding, thus suggesting comparable roles for H3K36me3 in *Drosophila* and *S. cerevisae*. Intriguingly, knockdown of dMes-4 has the opposite effect as acetylation of H4K16 is decreased at transcribed regions. Together, these data are in agreement with a step-wise mechanism of dMes-4 mediated H3K36 dimethylation, which in turn serves as a substrate for dHypb-dependent trimethylation. Moreover, displaying opposite crosstalk to H4K16ac, the two methylation states appear to serve distinct functions which might enable dynamic fine tuning of chromatin compaction during transcription elongation in *Drosophila*.

3.2.2. Published Manuscript

3.2.3. Supplementary Data

3.2.3.1. Supplementary Figures



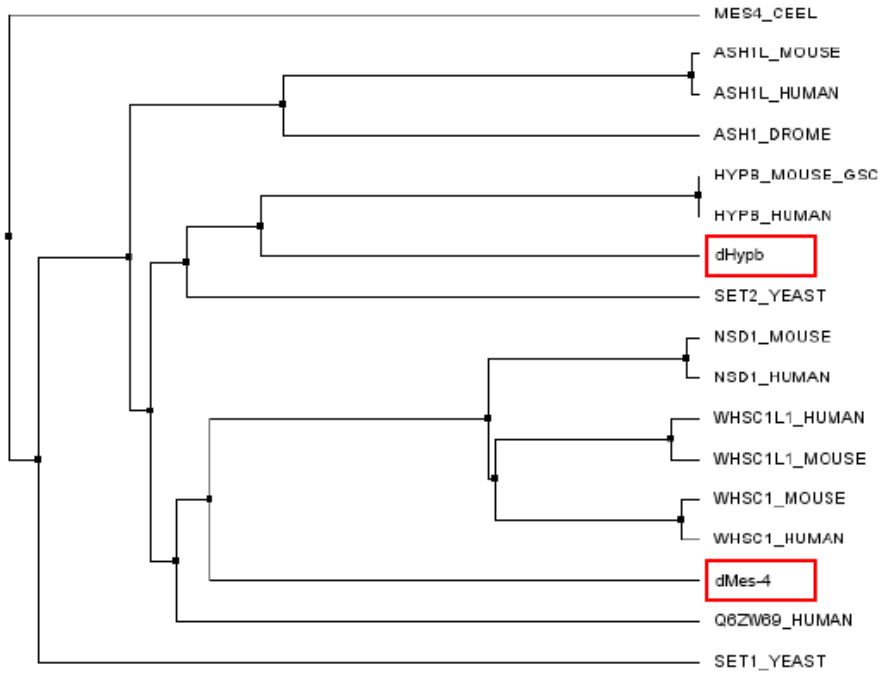
Supplementary Figure 1: Characterization of H3K36 methylation state-specific antisera **(A)** Indicated amounts of either modified or unmodified H3 peptide (residues 25-45) were spotted onto polyvinylidene difluoride membranes and probed with commercial rabbit antisera directed against H3K36me2 (Upstate#369, Upstate#247, see Material and Methods) or H3K36me3 (Abcam) at 1:1000 dilution each. The antibodies used in this study (Upstate#369 against H3K36me2 and Abcam ab9050 against H3K36me3) are highly specific in this analysis, whereas Up#247 shows considerable cross-reactivity. We have generated a monoclonal antibody directed against H3K36me2 which shows high specificity towards H3K36me2 peptide. (B) Western blot analysis using the monoclonal H3K36me2 antibody validates this specificity as it detected the reduction of dimethylation but not trimethylation upon knockdown of dMes-4 and dHypb, respectively. (C) Specificity to H3K36 in the context of full-length histone H3. Histones isolated from cells expressing either wild-type H3.3

(H3K36) (Wirbelauer et al., 2005) or H3.3 in which Lysine 36 has been mutated to Alanine (H3K36A) were tested with the specific antibodies. Lack of signal in mutant H3.3 confirms high specificity for H3K36 in the context of histone H3. Detection of the V5 epitope of the ectopically expressed histones serves as loading control.

A

Gene ID	Gene name	Sequence position	E-value	% Identity
CG1716	dHypb	1380-1550	1.00E-84	49
CG8887	Ash1	1348-1520	2.00E-75	39
CG4976	dMes-4	1202-1374	2.00E-74	34
CG3848	Trx-related	2260-2409	1.00E-59	34
CG8651	Trx	3680-3827	6.00E-58	33
CG6502	Ez	567-747	1.00E-53	37
CG6476	Su(var)3-9	471-634	3.00E-43	31
CG40351	SETD1	1498-1640	9.00E-40	31
CG3307	SETD8	565-675	9.00E-40	32
CG2995	-	1437-1600	1.00E-28	32

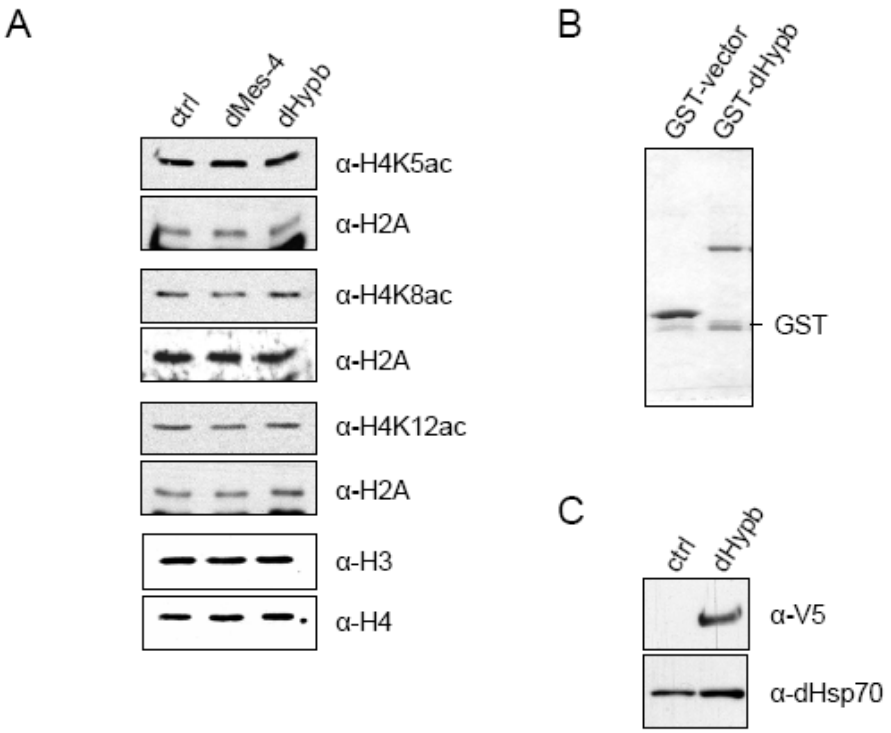
B



Supplementary Figure 2: Homology comparison of dMes-4 and dHypb

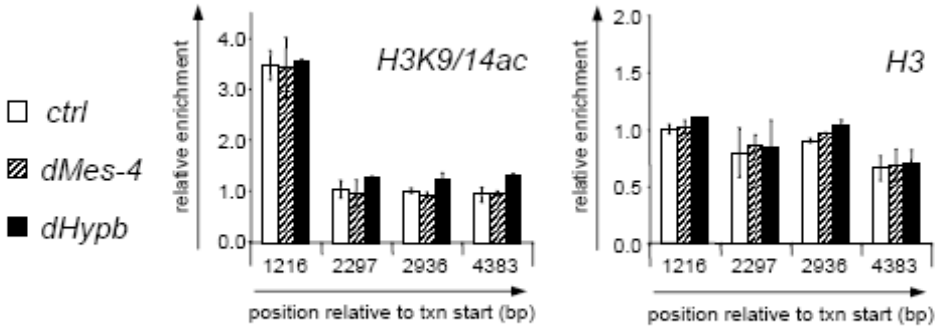
(A) PsiBlast identifies *Drosophila* proteins with homology to the SET domain of *S. cerevisiae* Set2 (aa 63-260). SET domains including Pre-SET, SET and Post-SET were aligned in order to determine the degree of sequence conservation. (B) PsiBlast similarity search using the

conserved SET domains of dHypb (aa 1372-1557) or dMes-4 (aa 1154-1427) predicts multiple H3K36 HMTs in human and mouse. Sequence alignment of selected homologues indicates that dHypb shares high similarity with yeast Set2 and human HYPB, while dMes-4 is closely related to human and mouse NSD proteins. The presence of multiple homologues of these *Drosophila* H3K36 HMTs suggests a conservation of the H3K36 pathway in mouse and human.

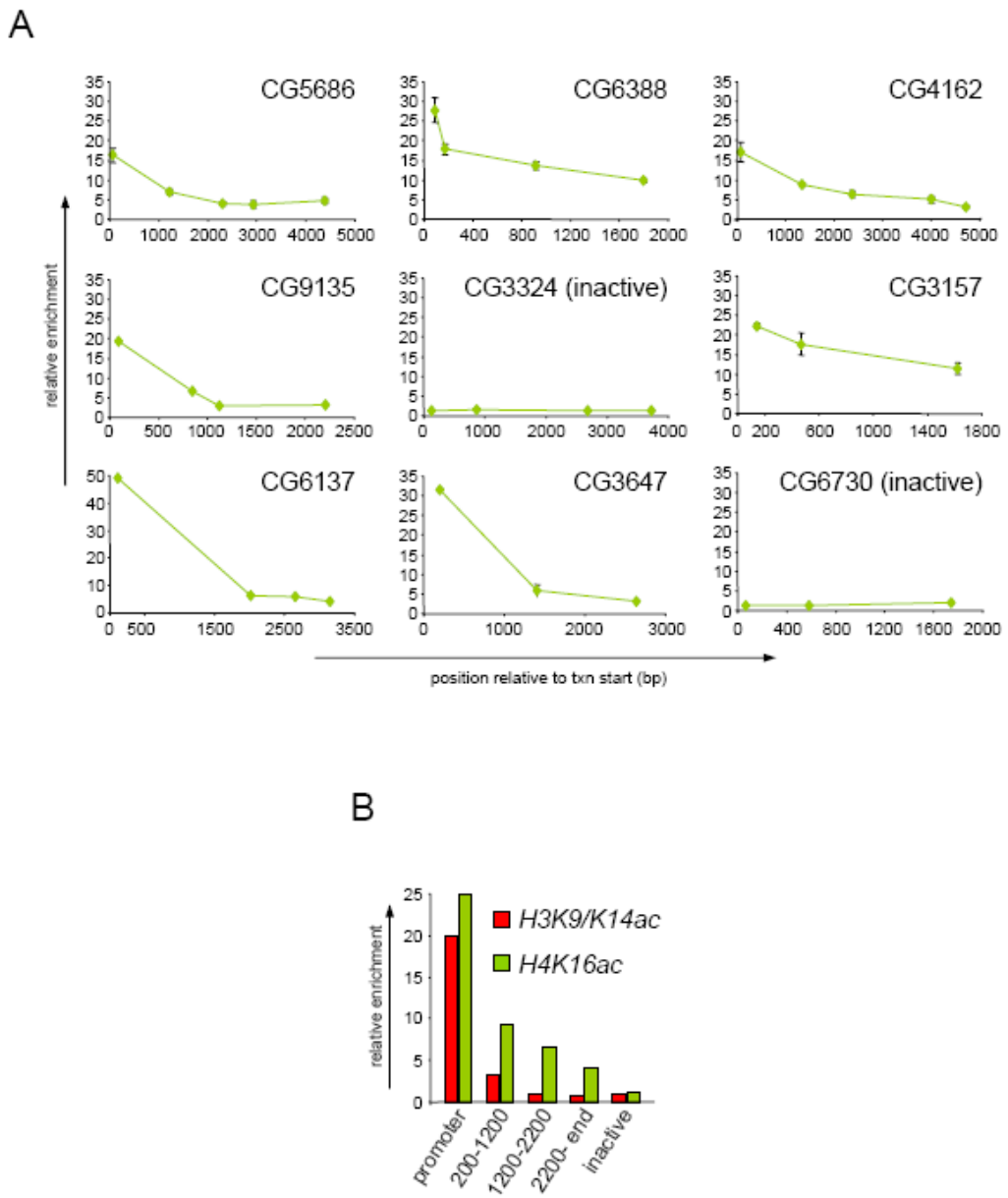


Supplementary Figure 3: Changes in H3K36 methylation states do not exert a global effect on histone acetylation other than H4K16

(A) Western blot analysis of bulk acetylation levels of specific residues on histone H4 compares RNAi and untreated control Kc cells. Detection of H2A, H3 and H4 serve as loading controls. (B) Coomassie-stained SDS PAGE gel shows purified GST-vector and GST-Hypb fragment used for *in-vitro* analysis of HMTase activity. (C) Western blot using anti-V5 antibody shows expression level of epitope-tagged dHypb after transfection in *Drosophila* Kc cells.



Supplementary Figure 4: Comparison of acetylation levels of H3K9/14 and total histone H3 occupancy between control and RNAi in Kc cells by ChIP and Real-time PCR. No effect upon changes in H3K36 methylation is observed.



Supplementary Figure 5: H4K16 acetylation is enriched at promoters and in coding regions of actively transcribed genes. (A) Steady state distribution of H4K16 acetylation along several genes was measured by ChIP and quantified Real-time PCR (see above) in Drosophila Kc cells. Shown is the average and standard deviation from at least three independent repeats starting with cells at different passages. X-axis reflects the base-pair position relative to the transcriptional start site. Y-axis reflects enrichment (bound/input normalized to an intergenic control). Numbers in graphs are gene IDs according to Flybase. (B) Comparison of the distributions of H4K16ac with H3K9/K14ac at autosomal genes (7 active and 2 inactive genes). Tested amplicons shown in (A) are grouped similar to Figure 2B. H4K16ac shows a promoter proximal bias similar to H3K9/K14ac yet is more abundant throughout coding regions when compared to inactive genes.

3.2.3.2. Supplementary Materials and Methods

Antibodies

Purified bacterially expressed protein fragments were used to generate mouse monoclonal (pMalC2-dHypb=aa 1-436 and pMalC2-dMes-4=aa 412-651) and rabbit polyclonal (pMalC2-dHypb=aa 1-436, pMalC2-dHypb=aa 919-1135, pMalC2-dHypb=aa 2040-2363 and pMalC2-dMes-4=aa 997-1016) antibodies according to standard procedures. Synthetic histone H3 tail peptide containing dimethylated lysine 36 was used to a generate mouse monoclonal antibody. Hsp70 (mouse monoclonal, StressGen), total H3 and H4 antibodies (Upstate), H2A (Upstate 07-146), H3K36me2 (Upstate 07-369), H3K36me3 (Abcam ab9050), H3K4me3 (gift from Bryan Turner, described in Schubeler et al., 2004), H3K79me2 (Upstate 07-366), H3ac (Upstate 06-599), H4ac (Upstate 06-598), H4K5ac (Upstate 07-327), H4K8ac (Upstate 07-328), H4K12ac (Upstate 07-595), H4K16ac (Upstate 07-329), MOF (gift from Asifa Akhtar (Mendjan et al, 2006)).

Immunofluorescence

Immunofluorescent staining of *Drosophila* Kc cells was carried out essentially as described (Wirbelauer et al, 2005) using the respective antibody at a dilution of 1/200.

Chromatin-Immunoprecipitation (ChIP)

ChIPs of histone modifications were carried out as described (Schubeler et al, 2004) with minor modifications. Cells (1×10^8) were cross-linked with formaldehyde for 8 min.. Sonication was performed for 3 x 15 sec at 70% (Branson Digital Sonfier) in lysis buffer (50 mM HEPES/KOH at pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% DOC, 0.1% SDS + Complete protease inhibitors (Roche)). 75µg chromatin and 3-5 µg antibody was used per IP. Immuno-complexes were isolated by adding protein A-Sepharose followed by four washing steps: 2x lysis buffer, 1x DOC buffer (10 mM Tris at pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% DOC, 1 mM EDTA), 1x TE at pH 8. Reversal and DNA purification was as described (Schubeler et al, 2004).

ChIPs of dMes-4 and dHypb were performed as described (Adelman et al, 2005) with minor modifications. Cells (1.5 x 10^8) were cross-linked for 10 min. Sonication was performed for 3 x 20 sec at 70% in sonication buffer (0.5% SDS, 20mM Tris at pH 8, 2mM EDTA, 0.5mM EGTA, 0.5mM PMSF, Complete protease inhibitor (Roche)). Chromatin was diluted 1:10 with ChIP dilution buffer (1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris at pH 8, 167 mM NaCl, 0.01% SDS) and 1ml was used per IP and 6µg antibody. Immunocomplexes were isolated by adding protein A-Sepharose followed by nine washing steps: 3x low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris at pH 8, 150mM NaCl), 3x high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris at pH 8, 500mM NaCl), once LiCl buffer (1mM EDTA, 10mM Tris at pH 8, 250mM LiCl, 1% NP40, 1% Sodium Deoxycholate), 2x TE. Elution, reversal and DNA purification as described (Schubeler et al, 2004).

Real-time PCR

PCR conditions and primer sequences were as described (Wirbelauer et al., 2005) except for the following additional primers at gene CG9135 (middle position = 1328 bp and size amplicon = 206 bp) were used for the analysis:
(FP) 5'-ACCGTTTATGCGAAGCTGAG-3'
(RP) 5'-CGCGCACTGTTTATGTTT-3'.

RNA interference in cultured cells

dsRNA for RNAi knockdown of *Drosophila* dMes-4 mRNA (bp 4507-5214), dHypb mRNA (bp 3236-3944) and GFP mRNA was generated according to Ambion MEGAscript manual instructions. 1.5x10^6 Kc cells were plated in 2ml medium and treated with 70µg dsRNA for 48h. Treatment was repeated after cell splitting for a total of 8 days before harvesting cells for subsequent analysis.

Tissue culture and transient transfection of Kc cells

Drosophila Kc cells were kept in HyQ-SFX (Hyclone). 2 x 10^6 cells were seeded and transfected with 3ug of plasmid DNA using Cellfectin (Invitrogen)

according to the manufacturer's protocol. Cells were harvested after 48h and extracts prepared.

Vector construction

Information will be provided upon request

SDS-Page and Western blot analysis

Histone preparation and separation on SDS gel were as previously described (Wirbelauer et al., 2005). For detection of endogenous dHypb and dMes-4, nuclei-extracts from $1x10^6$ cells were loaded on 3-8% NuPAGE Novex Tris-Acetate Mini Gels (Invitrogen) using reducing conditions according to manufacturer's instructions. Proteins were transferred to Hybond P membrane (Amersham) using the NuPAGE system (Invitrogen) followed by detection with monoclonal antibodies against dHypb and dMes-4.

Preparation of Kc cell nuclei

Kc cells were resuspended in solution I (10mM Tris pH8,10mM EDTA, 0.5mM EGTA, 0.25% Triton X 100, 1mM DTT, Complete protease inhibitor (Roche)) and incubated for 5 min on ice, followed by cold centrifugation for 5 min at 3000 rpm. After additional washing in solution I, the pellet was resuspended in lysis buffer (50mM Tris pH7,5, 250mM NaCl, 5mM EDTA pH 8.0, 0,5% NP40, 50mM NaF, 10% Glycerol, 0,25% SDS, 1mM DTT, Complete Protease inhibitor (Roche)).

Fly strains and transgenes

RNAi expression transgenes for dMes-4 and dHypb were produced by cloning PCR products corresponding to coordinates dMes-4 (4507-5214 bp) and dHypb (3236-3944 bp) and transformed using standard methods (Lee and Carthew, 2003). All other strains were obtained from the Bloomington Stock Center. Phenotypes were assessed by crossing transgene insertion strains to Tubulin-Gal4/TM6B, Tb Hu e driver strain and comparing the survival of Tubby vs. non-Tubby progeny. Lethality was observed for 4 independent lines of dHypb (7A2, 20A3, 23B3, and 28A). No lethality was observed for two

independent lines of dMes-4 (25A3 and 54A), which however showed weaker knockdown.

Average distance tree

A similarity search (PsiBLAST) was carried out in MyHits (Swiss Institute of Bioinformatics) using the conserved SET domains of *S. cerevisae* Set2 (63-260 bp) and *D. melanogaster* CG1716 (1372-1557 bp) and CG4976 (1154-1427 bp). Homologues were chosen manually and aligned (ClustalX). Alignment was employed to create BLOSUM62 average distance tree.

Histone-Methyltransferase assay using calf thymus histones

GST-tagged recombinant fragments including pre-SET, SET and post-SET domains from dHypb (aa 1351-1553) were purified from baculovirus infected SF9 cells. 4µg protein were incubated with 2µg calf thymus core histones (Roche) along with 1µCi/µl S-Adenosyl-L (methyl-3H) methionine (^{3}H-SAM, Amersham TRK865) in methyltransferase reaction buffer (final conc.: 50mM Tris/HCl, 50mM NaCl and 1mM DTT at pH 8.5) for 1h at 30ºC in a total volume of 20µl. The reaction was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by semi-dry transfer to membrane (Hybond P (Amersham)) and exposure to film.

Preparation of yeast nuclear extracts

Nuclear extracts were prepared from yeast nuclei of the following strains (NKI3041: MATa leu2d0 lys2d0 ura3d0 dot1::NATMX set1::HPHMX; UCC7361: MATa leu2D0 lys2D0 ura3D0 dot1::NATMX set1::KANMX) in (25 mM K2SO4, 30 mM Hepes pH 7.6, 5 mM MgSO4, 1 mM EDTA, 10% glycerol, 0.5% NP40). Extracts were briefly sonicated to shear chromatin.

Histone-Methyltransferase assay using yeast nuclear extracts

Recombinant baculoviruses containing full-length dHypb (pVl1393-dHypb) were generated using the BaculoGold Tranfection Kit (Pharmingen), according manufacturer's instructions. $5x10^6$ infected SF9 insect cells were lysed in 800µl TNN buffer (50mM Tris pH7.5, 250mM NaCl, 5mM EDTA pH 8.0, 0.5% NP40, 50mM NaF, 1mM DTT, Complete protease inhibitor (Roche))

and incubated for 20 min. on ice followed by 15 min. cold centrifugation at 13000 rpm. Full-length Hypb was immuno-purified from supernatant using a monoclonal anti-dHypb antibody (2h at 4˚C) and 40µl 50% ProteinA-Sepharose slurry (1h at 4˚C) (GE Healthcare). Baculovirus infection with an empty vector (pV11393) and subsequent IP with a monoclonal anti-HA antibody (12CA5) served as control. Sepharose-bound immuno-complexes were washed 3 times in methyltransferase reaction buffer. HMTase assay was performed by incubating beads with 40µl reaction mix (4 µl of 10X methyltransferase reaction buffer with 2mM beta Mercaptoethanol, 500ng of yeast lysate and S-adenosylmethionine (final conc. 20µM)) for 1h at 30ºC. The reaction was terminated with 10µl of 5x SDS loading buffer, boiling for 10 min. and subsequent analysis by SDS PAGE and Western blot.

3.3. Transcription-coupled methylation at lysine 36 of histone H3 regulates dosage compensation by enhancing recruitment of the MSL complex in *Drosophila*

Oliver Bell, Thomas Conrad, Jop Kind, Christiane Wirbelauer, Asifa Akhtar and Dirk Schübeler

3.3.1 Summary

Our study of H3K36 methylation in female *Drosophila* cells revealed an intricate histone-tail crosstalk to acetylation of lysine 16 at histone H4. Acetylation at this residue is particularly enigmatic since it has been shown to directly influence packaging of higher-order chromatin (Dorigo et al, 2003; Shogren-Knaak et al, 2006) and to be required for dosage compensation at the X-chromosome in male flies (Akhtar & Becker, 2000).

MSL3 is part of the dosage compensation complex (DCC) and required for H4K16 hyperacetylation of the single male X chromosome (Gu et al, 1998). Intriguingly, MSL3 is one of the *Drosophila* homologues of the yeast H3K36me binding protein Eaf3 (Eisen et al, 2001) and recent reports demonstrated that DCC members localize to the 3' end of dosage-compensated genes (Alekseyenko et al, 2006; Gilfillan et al, 2006). Based on these findings, we investigated the role of H3K36 methylation in DCC targeting. We find that dHypb-dependent reduction in H3K36 trimethylation has an X-chromosome specific effect by reducing H4K16 hyperacetylation at dosage compensated genes. This effect reflects compromised MSL1 and MOF recruitment and coincides with failure to upregulate transcription of several target genes. Thus, we propose that in analogy to K36me in budding yeast, H3K36me3 specifically binds MSL3 and in turn recruits MOF and other members of the DCC to the 3' end of X-linked target genes. This model is in agreement with recent evidence of MSL3 binding Set2-methylated nucleosomes *in vitro* (Larschan et al, 2007).

Importantly, despite the chromosome-specific role of trimethylation, H3K36 methylation patterns do not appear to be different on the male X. Thus the same modification that reduces acetylation at autosomal genes signals to enhance acetylation at dosage compensated genes. One potential explanation for the differential readout of H3K36me3 is the association with complexes bearing alternative methyl-binding proteins. Collectively, these results indicate a context-specific readout of H3K36 methylation states at autosomes and the male X. This predicts that euchromatic histone modifications simply serve as characteristic landmarks to signal for recruitment of proteins with diverse functions to the body of active genes.

3.3.2. Submitted Manuscript

3.3.3. Supplementary Data

3.3.3.1. Supplementary Figures

3.3.3.2. Supplementary Materials and Methods

4. GENERAL CONCLUSIONS

Our studies reveal an intricate landscape of euchromatic histone modification along the body of active genes in *Drosophila*. Methylation of lysine 4 and lysine 79 of histone H3 localized adjacent to promoters whereas H3K36me2 peaked mid-genic and H3K36me3 accumulated at 3' ends of transcribed regions. This is similar to high-resolution profiles of trimethylation at K4, K36 and K79 in other species (Barski et al, 2007; Mikkelsen et al, 2007; Pokholok et al, 2005) indicating that the complex pattern of tail modifications along active genes is not limited to the *Drosophila* genome. In contrast to the topographic differences of tail modifications, deposition of histone variant H3.3 was uniform throughout transcribed regions. While this agrees with the fact that this variant is enriched for all tested modifications, it argues against an upstream role of H3.3 to determine a particular chromatin state. Instead, the targeting of individual histone marks most likely relies on separate mechanisms coupled to different forms of elongating RNA polymerase (Li et al, 2007a).

We identified two distinct HMTs involved in the methylation of lysine 36 of histone H3 in *Drosophila*. dMes-4 is required for both di- and trimethylation while dHypb is responsible for trimethylation only. Importantly, reduction of trimethylation by knockdown of dHypb resulted in increased levels of K36me2 in the 3' end of active genes. Based on this and the fact that dHypb required premethylated K36 for activity *in vitro*, we propose a step-wise mechanism in which dMes-4 mediates dimethylation which is substrate for dHypb-dependent trimethylation. Such model implies specific recruitment of dHypb to the 3' end of transcribed genes. In budding yeast, Set2-directed methylation relies on an interaction with the serine-2-phosphorylated CTD of RNA polymerase (Kizer et al, 2005). An interaction with hyperphosphorylated polymerase has been recently reported for dHypb (Stabell et al, 2007) indicating that a similar mechanism could be responsible for targeting the trimethylase to promoter-distal regions of *Drosophila* genes. Conversely, it is conceivable that dMes-4 interacting with serine-5-phosphorylated RNA polymerase at promoter-proximal sites could account for the different spatial localization of H3K36 dimethylation.

Specific localizations not only reflect independent targeting mechanisms but also suggest separate functions of histone marks during the

course of transcription. Indeed, at autosomes H3K36 di- and trimethylation states display opposite crosstalk to histone acetylation. This is reminiscent of the function in budding yeast, where methylation of lysine 36 signals for removal of transcription-coupled histone acetylation through recruitment of the Rpd3S deacetylase complex (Carrozza et al, 2005b; Joshi & Struhl, 2005; Keogh et al, 2005). In *Drosophila*, reduction of H3K36 trimethylation at autosomal genes coincided with hyperacetylation preferentially at H4 lysine 16. Acetylation of this residue has been shown to prevent higher-order chromatin folding, thus suggesting a role for H3K36me3 in recruitment of an HDAC activity, analogous to *S. cerevisae*. Intriguingly, knockdown of dMes-4 and subsequent loss of di- and trimethylation had the opposite effect as acetylation of H4K16 was decreased at transcribed regions. Thus, the increase of dimethylation in the dHypb knockdown is required for hyperacetylation of H4K16, indicating that dimethylation has a function distinct from trimethylation and possibly recruits a histone acetylase activity. In the 3' end, H3K36me3 antagonizes hyperacetylation of H4K16 thereby mediating a more compact chromatin structure.

Combined with recent evidence of H3K4me3 interacting with HATs (Pray-Grant et al, 2005) and nucleosome remodelers (Wysocka et al, 2006) at active promoters, these data imply that tail modifications function to mediate remodeling of the nucleosomal template at all steps of the transcription cycle. Importantly, they do not only signal to "loosen" chromatin in front of elongating polymerase (Schwabish & Struhl, 2004) but also to reestablish compact structure behind and thus prevent subsequent transcription from cryptic internal start sites (Carrozza et al, 2005b; Joshi & Struhl, 2005; Kaplan et al, 2003; Schwabish & Struhl, 2006).

Notably, disruption of H3K36 methylation in yeast revealed that abberant transcription frequency directly correlates with the length of hyperacetylated ORFs (Li et al, 2007c). Given the larger average gene length in higher eukaryotes, this would predict that H3K36 trimethylation plays a more critical role in suppressing cryptic transcripts generated from intragenic regions in *Drosophila*. Nevertheless, we have not obtained any evidence for increased accumulation of aberrant mRNAs upon dHypb-dependent hyperacetylation. While this might indicate that the increase of acetylation is

insufficient to disrupt chromatin structure and expose internal start sites, it is also possible that in *Drosophila* improper transcripts are more rapidly removed by the mRNA surveillance machinery (Andrulis et al, 2002). Combined knockdown of the H3K36-trimethylase and components of the responsible exosome complex might be able to resolve the apparent paradox.

Additional complexity for the functional readout of euchromatic histone modifications was revealed by the observation that in *Drosophila* H3K36me3 has a chromosome-specific role to enhance hyperacetylation on the single male X chromosome. This separate function is not reflecting different methylation patterns on the X chromosome but instead appears to involve context-specific interaction with the Eaf3 homologue MSL3 as part of the dosage compensation complex. Indeed, reduction of H3K36 trimethylation and subsequent decline in H4K16 acetylation coincided with compromised recruitment of MOF, MSL1 (in this study) and MSL3 (Larschan et al, 2007) at X-linked target genes. Thus, the same modification that reduces acetylation at autosomal genes mediates binding of the MSL HAT complex to enhance acetylation at dosage-compensated genes. This model is supported by recent evidence showing preferential interaction between MSL3 and Set2-methylated nucleosomes *in-vitro* (Larschan et al, 2007). Importantly, diminished MSL complex association upon dHypb RNAi resulted in failure to upregulate transcription of target genes. Together, these results strongly suggest separate context-specific roles of H3K36 trimethylation to modulate levels acetylation at transcribed autosomal regions and at dosage-compensated genes on the male X chromosome.

Finally, these findings highlight that euchromatic tail modifications provide general signaling platforms, which impart their specificity only by marking characteristic positions along the body of the transcription unit. The targeting chromatin modifications to these sites is achieved through association of the responsible enzymes with different phosphorylated forms of elongating RNA polymerase (reviewed by (Li et al, 2007a). We propose that the resulting layout of spatial landmarks is important to integrate different aspects of the transcription process and to robustly control the accessibility of the chromatin template to the polymerase machinery.

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6. CURRICULUM VITAE

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08/2003 – 01/2008	Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland PhD studies in the laboratory of Dr. Dirk Schübeler: "Localization and Function of Histone Methylation at Active Genes in *Drosophila*"
10/1999 – 06/2003	Mannheim University of Applied Sciences, Mannheim, Germany BSc (Honours) in Biotechnology
10/2002 – 06/2003	European Molecular Biology Laboratories (EMBL), Heidelberg, Germany Final Thesis in the laboratory of Dr. Matthias W. Hentze "Characterization of the Iron Homeostatic Machinery in a Mouse Model of Hemolytic Anemia"
02/2002 – 06/2002	University of Teesside, Middlesbrough, UK Theoretical semester abroad
08/2001 – 01/2002	Scripps Institution of Oceanography, La Jolla, USA Practical internship semester
09/1998 – 06/1999	California State University, Northridge, USA BSc in Biology with emphasis on Medical Technology

08/1997 – 06/1998 Simi Valley High School, Simi Valley, USA

Academic exchange program organized by
Parlamentarisches Patenschaftsprogramm der
Bundesrepublik Deutschland and the US Congress

08/1992 – 06/1997 Coubertin Gymnasium, Berlin, Germany

Publications

Bell O, Conrad T, Kind J, Wirbelauer C, Akhtar A, Schubeler D (2008) Transcription-coupled methylation of histone H3 at lysine 36 regulates dosage compensation by enhancing recruitment of the MSL complex in *Drosophila melanogaster*. *Mol Cell Biol* **28**(10)**:** 3401-3409

Bell O, Wirbelauer C, Hild M, Scharf AN, Schwaiger M, Macalpine DM, Zilbermann F, van Leeuwen F, Bell SP, Imhof A, Garza D, Peters AH, Schubeler D (2007) Localized H3K36 methylation states define histone H4K16 acetylation during transcriptional elongation in *Drosophila*. *Embo J* **26**(24): 4974-4984

Galy B, Ferring D, Minana B, **Bell O**, Janser HG, Muckenthaler M, Schumann K, Hentze MW (2005) Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2). *Blood* **106**(7)**:** 2580-2589

Wirbelauer C, **Bell O**, Schubeler D (2005) Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active histone modifications show a promoter-proximal bias. *Genes Dev* **19**(15)**:** 1761-1766

Presentations

09/2007 Cold Spring Harbor Meeting on Mechanisms of Eukaryotic Transcription

Poster presentation

05/2007 EMBO Conference on Chromatin an Epigenetics

Poster presentation

09/2006 TriRhena Chromatin & Transcription Meeting

Oral presentation

04/2006 Keystone Meeting on Regulation of Eukaryotic Transcription: from Chromatin to mRNA

Poster presentation

03/2006 FMI – Novartis Institutes for Biomedical Research (NIBR)
Epigenetics Symposia

Oral presentation

09/2005 FMI Annual Meeting

Oral presentation

Fellowships and Awards

04/2006	Keystone Symposia Scholarship
08/2003	FMI International PhD Program Scholarship
02/2003	Young Innovator Award of Baden-Würtemberg, Business Start-ups from Universities and Research Institutes
08/1997	Fellowship of the Parlamentarisches Patenschaftsprogramm der Bundesrepublik Deutschland and the US Congress

Voluntary Team Work

- Organization of the Career Guidance Conference in Life Sciences (2005) (www.cgc2005.com)
- Organization of the FMI Student Science Colloquia Series
- FMI student representative (www.fmi.ch/student)

Hobbies

- Member of Therwil men's Volleyball team competing in NLB (division II)