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DISSERTATION

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Investigation of a phospho-histone H3 based histone code during transcriptional activation

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Abstract

Within eukaryotic nucleus, DNA is organized into a structural dynamic nucleoprotein complex designated “chromatin”. The fundamental subunit of the chromatin polymer is constituted by the nucleosome, an octameric protein-DNA complex composed of four different histone species, histone H2A, H2B, H3 and H4.

Posttranslational modifications (PTM) of histone amino-terminal tails such as methylation, phosphorylation or acetylation are pivotal elements in chromatin biology. According to the “histone code” hypothesis, PTMs exert their function *via* the recruitment of modification dependent binding factors (detector proteins) that finally modulate chromatin architecture.

In interphase phosphorylation of serine10 at histone H3 correlates with transcriptional activation of a subset of mammalian genes, including the HDAC1 gene. Further this particular modification frequently coincides with lysine acetylation on the same histone H3 tail, and the dual modification is frequently referred to as phosphoacetylation.

The aim of this thesis was to investigate the role of histone H3 phosphorylation/ phosphoacetylation during transcriptional activation and to examine whether multiple different modifications cooperate during this process.

14-3-3 proteins were identified as detector proteins for phosphorylated histone H3. *In vitro* interaction studies indicate that the weak interaction of 14-3-3 with serine10 phosphorylated histone H3 is susceptible to modulation *via* combinatorial modification patterns: phosphorylation is necessary for significant interaction but additional histone H3 lysine9 or lysine14 acetylation (phosphoacetylation) increases the affinity of 14-3-3 for the H3 tail. Besides acetylation, lysine9 is also methylated in transcriptional repressed chromatin. Mass spectrometry analysis reveals that phosphoacetylation coincides with lysine9 methylation *in vivo*. This triple modified histone H3 species is efficiently bound by 14-3-3 proteins *in vitro,* suggesting that histone phosphorylation and acetylation can cooperate to temporarily “fade out” transcriptional repressive lysine methylation whilst retaining epigenetic information. Chromatin immunoprecipitation experiments demonstrate the localization of 14-3-3 proteins to the HDAC1 promoter region in a histone H3 serine10 phosphorylation dependent manner. This recruitment is reinforced by additional histone acetylation and correlates with the dissociation of the transcriptional repressive module HP1γ. siRNA-mediated depletion of 14-3-3 proteins abolishes the transcriptional activation of HDAC1, suggesting that 14-3-3 proteins relay the phosphoacetylation signal to the transcriptional machinery. Together, the presented data indicate that 14-3-3 proteins are central mediators of histone H3 phosphoacetylation signals during transcriptional induction.

Zusammenfassung

DNA liegt innerhalb des eukaryontischen Zellkerns in Form eines dynamischen Nukleoprotein-Komplexes vor, der als „Chromatin“ bezeichnet wird. Die elementare Untereinheit des Chromatinpolymers ist das Nukleosom, ein DNA-Protein-Komplex, welcher je zwei Kopien der vier Histone H2A, H2B, H3 und H4 beinhaltet.

Post-translationelle Modifikationen (PTM) der N-Termini von Histonen, wie zum Beispiel Methylierung, Azetylierung oder Phosphorylierung stellen zentrale Elemente in der Chromatin-Biologie dar. Entsprechend der „Histonecode“-Hypothese vermitteln PTM diese Funktion durch die Rekrutierung von modifikationsabhängigen Faktoren (Detektorproteinen), durch die schließlich die Chromatin-Architektur verändern.

Während der Interphase korreliert die Phosphorylierung von Serin10 an Histone H3 mit der Transkription einer spezifischen Gruppe von Genen, einschließlich des HDAC1-Gens. Außerdem trifft diese spezielle Modifikation häufig mit Azetylierung von Lysin9 oder 14 desselben Histon H3 Moleküls zusammen, weswegen diese Doppel-Modifikation auch oft als „Phosphoazetylierung“ bezeichnet wird.

Ziel dieser Arbeit war es, die Rolle der Phosphorylierung/ Phosphoazetylierung von Histon H3 während der Transkription zu untersuchen und zu prüfen, ob mehrere unterschiedliche Modifikationen in diesem Prozess zusammenwirken.

14-3-3 Proteine wurden als „Detektor Proteine“ für phosphoryliertes Histon H3 identifiziert. Interaktionsmessungen *in vitro* zeigten, dass die schwache Interaktion zwischen 14-3-3 und Serin10 phosphoryliertem Histon H3 durch kombinatorische Modifikationsmuster moduliert wird: Serin10 Phosphorylierung ist für eine signifikante Interaktion notwendig, aber zusätzliche Azetylierung von Lysin9 oder Lysin14 erhöht die Affinität von 14-3-3 für Histon H3. Lysin9 wird neben Azetylierung auch in transkriptionell unterdrücktem Chromatin methyliert. Massenspektroskopische Studien zeigen, dass Phosphoacetylierung mit Lysine9 Methylierung *in vivo* zusammentrifft. Diese dreifach modifizierte Spezies wird *in vitro* effizient durch 14-3-3 gebunden. Dies lässt den Schluß zu, dass Histone Phosphorylierung und Azetylierung kooperieren, um temporär die reprimierende Lysine9 Methylierung „auszublenden“, während die epigenetische Information dieser Modifikation erhalten bleibt. Chromatinimmunopräzipitations-Experimente zeigen, dass die Lokalisierung von 14-3-3 Proteinen am HDAC1 Promoter abhängig von Histone H3 Phosphorylierung ist. Diese Rekrutierung wird durch zusätzliche H3 Azetylierung verstärkt und korreliert mit der Dissoziation des transkriptionellen Repressors HP1γ. siRNA vermittelte Abreicherung von 14-3-3 Proteinen unterbindet die transkriptionelle Induktion von HDAC1, was darauf hindeutet, dass 14-3-3 Proteine das Phosphoazetylierungs-Signal an die Transkriptions-Maschinerie weiterleiten. Zusammenfassend zeigen die präsentierten Daten, dass 14-3-3 Proteine zentrale Mediatoren des H3 Phosphoazetylierungs-Signals während der transkriptionellen Induktion sind.

Content

1. Introduction

1.1 Eukaryotic chromatin

The enormous length and complexity of eukaryotic genomes confronts the cell with several obstacles. Genomic DNA must be assembled into the nuclear sub-cellular compartment and despite over then thousand fold compaction, genetic information has to be readily accessible. A further difficulty arose with the evolution of multi-cellular organisms. Multicellularity requires functional specialization of individual cells and therefore differential gene expression. The cell meets these requirements by organizing genomes into a compact but dynamic nucleoprotein complex called chromatin.

The addition of a protein component to the genomic DNA allows for efficient genomic length reduction and regulation of access to the underlying genetic information. The structural traits of the chromatin polymer are locally restricted and characteristic for a particular function or gene status. The extent of such spatial restrictions can vary considerably depending if special structures like centromeres or telomeres or actively transcribed regions are assembled. The plethora of factors that regulate chromatin structure and function includes DNA sequence and modifications, RNAs, DNA binding proteins and histones and their post-translational modifications (Gelato and Fischle, 2008).

1.2 Structural organization of chromatin and sub-components

The basic repeating unit of chromatin is the nucleosome, which consists of 146 base pairs (bps) of DNA wrapped around an octameric protein complex composed of four different histone proteins (Luger et al., 1997). The nucleosome contains two molecules of histones H2A, H2B, H3 and H4. These histone proteins are assembled into a tripartite structural histone octamer containing a central $(H3/H4)_2$ tetramer and two H2A/H2B dimers (Figure 1-1). Coiling of DNA around the histone octamer may comprise an energetically unfavorable condition that is compensated by the formation of a multitude of direct or water mediated histone DNA interactions (Luger and Richmond, 1998a; Langst and Becker, 2004). The multitude of these DNA-histone interactions renders the nucleosome particle a relatively stable structural entity. Alternating nucleosomes form a “beads on a string” like structure with an average of 54bp of intervening DNA (Butler and Thomas, 1980; Felsenfeld and McGhee, 1986). This low compaction state of the chromatin polymer is also referred to as the 10nm fiber.

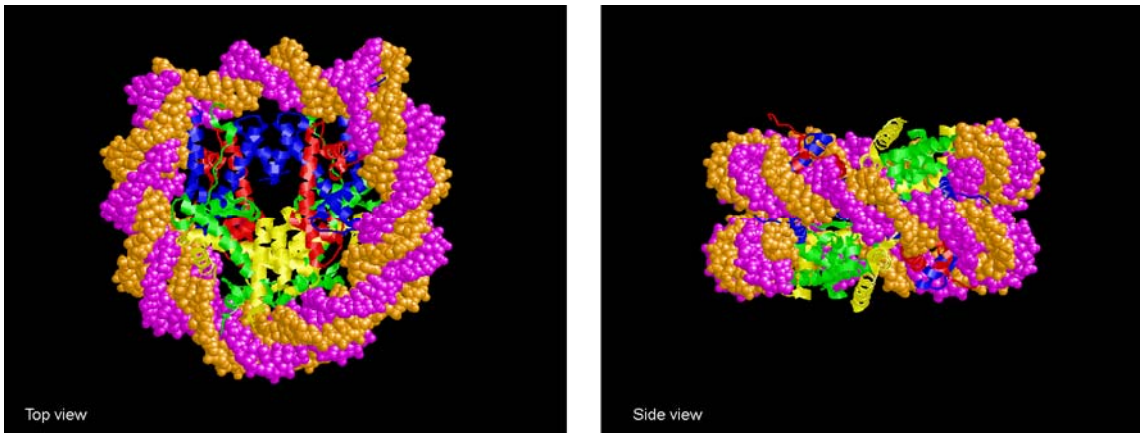

Figure 1-1 Structure of the nucleosomal core. Pictures were rendered using RasMol software on the PDB data file 1KX3. Both left-handed DNA strands are depicted in "spacefill" view and are colored orange and magenta respectively. Histone molecules are depicted as secondary structure cartoons. The following color code was used for this graphic: Histone H3: blue, Histone H4 red, Histone H2A: green and Histone H2B: yellow. The left panel shows a top view on the nucleosomal core. The right panel shows a 90° horizontal flip of the left panel designated as side view. The 146bp of DNA are wrapped 1.7 times around the nucleosomes forming the 10nm diameter nucleosomal core.

Besides the core histones, linker histones such as H1 were identified in metazoans. Binding of this histone species to nucleosomal arrays causes compaction of the linear "beads on a string" structure to a "30nm fiber" conformation (Allan *et al.*, 1980; Thomas and Butler, 1980; Allan *et al.*, 1981; Felsenfeld and McGhee, 1986; Graziano *et al.*, 1996; Ramakrishnan, 1997; Wedemann and Langowski, 2002). The folding of chromatin into the higher order "30nm fiber" structure is supported by X-ray crystallography and electron microscopy studies but has not yet been directly confirmed *in vivo* (Dorigo *et al.*, 2004; Schalch *et al.*, 2005; Robinson *et al.*, 2006).

Positioning of nucleosomes relative to the underlying DNA is influenced by nucleotide sequence preferences (Travers and Klug, 1987) and the read out of DNA-sequence features by chromatin-remodeling complexes (Rippe *et al.*, 2007). The rotational position is mainly determined by the preference of the minor groove DNA to contact the nucleosomes via A-T rich motifs and translational positioning is dependent on local DNA bendability (Rhodes and Klug, 1980; Pryciak and Varmus, 1992). Furthermore, the association with the octamer confers an unusual curvature to the DNA-base pair geometry which has important implications in the sequence-dependent protein recognition, nucleosomes positioning and mobility (Richmond and Davey, 2003). Genome wide mapping approaches in S*accharomyces Cerevisiae* and *Drosophila melanogaster* revealed that there are also differences in nucleosome positioning between major eukaryotic lines with respect to the transcriptional start site (TSS) and histone variant incorporation (Albert et al., 2007; Mavrich et al., 2008).

Histones constitute the fundamental protein units of the nucleosomal octamer (Figure 1-1). Structurally eukaryotic histones are bipartite proteins, composed of a carboxy-terminal globular histone fold domain and less structured amino-terminal tails. The histone fold domains are essential for the structural integrity of the nucleosome. These structural components organize the nucleosomal DNA and mediate intranucleosomal and secondary internucleosomal interactions (Travers and Klug, 1987; Luger *et al.*, 1997; Luger and Richmond, 1998a; Richmond and Davey, 2003; Hendzel *et al.*, 2004; Luger, 2006) (Figure 1-1). The amino-terminal tails are thought to be mainly unstructured (Luger and Richmond, 1998b) although computational prediction of the solution structure of the histone H3 tail suggested that several stretches are prone to adopt an α-helix conformation (Eberlin *et al.*, 2008; Liu and Duan, 2008) (Figure 1-2). Importantly such secondary structures would have important implications on the interaction of histone tails with modification dependent binding proteins (see sections 3.3 Presented Publication 2. “Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns”).

1.3 Chromatin assembly

The organization of DNA into the chromatin polymer poses some obstacles for several DNA based procedures like DNA replication, transcription or DNA damage repair.

Assembly of chromatin occurs in two distinct modes. During DNA synthesis, the newly synthesized DNA is packaged into chromatin at the replication fork in a mechanism depending on chromatin assembly factors, remodeling complexes and histone chaperones (Groth et al., 2007). *De novo* Histone deposition occurs in a well-defined order with the initial deposition of two histone H3/H4 dimers to form the H3/H4 tetramer and subsequent addition of two histone H2A/H2B dimers (Wolffe and Schild, 1991; Groth *et al.*, 2007). The majority of histone proteins are exclusively expressed in S-phase of the cell cycle to provide the material for replication coupled (RC) chromatin assembly. One major demand during DNA replication is the propagation of local epigenetic states to the daughter strands. The parental nucleosomes are disassembled ahead of the replication fork into H2A/H2B dimers and H3/H4 tetramers. The tetrameric subunits are transferred to the daughter strands behind the replication fork in a random manner that, together with *de novo* nucleosomes assembly maintains local patterns of histone variants and post translational modifications (Groth et al., 2007). During transcription, nucleosomes are assembled in a replication independent (RI) manner that involves the “histone regulator A” (HIRA) complex and the histone variant H3.3 (Ahmad and Henikoff, 2002b; Ahmad and Henikoff, 2002a; Henikoff *et al.*, 2004a; Henikoff *et al.*, 2004b) (see section 1.5.1 Histone H3 variants).

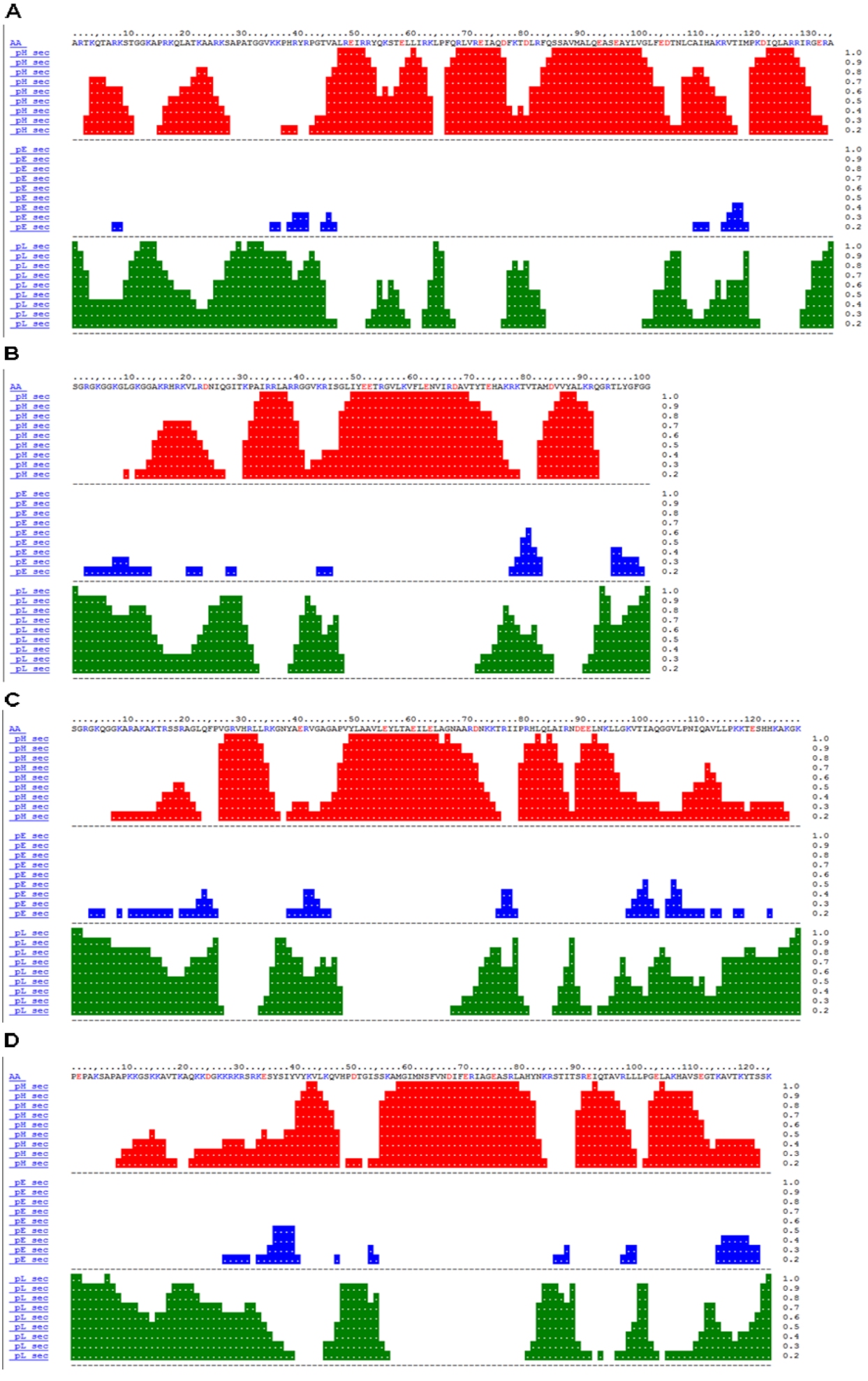
A
B
C
D
AA
pH sec
pE sec
pL sec

Figure 1-2 Prediction of secondary structure elements in the four core histone species. The probability (1= high, 0= low) for assigning α-helical conformation (pH sec, red squares), strand conformation (pE sec, blue squares) or neither helix nor strand conformation (pL sec, green squares) was calculated using the algorithm provided at the predictprotein server (www.predictprotein.org) (Rost et al., 2004). Predictions were run on the amino-acid sequences for human histone H3.2 (A), histone H4 (B), histone H2A (C) and histone H2B (D). Several stretches of the amino–terminal tails could potentially form α-helices, which would significantly impact the accessibility to additional factors.

1.4 Hallmarks of large scale chromatin elements

Within a eukaryotic cell the overall arrangement of chromatin is not uniform but ordered into particular structures and superstructures. These spatial (and sometimes even temporal) restricted structural elements are distinguishable by characteristic hallmarks like incorporation of non-canonical histone variants, particular histone post-translational modifications, chromatin compaction status and locally restricted non-histone protein components.

The simplest (and not always valid) classification of large scale chromatin elements discriminates two general components, euchromatin and heterochromatin. The first one is characterized by low condensation states and enrichment for actively transcribed genes. Also particular histone post-translational modifications like lysine acetylation and histone variants are associated with this “active” state of chromatin (reviewed in (Gelato and Fischle, 2008) see also 1.5 Histone variants, 1.6 Histone Post-translational modifications (excerpt from (Winter et al., 2007), 1.7 Impact of post translational modifications on chromatin states.). In contrast, heterochromatic structures comprise highly condensed and transcriptional inert genomic regions, associated with a different set of modifications and histone variants. As further hallmark it was found that heterochromatic structures replicate later during S-phase than euchromatic regions. Heterochromatin is further classified as facultative or constitutive. Transcriptional repression in facultative heterochromatin is dependent on proper signals like cell cycle stage, extracellular stimulation or developmental stage. One prominent example for facultative (large scale) heterochromatin formation is the X-inactivation in female mammals required for dosage compensation. The resulting Barr body is highly enriched in heterochromatic structures and transcriptionally down-regulated compared to the active X-chromosome. DNA transcription within constitutive heterochromatin is permanently silenced (or strongly reduced) and frequently contains highly repetitive DNA elements (compare also 1.9 RNA as chromatin component).

In addition, specialized structural elements like centromeres and telomeres are formed, which are required to organize chromosomes within the nucleus and to ensure proper replication and distribution during cell cycle progression. These structures are also characterized by the presence of particular histone variants such as the histone H3 variant

"centromeric protein A" (CENP-A) (Hake and Allis, 2006; Pandita *et al.*, 2007; Gelato and Fischle, 2008).

In addition, regulation of expression of genes sharing common factors may occur at the level of chromatin elements. Insulators were found to constitute important elements in the local restriction of euchromatin or heterochromatin. These are DNA elements defined by their ability to impact spreading of chromatin states (Dorman *et al.*, 2007). Based on their activity these insulators are classified as enhancer-blocking or barrier insulators (also referred to as boundary elements). Enhancer-blocking insulators inhibit stimulation of a promoter by an enhancer when it is positioned between them. Barrier insulators block the spreading of heterochromatin, thereby suppressing position effect variegation (PEV). Importantly some insulators combine the activities of both of these elements.

Although overlaps in the molecular mechanisms used by both elements exist, it is thought that the exact mode action is different between the two types of insulators. Barrier insulators are thought to target chromatin structure on the level of post-translational modifications. This has been demonstrated for the HMR mating type locus barrier insulator, which contains the $tRNA^{Thr}$ gene in *Saccharomyces cerevisiae*. The activity of the insulator requires the Sas2p and the Gcn5p acetyltransferases along with RNA polymerase III mediated transcription of the $tRNA^{Thr}$ gene (Donze *et al.*, 1999; Donze and Kamakaka, 2001). Spreading of telomeric heterochromatin in yeasts is restricted via anti-silencing regions and recruitment of the transcription factors Reb1p and Tbf1p to these sites. Also the methylation of histone H3K4 has been demonstrated to restrict heterochromatic spreading from telomeres (Nishioka *et al.*, 2002; Zegerman *et al.*, 2002; Santos-Rosa *et al.*, 2004). In vertebrates the 5'HS4 element in the β-globin locus unites the activities of enhancer-blocking and barrier insulators. The respective activities are associated with binding of different factors to this element as CTCF is required for the enhancer-blocking function and the binding sites for USF1 and USF2 for barrier activity (reviewed in (Dorman *et al.*, 2007)).The latter proteins were demonstrated to interact with the histone H3K4 specific methyltransferase Set7/9 and the histone acetyltransferase PCAF. Knock down of USF1 results in reduced H3K4me3 and histone acetylation but increased H3K9me3 at the 5'HS4 element (West *et al.*, 2004). Also nucleosome positioning has been implicated in barrier insulator activity (Dorman *et al.*, 2007).

In addition, the activity of barrier insulators has been linked to structural compartmentalization of chromatin. However, it is well established that enhancer-blocking insulators organize chromatin into structural loops with insulator proteins shaping the loop-base. The loop base can either form clusters and/or be tethered to different nuclear structures like nuclear pores, nuclear lamina or the nucleolus (Labrador and Corces, 2002). Clustering of insulators leads to the formation of insulator bodies, which may also be

dependent on BTB/POZ domain containing proteins as has been suggested based on studies on the *Drosophila* Su(Hw) insulator complex (Gause *et al.*, 2001; Ghosh *et al.*, 2001; Pai *et al.*, 2004; Ramos *et al.*, 2006). In vertebrates tethering of insulator elements to nuclear components has been reported for the CTCF factor. CTCF binds to almost all known enhancer-blocking elements in vertebrates (West *et al.*, 2004) and was found to interact with nucleophosmin, a prominent component of the nucleolus thereby suggesting that nucleolar localization may play a role in insulation.

Tethering of a chromatin loop appears to block the propagation of chromatin states originating either from heterochromatin or an enhancer element. Therefore loop formation comprises an important element in enhancer-blocking as well as barrier insulator function. Interestingly, screens for insulator proteins in yeast identified many proteins related to nuclear transport (Ishii *et al.*, 2002). It is not yet fully clarified how loop formation would restrict spreading of chromatin states; however, the clustering and loop formation appears to be a common mechanism for enhancer-blocking and barrier-insulators in all eukaryotes. One mechanism may include the formation of physical barriers that block the propagation. Also the sequestration of chromatin loops to nuclear regions corresponding to particular transcription states (like RNA polymerase II transcription machineries) has been suggested (Dorman *et al.*, 2007). According to this model the transcription of boundary elements would allow to discriminate from enhancer-blocking insulator organized loops. In addition chromatin loops could facilitate the physical contact between enhancer elements and promoters. In this way insulator bodies would organize the genome in the nuclear compartment according to differentiation states of the cell (Dorman *et al.*, 2007). This would also imply that different cell types would maintain different insulator body mediated nuclear organization.

1.5 Histone variants

In addition to the canonical forms, specialized non-allelic histone variants have evolved. Incorporation of specialized histone isoforms confers particular properties to the chromatin polymer corresponding to distinct epigenetic and transcription states (Table 1).(Hake and Allis, 2006) The canonical histone variants are only expressed and incorporated during S-phase (replication coupled incorporation RC), while specialized replacement variants are synthesized and incorporated throughout the cell cycle (replication independent incorporation, RI). Only two replacement histone variants are expressed in all eukaryotic cells: a centromere specific isoform of histone H3 and a variant of histone H2A designated H2A.Z (Htz 1 in yeast).

1.5.1 Histone H3 variants

Besides a centromere specific form of histone H3, centromeric protein A (CenpA in mammals, CID in *Drosophila* and Cse4 in *Saccharomyces cerevisiae*), in mammals three additional isoforms of histone H3 are known. Two of these variants, designated H3.1 and H3.2 are the major forms and synthesized and incorporated only during S-phase and assembled into chromatin in a RC manner. The H3.2 variant appears to be specific for higher eukaryotes and is found in plants, frogs, flies and birds but absent in yeasts. Histone H3.1 is only found in mammals and absent in other eukaryotes. RC histone deposition occurs via a replication specific complex that involves the chromatin assembly factor 1 (CAF 1). The third isoform, H3.3 is synthesized throughout the cell cycle and incorporated in a RI manner. Besides variants in amino acid sequence, the non-allelic H3.3 variant differs from its S-phase specific counterparts as the coding sequences contain introns and encode poly-adenylated mRNAs (Thatcher *et al.*, 1994). Analysis of codon usage and intron positioning in H3 genes in vertebrates led to the hypothesis that the H3.3 variant is the ancestral form (Wells et al., 1986). Indeed there is no known genome encoding only H3 and no H3.3 (Malik and Henikoff, 2003). In this way RC variants would have evolved via loss of intronic DNA and polyadenylation signals early in evolution. The fact that other species such as *Drosophila* contain an H3.3 variant identical to that of vertebrates further supports this scenario. However, sequence analysis of histone H3 genes from several different species rather indicates multiple origins of replication independent H3 replacement variants (Thatcher *et al.*, 1994; Malik and Henikoff, 2003). The “invention” of multiple H3.3s during species separation would imply that organisms require a histone H3 variant which is expressed independent of DNA replication. Similar changes in key amino acids in *Tetrahymena* and animal histone H3.3 variants together with a strong selection for protein length suggest that also unique structural features are important for the RI variant (Thatcher *et al.*, 1994). Although these changes affect homologous amino acid position they are not convergent and different substitutions are found in different species (Thatcher *et al.*, 1994). In *Tetrahymena termophila* the constitutive expression of the replacement variant hv2 (H3.3) seems to be more important than a particular primary sequence (Yu and Gorovsky, 1997). However, deposition of *Drosophila* H3.3 *via* the histone regulator A (HIRA) complex is dependent on critical amino acid changes in the histone fold domain (HFD) (together with its constitutive expression) (Ahmad and Henikoff, 2002b). In *Ascomycetes* only one histone H3 gene is retained corresponding to the vertebrate H3.3 variant. Since there is little doubt regarding multiple origins of H3.3, it appears that a replacement variant was present in an ancestral small and transcriptionally active genome. The fast expansion of eukaryotic genomes, together with the evolution of multicellularity, which requires transcriptional silencing of large genomic portions, may have favored the acquirement of canonical H3 variants (Malik and

Henikoff, 2003). Besides these three histone H3 variants, a testis specific form of histone H3 has been found in mammals, demonstrating that also tissue specific histone variants exist.

As mentioned, the three histone H3 variants, H3.1, H3.2 and H3.3 differ in only a few amino acid positions, which, besides the temporal expression differences, appear to be already sufficient to confer the differences in spatial incorporation (Figure 1-3). The H3.3 variant differs from H3.1 and H3.2 in the globular domain, at position 87, 89 and 90. These three amino acid changes were found to be crucial to mediate the RI-incorporation via the HIRA complex (Ahmad and Henikoff, 2002b). Another difference between H3.3 and the two RC H3 variants concerns the amino-terminal portion of the molecule at position 31. H3.1 and H3.2 contain an alanine at this site whereas H3.3 contains a serine, which becomes phosphorylated during prometaphase and metaphase near to centromeric regions (Hake et al., 2005). Another difference is found at position 96. Histones H3.2 and H3.3 contain a serine at this site, whereas H3.1 contains a cysteine. The only cysteine common for all three histone H3 variants is located at position 110 (Figure 1-3). This particular position was found to be inaccessible in transcriptionally silent chromatin (Allfrey et al., 1964). It was speculated that cysteine 110 is involved in the formation of intramolecular disulfide bond, corresponding to a reversible transcriptional repressed state. The histone H3.1 specific cysteine 96 was proposed to form intermolecular disulfide bonds to neighboring nucleosomes or components of the nuclear envelope, like the lamin B receptor (Hake and Allis, 2006).

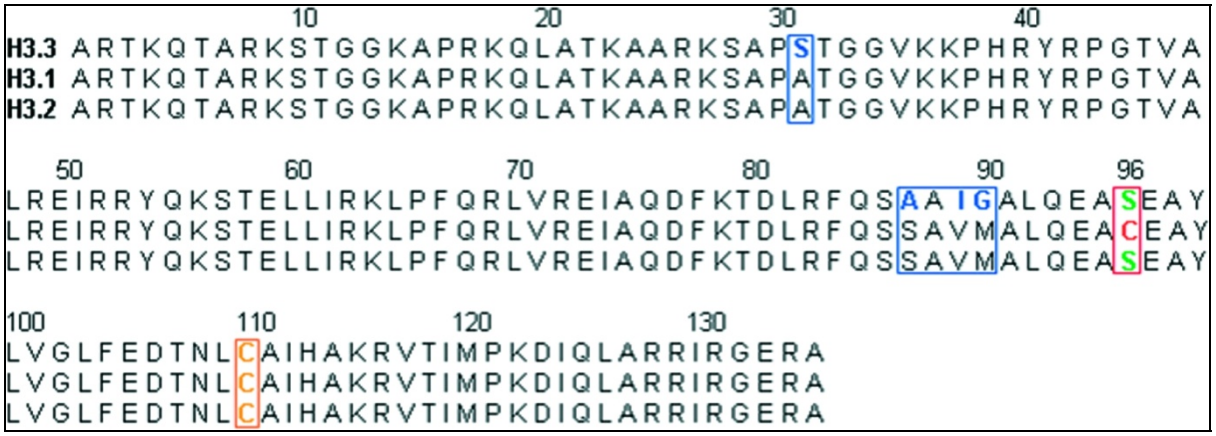

Figure 1-3 Sequence alignment of histone H3 variants (taken from (Winter *et al.*, 2007)). The three canonical histone H3 variants, H3.1, H3.2 and H3.3 differ only in a few but critical amino acids. H3.3 contains a serine at position 31, which is phosphorylated during mitosis. Three alterations at position 87, 89 and 90 were found to be sufficient for replication independent incorporation of H3.3 via the HIRA complex (Ahmad and Henikoff, 2002b). All three histone variants possess a cysteine at position 110, which is thought to form internucleosomal disulfide bonds. H3.1 contains a unique cysteine at position 96, which constitutes the sole difference between H3.1 and H3.2. This residue may be involved in the formation of intramolecular disulfide bonds.

Such intermolecular “cross-linking” via disulfide bonding would generate long-term (constitutive) transcriptional repressive heterochromatin (Hake and Allis, 2006). Up to now it is not clear why the cell distinguishes between replication specific and replication independent histone variants. The few amino acid differences between the three different histone H3 variants are not very likely to severely change the overall structure of the nucleosome although they were found essential for RI deposition (Ahmad and Henikoff, 2002b).

Some reports however indicate a clear difference in the biochemical properties of nucleosomes containing different histone H3 variants. Recent observations suggest that the overall dynamics in histone mobilization and nucleosome stability are dependent on the particular histone H3 variant incorporated. In this context the continuous activity of chromatin remodeling machineries (see below) act on the transcription related H3.3 containing nucleosomes. Probably as a consequence of this remodeling activity it was found that H3.3 containing nucleosomes tend to loose or exchange their H2A/H2B dimers more easily than H3.1 containing nucleosomes (Steven Henikoff, unpublished data, Keystone Symposium on molecular basis for epigenetic phenomena April, 2008).

Another difference concerns the distribution of post-translational modifications (PTMs) on different histone H3 variants (see also section 1.6 Histone Post-translational modifications (excerpt from (Winter et al., 2007)). In general, histone H3.3 is enriched for PTMs associated with transcriptionally active chromatin like lysine 4 methylation, whereas H3.2 is mainly decorated with modifications corresponding to facultative heterochromatin and H3.1 contains activating plus silencing modifications, although the latter are different from the modifications mapped for histone H3.2 (McKittrick *et al.*, 2004; Hake *et al.*, 2006; Loyola *et al.*, 2006). It was suggested that the basis for such isoform specific modification patterns is provided by different nucleosome assembly pathways using either histone H3.3 at actively transcribed genes and histones H3.1 and H3.2 at the replication fork (McKittrick et al., 2004). Up to now it is not clear whether separate assembly pathways exist for the replication coupled histone H3.1 and H3.2 isoforms.

1.5.2 Histone H2A variants

The histone H2A family represents the most heteromorphous group of histone variants. In contrast to the histone H3 variants, which differ in only a few amino acid positions (Figure 1-3), the H2A variants differ considerably with regard to whole protein domains (Table 1).

One particular variant, histone H2A.Z was strongly correlated with transcriptional regulation in several different organisms. Histone H2A.Z (Htz1 in yeast, H2Av in *Drosophila*) shows only about 60% sequence identity with canonical (replication coupled) histone H2A. However, this particular histone variant is highly conserved throughout evolution, even more than the canonical histone H2A (Thatcher and Gorovsky, 1994).

Biophysical and biochemical studies indicated that H2A.Z incorporation confers some altered properties to nucleosomes and higher order chromatin structures. Incorporation of H2A.Z into nucleosomal arrays facilitates intramolecular folding but inhibits the generation of highly condensed structures (Fan et al., 2002). Also the incorporation of heterochromatic components like HP1α was found more efficient for H2A.Z containing arrays, which may have implications in the formation of constitutive heterochromatin (Fan et al., 2004).

Deposition of this particular isoform has been correlated with actively transcribed genes (Santisteban *et al.*, 2000; Adam *et al.*, 2001; Bruce *et al.*, 2005). About 214 genes were found to be down-regulated in yeast *Htz1* mutants whereas 109 genes were up regulated (Meneghini et al., 2003). Interestingly, it was found that down-regulated genes cluster near to telomeres and are organized in H2A.Z activated domains (HZADs). It was suggested that transcriptional activation of these genes might be maintained *via* an anti-silencing effect of H2A.Z by restriction of telomeric heterochromatin spreading. Deletion of the silent information regulator 2 (SIR2), an important factor in the formation of telomeric heterochromatin, can suppress the down regulation of genes in Htz1 mutants (Meneghini et al., 2003). In this way, H2A.Z deposition functions as “barrier” to the spreading of telomeric heterochromatin, which appears to be also dependent on H4K16 acetylation.

So far all genome wide mapping approaches performed to determine the distribution of H2A.Z within the genome concluded that this variant is in particular enriched at gene promoters (Guillemette *et al.*, 2005; Li *et al.*, 2005; Raisner *et al.*, 2005; Zhang *et al.*, 2005; Millar *et al.*, 2006). Although this particular distribution of H2A.Z at the 5’ end of genes appears to account mainly for transcriptional inactive promoters and actively transcribed genes are in general slightly depleted in H2A.Z (Guillemette *et al.*, 2005; Li *et al.*, 2005; Zhang *et al.*, 2005; Millar *et al.*, 2006), it is believed that this particular variant may be important to “poise” genes for transcription. Comparison of genome wide mapping data of histone H2A.Z distribution and nucleosome positioning demonstrated that H2A.Z containing promoters have a highly positioned nucleosomes and a well defined nucleosome free region (Guillemette et al., 2005). This nucleosome positioning may in part be dependent on H2A.Z incorporation and contribute to the transcription “poising” effect of H2A.Z (Guillemette et al., 2005) by maintaining an environment accessible for transcription factors .

In addition, H2A.Z may lower the stability of the nucleosome either indirect or via specific remodeling factors (Zhang et al., 2005) which may facilitate the initial steps of transcriptional activation (reviewed in reference (Guillemette and Gaudreau, 2006)). In addition it was found that acetylation of H2A.Z lowers the stability of the interaction with the nucleosome and post-translational modifications may therefore contribute to the roles of H2A.Z in transcriptional regulation (Thambirajah et al., 2006).

Incorporation of histone H2A.Z is mediated by the SWR1 complex that can specifically exchange canonical H2A by the histone H2A.Z variant in nucleosomes (Krogan *et al.*, 2003b; Kobor *et al.*, 2004; Mizuguchi *et al.*, 2004). The SWR1 complex is named after its catalytic domain containing an ATPase/Helicase domain homologous to Swi2/Snf2 and contains 12 or 13 proteins including histone H2A.Z (Mizuguchi et al., 2004). In yeast the SWR1 complex shares several components with the histone acetyl transferase (HAT) complex NuA4 (Kobor et al., 2004; Krogan et al., 2004) and in higher eukaryotes both appear to be assembled in the Tip60 complex (Doyon and Cote, 2004). In human cells two complexes have been identified that may mediate the exchange of histone H2A.Z. The Tip60 complex contains the SWR1 homologue p400 and Esa1, a homologue of the Tip60 HAT. In addition, the SWI2/SNF2 related CBP activator protein (SRCAP) can incorporate histone H2A.Z/H2B dimers into chromatin *in vitro* (Ruhl et al., 2006).

The targeting of histone H2A.Z to promoter regions is not yet fully clarified. Histone acetylation has been hypothesized as a potential targeting mechanism as one component of the yeast SWR1 complex, the Bdf1 bromodomain containing protein, is required for H2A.Z localization to gene promoters (Raisner et al., 2005; Zhang et al., 2005). In addition, SWR1 may be recruited by transcription factors as demonstrated for the Reb1 binding sequence in the yeast SNT1 gene (Raisner et al., 2005).

Besides, an implication in gene transcription, histone H2A.Z incorporation has also been linked to the formation of heterochromatic structures. During mouse development H2A.Z becomes enriched at the pericentromeric heterochromatin and constitutes an important factor for the recruitment of HP1α and proper chromosome condensation (Rangasamy et al., 2004). And loss of Htz1 in yeast causes loss of subtelomeric silencing and chromosome instability (Krogan et al., 2004).

Histone	variant	Localization	Features	Functions
H3	H3.1	constitutive heterochromatin?	C96	RC deposition
	H3.2	facultative heterochromatin?	S96	RC deposition
	H3.3	transcribed region	S31, G90, I89, A87, S96	RI deposition
H2A	macro-H2A	inactive X-chromosome	C-terminal macro-domain essential for most of its functions	Represses transcriptional initiation. interferes with histone acetylation by p300 blocks sliding by ACF and remodeling by Swi/Snf inhibits transcription factor binding (NFkB).
	H2A.Z	promoter heterochromatin boundary	Loop1 different from canonical H2A	Facilitates TBP binding prevents elongation associated modification and remodeling at promoter
	H2A.Bbd	active X chromosome and autosomes	lack of C-terminal parts, nucleosomes organize only 118-130bp of DNA	confers lower stability to the nucleosome
	H2A.X	generally distributed	A conserved C-term SQ(E/D) motif that becomes phosphorylated upon DNA damage.	Phosphorylated during DNA damage, required for recruitment of DNA repair machinery

Table 1 Histone H3 and Histone H2A variants (adapted from (Li et al., 2007))

1.6.0 Preface to chapter 1.6 Histone post-translational modifications

Post-translational modifications (PTM) of histones and other proteins are recognized as important regulators of protein function and stability, protein-protein interactions or subcellular localization (Yang, 2005). Histone proteins are subjected to a multitude of different PTMs including phosphorylation, acetylation, methylation, ADP-ribosylation, ubiquitinylation or SUMOylation with differential impacts on transcriptional regulation.

The temporal overlap of various different PTMs on histone amino-terminal tails discloses the possibility of combinatorial modification effects. This idea has been termed the “histone code hypothesis” (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Several experimental data demonstrate indeed combinatorial effects for several overlapping histone modifications however, the evidence for a universal “histone code” is apparently lacking.

Per definition a code constitutes an ”unvarying rule for replacing a piece of information (such as a letter, word, or phrase) with an arbitrarily selected equivalent (Encyclopedia Britannica) or in other words, an invariant system for converting information into another form. During the process of encoding the original information is converted (code symbols), according to the rules of the code. This encoded information can then be reconverted during the process of decoding into the original information, which is then accessible to the receiver.

One well established example for a code-system in organisms is comprised by the genetic code. The encoded information is contained within the sequence of nucleobase triplets (codons) of genetic DNA, which are finally translated (decoded) into the particular amino acids. Importantly, the genetic code is redundant and several base triplets can encode the same amino acid. Although the genetic code is in general universal it is important to mention that there are differences in codon usage between different organisms. In this case the components of the “code-system” are easy to assign: the base-sequence of the DNA contains the encrypted information in the form of codon triplets. The reading/receiving units are the RNA polymerase machinery together with the translation apparatus that decodes the information, which is essentially the amino acid composition of a particular protein.

In terms of the histone code hypothesis, the PTMs are the code symbols that epitomize the “encoded” information, such as transcription state of the decorated chromatin. The process of “encoding” would then be mediated via the enzymatic machineries that place or remove particular modifications and the encoding machinery is comprised by modification dependent interaction proteins (detectors, effectors) that “translate“ modifications thereby mediating a biological effect. In this context it would be justified to use the term “code”. However, in the case of histone modifications, it appears that the effect of a particular modification is not universal but rather context dependent. (This will be discussed in more detail in the 1.7 Impact of post translational modifications on chromatin states.

The assertions stated above should point to a certain problem with the term “histone code”. Particular modifications or patterns of modifications must mediate a particular effect to justify the term code, however for several modifications it appears that their “readout” is rather context dependent and therefore hardly compatible with the picture of universal histone code (Shiv Grewal, Keystone Symposium on molecular basis for epigenetic phenomena 2008).
Also for some histone modifications it is not fully understood if their presence at a particular genomic region is rather cause or consequence of a particular chromatin based events.
One further assumption of the histone code proposes the interdependency of histone PTMs. In contrast to the picture of a universal histone code, there is good evidence for “cross talk” between histone modifications (Fischle *et al.*, 2003a; Fischle *et al.*, 2003b; Nightingale *et al.*, 2006b; Winter *et al.*, 2007). The following section focuses in particular on examples of interdependent histone modifications as this is one major background for the project of the thesis.

1.6 Histone post-translational modifications (excerpt from (Winter Stefan *et al.*, 2007)

The cellular response to environmental cues such as cellular stress, change of metabolic conditions, or differentiation signals is mediated via the activation of signal transduction cascades (Downward, 2001). Stimulation of such pathways entails an alteration and adaptation of the gene expression profile to equip the cell for a proper response to novel and sometimes menacing demands. During the last decades numerous cellular signal transduction pathways and molecules involved have been identified and characterized, expanding our knowledge on how the cell recognizes signals and relays them. To access the biological information embedded in the genome, genes have to be transcribed in the context of a debilitating chromatin environment and signal transduction frequently affects the chromatin level.
Therefore, to initiate and conduct transcription the chromatin polymer is subjected to dynamic structural alterations that finally confer the formation of a transcriptional competent environment. Different mechanisms like the post-translational modification (PTM) of histones, chromatin remodeling via ATP-dependent machineries and the incorporation of particular histone-variants lead to a preparation and adaptation of the chromatin environment to transcription.

1.6.1 Histone post-translational modifications and modification cross-talk

The first observations that acetylation and methylation of histone molecules impact RNA synthesis emphasized the role of these molecules and their PTMs in the process of transcription (Allfrey et al., 1964). With the establishment of histone acetyltransferases (HATs) as transcriptional co-activators (Brownell et al., 1996; Mizzen et al., 1996) and histone deacetylases (HDAC) as co-repressors (Taunton et al., 1996), post-translational

histone modifications were ultimately stated as a pivotal point in the modulation of genome accessibility. The repertoire of known histone PTMs has since been considerably expanded and modifications like lysine or arginine methylation, lysine acetylation, ADP-ribosylation and ubiquitination or serine and threonine phosphorylation have been correlated with transcriptional active or repressive chromatin states. The plethora of possible histone modification patterns epitomizes the enormous potential information content that may expand the genetic information and defines local and global chromatin states via an additional level termed the histone code (see also section 1.6.0 Preface to chapter 1.6 Histone Post-translational modifications) (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

1.6.2 Epigenetic crosstalk: stable versus dynamic modifications

The term epigenetic defines heritable changes in gene expression that do not involve changes of the DNA sequence. Such epigenetic traits are established during differentiation and comprehend DNA methylation, RNA-induced silencing and histone PTMs. These marks are essential for defining and maintaining cellular identity (Turner, 2002; Egger *et al.*, 2004), which is also emphasized by the observation that tumor cells display far-reaching epigenetic alterations during transformation (Fraga and Esteller, 2005).

Histone PTMs are now recognized as important mediators of epigenetic traits that are crucial for defining specialized chromatin states like constitutive or facultative heterochromatin and the establishment and maintenance of cell-type specific gene expression profiles. There is accumulating evidence that different histone PTMs occur as interdependent events and frequently one initial modification may stipulate the context for subsequent events. The basis for such modification based crosstalk may originate either (1) from the assembly of multi-protein complexes that amalgamate several distinct chromatin modifying activities, (2) by supporting or hampering of protein-protein interactions or (3) via a direct influence of combinatorial PTMs on enzymatic activities. Therefore, the modification “make-up” of a histone molecule prior to a particular stimulus may provide the context for consecutive modification events. In addition, stimulus induced PTMs may provide the basis for subsequent “delayed”-modifications and may themselves be biased by local pre-modifications. Such PTM directed intramolecular signaling is not limited to histone modifications but rather appears as an important mechanism in the regulation of various cellular processes (Yang, 2005). These considerations are even meaningful for the definition of epigenetic marks: modifications with high turnover rates such as lysine acetylation are often considered as irrelevant for epigenetic mechanisms due to their dynamic turnover and ephemerality. Given the interdependency and sequential placement of PTMs, even rapidly processed modifications could epitomize a particular epigenetic status possibly dependent on other more stable marks, but with reciprocal connection. Since lysine acetylation is in general associated with transcriptional active regions, the local under-representation of this

modification (hypoacetylation) could be interpreted as transcriptional silent hallmark, constituting an epigenetic state dependent on a previously established repressive environment via more stable histone modifications. Such repressive heterochromatic states are established during differentiation via relatively stable marks such as CpG island methylation and methylation of histone H3 at K9 and K27 in conjunction with heterochromatin protein1 (HP1) or Polycomb-group proteins (PcG), respectively (Lachner et al., 2003). Nevertheless, the view of K9 methylation and HP1 recruitment as a sole repressive hallmark has been questioned by reports on the localization of both factors to transcribed regions and reports on a H3K9me3 independent recruitment of HP1beta (Vakoc *et al.*, 2005; Dialynas *et al.*, 2006).

In contrast to methylation, acetylation of histones comprises a modification with rapid turnover rates, and transcriptional active regions are in general enriched for acetylated nucleosomes (hyperacetylation) whereas transcriptional suppressed areas are mainly hypoacetylated. Studies on the genome wide distribution of histone acetylation suggest a two-phased distribution of this modification. Firstly, in a local manner, where acetylation is targeted to neighboring nucleosomes of regulatory elements, in particular at enhancers and promoters but also at the most 5'-transcribed sequences. Secondly, in a less pronounced manner during the establishment of vast chromatin regions, which display an opened chromatin conformation (Calestagne-Morelli and Ausio, 2006). The fact that such cell-type and context dependent domains of histone acetylation are strictly conserved even between orthologous loci further suggest a strong selective pressure for proper inheritance of these PTM systems.

Importantly, it appears that the formation of silent chromatin regions is also dependent on the removal of active marks in such areas since the enzymes responsible for the formation of transcriptional repressive histone modifications, Suv39h1 and G9a for K9 methylation as well EED/EZH for K27 methylation and the silencing via CpG methylation are associated with HDAC activity (Jones *et al.*, 1998; Nan *et al.*, 1998; van der Vlag and Otte, 1999; Fuks *et al.*, 2000; Czermin *et al.*, 2001; Vaute *et al.*, 2002; Shi *et al.*, 2003). Although HDAC inhibition is in most cases apparently not sufficient for reactivation of elements silenced by CpG methylation, inhibition of DNA methyltransferases was found to synergize with HDAC inhibitors (HDI) in the activation of such elements, suggesting a requirement for deacetylation in this silencing process (Kaslow and Migeon, 1987; Cameron *et al.*, 1999; Csankovszki *et al.*, 2001). Further the maintenance of pericentric heterochromatin appears dependent on the suppressions of hyperacetylation, as HDAC inhibition results in delocalization to the nuclear periphery and loss of HP1 retention (Taddei et al., 2005). Together these observations demonstrate an interplay between the placing of repressive marks and targeted suppression

of activating marks in the formation and maintenance of transcriptional repressive chromatin states.

1.6.3 Interdependency between transcriptional activating lysine methylation and histone acetylation

Lysine methylation can also be associated with transcriptional active regions, as found for lysines 4, 36, 79 of histone H3 (Strahl *et al.*, 1999; Santos-Rosa *et al.*, 2002). Also in the case of activating methyl-lysine marks, methylation appears to be directly linked to acetylation. Recent reports demonstrate a tight coupling between methylation of K4 in histone H3 and lysine acetylation: Mass spectrometric studies indicated a preferential localization of K4 methylation (mono-, di- and trimethylation) at penta-acetylated histone H3, whereas K9 methylation coincides mainly with mono-acetylation (Zhang et al., 2004). Hazzalin *et al.* reported a continuous turnover of histone acetylation at all K4 trimethylated histone H3 molecules, even during transcriptional silent states, which therefore renders these molecules hypersensitive to HDI mediated hyperacetylation (Hazzalin and Mahadevan, 2005; Clayton *et al.*, 2006). Further, transcriptional induction of the immediate early (IE) genes *c-fos* and *c-jun* required this continuous turnover rather than stable histone acetylation, which is in general associated with transcriptional active states, and interruption of the HAT/HDAC equilibrium ceased stimulus dependent induction. HDAC inhibition and the resulting increase in acetylation did not affect the H3K4 trimethylation levels at these genes suggesting that promoter associated nucleosomes are pre-modified to target acetylation turnover.

Nightingale *et al.* reported a global increase in H3K4 trimethylation upon HDAC inhibition and identified MLL4 as the responsible histone methyltransferases (HMTase) (Nightingale *et al.*, 2006a). Importantly, this stabilizing effect of histone acetylation on H3K4 methylation was linked to an increased substrate-attraction of the H3K4 HMTase MLL1/4 by acetylated and phosphorylated histone H3 *in vitro*, whereas the activity of Set7, a HMTase mainly associated with H3K4-monomethylation was not affected (Milne *et al.*, 2002; Xiao *et al.*, 2003; Nightingale *et al.*, 2006a). The preference of MLL1/4 for acetylated substrates was demonstrated *in vitro* and in the absence of additional factors suggesting a direct impact of PTMs on the enzymatic activity (Nightingale *et al.*, 2006a). These findings are particular interesting as they demonstrate a reciprocal crosstalk between H3K4 methylation and lysine acetylation although in the case of *c-fos* and *c-jun* H3K4 methylation appears clearly independent of acetylation (Hazzalin and Mahadevan, 2005). Strikingly, the HDI mediated increase in H3K4 trimethylation appears to be reversible upon loss of acetylation as removal of the HDI resulted in rapid deacetylation accompanied by a slower decline of H3K4me3 with a half life of roughly one hour suggesting the existence of a yet to be identified histone demethylase for this isoform (Fig 1-4). Methylation of H3K4 was demonstrated to recruit several enzymatic activities (Sims and Reinberg, 2006) including HAT and chromatin

remodeling activity via Chd1, the BPTF subunit of the NURF complex and WDR5, which is also required for MLL activity (Santos-Rosa *et al.*, 2003; Dou *et al.*, 2005; Pray-Grant *et al.*, 2005; Wysocka *et al.*, 2005; Wysocka *et al.*, 2006). Also the recently discovered H3K9/K36me3 histone demethylase JMJD2A is specifically recruited by H3K4me2/3 and H4K20me2/3 (Huang et al., 2006) and the PHD finger containing ING2 protein was identified as specific detector-protein for H3K4me2/3 (Pena et al., 2006; Shi et al., 2006).

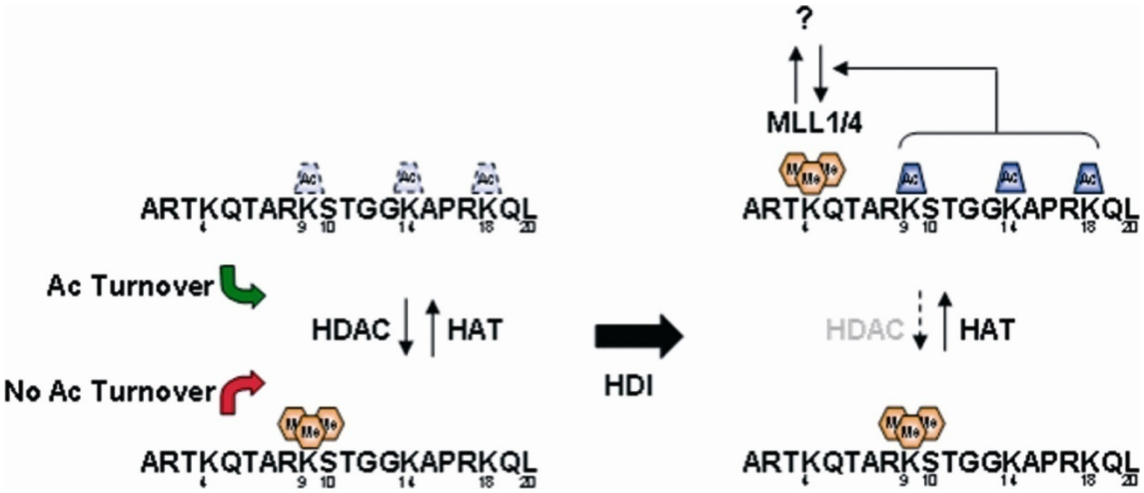

Figure 1-4 Dynamic acetylation turnover at K4 methylated histone H3 (Winter *et al.*, 2007). The continuous reciprocal activities of HATs and HDACs are targeted to K4 methylated but not to K9 methylated histone H3. The former species displays therefore a hypersensitivity to HDAC inhibitor mediated hyperacetylation, whereas K9 methylated histone H3 is less susceptible due to the missing activity of HATs. Histone acetylation in turn can exert a stabilizing effect on K4 methylation by increasing the efficiency of MLL1/4 mediated K4 methylation, which can be reversed via a yet unidentified trimethyl-K4 specific demethylase.

Besides the recruitment of transcriptional stimulating factors, H3K4 methylation can abolish the association or activity of transcriptional repressors such as the nucleosome remodeling and deacetylase complex (NuRD) or the H3K9 methyltransferase Suv39h1. Further, H3K4 methylation is essential for the restriction of Sir1p to heterochromatic regions, providing an additional level how this modification contributes to transcriptionally active chromatin (Nishioka *et al.*, 2002; Zegerman *et al.*, 2002; Santos-Rosa *et al.*, 2004). Importantly, the ING proteins are associated with HDAC and HAT activity and can therefore recruit both transcriptional repressive and activating activities to me2/3K4 modified histone H3 (Doyon et al., 2006). Another example for the interaction with enzymatic activities that are normally associated with differential transcriptional effects is the co-localization of HCF-1 with a Set1/Ash2 related HMTase complex as well as the Sin3 HDAC complex (Wysocka et al., 2003).

1.6.4 Histone phosphoacetylation a special relation *in cis*

Histone phosphorylation comprises a particular interesting modification with extremely opposite characteristics in mitosis or interphase, respectively. Mitotic phosphorylation of histone H3 at serine10 (H3S10ph) occurs in a global manner, first appearing in late G2 phase at pericentromeric heterochromatin and propagating throughout the whole chromosome until late prophase. Hyperphosphorylation is maintained during metaphase and dephosphorylation is initiated concomitant with anaphase and completed at telophase (Hendzel et al., 1997). This mitosis specific phosphorylation of histone H3 is mediated by the Aurora B kinase and is required for the displacement of HP1 proteins from mitotic condensed chromatin (Mateescu *et al.*, 2004; Fischle *et al.*, 2005; Hirota *et al.*, 2005).

In contrast, interphase phosphorylation of histone H3 is a much more restricted event since only a few genomic regions are targeted by this modification. Importantly, this limited interphase histone phosphorylation correlates with transcriptional activation rather than chromatin compaction suggesting that the phosphorylation event itself does not directly initiate major alterations in chromatin structure.

Histone H3 phosphorylation was first discovered in the Sixties to be mediated by both cAMP dependent and independent kinase activity and Mahadevan and co-workers described the phosphorylation of histone H3 as one of the earliest events in quiescent cells concomitant with the induction of the proto-oncogenes *c-fos* and *c-jun* upon growth factor or stress stimulation (Gutierrez and Hnilica, 1967; Langan, 1968; Shoemaker and Chalkley, 1978; Mahadevan *et al.*, 1991). This rapid and transient phosphorylation event on histone H3 is now referred to as the “nucleosomal response”.

Since these reports histone H3 phosphorylation was found as a downstream target of several signal transduction cascades including the transcriptional activation of IE genes *c-fos* and *c-jun* as well as the late-induced *HDAC1* gene via MAP-kinase stimulation or the cytokine induced H3 phosphorylation via IKK-α (Thomson *et al.*, 1999; Cheung *et al.*, 2000b; Clayton *et al.*, 2000; Hauser *et al.*, 2002; Anest *et al.*, 2003; Yamamoto *et al.*, 2003). Knockout studies indicate that H3S10 phosphorylation is mainly mediated via the activity of the downstream effector kinases MSK1/2. Besides other reports on the induction of histone H3 phosphorylation it was demonstrated that also the progesterone receptor (PR) mediated transcriptional induction of the mouse mammary tumor virus (MMTV) promoter involves the phosphorylation of H3 in an ERK and Msk1 dependent manner (Vicent et al., 2006). In this report the authors demonstrated that the PR forms a ternary complex with ERK and MSK1 upon hormone stimulation, which in turn is rapidly recruited to the MMTV promoter to mediate H3S10 phosphorylation at histone H3. Disturbance of this cascade and loss of histone phosphorylation resulted in impaired recruitment of the transcriptional co-activators

Brg1 and PCAF and reduced localization of RNA-polymerase II (RNAP II) suggesting a role for the phS10 mark during the initiation phase of transcription.

Recently, another study investigated the osmotic-stress dependent repression of hormone-dependent transcription of the MMTV promoter. In this system, hormonal induction of MMTV promoter mediated transcription was repressed by osmotic shock in UL3 cells. Osmotic-shock mediated displacement of the glucocorticoid receptor (GR) temporarily and spatially coincided with phosphorylation of S10 and S28 at histone H3 suggesting that interphase phosphorylation of H3 can also occur during transcriptional repressed states in this system (Burkhart et al., 2007).

An interesting feature of H3S10 phosphorylation during interphase is a strong link with acetylation events on neighboring lysines at position 9 and 14 (H3K9/14ac), which finally gave rise to the neologism “histone H3 phosphoacetylation” (H3S10phK14ac) (Cheung *et al.*, 2000b; Clayton *et al.*, 2000). The explanation for the close co-existence of both PTMs is controversial. The observation that the transcriptional co-activator and HAT Gcn5 displayed a strongly increased preference for histone H3 peptides when they were phosphorylated at S10, and Snf1 kinase mediated H3S10 phosphorylation was required for the additional acetylation of H3K14 at the INO1 promoter led to the proposal of a “synergistic coupled model” (Cheung *et al.*, 2000b; Lo *et al.*, 2000; Lo *et al.*, 2001). According to this scenario, the preceding phosphorylation increases the affinity of HAT-enzymes for the histone H3 tail and is obligate for the subsequent acetylation event to generate the phosphoacetylated isoform. Indeed studies on the substrate preferences of recombinant Gcn5 with uniformly H3S10 phosphorylated nucleosomal arrays confirmed these results. However, this preference for H3S10ph nucleosomes was abolished when Gcn5 was assembled into the native SAGA-complex (Shogren-Knaak et al., 2003). Studies on two Snf1 regulated targets genes, INO1 and GAL1 revealed a gene specific validity of this model. Whereas H3S10ph at the INO1 promoter was required for recruitment of the SAGA complex and subsequent acetylation of H3K14, at the GAL1 promoter both events are independent and SAGA is recruited also in the absence of H3S10ph (Lo et al., 2005). Importantly, the relevant activators showed different abilities to interact with the co-activators Snf1 and SAGA, respectively; INO1 activator can solely recruit Snf1 kinase activity, whereas GAL4 activator can interact with both factors explaining the uncoupling of H3S10ph and H3K14ac at the GAL1 promoter. However, despite this lack of direct dependency, H3S10ph was still required to mediate transcriptional induction of the GAL1 and the INO1 gene suggesting a cooperation of H3S10ph and H3K14ac in transcriptional activation (Figure 1-5).

Additional support for an independent recruitment of both modifications comes from detailed studies on the IE *c-fos* and *c-jun*. Upon MAP-kinase stimulation via growth factors or stress inducers like anisomycin, only a minute fraction of nucleosomes becomes phosphorylated in

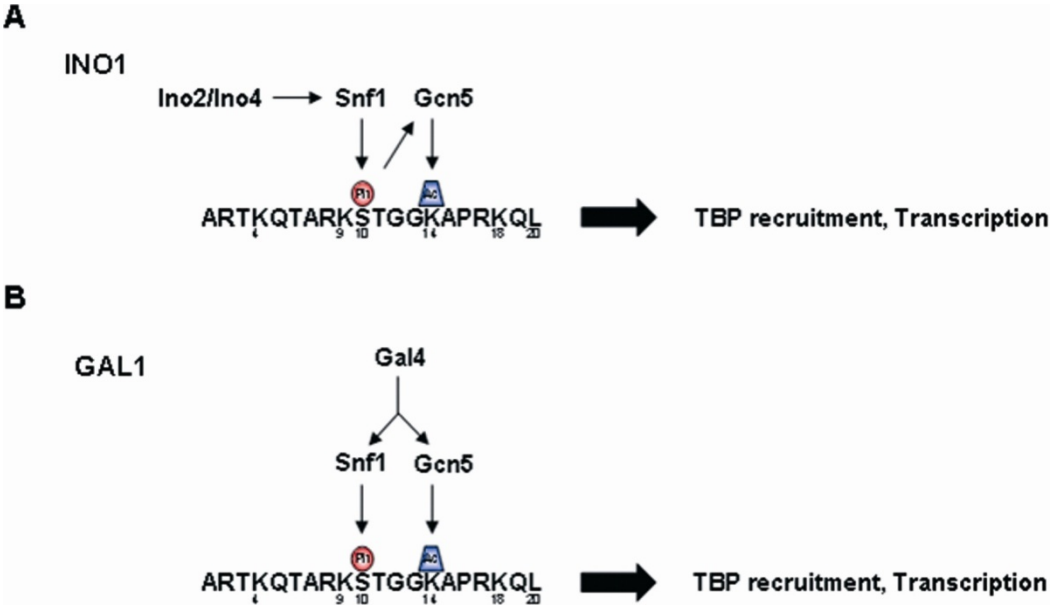

Figure 1-5 Promoter specific crosstalk between histone H3 S10 phosphorylation and K14 acetylation (taken from (Winter, 2007)). Transcriptional induction of the *INO1* and the *GAL1* genes involves phosphoacetylation of histone H3. (**A**) The Ino2/4 heterodimer interacts with the Snf1 kinase and recruits the enzyme to the *INO1* TATA sequence where it phosphorylates histone H3 at S10. The phosphorylation event is required for the subsequent recruitment of the Gcn5 HAT and acetylation of K14. (**B**) In contrast to the *INO1* activator Ino2/4, the *GAL1* activator Gal4 can interact with both Snf1 and Gcn5 and therefore acetylation of K14 at the *GAL1* promoter is independent of S10 phosphorylation. Despite this uncoupling, both histone modifications are required for TBP recruitment and transcriptional induction at both promoters.

quiescent cells which is particular susceptible to HDI induced hyperacetylation (Barratt et al., 1994).

The nucleosomes of the *c-jun* promoter region are rapidly and transiently phosphoacetylated and appearance of this dual-modified isoform is strongly correlated with transcriptional induction. Thomson and co-workers found that the regulation of S10ph and K9/14ac is dynamically and independently regulated at the promoters of these protooncogenes (Thomson et al., 2001). Both modification events occur independently as inhibition of p38 or ERK MAP-kinase activity abolished H3S10ph and H3S10phK14ac but did not alleviate anisomycin induced hyperacetylation (Thomson *et al.*, 2001; Mahadevan *et al.*, 2004) (Figure 1-6). This suggests further that another stress-responsive pathway mediates the local equilibrium-shift between HAT and HDAC activity. Importantly, p38 MAP-kinase stimulation did not influence bulk histone acetylation levels, implying the strict local delivery of this effect (Hauser et al., 2002). In addition HDI induced histone H3 hyperacetylation was observed in G_0 cells at the bulk chromatin level and the *c-jun* promoter, demonstrating a continuous turnover of this modification even in the absence of H3S10ph (Thomson *et al.*, 2001; Hauser *et al.*, 2002). Despite a stable bulk histone hyperacetylation in the presence of TSA, histone acetylation at the IE genes is transient, and may therefore involve a TSA-insensitive deacetylase (Thomson et al., 2001). A striking feature of this continuous acetylation turnover

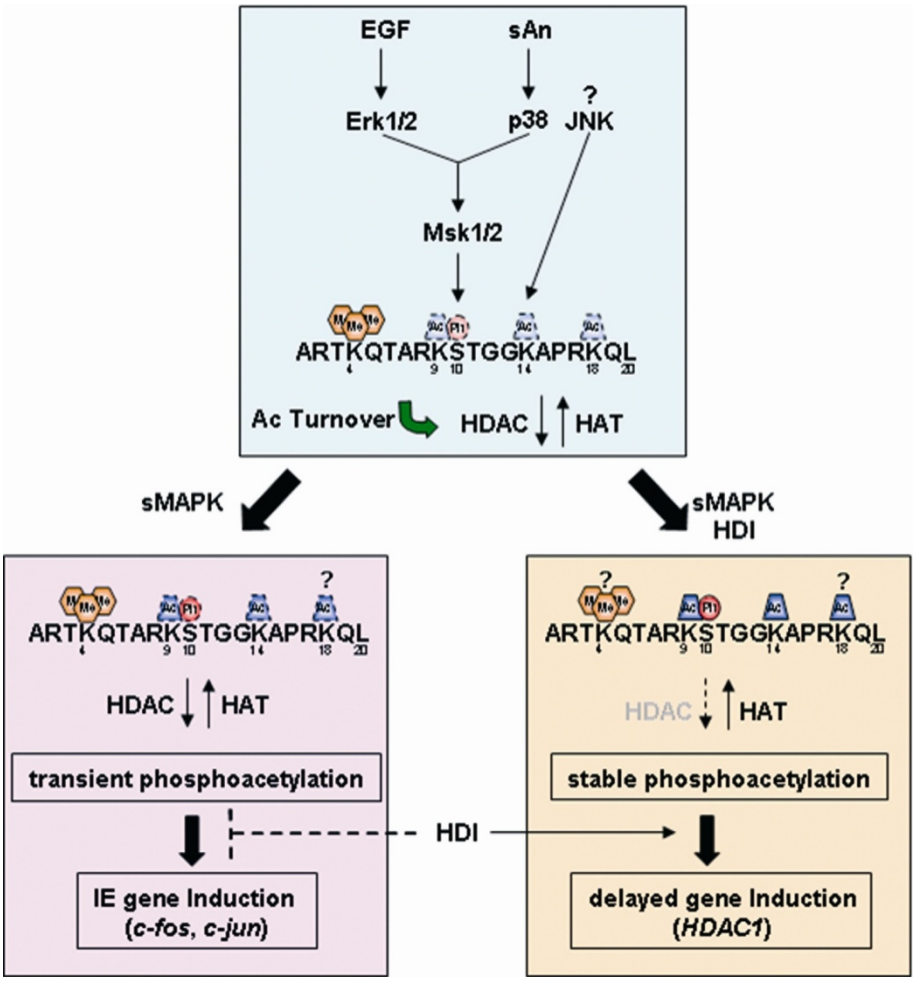

Figure 1-6 Phosphoacetylation of histone H3 impacts transcription by gene specific mechanisms (taken from (Winter, 2007)). (upper panel) Two MAP kinase cascades, the ERK and p38 pathways are upstream of S10 phosphorylation. ERK can be activated by growth factors like EGF and p38 by stress inducers like anisomycin. Both cascades can mediate activation of the effector kinase MSK1/2 that directly phosphorylates histone H3 (red circle). An alternative effector kinase may be Rsk2, which is only activated via the ERK pathway. S10 phosphorylation is targeted to a minute fraction of nucleosomes that are highly susceptible to lysine acetylation and in the case of *c-fos* and *c-jun* both modifications are targeted to K4 methylated nucleosomes, which are subjected to continuous acetylation turnover (blue trapezium) (see also **Fig. 1-5**). In contrast to ERK and p38, the JNK pathway does not mediate S10 phosphorylation. However, it was suggested that JNK could mediate the increase in lysine acetylation at the *c-fos* and *c-jun* promoters observed upon JNK/p38 stimulation independently of S10 phosphorylation. **(Left panel)** Transcriptional induction of IE genes, which are "poised to transcribe", is mediated via transient phosphoacetylation mediated by MAP-kinase stimulation. S10 phosphorylation and acetylation are independently localized at K4 methylated nucleosomes and blockade of acetylation turnover via HDI ceases transcriptional induction. **(Right panel)** Transcriptional induction of the non-IE phosphoacetylation target gene *HDAC1* requires both modifications in a stable manner. Histone phosphoacetylation can be stabilized via additional HDAC inhibition (HDI) and both modifications show synergy in the transcriptional induction of *HDAC1* (for details see text). The role of K4 methylation has not yet been determined in this system.

at the *c-fos* and *c-jun* promoters was described recently: ablation of this turnover via HDI ceases MAP-kinase mediated transcriptional induction of these genes, demonstrating that constant acetylation/deacetylation is required for activation rather than a highly acetylated state (Hazzalin and Mahadevan, 2005).

This requirement of acetylation turnover appears to be a specific trait of the IE genes as deacetylase inhibition mainly exerts a positive transcriptional response, although some genes clearly require HDAC-activity for expression (Zupkovitz et al., 2006). Although in the case of IE genes, phosphorylation and acetylation of histone H3 constitute two independent events, both modifications were found to synergize in the transcriptional activation of the late inducible HDAC1 gene, which is therefore differentially regulated as the IE genes (Hauser et al., 2002). HDAC1 is repressed via a negative feedback mechanism and not expressed in serum starved G0 cells (Hauser et al., 2002; Schuettengruber et al., 2003). The local HDAC predominance can be overcome, by HDI treatment and was sufficient to induce low levels of HDAC1 expression. Although MAP-kinase activation leads to transient phosphoacetylation at the HDAC1 promoter, this short-term alteration in chromatin structure is not sufficient to overcome the transcriptional repressive, local predominance of HDACs. Upon additional inhibition of deacetylase activity phosphoacetylation is stabilized and shows synergistic effects on the transcriptional induction (Hauser et al., 2002) (Figure 1-6). This study did not address the question concerning coupled or uncoupled phosphoacetylation, but clearly demonstrates that histone phosphorylation and acetylation can have synergistic effects in transcriptional activation.

Another interesting feature of H3S10 phosphorylation is the strong inhibition of the H3K4 lysine specific demethylase LSD1. The observations that LSD1 mediated H3K4 demethylation is strongly dependent on removal of other PTMs suggests that several enzymatic activities cooperate during the formation of transcriptional repressive states (Forneris *et al.*, 2005; Forneris *et al.*, 2006). The function of histone phosphorylation in transcriptional induction is still not understood. However, due to the extreme local restriction of this modification, and the variety of signal-transduction systems that have been reported to mediate histone phosphorylation it is likely that this PTM can mediate transcriptional induction via several different mechanisms. Therefore, the genomic context of H3S10 phosphorylation may be a critical factor for the molecular events, initiated by this modification.

1.6.5 Histone ubiquitination and modification cross talk *in trans*

Ubiquitination of histone molecules was found for histones H2A, H2B, H3, H4, H2A.Z, macroH2A and H1(Zhang, 2003; Kinyamu *et al.*, 2005; Osley *et al.*, 2006). In most cases only a single ubiquitin molecule is attached to histones, which is not sufficient for targeting via the 26S proteasome.

Ubiquitination of Histone H1 is mediated via the TAF1 ($TAF_{II}250$) component of the TFIID complex and is linked to the transcription of a subset of genes. Ubiquitination via TAF1 is particular interesting as it possesses both, ubiquitin activating (E1) and conjugation (E2) activity (Pham and Sauer, 2000). Histone H2A, the first protein found to be ubiquitinated (Goldknopf et al., 1975), is modified at position K119 within the C-terminal globular histone fold domain. Ubiquitinated H2A (uH2A) comprises approximately 5-15% of total cellular H2A molecules but seems to be restricted to higher eukaryotes and is apparently absent in *Saccharomyces cerevisiae*. Histone H2A ubiquitination is mediated via RING-domain containing components of the Polycomb-group complex PRC1 and was functionally linked to Polycomb silencing and X-chromosome inactivation (de Napoles *et al.*, 2004; Fang *et al.*, 2004; Wang *et al.*, 2004a). On the other hand the same modification was found to correlate with transcriptionally active chromatin states, exemplified by an enrichment of uH2A on the nucleosomes of the *hsp70* promoter and a depletion at nontranscribed satellite DNA (Levinger and Varshavsky, 1982). Further, ubiquitination of H2A and H2B was shown to be enriched at actively transcribed regions in different systems like bovine thymus, chicken erythrocytes and macronuclei of *Tetrahymena* (reviewed in (Zhang, 2003; Osley *et al.*, 2006)).

Ubiquitination of H2B was found at K123 in yeast and at K120 in other eukaryotes. In contrast to uH2A, 2HB ubiquitination is much less abundant (1-2% of total H2B molecules) but more widely distributed within eukaryotes. The yeast enzymes responsible for H2B ubiquitination have been identified as the E2 ligase Rad6/Ubc2 and the E3 ligase Bre1, and both these enzymes have human homologues, UbcH6 and RNF20/RNF40, respectively (Zhang, 2003; Osley *et al.*, 2006) (Figure 1-7).). Rad6/Ubc2 represents a multifunctional E2 enzyme with several different cellular substrates that are specifically targeted via the interaction with different E3 ligases. Bre1 is an important co-factor for Rad6 mediated ubiquitination, as it is also required for the recruitment of Rad6 to promoters, as well as its' association with the elongating RNAP II and spreading into the coding region (Henry et al., 2003; Wood et al., 2003). A multitude of additional factors that promote H2B ubiquitination have been described and a majority of them are involved in transcriptional regulation as will be discussed below.

It is noteworthy that both histone H2A and H2B constitute (although inefficient) targets for Mdm2 E3 ligase mediated mono-ubiquitination and therefore alternative E3 ligases may provide a gene-specific context for H2A/H2B ubiquitination (Minsky and Oren, 2004). The responsible enzyme for removing ubiquitin from H2B in euchromatic regions was identified as Ubp8 a component of the SAGA acetyltransferase complex, which therefore combines deubiquitination and acetylation activities (Henry *et al.*, 2003; Daniel *et al.*, 2004) (Figure 1-7). Importantly, efficient transcriptional initiation requires sequential ubiquitination and

deubiquitination as presence of uH2B coincides with the localization of RNAP II and absence of this modification results in a delay of this recruitment. Further, it was found that the loss of Ubp8 results in an inverse imbalance of H3K4 and H3K36 methylation patterns, which may also perturb transcription (Henry et al., 2003).

Another ubiquitin protease that targets uH2B is Ubp10, which appears to primarily act on subtelomeric regions adjacent to heterochromatin, although a role in euchromatic repression has been observed (Emre et al., 2005; Gardner et al., 2005) (Figure 1-7). Genetic analysis of yeast mutants displaying defects in H2B ubiquitination revealed an amazing dependency of histone H3 K4 and K79 methylation on this modification. Mutations that abolish ubiquitination of H2B also globally affected H3K4 and H3K79 methylation and caused telomeric and mating type locus silencing defects. This loss of heterochromatic structures was linked to the decreased repulsion of Sir proteins from euchromatic regions due to impaired H3K4 methylation (Briggs *et al.*, 2002; Dover *et al.*, 2002; Sun and Allis, 2002). Recent data indicate that mono-methylation of H3K4 is independent of H2B ubiquitination whereas the di- and tri-methylated states are preferentially regulated *in trans* (Dehe et al., 2005; Shahbazian et al., 2005). One key component in this modification crosstalk seems to be the transcription elongation regulator complex PAF and the association of HMTases and ubiquitin ligases with elongating RNAP II (Krogan et al., 2003a; Ng et al., 2003). Importantly, uH2B is not determinant for the recruitment of Set1 and Dot1 HMTases, but required for the localization of the proteasomal ATPases Rpt4 and Rpt6, which are in turn essential for histone H3 methylation (Ezhkova and Tansey, 2004). Further the Rpt6 ATPase-activity of the 19S proteasome regulatory particle (19S RP) was found to be necessary for optimal recruitment of the SAGA-complex to the GAL1-GAL10 promoters, which demonstrates a non-proteolytic role for proteasomal components in transcriptional activation (Lee *et al.*, 2005a). SAGA associates with the chromodomain protein Chd1 that binds methylated K4, and the complex integrates histone acetyltransferase and ubiquitin protease activities via the components Gcn5 and Ubp8. Both histone ubiquitination and acetylation are dynamically regulated during transcriptional initiation by SAGA demonstrating the importance of the 19S RP mediated regulation of SAGA recruitment.

Besides the PAF elongation complex the transcription elongation factor BUR1, a cyclin-dependent type kinase (cdk), in conjunction with the cyclin-BUR2, was found as an essential component for histone H2B ubiquitination, H3K4 trimethylation and mediators for the interaction with the PAF-complex (Laribee et al., 2005; Wood et al., 2005). Interestingly, mutation of H3K36 to arginine or defects in the K36 specific HMTase Set2, were identified as origin of the growth defect displayed by BUR1/BUR2 mutants. These mutants also displayed reduced levels of trimethylated H3K36 suggesting that the regulatory kinase BUR1/BUR2 also influences transcription dependent H3K36 tri-methlyation via Set2 (Chu et al., 2006).

How exactly ubiquitination impacts transcription is still elusive since some defects displayed in uH2B mutants may be contributed by the crosstalk with H3K4/79 methylation. However, there is accumulating evidence for a function of uH2B in transcriptional initiation and elongation some of which may also be mediated via downstream methylation and some directly contributed via the ubiquitin mark. One model for the role of histone ubiquitination proposes that the effects of this modification are mainly exerted via the bulky ubiquitin moiety and the impact of this residue on the overall chromatin structure. However, *in vitro* studies failed to detect any major alteration in the structure of nucleosomal arrays. The second model proposes that ubiquitin serves as binding site for modification dependent detector proteins, which recognize the ubiquitin moiety and lead to the recruitment of further transcriptional activators.

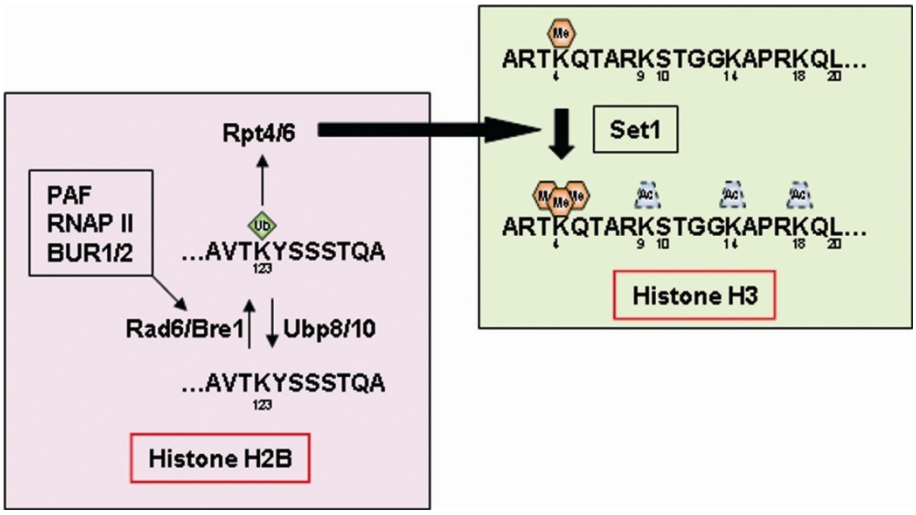

Figure 1-7 Modification crosstalk in trans between H2B ubiquitination and H3 methylation (taken from (Winter, 2007)). Ubiquitination of H2B at K123 in yeast (K120 in human) was found as essential upstream event for methylation of K4 at histone H3. Rad6/Bre1 were identified as responsible E2 and E3 ligases, respectively. The isopeptidase Ubp8 removes ubiquitin in euchromatic regions, whereas Ubp10 mainly acts on heterochromatic chromatin. Rad6/Bre1 mediated ubiquitination was found to be dependent on various additional factors including the RNAP II/PAF elongation complex, as well as the activity of BUR1/2 kinase. Monoubiquitination of H2B is not required for recruitment of the K4 HMTase Set1, but for the localization of the proteasomal components Rpt4/6, which in turn are required for processive di- and trimethylation of K4. K4 methylation again may direct acetylation turnover to the H3 tail in cis

1.7 Impact of post translational modifications on chromatin states

Two overlapping models how histone PTMs can impact the chromatin structure have been considered. In the first scenario histone modifications directly impact the compaction status of the chromatin polymer. This has been most extensively discussed for serine phosphorylation and lysine acetylation, as both modifications alter the overall charge of the molecule and could therefore attenuate electrostatic interactions between negatively charged DNA and positively charged histone tails (Ura *et al.*, 1997; Wolffe and Hayes, 1999; Cosgrove *et al.*, 2004; Ahn *et al.*, 2005; Shogren-Knaak *et al.*, 2006). However, some reports concerning the electrostatic induced conformational alteration by modifications are contradictory (McGhee *et al.*, 1983; Prigent and Dimitrov, 2003) and histone methylation does not alter the lysine charge but is well established to impact chromatin conformation. Nevertheless, one noteworthy histone modification, the acetylation of lysine 16 on histone H4, was demonstrated to directly impact chromatin structure *in vitro*. Incorporation of K16 acetylated histone H4 was found to inhibit 30nm fiber folding of nucleosomal arrays and cross-fiber interactions (Shogren-Knaak *et al.*, 2006). Also the mobilization of nucleosomes via the ACF chromatin remodeling enzyme was found to be inhibited by H4K16 acetylation, indicating that also interaction with proteins may be modulated by this particular modification (Shogren-Knaak *et al.*, 2006).

Genetic studies in budding yeast including all different combinations for lysines 5, 8, 12 and 16 of histone H4, demonstrated that H4K16 showed specific non-cumulative (additive) effects on gene expression, in contrast to the other three lysines, for which only cumulative effects have been observed (Dion *et al.*, 2005). These findings further underscore the outstanding importance of K16 acetylation for regulation of chromatin structure. So far H4K16ac is the only (single) modification known capable to directly impact chromatin folding even in the absence of additional factors. The molecular basis for this may originate from the positioning of the K16 moiety within the nucleosome. The amino acids 14-19 of histone H4 interact directly with an “acidic batch” in the histone H2A/H2B dimer interface and this interaction may be sensitive to acetylation. Several reports like the observation that the histone H4 tail is required for chromatin compaction *in vitro* (Gordon *et al.*, 2005) and gene expression but not for cell viability (Durrin *et al.*, 1991; Ling *et al.*, 1996) support such a scenario.

In the second “effector mediated” model, histone PTMs are recognized by specific protein modules that mediate the recruitment of further complex components to impact chromatin structure. A multitude of reports on the identification and characterization of modification dependent protein domains exits that recognize PTMs and thereby regulate cellular behavior (Seet *et al.*, 2006). Structural analysis of binding modules for modified histone tails suggest that such interactions are not only dependent on the modification state but also on the

sequence context of the histone tail (Taverna *et al.*, 2007). Importantly such modification dependent adaptor proteins constitute a prerequisite for the “histone code”- hypothesis as binding of such factors would reflect the unique modification composition presented on a histone tail (Cheung *et al.*, 2000a). Several different protein domains recognizing different PTMs on histone tails have been identified and extensively characterized on biological, biochemical and structural levels (Taverna *et al.*, 2007).

1.7.1 Histone acetylation is recognized by bromodomains

Besides the direct impact of histone acetylation on nucleosome structure and secondary interactions, acetylated lysines were found to constitute binding platforms for bromodomain containing proteins. Bromodomains are found in a variety of proteins which are frequently associated with chromatin, such as the nuclear acetyltransferase Gcn5. Functional bromodomains are essential components for the transcription associated functions of these factors. The prototypical bromodomain of the Gcn5 homologue PCAF (p300/CBP associated factor) was the first modification dependent histone binding module to be structurally characterized (Dhalluin *et al.*, 1999a; Dhalluin *et al.*, 1999b). These studies demonstrated a left-handed, antiparallel four helix bundle topology and outlined the importance of hydrophobic interactions for acetyl-lysine binding of a histone H4K16ac peptide. In addition, further variations of bromodomain organization have been characterized, like the double bromodomains of the hTAF1 histone acetyltransferase (formerly $TAF_{II}250$), a component of the TFIID complex (Jacobson *et al.*, 2000; VanDemark *et al.*, 2007). These double bromodomains can bind to dual acetyl-lysines like an H4K5acK12ac peptide with high affinity and more weakly to single acetylation marks. The spatial spacing of the two bromodomains by about 25Å suggests binding of dual acetyl-lysines that are separated by about seven amino acids, which matches for the major acetylation sites in histone H4, lysines 5, 8, 12 and 16. However, the structure of the TAF1 double bromodomains in complex with multiple acetylated histone H4 peptides has not yet been determined and therefore the molecular basis for interaction with dual or multiple acetylated peptides is not known. Also the Rsc4p component of the yeast RSC chromatin remodeling complex contains two bromodomains, which were recently shown to adapt a different conformation than the TAF1 double bromodomains. Instead of the independent folding observed in TAF1, the Rsc4p bromodomains fold as an single unit with same orientation, resulting in a more compact folding and only about 20Å spacing (VanDemark *et al.*, 2007). Interestingly, the second bromodomain of Rsc4p was demonstrated to be involved in the recognition of H3K14ac peptides, whereas the first bromodomain is involved in the recognition of an autoregulatory acetyl-lysine (K25) that inhibits the binding of the H3K14ac to the second bromodomain (VanDemark *et al.*, 2007).

This mechanisms points to a not yet well developed field of interest, the regulation of PTM dependent histone binding by autoregulatory modifications.

1.7.2 Recognition of histone methyl-lysine- marks

In contrast to lysine acetylation, lysine methylation occurs in different degrees as mono- (me1), di- (me2) or trimethylation (me3) and is recognized *via* a much more diverse range of protein domains. Detector modules for methylated lysines are grouped into the royal superfamily which includes chromodomains, double chromodomains, chromo barrels, malignant brain tumor (MBT) domains and double and tandem tudor domains. In addition methylated lysines are bound via PHD fingers and WD40 repeat containing proteins (see Table 2).

Whereas lysine acetylation ablates the positive charge of the ε-amino group, mono-, di- and trimethylated lysines are predicted to be positively charged at physiological pH. Increasing degrees of lysine methylation result in increased hydrophobicity and cation radius of the methylammonium group, whereas the ability for hydrogen bond formation decreases. Therefore, the binding of methylated lysines requires a hydrophobic interaction environment that can also accommodate the positive charge. A general feature of binding modules to methylated lysines, appears to be the fitting of the methylammonium group into an “aromatic cage”, composed of two up to four aromatic amino acids. Thereby the methylammonium group is positioned between the π-electron systems of the aromatic residues and stabilized via π-cation-type interactions. In addition also hydrophobic effects stabilize the interaction although to a lesser extent (Hughes *et al.*, 2007).

The first screens for modules that bind to H3K9me3 demonstrated that “heterochromatin protein 1” (HP1) binds to H3K9me3 via its “chromatin organization modifier” domain (chromodomain) (Lachner *et al.*, 2001; Jacobs and Khorasanizadeh, 2002; Nielsen *et al.*, 2002). The chromodomain of HP1 was found to be similar to a region of the *Drosophila* Polycomb, which was shown to bind to H3K27me3. The basis for the separate interaction of HP1 chromodomain with histone H3 in a K9me2/3 context and Polycomb chromodomain in a K27me3 context has been linked to an extended interaction groove of the Polycomb chromodomain that allows for additional amino acids contacts specific for the H3K27 context (Fischle *et al.*, 2003c). Extensive characterization of the HP1 chromodomain *via* crystallography and NMR revealed an incomplete β-barrel conformation (Jacobs and Khorasanizadeh, 2002; Nielsen *et al.*, 2002). Upon binding, the histone peptide adopts an induced fit β-strand conformation thereby completing the β-barrel. The methyl-lysine is projected into a three amino acid aromatic “cage” positioned at one end of the chromodomain β-barrel.

A variation of the chromodomain interaction is observed for the double chromodomains of the “chromo helicase DNA binding protein” (CHD1). Besides a central SWI2/SNF2 helicase

and a carboxy-terminal DNA binding domain, CHD1 contains two amino-terminal chromodomains and has been implicated in transcriptional activation as well as nucleosome assembly and histone H3.3 incorporation during elongation (Sims *et al.*, 2004; Lusser *et al.*, 2005; Konev *et al.*, 2007). In the human CHD1 protein the two chromodomains are bridged by a helix linker and cooperate in the binding of K4 methylated histone H3 peptides (Flanagan *et al.*, 2005). Interestingly one of the caging tryptophans is sandwiched between H3R2 and the methylammonium group and this residue is replaced with glutamate in the yeast CHD1 excluding a similar binding mode.

The histone H3K9 and H3K36 specific demethylase JMJD2A (JHMDM3A) contains double tudor domains that are involved in the interaction with K4 methylated histone H3 (Huang *et al.*, 2006). Only the second tudor domain is involved in binding of an histone H3K4me3 peptide which is due to a more negative electrostatic potential and the ability to form hydrogen bonds with H3R2 and H3T3.

In addition the tandem tudor domains of the p53 associated protein 53BP1 were shown to bind H4K20me1/2 but not the trimethylated state (Botuyan *et al.*, 2006). The specific recognition of lower methylation states has been attributed to a hydrogen bond formed between an aspartate lining the binding site and the dimethylammonium proton and steric exclusion of the trimethylammonium group (Botuyan *et al.*, 2006). The amino acids involved in complexing the dimethylammonium group are also required for targeting 53BP1 to DNA double strand breaks *in vivo* (Botuyan *et al.*, 2006). Interestingly, 53BP1 can also interact with p53 dimethylated at K370 although the amino acid context is different from H4K20 (Huang *et al.*, 2007).

The specific recognition of lower lysine methylation states, like mono- or dimethylation has also been attributed to malignant brain tumor (MBT) repeat containing proteins. These factors form a group of proteins defined by a variable number of tandem MBT repeats spanning about seventy amino acids. Originally, the founding member of this family, L(3)mbt (L3MBTL1 in human) was identified in *Drosophila* by mutations causing malignant transformation of larval brain cells (Gateff, 1982). In flies two additional MBT repeat proteins, “Sexcomb on midleg” (Scm) (Grimm *et al.*, 2007) and “Sexcomb with four MBT domains” (Sfmbt) (Klymenko *et al.*, 2006) were identified that both belong to the Polycomb group family of developmentally important gene regulators. The MBT family members function as transcriptional repressors and the polycomb group proteins stably maintain transcriptional silencing over many cell generations (Schuettengruber *et al.*, 2007). The human genome contains at least ten genes encoding proteins with two up to four malignant brain tumor domains. Using peptide array approaches and *in vitro* reconstituted chromatin to pinpoint interaction motifs, H4K20me1/2 and H1bK26me1/2 were identified as modifications bound by L3MBT1 (Kim *et al.*, 2006; Trojer *et al.*, 2007). In contrast to other binding domains for

methylated lysines, MBT proteins only bind the mono- and dimethylated states. This finding has important implications concerning the readout of different degrees of lysine methylation on histones. A particular lysine residue could therefore mediate different biological effects depending on the degree of methylation and the particular degree-dependent binding module. The interaction of a protein containing the three MBT domains of L3MBTL1 to methylated oligonucleosomes resulted in chromatin compaction and therefore would be correlated with transcriptional repression of the bound genomic regions *in vivo* (Trojer *et al.*, 2007; Trojer and Reinberg, 2008). Interestingly, also hyperacetylated nucleosomal arrays were compacted via L3MBTL1 suggesting that H4K20me1/2 and H1bK26me1/2 can overcome histone acetylation (Trojer *et al.*, 2007).

The three MBT repeats of L3MBTL1 display a tripartite propeller like architecture (Wang *et al.*, 2003). Each repeat contains an aromatic cage composes of three amino acids and an aspartate. The overall arrangement of the binding pockets explains the selection of mono- and dimethylated substrates. The insertion mode of factors specific for lower lysine methylation states such as 53BP1 *via* its tandem tudor domains (see above) or L3MBTL1 is now referred to as “cavity insertion” (Taverna *et al.*, 2007). In contrast, binding modules for higher methylation degrees such as HP1 chromodomain or CHD1 double chromodomains use a “surface groove” interaction motif. In the “cavity insertion” mode the methylammonium group is inserted into a narrow protein cleft that also potentially poses steric hindrance to higher methylation states. In addition, the ability of mono- and dimethylammonium groups to form hydrogen bonds with acidic residues lining the binding pocket also contributes to the selective coordination of lower lysine methylation states in L3MBTL1 and 53BP1 (Taverna *et al.*, 2007). “Surface groove” recognition accommodates the bound peptide into a wider and more accessible binding pocket on the protein surface. In this mode the methyl-lysine is arranged along the protein surface thereby posing less steric restriction to the “caging” of the trimethylammonium group.

Whereas there is already a multitude of reports exploring the structural basis for the interaction of MBT proteins with their substrates, relatively little is still known about their biological function. The fact that L3MBTL1 binds to mono- and dimethyl-lysine in many sequence contexts poses some difficulties on the identification of biological relevant targets. The purification of L3MBTL1 associated proteins identified (besides core histones and histone H1b) HP1γ, Retinoblastoma protein 1 and the sequence dependent transcription factors TEL and E2F2 (Boccuni *et al.*, 2003; Lewis *et al.*, 2004; MacGrogan *et al.*, 2004; Trojer *et al.*, 2007; Kalakonda *et al.*, 2008). This links the biological functions of L3MBTL1 to the formation of facultative heterochromatin as well as regulation of cell cycle progression. Further, the recruitment of L3MBTL1 to metaphase also suggests a role during G2/M transition (Koga *et al.*, 1999).

In addition to the double chromodomain of CHD1 and the double tudor domain of JMJD2A, it was demonstrated that also plant homeodomain (PHD) finger containing proteins recognize histone H3K4me3. The BPTF PHD finger component of the “nucleosome remodeling factor” (NURF) was shown to interact with histone H3K4me2/3 peptides and also with chromatin in an H3K4me3 dependent manner (Li *et al.*, 2006; Wysocka *et al.*, 2006). This interaction also helped to understand the high correlation of histone H3K4me2/3 with transcriptional active genes as NURF was demonstrated to faciliate transcription of chromatin templates (Tsukiyama and Wu, 1995; Mizuguchi *et al.*, 1997; Mizuguchi and Wu, 1999). In the BPTF PHD finger the methylammonium group is caged by four aromatic amino acids and separated from H3R2 via a tryptophan indole group reminiscent of interaction between the CHD1 double chromodomain with this modification (see above). In addition BPTF contains a bromodomain and therefore the presence of multiple domains may allow for simultaneously binding of H3K4me3 and H4Kac (Taverna *et al.*, 2007).

In addition the Yng1p PHD finger component of the NuA3 histone acetyltransferase complex was demonstrated to bind H3K4me3. The functional PHD finger of Yng1p was required for the increased substrate preference of the NuA3 complex towards H3K4me3 peptides (Martin *et al.*, 2006; Taverna *et al.*, 2006). Mutation of this PDH finger also resulted in decreased acetylation of NuA3 target ORFs suggesting a hierarchical order of events.

Furthermore, it was reported that the TAF3 subunit of the TFIID complex binds to H3K4me3 via a PHD finger motif (Vermeulen *et al.*, 2007) which may therefore contribute to localization of transcriptional start sites cooperative with the recognition of double acetylation marks via the dual bromodomains of the TAF1 subunit.

Another example, the ING2 PHD finger containing proteins that specifically recognize H3K4me3 is discussed in 1.6.3 Interdependency between transcriptional activating lysine methylation and histone acetylation.

Besides the specific recruitment of modules *via* methylation, it was also demonstrated that some factors initially bind the unmodified form and methylation greatly reduced the binding affinity. This was shown for the PHD finger protein BHC80, a component of the co-repressor complex. The PHD finger recognizes the H3 peptide in a lysine 4 context via a hydrogen bond cage. Also the ζ-ammonium group of the unmethylated lysine forms two hydrogen bonds with acceptors in the PHD finger binding pocket. The reduced hydrogen bonding potential of higher methylation states together with steric exclusion dictated by binding cleft geometry may provide the basis for reduced interaction potential upon H3K4 methylation (Lan *et al.*, 2007).

Also the interaction of the cysteine rich zinc binding domain of DNMT3L was demonstrated to interact with the histone H3 amino terminal tail but only in the unmethylated form, suggesting a reciprocal link between histone modifications and DNA methylation (Ooi *et al.*, 2007).

The recognition of unmodified histone H3R2 by the WD40 repeat protein WDR5 has been demonstrated (Couture *et al.*, 2006; Han *et al.*, 2006; Ruthenburg *et al.*, 2006; Schuetz *et al.*, 2006). Loss or mutation of WDR5 causes loss of H3K4me3, without affecting the levels of mono- and dimethylation or stability of the relevant MLL1 histone methyltransferase complex. Therefore it was suggested that WDR5 binding to H3R2 may exert a "presenter" function and facilitate downstream H3K4 trimethylation.

Domain		Modification recognized
PHD finger		H3K4me3, H3K4me0, H3K9me3, H3K36me3
WD40 repeat		H3R2me2, H3K4me2
Royal Superfamily	Chromodomain	H3K9me2/3, H3K27me2/3
	Double Chromodomain	H3K4me1/2/3
	Chromo barrel	H3K36me2/3
	Tudor	Rme2s
	Tandem / Double Tudor	H3K4me3, H3K4me0, H4K20me1/2
	MBT	H4K20me1/2, H1K26me1/2, H3K4me1, H3K9me1/2

Table 2 Binding domains for methylated lysines/ arginines in histones. Members of the royal superfamily are marked by grey background.

1.7.3 Recognition of histone phospho-serine marks

Introductory annotation: as major topic of the thesis the phosphorylation of serines 10 and 28 of histone H3 and their recognition by 14-3-3 proteins will is discussed separately in the sections 1.6.4 Histone phosphoacetylation a special relation in cis and section 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns". Also a discussion of the 14-3-3 protein family is provided in this section.

Induction of DNA double strand breaks prompts cells to initiate damage response pathways in order to coordinate DNA repair pathways and cell cycle arrest. One initial and crucial event for targeting of the double strand break repair machinery to relevant genomic regions involves the phosphorylation of the histone H2A variant H2A.X. Upon occurrence of double strand breaks, histone H2A.X becomes phosphorylated at S139 (known as γH2A.X) by the "ataxia telangiectasia mutated" kinase (ATM), (Rogakou *et al.*, 1998; Burma *et al.*, 2001). The "mediator of DNA checkpoint protein 1" (MDC1) was found to localize to DNA double strand breaks in a manner depending on γH2A.X (Peng and Chen, 2003). Subsequent

studies demonstrated that MDC1 directly binds to γH2A.X via its tandem BRCT domains thereby targeting the DNA double strand break repair machinery to relevant lesions (Lee *et al.*, 2005b; Stucki *et al.*, 2005). The importance of this interaction for downstream DNA repair machineries was also demonstrated, as in H2A.XS139A mutant cells MDC1 and also downstream proteins like 53BP1 are no longer recruited to DNA lesions (Stucki *et al.*, 2005).

1.7.4 Recognition of methylated arginines

Arginine methylation *via* the co-activators and arginine-methyltransferases CARM1 and PRMT1 has been implicated in stimulation of nuclear-receptor mediated and p53 dependent transcription (Strahl *et al.*, 2001; Wang *et al.*, 2001; Cuthbert *et al.*, 2004; Wang *et al.*, 2004b; Scoumanne and Chen, 2008). CARM1 targets arginine 17 in histone H3 and PRMT1 methylates histone H4R3 and both modifications are reversible by demethylation and deimination (Cuthbert *et al.*, 2004; Wang *et al.*, 2004b). Arginine can be either mono- or dimethylated at and the latter modification degree can be either symmetric or asymmetric.
The insight into the molecular basis, how histone arginine methylation impacts transcription, is relatively limited as no structural data on are available. However, it was suggested that symmetrical methylated arginines may be recognized by tudor domain containing proteins.

1.8 Chromatin remodeling

In addition to post-translational modifications of histone molecules that regulate chromatin folding (Jenuwein and Allis, 2001), the structural organization on the level of the chromatin fiber is impacted *via* chromatin remodeling enzymes. These remodeling factors have been implicated in chromatin based processes including gene transcription or repression, DNA repair and recombination, chromatin assembly or maintenance of higher structure chromatin folding (Becker, 2002).
As within the quite stable nucleosomal core particle, DNA contacts the histone octamere via several direct and water mediated interactions ((Luger and Richmond, 1998a) and section 1.2 Structural Organization of Chromatin and sub-components) break down of the octamer-DNA contact requires a net energy input. Chromatin remodeling enzymes couple the energy released from ATP hydrolysis to the temporal disruption of histone-DNA interactions, thereby facilitating the accessibility of the underlying DNA to DNA-binding proteins (Varga-Weisz *et al.*, 1995; Becker, 2002; Narlikar *et al.*, 2002; Langst and Becker, 2004).
These enzymes belong to the superfamily II of helicases (SF2) and within this group mainly to DEAD/H ATPases (Eisen *et al.*, 1995; Muchardt and Yaniv, 1999; Havas *et al.*, 2001). This fraction can be further classified into sub-families based on unique structural features of the ATPase component, complex composition and biological function (Becker, 2002; Corona and

Tamkun, 2004; Langst and Becker, 2004; Bouazoune and Brehm, 2006; Saha *et al.*, 2006; Winter *et al.*, 2007).

The first class of Swi2/Snf2 (switch2/sucrose-non-fermenting2) enzymes is characterized by a bromodomain and includes the yeast complexes SWI2/SNF2 (ySWI/SNF) and the "remodels the structure of chromatin" (RSC) complex. In higher eukaryotes the *Drosophila* Brahma complexes BAP and PBAP, the human BRM (hBRM) and BRG1 (hBRG1), and the mouse BRG1 (mBRG1) complexes belong to this class (Aoyagi *et al.*, 2002).

The "imitation switch" (ISWI) ATPases comprise the *Drosophila* "ATP-utilizing chromatin assembly and remodeling factor" (ACF), the "nucleosome remodeling factor" (NURF) and the "chromatin accessibility complex" (CHRAC). The ISWI ATPases are characterized by presence of SANT and SLIDE-domains (Tsukiyama and Wu, 1995; Ito *et al.*, 1997; Varga-Weisz *et al.*, 1997; Langst *et al.*, 1999; Grune *et al.*, 2003; Corona and Tamkun, 2004; Mellor and Morillon, 2004).

The CHD/Mi-2 family members are characterized by a chromodomain and PHD fingers. The well-characterized "nucleosome remodeling and deacetylase" (NURD) complex contains HDACs 1 and 2 together with the associated "retinoblastoma binding proteins" (RbAp) 48 and 46 and the CHD4 ATPase (Tong *et al.*, 1998).

INO80 related enzymes are characterized by a split ATPase domain. This group has been originally known and characterized in yeast and also a human homologue of the yeast INO80 complex has been described (Jin *et al.*, 2005).

The SWR1 chromatin remodeling complex has been implicated in the deposition of the histone H2A.Z variant and is also discussed in the section 1.5.2 Histone H2A variants.

Despite the overall stability of the nucleosome particle, passive movement at moderately elevated temperature and/or ionic strength has been reported (Beard, 1978; Pennings *et al.*, 1991; Meersseman *et al.*, 1992). The initial action of chromatin remodeling enzymes may be the lowering of the energy barrier and enhancement of the intrinsic properties of the nucleosome to change translational position along the DNA (Langst and Becker, 2004).

Several models for the action of chromatin remodeling enzymes have been discussed (reviewed in (Winter *et al.*, 2007)). Based on multiple *in vitro* and *in vivo* studies it was suggested that qualitative differences observed in chromatin remodeling phenomenology may be due to variation of kinetic and geometric parameters of the same basic mechanism referred to as "Loop recapture" model (Langst and Becker, 2004). According to this scenario distortion of DNA at the nucleosomal edge would disrupt DNA histone interactions and loop out a stretch of DNA. Subsequently, linker DNA would be "pulled" into direction of the nucleosome, thereby propagating the loop and therefore DNA displacement. Experimentally observed chromatin remodeling phenomenology differs between particular complexes like the ISWI and Snf2 classes. This may reflect a variation in parameters of the "loop recapture

model" rather than fundamental differences in the remodeling mechanism (Langst and Becker, 2004). Cross-linking of the histone octamer does not prevent SWI/SNF catalyzed nucleosome movement (Singleton and Wigley, 2002; Kassabov *et al.*, 2003; Smith *et al.*, 2003). Therefore, the activity for chromatin remodeling complexes may impinge mainly on DNA and not on the octamere conformation. However some chromatin remodeling complexes appear to mediate the exchange of canonical versus specialized histone variants demonstrated for the SWR1 complex and histone H2A.Z incorporation (see 1.5.2 Histone H2A variants) and also the replication independent deposition of H3.3 was found to be partially dependent on the SWI2/SNF2 type CHD1 helicase (Konev *et al.*, 2007).

Chromatin remodelers can distort DNA by inducing negative supercoils (Havas *et al.*, 2000) and the activity of DEAD/H ATPases is stimulated by nucleic acids. However, diverse remodeling complexes differ with respect to their "preferred" substrates. Yeast SWI/SNF complex and Brg1 are stimulated by free DNA whereas ISWI is fully stimulated only by nucleosomal substrates and Mi-2 requires the histone H4 amino-terminal tail for full activity. Because of the close relationship of chromatin remodeling enzymes with SF2 helicases nucleosome "sliding" may resemble the activity of ATP-dependent DNA translocases (Langst and Becker, 2004). In this way chromatin remodeling enzymes would function as fixed DNA translocases that would detach DNA from the nucleosomal edge resulting in a "loop recapture"-mechanism that would finally lead to translational deposition of the nucleosome. In support of this scenario it was found that the histone chaperon HMGB1, which promotes DNA bending, facilitates ACF mediated chromatin remodeling (Bonaldi *et al.*, 2002).

Conversely it may be expected that the degree of chromatin compaction impacts nucleosome mobility. Histone acetylation has been in general linked to decondensed chromatin states (see 1.6.3 Interdependency between transcriptional activating lysine methylation and histone acetylation and 1.7.1 Histone acetylation is recognized by bromodomains.) and appears to be also functionally linked to chromatin remodeling (Becker and Horz, 2002). The incorporation of linker histones like histone H1 into nucleosomal arrays causes compaction into a 30nm fiber conformation (see 1.2 Structural organization of chromatin and sub-components). In addition histone H1 association restricts passive and active nucleosome mobilization probably by blocking access to the critical edge of the nucleosomal DNA binding (reviewed in (Becker, 2002). The incorporation of linker histones, which cause condensation of nucleosomal arrays, may therefore restrict the activity of remodeling enzymes whereas factors that promote chromatin decondensation may cooperate with the activity of remodeling machineries.

1.9 RNA as chromatin component

Besides the DNA and protein components several studies demonstrate that also non-coding RNAs constitute structural or targeting elements of chromatin, thereby contributing to epigenetic regulation. Such RNA impacted epigenetic phenomena include dosage compensation in flies and mammals, imprinting, gene silencing and also formation of constitutive heterochromatin by RNA interference (Bernstein and Allis, 2005).

Dosage compensation is required for equalization of transcription from different numbers of sex-chromosomes in male and female organisms. In *Drosophila* expression of genes on the male X chromosome is upregulated about twofold to reach expression levels of the two female X chromosomes. Mutations that cause loss of male dosage compensation result in a male specific lethal (MSL) phenotype. The “dosage compensation” complex (DCC) or “male specific lethal” complex (MSL) is required for male specific X chromosome hyper-activation. This ribonucleoprotein complex comprises the male specific lethal proteins MSL1, MSL2, MSL3, the RNA helicase MLE, the histone H4K16 specific acetyltransferase MOF and the histone H3S10 kinase JIL1. Some of these components like MSL2 are male specific whereas others like MOF are also expressed in females. The DCC complex is specifically targeted to the male X chromosome and crucial for the transcriptional upregulation. Interestingly, two functional redundant non-coding RNAs referred to as *roX1* and *roX2* are components of the MSL-complex. These two RNAs are required for proper targeting and assembly of the complex to the male X chromosome (Gu *et al.*, 1998; Kageyama *et al.*, 2001; Meller and Rattner, 2002; Gilfillan *et al.*, 2004; Rattner and Meller, 2004). The MOF and MSL3 components of the MSL-complex contain chromodomains that were shown to bind RNAs *in vitro* and *in vivo* (Akhtar *et al.*, 2000). This RNA binding capacity of the chromodomains may contribute to a targeting mechanism of the MSL-complex to the male X chromosome via *rox*-RNAs (Akhtar *et al.*, 2000).

In contrast to the dosage compensation mechanism utilized by flies, female mammals largely inactivate transcription of one X chromosome to equalize expression levels relative to male organisms. Interestingly X-inactivation in the embryo is random in eutherian (placental) mammals like mice, whereas metatherian (marsupial) mammals show a non-random inactivation of the paternal X chromosome. Inactivation has been linked to a single genomic region on the X chromosome designated “X-inactivation center” (Xic) (Brockdorff *et al.*, 1991; Kay *et al.*, 1993; Penny *et al.*, 1996). The Xic center produces several non-coding RNAs the most prominent ones being the *Xist* RNA and its’ antisense product *Tsix*. Expression of the *Xist* RNA is restricted to the inactive X chromosome (which is coated by the RNA) and required for initiation but not maintenance of X-inactivation. In contrast, expression of *Tsix* is thought to counteract *Xist* expression (Heard *et al.*, 2004). Once silencing is initiated via the *Xist* RNA, a multitude of chromatin remodeling events lead to the formation of the Barr body

facultative heterochromatin (see also 1.4 Hallmarks of large scale chromatin elements). These chromatin alterations include histone H3/H4 hypoacetylation, methylation of histone H3K9/K27 and histone H4K20, incorporation of macro-H2A and DNA methylation. Interestingly it has been demonstrated that also localization of some member of the polycomb group one complex (PRC1) to the inactive X chromosome may not only depend on histone H3K27 methylation but also on expression of *Xist*. However the functional basis that links *Xist* expression to chromatin remodeling of the inactive X chromosome is not known.

Another strong implication for RNA as inducer of heterochromatic structures originates from observations during RNAi mediated transcriptional gene silencing. The action of RNA mediated transcriptional silencing can impinge on different levels comprising translational inhibition, RNA degradation and changes in chromatin organization. Importantly, transcriptional gene silencing effects in fission yeast have been demonstrated to be mainly mediated *via* formation of heterochromatin (Lippman *et al.*, 2004). The purification of the "RNA induced initiation of transcriptional gene silencing" (RITS) complex from fission yeast demonstrated a targeting mechanism for small RNAs produced by Dicer in the localization and activity of RITS (Ekwall, 2004; Verdel *et al.*, 2004). The RITS complex is localized to all known heterochromatic regions like centromeres, telomeres or the yeast mating type locus in a histone H3K9 methylation dependent manner (Volpe *et al.*, 2002). In addition, RNA mediated heterochromatin formation is also required for the localization of "centromeric protein A" and kinetochore formation (Folco *et al.*, 2008). The loading of RITS on these heterochromatic structures is not only dependent on key enzymes of the RNAi machinery and siRNAs but also required for the formation of additional siRNAs, which in turn mediate the propagation of the heterochromatic state (Volpe *et al.*, 2002). Further a second complex has been purified that has been designated "RNA-dependent RNA polymerase complex" which interacts with RITS and is required for the localization to centromeric heterochromatin (Motamedi *et al.*, 2004). Recent data also suggest that RITS may initially interact with the nascent transcript rather than processed siRNAs (Buhler *et al.*, 2006).

Based on these observations it was suggested that RNAi exerts a critical function in the formation of heterochromatin. This is also supported by reports demonstrating that nuclear RNAs are required to maintain the structural organization of pericentromeric heterochromatin (Maison *et al.*, 2002; Muchardt *et al.*, 2002) and loss of centromeric silencing in cells deficient for Dicer (Kanellopoulou *et al.*, 2005).

2. Results (Part 1)

2.1 Introduction

As discussed in section 1.6.1 Histone post-translational modifications and modification cross-talk, histone post-translational modifications are important factors in the regulation of chromatin structure and function. Several observations that different PTMs on a histone tail can influence each other in a reciprocal manner led to the suggestion of the "histone code" hypothesis (Strahl and Allis, 2000; Jenuwein and Allis, 2001) (see also 1.6.0 Preface to chapter 1.6 Histone Post-translational modifications).

The global phosphorylation of histone H3 at serine 10 has originally been associated with mitotic progression in all eukaryotes except plants where the kinetics and extent of this modification are different. During late G2 phase histone H3S10 phosphorylation accumulates at pericentromeric heterochromatin. In the course of mitotic progression and increasing chromosome condensation the modification spreads along chromosome arms and reaches maximum levels approximately at metaphase (Hendzel *et al.*, 1997). At the onset of anaphase, serine 10 phosphorylation steadily declines and is almost completely reduced at telophase.

The major kinase responsible for the mitosis specific phosphorylation of histone H3 at serine 10 appears to be the Aurora B component of the chromosomal passenger complex (Hsu *et al.*, 2000; Crosio *et al.*, 2002; Goto *et al.*, 2002; Vader *et al.*, 2006). However, loss of Aurora B does not completely abolish mitotic histone H3S10 phosphorylation and several additional kinases involved in mediating this modification have been identified. This group of mitosis specific enzymes includes the NIMA kinase in *Aspergillus nidulans* (De Souza *et al.*, 2000), *Drosophila* NHK1 (VRK1 in mammals) (Cullen *et al.*, 2005; Kang *et al.*, 2007) as well as the NIMA-related kinase Nercc1 in mammals (Roig *et al.*, 2005). On the other hand, the phosphatase PP1 has implicated in the removal of mitosis specific histone H3S10 phosphorylation (Hsu *et al.*, 2000).

The function of this histone modification has been an enigma since *Tetrahymena* cells with serine 10 to alanine mutation displayed severe defects in chromosome condensation and segregation (Wei *et al.*, 1999) whereas equivalent yeast mutants did not display mitotic perturbations (Hsu *et al.*, 2000). Therefore, a direct requirement for histone H3S10 phosphorylation to mediate mitotic chromatin condensation is unlikely and alternative models have been discussed (Hans and Dimitrov, 2001; Prigent and Dimitrov, 2003) (see also 4. Final discussion and additional results). Advances in the understanding of mitotic histone H3S10 phosphorylation came from the observation that this modification coincides with histone H3K9 methylation during mitosis (Fischle *et al.*, 2003a). Although initial studies using modification-specific antibodies suggested an exclusive nature of histone H3K9 methylation

and H3S10 phosphorylation (Rea *et al.*, 2000), mass spectrometry approaches unequivocally demonstrated the coexistence of both modifications on the same histone tail (Fischle *et al.*, 2005; Winter *et al.*, 2008b). The interaction of heterochromatin protein 1 (HP1) with lysine 9 methylated histone H3 is abolished upon phosphorylation of serine 10 leading to its displacement from mitotic chromosomes (Mateescu *et al.*, 2004; Fischle *et al.*, 2005; Hirota *et al.*, 2005; Terada, 2006). Thereby, histone H3S10 phosphorylation contributes to the regulation of genome accessibility and chromatin function during mitosis (Dormann *et al.*, 2006). One major effect of the mitosis specific dissociation of HP1 proteins in response to histone H3S10 phosphorylation has been described recently. Withdrawal of the yeast HP1 homologue Swi6 from mitotic chromosomes *via* histone H3S10 phosphorylation was found to be required for transcription of heterochromatic repeats in S-phase. The RNAs produced from this transcription event direct the subsequent silencing of these repeats via RNAi directed heterochromatin formation (Kloc *et al.*, 2008). Therefore histone H3S10 phosphorylation may contribute to epigenetic inheritance of constitutive heterochromatin by dynamically displacing HP1 proteins in a cell cycle specific manner.

In contrast to the mitotic distribution of histone H3 phosphorylation, this modification is much more restricted during interphase (in particular G1 phase of the cell cycle) (see also section 1.6.4 Histone phosphoacetylation a special relation in cis). During mitosis the phosphorylation is present under conditions of strong chromatin compaction, whereas for the period of interphase this modification has been correlated mainly with transcriptional activation of a restricted subset of genes (reviewed in (Winter *et al.*, 2007)). Interestingly, several reports also suggest that histone H3S10 phosphorylation can also correspond to transcriptional repressed states (Burkhart *et al.*, 2007; Sabbattini *et al.*, 2007; Komiya *et al.*, 2008). Together, these observations point to a context-dependent function of histone H3S10 phosphorylation suggesting the absence of a generalized “histone code” (see also section 1.6.0 Preface to chapter 1.6 Histone Post-translational modifications). Instead the studies discussed above and in section 1.6.4 Histone phosphoacetylation a special relation in cis rather suggest a dynamic impact of this modification on local chromatin function depending on spatially restricted biochemical parameters. In this note the interpretation of the “histone H3S10ph histone code” would depend on the particular genomic region, the stage of the cell cycle and additional modifications.

2.2 Aims of the project and summary of the presented publication

Activation of the “extracellular signal regulated kinase” (ERK)- or p38-“mitogen activated protein” (MAP) kinase cascades can mediate the phosphorylation of histone H3S10 *via* the downstream kinases MSK1/2 (Figure 2-1). This phosphorylation event has been designated “nucleosomal response” and was correlated with transcriptional activation of immediate early

genes like *c-fos* and *c-jun* in interphase cells (Thomson *et al.*, 1999; Cheung *et al.*, 2000b; Clayton *et al.*, 2000). Phosphorylation of serine 10 frequently coincides with acetylation of neighboring lysines 9 and 14 leading to the term “phosphoacetylation” (Mahadevan *et al.*, 1991; Cheung *et al.*, 2000b; Clayton *et al.*, 2000) (see section 1.6.4 Histone phosphoacetylation a special relation in cis). Besides immediate early genes it was reported that the gene encoding histone deacetylase 1 (HDAC1) is regulated by cooperative histone H3 phosphorylation and acetylation (stable phosphoacetylation) suggesting a different mechanism than for the immediate early genes, which are induced by transient histone H3 phosphoacetylation (Hauser *et al.*, 2002). Therefore, the dual modification rather than the single modifications appears to be the biological relevant factor for induction of the HDAC1 gene.

According to the “histone code” hypothesis the PTMs of histones constitute binding sites for modification dependent interaction modules (also referred to as detector or effector proteins) (see also section 1.7 Impact of post translational modifications on chromatin states.). Based on this assumption the working hypothesis for the project was that a particular factor (or factors) would bind to phosphoacetylated histone H3 tails and mediate transcriptional induction of HDAC1. In order to identify nuclear proteins that specifically interact with the phosphoacetylated form of the histone tail, an affinity chromatography approach was set up using histone H3 amino-terminal tail peptides as bait. By means of this methodology the ε and ζ isoforms of the 14-3-3 protein family were identified to specifically bind to the phosphorylated and phosphoacetylated histone H3 tail. 14-3-3 proteins were the first phospho-serine binding factors that were identified (Muslin *et al.*, 1996). Based on this background and after preliminary observations that 14-3-3 proteins indeed interact with chromatin in a histone H3 phosphorylation dependent manner, the role of this interaction was further analyzed.

To characterize the interaction between 14-3-3 proteins and histone H3 in detail, *in vitro* interaction studies were conducted. As basis for these experiments the modification “make up” of serine 10 phosphorylated histone H3 molecules (under different MAP-kinase activation conditions) was investigated via mass-spectrometry in interphase cells. This analysis demonstrated that serine 10 phosphorylation can occur as single histone H3 modification in interphase but frequently coincides with additional modifications like lysine 9 methylation and lysine 9/14 acetylation (for details refer to table II in the presented publication).

On basis of these mass spectrometry derived results a possible impact of different modification patterns on the interaction between 14-3-3 proteins and the histone H3 tail was analyzed *in vitro*. These experiments pointed out that initial binding of 14-3-3 proteins to histone H3 requires phosphorylation of the latter but was significantly enhanced when an acetyl group was present on either lysine 9 or 14. Methylation of histone H3 lysine 9 exerted

no major impact on the phosphorylation dependent interaction with 14-3-3. However, binding to a triple modified peptide (H3K9me2S10phK14ac) was again significantly enhanced relative to a “phosphomethylated” state (H3K9me2S10ph).

Chromatin immunoprecipitation analysis demonstrated that 14-3-3 proteins were also recruited to the HDAC1 promoter region in a histone H3 phosphoacetylation rather than phosphorylation dependent manner. This observation suggests that also *in vivo* the dual modification constitutes the preferred substrate for the interaction. The recruitment of 14-3-3 proteins also correlated with displacement of the transcriptional repressive module HP1 and conversion of dimethylated histone H3 in a phosphomethylated state (H3K9me2S10ph). Based on the results from the mass spectrometry analysis it is tempting to suggest that dimethylated histone H3 is converted into a triple modified state (H3K9me2S10phK14ac) which blocks HP1 binding but allows for efficient recruitment of 14-3-3 proteins.

The putative role of 14-3-3 localization to the promoter region in transcriptional activation of the HDAC1 gene was investigated by RNAi mediated depletion of the 14-3-3 ε and ζ isoforms. Upon depletion of the 14-3-3 ζ isoforms a complete loss of HDAC1 transcriptional induction was observed suggesting a role for 14-3-3 proteins during transcriptional activation.

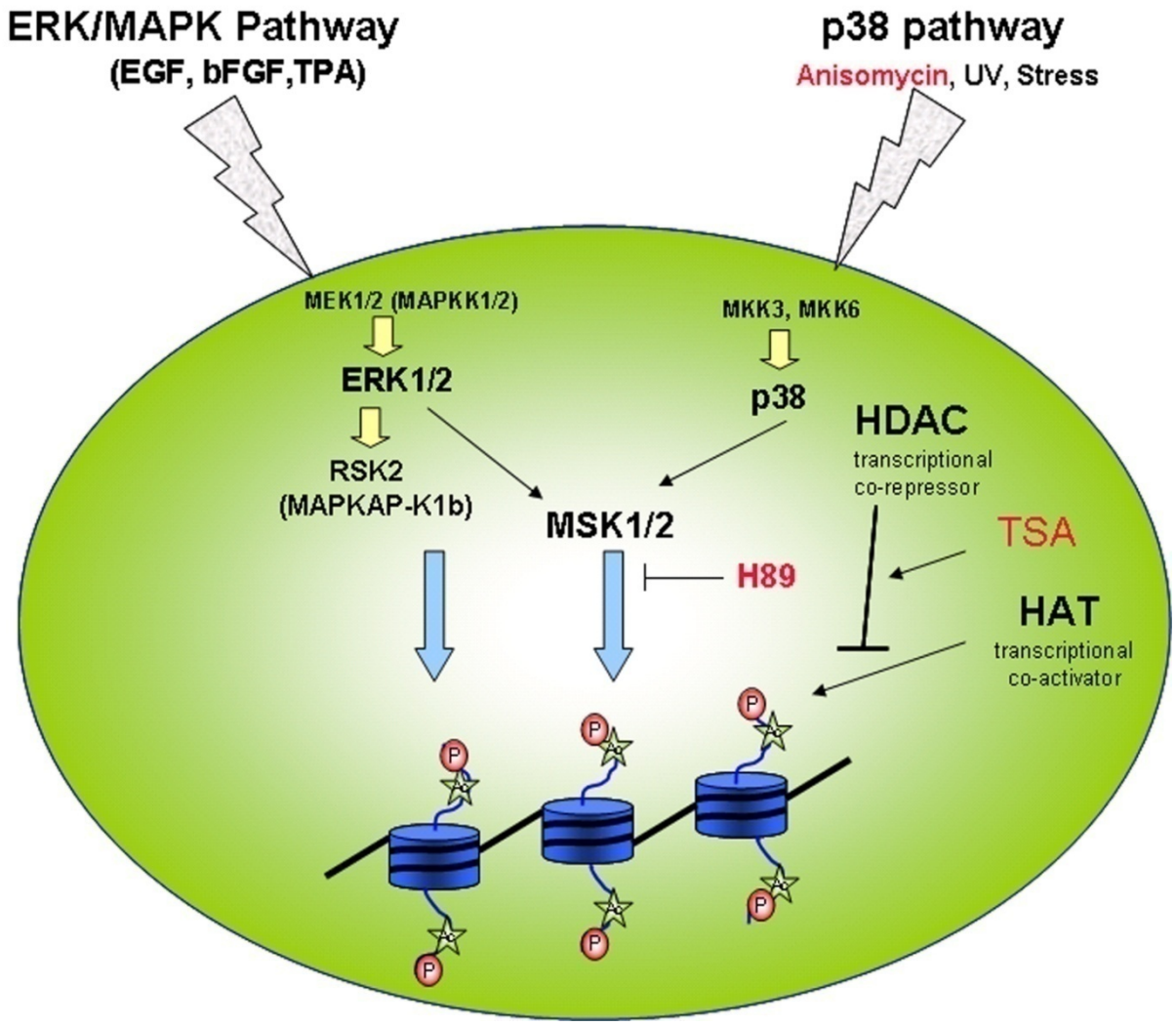

Figure 2-1 MAP-kinase cascades mediating histone H3 serine 10 phosphorylation.

Two different MAP-kinase pathways were identified that can mediate the phosphorylation of histone H3S10: the mitogen activated ERK (p42/44) pathway and the stress induced p38 pathway. ERK is stimulated by growth factors (FCS) whereas p38 is stress responsible and activated by the drug anisomycin. Downstream effector kinases, which were found to mediate histone phosphorylation are Rsk2 (MAPKAP-k1b) and MSK1/2. The latter enzyme can be efficiently inhibited by the compound H89 which is sufficient to block histone phosphorylation under active ERK- or p38- MAP kinase signaling. Acetylation is regulated by the interplay between HATs and HDACs, which are prominent components of transcriptional co-activator or co-repressor complexes respectively. HDACs are blocked by the wide range inhibitor of deacetylase activity trichostatin A (TSA), thereby inducing hyperacetylation of HDAC target proteins.

2.3 Presented Publication 1 (Winter *et al.* 2008). "14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation".

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14-3-3 Proteins recognize a histone code at histone H3 and are required for transcriptional activation

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Interphase phosphorylation of S10 at histone H3 is linked to transcriptional activation of a specific subset of mammalian genes like HDAC1. Recently, 14-3-3 proteins have been described as detectors for this phosphorylated histone H3 form. Here, we report that 14-3-3 binding is modulated by combinatorial modifications of histone H3. S10 phosphorylation is necessary for an interaction, but additional H3K9 or H3K14 acetylation increases the affinity of 14-3-3 for histone H3. Histone H3 phosphoacetylation occurs concomitant with K9 methylation *in vivo*, suggesting that histone phosphorylation and acetylation can synergize to overcome repressive histone methylation. Chromatin immunoprecipitation experiments reveal recruitment of 14-3-3 proteins to the HDAC1 gene in an H3S10ph-dependent manner. Recruitment of 14-3-3 to the promoter is enhanced by additional histone H3 acetylation and correlates with dissociation of the repressive binding module HP1γ. Finally, siRNA-mediated loss of 14-3-3 proteins abolishes the transcriptional activation of HDAC1. Together our data indicate that 14-3-3 proteins are crucial mediators of histone phosphoacetylation signals.

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Introduction

The unstructured N-terminal tails of histone proteins are targeted by various different post-translational modifications (PTMs) like acetylation, methylation, phosphorylation or ADP ribosylation. These PTMs are critical factors in the regulation of local and global chromatin function and confer distinct properties to regions of the genome that finally modulate chromatin-associated processes such as transcription. It was suggested that the specific modification make-up constitutes a 'histone code', which is recognized via a 'decoding machinery' comprised by modification-dependent, chromatin-associated polypeptides (Strahl and Allis, 2000; Fischle *et al*, 2003). One particular PTM, the phosphorylation of histone H3 at S10, emerges in two distinct phases of the cell cycle, with considerable differences in dynamics and abundance (Johansen and Johansen, 2006; McManus and Hendzel, 2006). Global mitosis-specific histone H3 phosphorylation is mediated by the Aurora B kinase (Hsu *et al*, 2000) and is required for the displacement of HP1 proteins (Mateescu *et al*, 2004; Fischle *et al*, 2005; Hirota *et al*, 2005). During interphase, H3S10 phosphorylation is targeted to only a minute fraction of nucleosomes and is tightly linked to acetylation of H3K9 and H3K14 (phosphoacetylation) (Mahadevan *et al*, 1991; Cheung *et al*, 2000b; Clayton and Mahadevan, 2003). Histone H3 phosphoacetylation can be mediated by kinases MSK1/2 that are downstream of the ERK (p42/44) or the p38 mitogen-activated protein (MAP) kinase pathways and has been correlated with transcriptional induction of the immediate-early (IE) genes *c-fos* and *c-jun* (Clayton *et al*, 2000; Cheung *et al*, 2000b; Thomson *et al*, 2001; Clayton and Mahadevan, 2003; Soloaga *et al*, 2003; Mahadevan *et al*, 2004).

The concept of gene activation by histone phosphorylation was extended to a variety of mammalian genes (Strelkov and Davie, 2002; Clayton and Mahadevan, 2003; Vicent *et al*, 2006). For instance, we reported the regulation of the HDAC1 gene by cooperative histone H3 phosphorylation and acetylation (Hauser *et al*, 2002). In contrast to the rapid and transient phosphoacetylation associated with IE gene activation, transcriptional induction of HDAC1 requires stable phosphoacetylation, which is achieved by stimulation of MAP kinase pathways and fine tuned via histone acetylation in an autoregulatory loop (Bartl *et al*, 1997; Schuettengruber *et al*, 2003).

In addition, detector proteins that specifically recognize PTMs on histones play a key role in the regulation of chromatin-associated events. For example, the bromodomains of GCN5 and TFIID250 have been shown to specifically associate with acetylated histones, while chromodomain proteins exemplified by the heterochromatin protein 1 (HP1) interact with specific methylated forms (Lachner *et al*, 2003). Recently, 14-3-3 proteins, which are well-established phospho-serine adaptor molecules (Muslin *et al*, 1996; Yaffe *et al*, 1997), have been described as detectors for phosphorylated histone H3 (Macdonald *et al*, 2005). However, the role of this interaction in the context of transcription is unclear.

Here, we report that interaction of 14-3-3ε and ζ with histone H3 is modulated by combinatorial PTM patterns. Binding of these proteins to phosphorylated H3S10 is stabilized by additional lysine acetylation. Phosphoacetylation of histone H3 at the HDAC1 promoter leads to the recruitment of 14-3-3 proteins concomitant with dissociation of HP1.

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As detector for specific active histone marks, we found that 14-3-3ζ is necessary for activation of the HDAC1 gene. Finally, we identify 14-3-3 as counterpart of the repressive binding module HP1, with reciprocal binding affinities for the modified histone H3 tail.

Results

Purification of 14-3-3 proteins as phosphoacetylated histone H3-binding factors

Histone H3 phosphorylation and acetylation synergize in transcriptional activation of the late inducible HDAC1 gene (Hauser *et al*, 2002), implying that phosphoacetylation is a biologically relevant PTM pattern. Therefore, we asked whether the phosphoacetylation mark is recognized by specific cellular factors. To this end, differentially modified matrix-coupled histone H3 peptides (Table I) were incubated with nuclear extracts from HeLa cells that were either left untreated or treated with the p38–MAP kinase activator anisomycin and the HDAC inhibitor trichostatin A (TSA). This combinatorial treatment was previously shown to stimulate histone H3 phosphoacetylation and HDAC1 gene expression (Hauser *et al*, 2002). Two modification-dependent factors of approximately 30 and 27 kDa were found that specifically interacted with the S10phK14ac (ph/ac)–histone H3 peptide (Supplementary Figure S1). These proteins were identified as the epsilon (ε) and zeta (ζ) isoforms of the 14-3-3 protein family via mass spectrometry. The presence of the two isoforms in HeLa nuclear extracts was verified by immunoblotting with isoform-specific 14-3-3 antibodies (Supplementary Figure S1B). In *in vitro* binding assays, 14-3-3ζ extracted either from untreated or anisomycin/TSA treated HeLa cells bound equally well to S10phK14ac H3 peptide, suggesting that activation of the MAP kinase pathway or HDAC inhibition does not alter the affinity of 14-3-3 for the modified histone H3 tail (Supplementary Figure S1C). Although 14-3-3 proteins have been recently shown to interact with phosphorylated histone H3 (Macdonald *et al*, 2005), the importance of this interaction for gene regulation is not yet clarified. We therefore examined the role of 14-3-3 proteins as detectors for ph/ac histone H3 and studied their role in the activation of transcription.

Table I Histone H3 peptides used in this study

Peptide	Sequence N–C
um	ARK STG GKA PRK QLC
K14ac	ARK STG GacKA PRK QLC
S10ph	ARK phSTG GKA PRK QLC
K9ac/S10ph	ARacK phSTG GKA PRK QLC
S10ph/K14ac	ARK phSTG GacKA PRK QLC
K9me^2/S10ph	Arme^{2}K phSTG GKA PRK QLC
K9me^2/S10ph/K14ac	ARme^{2}K phSTG GacKA PRK QLC
H3(1–20) um	ART KQT ARK STG GKA PRQ LC
H3(1–20) S10ph	ART KQT ARK phSTG GKA PRQ LC
H3(1–20) K9ac/S10ph	ART KQT ARacK phSTG GKA PRQ LC
H3(1–20) S10ph/K14ac	ART KQT ARK phSTG GacKA PRQ LC
H3(1–20) K9me^2/S10ph	ART KQT AR me^{2}K phSTG GKA PRQ LC
H3(1–20) K9me^2/S10ph/K14ac	ART QT AR me^{2}K phSTG GacKA PRQ LC
H3 um (25–38)	ARK SAP ATG GVK KPC
H3 S28ph (25–38)	ARK phSAP ATG GVK KPC

acK, acetylated lysine; me2K, dimethylated lysine; pS, phosphoserine.

14-3-3 Proteins interact with histone H3 in a modification-dependent manner

To verify that 14-3-3 proteins bind indeed to ph/ac histone H3, we performed GST pull-down assays with histones isolated from 3T3 mouse fibroblasts. To avoid mitotic S10 phosphorylation, we used resting cells, which show only low levels of ph/ac histone H3 and cells that were simultaneously treated with anisomycin and TSA to stimulate H3 phosphoacetylation (Figure 1A, panel i, lanes 1 and 2). After incubation with GST–14-3-3ζ or GST as control, bound histones were analyzed by immunoblotting. Probing of the blot with antibodies specific for S10phK14ac H3 revealed that this modification form could interact with GST–14-3-3ζ but not with GST (Figure 1A, panel i). The total amount of H3 associated with 14-3-3 proteins was increased upon TSA/anisomycin treatment as displayed with modification independent C-terminal H3 antibodies (Figure 1A, panel ii). A similar *in vitro* interaction was also found for 14-3-3ε (data not shown). These results indicate that 14-3-3ζ and ε bind to histone H3 in a modification-dependent manner.

Additional acetylation stabilizes the interaction between S10 phosphorylated histone H3 and 14-3-3 proteins

Histone proteins extracted from mammalian cells may carry in addition to phosphorylation and acetylation various other PTMs. To utilize a more defined set of modifications, we modified recombinant histone H3 *in vitro*. Phosphorylation or acetylation reactions were performed using MSK1 (Figure 1B, panel iii, lane 2) or the histone acetyltransferase PCAF, respectively (Figure 1B, panel ii, lane 4). Initial phosphorylation and subsequent acetylation reactions generated ph/ac histone H3 (Figure 1B, panel i, lane 3). As control, enzymes were omitted from reactions (Figure 1B, lane 1) and total amounts of H3 in the different modification reactions were visualized with the C-terminal H3 antibodies (Figure 1B, panel iv).

The interaction of *in vitro* modified H3 with 14-3-3ζ was analyzed in GST pull-down assays. As expected, phosphorylation led to association with GST–14-3-3ζ (Figure 1C, panel i), whereas acetylation by PCAF alone did not mediate any binding (Supplementary Figure S2C, and data not shown). Strikingly, 14-3-3ζ binding was stronger for phosphoacetylated than for phosphorylated H3, indicating that in the context of S10 phosphorylation acetylation exerts a stabilizing effect (Figure 1C, panel i).

Mass spectrometry analysis of MSK1-modified histone H3 revealed that not only S10 but also S28 was phosphorylated (Supplementary Figure S2D). 14-3-3 Proteins were previously shown to interact not only with H3S10ph but also with H3S28ph peptides (Macdonald *et al*, 2005). Furthermore, acetylation of the neighboring K23 residue was reported (Daujat *et al*, 2002). Therefore, we performed binding assays with different mutated histone H3 proteins. The efficiency of phosphorylation by MSK1 and acetylation by PCAF was monitored by immunoblot analysis and kinase assays with γ-^{32}P-ATP (Supplementary Figures S2A and B). Mutation of either S10 in combination with K14 or S28 and K23 led to 55–60% reduction in ^{32}P incorporation, while the quadruple

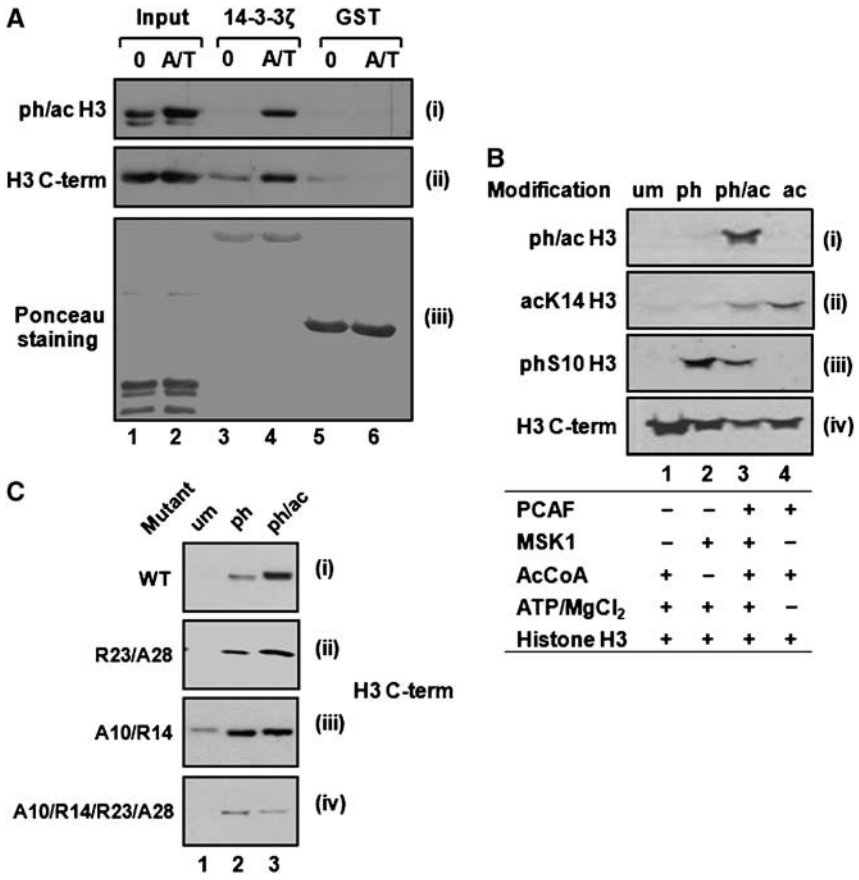


Figure 1 14-3-3 Binding to histone H3 is dependent on H3 phosphorylation and stabilized by additional acetylation. (**A**) Induction of phosphoacetylation increases histone H3 interaction with 14-3-3. Histones were isolated from resting 3T3 fibroblasts that were either left untreated (0) or stimulated for 1 h with anisomycin and TSA (A/T) and incubated with GST or GST–14-3-3ζ. Bound histones were analyzed by immunoblotting with antibodies against ph/ac histone H3 (panel i) and C-terminal histone H3 (H3 C-term) (panel ii). Loading of GST and GST–14-3-3 was monitored by Ponceau staining (panel iii). (**B**) *In vitro* modification of histone H3. Recombinant histone H3 was phosphorylated by MSK1 (lane2), acetylated by PCAF (lane 4) or phosphoacetylated with both enzymes (lane 3). Enzymes were omitted in control reactions (lane 1). The modification status was analyzed by sequential immunoblotting with the indicated antibodies. Corresponding modifications are denoted at the top. (**C**) Acetylation effects on the 14-3-3/histone H3 interaction are more dominant for the R23A28 mutant than for the A10R14 mutant. The indicated histone H3 mutants were *in vitro* modified as indicated and incubated with GST–14-3-3ζ proteins. Bound histone H3 proteins were analyzed by immunoblotting with C-terminal histone H3 antibodies. The panel shows one representative experiment for each mutant or WT histone H3.

mutant displayed about 20% residual phosphorylation (Supplementary Figure S2B). According to the mass spectrometry analysis, histones H3 becomes phosphorylated at T45 and S57 in the absence of both serines. Surprisingly, a K9R mutation significantly increased ^{32}P incorporation by MSK1 and therefore these mutants were omitted from further analysis.

Pull-down assays with histone H3 bearing K23R/S28A double mutations (R23/A28) revealed an increased interaction of 14-3-3ζ with the phosphoacetylated than to the phosphorylated form (Figure 1C, panel ii). Mass spectrometry analysis confirmed that this mutant was predominantly phosphorylated on S10. In contrast, 14-3-3ζ binding to the S10A/K14R mutant (A10/R14) was similar in the presence and absence of acetylation (Figure 1C, panel iii), and loss of both serines in the quadruple mutant resulted in only weak interaction with 14-3-3ζ. Taken together, these data indicate that the binding affinity of 14-3-3ζ for S10-phosphorylated histone H3 is significantly enhanced by additional lysine acetylation. Since combinatorial binding of 14-3-3 to target factors has not been reported, we decided to investigate this effect in more detail.

Interphase phosphorylation of histone H3 occurs in the context of additional PTMs

The efficiency of phosphorylation-mediated binding of 14-3-3 to target proteins is strongly dependent on the amino-acid environment around the phosphorylated residue (Yaffe *et al*, 1997; Uchida *et al*, 2006). As a basis for studying the impact of additional modifications on the interaction with 14-3-3, we determined PTM patterns present on S10-phosphorylated N-terminal tails of histone H3 via a mass spectrometry approach. As already mentioned, interphase phosphorylation of histone H3 affects only a small sub-fraction of all nucleosomes (Barratt *et al*, 1994). Our mass spectrometry analysis clearly indicates the presence of various different modifications like lysine methylation and acetylation in addition to H3S10ph (Table II). In agreement with previously published data (Dyson *et al*, 2005), we observed some residual histone H3 phosphorylation in samples derived from resting and untreated cells.

Anisomycin treatment increased the S10ph histone H3 pool, as well as the complexity of modification patterns (Table II). Besides single phosphorylated histone H3 and H3K9me1/2/3/S10ph forms, we identified a

Table II PTMs on the S10-phosphorylated tryptic histone H3 peptide $K_9STGGKAPR_{17}$

Condition	Peptide sequence	MH+	LTQ-FT	LTQ
Resting	R.KpSTGGKAPR.K	1093.5	0/3	1/1
	R.me^{1}KpSTGGKAPR.K	1107.556	1/3	0/1
	R.me^{2}KpSTGGKAPR.K	1065.545	2/3	0/1
	R.me^{3}KpSTGGKAPR.K	1079.561	2/3	1/1
	R.KpSTGGacKAPR.K	1079.524	1/3	0/1
	R.acKpSTGGme^{3}KAPR.K	1065.545	1/3	0/1
	R.me^{3}KpSTGGacKAPR.K			
sAn	R.KpSTGGKAPR.K	1093.540	1/3	1/1
	R.me^{1}KpSTGGKAPR.K	1107.556	2/3	1/1
	R.me^{2}KpSTGGKAPR.K	1065.545	3/3	1/1
	R.me^{3}KpSTGGKAPR.K	1079.561	3/3	1/1
	R.KpSTGGacKAPR.K	1079.525	2/3	1/1
	R.me^{1}KpSTGGacKAPR.K	1093.540	1/3	0/1
	R.me^{2}KpSTGGKacAPR.K	1051.530	1/3	1/1
	R.acKpSTGGme^{3}KAPR.K			
	R.me^{3}KpSTGGacKAPR.K	1065.5	0/3	1/1
	R.me^{3}KpSTGGme^{3}KAPR.K			
SAn/TSA	R.KpSTGGKAPR.K	1093.540	1/3	1/1
	R.me^{1}KpSTGGKAPR.K	1107.556	1/3	0/1
	R.me^{2}KpSTGGKAPR.K	1065.545	2/3	1/1
	R.me^{3}KpSTGGKAPR.K	1079.561	3/3	1/1
	R.KpSTGGacKAPR.K	1079.524	2/3	0/1
	R.acKpSTGGacKAPR.K	1065.509	2/3	1/1
	R.me^{1}KpSTGGacKAPR.K	1093.540	3/3	1/1
	R.me^{2}KpSTGGacKAPR.K	1051.530	2/3	1/1
	R.acKpSTGGme^{3}KAPR.K	1065.545	1/3	1/1
	R.me^{3}KpSTGGacKAPR.K			

acK, acetylated lysine; LTQ-FT/LTQ, the value indicates the frequency of peptide recovery either on the LTQ-FTICR hybrid instrument or the LTQ mass spectrometer (e.g., 2/3 meaning two times out of three experiments); me1K, monomethylated lysine; me2K, dimethylated lysine; me3K, trimethylated lysine; MH+, monoprotonated mass; pS, phosphorylated serine; PTM, post-translational modification; sAn, anisomycin; TSA, trichostatin A. Vertical lines indicate one peptide species where modifications were not unequivocally assigned to a particular position.

phosphoacetylated species with the acetyl moiety at position 14 (S10phK14ac). In addition, we identified a triple modified H3 peptide with the modification status K9me2S10phK14ac. This form is particular interesting as it carries an active and a repressive modification in addition to H3S10ph.

Additional treatment with TSA led to further changes in the phospho-form composition and gave also rise to a K9K14 diacetylated ph/ac form (K9acS10phK14ac). While TSA treatment had no effect on overall S10 phosphorylation (Supplementary Figure S3A, panel i, lanes 5 and 6 and Supplementary Figure S3B), the abundance of the S10phK14ac epitope was almost doubled (Supplementary Figure S3A, panel i, compare lanes 2 and 3). These results suggest that p38 MAP kinase activation leads to the formation of several different phospho-histone H3 forms and the composition of this pool is altered by additional TSA treatment. Taken together, interphase H3S10 phosphorylation occurs as single modification, but frequently coincides with lysine methylation and acetylation on the same histone H3 tail.

Additional histone modifications affect the 14-3-3/histone H3 interaction

To investigate the impact of lysine acetylation and methylation on the interaction between 14-3-3 and histone H3, we performed *in vitro* peptide pull-down assays. This experimental setup also allowed us to use a homogenously modified system for the interaction studies. Differentially modified histone H3 peptides were synthesized on the basis of the mass spectrometry results (Table I). Since we are interested in the role of H3S10ph during transcriptional activation, we focused on modifications that are known to prevalently reside in euchromatin and excluded H3K9me3, the archetype of heterochromatic histone modifications. Equal amounts of the differentially modified immobilized H3 peptides were incubated with *in vitro* translated (IVT) 14-3-3ζ protein. Phosphorylation of H3S10 was required for significant interaction with 14-3-3 (Figure 2A, lane 2), whereas only slight background signals were observed for the unmodified or the H3K14ac peptide (Figure 2A, lane 1, and data not shown).

Acetylation of H3K9 caused a moderate but reproducible reinforcement of the interaction (Figure 2A, lane 3). Remarkably, additional acetylation of H3K14 strongly increased the interaction, supporting the results from the GST pull-down experiments (Figure 2A, lane 4 and Figure 1C). To rule out the possibility that the increased binding of 14-3-3ζ to the H3S10phK14ac peptide is a unique property of IVT or recombinant proteins, we confirmed this effect with endogenous 14-3-3 present in HeLa nuclear extracts (Figure 2B).

In contrast, binding to the H3K9me2S10ph peptide was slightly reduced compared with the S10ph peptide (Figure 2A, compare lanes 2 and 5). Our mass spectrometry analysis revealed the presence of a K9me2S10phK14ac histone H3 form (Table II). Binding studies with the corresponding triple modified peptide (K9me2S10phK14ac) demonstrated that additional acetylation of K14 also increased binding of 14-3-3ζ to the phosphomethylated H3 peptide (Figure 2A, lane 6).

To further confirm the stabilizing effect exerted by H3K14 acetylation, we performed competition assays using the H3S10phK14ac peptide as bait and free unmodified, H3S10ph and H3S10phK14ac peptides as competitors (Figure 2C, lanes 4–6). Addition of the H3S10ph peptide reduced binding of 14-3-3ζ to the H3S10phK14ac peptide to approximately 65% compared with non-competed binding, whereas the unmodified peptide had no effect (Figure 2C, lanes 4 and 5). Importantly, the H3S10phK14ac peptide was found to be a much more potent competitor with an average reduction of binding to about 15% of non-competed assays (Figure 2C, lane 6). These data also demonstrate that the binding of 14-3-3ζ to the phosphoacetylated histone H3 tail is highly dynamic and reversible.

Together, our data suggest that significant binding of 14-3-3 to the histone H3 tail requires initial phosphorylation but is susceptible to the presence of additional PTMs.

To quantify the binding of 14-3-3 to various combinations of H3 modifications, we determined dissociation constants of the interactions. Therefore, we performed fluorescence polarization measurements using recombinant 14-3-3 in combination with fluorescinated, differentially modified H3 peptides (Figure 3). In concordance with the peptide pull-down assays, we detected strong interaction of 14-3-3 with the histone H3 peptide upon phosphorylation of S10, whereas only very weak interaction with the unmodified peptide was observed. The H3K9me2S10ph peptide was bound with comparable strength as the H3S10ph peptide, suggesting that K9me2 does not significantly impair the binding of 14-3-3. The affinity of 14-3-3 for the H3S10ph and the H3K9me2S10ph

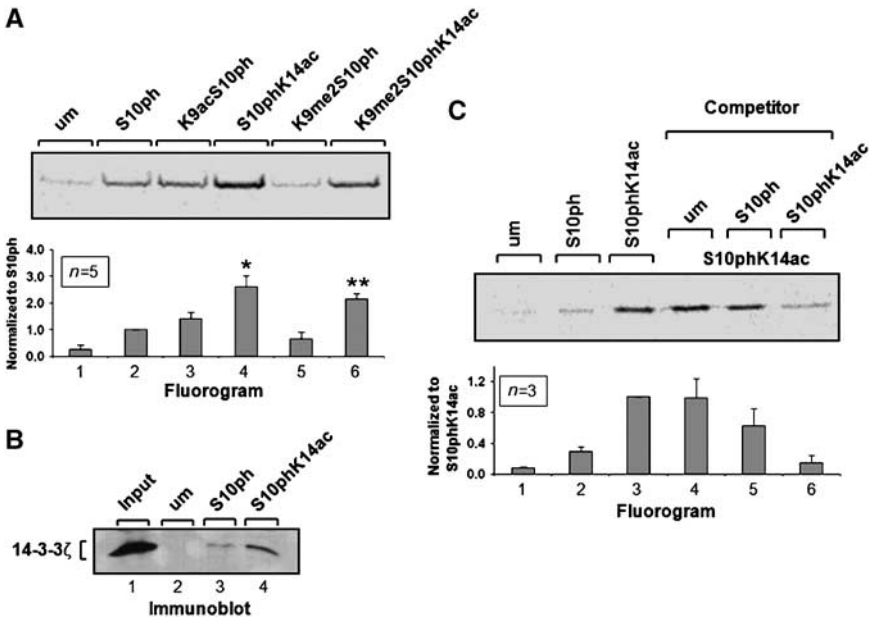


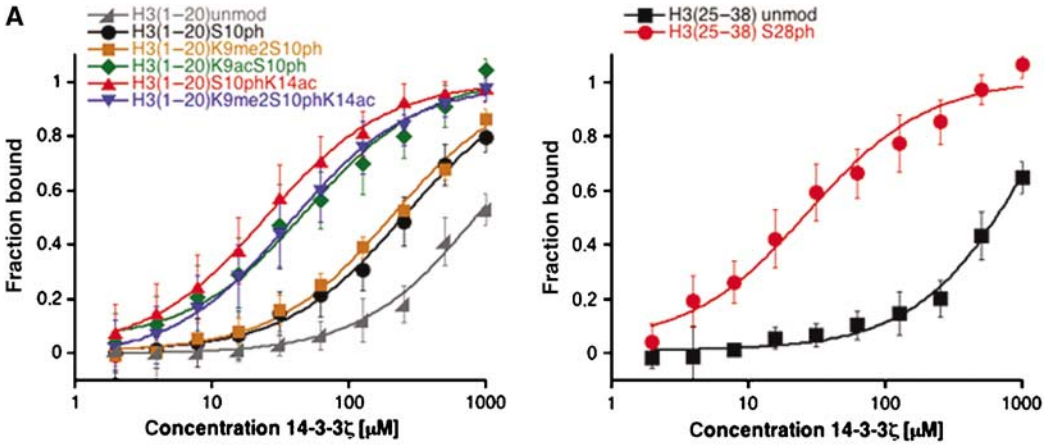
Figure 2 Modulation of the histone H3/14-3-3 interaction by additional modifications. (**A**) Histone H3/14-3-3 interaction is modulated by additional lysine acetylation. IVT ^{35}S-methionine-labeled 14-3-3ζ was incubated with differentially modified gel-coupled histone H3 peptides. Bound proteins were analyzed by SDS–PAGE and fluorography. The panel shows one representative experiment. The signal intensity for each band was quantified and is depicted as summary of five independent measurements (mean ± s.d.). Values were normalized relative to H3S10ph peptide-bound fraction (lane 2). Additional acetylation increased the association with 14-3-3 proteins (lane 4, $*P = 0.001$, *t*-test). A similar effect of H3K14 acetylation was observed for the H3K9me2/S10ph peptide (lane 7, $**P < 0.001$, *t*-test). (**B**) Nuclear 14-3-3 proteins preferentially bind to the S10phK14ac histone H3 peptide. Nuclear extracts were incubated with unmodified, S10ph or S10phK14ac H3 peptides. An aliquot of the nuclear extracts was used as input control. Bound proteins were analyzed on immunoblots with 14-3-3ζ antibodies. (**C**) Additional K14 acetylation increases the competitor potential of the S10ph histone H3 peptide. Binding reactions were performed as described for panel A (lanes 1–3). In addition, binding reactions on the ph/ac peptide were performed in the presence of a 20-fold molar excess of unmodified, H3S10ph or H3S10phK14ac free competitor peptides. Bound 14-3-3ζ proteins were analyzed by SDS–PAGE and fluorography. Each signal was normalized to the non-competed H3S10phK14ac peptide binding (lane 3) and is depicted as histogram showing the average of three independent experiments (mean ± s.d.).

peptide was further enhanced by additional acetylation. This effect was slightly more pronounced for K14ac ($K_d = 49\,\mu M$ for K9ac/S10ph and $K_d = 35\,\mu M$ for S10ph/K14ac). The H3S28ph peptide was bound with much higher initial affinity than the H3S10ph peptide ($K_d = 30\,\mu M$), which may possibly be attributed to the proline at position 30 that can also be found in one of the high-affinity 14-3-3-binding motifs RSXSpXP, where a proline is located at position $n + 2$ from the phosphorylated serine (Yaffe *et al,* 1997). Importantly, similar results were also obtained with 14-3-3ζ without GST-tag and 14-3-3ε (Figure 3B).

In conclusion, our biochemical and biophysical studies indicate a function of the double and triple modified histone H3 forms in the recruitment of 14-3-3 proteins: interaction between histone H3 and 14-3-3 is mediated by S10 phosphorylation and acetylation of K9 or K14 significantly increases the affinity of 14-3-3 for the histone H3 tail.

14-3-3 Proteins are recruited to the promoter region of the HDAC1 gene by histone H3 phosphoacetylation

Next, we sought to determine whether 14-3-3 proteins associate with the HDAC1 gene in a histone H3 phosphoacetylation-dependent manner. As shown previously (Hauser *et al*, 2002), HDAC1 expression in resting 3T3 fibroblasts was low but could be efficiently stimulated by combinatorial treatment with anisomycin and TSA (Figure 4A). In contrast, anisomycin alone did not induce HDAC1 transcription and TSA had an intermediate effect. A possible recruitment of 14-3-3 proteins to the HDAC1 promoter region was investigated by ChIP assays of resting and stimulated 3T3 fibroblasts (Figure 4B; Supplementary Figure S4B). Histone H3 phosphoacetylation was absent from the HDAC1 promoter region in resting cells, both in the presence and absence of TSA (Figure 4B, panel iv). Anisomycin treatment moderately elevated the levels of ph/ac histone H3, whereas additional treatment with TSA led to high levels of phosphoacetylation that are linked to transcriptional induction of the HDAC1 gene (Figure 4A and B; Hauser *et al*, 2002). Interestingly, the recruitment of 14-3-3ζ to the HDAC1 promoter was in strong correlation with the levels of ph/ac histone H3 (Figure 4B, panel v, lanes 1–4). In contrast, H3 phosphoacetylation and 14-3-3 recruitment were not observed at control genes such as β-actin (Figure 4B, panel v, lanes 5–8) or histone H4 (data not shown). As transcriptional activation can be accompanied by a reduction of nucleosome density within the proximal promoter region (Yuan *et al*, 2005), ChIP assays with C-terminal histone H3 antibodies showed that activation of the HDAC1 promoter resulted in only a slight reduction in histone occupancy (Figure 4B, panel iii; Supplementary Figure S4B). However, similar changes in nucleosome density upon anisomycin/TSA treatment were also observed for the actin control region. In accordance with the data from the mass spectrometry analysis, TSA treatment had no effect on the anisomycin-mediated H3S10 phosphorylation on the HDAC1 promoter (Figure 4C, panel iv). ChIP assays with H3K9me2 antibodies showed reduced dimethylation in response to



B

Peptide (H3 1–20)	14-3-3ζ-GST	14-3-3ζ	14-3-3ε-GST	GST
um	418±81	>1000	840±130	nb
S10ph	137±20	240±39	222±35	nb
K9me2	378±121	>800	473±104	nb
K9me2S10ph	161±11	200±18	211±21	nb
K9acS10ph	49±18	42±6	116±26	nb
S10phK14ac	35±13	26±9	73±19	>700
K9me2S10phK14ac	61±12	40±10	121±23	>700
um (25-38)	590±96	>1000	990±189	>1000
S28ph (25-38)	30±7	23±4	55±14	>500

Figure 3 Binding of 14-3-3 to an H3S10ph peptide is enhanced by additional lysine acetylation. (**A**) Lysine acetylation increases the affinity of 14-3-3 for the phosphorylated H3 peptide. Binding of 14-3-3ζ to the indicated H3 peptides was analyzed by fluorescence polarization measurements. The panel shows the average of at least three independent measurements (mean ± s.d.). (**B**) Dissociation constants (K_d in μM) for the interaction of different 14-3-3 isoforms with the indicated histone H3 peptides determined by fluorescence polarization measurements. Values are average (mean ± s.d.) of at least three independent measurements.

anisomycin and in particular to anisomycin and TSA (Figure 4C, panel iii). However, dot blot analysis with different modified histone H3 peptides revealed that additional S10 phosphorylation reduced the binding affinity of the H3K9me2 antibodies (Supplementary Figure S4). Therefore, we cannot deduce a reduction in H3K9 dimethylation in response to stimulation by anisomycin. To circumvent this problem, we used in the following experiment antibodies directed against H3K9me2S10ph (Supplementary Figure S4A).

Our *in vitro* studies show that 14-3-3ζ is a high-affinity detector protein for ph/ac histone H3. To confirm that 14-3-3ζ localization to the HDAC1 promoter is indeed dependent on ph/ac histone H3, we used the kinase inhibitor H89, a potent suppressor of the nucleosomal response (Thomson *et al*, 1999). Pretreatment of cells with H89 abolished anisomycin/TSA induced H3 phosphoacetylation at the HDAC1 promoter (Figure 4E, panel iii; Supplementary Figure S4B) and significantly reduced transcriptional activation (Figure 4D). Importantly, the recruitment of 14-3-3ζ to the promoter was strongly impaired upon inhibition of the nucleosomal response (Figure 4E, panel iv). Phosphomethylation of histone H3, which was increased upon anisomycin/TSA treatment, was also sensitive to H89 (Figure 4E, panel v). In agreement with data from the mass spectrometry analysis, this finding indicates that at responsive loci the K9 methylated histone H3 is converted into a multiple modified form. HP1 proteins recognize and bind methylated K9 at histone H3 and were previously shown to be involved in the repression of euchromatic genes (Nielsen *et al*, 2001; Ogawa *et al*, 2002; Hediger and Gasser, 2006). Therefore, we analyzed the effect of H3 phosphorylation on the binding of HP1 in a euchromatic environment. ChIP assays revealed that HP1γ was located at the HDAC1 promoter in resting cells, but was released upon induction of phosphoacetylation and phosphomethylation (Figure 4E, panel vi, lanes 1 and 2). HP1 dissociation was blocked by H89, indicating that the nucleosomal response is important not only for the recruitment of 14-3-3ζ but also for the release of HP1γ (Figure 4E, panel vi, lane 3). We conclude that activation of the HDAC1 gene is associated with differential localization of two histone H3-binding modules HP1 and 14-3-3. These show opposing binding affinities for combinatorial modifications of histone H3. The transcriptional 'off-state' is characterized by H3K9me2 and HP1γ binding, while activation is linked to phosphoacetylation/phosphomethylation, recruitment of 14-3-3ζ and concomitant HP1γ displacement.

14-3-3ζ Is required for transcriptional induction of the HDAC1 gene

Given that 14-3-3ζ is recruited to the HDAC1 promoter region in a phosphoacetylation-dependent manner, we wanted to determine whether this recruitment has an impact on transcriptional induction. To address this question, we used siRNA-mediated depletion of 14-3-3ε and ζ proteins in HeLa cells, as pilot experiments indicated that 14-3-3 knockdown was most efficient in this cell system. In addition, several phosphoacetylation target genes that were previously identified in 3T3 mouse fibroblasts were confirmed to be activated

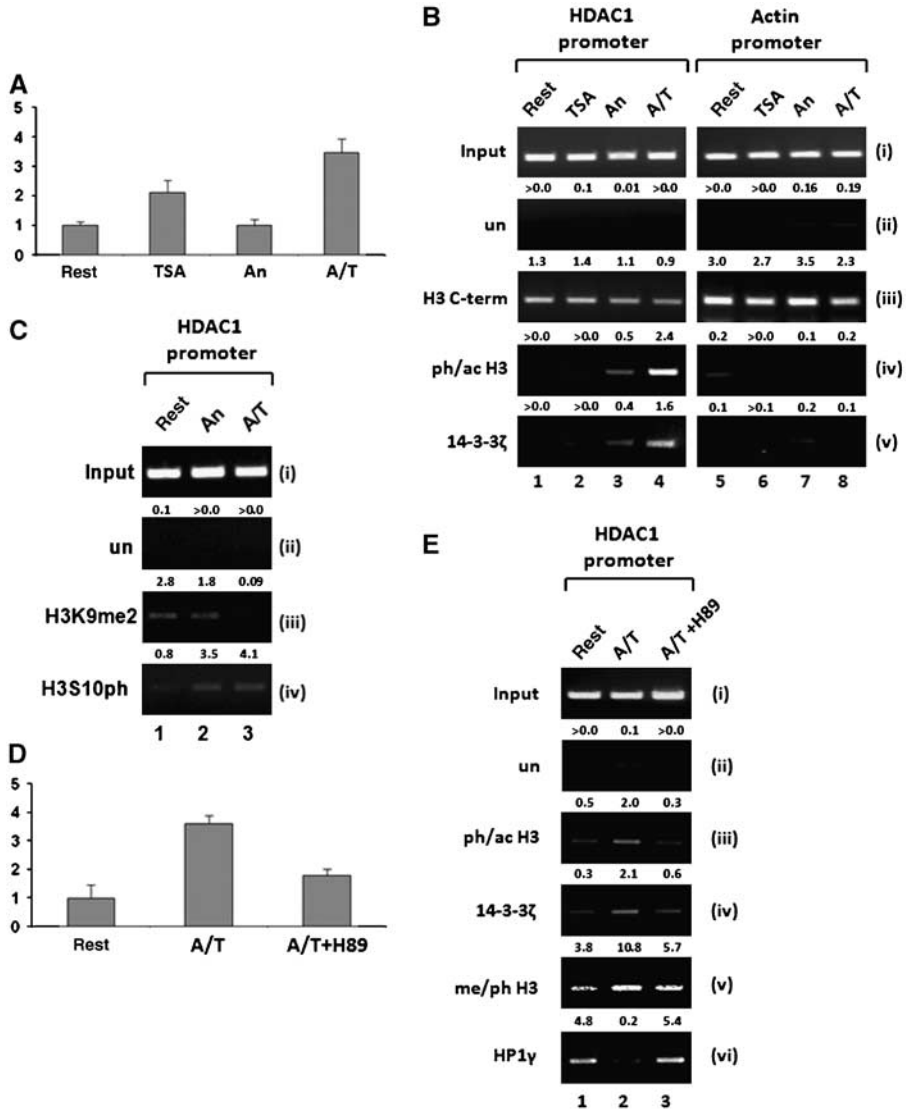


Figure 4 Localization of 14-3-3 proteins to the HDAC1 promoter correlates with histone H3 phosphoacetylation. (**A**) Phosphorylation and acetylation of histone H3 synergize in HDAC1 gene expression. Resting 3T3 fibroblasts were either left untreated (rest) or stimulated with TSA, anisomycin (An) or both drugs simultaneously (A/T) for 6 h. HDAC1 mRNA expression was analyzed by quantitative real-time RT–PCR and normalized to β-actin. Data are depicted relative to the untreated control as average of two independent experiments (mean ± s.d.). (**B**) Localization of 14-3-3ζ to the HDAC1 promoter correlates with phosphoacetylation of histone H3. Chromatin was prepared from resting and TSA-, anisomycin- or TSA/anisomycin-stimulated 3T3 fibroblasts and ChIP assays were performed with the indicated antibodies. Rabbit pre-immune serum was used as unspecific antibody control (un). Precipitated and input DNAs were analyzed by semi-quantitative PCR with primers specific for the HDAC1 promoter and the β-actin promoter. Intensities were quantified relative to input DNA (arbitrary units) and are indicated above the panels. (**C**) The H3K9me2 and H3S10ph epitopes are inversely present at the HDAC1 promoter. Induction of histone H3 phosphoacetylation via anisomycin (An) or combinatorial treatment (A/T) leads to reduced epitope availability for the H3K9me2 antibody whereas the H3S10ph antibody is only slightly affected. (**D**) Transcriptional super-induction of HDAC1 requires the nucleosomal response. Resting 3T3 fibroblasts were treated with anisomycin and TSA (A/T), or in addition pretreated with H89 (10 μM) 15 min before A/T treatment (A/T + H89). Expression analysis was performed as described for panel A. (**E**) The nucleosomal response is required for localizing 14-3-3ζ to the HDAC1 promoter and to generate the phosphomethyl dual mark. ChIP analysis with antibodies specific for modified histone H3, 14-3-3ζ and HP1γ was performed as described for panel B.

by anisomycin and TSA in this system (data not shown). Protein and mRNA levels of 14-3-3ε and ζ were efficiently reduced by siRNA-mediated gene silencing, while the levels of both isoforms were unaffected by an unspecific control siRNA (Figure 5A, panels i and iii, lanes 1–4; Supplementary Figure S5A). Anisomycin and TSA treatment did not affect the efficiency or specificity of the knock down (Figure 5A, panels i and iii, lanes 5–8; Supplementary Figure S5A).

Next, we examined the impact of 14-3-3 depletion on H3 phosphoacetylation. Since 14-3-3 proteins are important components of MAP kinase pathways (Xing *et al*, 2000), we first analyzed whether the signal transduction cascades mediating H3 phosphoacetylation remain intact in cells depleted for particular isoforms. No significant change in bulk histone phosphoacetylation was observed in 14-3-3 knockdown cells (Supplementary Figure S5B), indicating that the p38 MAP kinase pathway remains fully functional. Furthermore, ChIP experiments of 14-3-3 knockdown cells confirmed that phosphoacetylation at the HDAC1 promoter was not impaired upon loss of either of the two 14-3-3 proteins (Figure 5B, panel iii).

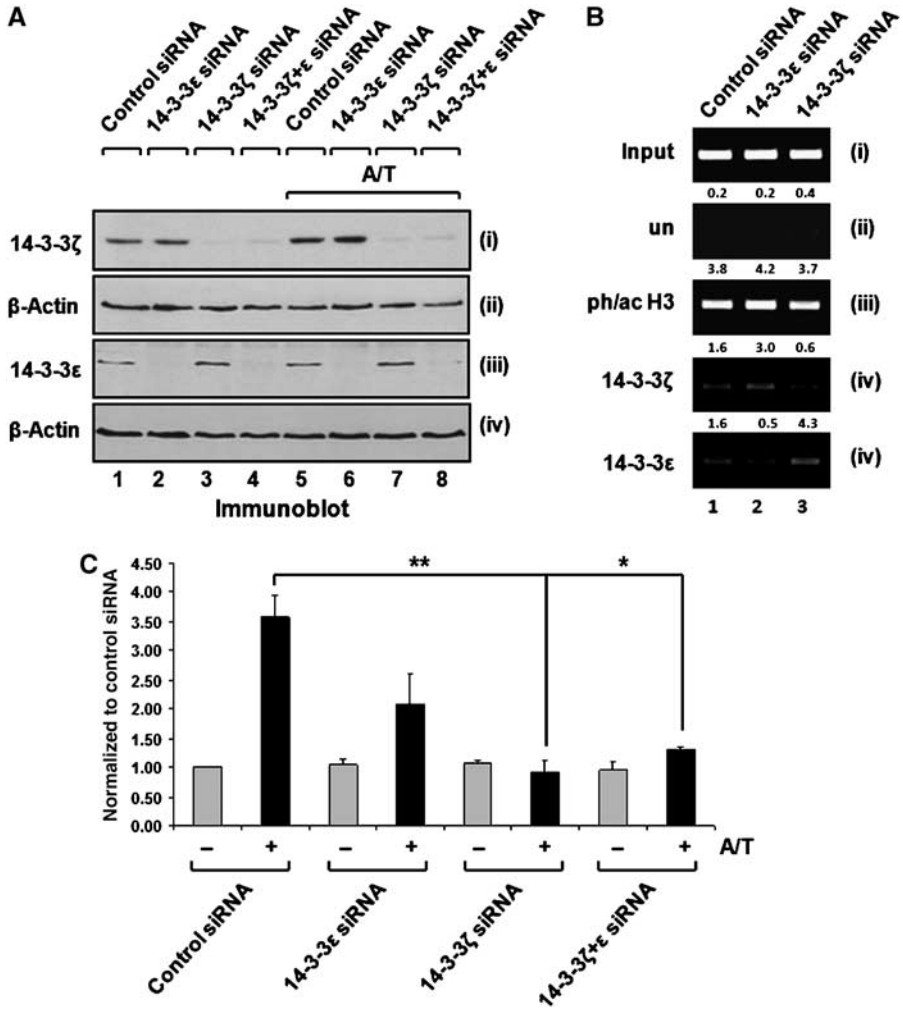


Figure 5 14-3-3ζ Is required for transcriptional activation of the HDAC1 gene by anisomycin and TSA. (**A**) Isoform-specific depletion of 14-3-3 proteins by RNA interference. HeLa cells were transfected with siRNAs against 14-3-3ε or ζ and either left untreated or stimulated with anisomycin and TSA (A/T). Whole-cell lysates were prepared and analyzed for 14-3-3 protein levels by immunoblotting. Equal loading was confirmed with a β-actin antibody. (**B**) 14-3-3 Depletion has no impact on the phosphoacetylation status of histone H3. HeLa cells, depleted of 14-3-3ε or ζ isoforms, and control cells were stimulated with TSA and anisomycin (sAn) for 1 h. ChIP assays were performed with the indicated antibodies. (**C**) Depletion of 14-3-3ζ interferes with transcriptional activation of the HDAC1 gene. HeLa cells were transfected with siRNAs as described for panel A. HDAC1 expression levels were determined by real-time RT–PCR, normalized to GAPDH levels and are depicted as fold increase compared with untreated samples, which were transfected with control siRNA. Values represent three independent experiments (mean ± s.d.). Depletion of 14-3-3ζ interferes with the induction of HDAC1 by anisomycin and TSA treatment (lane 6, $^{**}P = 0.002$ and lane 8, $^{*}P = 0.01$, *t*-test), whereas 14-3-3ε depletion caused a more moderate reduction (lane 4, $P = 0.07$, *t*-test).

Finally, we examined the effect of 14-3-3 knockdown on the induction of the HDAC1 gene, which can be induced by combinatorial treatment with anisomycin and TSA in proliferating HeLa cells (Figure 5C, lanes 1 and 2). Depletion of 14-3-3ε had a negative impact on HDAC1 activation (Figure 5C, lanes 3 and 4). However, this effect was not statistically significant ($P = 0.07$) and therefore a direct role for 14-3-3ε in HDAC1 expression cannot be reliably inferred. However, loss of 14-3-3ζ strongly interfered with transcriptional induction, suggesting an activating role of 14-3-3ζ at the HDAC1 promoter (Figure 5C, lanes 5 and 6). The same effect was observed for the 14-3-3ε and ζ double knockdown (Figure 5C, lanes 7 and 8).

Altogether, these data indicate that 14-3-3 proteins recognize ph/ac histone H3 and are required for the induction of the phosphoacetylation target gene HDAC1.

Discussion

Additional PTMs modulate the phosphorylation-dependent interaction of histone H3 with 14-3-3

In this report we investigated the function of 14-3-3ε/ζ as detector proteins for phosphoacetylated histone H3 and their roles in transcriptional regulation. The first important finding of this study is the modulation of the phosphorylation-dependent interaction between 14-3-3 and the histone H3 tail. Although the binding affinities of 14-3-3 proteins were correlated with the amino-acid sequence, flanking the phosphorylated residue (Yaffe *et al*, 1997; Uchida *et al*, 2006), this is to our knowledge the first report demonstrating that this interaction can be affected by PTMs. Phosphorylation of histone H3 is clearly required to mediate interaction of histone H3 with 14-3-3. In contrast, acetylation as single

modification does not result in significant interaction. However, in the context of H3S10 phosphorylation, additional acetylation causes a significant shift in the equilibrium toward the bound state (Figures 2 and 3). This effect was observed in a more pronounced manner for S10 phosphorylation rather than for S28 (Figure 1C, and data not shown), which may be addressed to the initial higher affinity of 14-3-3 for the S28ph peptide (Macdonald *et al*, 2005; Figure 3). In this scenario, lysine acetylation could function as an 'auxiliary modification' that supports the relatively weak interaction of 14-3-3 with H3S10ph, whereas the significantly stronger interaction with H3S28ph is less reliant on additional modifications. In addition, the dual modification would allow for an additional level of regulation. By relying on two modifications for complete stimulation, a more fine-tuned response could be achieved. Especially since the machineries laying down the two marks are different and respond to distinct signaling pathways. Although a previous study indicated no significant effect of dual H3K9/K14 acetylation on this interaction (Macdonald *et al*, 2005), our data set clearly indicates a modulation of the 14-3-3/histone H3 interaction by single acetylation. One explanation could be that the positive effect of single acetylation observed in our study is neutralized by the dual acetylation mark. It might be that a single acetylation helps stabilizing the interaction by generating additional contacts within the phosphoacetyl peptide and also with 14-3-3. These findings were also corroborated by *in vivo* experiments. MAP kinase activation resulted in low levels of ph/ac histone H3 and minor recruitment of 14-3-3ζ to the HDAC1 promoter region; additional TSA treatment increased local phosphoacetylation and 14-3-3 recruitment (Figure 4B). Given that the deacetylase inhibitor does not affect the abundance of the H3S10ph modification *per se* (Hauser *et al*, 2002; Supplementary Figure S3), these data suggest that the phosphoacetylated form is also preferred in native chromatin.

Since several 14-3-3-interacting proteins such as HMGN1 or p53 are targeted by multiple different PTMs, this finding is of particular interest regarding 14-3-3 biology. We hypothesize that additional PTMs could also modulate 14-3-3 binding to other proteins. Since the amino-acid composition of histone H3 surrounding S10 does not match one of the high-affinity 14-3-3-binding motifs, it is possible that the postulated modulation of binding may only be relevant for a specific subset of 14-3-3-associated proteins with initial low-affinity binding.

Combinatorial histone modifications and detector proteins

While in mitosis the majority of histone H3 is decorated by the S10ph mark, activation of the MAP kinase pathway in resting cells leads to the phosphorylation of a minute fraction of H3 molecules. Our mass spectrometry analysis reveals the presence of additional lysine methylation and acetylation besides HS10ph in particular upon combinatorial treatment of cells (Table II). We did not observe an H3K9acS10ph form in anisomycin-stimulated cells, although previous research clearly demonstrated the generation of this species under these conditions. One possible reason may be the extremely low abundance of this species that could be picked up by high-affinity antibodies but not by bulk analysis using mass spectrometry. The potential modulation of protein–histone interactions by different PTM combinations was formulated as the histone code hypothesis (Cheung *et al*, 2000a; Strahl and Allis, 2000), postulating that PTMs can cooperate in directing the accessibility of chromatin. In agreement with this hypothesis, 14-3-3 binding to H3S10ph is enhanced by additional active marks (K14ac or K9ac). Interestingly, HP1γ as a detector of the repressive H3K9me2 mark shows a reciprocal affinity: phosphorylation or phosphoacetylation of histone H3 triggered the displacement of HP1 from mitotic chromatin (Mateescu *et al*, 2004; Fischle *et al*, 2005; Hirota *et al*, 2005). It is important to mention that 14-3-3ε and ζ are globally excluded from mitotic chromosomes (Macdonald *et al*, 2005, and data not shown), which may be attributed to the generation of a '14-3-3 sink' during mitosis (Margolis *et al*, 2006). Thus it appears that 14-3-3 proteins are specific detectors for phosphorylated histone H3 during interphase.

14-3-3 Proteins are required for transcriptional induction of the HDAC1 gene

Based on the diametrical binding affinities of 14-3-3 for ph/ac histone H3 and HP1γ for H3K9me2, it is tempting to speculate that the two factors epitomize different transcriptional states in the regulation of particular target genes. This concept is supported by a recent report, which demonstrated HP1γ displacement during transcriptional initiation concomitant with H3S10ph during progesterone receptor-mediated gene activation (Vicent *et al*, 2006).

We observed H3K9me2 and localization of HP1γ at the promoter of the transcriptional silent HDAC1 gene (Figure 4C and E). Stimulation of H3 phosphoacetylation resulted in the recruitment of 14-3-3ζ (Figure 4B and E), which correlated with the displacement of the transcriptional repressor HP1γ (Figure 4E). Inhibition of the nucleosomal response resulted in reduced transcriptional responsiveness accompanied by HP1γ retention and absence of 14-3-3 (Figure 4E). Therefore, one particular function of histone phosphorylation in this context may be replacement of the transcriptional repressive module. We cannot completely rule out that lack of 14-3-3 recruitment and persistence of HP1γ at the HDAC1 promoter may be caused by reduced transcriptional responsiveness of the promoter upon kinase inhibition. However, the adaptations of the chromatin embedding the HDAC1 promoter region occur much earlier than transcriptional initiation (Hauser *et al*, 2002). Together with the contrary binding affinities of HP1 and 14-3-3 for phosphomethylated/acetylated histone H3 and the appearance or absence of these marks in the different treatment conditions, we consider a direct effect via the reduction of H3S10 phosphorylation more likely than an indirect via reduced transcriptional responsiveness.

Our ChIP experiments suggest that the H3K9me2 mark at the HDAC1 promoter is converted to K9me2S10ph rather than being actively removed (Figure 4E). This scenario is also supported by mass spectrometry results where corresponding double and triple modified states of histone H3 have been identified (Table II). These observations expand the binary switching model (Fischle *et al*, 2003) from a mitosis specific mechanism to interphase transcription and implicate a particular function of histone H3 phosphorylation in this process.

The 14-3-3 RNAi experiments demonstrate a requirement for 14-3-3 proteins in the transcriptional induction of HDAC1 (Figure 5C). In agreement with earlier studies on the function

of 14-3-3 proteins in MAP kinase cascades (Xing *et al*, 2000), a reduction did not impair H3 phosphoacetylation. However, depletion of 14-3-3ζ almost completely abolished the transcriptional induction of HDAC1. Thus, 14-3-3 proteins are crucial factors for mediating the switch from transcriptional repressive to active chromatin *in vivo*, although they appear not essential for establishing histone phosphoacetylation *per se*.

Based on our analysis, we suggest the model shown in Figure 6 for induction of the HDAC1 gene. The transcriptional silent state encountered in resting cells is epitomized by the presence of H3K9me2 bound by HP1γ and the absence of ph/ac histone H3. Stimulation of histone H3 phosphoacetylation leads to HP1γ displacement (Figure 4B and E; E Simboeck and C Seiser, unpublished observations) and consolidated binding of 14-3-3, which can now trigger the switch to transcriptional competent chromatin environment in the presence of H3K9me2. Binding of 14-3-3, in turn, might stabilize the ph/ac mark on histone H3. We also hypothesize that the activity of phosphatases and deacetylases may be required for the conversion of me2/ph and me2/ph/ac histone H3 into a K9me2 form, which could trigger subsequent relocalization of HP1 to the promoter region and transcriptional 'shut-down' of the target.

To examine a more general role of the dual H3 modification in gene activation, we have recently identified a set of novel phosphoacetylation target genes in mouse fibroblasts (E Simboeck, C Hauser and C Seiser, unpublished observations). Importantly, all tested target genes showed recruitment of 14-3-3 to their promoters and a requirement for 14-3-3ζ for full gene activation. These results suggest that 14-3-3 proteins have a more general role in the regulation of genes targeted by histone H3 phosphoacetylation.

Materials and methods

Plasmid construction and site-directed mutagenesis

Expression vectors for N-terminal GST-tagged mouse 14-3-3ε and 14-3-3ζ were produced by conventional PCR cloning of cDNA into the pGEX-5X2 vector (Amersham Biosciences). For T7 promoter-driven *in vitro* transcription/translation reactions, 14-3-3ε and 14-3-3ζ cDNAs were cloned into the pCINeo vector (Promega). Histone H3 mutant constructs were generated in the pET-3d vector using the QuickChange site-directed mutagenesis kit II (Stratagene). All constructs were verified by DNA sequencing. Specific sequence data are available upon request.

Cell culture, transfection and reagents

Swiss 3T3 mouse fibroblasts and HeLa human cervix carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum (FCS). 3T3 cultures were rendered quiescent by incubation in DMEM containing 0.2% (v/v) FCS for 72 h. The siRNAs specific for human 14-3-3ε (sc-29588) and 14-3-3ζ (sc-29583), and unspecific control siRNA (sc-37007), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with siRNAs at a final concentration of 24 nM using Lipofectamine 2000 (Invitrogen) and grown for additional 72 h. Control transfection experiments were carried out using an unspecific control siRNA at a final concentration of 48 nM.

The following drugs were used in this study: TSA (165 nM; Wako Pure Chemical Industries), anisomycin (180 nM; Sigma) and H89 (10 μM; Alexis Biochemicals).

RNA isolation and real-time RT–PCR analysis

Total RNA was isolated with TRIZOL reagent (Invitrogen) as specified by the manufacturer. For cDNA, 1 μg of total RNA was reverse transcribed with iScript cDNA synthesis kit (BioRad). Real-time RT–PCR reactions were performed with 0.5 μl of the RT reaction by iCycler iQ system (BioRad) using SYBER green (Molecular probes) for labeling. Primer sequences are available upon request.

Western blot analysis and antibodies

Histone preparation and western blot analyses were performed as previously described (Hauser *et al*, 2002), or using the Odyssey® infrared imaging system (LI-COR Biosciences). The following antibodies were used in this study: modification-specific histone antibodies from Upstate Biotechnology (Lake Placid, NY, USA), C-terminal histone H3 antibody from Abcam; 14-3-3ε (T16) and 14-3-3ζ (C16) were purchased from Santa Cruz Biotechnology and affinity-purified via recombinant GST–14-3-3ε and ζ proteins, respectively.

GST pull-down assay

Recombinant GST-tagged proteins were expressed in and purified from the *Escherichia coli* strain BL21 RIL and GST pull-down experiments were performed as described previously (Doetzlhofer *et al*, 1999) and in Supplementary data.

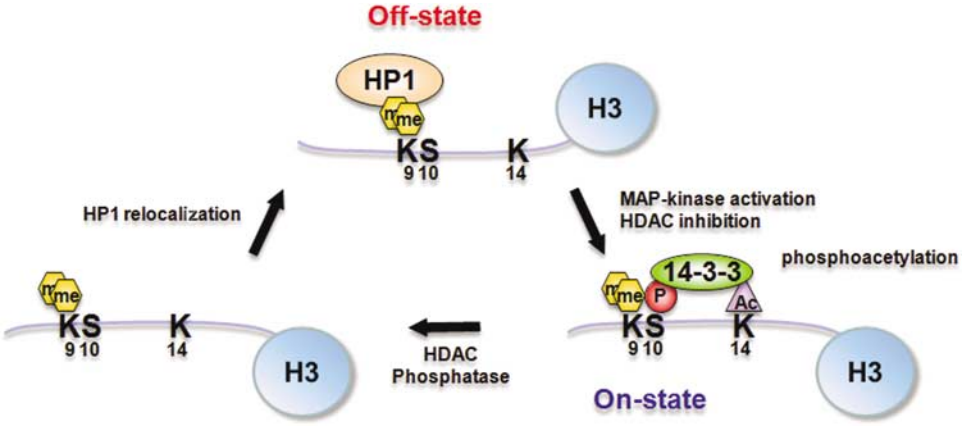


Figure 6 Model for the role of histone H3 modifications in the regulation of HP1 and 14-3-3 binding during transcriptional induction of the HDAC1 gene. In the transcriptional silent state, the HDAC1 promoter region is occupied by H3K9me2-modified nucleosomes and HP1γ. Activation of MAP kinase signaling via anisomycin and inhibition of HDAC activity via TSA leads to the formation of triple modified histone H3, stable binding of 14-3-3 and transcriptional induction of the HDAC1 gene. Removal of the phosphorylation and acetylation marks via phosphatases and deacetylases, respectively, can regenerate K9 dimethylated histone H3 and allow the re-association of HP1 proteins.

Preparation of nuclear extracts

Extracts were prepared as described at http://www.celldeath.de/apometh/emsa.html, except that all buffers were supplemented with Complete-Protease inhibitor cocktail (Roche), phosphatase inhibitors (PPI): 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 50 mM sodium fluoride, 20 mM sodium pyrophosphate and 10 mM sodium butyrate.

In vitro peptide pull-down assay

For a detailed description, see Supplementary data. Histone H3 peptides were purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). Peptides were covalently coupled to agarose beads and 10 μl of peptide slurry were incubated with 2.5 μl IVT, ^{35}S-methionine-labeled 14-3-3 proteins (TNT rabbit reticulocyte lysate system; Promega) in 50 μl total reaction volume.

Fluorescence polarization binding measurements

Measurements were performed as described previously (Jacobs *et al*, 2004) and in Supplementary data.

Chromatin immunoprecipitation assays

Preparation of soluble chromatin and chromatin immunoprecipitation assays were carried out as described previously (Hauser *et al*, 2002).

PCR analysis of immunoprecipitated DNA

All PCRs were performed on a Biometra D3 thermocycler, using Eppendorf PCR Master Mix. Primer sequences are available upon request. The linear range for each primer pair was determined empirically using different amounts of input DNA. PCRs with increasing amounts of genomic DNA were carried out along with the PCRs of the immunoprecipitated DNA. PCR products were resolved on 2% agarose–TAE gels.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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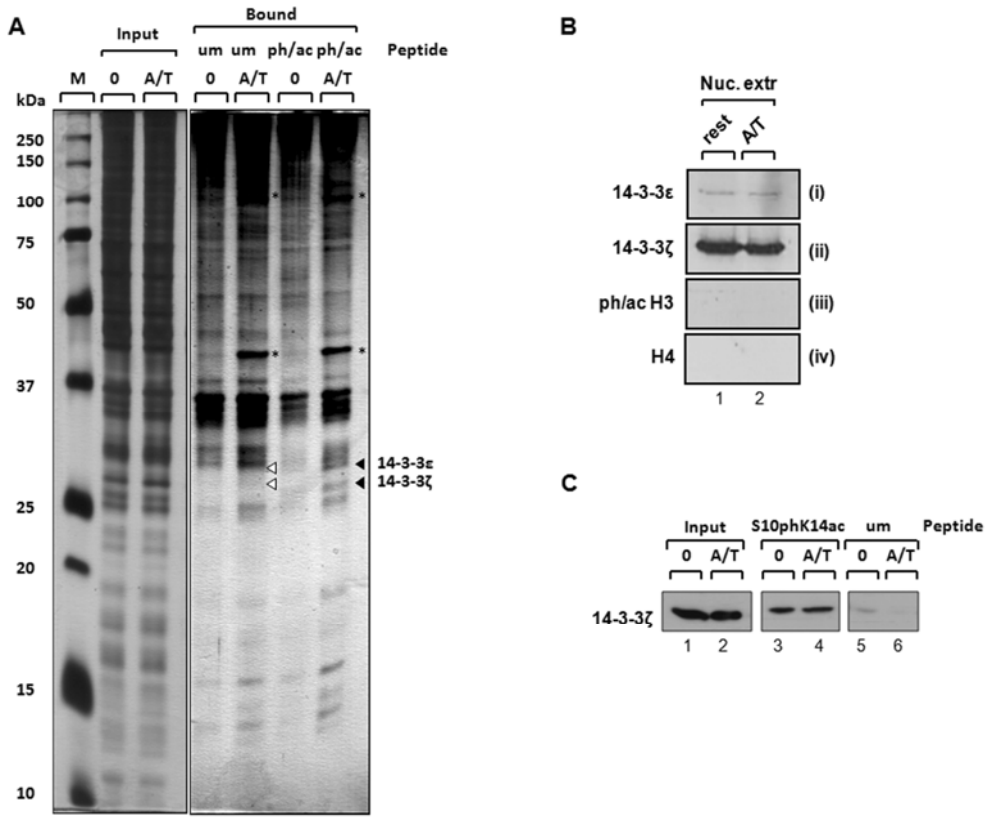
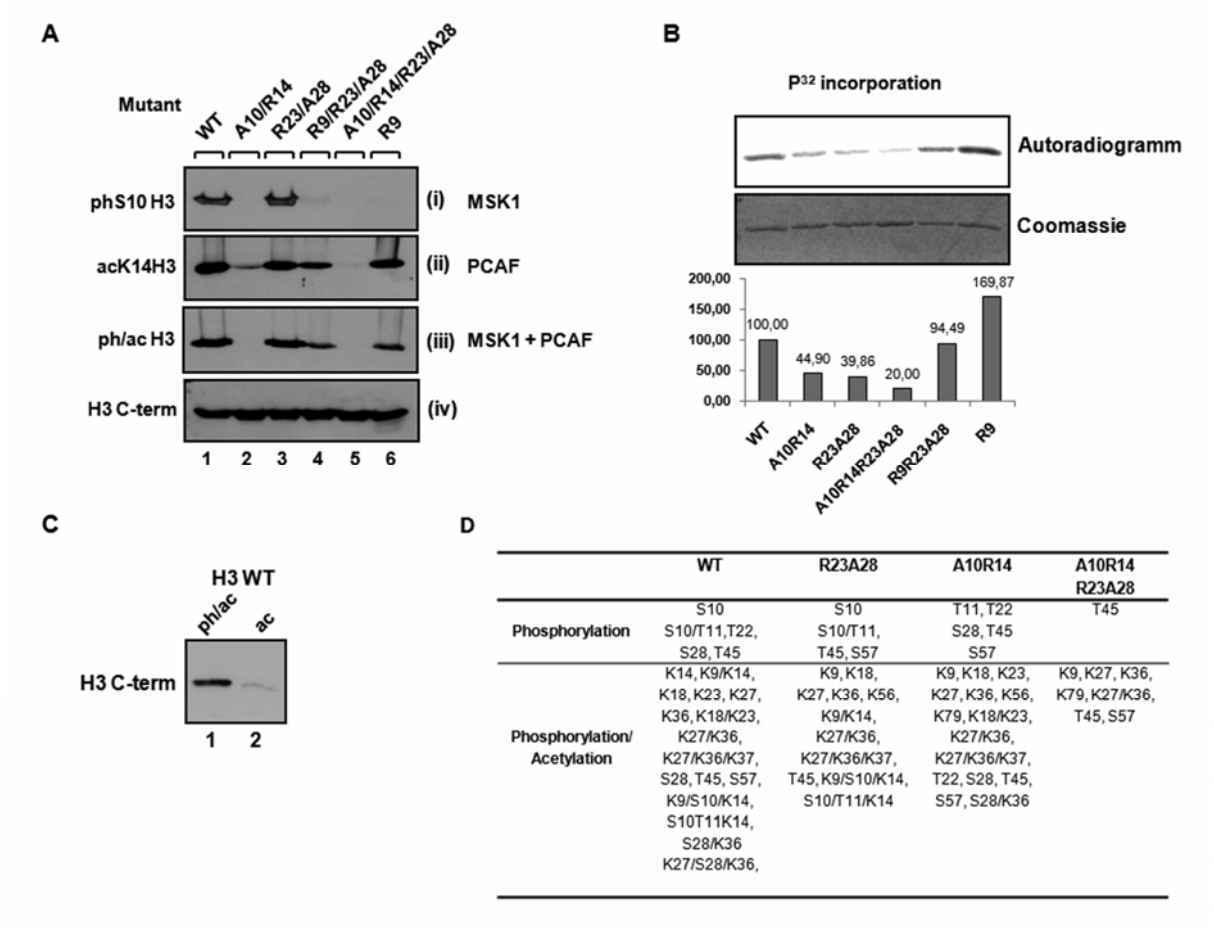


Figure S1. Affinity purification of 14-3-3 proteins as phosphorylation dependent histone H3 interacting factors.

(A) Nuclear extracts from proliferating HeLa cells which were either left untreated (lanes 2, 4 and 6) or treated with anisomycin and TSA (50 ng/ml) (lanes 3, 5 and 7) for one hour were incubated with unmodified (um) or H3S10phK14ac (ph/ac) histone H3 peptides. Bound proteins were separated by SDS-PAGE and visualized by silver staining. Factors specifically interacting with the S10phK14ac peptide (black triangles) were excised from the gel and identified by mass spectrometry. The corresponding regions of the unmodified peptide incubations were used as control (white triangles). The asterisks denote factors that showed modification independent peptide interactions only after anisomycin and TSA treatment.

(B) Analysis of 14-3-3 protein localization. 3T3 mouse fibroblasts were arrested by serum deprivation for 72h (lane 1) and were stimulated with anisomycin and TSA (lane 2). Nuclear extracts were prepared and analyzed by SDS-page and immunoblotting with antibodies specific for 14-3-3ε (panel i), 14-3-3ζ (panel ii), ph/ac histone H3 (panel iii) and histone H4 (panel iv). Both 14-3-3 proteins are clearly detected in nuclear extracts (lanes 4 and 5).

(C) Anisomycin and TSA treatment does not significantly alter the binding properties of 14-3-3 proteins to the unmodified or the H3S10phK14ac histone H3 peptide. Whole cell lysates were prepared from HeLa cells treated as described for panel A and incubated with the unmodified or the S10phK14ac peptide. Binding of 14-3-3ζ from either untreated (lanes 3 and 5) or stimulated (lanes 4 and 6) cells was analyzed by immunoblotting. An input aliquot of either non-treated (0) (lane 1) or stimulated (AT) (lane 2) lysate was used to control equal loading. One representative experiment is shown indicating that the binding of 14-3-3ζ is not considerably changed upon drug treatment.



	WT	R23A28	A10R14	A10R14 R23A28
Phosphorylation	S10 S10/T11,T22, S28, T45	S10 S10/T11, T45, S57	T11, T22 S28, T45 S57	T45
Phosphorylation/ Acetylation	K14, K9/K14, K18, K23, K27, K36, K18/K23, K27/K36, K27/K36/K37, S28, T45, S57, K9/S10/K14, S10T11K14, S28/K36 K27/S28/K36,	K9, K18, K27, K36, K56, K9/K14, K27/K36, K27/K36/K37, T45, K9/S10/K14, S10/T11/K14	K9, K18, K23, K27, K36, K56, K79, K18/K23, K27/K36, K27/K36/K37, T22, S28, T45, S57, S28/K36	K9, K27, K36, K79, K27/K36, T45, S57

Figure S2. Analysis of *in vitro* modification of Histone H3 mutants.

(A) Wildtype histone H3 (lane 1) or the indicated histone H3 mutants (lanes 2-6) were in vitro modified as described for figure 1B. *In vitro* modification was monitored by immunoblotting with the indicated antibodies. Mutation of lysine 9 to arginine (lanes 4 and 6) resulted in impaired recognition by the H3S10phspecific antibody and reduced recognition of the H3K14ac and ph/ac specific antibodies.

(B) Incorporation of ^{32}P via MSK1 mediated phosphorylation of WT and mutant histone H3. The indicated H3 mutants were phosphorylated using P^{32}-ATP (1µCi/µl final concentration) and P^{32} incorporation was normalized to total histone H3 loading. The histogram depicts normalized incorporation efficiencies relative to wild type histone H3. Mutation of lysine 9 to arginine resulted in strongly increased ^{32}P incorporation despite the reduced affinity of modification specific antibodies as described for panel A, suggesting that this mutation increases MSK1 mediated phosphorylation *in vitro*.

(C) Histone H3 acetylation via PCAF does not mediate binding to 14-3-3 in the absence of additional phosphorylation. WT recombinant histone H3 was *in vitro* phosphoacetylated (lane 1) or acetylated only (lane 2) as described in material and methods section and incubated with GST-14-3-3ζ. Bound histones were analyzed by immunoblotting with C-terminal H3 antibodies.

(D) Mass spectrometry analysis of amino acid positions phosphorylated by MSK1 or acetylated by PCAF in histone H3 mutants *in vitro* (S: serine, T: Threonine, K: Lysine)

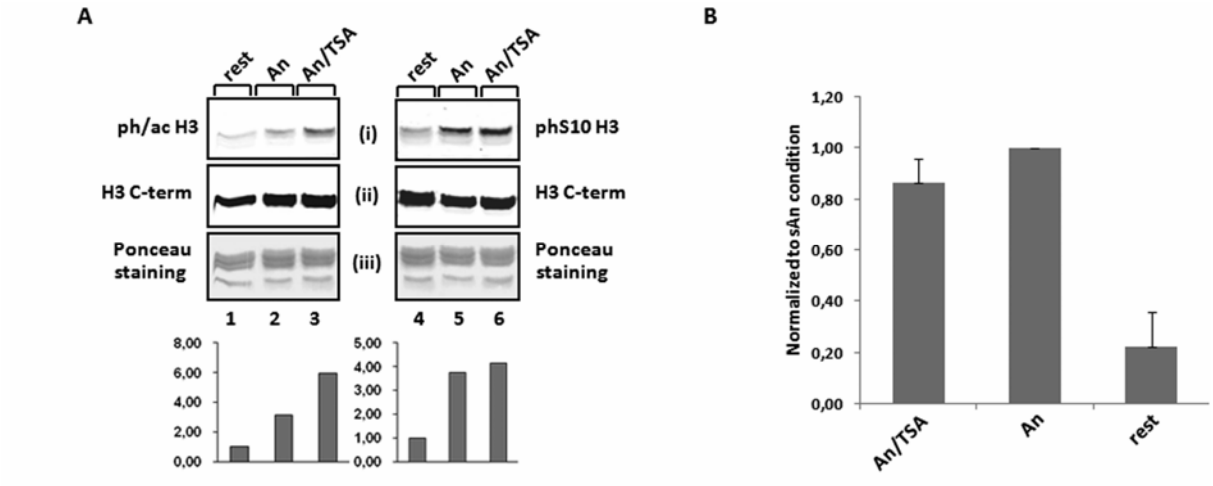


Figure S3. Anisomycin mediated phosphorylation of H3S10 is not increased by TSA.

(**A**) Quantitative analysis of histone H3 phosphorylation and phosphoacetylation upon differential stimulation. Histones were extracted from resting 3T3 fibroblasts that were left untreated (rest) or treated with anisomycin (An) or anisomycin plus TSA (An/TSA). Levels of histone H3 phosphorylation and phosphoacetylation were analyzed by quantitative immunoblotting with the indicated antibodies (panel i). Signals for histone H3 phosphorylation and phosphoacetylation were normalized to histone H3 C-terminal antibodies signals (panel ii). As additional control Ponceau staining of the blot is shown (panel iii). The panel depicts one representative experiment and relative levels of histone modifications are depicted as histogram under the panel.

(B) Additional TSA treatment does not increase the anisomycin generated phospho-histone H3 pool. Histones were isolated as described for panel A and histone H3 was used for mass spectrometry analysis. Intensities for all identified S10 phosphorylated histone H3 peptides (neglecting additional modifications) were summarized and normalized to an unmodified H3 control peptide. Values are depicted relative to the anisomycin condition as average of four independent measurements (mean±SD).

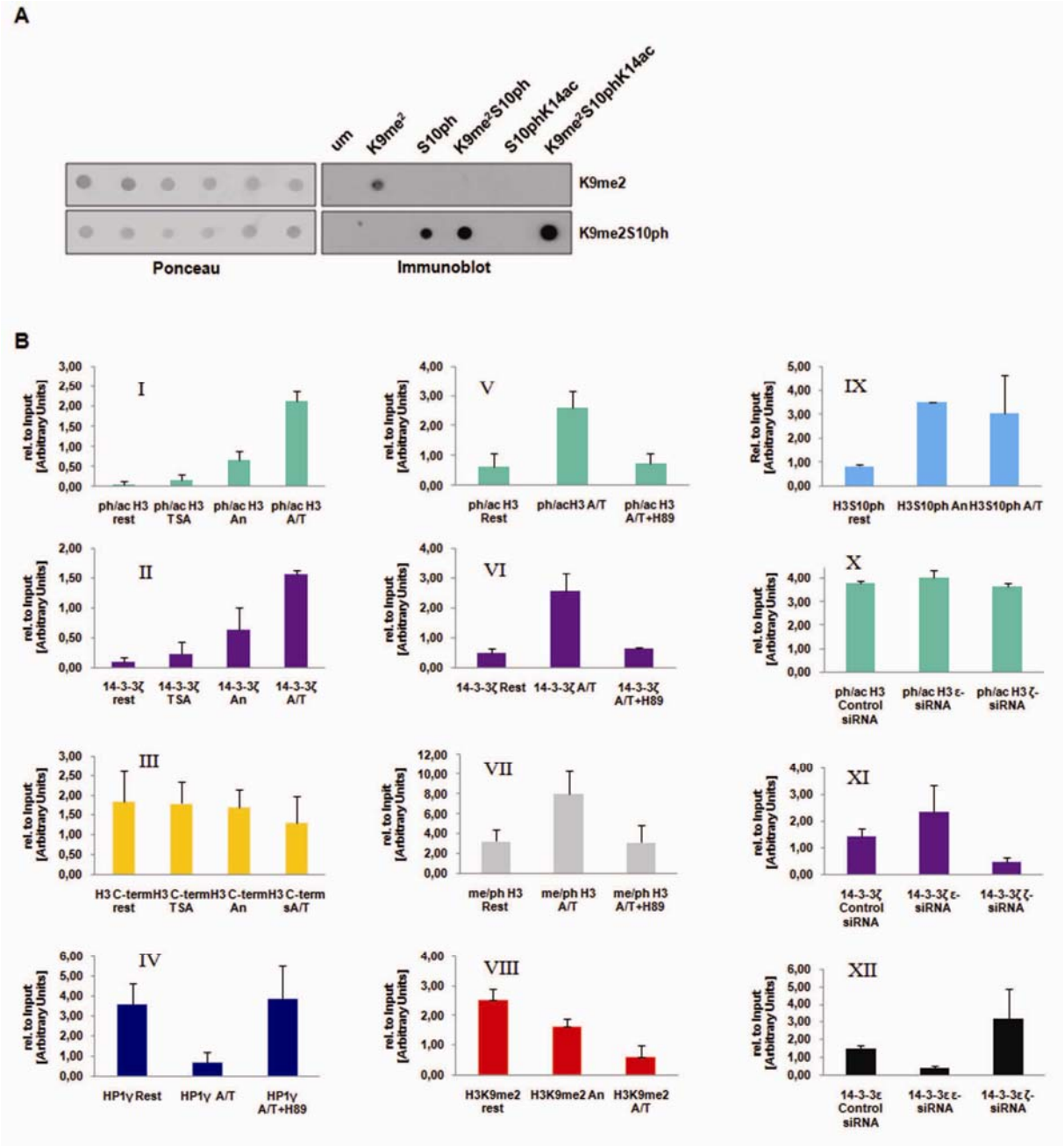


Figure S4: (A) Anti-me^2 antibody is occluded by additional S10 phosphorylation. The reactivity of the indicated antibodies towards differentially modified H3 peptiodes was investigated by dot blotting.

(B) Summary of ChIP-assays performed in the study. The panels indicate the relative amount of precipitated DNA relative to the Input. (I) ph/ac H3 n=3 (II) 14-3-3ζ n=3 (III) H3 C-term. N=2 (IV) HP1γ n=3 (V) ph/ac H3 n=5 (VI) 14-3-3ζ n=2 (VII) me/ph H3 n=4 (VIII) H3K9me2 n=2 (IX) H3S10ph n=2 (X) ph/ac H3 n=2 (XI) 14-3-3ζ n=2 (XIII) 14-3-3ε n=2.

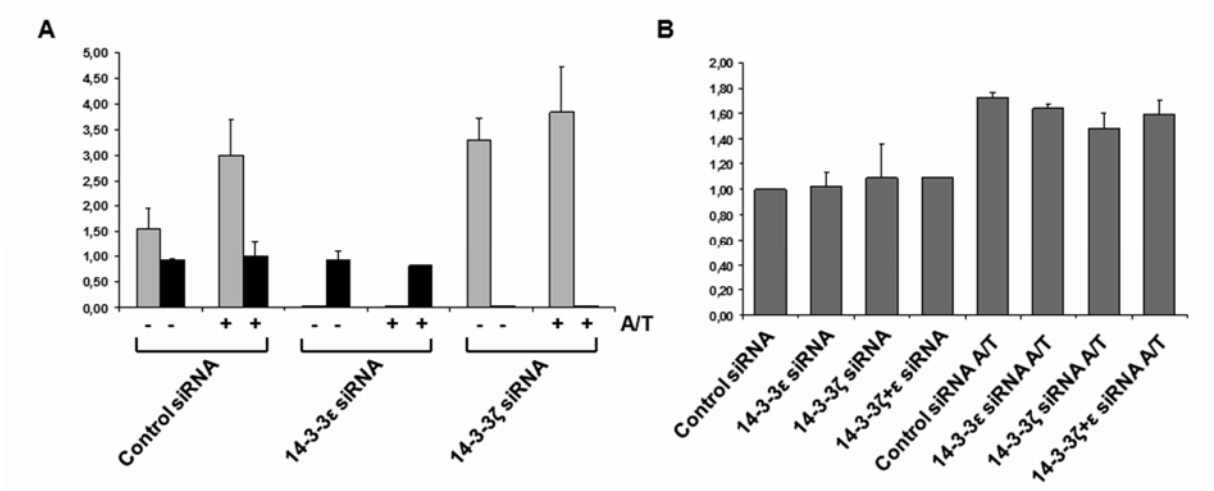


Figure S5. 14-3-3 knock down specificity and histone H3 phosphoacetylation in 14-3-3 knock down cells.

(**A**) HeLa cells were transfected with siRNAs as described for Fig. 5A and RNA was prepared as described for Fig. 5C. 14-3-3 expression levels were analyzed by quantitative Real-time PCR and normalized to GAPDH expression levels. Expression of 14-3-3ε (grey bar) and ζ (black bar) are depicted as diagram. Transfection with 14-3-3ε siRNA did not interfere with 14-3-3ζ expression and *vice versa* indicating strong isoform specificity of the knock down (compare lanes 1, 3 and 5). Treatment with anisomycin and TSA did not affect efficiency or specificity of the knock down (lanes 2, 4 and 6).

(**B**) 14-3-3 knock down does not influence histone H3 phosphoacetylation. Histones prepared from 14-3-3 knock down cells were analyzed for presence of phosphoacetylated histone H3 by quantitative immunoblotting. Results are depicted as diagram, summarizing three independent experiments (mean±SD).

3. Results (Part 2)

3.1 Introduction

14-3-3 proteins are a ubiquitously expressed family of acidic proteins comprising seven family members in mammals and at least two members in all eukaryotes (see also section 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"). In general, two U-shaped 14-3-3 monomers dimerize to form a cup shaped, rigid and stable 14-3-3 homo- or heterodimer (Figure 3-2). Dimeric binding of 14-3-3 proteins may be important for biological functions. According to the "gatekeeper" hypothesis (Yaffe, 2002), phosphorylation dependent interaction of 14-3-3 with a target protein would involve two events; one binding to a high affinity mode 1 or mode 2 consensus motif (the gatekeeper residue) that allows for efficient binding of 14-3-3 and an additional interaction event normally not within a high affinity consensus. Due to the interaction of one monomer with the high affinity motif the efficiency for the subsequent interaction with the low affinity motif is strongly reinforced. However, the biological relevance of either homo- or heterodimer formation is not well understood but was discussed to have strong implications for the regulation of the biological function of 14-3-3 proteins. For example the monomer combinations of ββ, βζ, βτ and ζτ were found to be able to scaffold c-Raf and Bcr whereas other combinations fail to (Braselmann and McCormick, 1995). Attempts to determine the *in* vivo preference of particular isoforms for homo- or heterodimer formation demonstrated an intrinsic preference for particular dimerization partners, independent of cellular conditions (Aitken, 2002; Aitken *et al.*, 2002). In addition, the preferential homodimer formation of 14-3-3σ was found to be dependent on three critical residues (Ser5, Glu20 and Glu80) (Verdoodt *et al.*, 2006). Together these observations suggest that the observed preferential dimer-combinations are compelled via a structural impact of the dimerization platform.

14-3-3 proteins were the first phospho-serine dependent adaptor molecules to be identified (Muslin *et al.*, 1996). Each of the 14-3-3 monomers contains one binding cleft to accommodate one phospho-serine (see 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"). Therefore one dimer can simultaneously bind two phosphorylated residues (see also above). Screening of peptide libraries demonstrated that two optimal consensus motifs, referred to as mode 1 and mode 2, are bound with high affinity by all 14-3-3 proteins (Yaffe *et al.*, 1997; Rittinger *et al.*, 1999). The mode 1 consensus motif contains the amino acid sequence RSXSphXP and the mode 2 consensus is of the sequence RXXXSphXP (Sph phosphorylated serine, X any amino acid except cysteine with different

preferences depending on position and particular 14-3-3 isoform (Yaffe *et al.*, 1997; Rittinger *et al.*, 1999) (Figure 1 in 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"). The crystal structures for 14-3-3 proteins in complex with either mode 1 or mode 2 binding peptides demonstrated the importance of proline at the critical position +2 from the phosphorylated serine (Rittinger *et al.*, 1999). This site is crucial to mediate the exit of the peptide from the 14-3-3 binding cleft. It was reported that there is strong selection for "turn-forming" amino acids at this position yet proline is strongly preferred as it can most efficiently direct the peptide outward (see 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"). Despite the superimposable overall structural organization of different 14-3-3 dimers and the excessive similarity in the high affinity consensus selection for different family members (Yaffe *et al.*, 1997; Rittinger *et al.*, 1999), isoform specific interactions with target proteins have been reported (Vincenz and Dixit, 1996; Prymakowska-Bosak *et al.*, 2002; Hermeking and Benzinger, 2006). One example for these isoform specific functions is provided by the cell cycle regulatory functions of 14-3-3 proteins (reviewed in (Hermeking and Benzinger, 2006). The epithelial restricted 14-3-3σ isoform is a crucial factor in the maintenance of G2/M arrest. 14-3-3σ is directly regulated by p53 and BRCA1 upon induction of DNA damage (Hermeking *et al.*, 1997) and cells deficient for this isoform can initiate but not maintain G2/M arrest (Chan *et al.*, 1999). The importance of 14-3-3σ for proper regulation of G2/M progression is also underlined by the observation that this isoform is frequently silenced *via* DNA methylation during malignant transformation (Ferguson *et al.*, 2000). In addition, G2/M arrest is also regulated by additional 14-3-3 proteins and the major target in this pathway is the CDC25C phosphatase (Kumagai *et al.*, 1998; Kumagai and Dunphy, 1999; Lopez-Girona *et al.*, 1999). Importantly CDC25C is not bound by 14-3-3σ and the major target for G2/M arrest mediated by this isoform appears to be the cyclinB/cdc2 complex. Another hint for isoform specific functions of 14-3-3 proteins is provided by reports demonstrating that 14-3-3β overexpression promotes cell growth and tumor formation in nude mice, whereas 14-3-3σ is mainly associated with tumor suppressive functions (Takihara *et al.*, 2000; Sugiyama *et al.*, 2003; Hermeking and Benzinger, 2006). Interestingly, the transformation promoting capacity of 14-3-3β has been linked recently to its potential as transcriptional repressor (Komiya *et al.*, 2008). This suggests that besides functions as transcriptional activators for phosphoacetylation target genes (Winter *et al.*, 2008b) 14-3-3 proteins can also function as repressors in an isoform specific context. Importantly this transcriptional repressive effect was demonstrated for the gene encoding the "mitogen activated protein kinase phosphates" (MKP1), which was shown previously to be regulated by histone H3 phosphoacetylation (Li *et al.*, 2001). The requirement for 14-3-3 proteins for transcriptional activation of MKP1 has

not yet been demonstrated. However, based on the data discussed above it is tempting to speculate that phosphoacetylation target genes may be regulated by 14-3-3 proteins in a dual- and isoform specific manner. In this scenario particular isoforms like 14-3-3β would maintain transcriptional repression whereas other isoforms like 14-3-3ζ or ε would be recruited via histone H3 phosphoacetylation and mediate transcriptional activation.

3.2 Impact on target proteins upon interaction with 14-3-3 proteins

Binding of 14-3-3 proteins to its target frequently involves phosphorylation of a serine in the context of either mode 1 or mode 2 consensus motifs. However, several 14-3-3 associated proteins do not contain either of these motifs or do not require serine/threonine phosphorylation for an interaction at all (reviewed in (Yaffe, 2002; Hermeking, 2003; Hermeking, 2006)). Several hundred 14-3-3 interaction partners have been described and the effects of 14-3-3 binding to a target protein can differ considerably. The modes how 14-3-3 binding impacts the behavior of the target protein have been classified into five distinct groups.

One well established function for 14-3-3 interaction is the sequestration into a different sub-cellular compartment of a protein like cytoplasmic nuclear shuttling. In this way normally 14-3-3 proteins retain their bound substrate in the cytoplasm thereby inactivating any nuclear functions of the bound factor. Examples for cytoplasmic retention by 14-3-3 are the M-phase specific phosphatase CDC25C, the pro-apoptotic transcription factors FKHRL1 and DAF16, the class II HDACs 4 and 5 and the high mobility group chromatin protein HMGN1 (Kumagai and Dunphy, 1999; Zeng and Piwnica-Worms, 1999; Brunet *et al.*, 2002; Prymakowska-Bosak *et al.*, 2002; Ellis *et al.*, 2003; Xiao *et al.*, 2003).

In addition 14-3-3 proteins can directly impact enzymatic activity of bound proteins like the tyrosine/ tryptophan hydroxylase (AANAT), which is hyperactivated upon interaction with 14-3-3 proteins (Ichimura *et al.*, 1987; Ichimura *et al.*, 1995). A prominent example for regulation of enzymatic activity by 14-3-3 proteins is the c-Raf kinase. 14-3-3 proteins regulate c-Raf in a complex manner. Firstly, in the absence of GTP-bound Raf, the interaction with 14-3-3 impairs enzymatic activity, whereas upon stimulation of the pathway 14-3-3 is required for full kinase activity. Also the DNA binding capacity of the p53 tumor suppressor was found to be strongly dependent on interaction with 14-3-3 proteins (Hermeking *et al.*, 1997; Hermeking and Benzinger, 2006). In addition the DNA binding capacity of the pro-apoptotic transcription factor DAF16 is strongly impaired upon Akt mediated phosphorylation and 14-3-3 binding.

Due to their dimeric nature 14-3-3 proteins can also function as phosphorylation dependent adaptor molecules. Thereby 14-3-3 bridges two factors that would otherwise not interact with each other. This function of 14-3-3 association has been extensively described for the c-Raf

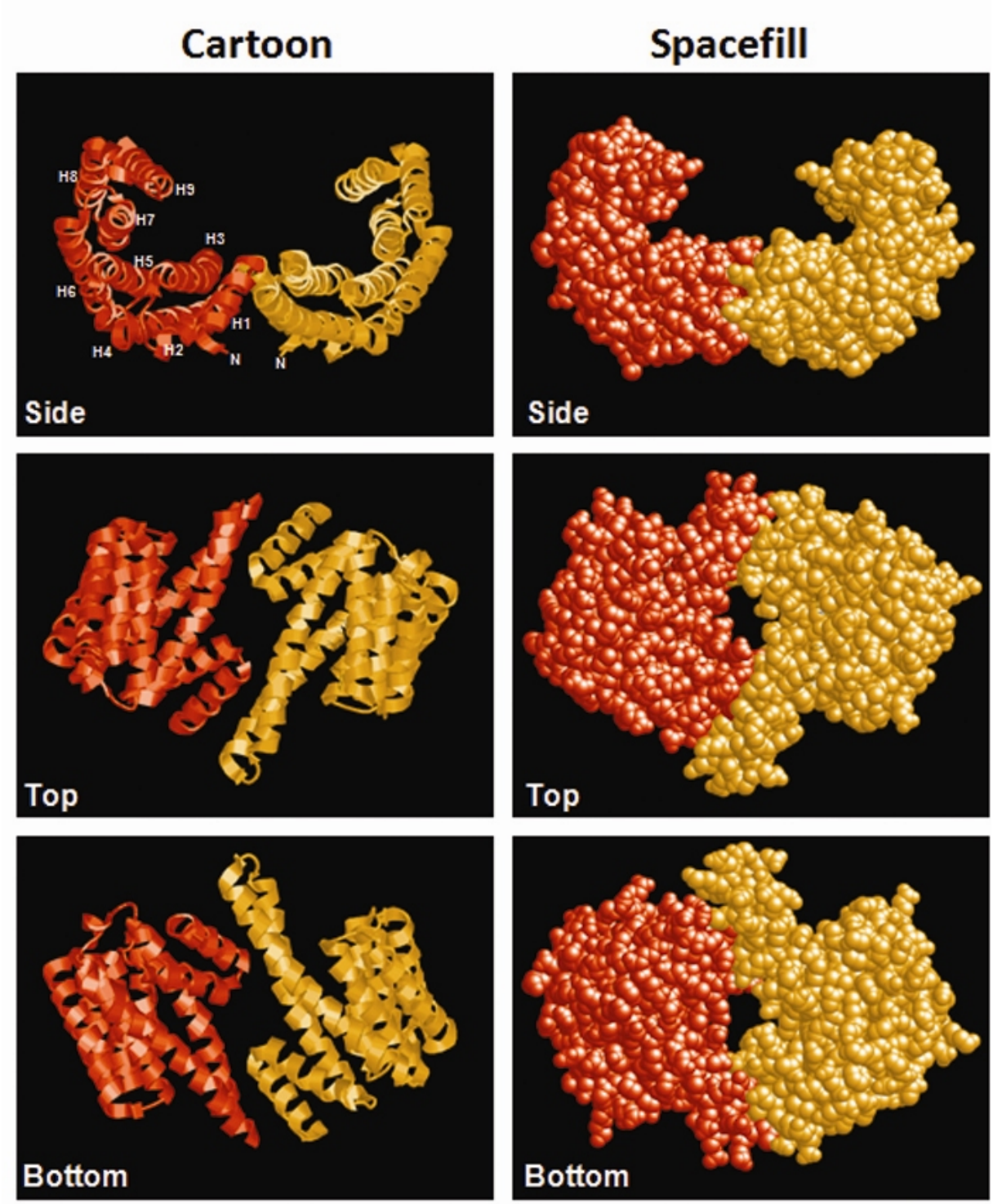


Figure 3-2 Structure of the 14-3-3 Zeta homodimer. Pictures were rendered using RasMol software on the PDB data file 1QJA. One 14-3-3 monomer is depicted in orange the other one in redorange. Pictures on the left side show cartoon view, which are viewed as “spacefill” view (atom Van der Waals radii) on the right side. The nine antiparallel α-helices are designated H1-9 (indicated in the upper left panel). The five amino-terminal helices show a rectangular orientation relative to the four carboxy-terminal helices. Helices H3, H5, H7 and H9 form an amphipathic groove which contains the most invariant residues, whereas variant residues are located on the outside of the protein. Phosphoserine binding is mediated by a basic pocket formed by amino acids Lys49, Arg56, Arg127 and Tyr128. The helices H1, H3 and H4 contribute to the dimer interface between the two monomers.

kinase and its bridging to factors like Bcr, PKC or A20 (Braselmann and McCormick, 1995; Vincenz and Dixit, 1996; Van Der Hoeven *et al.*, 2000). Interaction with 14-3-3 proteins may also inhibit protein-protein interactions as has been demonstrated for the E3 ligase, CBL and the scaffolding factor IRS1 (Craparo *et al.*, 1997; Liu *et al.*, 1997).

Another mode of action is provided by protection from post-translational modification or "demodification" (like dephosphorylation or proteolysis) due to interaction with 14-3-3 proteins. This mechanism has been demonstrated for several proteins like histones, c-Raf and Bad (Chen and Wagner, 1994; Dent *et al.*, 1995; Chiang *et al.*, 2001).

Together these examples demonstrate the wide spectrum how 14-3-3 binding can influence the properties of the associated partner.

3.2 Aims of the project and summary of the presented publication

The interaction between histone H3 and 14-3-3 proteins can be mediated *via* the phosphorylation of two serines in the amino-terminal tail (Macdonald *et al.*, 2005; Winter *et al.*, 2008b). The amino acid sequence of both residues in the amino-terminal portions is identical but the carboxy-terminal amino acids differ considerably (see Figure 1 in section. 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"). Quantitative measurements demonstrated that the interaction between 14-3-3 and serine 28 phosphorylated histone H3 is significantly stronger than for serine 10 (Macdonald *et al.*, 2005; Winter *et al.*, 2008b). Importantly serine 28 contains a proline at position P+2 (H3P30) that is strongly selected in the 14-3-3 high affinity consensus motifs (see above). Serine 10 contains tandem glycines at the P+2 and P+3 positions that were demonstrated to mediate the exit of the histone H3S10ph peptide from the 14-3-3 binding cleft (Macdonald *et al.*, 2005). The crucial role of proline 30 for strong interaction with 14-3-3 proteins was demonstrated by analyzing the interaction with several histone mutants containing proline or alanine at the +2 position from either serine 10 or serine 28.

Importantly, the relatively weak interaction between serine 10 phosphorylated histone H3 and 14-3-3 is susceptible to additional acetylation on either lysines 9 or 14 (Walter *et al.*, 2008; Winter *et al.*, 2008b). However, another report investigating the interaction with phosphorylated and double acetylated (lysines 9 and 14) failed to detect any significant impact on the interaction (Macdonald *et al.*, 2005). To sort out this controversy the interaction of 14-3-3 with this particular modification state was reevaluated. These experiments demonstrated that indeed a histone H3 peptide with the modification state K9acS10phK14ac is bound with similar affinity than a histone H3S10ph peptide; however single acetylation on either K9 or K14 increases the affinity. Therefore the interaction is stabilized by single acetylation whereas double acetylation does not significantly affect the interaction.

The high affinity binding to either the serine 28 or a serine 10 phosphorylated peptide that contains a proline at position P+2 (instead of glycine) was found not susceptible to single acetylation. This observation suggests that single acetylation may probably help to modulate the organization of the peptide within the binding cleft, possibly by supporting the exit from the cleft (details see text). The implications of this modulation and the different affinities of 14-3-3 for either serine 10 or serine 28 phosphorylated histone H3 are discussed in detail. Also the possible evolutionary requirement for such a multiple modification dependent interactions is reviewed.

3.3 Presented Publication 2 (Winter *et al.* 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"

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Extra View

Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns

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Post-translational modifications of histones are determining factors in the global and local regulation of genome activity. Phosphorylation of histone H3 is globally associated with mitotic chromatin compaction but occurs in a much more restricted manner during interphase transcriptional regulation of a limited subset of genes. In the course of gene regulation, serine 10 phosphorylation at histone H3 is targeted to a very small fraction of nucleosomes that is highly susceptible to additional acetylation events. Recently, we and others have identified 14-3-3 as a binding protein that recognizes both phosphorylated serine 10 and phosphorylated serine 28 on histone H3. In vitro, the affinity of 14-3-3 for phosphoserine 10 is weak but becomes significantly increased by additional acetylation of either lysine 9 or lysine 14 on the same histone tail. In contrast, the histone H3S28 site matches elements of 14-3-3 high affinity consensus motifs. This region mediates an initial stronger interaction that is less susceptible to modulation by "auxiliary" modifications. Here we discuss the binding of 14-3-3 proteins to histone H3 in detail and putative biological implications of these interactions.

Intrinsic Factors Influencing 14-3-3 Histone H3 Interaction

14-3-3 proteins comprise a highly conserved protein family with at least two isoforms expressed in lower eukaryotic organisms and up to 15 in plants. In mammals the 14-3-3 family comprises seven members (β, γ, ε, η, τ/θ, ζ and σ) each encoded by a distinct gene. Despite considerable variability in the coding sequences, 14-3-3 proteins display a high degree of overall conservation in primary and tertiary protein structure. Although most isoform are ubiquitously expressed, 14-3-3σ expression appears restricted to epithelial tissue. Further, spatial and temporal patterns of isoforms expression occur during developmental progression.[1-3] Differential post-translational modifications of particular isoforms have also been reported.[4-6]

Pioneering research revealed the dimeric nature of this protein class[7,8] as an important hallmark of 14-3-3 biology.[9,10] Moreover, 14-3-3 proteins were identified as the first phosphoserine/threonine dependent adaptor molecules.[11] Detailed investigations on substrate preferences demonstrated that 14-3-3 proteins recognize two internal consensus motifs: the sequences RSXS/TphXP (mode 1) and RXXXS/TphXP (mode 2) where S/Tph indicates phosphorylated serine or threonine and X any amino acid except cysteine with position dependent preferences.[11-13] Further, a carboxy-terminal consensus referred to as mode 3 has been identified.[14-16] Although one of these consensus sequences is frequently found within 14-3-3 associated proteins, several interaction partners contain variations of this motif or do not require phosphorylation for binding at all.[17-22]

Recently, 14-3-3 proteins were reported to interact with phosphorylated histone H3.[23-25] These studies indicated a function of this association in transcriptional activation.[25] The two phosphorylated serines within histone H3, S10 and S28 that were shown to mediate interaction with 14-3-3, do not perfectly match one of the two consensus motifs (Fig. 1A). In vitro, H3S28ph however mediates a significantly stronger interaction with 14-3-3 than H3S10ph (Fig. 1B).[23,25]

Both mode 1 and mode 2 consensus motifs contain proline at position P + 2 which adopts either *cis* conformation in mode 1 or *trans* conformation in mode 2.[12] In general there is a strong selection for turn-forming residues at this position.[12,13] Histone H3S10 and H3S28 are preceded by the same amino acid motif ARK. The carboxy-terminal sequence however differs considerably between the two sites (Fig. 1A). H3S10 is followed by an additional phosphorylatable threonine at P + 1. Tandem glycine residues follow at P + 2 and P + 3. In the crystal structure of 14-3-3ζ bound to the phosphorylated H3 tail these residues allow the H3 peptide to exit the binding cleft (Fig. 3).[23] In contrast, H3S28 is followed by an alanine and contains proline at position 30 (H3P30) matching the strongly preferred proline at position P + 2 contained within the two 14-3-3 consensus motifs (Fig. 1A).[12,13] The presence of proline at P + 2 appears to be favorable over tandem glycines as indicated by significantly stronger interaction of 14-3-3 with the H3S28 site compared to the H3S10 site (Fig. 1B).[23,25] Further, mutation of H3P30 to alanine (H3P30A) significantly decreased the affinity of 14-3-3ζ for the H3 tail (Fig. 1C). Conversely, exchange of glycine at position 12 by proline (H3G12P) resulted in enhanced 14-3-3ζ binding to the H3S10ph peptide (Fig. 1D). Therefore and in agreement with profound structural data,[12,13,23] H3P30 appears to be a

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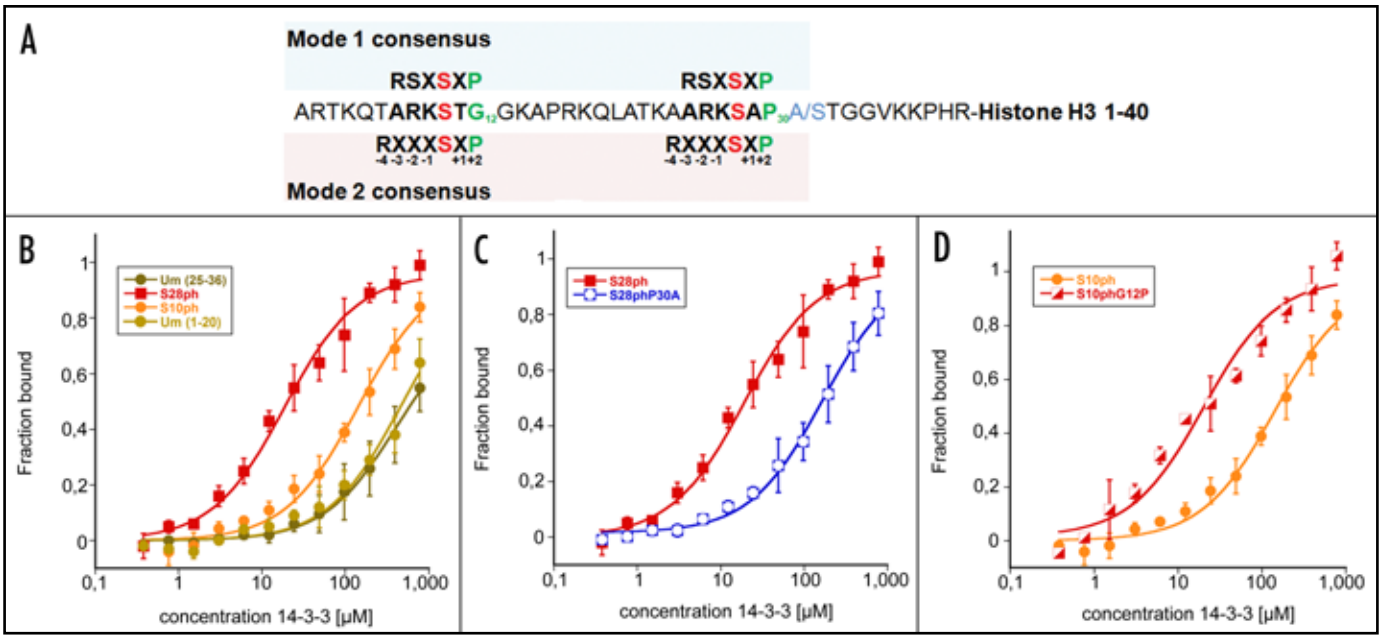

Figure 1. Intrinsic factors influencing the interaction of 14-3-3 with histone H3. (A) Sequence alignment of high affinity 14-3-3 consensus motifs of mode 1 (upper box) and mode 2 (lower box) with histone H3. The critical position at P + 2 (green residues) from the phosphorylated serines (red residues) is formed by glycine 12 for serine 10 and proline 30 for serine 28, respectively. Histone H3.3 contains a serine at position 31 (blue residue) whereas histones H3.1 and H3.2 contain an alanine at this site. (B) Serine 28 phosphorylated histone H3 is bound by 14-3-3ζ with higher affinity than H3 phosphorylated at serine 10. Binding curves determined by fluorescence polarization measurement are shown.[60] Binding assays were performed for H3S28ph peptide, H3S10ph peptide and the respective unmodified controls. Data points of at least three independent measurements were averaged. Binding curves were fitted using least square algorithm. Dissociation constants (Kd) values are summarized in Table 1. (C) Proline 30 constitutes an important factor for the higher affinity of the H3S28ph peptide. Proline 30 was mutated to alanine (H3S28phP30A) and affinity for 14-3-3 binding was determined. (D) Proline at position 12 enhances binding to the H3S10ph peptide. The P + 2 position was changed from glycine to proline (H3S10phG12P) and binding assays were performed as described for (B). (D) Proline 30 constitutes an important factor for the higher affinity of the H3S28ph peptide. Proline 30 was mutated to alanine (H3S28phP30A) and affinity for 14-3-3 binding was determined.

crucial residue in mediating the high affinity of 14-3-3 towards the S28 phosphorylated H3 tail.

Another important parameter of 14-3-3 interaction with histone H3 peptides is a conformational stabilization of the peptide by several intramolecular interactions.[23] The phosphate oxyanion forms interactions with the H3G12 backbone amide. In addition, an intramolecular salt bridge is formed between arginine 8 (P - 2) and the phosphate oxyanion of serine 10. This is analogous to the interaction of 14-3-3 with the mode 2 consensus peptide where the guanidine group of the P - 4 arginine forms a salt bridge with the phosphate oxyanion. This conformation is not observed for the P - 3 arginine in mode 1 binding.[12,23] Therefore, the interaction between H3S10ph and 14-3-3 exhibits structural features of mode 2 binding. However, in this case the exit of the peptide from the binding cleft is not mediated by the P + 2 proline but via the tandem glycine residues at P + 2 and P + 3. To this point, there are no structural data on the interaction between H3S28ph and 14-3-3. Given the identical amino acid composition amino-terminal of H3S10 and H3S28 it is likely that arginine 26 (P - 2) adopts a similar conformation as arginine 8 (P - 2) and forms a salt bridge with the phosphate oxyanion. This mode of interaction would imply the P + 2 proline (H3P30) adopting *trans* conformation thereby allowing the peptide to exit the binding cleft.[12]

Does isomerisation of prolines in the histone H3 tail therefore participate in the regulation of 14-3-3 binding to the histone H3 tail? Proline isomerisation constitutes an important factor in regulation of protein folding. For 14-3-3 interaction with target proteins, proline is strongly preferred at position P + 2 and adopts either *cis* conformation in mode 1 and *trans* conformation in mode 2 binding.[12] In solution the *cis* conformation is relatively abundant (5–10% of peptidyl-prolyl bonds) compared to other non-prolyl peptide bonds and proline isomerisation events constitute an important factor for secondary structure formation.[26] Peptidyl-prolyl isomerisation has been reported for histone H3P30 and H3P38 via the FKBP proline isomerase family member Fpr4.[27] In this study histone H3P30 isomerisation was demonstrated to directly impact Set2 mediated K36 methylation. Therefore, it will be interesting to investigate whether H3P30 isomerisation could impact 14-3-3 binding to phosphorylated histone H3.

Extrinsic Factors Influencing 14-3-3 Histone H3 Interaction

Histone proteins are subject of an extensive and steadily expanding list of post-translational modifications (PTMs).[28-30] Several reports indicate interphase H3S10 phosphorylation frequently coinciding with adjacent acetylation events (phosphoacetylation). This process has been investigated in particular for H3K9acS10ph and H3S10phK14ac phosphoacetylation.[25,30-35] Studies using antibodies directed against double modified H3 species and mass spectrometry based approaches also demonstrated H3S10 phosphorylation co-existing with neighboring lysine methylation (mono-, di- and trimethylation) on H3K9. In addition, triple modified forms (H3K9meS10phK14ac) were identified.[25,36,37]

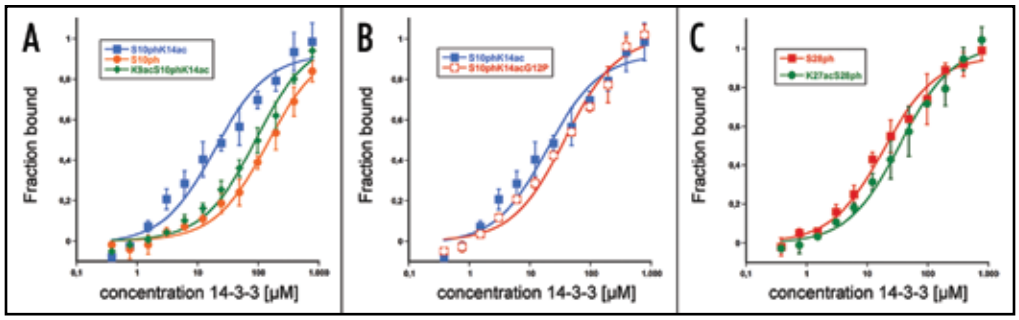

Figure 2. Extrinsic factors influencing the interaction of 14-3-3 with histone H3. (A) Discrete degrees of acetylation result in different effects on the interaction with 14-3-3ζ. Binding curves for the phosphorylated histone H3 peptide (H3S10ph), the single phosphoacetylated peptide (H3S10phK14ac) and the double phosphoacetylated peptide (H3K9acS10phK14ac) were determined as described for Figure 1. Single acetylation of H3K9 or H3K14 results in increased affinity of 14-3-3 for the H3S10ph peptide[24,25] whereas for the double phosphoacetylated peptide (H3K9acS10phK14ac) this effect is abolished. (B) Acetylation cannot further increase the affinity for the H3S10phK14ac when proline is at position P + 2 from serine 10. (C) Acetylation of H3K27 does not increase binding to the H3S28ph peptide, which already contains a proline at P + 2.

Recent evidence indicates that additional histone modifications modulate interaction between S10 phosphorylated histone H3 and 14-3-3. Additional acetylation on either H3K9 or H3K14 significantly increases the affinity for the S10 phosphorylated H3 tail.[24,25] Importantly, this enhancing effect is only observed for single acetylation events, as an H3K9acS10phK14ac peptide showed interaction parameters similar to the single phosphorylated histone H3 tail (Fig. 2A and ref. 23). This demonstrates that discrete degrees of histone H3 acetylation result in different affinities for the interaction with 14-3-3.

Since the crystal structure of 14-3-3ζ in complex with the histone H3 tail has been determined with the phosphorylated and double phosphoacetylated form, it is unclear how single acetylation may increase the affinity of 14-3-3. The H3K14 side chain is directed outward of the 14-3-3 binding cleft, but the acetyl moiety is not visible in the crystal structure suggesting a flexible state of this group.[23] In contrast, the acetyl group at H3K9 folds back and forms a hydrogen bond with the backbone amide of alanine 7, causing some minor reorganization of the peptide backbone. The selectivity of the 14-3-3 consensus motifs is restricted to the sequence from position P - 4 to P + 2.[13] Thus, it is likely that acetylation of H3K14 causes increased affinity via a peptide intramolecular interaction rather than by direct interaction with 14-3-3. The exit of the histone H3S10ph peptide from the binding cleft is mediated by the tandem glycines at P + 2 and P + 3. This structural feature appears to be not optimal for the interaction with 14-3-3 and proline at P + 2 would be clearly favorable (Figs. 1C, D and 3).[13,23] Therefore, we speculate that one mode how lysine acetylation could cause increased affinity may be via the stabilization of a kinked structure that might improve the exit of the peptide from the binding cleft directing it outwards analogously to the P + 2 proline in mode 2 consensus motif. Putatively, the peptide may only assume this conformation upon fitting into the cleft, which requires an extended conformation.[23] This hypothesis is supported by the observation that mutation of H3G12 to proline results in increased affinity of 14-3-3 for the peptide (Fig. 1D) that is not further increased by additional acetylation of H3K14 (Fig. 2B). Similarly, the high binding affinity of 14-3-3 for H3S28ph is also not affected by additional acetylation of H3K27 (Fig. 2C).

Structural analysis shows the H3K9 acetyl group forming a hydrogen bond with the backbone amide of H3A7. This residue points outward the binding cleft and is not involved in intramolecular interactions in the non-acetylated peptide.[23] Further, the acetyl group of H3K14 appears not to participate in any interactions in the double acetylated peptide and remains flexible.[23] Interestingly, double H3K9/K14 acetylation does not significantly increase the interaction with the H3S10ph peptide (Fig. 2A and ref. 23). Likewise, dimethylation of H3K9 has no significant effect on the affinity of 14-3-3 for the peptide.[25] These observations imply that charge neutralization via acetylation of one lysine (H3K9 or H3K14) is favorable for the interaction with 14-3-3, whereas charge neutralization of both lysines (H3K9 and H3K14) abrogates the enhancing effect. Based on these observations it is tempting to speculate that acetylation of one lysine may result in the formation of either an inter- or intramolecular interaction (see above), that supports the organization of the peptide in the 14-3-3 binding cleft. If both lysines are acetylated, this interaction cannot be established. The acetyl group of H3K9 folds back to form the hydrogen bond with the backbone amid of alanine 7, while the acetyl group on H3K14 adopts a flexible conformation.[23]

Another possible way how PTMs may facilitate the interaction with 14-3-3 comes from structural predictions of the histone H3 tail. The histone H3 amino-terminal region is assumed to be mainly unstructured. Several stretches were recently predicted to have a high probability of adopting α-helical conformation, in particular the stretch from H3T3 to H3S10.[38] Computational simulation showed that the stability of this helical population may be significantly reduced upon lysine acetylation,[38] whereas serine phosphorylation was predicted to stabilize helical conformations.[39] In addition, the computer model predicts that single dimethylation of H3K9 shows no major shift in α-helix population, but in concert with H3K4 dimethylation leads to reduced stability of the α-helical conformation.[38] In general, the interaction of proteins with the histone H3 tail requires an extended conformation. The relaxation of putative α-helical structures by PTMs could therefore enhance the contact with binding proteins by facilitating a shift in the equilibrium between the helical and non-helical conformation states. Although, the impact of H3K9/H3K14 double acetylation was not simulated, it appears that these effects are rather cumulative. Such an interpretation is therefore hardly compatible with

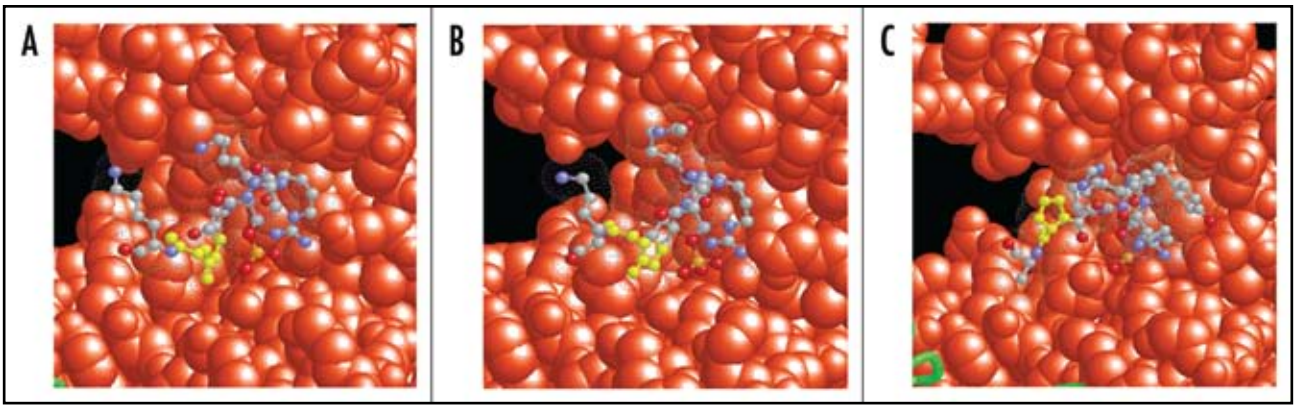

Figure 3. Critical amino acids at position P + 2 mediate the exit of the peptide from the 14-3-3 binding cleft. (A) H3S10ph histone H3 peptide (ball and stick view with dotted van der Waals radii) located within the 14-3-3 binding cleft (spacefill view, orange atoms) (PDB entry 2C1N).[23] The tandem glycine residues that mediate exit of the peptide from the binding cleft are highlighted in yellow. (B) Representative view of the H3K9acS10phK14ac histone H3 peptide (PDB entry 2C1J) arranged as described for panel A.[23] Representative view of the mode 2 binding peptide (PDB entry 1QJA) the proline at position P + 2 that mediates the exit from the binding cleft adopts *trans* conformation and is highlighted in yellow.[12] Figures were rendered using RasMol software on the designated PDB-data files.

the observation that double acetylation abolishes enhanced 14-3-3 binding (Fig. 2A).

Besides the discussed examples, the impact of several other possible modifications on the interaction between histone H3 and 14-3-3 has not yet been investigated. For example, methylation of arginine 8 (P - 2) could probably impact the interaction with the phosphate-oxyanion (see above). Also, phosphorylation of threonine 11 might modulate the accessibility of phosphorylated serine 10. However, it is not clear whether such hypothetical modification patterns are indeed established in vivo.

Implications for Combinatorial Modification Patterns

Several examples for modulation of protein binding via combinatorial modification patterns have been described,[24,25,36,37,40-43] suggesting that histone modifications are frequently cooperative. A biological effect of a PTM might rather depend on the complete modification "make-up" of the histone tail or even entire nucleosomes than on a singular readout.

One obvious advantage of modulating the binding of PTM detector proteins to substrates via combinatorial modification patterns is the increased ability to fine tune the interaction and gain additional control levels. In the case of histone H3S10 phosphorylation the interplay with the binding protein of H3K9 methylation, heterochromatin protein 1 (HP1), and 14-3-3 proteins constitutes a reciprocal system. HP1 is displaced by additional S10 phosphorylation[36,41] or phosphoacetylation[44] while 14-3-3 is recruited at the same time (Fig. 4).[25] Additional acetylation of H3K14 increases the affinity of 14-3-3 for H3K9me2S10ph histone H3 thereby supporting recruitment to relevant genomic regions. Hence, the triple modified form allows to efficiently "override" the transcriptional repressive H3K9 methylation not only by HP1 displacement but also via 14-3-3 recruitment.[25] Since H3S10ph already leads to displacement of HP1, why should "overriding" of H3K9 methylation be important for transcriptional activation?

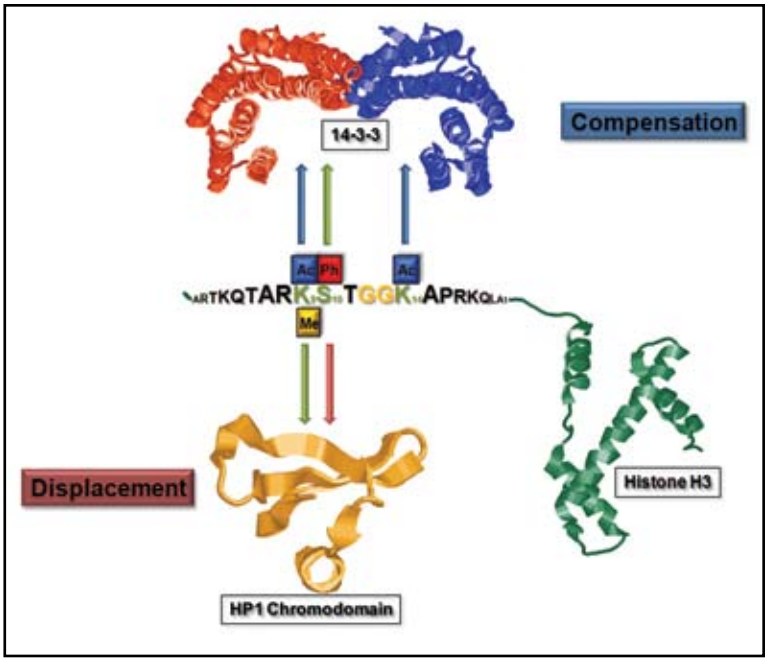

Figure 4. Modulation of detector protein binding by combinatorial modification patterns. The panel depicts two distinct modes of action for combinatorial modification patterns. In the case of the 14-3-3 histone H3 interaction phosphorylation of serine 10 (green residue) is the initial trigger for binding (green arrow upwards), which is of low affinity. Additional acetylation of lysine 9 or 14 compensates for the non-optimal interaction platform provided by the P + 2/3 tandem glycine backbone (orange residues) and supports the interaction between histone H3 and 14-3-3 (blue arrows up). The binding of the HP1 chromodomain exemplifies another mode how complex modification patterns can impact the interaction of modification dependent binding proteins. The initial trigger for the interaction with the chromodomain is di- or trimethylation of H3K9 (green arrow down), which in general generates transcriptional repressive chromatin. The additional phosphorylation of serine10 results in displacement of HP1 and allows for subsequent interaction with 14-3-3 proteins (upper part).

Knockdown of particular 14-3-3 isoforms resulted in reduced transcriptional induction of genes regulated by histone H3 phosphoacetylation.[25] Therefore, it appears that establishment of H3

Table 1 Dissociation constants in μM for differentially modified histone H3 peptides determined by fluorescence polarization measurements

Peptide	Kd [μM]
H3Um (1–20)	450,86 ± 158,67
H3S10ph (1–20)	140,68 ± 22,68
H3S10phG12P (1–20)	19,92 ± 5,36
H3S10phK14ac (1–20)	19,28 ± 5,6
H3S10phK14acG12P (1–20)	39,98 ± 7,9
H3K9acS10phK14ac	108,88 ± 18,6
H3Um (25–38)	403,55 ± 51,55
H3S28ph (25–38)	18,65 ± 2,9
H3K27acS28ph (25–38)	34,86 ± 5,68
H3S28phP30A (25–38)	162,0 ± 18,56

Values are average of at least three independent measurements (Kd [μM]) and Standard deviation is indicated.

phosphoacetylation and displacement of HP1 is not sufficient for gene activation. An H3S10ph binding protein with activator function seems to be required.

Shut-down of transcription in this system might involve the activity of histone deacetylases (HDACs) as well as serine/threonine phosphatases. These enzymes re-establish the repressive H3K9me2 signature without the requirement of histone methyltransferases and allow for re-association of HP1, provided that the promoter-associated nucleosomes are not exchanged during transcription.

Active deprivation of H3K9 methylation was demonstrated for other systems such as androgen receptor mediated transcriptional activation.[45-47] In the case of some phosphoacetylation target genes H3K9 methylation is rather transformed into complex modification forms like H3K9meS10ph or H3K9meS10phK14ac.[25,48] Why then is H3K9 methylation not always removed? The repressive effect of H3K9 methylation is in these modification states "ignored" since both activating modifications are efficiently bound by 14-3-3.[25] Obviously, cellular regulation relies on memory systems and certain histone methylation signals must be retained. In these cases, the readout is rather regulated than the mark itself. Removal of phosphorylation and acetylation signals suffices for re-establishing a transcriptional repressive environment.

Two enzymes are able to remove methylation of H3K9. In the context of androgen receptor mediated transcriptional induction LSD1 resolves the dimethylated and monomethylated states. JmjC-domain containing demethylases like JMJD2C are active on trimethylated H3K9.[45,47,49] Interestingly, both enzymes appear to be excluded from their substrates when H3S10 is phosphorylated. Also, the activity of LSD1 on hyperacetylated nucleosomal substrates is reduced.[50,51] Obviously, phosphorylation and acetylation signals therefore can protect H3K9me against demodification. Interestingly, phosphorylation of threonine 11 was recently demonstrated to stimulate H3K9 demethylation and to facilitate androgen receptor mediated transcription[46] suggesting that histone phosphorylation can also propagate demethylation depending on the particular modified residue.

Implications for Intrinsic Factors

All different enzymes adding or removing diverse but spatial closely located modifications on histones have to recognize and act on the same amino acid "platform" (apart from previously positioned modifications). Therefore, evolutionary constraints imposed on the amino-terminal tails of histones may have favored the generation of multi-modifiable patches, accessible to a vast variety of different enzymatic machineries, probably at the cost of substrate efficiency.

The same restrictions might limit the interaction with PTM-dependent binding proteins. One concrete example, the interaction between 14-3-3 and phosphorylated histone H3 was discussed in this article. H3S10 phosphorylation mediates only weak interaction with 14-3-3 proteins and one particular factor for this low affinity is the lack of the P + 2 proline, which is functionally replaced by tandem glycine residues (Fig. 1A). Substitution of the P + 2 position by proline would be clearly favorable for 14-3-3 binding (Fig. 1D), but evolutionary constraint retained tandem glycines, indicating that these residues may be important for other interactions and therefore indispensable.[51] The insufficiency of the amino acid patch surrounding H3S10, to mediate strong initial interaction with 14-3-3 can be compensated by additional acetylation of either H3K9 or H3K14.[24,25] One particular function of combinatorial modification patterns for this interaction may therefore be compensation of non-optimal interaction platforms (Fig. 4). Such non-optimal motifs may have originated from evolutionary constraint amino-acid mutability required to maintain modification versatility. However, acetylation of H3K9 or H3K14 has additional effects than modulating 14-3-3 binding. Also this modification is much more abundant than interphase histone phosphorylation. Therefore direct coevolution of both events appears unlikely.

Histone H3S10 phosphorylation can be mediated via mitogen activated protein (MAP) kinase pathways and some immediate early genes (IE) are rapidly and transiently induced by stress stimuli.[33] It however appears desirable to restrict the plethora of potentially activated genes in a manner adequate to the precise stimulus. One possibility for such tight control is to limit the kinase substrate interaction. Indeed it was demonstrated that overexpression of the histone H3S10 kinase MSK1 does not change either distribution or overall amounts of histone H3S10 or H3S28 phosphorylation, despite full activation of the exogenous kinase.[52] Obviously additional factors are critical to allow for the placement of either histone H3S10 or H3S28 phosphorylation. Not all genes targeted by histone H3S10 phosphorylation are activated upon transient MAP kinase stimulation but rather require complex modification patterns.[25,34] The requirement for a dual modification also allows for a more refined binding regulation and transcriptional activation. Expression of these genes is more tightly regulated as two distinct pathways are required for full transcriptional activation.

Another example on the function of combinatorial modification patterns was provided by studies on the displacement of HP1 proteins bound to H3K9me2/3 via H3S10 phosphorylation (Fig. 4).[36,41] In this context the combinatorial modification obviously does not compensate for non-optimal binding conditions but rather provides a rapid displacement of the binding protein without need to "erase" the epigenetic information of H3K9 methylation.[53] Obviously this epigenetic "memory maintenance" system is extremely valuable during mitotic progression.

These examples demonstrate possible functional outcomes of combinatorial modification patterns: positive compensation for non-optimal binding motifs and increased regulatory control in the case of the 14-3-3 and H3S10ph interaction, or generation of unfavorable binding platforms epitomized by H3K9me2/3S10ph in the case of HP1 proteins (Fig. 4). It is important to mention that in vivo the complexity of these events may be significantly expanded by the putative contribution of additional factors and also the more limited access to nucleosomal histones.

Consideration of Histone Variants

Posttranslational modification of histone amino-terminal tails constitutes an important mechanism for the regulation of genome accessibility.[54] Within the nucleosomal core histones specialized variants have evolved. These take over particular functions in genome organization like centromere maintenance or constitutive heterochromatin formation.[30,54] Besides the centromere specific isoform, Centromeric protein A (CenpA), three additional histone H3 isoforms are expressed in mammals, designated as H3.1, H3.2 and H3.3.

Concerning the transcription-associated interaction between histone H3 and 14-3-3 proteins the latter isoform H3.3 is particular interesting. Histone H3.3 can be incorporated into chromatin outside of S-phase in a replication independent manner (RI), which is important for nucleosome exchange during transcription.[55] Phosphorylation of H3S10 and H3S28 may be spatially separated[52,56] and asymmetrically targeted to specific isoforms, as H3.3 was found to be the main species phosphorylated at serine 28 in chicken erythrocytes.[57]

Because of the spatial separation in interphase cells, H3S10ph and H3S28ph may correlate with transcriptional activation of distinct target genes. As interaction between 14-3-3 and H3S28ph is significantly stronger,[23,25] target genes for H3S28 phosphorylation may be less dependent on additional histone acetylation to enable 14-3-3 binding. For genes targeted by serine 10 phosphorylation, additional lysine acetylation, besides other functions, is important to stabilize the interaction with 14-3-3.[25]

Although H3S28 phosphorylation may not require additional acetylation for 14-3-3 binding, the modification co-exists with additional histone H3 acetylation in vivo and is even stimulated by preceding HDAC inhibition.[52] This indicates that histone acetylation may be nevertheless important for transcriptional activation of H3S28ph targets but in a different context than reinforcement of 14-3-3 binding. Based on the in vitro interaction studies the reason for this may differ between both systems. In the case of H3S10 phosphorylation, binding of 14-3-3 is stabilized by additional acetylation (H3K9 or H3K14). The interaction with the H3S28 phosphorylated histone H3 tail is not modulated (Fig. 2C). However, increased acetylation supports phosphorylation of H3S28[52] and thereby creation of an efficiently bound 14-3-3 substrate. Therefore, the recruitment 14-3-3 to H3S10ph and H3S28ph, may be modulated by additional acetylation. H3S28 phosphorylation is more abundant in the context of hyperacetylated histone H3 and this may directly correlate with increased recruitment of 14-3-3 proteins and transcriptional activation.

It will be important to determine whether histone H3 acetylation directly mediates increased S28 phosphorylation, or if HDAC inhibition modulates the activity of H3S28 kinase activity.

Conclusions

The high degree of conservation and slow evolution of histone molecules emphasizes the evolutionary constraints imposed on these proteins that provide the structural basis for genome organization.[54] Besides specialized histone variants, PTMs provide an additional tool for the generation of diversity in a more dynamic manner. Several studies indicate that PTMs can operate as combinatorial rather than single entity. These investigations demonstrated different readouts of combinatorial modifications either by generating positive or repulsive effects. The advantages of combinatorial modification patterns discussed here are multi-layered, which is emphasized by the different effects of known combination systems. These include increase in binding affinity and thereby gain of control options, as demonstrated for 14-3-3, or reduced affinity as demonstrated for HP1.[24,25,36,41]

A major step to investigate this interplay is the mapping of combinatorial modification patterns in vivo. Mass spectrometry based approaches are promising tools towards a profound understanding in combinatorial modification patterns.[25,36,58-63] Such studies may also provide a basis for the generation of antibodies against complex PTM patterns and genome wide mapping approaches.

Complex PTM patterns may constitute biological relevant factors and yield a single readout rather than simple additive effects (e.g., the more acetylation the more binding the more transcription). This is supported by the observation that increased affinity of 14-3-3 for histone H3S10ph is mediated by single acetylation, but abandoned by double acetylation (Fig. 2A). Different "forms" of phosphoacetylation therefore result in different impacts on the interaction with 14-3-3 and not in simple additive effects.

Also the displacement of HP1 proteins from H3K9me2/3 by H3S10 phosphorylation constitutes a specific non-additive event. The obvious advantage of this system is the retention of epigenetic information. This appears to be desirable not only during mitotic progression[36,41,44,53] but also during transcriptional activation of particular target genes.[25,48] The generation of multiple modified histone forms may comprise an important tool to temporarily switch from an epigenetically determined state to another. The epigenetic information is not removed but temporarily "faded out" by assembly into complex PTM patterns. The generation of complex PTM combinations may therefore provide an elegant system to dynamically regulate the maintenance of cellular memory.

Acknowledgements

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4. Final discussion and additional results

Context specific functions of histone H3 serine 10 phosphorylation

Phosphorylation of histone H3 at serine 10 is placed under different cellular conditions with remarkable differences in abundance, kinetics and associated biological function (see also section 2.1 Introduction).

Firstly, the modification is regulated in a cell cycle dependent manner and decorates the condensed mitotic chromosomes with increasing abundance from late G2 phase until early telophase (Hendzel *et al.*, 1997) (Figure 4-1). In contrast, in interphase cells serine 10 phosphorylation is much less abundant and associated with transcriptional activated regions (Mahadevan *et al.*, 1991).

Secondly, it was demonstrated that phosphorylation of serine 10 together with methylation of lysine 9 is placed along large genomic domains spanning genes which are transcriptionally silenced during terminal differentiation of mesenchymal stem cells (Sabbattini *et al.*, 2007). In addition, histone H3S10 and H3S28 phosphorylation were found to coincide with osmotic stress mediated transcriptional repression of the “mouse mammary tumor virus” (MMTV) promoter. The fundamental differences of these diverse biological effects associated with histone H3 phosphorylation evoke the conclusion that the impact of this modification does not directly impinge on the structural level of chromatin but is rather mediated *via* modification dependent binding proteins (see also 1.7 Impact of post translational modifications on chromatin states.). However, the events mediated by histone H3S10 phosphorylation during these different biological processes may probably involve common “traits” which will be discussed below.

The differences in abundance and kinetics may be an attribute of the particular enzymatic machinery that places histone H3S10 phosphorylation. In case of the cell cycle regulated mitosis specific phosphorylation the Aurora B kinase was identified as the major enzyme. Expression and activity of Aurora B is restricted to late G2/M transition and tightly associated with global histone H3S10 phosphorylation (Hsu *et al.*, 2000; Crosio *et al.*, 2002) (see also 2.1 Introduction and Figure 4-1). Several different signal transduction pathways were

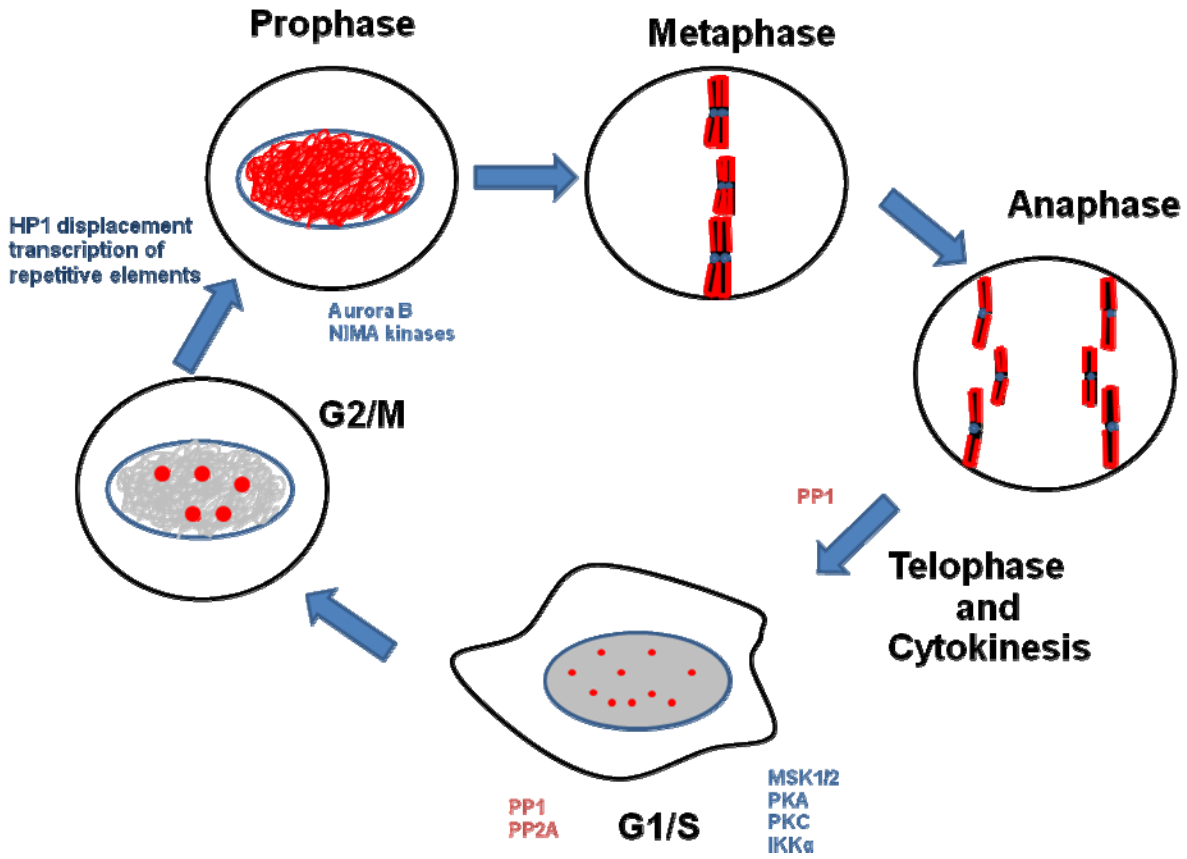


Figure 4-1 Mitotic and Interphase phosphorylation of histone H3. During interphase of the cell cycle (G1/S) histone H3S10 phosphorylation is very low abundant and targeted into small speckles (red dots). During this stage of the cell cycle the modification is mediated via several different signal transduction pathways. Responsible histone H3S10 kinases are indicated in blue, phosphatases active in G1 phase are indicated in red. During G2/M transition abundant serine 10 phosphorylation firstly localizes to the pericentromeric heterochromatin and is massively expanding until prophase. High levels of histone H3S10 phosphorylation are maintained until telophase and then rapidly removed by PP1. One function of this mitosis specific modification demonstrated in fission yeast is the displacement of HP1 proteins, which in turn allows for transcription of repetitive elements during S-phase. These transcripts activate the RNAi response pathway, which in turn directs again heterochromatin formation and epigenetic inheritance of these structures.

demonstrated to mediate the “nucleosomal response” during transcriptional activation including MSK1/2, PKA, IKKα and PKC (see Figures 2-1, 4-1 and 1.6.4 Histone phosphoacetylation a special relation in cis). Importantly, the placement of this modification in interphase appears to be a highly regulated event as overexpression of the MSK1 kinase does not alter the steady state levels of this modification (Dyson *et al.*, 2005). In addition, promoter elements of histone H3S10ph target genes cannot be activated in reporter assays by stimulation of either ERK nor p38-MAP kinase activity (E. Simboeck, B. Schuettengruber, C. Seiser unpublished data) suggesting also a spatial component in histone H3S10ph restriction.

Effects of mitotic and interphase histone H3S10 phosphorylation may be mediated by similar but context dependent effects- 1. Displacement of proteins

In addition to the quantitative differences also qualitative deviations between different histone H3S10 phosphorylation events exist. The requirement of this “mitotic marker” modification for M-phase progression has been analyzed in strains of the ciliate *Tetrahymena termophila* and budding yeast carrying mutations of serine 10 to alanine (S10A) (Wei *et al.*, 1999; Hsu *et al.*, 2000). Confusingly, the effects of the histone H3S10A mutation on cell viability and mitotic progression are significantly different between both organisms as *Tetrahymena* strains lacking S10 display chromosome segregation defects, whereas Saccharomyces *cerevisiae* strains were not affected. The reason for this discrepancy is unknown. However, recent data on the function of mitotic histone H3S10 phosphorylation in fission yeast could help to develop a working hypothesis for this problem. One major difference between both organisms concerns the formation of heterochromatic structures. Heterochromatin formation organization is significantly different between both organisms. siRNA mediated heterochromatin formation was demonstrated as essential component for the organization of pericentromeric and centromeric heterochromatin in fission yeast (see section 1.9 RNA as chromatin component). However, the genome of Saccharomyces *cerevisiae* does not encode any component of the RNAi machinery and this organism also lacks histone H3K9 and H3K27 methylation. In this system heterochromatin formation and silencing is mediated by different mechanism involving in particular deacetylases of the Sirtuin class (Pirrotta and Gross, 2005). In contrast the *Tetrahymena* genome encodes three different Dicer like enzymes (DCL1-3) and DCL1 was demonstrated to be involved in genome rearrangement, chromosome segregation and meiosis (Mochizuki and Gorovsky, 2005).

Methylation of histone H3K9 in *Tetrahymena* was so far only observed on heterochromatic structures that are formed during the siRNA (scan-RNA) targeted DNA elimination occurring during macronuclear maturation (Liu *et al.*, 2004). However, siRNA directed histone H3K27 methylation was observed as hallmark for heterochromatin in this organism which also regulates H3K9 methylation in the context of scan-RNA dependent DNA elimination (Liu *et al.*, 2007). Both modifications are bound by an “effector” chromodomain protein Pdd1p. This clearly demonstrates a siRNA mediated component of genome regulation in *Tetrahymena*.

Importantly, one established function of mitosis specific histone H3S10 phosphorylation is the displacement of HP1 proteins from Chromatin (Mateescu *et al.*, 2004; Fischle *et al.*, 2005; Hirota *et al.*, 2005; Dormann *et al.*, 2006; Terada, 2006; Vicent *et al.*, 2006; Sabbattini *et al.*, 2007; Winter *et al.*, 2008b). It was also demonstrated that this histone H3S10ph mediated HP1 displacement is important to allow for transcription of heterochromatic repeats in fission yeast which in turn leads to siRNA mediated silencing of these elements and maintenance of constitutive heterochromatin (Kloc *et al.*, 2008). Therefore, one function of

mitotic histone H3 phosphorylation appears to be the removal of repressive proteins to allow for transcription of these regions, which subsequently leads to siRNA mediated heterochromatin formation and epigenetic inheritance. The absence of the RNAi machinery in budding yeast could therefore be a possible reason for the lack histone H3S10 mutant effects. It would be interesting to determine if the facultative heterochromatin formation that was correlated with histone H3 phosphomethylation in terminally differentiated post-mitotic cells (Sabbattini *et al.*, 2007) would be dependent on a functional RNAi machinery.

Despite these differences of heterochromatin formation in both organisms, mitotic histone H3S10 phosphorylation is present in both of them. This suggests (although not compellingly) that nevertheless histone H3S10 phosphorylation may also be an important component in regulating epigenetic maintenance in *Saccharomyces cerevisiae*, however with a different function than displacement of HP1 proteins. The lack of repressive histone methylations like histone H3K9/K27 methylation and RNAi machineries in budding yeast stipulates that the target for histone H3S10 phosphorylation must be different. In principle two different scenarios for the action of a histone modification are possible: either recruitment (see below) or inhibition of binding of a modification dependent detector protein. It was suggested that in addition to placing repressive histone modifications, the absence of activating modifications (like histone acetylation, or H3K4/K79 methylation) could epitomize a transcriptional repressive (epigenetic) state (Pirrotta and Gross, 2005; Winter *et al.*, 2007). Indeed in budding yeast the presence of activating histone modifications like H3K4/K79 methylation or H4K16 acetylation restricts the binding of Sirtuin containing repressive complexes. In addition, it is well established that SIRT2 is a critical factor for mitotic progression by deacetylating tubulin[1] and histone H4K16 (Dryden *et al.*, 2003; Vaquero *et al.*, 2006; Vaquero *et al.*, 2007). It is not yet known if histone H3S10 phosphorylation would also impair the binding of Sirtuins to chromatin but one possible function of mitotic histone H3S10 phosphorylation in budding yeast may be the regulation of genome accessibility to repressive machineries.

Another hint towards such a probable “restrictor” function of histone H3S10 phosphorylation comes from interaction studies between differentially modified histone H3 tails and nuclear proteins. The interaction of HDAC containing complexes with histone H3 amino terminal tails was investigated via pull down assays and immunoblotting against core components of transcriptional repressor complexes like HDAC1, HDAC2 and RbAp48 (Figure 4-2A). Interestingly, the binding of all three HDAC complex components to the histone H3 amino terminal tail can be established in an HP1 independent manner, as they also interact with the unmodified tail (Figure 4-2A, lane 3). Interestingly, this interaction is almost completely

[1] Tubulin appears not to be acetylated in yeast suggesting that the major mitotic SIRT2 target is histone H4K16 **Polevoda, B., and Sherman, F.** (2002). The diversity of acetylated proteins. Genome Biol *3*, reviews0006.

abolished upon phosphorylation of serine 10 without regard of the additional modifications like methylations and acetylations (Figure 4-2A, lanes 4, 6, 7, 9, 10, 12, 14, 15, 17, 18). In agreement with earlier results (2.3 Presented Publication 1 (Winter et al. 2008). “14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation”.) , under these experimental conditions the interaction between 14-3-3ζ was only stable with the phosphoacetylated forms of the histone H3 tail (Figure 4-2A, lanes 7, 10, 15, 18). This demonstrates that in addition to the displacement of HP1 proteins, histone H3S10 phosphorylation may also lead to the displacement of HDAC containing complexes. Initially the interaction between the Sin3 HDAC complex and histones was attributed to the interaction between RbAp48 and the histone H4 amino-terminal tail (Vermaak *et al.*, 1999). However, RbAp48 can also interact directly with the histone H3 amino-terminal tail ((Wysocka *et al.*, 2006) and Figure 4-2A). Importantly, the contact between RbAp48 and the histone H3 amino-terminus is abolished upon methylation of H3K4 (Wysocka *et al.*, 2006) another histone modification that is mainly associated with transcriptional active promoters.

Despite the complete loss of interaction between the histone H3 amino-terminal tail and HDAC complexes (Figure 4-2A), the interaction in a nucleosomal context may be less susceptible to this effect due to additional interaction platforms like histone H4. It has not yet been tested if histone H3 phosphorylation would also alleviate HDAC binding to nucleosomal histones or arrays. However, some hints exists that histone H3S10 phosphorylation may also impact binding of HDAC complexes to native structured chromatin. It was demonstrated that HDAC- and HAT complexes are globally displaced from mitotic condensed chromosomes similar to HP1 proteins (Kruhlak *et al.*, 2001; Cimini *et al.*, 2003). Indirect immunofluorescence analysis in 3T3 fibroblast with antibodies against serine 10 phosphorylated histone H3 and HDAC1 raise the possibility that regions of strong histone H3S10ph in late G2 cells (G2 speckles, beginning phosphorylation of pericentric heterochromatin, see also Figure 4-1) are indeed depleted for HDAC1 (Figure 4-2B).

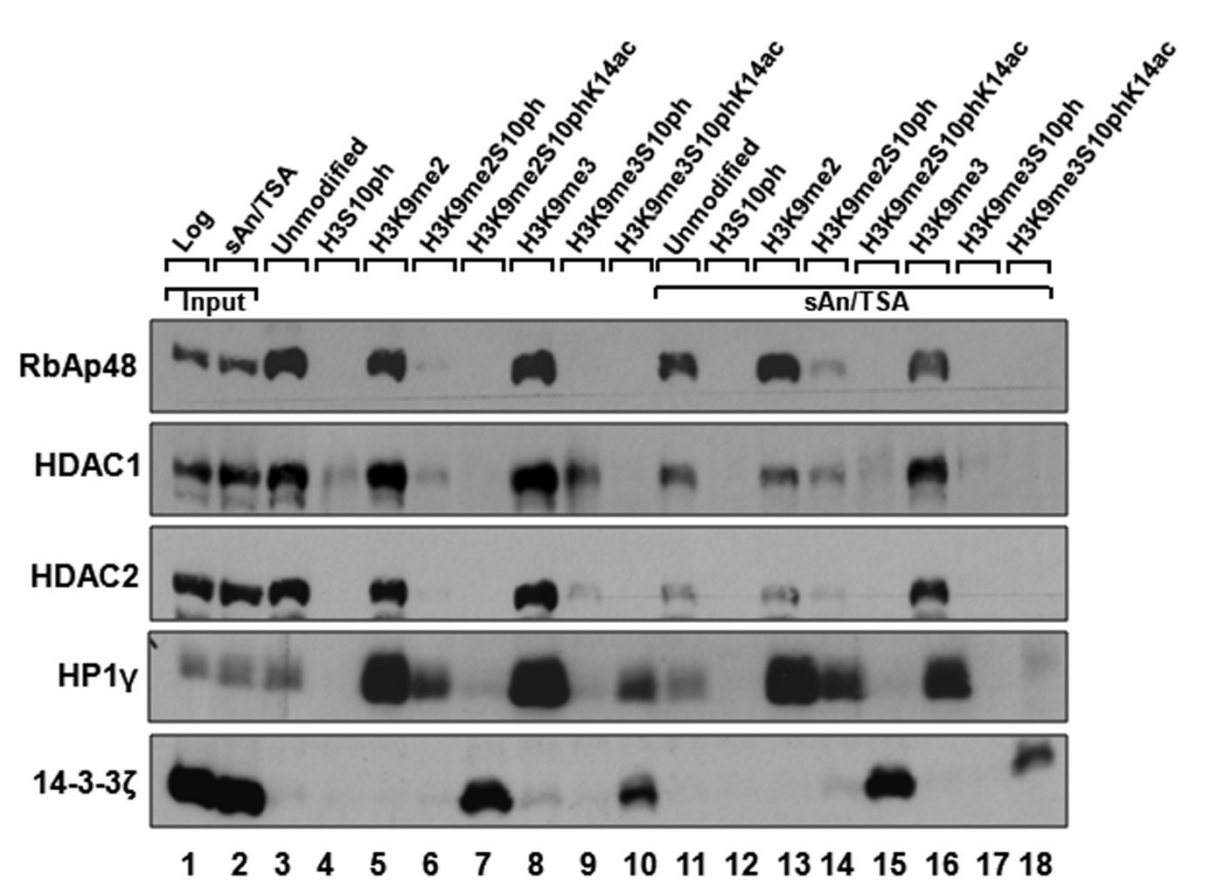
Treatment of the cells with the activator of the p38 MAP-kinase pathway, anisomycin caused increased phosphorylation of histone H3 in small speckles which do also in general not overlap with HDAC1 containing speckles (Figure 4-2B). Importantly, for these experiments cells were pre-extracted thereby removing nuclear components that are not stably associated with either nuclear matrix or chromatin components. However, additional studies and experimental set ups, including different immunofluorescence staining protocols combined with profound statistical analysis will be required to assure this observation. In addition, chromatin immunoprecipitation assays should be used to investigate the working hypothesis at increased resolution.

Nevertheless based on the observations so far one additional function of histone H3S10 phosphorylation may be the displacement of particular (transcriptional repressive) modules

like HP1 proteins or HDAC complexes from chromatin. This displacement may allow for transcription of the corresponding genomic regions. However the exact effect would be stipulated by the particular region transcribed. In case of the repetitive elements within pericentromeric repeats, transcription results in activation of the RNAi machinery and subsequent siRNA mediated heterochromatin formation. In the case of budding yeast where heterochromatin formation occurs in an RNAi independent manner via class III HDACs, histone H3S10 phosphorylation may also support the exact regulation of accessibility of these factors to the genome.

Thereby in addition to inducible gene regulation, dynamically regulated modifications like histone H3 phosphorylation may also contribute to more long term epigenetic phenomena like the maintenance and inheritance of heterochromatin. Importantly such a mechanism would depend on the generation of multiple modified histone species containing highly dynamic modifications, like acetylation or phosphorylation and more stable marks like lysine methylations. As support for this hypothesis, corresponding complex modification states are well documented in distinct stages of the cell cycle (see 2.3 Presented Publication 1 (Winter et al. 2008). “14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation”. and below).

A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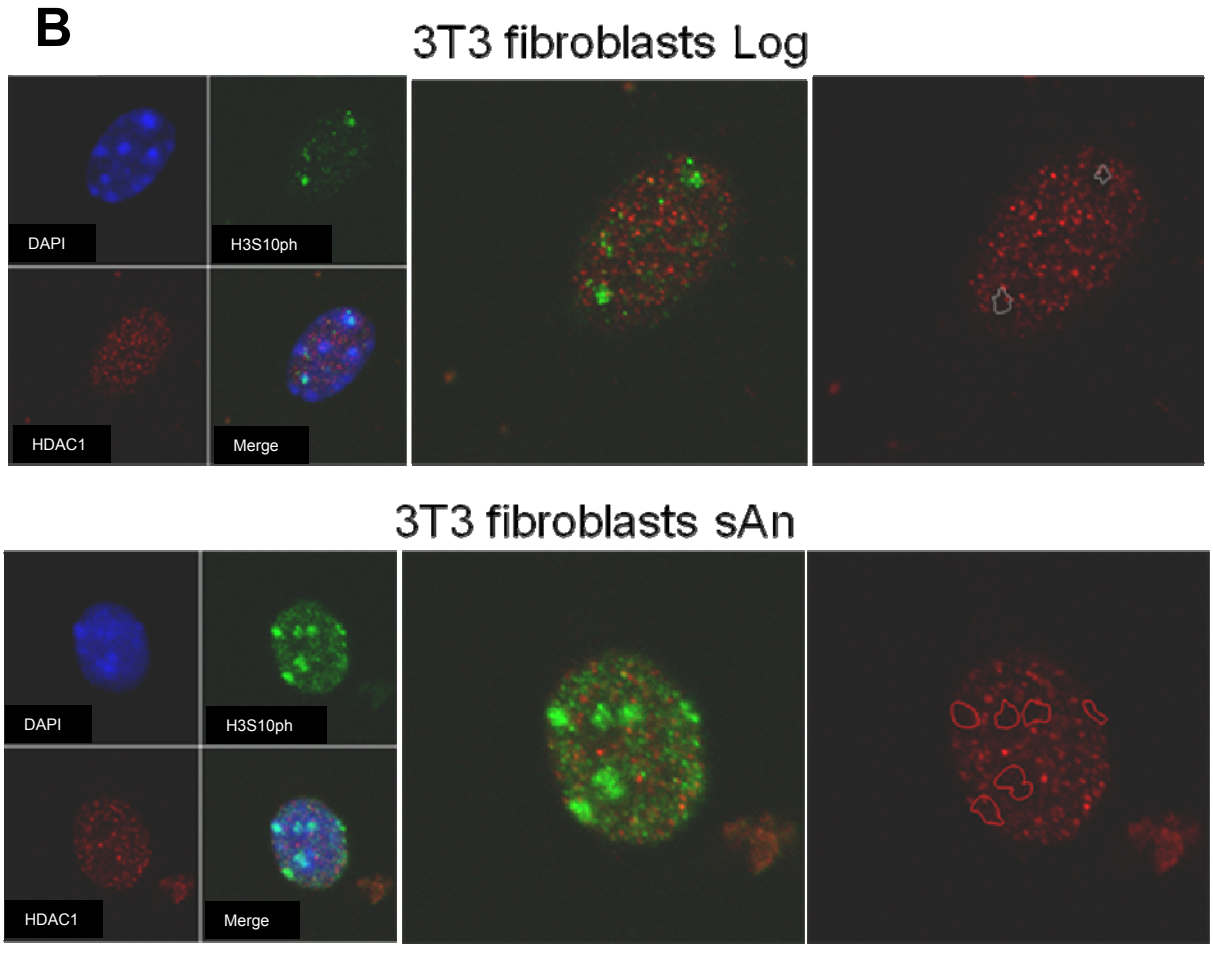
B


3T3 fibroblasts Log
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H3S10ph
HDAC1
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3T3 fibroblasts sAn
DAPI
H3S10ph
HDAC1
Merge

Figure 4-2 Interaction of HDAC complex components with histone H3 tails is blocked by serine 10 phosphorylation. (A) Nuclear extracts from HeLa cells either logarithmically growing (lanes 1, 3-10) or stimulated with anisomycin and TSA for 1h (2, 11-18) were incubated with differentially modified histone H3 peptides. After washing of the binding reactions bound proteins were analyzed by immunoblotting with the indicated antibodies. The blot was probed sequentially with anti-RbAp 48 (Upstate, 13D10), anti-HDAC1 (10E2), anti-HDAC2 (3F3), anti-HP1γ Upstate, 42s2) and anti-14-3-3ζ (Santa Cruz, C16, affinity purified). **(B)** Confocal immunofluorescence microscopy of pre-extracted Swiss 3T3 mouse fibroblasts in late G2 phase. The left panel shows the single channels for either DNA (DAPI staining, blue), anti-histone H3S10ph (Upstate, green) and anti-HDAC1 (10E2, red) or three channel overlay (lower right). Middle panel shows a magnification of the red/green channel overlay. In the right panel the green channel is faded out and areas of massive histone H3 phosphorylation are marked in white (Log cells) or red (anisomycin treated cells). Note that HDAC1 and H3S10ph do mainly not colocalize to the same regions (yellow spots in red green overlay).

Effects of mitotic and interphase histone H3S10 phosphorylation may be mediated by similar but context dependent effects- 2. Recruitment of proteins

The displacement of transcriptional repressive proteins from chromatin *via* histone H3S10 phosphorylation would suggest that the general outcome of this modification may be transcription of the decorated genomic region. However, in the case of some genes it appears that the presence of this modification and the displacement of repressive modules is not fully sufficient for transcription (Winter *et al.*, 2008b). Instead additional modules appear to be required which may be recruited by preceding phosphorylation of serine 10.

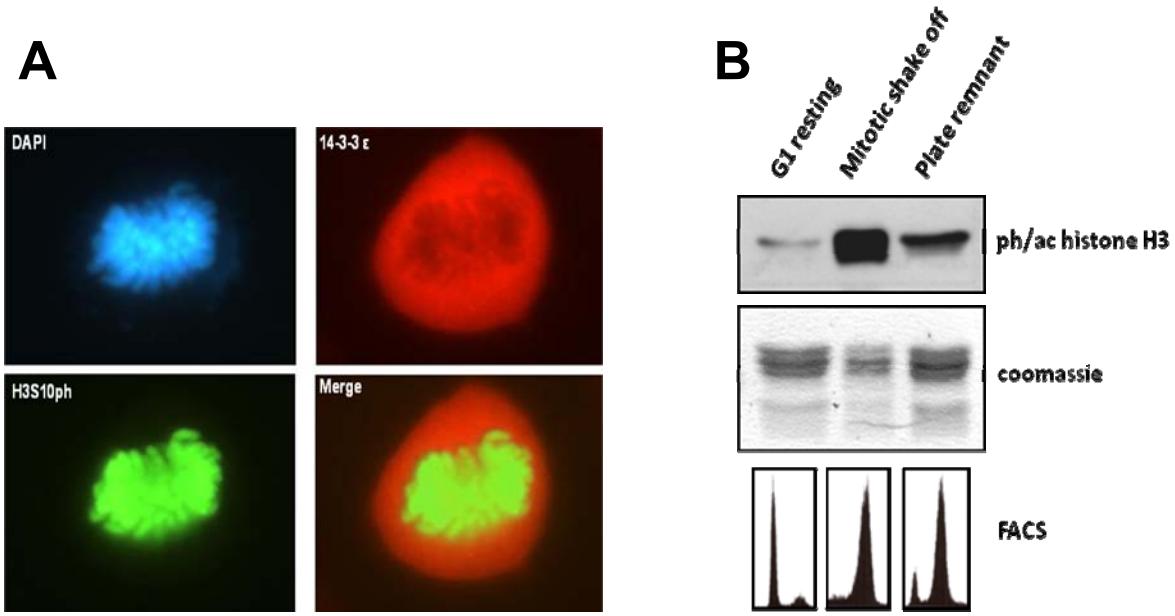
One early function that has been assigned to histone H3S10 phosphorylation described a preference of the histone acetyltransferase Gcn5 for histone H3 when phosphorylated at serine 10 (Cheung *et al.*, 2000b). This observation tempted to explain the strong correlation between histone H3S10 phosphorylation and K9/14 acetylation based on increased enzymatic activity of Gcn5 on histone H3S10ph tails. However, the increased enzymatic activity of Gcn5 was demonstrated to be lost when the enzyme is assembled into the native SAGA complex (Shogren-Knaak *et al.*, 2003) and additional reports implied an independent targeting of both modifications to the same histone H3 tail (Thomson *et al.*, 2001). In addition, it was demonstrated that the recruitment of Gcn5 activity by histone H3S10 phosphorylation is mediated by promoter specific mechanisms depending on the particular transcription factor involved (Lo *et al.*, 2005) (Figure 1-5 and section 1.6.4 Histone phosphoacetylation a special relation in cis). Importantly, the observation that H3S10 phosphorylation may interfere with the accessibility of the amino-terminal tail for HDAC containing complexes raises the possibility that phosphorylation may stabilize nearby acetylations (Figure 4-2A). However, whether only the interaction or also the enzymatic activity of HDACs is blocked by histone H3S10 phosphorylation is not yet clear and currently under investigation.

An event downstream from placing of histone H3 phosphoacetylation, the recruitment of 14-3-3 proteins to the promoter regions of target genes was now demonstrated in several systems (Macdonald *et al.*, 2005; Walter *et al.*, 2008; Winter *et al.*, 2008b). 14-3-3 proteins were established as the first class of proteins that recognize histone H3S10 phosphorylation during interphase. Although the factors downstream of 14-3-3 binding have not yet been explored in detail, it appears that their recruitment contributes to transcriptional activation as siRNA mediated depletion of particular isoforms is sufficient to block induction of the HDAC1 phosphoacetylation target gene (Winter *et al.*, 2008b).

The “displacement function” of mitotic histone H3S10 phosphorylation and the possible therewith associated epigenetic regulation of heterochromatin inheritance appears a well established function. However, it is not clear if additional factors are concomitantly recruited to mitotic chromosomes.

14-3-3 proteins are important in the regulation of cell cycle progression (Hermeking and Benzinger, 2006). Various crucial factors for G2/M transition like CDC25C or cyclinB/cdc2 complexes are regulated by their association with 14-3-3 proteins. However, 14-3-3 does not interact with mitotic condensed chromosomes (Figure 4-3A) despite the massive presence of the preferred post-translational modifications for the interaction with histone H3S10 phosphorylation in conjunction with K9 or K14 acetylation and S28 phosphorylation (Figures 4-3B and C and (Macdonald *et al.*, 2005)). The reason for this exclusion of 14-3-3 from mitotic condensed chromosomes may be attributed to a huge excess of phosphorylated vimentin and keratin intermediate filaments that were shown to create a “14-3-3 sink” during G2/M transition thereby activating key factors for mitotic progression including CDC25C (Margolis *et al.*, 2006).

So far it is not clear if in addition to the displacement function of mitotic histone H3S10 phosphorylation additional factors are also recruited by this modification. One proteins whose localization to condensing chromosomes has been linked Aurora B kinase activity and histone H3S10 phosphorylation is the Barren condensin in *Drosophila* (Giet and Glover, 2001). In addition, it appears that histone H3S10 phosphorylation precedes the condensin mediated chromosome condensation (Cabello *et al.*, 2001) which would be prerequisite for a mechanism where histone H3S10ph recruits condensins. However if there is a direct modification dependent recruitment or a functional link between HP1 displacement and the recruitment of condensin is still unknown (Dormann *et al.*, 2006)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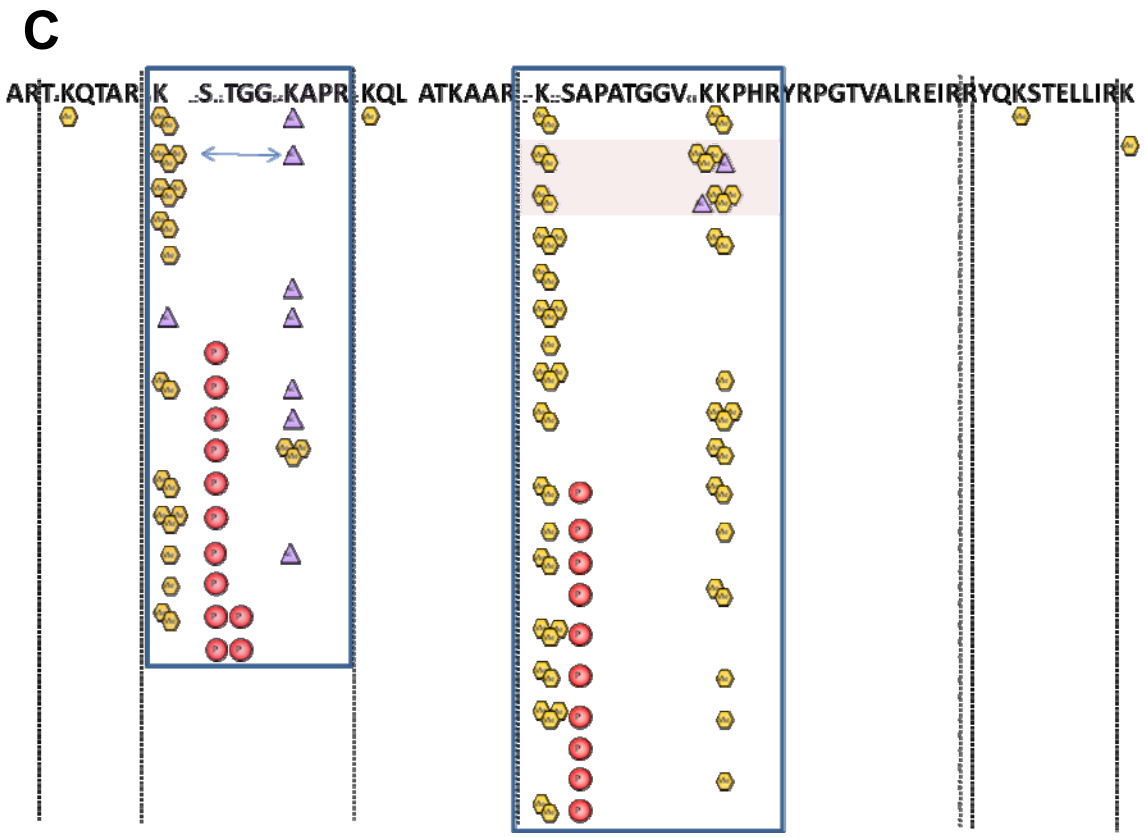
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ARTKQTARKSTGGKAPRKQL ATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTELLIRK

Figure 4-3 14-3-3 proteins are displaced from mitotic condensed chromosomes despite massive formation of phosphoacetylated and phosphorylated histone H3. (A) Indirect immunofluorescence of anaphase 3T3 fibroblasts showing DNA staining (DAPI, blue), anti-histone H3S10ph (upstate, green) and 14-3-3 Epsilon (Santa Cruz T16, red); picture was produced by Thomas Machacek. **(B)** Immunoblot to analyze histone H3 phosphoacetylation (H3S10phK14ac) in mitotic shake-off cells. 3T3 fibroblasts were arrested in G1 phase by serum deprivation for 72h or in mitosis by nocodazole (1µM) treatment for 20h. Less stably attaching mitotic cells were carefully washed from the plate. Samples were split and cell cycle stage distribution was analyzed by fluorescence activated cell sorting (FACS). Mitotic shake-off population contained >90% G2 cells. Histones were prepared as described in 2.3 Presented Publication 1 (Winter et al. 2008). "14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation" and the presence of H3S10phK14ac marks was analyzed by immunoblotting. Loading was controlled by Coomassie staining of a duplicate gel. **(C)** Histone H3 bands from the Coomassie gel shown in (B) were excised and subjected to mass spectrometry analysis. The figure designates the amino acid sequence of histone H3 until position 64. Dotted vertical lines indicate the cleavage sites for trypsin the blue boxes enclose the two peptides formed after tryptic digest that contain serine 10 and serine 28 respectively. Modifications that were found in combination are presented in the same line (meaning each line represents a different modification status of the peptide); methylation: orange hexagons, phosphorylation: red circle, acetylation: blue triangle. Double headed arrow: for this species it was not possible to unequivocally assign the trimethyl-group and the acetyl-group to either position 9 or 14. Red rectangle: the peptides in these two lines were not unequivocally separated.

Modulation of detector protein interaction with histones by multiple post-translational modifications

The evolution of different histone variants, in particular found in organisms with more complex genome organization (Hake and Allis, 2006), suggests that the "common theme" of genome packing exerted by all histones, requires versatility and variation (see section 1.5 Histone variants). Depending on the particular genomic region to be organized, different requirements are posed on the protein component of chromatin. One clear cut example is provided by the incorporation of centromere specific histone H3 variant, which is found in all eukaryotic organisms (also in organisms with holocentric chromosomes (Nagaki *et al.*, 2005)). Apparently, the specialized structural requirements posed on centromeric chromatin are not compatible with the traits of canonical histone H3 and variation of the "standard packing" function is required.

In addition to the incorporation of histone variants, post-translational modifications provide the possibility to generate variation in a more dynamic manner. These modifications are well established in their function as platforms for the interaction with modification dependent proteins (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Fischle *et al.*, 2003b; Taverna *et al.*, 2007; Gelato and Fischle, 2008). Several different protein domains that provide the molecular basis for modification dependent interaction with histones have been excessively elaborated and expanded the understanding how modifications contribute to biological phenomena (Taverna *et al.*, 2007).

The amino terminal tail of histone H3 is subjected to a multitude of different post-translational modifications (Figure 4-4) which are placed, recognized and removed by various different enzymes and “detector” proteins. Despite fundamental biochemical differences in enzymatic reactions and interaction parameters, different factors modifying identical or closely spaced residues encounter an identical amino acid backbone. The same considerations may be also relevant for the detection of post-translational modifications via modification dependent interaction domains (see section 1.7 Impact of post translational modifications on chromatin states.).

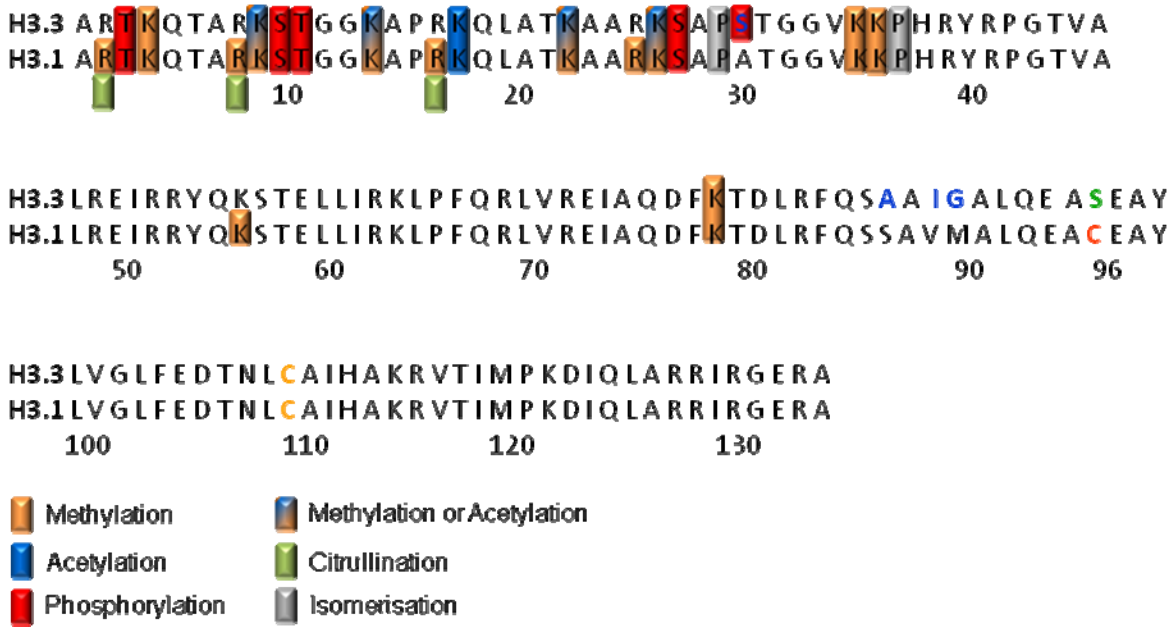

Figure 4-4 Histone H3 post-translational modifications. The different possible post-translational modifications are indicated for either histone H3.1 or histone H3.3. Differences in the amino acid sequence between both isoforms are indicated in color.

For this reason it has been suggested that evolutionary constraints posed on the amino acid sequence of histone amino-terminal tails favored the generation of “platforms” that are accessible to a wide range of different post-translational modifications (Winter *et al.*, 2008a) (see section 3.3 Presented Publication 2 (Winter et al. 2008). “Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns”). Such versatility constraints may most probably help to meet the requirements of a wide range of different enzymes and binding proteins, even though this may occur at the cost of substrate efficiency (Winter *et al.*, 2008a). Therefore, one may speculate that evolutionary optimization may affect chromatin modifying enzymes and binding proteins rather than histone molecules.

Histone modifying enzymes differ considerably with regard to their substrate specificity. For example some histone acetyltransferases can modify a wide range of histones i*n vitro*, like PCAF which can acetylate all four different core histones (Schiltz *et al.*, 1999) whereas others like MOF are specific for a particular histone residue (Akhtar and Becker, 2000). In the first case, specificity *in vivo* may be either not required or may be posed upon assembly into multi-protein complexes. In the first scenario the task of the enzyme would be to acetylate as many sites as possible, thereby contributing to transcriptional activation. In case of histone methyltransferases and demethylases it appears that in general these enzymes show relatively high levels of specificity for particular residues suggesting a backbone driven interaction constraint (Lachner *et al.*, 2003; Klose *et al.*, 2006; Klose and Zhang, 2007; Tian and Fang, 2007). The interaction between histone H3 and some modification dependent binding proteins appears to be "optimal" with regard to the interaction parameters. For example single amino acid substitutions within the histone H3 tail cause already a severe reduction in binding affinity of HP1 proteins to methylated K9 (Fischle *et al.*, 2005). Similar observations where made with several other modification depending binding modules (Taverna *et al.*, 2007).

In case of histone H3S10 and S28 phosphorylation, interaction with 14-3-3 proteins is significantly different. The interaction with S28 phosphorylated H3 molecules is much more stable than with S10 (Macdonald *et al.*, 2005; Winter *et al.*, 2008b). The reason for this difference has been attributed to the lack of a proline at position P+2 from the phosphorylated serine (see section 3.3 Presented Publication 2 (Winter et al. 2008). "Modulation of 14-3-3 interaction with phosphorylated histone H3 by combinatorial modification patterns"). Therefore, in the context of S10, 14-3-3 encounters a non-optimal binding motif whereas S28 provides the critical P+2 proline and therefore high affinity interaction. One major difference concerning the 14-3-3 and histone H3 interaction with regard to other modification-dependent binding proteins concerns overall substrate specificity. Whereas, many binding proteins are specific for histones or few other proteins, 14-3-3 proteins bind to several hundreds of factors with a wide range of possible biological effects (see section 3.2 Impact on target proteins upon interaction with 14-3-3 proteins). This multitude of interaction partners may limit the possible adaptations within 14-3-3 proteins to optimize their interaction with one particular factor and may favor the most successful configuration for wide range binding partners.

The placement of histone post-translational modification provides a dynamic system for the generation of versatility. In addition, the formation of multiple modified species may help to fine tune the "function" of a given modification by adding a context dependent component. Two examples for histone H3S10 phosphorylation in combination with other modifications are now well established: the displacement of HP1 proteins from K9 methylated histone H3

and the increase in binding affinity of 14-3-3 proteins for histone H3S10phK9/14ac. In combination with H3K9 methylation, H3S10 phosphorylation leads to displacement of HP1 proteins. Single histone H3S10 phosphorylation causes low 14-3-3 interaction affinities due to the lack of P+2 proline. However, placing of single acetyl groups compensates for this non-optimal binding motif (Walter *et al.*, 2008; Winter *et al.*, 2008a; Winter *et al.*, 2008b). Importantly, the substitution of the tandem glycines that mediate the exit of the histone H3 tail from 14-3-3 would severely affect the activity of the histone demethylase JMJD2A (Ng *et al.*, 2007) and suggesting that adaptations contributing to high affinity 14-3-3 binding may not be possible on the histone H3S10 backbone level due to interference with the function of other histone H3 modifying/binding factors. In this context combinatorial modification patterns would support the interaction with a protein that would otherwise not engage in a stable interaction. This is supported by the observation that stable histone H3 phosphoacetylation at the HDAC1 promoter is required for 14-3-3 recruitment and transcriptional induction and H3K14 is critical for the localization of 14-3-3 in yeast (Walter *et al.*, 2008; Winter *et al.*, 2008b).

Conclusion

Histone H3S10 phosphorylation is placed under different cellular conditions depending on cell cycle or differentiation stage and signal-transduction pathway activation. Because of the considerable differences in chromatin conformations between these diverse conditions, it is unlikely that histone H3S10 phosphorylation may directly impact the structural level of chromatin, but rather affects the affinity of modification dependent binding proteins. So far two functional outcomes of interphase serine 10 phosphorylation have been described. Firstly, the disruption of the transcriptional repressive binding module HP1 with K9 methylated histone H3 and secondly the recruitment of 14-3-3 proteins. The binding of 14-3-3 proteins to serine 10 phosphorylated histone H3 is of low affinity but additional acetylation of either lysine 9 or 14 (phosphoacetylation) significantly reinforces the interaction. Surprisingly, this effect is abandoned by simultaneous double acetylation of both lysines whereas H3K9 methylation does not affect the interaction. This suggests that only discrete modification-species are bound with high affinity.

Whereas HP1 displacement appears to be relevant during all stages of the cell cycle, the phosphoacetylation dependent recruitment of 14-3-3 proteins does not occur during mitosis. This implies that other proteins may be recruited by mitosis specific serine10 phosphorylation or that the displacement of HP1 proteins is “sufficient” for the required biological effect. Importantly, mitosis specific and interphase phosphorylation have now been linked to transcription of the decorated genomic regions. The mitosis specific HP1 displacement has been correlated with transcription of repetitive elements, which is required for subsequent

RNAi mediated epigenetic inheritance of heterochromatic structures. Also interphase histone H3S10 phosphorylation was demonstrated to mediate HP1 displacement at relevant promoter regions. However, it appears that the displacement event is not sufficient for transcriptional activation of some genes and the recruitment of 14-3-3 proteins is required.

Despite the presence of histone H3 serine10 phosphorylation has been associated with transcriptional activation, the displacement of HP1 proteins by histone H3 phosphorylation/methylation was also demonstrated for huge genomic regions during terminal differentiation. Although it is not yet clear whether this silencing event is somehow related to transcription of sequences that may target RNAi mediated heterochromatin formation to these sites, histone H3S10 phosphorylation definitely exerts several different context dependent functions.

This implies the absence of a general “histone code” for this particular modification but rather favors the view of a “context dependent histone code”, which appears to be an attractive model for the function of several different histone modifications.

The “histone code” of 14-3-3 binding to serine 10 phosphorylated histone H3 and a possible relevance in transcription has been investigated in the course of this thesis. Surprisingly, the conducted experiments demonstrated that the interaction is dependent on an auxiliary acetylation, indicating that only distinct histone H3 modification species constitute targets of varying affinity for 14-3-3 proteins. This adds a certain level of complexity to the role of 14-3-3 proteins in chromatin biology as binding may be regulated by two different modifications. An open question remains the distribution of complex modification species that are high affinity sites for 14-3-3 proteins within the genome. Genome wide mapping approaches for 14-3-3 proteins and complex histone modification species, using ChIP on chip and Solexa technology are valuable tools in the field of chromatin research. Finally such studies would aim to decipher the functional role of 14-3-3 proteins in chromatin biology and expand our understanding for the requirement of complex post-translational modification patterns.

5. Material and Methods

5.1 Tissue Culture

5.1.1 Cell cultivation

Swiss 3T3 mouse fibroblasts and HeLa human cervix carcinoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% (vol/vol) fetal calf serum (FCS) at 37°C and in an atmosphere of 7.5% CO_2. 3T3 cultures were arrested in G1 phase by incubation in DMEM containing 0.2% (vol/vol) FCS for 72 h. Cells were cultured in 10-15 cm petridishes with 10-20 ml DMEM medium. After reaching a critical density (subconfluent), the cells were split and supplied with fresh medium: cells were washed twice with 1x PBS and incubated with some drops of TE at 37°C for several minutes. When the cells detached the surface, they were resuspended in an appropriate amount of medium and split to fresh petridishes in adequate dilution.

5.1.2 Reagents for tissue culture

Trichostatin A (TSA) (50 ng/ml [165 nM]; *Wako Pure Chemical Industries*), anisomycin (50 ng/ml [180 nM]; *Sigma*), and H89 (10 µM; *Alexis Biochemicals*).

5.1.3 siRNA transfection

The siRNAs specific for human 14-3-3ε (sc-29588) and 14-3-3ζ (sc-29583) and unspecific control siRNA (sc-37007) were purchased from *Santa Cruz Biotech*. For siRNA transfection 2.5 X 10^5 cells were plated in 6-well culture dishes, 12h prior to standard lipofectamine 2000 (*Invitrogen*) transfection. Cells were transfected with siRNAs against 14-3-3ε or 14-3-3ζ at a final concentration of 24nM. Control transfections were carried out, using an unspecific control siRNA at a final concentration of 48nM. Medium was changed after 8 hours complex incubation and cells were grown for additional 72h.

5.1.4 Mitotic arrest

HeLa cells were grown to ~70% confluence and treated with taxol or nocodazol with the indicated concentrations (see corresponding figures). Protein and FACS samples were prepared 20h after drug treatment. Control samples were treated with the same volume of DMSO as drug treated samples.

5.1.5 Freezing and thawing of cells

Freezing of Cells

Logarithmically growing cells were trypsinized, resuspended in 10 ml medium and collected by centrifugation at 1.200 rpm for 5 min. The resulting cell pellet was dissolved in 90% FCS and 10% DMSO, mixed well and added into a freezing-ampoule (*cryo tube, Nunc*). After 30 min incubation on ice, the freezing-ampoule was cooled to -80°C for three days and finally for long term storage transferred to liquid nitrogen.

Thawing of Cells

For thawing of cells stored in liquid nitrogen, they were quickly thawed at 37°C in a water bath for 2 -3 min. Then cells were transferred to a fresh petridish containing a 10 ml CO_2 saturated DMEM medium.

5.2 Bacterial culture

5.2.1 Bacterial strains

XL1

This strain contains the F'-plasmid and therefore allows a blue/white selection on X-gal plates. Superinfection with M13 is possible after tetracycline selection.

Genotype: sup E44, hsd R17, rec A1, end A1, gyr A46, thi, rel A1,

lac^-, F' [$proAB^+$, $lacI^q$, lacZ, DM15, Tn10(tet^r)].

XL1 Blue

Genotype: recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F´ proAB $lacI_qZ\Delta M15$ $Tn10\ (Tet_r)$]

DH5αF'

Genotype: F', endA1, hsdR17 (rK^-mK^+), supE44, thi-1, recA, gyrA

(Nal^r), relA1, Δ(lacZYA-argF) U169, deoR [ϕ80dlcΔ(lacZ)M15].

BL21-Gold(DE3)-RIPL strain (*Stratagene*)

Genotype: F– ompT hsdS(rB– mB–) dcm+ Tetr gal λ(DE3), endA, Hte

5.2.2 Media and solution and culture of bacteria

Liquid cultures were inoculated with bacteria either from frozen bacterial cultures or from cultures from agar plates stored at 4°C. A colony was picked with a sterile toothpick, transferred into the appropriate liquid medium and incubated at 37°C under agitation overnight.

Antibiotic Stock Solutions

Ampicillin 100 mg/ml, Tetracyclin 12.5 µg/ml, Kanamycin 50 µg/ml

Luria-Bertani Liquid Medium (LB-Medium) (1L)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar (for LB-plates only)
autoclave
pH to 7.0
10 ml of 2 M glucose (filter sterilized)

NZY+ Broth (1L)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
pH 7.5 (using NaOH)
autoclave
12.5 ml of 1 M MgCl2 (filter sterilized)
12.5 ml of 1 M MgSO4 (filter sterilized)
10 ml of 2 M glucose (filter sterilized)

SOB Medium (1L)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
autoclave
10 ml of 1 M MgCl2 (filter sterilized)
10 ml of 1 M MgSO4 (filter sterilized)

SOC Medium (100 ml)

99ml SOB medium
1ml of 2 M glucose (filter sterilized)
Filter sterilize again

5.2.3 Preparation of frozen *E. coli* stocks

2x Freezing Buffer

Per liter:
K_2HPO_4 12.60 g
KH_2PO_4 3.60 g
Na citrate 0.90 g

$MgSO_4$ 7 H_2O 0.18 g
$(NH_4)_2SO_4$ 1.80 g
Glycerol 88.00 g
Buffer was autoclaved and stored at 4°C. Bacterial cultures were mixed 1:1 with freezing buffer and stored at -80°C.

5.2.4 Bacterial transformation

Transformation of DH5α, XL1 and XL1-Blue cells

Cells were gently thawed on ice and aliquoted at 50 µl per transformation reaction in a prechilled 14-ml BD Falcon polypropylene round-bottom tube. An appropriate aliquot of DNA was added and samples were mixed and incubated for 30 minutes on ice. Transformation reactions were heat pulsed at 42°C for 45 seconds and immediately chilled on ice for 2 minutes. 0.5 ml of LB-medium *(DH5α)* or NZY+ (XL1 and XL1-Blue) broth preheated to 42°C was added and cells were incubated at 37°C for 1 hour under agitation. Appropriate aliquots were placed on LB-agar plates containing appropriate antibiotics for selection of successful transformants. Plates were incubated overnight at 37°C.

Transformation of cells BL21-Gold(DE3)-RIPL

Cells were gently thawed on ice and aliquoted at 100 µl per transformation reaction in a prechilled 14-ml BD Falcon polypropylene round-bottom tube. B-mercaptoethanol was diluted with sterile ddH_2O 1/10 and 2 µl were added per reaction. Cells were incubated for further 10 minutes on ice with shaking every 2 minutes. 4-50 ng of plasmid DNA were added and reactions incubated on ice for 30 minutes. Transformation reactions were heat pulsed at 42°C for 20 seconds and immediately chilled on ice for 2 minutes. 0.9 ml of SOC-medium preheated to 42°C were added and cells were incubated at 37°C for 1 hour under agitation. Appropriate aliquots were placed on LB-agar plates containing appropriate antibiotics for selection of successful transformants. Plates were incubated overnight at 37°C.

5.3 DNA analysis

5.3.1 DNA preparation

Wizard DNA mini-preparation

The Wizard Plus SV Miniprep DNA Purification System (*Promega)* was used to obtain high quality plasmid DNA. All steps were performed according to the manufacture specifications. 2-10 ml of an overnight culture were pelleted by centrifugation at 14.000 rpm for 5 minutes. Cells were resuspended in 250 µl Cell Resuspension Solution. 250 µl of Cell Lysis Solution were added and the mixture was incubated for 5 minutes at RT. Following addition of 10 µl

Alkaline Protease Solution and incubation a further 5 minutes the lysate was neutralized with 350 µl Neutralization Solution. Cell debris and chromosomal DNA were pelleted by centrifugation at 14000 rpm for 10 minutes and the supernatant, containing the plasmid DNA, was loaded onto a Miniprep Spin Column. In a series of centrifugation steps the DNA from the lysate was bound to the column, washed and finally eluted in 20-50 µl H_2O.

Preparation of large DNA quantities

To isolate high yields of pure plasmid DNA the "*QIAGEN* Plasmid Midi Kit" was used according to the manufacture instructions. 100 ml overnight culture were pelleted by centrifugation and the cell pellet resuspended in 4 ml Buffer P1. Next 4 ml Buffer P2 were added, mixed and incubated at RT for 5 minutes. After addition of 4 ml Buffer P3 (pre-chilled on ice) the lysate was transferred to the cartridge and incubated for an additional 10 min at RT. Equilibration of a *QIAGEN*-tip 100 was performed by applying 4 ml Buffer QBT. After the filtration of the cell lysate into the tip, the clear cell lysate entered the resin by gravity flow. The column was washed twice with 10 ml Buffer QC and the plasmid DNA was eluted with 5 ml Buffer QF. The isolated plasmid DNA was precipitated with 3.5 ml isopropanol and pelleted by centrifugation at 16000 rpm for 30 min at 4°C. The DNA pellet was washed with 70% ethanol dried and dissolved in 200 µl TE or H_2O.

5.3.2 Enzymatic DNA reaction

Digestion of DNA by Restriction Endonucleases

DNA was digested with the restriction endonucleases (*New England biolabs*) for 2 hours for analytical purpose up to overnight digestion for cloning of the digested fragments. An aliquot of DNA preparation was with mixed with 1x appropriate buffer and the recommended amount of the desired enzyme at the respective optimal temperature (in general 37°C). Acetylated BSA was added if recommended by the manufactures.

Dephosphorylation of DNA 5'-ends

For dephosphorylation of DNA 5'-ends 1µg of DNA was incubated with 1x Antarctic phosphatase reaction buffer and 5 units Antarctic phosphatase (*New England biolabs*). Reactions were incubated for 15 minutes at 37°C and the enzyme was inactivated by incubation at 65°C for 5 minutes.

Ligation of DNA Fragments with T4-DNA Ligase

Blunt and recessed DNA fragments were ligated in appropriate DNA vectors for up to 2 hours at RT or overnight at 16°C. The ligation mix was precipitated with 1 µl glycogen, and 100 µl 96% ethanol. After washing and drying the DNA pellet was dissolved in 10 µl H_2O and stored at -20°C. 5 µl of the purified ligation mix was used for transformation

5.3.3 Cloning of 14-3-3 expression constructs

Total RNA was isolated from swiss3T3 mouse fibroblasts and reverse transcribed into cDNA using the i-script™ cDNA synthesis kit (*BioRad*). 14-3-3ε, ζ or β encoding cDNA were amplified with specific primer pairs. For cloning of 14-3-3σ, cDNA from mouse embryonic stem cells which were differentiated into an epithelial lineage was used (kindly provided from Dr. Andreas Eger). The following primers were used for specific amplification (red residues: restriction sites, green residues: filling nucleotides to restore correct reading frame):

14-3-3ε:

ε-Eco-S: 5'-CGGAATTCATGGATGATCGGGAGGATCTGG-3'
ε-Xba-AS: 5'-GCTCTAGATCACTGATTCTCATCTTCCACATCC-3'
ε-BamHI-S: 5'-CGGGATCCCGATGGATGATCGGGAGGATCTGG-3'
ε-Eco-AS: 5'-GCGAATTCTCACTGATTCTCATCTTCCACATCC-3'

14-3-3ζ:

ζ-Eco-S: 5'-CGGAATTCATGGATAAAAATGAGCTGGTGCAG-3'
ζ-Xba-AS: 5'-GCTCTAGATTAATTTTCCCCTCCTTCTCCTGC-3'
ζ-BamHI-S: 5'-CGGGATCCCGATGGATAAAAATGAGCTGGTGCAG-3'
ζ-Eco-AS: 5'-CGGAATTCTTAATTTTCCCCTCCTTCTCCTGC-3'

14-3-3 β:

β-Eco-S: 5'-CGGAATTCATGACCATGGATAAGAGTGAGCTGG-3'
β-Xba-AS: 5'-GCTCTAGATTAGTTCTCTCCCTCTCCAGCATC-3'
β-Bam HI-S: 5'-CGGGATCCCGATGACCATGGATAAGAGTGAGCTGG-3'
β-Eco-AS: 5'-CGGAATTCTTAGTTCTCTCCCTCTCCAGCATC-3'

14-3-3σ:

σ-Eco-S: 5'-CGGAATTCATGGAGAGAGCCAGTCTGATCC-3'
σ-Xba-AS: 5'-GCTCTAGATCAGATGTGGGGGTCATCCGGAGC-3'
σ-BamHI-S: 5'-CGGGATCCCGATGGAGAGAGCCAGTCTGATCC-3'
σ-Eco-AS: 5'-CGGAATTCTCAGATGTGGGGGTCATCCGGAGC-3'

Identity of amplified fragments was controlled by specific restriction enzyme digestion and agarose gel electrophoresis. After confirmation fragments were digested with EcoRI and XbaI for cloning into pCI-Neo expression vector or BamHI and EcoRI for cloning into the pGEX5x2-vector Figure 5-1. All constructs were verified by sequencing of the insert.

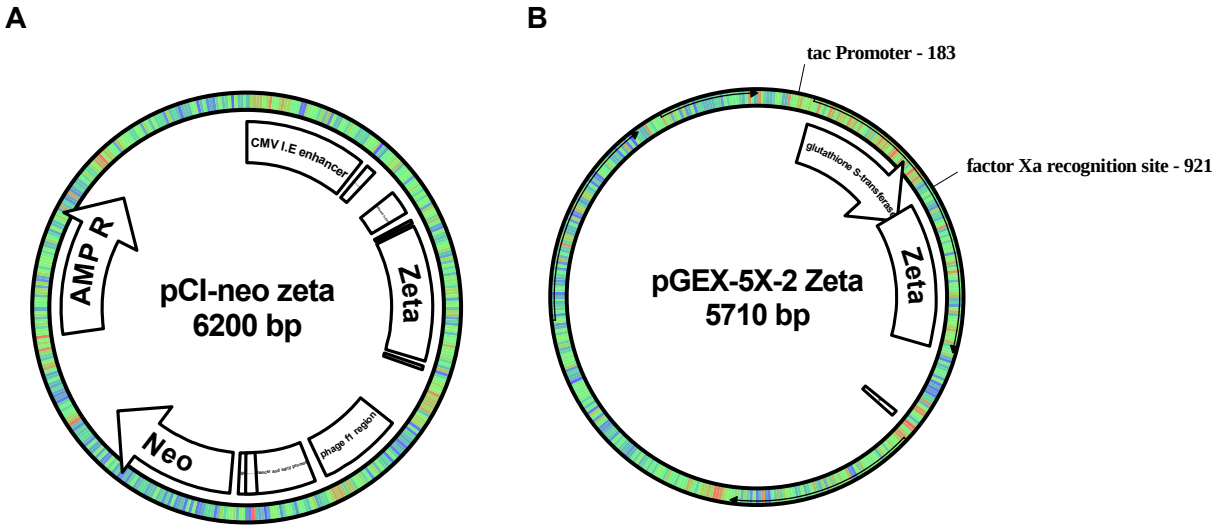

Figure 5-1 Maps for pCI-neo/14-3-3zeta and pGEX5X2-GST/14-3-3zeta

5.3.4 Cloning of histone H3 mutants

Xenopus laevis histone H3.2 construct cloned in the pETD3d was kindly provided by Dr. Wolfgang Fischle (Reference (Luger *et al.*, 1997)). Site directed mutagenesis was performed using the Quick change site directed mutagenesis kit II (*Stratagene*) according to the manufacturers instruction. The following primer pairs were used (the WT sequence is indicated to highlight the mutated site); green residues: Lysine 9 and 14 or 23 respectively, pink residues: serine10 or serine 28 respectively, red residues: mutated codon.

Histone H3K9R:

5'-G CAG ACC GCC CGT AAA TCC ACC GGA GGG AAG G-3' (WT)

5'-G CAG ACC GCC CGT aga TCC ACC GGA GGG AAG G-3' (Mut)

5'-C CTT CCC TCC GGT GGA tct ACG GGC GGT CTG C-3' (Mut_{rev})

Histone H3K23R/S28A

5'-G CTG GCC ACC AAG GCA GCC AGG AAG TCC GCT CCT GCT ACC-3' (WT)

5'-G CTG GCC ACC *agg* GCA GCC AGG AAG gcc GCT CCT GCT ACC-3' (Mut)

5'-GGT AGC AGG AGC ggc CTT CCT GGC TGC cct GGT GGC CAG C-3' (Mut_{rev})

Histone H3K9R/K23R/S28A

Primer pairs for the H3K9R were used on the Histone H3K23R/S28A construct.

Histone H3S10A/K14R

5'-CC GCC CGT AAA TCC ACC GGA GGG AAG GCT CCC CGC-3' (WT)

5'-CC GCC CGT AAA gcc ACC GGA GGG *agg* GCT CCC CGC-3' (Mut)

5'-GCG GGG AGC cct CCC TCC GGT ggc TTT ACG GGC GG-3' (Mut_{rev})

Histone H3S10A/K14R/K23R/S28A

Primer pairs for the H3S10A/K14R were used on the Histone H3K23R/S28A construct. Annotation: due to unusual phosphorylation of the H3K9R and H3K9R/K23R/S28A mutants these proteins were not used for interaction studies with 14-3-3 proteins (see 2.3 Presented Publication 1 (Winter et al. 2008). "14-3-3 proteins recognize a histone code at histone H3 and are required for transcriptional activation".).

The following PCR conditions were used:

Step	cycle(s)	Temperature	Time
1	1	95°C	30 seconds
2	12-18	95°C	30 seconds
		55°C	1 minute
		68°C	5 minutes (1 minute/kb)

After completion of amplification parental (non-mutagenized) DNA was digested with *DpnI* for 1h at 37°C. 1µl of *DpnI* digested DNA was used for transformation of *E. coli XL-1* cells (see 5.2.4 Bacterial transformation). Successful transformants were selected on LB-Amp-Agar plates and five independent colonies were picked per construct and used for plasmid-mini preparation. All constructs were controlled by restriction enzyme analysis. Successful mutation was controlled by DNA sequencing and mass spectrometry of recombinant expressed histones (see below).

5.4 RNA Analysis

5.4.1 Isolation of total RNA

For the isolation of total RNA from tissue culture cells the TRIZOL®Reagent (GibcoBRL®) was used according the manufacture's instruction.

RNA concentration was determined by measuring the absorbance at 260 nm. Absorbance values were used to calculate concentration according to formula (1).

(1) $E_{260nm} \times (dilution)^{-1} \times e^{-1} = concentration\ \mu g/ml$

(e^{-1} = 40 for single stranded RNA)

To ensure equal loading, aliquots of the RNA were diluted in RNA sample buffer containing Ethidiumbromid and separated on MOPS/EDTA agarose gels.

5.4.2 RNA separation on MOPS/EDTAS gels

10x MOPS/EDTA

0.2M MOPS
50mM Na-acetate
10mM EDTA
pH 7.0

RNA Sample Buffer

Formamide	15 ml
10x MOPS	3 ml
37% Formaldehyde	4.8 ml
H_2O	2 ml
Glycerol	2 ml

5µl ethidium bromide (1 mg/ml)

MOPS/EDTA gel (100 ml)

Agarose	1.2 g
H_2O	85 ml
10x MOPS	10 ml
37% Formaldehyde	5 ml

RNA samples in sample buffer were heated to 65°C for 5 minutes and loaded on a 1.2% MOPS/EDTA gel.

5.4.3 Northern transfer of RNA

10x SSC

NaCl	1.5 M
Na-citrate	150 mM

Methylen blue staining solution

Na-acetate	0.5 M
Methylen blue	0.04%

The mRNA was separated in a 1.2% MOPS/EDTA gel without ethidium bromide and the gel was soaked for 15 minutes in 10x SSC to remove formaldehyde. After overnight transfer the nylon membrane was washed in 10x SSC for 1 minutes. RNA was cross-linked by UV

radiation (0.12 J, *Stratalinker*) and the membrane stained 5 minutes with Methylen blue. After washing with H_2O the visible rRNA bands (28S, 18S) were labeled and the membrane wrapped in Saran and stored at -20°C.

RNA-DNA Hybridization

Northern blots were hybridized by the sandwich method. To remove non-hybridized radioactive DNA probes, nylon membranes were washed 2 x 15 minutes at RT with 6x SSC + 0.1% SDS and 15 minutes at 55°C with 2x SSC + 0.1% SDS. Membranes were wrapped in Saran and exposed at -80°C.

Stripping of northern blots

Stripping buffer

SSC 0.1%

SDS 1%

To remove radioactive probes, nylon membranes were incubated three times with boiling stripping buffer.

5.5 Protein Analysis

5.5.1 Preparation of total protein extracts from mammalian cells

Cells were harvested either by trypsin or scraping and resuspended in DMEM. Cells were pelleted by centrifugation (1.000 rpm, 5 minutes) and washed once in PBS. After repeated centrifugation cells were resuspended in PBS, transferred to an Eppendorf tube and pelleted by centrifugation at 12.000 x g for 10 sec. The pellet was resuspended in 2 x pellet volume extraction buffer and immediately frozen in liquid nitrogen. Lysates were thawed at 37°C and refrozen in liquid nitrogen for an additional four times. Supernatant was cleared by centrifugation at 14.000 x g for 30 minutes and supernatant was directly used for SDS-PAGE.

Extraction Buffer

Tris-HCl ph 8.0	20 mM
NaCl	100 mM
EDTA pH 8.0	1 mM
NP-40	0.5 %

Protease inhibitors (Complete; Boehringer Mannheim)

PMSF	1mM

5.5.2 Inhibitors

Deacetylase inhibitors

Na-Butyrate (10mM final concentration)

Trichostatin A (165nM final concentration)

Phosphatase inhibitors

NaF	0.21g/100ml lysis buffer
Tetra-Sodium-pyrophosphate	0.9g/100ml lysis buffer
Na-orthovanadate (100×)	0.184g/10ml 50mM Tris pH7.5
β-glycerphosphate (100×)	2.16g/100ml H_2O

5.5.3 Histone isolation

Cells were trypsinized, resuspended in 10 ml DMEM and harvested by centrifugation (1000 rpm, 10 minutes). After washing with cold PBS and repeated centrifugation the pellet was resuspended by in 1 ml ice cold lysis buffer and again centrifuged. Three further wash steps with lysis buffer and one with Tris/ EDTA, each followed by centrifugation, resulted in a nuclear pellet, which was resuspended in 100 µl ice cold H_2O supplemented with H_2SO_4 to a final concentration of 0.4 N. The lysates were incubated on ice for at least 1 h and then centrifuged (4°C, 12.000 rpm, 10 minutes). The supernatant was mixed with 10 x volume acetone and the histones were precipitated overnight at -20°C. The next day the precipitates were collected by centrifugation (12.000 rpm, 10 minutes). The pellet was air dried and resuspended in a suitable volume of H_2O.

Lysis Buffer

Tris	10mM
Na-bisulfit	50mM
$MgCl_2$	10mM
Na-butyrate	10mM
Triton X-100	1%
Sucrose	8.6%

Adjust pH to 6.5

Wash Buffer

Tris	10 mM
Na_3EDTA	13 mM

adjust pH to 7.4

5.5.4 Preparation of nuclear extracts

Cells were harvested, resuspended in 1ml ice cold PBS and subsequently pelleted at 500 X g and 4°C for 5 minutes. PBS was aspirated and cells resuspended in 100µl lysis buffer per 1E+07 cells. Nuclei were pelleted at 500 X g and 4°C for 5 minutes. The cytoplasmic supernatant was removed and the nuclear pellet washed with wash buffer. Nuclei were pelleted at 500 X g and 4°C for 5 minutes and supernatant was discarded. Nuclei were resuspended in low salt buffer. Nuclear proteins were extracted by addition of equal volume high salt buffer and incubation at 4°C for 45 minutes on a roller. Insoluble material was pelleted at 14000 X g and 4°C for 30 minutes, the supernatant containing soluble nuclear proteins was used for further experimental procedures.

Lysis buffer

Sucrose	0.32 M
Tris-HCl pH 8.0	10 mM
$CaCl_2$	3 mM
MgOAc	2 mM
EDTA	0.1 mM
NP-40	0.5%
DTT	1 mM
PMSF	1 mM

Complete-Protease inhibitor cocktail (*Roche*)

Phosphatase Inhibitors: 20 mM β-glycerophosphate, 100 µM sodium orthovanadate, 50mM sodium fluoride, 20mM sodium pyrophosphate 10 mM sodium butyrate

Wash buffer = Lysis buffer without NP-40

Low salt buffer

HEPES pH 7.9	20 mM
$MgCl_2$	1.5 mM
KCl	20 mM
EDTA	0.2 mM
Glycerol	25% (v/v)
DTT	0.5 mM
PMSF	1 mM

Complete-Protease inhibitor cocktail (*Roche*)

Phosphatase Inhibitors: 20 mM β-glycerophosphate, 100 µM sodium orthovanadate, 50mM sodium fluoride, 20mM sodium pyrophosphate, 10 mM sodium butyrate)

High salt buffer

HEPES pH 7.9	20 mM
$MgCl_2$	1.5 mM
KCl	800 mM
EDTA	0.2 mM
Glycerol	25% (v/v)
DTT	0.5 mM
PMSF	1 mM

Complete-Protease inhibitor cocktail (*Roche*)

Phosphatase Inhibitors: 20 mM β-glycerophosphate, 100 µM sodium orthovanadate, 50mM sodium fluoride, 20mM sodium pyrophosphate, 10 mM sodium butyrate)

5.5.5 Affinity purification of phosphoacetyl histone binding proteins

Histone H3 peptides were purchased from *Peptide Specialty Laboratories GmbH* (Heidelberg, Germany). Peptides were covalently coupled to agarose beads via free sulfhydryl-groups (*SulfoLink Kit, Pierce Biotechnology*) at a concentration of 2.5 µg peptide per µl solid gel volume and stored as 50% slurry at 4°C. Coupling efficiency was monitored by peptide dot-blotting and *Ponceau* staining. For affinity purification 200-500µg of purified nuclear extracts were diluted 1/8 with ΔX buffer and incubated with 40µl of pre-equilibrated peptide-slurry over night (14h) at 4°C on a roller. Beads were collected by centrifugation and washed sequentially with ΔX buffer, RIPA buffer 300, RIPA buffer 500 and ΔX buffer. Bound proteins were resolved by SDS-PAGE and visualized by silver staining. Proteins specifically interacting with the phosphoacetylated histone H3 peptide were identified by mass spectrometry.

ΔX buffer

Tris-HCl pH 7.4	50 mM
NaCl	150mM
EDTA	10mM
PMSF	1 mM

Complete-Protease inhibitor cocktail (*Roche*)

Phosphatase Inhibitors: 20 mM β-glycerophosphate, 100 µM sodium orthovanadate, 50mM sodium fluoride, 20mM sodium pyrophosphate, 10 mM sodium butyrate)

RIPA buffer 300

NaCl	300 mM
Tris-HCl pH 8.0	50 mM
SDS	0.1%
sodium deoxycholate	0.5%
NP-40	0.1%

RIPA buffer 500 = RIPA buffer 300 with 500mM NaCl

5.5.6 Expression and purification of recombinant 14-3-3 GST-fusion proteins

BL21 cells were transformed with plasmids encoding GST or GST-tagged 14-3-3β, ζ, ε or σ. An overnight culture (100ml- 2000ml) grown in LB-Amp + 0.2% Glucose was diluted 1/10-1/20 with LB medium. Cells were grown to a density of 0.6-0.8 (OD600nm) and protein expression was induced by addition of IPTG to a final concentration of 1mM for 4 hours. Cells were collected at 4°C by repeated centrifugation at 4000rpm in a Sorvall centrifuge using an GS3 rotor. Pellet was resuspended in 1ml/100ml culture ΔX Buffer supplemented with Protease Inhibitors (*Boehringer Complete*). Cells were lysed by sonication in a maximum volume of 10ml by four continuous 25-30 second pulses at 70% output level on ice. NP-40 was added to a final concentration of 1% and samples were incubated on ice for 30 minutes. Lysate was cleared by centrifugation at 14.000 rpm for 25 minutes at 4°C and directly used for affinity purification via GST-agarose (Sigma) (500µl solid gel per 1L of culture). Binding was performed overnight at 4°C in an overhead mixer. Beads were collected by centrifugation (500g, 5 minutes at 4°C) and washed extensively with ΔX Buffer (5X 10-15 minutes with 10 gel volumes) Supernatant was saved.

5.5.7 Elution of GST-proteins with Glutathione

GST-Agarose bound 14-3-3-GST fusions were resuspended in Elution Buffer (50mM Tris-HCl pH9.5 10mM glutathione) using 1ml buffer per 500 µl solid gel and incubated on a roller at RT for 1h. Beads were collected by centrifugation and supernatant was collected as the first elution fractions. Elution was repeated with 500 µl buffer per 500 µl solid gel. Volume was adjusted to 2.2ml and buffer was exchanged via PD10 desalting columns. Samples were centrifuged at 14.000 rpm for 30 minutes and concentration was determined by measuring absorbance at 280nm (for 14-3-3Zeta GST: (ABS280nm)/70625= molar concentration). If required samples were concentrated using *Centricon* 10 kDa cut off spin-columns (*Millipore*).

5.5.8 Cleavage of 14-3-3-GST with FactorXa (*Cleavage Capture Kit Novagen*)

GST-fusion protein immobilized on beads was resuspended in appropriate volume of 1Xcleavage buffer and 0.5 up to 1U of FacorXa per 50 µg of protein was added. Samples were incubated at 20°C on a roller for 16-18h. Beads were collected by centrifugation and supernatant was cleared from FactorXa by Xarrest agarose.

5.5.9 GST-Pull down assay and in vitro modification of recombinant histone H3

Recombinant GST tagged proteins were expressed in and purified from the *Escherichia coli* strain BL21. Beads coated with GST fusion proteins (5 µg) were incubated with 20 µg acid extracted histone proteins in 200 µl ΔX buffer at 4°C for 2h on a roller. Reactions were washed sequentially with ΔX buffer, RIPA 300 buffer, RIPA 500 buffer and finally with ΔX buffer. Bound proteins were separated by 16% SDS-PAGE and visualized by Western blotting.

MSK1 kinase and PCAF histone acetyltransferase were purchased from *Upstate Biotechnology* (Lake Placid, N.Y.). Recombinant histone H3 was either purchased as well (*Upstate*) or expressed in *E.coli* BL21 cells (see below). *In vitro* histone modifications were performed according to the provider instructions. *In vitro* phosphoacetylation of histone H3 was carried out by initial phosphorylation for 45 minutes at 30°C and subsequent acetylation for 45 minutes at 30°C after adjustment of buffer conditions to PCAF assay requirements For monitoring modification status of histones, modification reactions were precipitated as described previously, resuspended in SDS sample buffer separated by SDS-PAGE and analyzed by immunoblotting.

5.5.10 Fluorescence polarization binding measurements

Histone peptides (residues 1-20 or 25-38) were labeled using fluorescein-5-EX succinimidyl ester (*Molecular Probes*). Fluorescinated peptides were purified by gel filtration and reversed phase chromatography. The identity and purity of all peptides was verified by mass spectrometry. After GSH Agarose affinity chromatography and cleavage of the GST tag with factor Xa protease, 14-3-3 zeta was further purified by anion exchange (Mono Q, *Amersham*), and gel filtration (Superdex 75, *Amersham*) chromatography. Fluorescence polarization binding measurements were performed under conditions of 50 mM HEPES pH 7.5, 50 mM NaCl, 2 mM DTT, and 100 nM fluorescein-labeled peptide (Fischle *et al.*, 2005) . Data were recorded on a *HIDEX Chameleon II* plate reader at room temperature. Anisotropy values were normalized to the bound and unbound state (fraction bound). Data sets obtained from at least three independent measurements were averaged.

5.5.11 *In vitro* peptide binding assay

10 µl of agarose coupled histone H3 peptides (see above) were equilibrated with 1 ml HERR-Buffer prior to incubation with 2.5 µl *in vitro* translated, ^{35}S labeled 14-3-3 proteins (*TNT* rabbit reticulocyte lysate system, *Promega*) in 50 µl total reaction volume. Binding reactions were incubated for 14h at 4°C on a roller. Reactions were washed twice with HERR buffer, twice with RIPA 300 buffer (see above) and finally twice with HERR buffer. Bound proteins were resolved by 16% SDS-PAGE. Gels were incubated with *ENHANCE*^{3} solution, dried under vacuum and proteins visualized by fluorography on storage phosphor plates. Signals were scanned with a Typhoon 8600 Imager (*Amersham*) and analyzed *Image Quant* software.

HERR buffer

HEPES pH 7.9	20mM
KCl	50mM
EDTA	2mM
NP-40	0.1%
Glycerol	10% (v/v)

Complete-Protease inhibitor cocktail (*Roche*),

Phosphatase Inhibitors: 20 mM β-glycerophosphate, 100 µM sodium orthovanadate, 50mM sodium fluoride, 20mM Sodium pyrophosphate

5.5.12 Immunoprecipitation Assay

Between 500-1000 µg protein extract was incubated with desired antibody overnight at 4°C under overhead mixing. Immune-complexes were harvested by adding 20 µl of protein A-agarose beads or 35 µl of G-beads for 2h at 4°C. The bound immune-complexes were pelleted for 5 minutes centrifugation at 4000 rpm. Beads were washed with five times IP-Lysis buffer and once with TBS (including all inhibitors as described above). For SDS-PAGE proteins were releases by boiling in SDS-sample buffer for 5 minutes.

IP-Lysis buffer

10% glycerol
20 mM Tris-Cl pH 8.0
135 mM NaCl
1% NP-40

TBS

25 mM Tris pH 7.4
137 mM NaCl
3 mM KCl

5.5.13 Expression and purification of recombinant histone H3

Recombinant WT and mutant histone H3 was expressed and purified as described (Himpel *et al.*, 1999). An overnight culture of BL21 (DE3) cells (normally 1ml) transformed with either wild type or mutant histone H3 was diluted 1/20 with LB-Amp containing 0.2% glucose. Cells were grown at 37°C to a density of 0.9 (OD600nm) before induction of protein expression by 0.1mM IPTG. Cells were incubated for an additional 2h at 37°C and then cooled on ice and collected by centrifugation. The pellet was frozen at -80°C and subsequently thawed in ice cold wash buffer and 0.5 mg lysozyme was added. The suspension was incubated for 5 minutes on ice under shaking and cells were lysed by sonication (four continuous 25-30 second pulses at 70% output level on ice). 300µl of 20% Triton X-100 were added and suspension was incubated for an additional 15 minutes. Inclusion bodies were pelleted at 12.000 rpm for 10 minutes at 4°C. The pellet was resuspended in 3ml wash buffer, 300µl of 20% Triton X-100 were added and 50µg DNAseI and RNAse A were added and suspension was incubated for 30 minutes at 37°C. Inclusion bodies were again collected by centrifugation at 14.000rpm and 4°C for 5 minutes. The pellet was resuspended in 2ml 7M guanidinium-HCl, pH 4.5, 0.1M DTT, by sonication and overhead mixing (40rpm) for 2h at 4°C. The denaturating buffer was exchanged via PD10 fast desalting columns (*Amersham*) equilibrated with 20mM HCl and 20mM HCl as eluant. Histones were aliquoted and lyophilized for long-term storage at -20°C. For reconstitution histones were resuspended in an appropriate volume of water, and centrifuged for 15 minutes at 4°C.

Wash buffer

NaCl 300mM

50mM Na–phosphate pH 7.8

5.5.14 SDS- Polyacrylamide gel electrophoresis (PAGE)

Acrylamide stock

Acrylamide	30 %
N,N´-methylene-bisacrylamide	0.8 %

A few spoons Mixed Bed Resin (ion-exchanger) were added. The stock was stored protected from light at 4°C.

Separating Gel Buffer

Tris-HCl pH 8.8	1.5 M
SDS	0.4 %

Stacking Gel Buffer

Tris-HCl pH 6.8	0.5 M
SDS	0.4 %

SDS Sample Buffer

Tris-HCl pH 8.8	100 mM
Glycerol	20 %
bromphenole blue	0.01 %
β-Mercaptoethanol	0.5%
SDS	4 %

10 x Running Buffer

Tris base	50 mM
Glycine	500 mM
SDS	0.2% (v/v)

5.5.15 Mass Spectrometry

Gel bands were excised and cut into smaller pieces. The gel pieces were washed with ultrapure water and destained with 44% acetonitrile in 50 mM $(NH_4)HCO_3$. Unmodified and mono-methylated lysine residues were propionylated as described (Peters *et al.*, 2003). Briefly, samples were incubated for 90 minutes at room temperature with 50 µl of a mixture of 70% propionic anhydrid in methanol and 20 µl 50 mM $(NH_4)HCO_3$, followed by two washing steps with 100 mM $(NH_4)HCO_3$. Disulfide bridges were reduced with DTT and alkylation of cysteine residues was performed using iodoacetamide. Proteins were digested with trypsin overnight at 37°C. Extracted peptides were separated on a reversed phase nano-HPLC (Ultimate 3000, Dionex, Sunnyvale CA, USA; trapping column: PepMap C18, 300 µm×5mm, 3µm, 100Å, flow rate 20 µl/minute; separation column: PepMap C18, 75 µm×150mm, 3 µm, 100Å, flow rate 300 nl/minutes, gradient: 0-25% B in 120 minutes, 25%-50%B in 60 minutes, 90% B for 18 minutes, solvent A: 5% acetonitrile, 0.1% formic acid in water, solvent B: 80% acetonitrile, 0.08% formic acid in water).

The HPLC was coupled online via a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark) to an LTQ or an LTQ-FT (Thermo Electron, Bremen, Germany) linear ion trap Fourier Transform hybrid mass spectrometer. The mass spectrometer was operated in data dependent mode, each high-resolution full scan (m/z 300 to 1800, resolution at m/z 400 set to 100.000) in the ICR cell was followed by 5 product ion scans in the linear trap, preferentially for precursor masses from a parent mass list containing the calculated masses of different modifications of the H3 K9-R17 peptide. For each MS2 scan the neutral loss algorithm in the *Xcalibur* 2.0 software was enabled. In this mode an MS3 scan is triggered, if

a neutral loss of phosphoric acid is detected among the 5 most intense fragment ions in the preceding MS2 scan. Dynamic exclusion (exclusion duration 30 s, exclusion window ±5 ppm) was enabled to allow detection of less abundant ions. Data analysis was performed using the SEQUEST search algorithm (BioWorks 3.2, Thermo Electron). A custom-built database, containing the histone variants of *Mus musculus* and common contaminants, was searched with a precursor mass accuracy of 20 ppm. Carbamidomethylation of cysteine and propionylation of lysine were set as static modifications, mono-methylation of lysine and arginine, acetylation, di- and tri-methylation of lysine, phosphorylation of serine, threonine and tyrosine as well as the loss of water from serine and threonine were set as variable modifications. All search results were subjected to stringent manual validation.

5.5.16 Silver staining of SDS-PAGE gels

Proteins were fixed in the gel by washing for 1h with fixer solution. The gel was subsequently washed with washing solution for two times 20 minutes and with water for 20 minutes or overnight. Sensitizing was performed for one minute prior to washing twice with water for one minute each. Staining with silver-nitrate was carried out by incubating the gel with the silver solution for 20 minutes at 4°C in the dark. After washing the gel twice with water, silver stained proteins were developed by gentle shaking in developing solution until desired staining is achieved. Development was terminated with stop solution 3 times for one minute. Silver stained gels were stored in storage solution at 4°C.

Fixer Solution

Ethanol	40 ml
acetic acid	10 ml
H_2O	ad 100 ml

Washing Solution

Ethanol	30 ml
H_2O	ad 100 ml

Sensitizing Solution

sodium thiosulfate	0.02 g
H_2O	ad 100 ml

Silver Solution

silver nitrate	0.1 g
H_2O	ad 100 ml

Developing Solution

sodium carbonate	3 g
formaldehyde 35%	40 µl
H_2O	ad 100 ml

Stop Solution

acetic acid	5 ml
H_2O	ad 100ml

Storage Solution

acetic acid	1 ml
H_2O	ad 100ml

5.5.17 Western blotting of SDS-PAGE gels

After proteins were separated on an SDS-gel, the stacking gel was removed and the separation gel was soaked in water. The support pads of the transfer unit, 3MM filter paper and nitro-cellulose membranes (*Schleicher & Schuell* BA 83) were also soaked in Harlow buffer. The sandwich was assembled in the following order: support pad, 2 sheets of 3 MM paper, gel, nitrocellulose membrane, 2 sheets of 3MM paper and again support pad. The sandwich was placed in the electrotransfer unit (*Hoefer*), filled with transfer buffer. Transfer to the membrane was performed at a constant current of 200 mA at 4°C for 1.5h under stirring with a magnet bar. The quality of the transfer was checked by staining the membrane with 1 x *Ponceau* for 1 minute. The excess *Ponceau* was removed by rinsing the membrane with distilled water.

Harlow Transfer Buffer

Tris base	25 mM
Glycine	190 mM
Methanol	20% (v/v)

10 x Ponceau S

Ponceau	2%
trichloroacetic acid	30%
sulfosalicylic acid	30%

5.5.18 Immunoblotting

Blocking Solution

PVP	1%
non fatty dried milk	1%
Tween 20	0.1%
NaN_3	0.01%
PBS ad 1L	

In order to block any unspecific binding of antibodies, the membrane was incubated with blocking solution for 1h at room temperature under gentle agitation. The incubation with the primary antibody (diluted in blocking solution) was carried out at 4°C overnight. After three washes with 1 x PBS, 0.1 % Tween-20, 5 minutes each, the blot was incubated with the secondary HPRD-antibody, diluted in 1 x PBS, 0.1 % Tween 20, for 1 hour at room temperature followed by another three washes.

5.5.19 Immuno-Detection by ECL (Enhanced Chemo-Luminescence)

ECL solution 1 and 2 (*Amersham*) were mixed at a 1:1 ratio. The blot was incubated with the mixed solution for 1 minute. Excess of ECL solution was removed; the membrane was packed into Saran wrap and exposed to X-ray films.

5.5.20 Stripping of Western blots

The blot was washed three times in PBS, 0.1% Tween 20. After incubation with stripping buffer at 50°C for 30 minutes, the blot was washed again three times with PBS, 0.1% Tween 20 and was then efficiency of stripping was checked with secondary antibody

Stripping Buffer (100 ml)

20% SDS	10 ml
1M Tris-HCl pH 6.7	6.25 ml
ß-mercaptoethanol	700 µl
H_2O ad 100ml	

5.5.21 Indirect Immunofluorescence

Cells were grown in coverslips placed in six-well tissue culture dishes. Medium was removed and cells were washed 3 times with PBS. If pre-extraction of cytoplasmic proteins was required cells were treated with PBS containing 0.1% Triton X-100 for 2 minutes prior to fixation with 4% paraformaldehyde in PBS for 20 minutes at RT. Cross-linker was quenched

by adding 50mM NH_4Cl for 5 minutes at RT. Cells were washed once with PBS containing 0.1% Triton X-100 and PBS-T (PBS containing 0.01% TX-100) and blocked with PBS-T/10% goat-serum for 1-2h at RT. Primary antibody was used at an appropriate dilution in PBS-T containing 10% goat serum overnight at 4°C in an humid chamber. Slides were washed 3 times with PBS-T prior to addition of secondary fluorescence-labeled antibody (dilution 1:1000 for FITC and Texas red and 1:1000 for Cy3 with PBS-T/10% goat-serum) in a dark and humid chamber for 1 hour at room temperature. After 3 washes with 1x PBS-T DNA was stained with (10-15 µl) DAPI in Vectashield (1 µg/ml final concentration), mounted with a coverslip and sealed with nail polish.

5.5.22 Chromatin Immunoprecipitation

Wash Buffer I

0.25%	Triton X-100
10mM	EDTA, pH 8
0.5 mM	EGTA, pH 7.5
10 mM	Hepes, pH 7.5

Wash Buffer II

0.2 M	NaCl
1mM	EDTA, pH 8.0
0.5 mM	EGTA, pH 7.5
10 mM	Hepes, pH 7.5

SDS Lysis Buffer

1 %	SDS
10 mM	EDTA
50 mM	Tris, pH 8.1

Dilution Buffer

0.01 %	SDS
1.1 %	Triton X-100
1.2 mM	EDTA
16.7 mM	Tris, pH 8.1
167 mM	NaCl

RIPA-Buffer

150 mM	NaCl
50 mM	Tris, pH 8.0
0.1%	SDS
0.5%	Na-Dodecylsulfate
1.0%	NP-40

High Salt wash

500 mM	NaCl
50 mM	Tris, pH8.0
0.1%	SDS
1.0%	NP-40

LiCl wash

250 mM	LiCl
50 mM	Tris, pH8.0
0.5%	Na-Dodecylsulfate
1.0%	NP-40

TE

10mM	Tris, pH 7.5
1mM	EDTA, pH 8.0

Preparation of sepharose bead suspension

Solid A-beads were pre-swollen in TE on ice for 30 minutes. Pre-swollen A-beads or G-beads which are provided by the manufacturer as a suspension, were washed three times with TE. Afterwards beads were combined with the following solutions per ml swollen beads.

20µl	sonicated Salmon Sperm DNA (10 mg/ml)
100µl	BSA (10 mg/ml)
50µl	NaAzid 2%
830µl	TE

Finally the beads were rolled for one hour at 4°C. Beads were stored at 4°C.

Formaldehyde fixation and preparation of soluble chromatin

Chemical cross link of chromatin was performed by addition of formaldehyde for 10 minutes at 37°C directly to the medium in the culture dish at a final concentration of 1%. Subsequent addition of glycine at a final concentration of 125 mM for 5 minutes at room temperature quenched the formaldehyde fixation. After washing the cells once with PBS they were scraped with a rubber policeman in PBS containing 1 mM PMSF. Cells were pelleted at 1.200 rpm at 4°C for five minutes. After washing the cell pellet with wash buffer I and II (both containing 1mM PMSF and aprotinin, pepstatin and leupeptin each at a final concentration of 1 µg/ml, 10mM Butyrate and phosphatase inhibitors) the cell pellet was resuspended in lysis buffer at a concentration of $2x10^7$ cells/ml containing inhibitors in the concentrations as indicated above. The lysate was incubated 30 minutes on ice. Chromatin was sheared to an appropriate size for chromatin immunoprecipitation by sonication of the cell lysate with 3 to 15 times burst of 15 seconds duration. The Bandelin UW 70 sonotrode used was equipped with a 2mm tip, set to 40% output and 90% duty cycle. After each sonication the lysate was kept on ice for 3 minutes. To pellet cellular debris, the cell lysate was centrifuged for 5 minutes at 4°C at 14.000 rpm.

Immunoprecipitation and isolation of precipitated DNA

The remaining supernatant was then aliquoted for different chromatin immunoprecipitations, diluted 10-fold in dilution buffer containing the specific inhibitors mentioned above and precleared with 20 µl of protein A/G sepharose slurry for one hour while rocking the tube at 4°C to reduce unspecific binding of chromatin to the protein A/G beads. After centrifugation for 5 minutes at 4°C at 4.000 rpm the supernatant was combined with the antibody in a new tube and rocked over night at 4 °C. To determine the remaining unspecific binding of chromatin a mock experiment without antibody was included. 30 µl of protein A/G sepharose slurry were added and rocked for at least one hour at 4°C.

Immune complexes bound to beads were collected by a centrifugation for 5 minutes at 4°C at 4.000 rpm. Pelleted beads were the successive washed one to two times with 1ml of each RIPA, high salt and LiCl buffer and two to four times with 1ml TE buffer. To elute the immune-complexes from the protein A/G beads they were shaken with 200 µl of 2% SDS, 0.1 M $NaHCO_3$, 10 mM DTT at room temperature for 15 minutes. After repeating this step, 20 µl of 4 M NaCl was added to the combined eluted samples. A 4 to 6 hour incubation of the eluted samples at 65°C was then performed to reverse the chemical cross-link. This step was followed by a proteinase K digestion of the samples. After addition of 8 µl 0.5 M EDTA pH 8.0 and 16 µl Tris pH 6.7 and 2 µl proteinase K (20mg/ml) the samples were incubated for one hour at 45°C.

To isolate the genomic DNA, bound by the antibody via the chromatin complexes a phenol-chloroform-isoamylalcohole extraction followed by a chloroform-isoamylalcohole extraction

was performed. The DNA in the recovered aqueous phase was then precipitated by adding 1/10th vol 3 M NaOAc, pH 5.2, 1 µl glycogen (20 mg/ml) and 2 vol of ice cold 96% ethanol at –80 °C. After centrifugation at 4 °C at 14.000 rpm for 15 minutes the pellet was washed with 70 % ethanol, air-dried an resolved in 40 µl water by shaking at room temperature.

Amplification of DNA by PCR

For quantification of the abundance of distinct DNA fragments in the chromatin immuno precipitation, semiquantitative PCR reactions were carried out. All PCR reactions were performed on a Biometra D3 thermocycler using Promega goTaq PCR Master Mix. The linear range for each primer pair was determined using different amounts of genomic DNA. PCR reactions with increasing amounts of genomic DNA were performed along with the immunoprecipitated DNA. PCR products were resolved on 2% agarose-TAE gels and quantified using the ImageQuant program (*Molecular Dynamics)*.

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