Role of Histone Deacetylases in Gene Expression and RNA Splicing

by

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Thesis Abstract

Histone deacetylases (HDAC) 1 and 2 play crucial role in chromatin remodeling and gene expression regimes, as part of multiprotein corepressor complexes. Protein kinase CK2-driven phosphorylation of HDAC1 and 2 regulates their catalytic activities and is required to form the corepressor complexes. Phosphorylation-mediated differential distributions of HDAC1 and 2 complexes in regulatory and coding regions of transcribed genes catalyze the dynamic protein acetylation of histones and other proteins, thereby influence gene expression.

During mitosis, highly phosphorylated HDAC1 and 2 heterodimers dissociate and displace from mitotic chromosomes. Our goal was to identify the kinase involved in mitotic phosphorylation of HDAC1 and 2. We postulated that CK2-mediated increased phosphorylation of HDAC1 and 2 leads to dissociation of the heterodimers, and, the mitotic chromosomal exclusions of HDAC1 and 2 are largely due to the displacement of HDAC-associated proteins and transcription factors, which recruit HDACs, from chromosomes during mitosis. We further explored the role of un- or monomodified HDAC1 and 2 complexes in immediate-early genes (IEGs), *FOSL1* (*FOS-like antigen-1*) and *MCL1* (*Myeloid cell leukemia-1*), regulation. Dynamic histone acetylation is an important regulator of these genes that are overexpressed in a number of diseases and cancers. We hypothesized that transcription dependent recruitment of HDAC1 and 2 complexes over the gene body regions plays a regulatory role in transcription and splicing regulation of these genes.

We present evidence that CK2-catalyzed increased phosphorylation of HDAC1 and 2 regulates the formation of distinct corepressor complexes containing either HDAC1 or HDAC2

homodimers during mitosis, which might target cellular factors. Furthermore, the exclusion of HDAC-recruiting proteins is the major factor for their displacement from mitotic chromosomes. We further demonstrated that un- or monophosphorylated HDAC1 and 2 are associated with gene body of *FOSL1* in a transcription dependent manner. However, HDAC inhibitors prevented *FOSL1* activation independently of the nucleosome response pathway, which is required for IEG induction. Interestingly, our mass spectrometry results revealed that HDAC1 and 2 interact with a number of splicing proteins, in particular, with serine/arginine-rich splicing factor 1 (SRSF1). HDAC1 and 2 are co-occupied with SRSF1 over gene body regions of *FOSL1* and *MCL1*, regardless of underlying splicing mechanisms. Using siRNA-mediated knockdown approaches and HDAC inhibitors, we demonstrated that alternative splicing of *MCL1* is regulated by RNA-directed localized changes in the histone acetylation levels at the alternative exon. The change in histone acetylation levels correlates with the increased transcription elongation and results in change in *MCL1* splicing by exon skipping mechanism.

Taken together, our results contribute to further understanding of how the multi-faceted HDAC1 and 2 complexes can be regulated and function in various processes, including, but not limited to, transcription regulation and alternative splicing. This can be an exciting area of future research for therapeutic interventions.

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In loving memory of my father

Late Nazibul Hussain Khan

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List of Abbreviations

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ADP	Adenosine diphosphate
APC1	Anaphase promoting complex 1
ASF/SF2	Alternative-splicing factor 1/Splicing factor-2
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
BIM	Bcl-2 interacting mediator of cell death
BME	β-mercaptoethanol
bp	Base pair
BRG1	Brahma-related gene-1
BSA	Bovine serum albumin
Cdc27	Cell division cycle protein 27
CDKN1A	Cyclin-dependent kinase inhibitor 1A
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/Enhancer-binding protein
CHD1	Chromodomain helicase DNA binding protein-1
ChIP	Chromatin immunoprecipitation

ChIP-seq	ChIP followed by high-throughput sequencing
CIP	Calf intestinal phosphatase
CK2	Casein Kinase 2
CLIP	UV cross-linking and immunoprecipitation
CLIP-seq	CLIP followed by high-throughput sequencing
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSP	Dithiobis[succinimidylpropionate]
EDTA	(Ethylenedinitrilo) tetraacetic acid
EGF	Epidermal growth factor
EGS	Ethylene glycol bis[succinimidylsuccinate]
ERK	Extracellular signal-regulated kinase
ESE	Exonic splice enhancer
ESS	Exonic splice silencer
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGFR2	Fibroblast growth factor receptor 2
FOSL1	FOS-related antigen-1
GTF	General transcription factor
H	Hours
H3K9ac	Histone H3 acetylation on lysine 9
H3K14ac	Histone H3 acetylation on lysine 14
H3 K9me3	Histone H3 trimethylation on lysine 9
H3S10ph	Histone H3 phosphorylation on serine 10
H3S28ph	Histone H3 phosphorylation on serine 28
HAT	Histone acetyltransferases
H3K4me3	Histone H3 trimethylation on lysine 4
H3K36me3	Histone H3 trimethylation on lysine 36

HCT116	Human colorectal cancer cell line
HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HDAC3	Histone deacetylase 3
HDLP	HDAC-related protein
HeLa	Henrietta Lacks (Human cervical cancer cell line)
HEK293	Human embryonic kidney 293 cell line
HIPK2	Homeodomain-interacting protein kinase 2
HP1	Heterochromatin protein-1
IB	Immunoblot
ID	Immunodepleted fraction
IEG	Immediate-early gene
IEF	Isoelectric focusing
ING	Inhibitor of growth
IP	Immunoprecipitated fraction
IPMK	Inositol polyphosphate multikinase
ISE	Intronic splice enhancer
ISS	Intronic splice silence
JNK	c-Jun N-terminal kinase
K562	Chronic myeloid leukemia cell line
KAT	Lysine acetyltransferases
KDa	Kilodalton
KMT	Lysine methyltransferase
LSD1	Lysine-specific histone demethylase-1
MAPK	Mitogen-activated protein kinase
MBD	Methyl-CpG-binding domain-containing protein
MCF7	Michigan Cancer Foundation-7
MCL1	Myeloid cell leukemia sequence 1
MCL1L	MCL1 long isoform
MCL1S	MCL1 short isoform

MiDAC	Mitotic deacetylase complex
Min	Minutes
miRNA	MicroRNA
MMTV	Mouse mammary tumour virus
MNase	Micrococcal nuclease
MPM-2	Mitotic protein monoclonal 2
MSK	Mitogen- and stress-activated protein kinase
MTA	Metastasis-associated protein
NAD	Nicotinamide adenine dinucleotide
NCAM	Neural cell adhesion molecule
NCoR	Nuclear receptor corepressor
ncRNA	Non-coding RNA
NF-κB	Nuclear factor-kappa B
NODE	Nanog and Oct4 associated deacetylase
NudC	Nuclear distribution protein C
NuRD	Nucleosome-remodeling and deacetylase repressor
p53	protein 53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ph	Phosphorylation
PIC	Pre-initiation complex
PKA	c-AMP dependent protein kinase A
PKC	Protein kinase C
Plk1	Polo-like kinase 1
PP	Protein phosphatase
PP1	Protein phosphatase 1
PP2	Protein phosphatase 2
PTGS2	Prostaglandin-endoperoxide synthase 2
PTM	Post-translational modification
qPCR	Real time PCR

RAR	Retinoic acid receptor
RAS	Rat sarcoma
RbAp	Retinoblastoma-associated protein
RE1	Repressor element-1
RNA	Ribonucleic acid
RNA-seq	Next-generation RNA-sequencing
RNAPII	RNA polymerase II
RNAPIIS5ph	RNA polymerase II phosphorylated at serine 5
RNase	Ribonuclease
RNP	Ribonucleoprotein
RRM	RNA recognition motif
RS domain	Arg/Ser-rich domain
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
Sec	Seconds
SF2/ASF	Splicing factor 2/Alternative splicing factor 1
siRNA	Small interfering RNA
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SOS	Son of sevenless
Sp1/Sp3	Specificity protein 1/3
snRNPs	Small ribonucleoprotein particles
SRE	Serum responsive element
SR proteins	Serine/arginine-rich proteins
SRSF1	Serine/arginine-rich splicing factor 1
SS	Splice site
SUMO	Small ubiquitin-like modifier
SWI/SNF	Switch/sucrose non-fermentable

TBB	4,5,6,7-tetrabromobenzotriazole
TBP	TATA-box binding protein
TBS	Tris buffered saline
TBP	TATA-binding protein
TE	Tris-EDTA
TFF1	Trefoil factor 1
TIP60	Tat-interactive protein 60
TLR	Toll-like receptor
TPA	12-*O*-tetradecanoate 13-acetate
TRE	TPA responsive element
Tris	Tris(hydroxylmethyl)aminomethane
TSA	Trichostatin A
TTBS	Tris buffered saline with Tween-20
U2AF	U2snRNP auxiliary factor
UPR	Upstream promoter region
UsnRNPs	Uridine-rich small ribonucleoprotein particles
UTR	Untranslated region
UV	Ultraviolet
VPA	Valproic acid
YY1	Yin Yang 1

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Chapter 1: Introduction

This chapter contains materials adapted in part, from the following publications:

*Delcuve GP, **Khan DH**, Davie JR. Targeting class I histone deacetylases in cancer therapy. Expert Opin Ther Targets. 17(1):29-41, 2013.*

*Delcuve GP, **Khan DH**, Davie JR. Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin Epigenetics. 4(1):5, 2012.*

***Khan DH**, Jahan S, Davie JR. Pre-mRNA splicing: role of epigenetics and implications in disease. Adv Biol Regul. 52(3):377-88, 2012.*

1.1 Chromatin structure and modifications

In eukaryotic cell nuclei, genetic information encoded in DNA is highly folded and compacted by histone and non-histone proteins in a dynamic nucleoprotein complex, called chromatin (Khorasanizadeh, 2004;Wolffe, 1994). Chromatin not only facilitates the packaging of DNA within the nucleus, but also serves as the major regulator of cellular functions and of gene expression (Dawson and Kouzarides, 2012;Kouzarides, 2007). The overall arrangement of chromatin in a cell is not uniform, rather dynamic in nature, and organized into particular structures and superstructures. In a simplistic term, chromatin is described by its state of condensation and is categorized into heterochromatin and euchromatin (Jenuwein and Allis, 2001;Trojer and Reinberg, 2007). The latter is characterized by low compaction, presence of certain post-translational modifications (PTMs) and non-canonical histone variants associated with active transcription, and accessibility to transcriptional regulators (Santos-Rosa and Caldas, 2005). In contrast, the heterochromatin is more condensed and is considered as transcriptionally silent or inactive, rich in repetitive sequences, which is also associated with a different set of PTMs and histone variants (Horn and Peterson, 2006;Santos-Rosa and Caldas, 2005). The biological outcomes at the cellular level are determined by the interplay between the different chromatin states. Therefore, alternations in chromatin structure and function by chromatin remodeling and/or histone modifications play an important role in eukaryotic functions.

1.1.1 The building block of chromatin: nucleosome

The basic repeat unit of chromatin is the nucleosome, comprised of 146 base pairs of DNA wrapped approximately 1.75 times around a histone octamer (Luger et al., 1997). The octamer is formed of four core histone proteins, H2A, H2B, H3 and H4 (**Figure 1.1**). The octamer is

arranged as a $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers (Davie and Spencer, 2000; Luger et al., 1997). Histones are small, evolutionarily conserved, basic proteins containing an unstructured C-terminal tail, a globular histone fold domain that functions in histone-histone and DNA-histone interactions, and a flexible charged N-terminal domain known as the histone tail, protruding from the nucleosomal core (Luger et al., 1997;Richmond and Davey, 2003). The N-terminal tails of histones are the targets for various PTMs, which are important for regulation of chromatin functions. Adjacent nucleosomes are joined by an intervening piece of linker DNA of variable length (10-50 bp) (Davie et al., 1999;Luger et al., 1997).

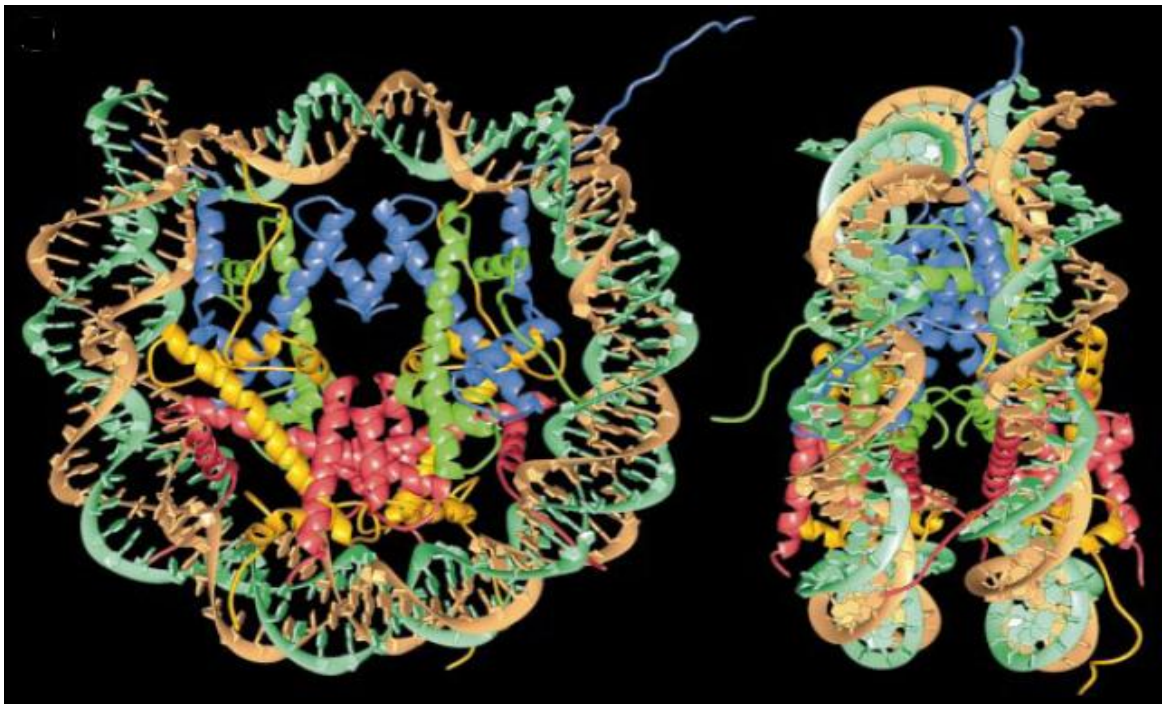

Figure 1.1: Nucleosome core particle
Ribbon traces for the 146-bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains are shown (blue: H3; green: H4; yellow: H2A; red: H2B. The views are down the DNA super helix axis for the left particle and perpendicular to it for the right particle. For both particles, the pseudo-twofold axis is aligned vertically with the DNA center at the top. *The figure and the text were reproduced with permission from Figure 1 (Luger et al., 1997).*

Linker histones, H1/H5, bind to the DNA entry/exit points of nucleosomes and to the linker DNA region between nucleosomes. Histone H1 has a different structure from the core histones, consisting of unstructured C-and N-terminal lysine rich domains linked via a globular helix domain. N-terminal tails of the core histones and the C-terminal tail of histone H1, are crucial for the formation of higher order chromatin structure (Davie and Spencer, 2000). This involves the extensive interactions between histone H1/H5 with DNA, and the inter- and intra-nucleosomal interactions mediated by histone tails and chromatin fiber-fiber interdigitation (Davie and Spencer, 2000;Luger et al., 1997;Richmond and Davey, 2003).

1.1.2 Chromatin modifications

The compact packing of genomic DNA into chromatin presents a major obstacle for various cellular processes such as transcription, replication, recombination and repair mechanisms (Horn and Peterson, 2006;Berger, 2007). To facilitate these events, chromatin structure undergoes structural changes or remodeling which helps to regulate the accessibility and recruitment of different factors responsible for each process, to the genomic DNA. The structure of the chromatin can be altered in different ways such as alteration in its components (e.g. the presence of repressors, activators, chromatin remodeling complexes, and/or incorporation of histone variants), and in covalent modifications of its constituents (such as DNA methylation at cytosine residues, and PTMs of histone tails) (Berger, 2007;Ellis et al., 2009). Hence, there are three main categories of chromatin-modifying enzymes: histone modifying enzymes, DNA modifying enzymes and ATP-dependent chromatin remodeling enzymes.

Histone modifying enzymes post-translationally modify the N-terminal tails of histone proteins to alter the structure and function of chromatin by recruiting other enzyme complexes and also provide binding sites for different regulatory proteins (Kouzarides, 2007). For example, bromodomain containing proteins can recognize the acetylated lysine residues and thus are targeted to specific sites on chromatin (Bottomley, 2004). DNA modifying enzymes methylate the CpG-rich sequences, which commonly involves a symmetrical conversion to 5-methyl cytosine on both strands of DNA (Bestor, 2000). DNA methylation represents the most stable epigenetic modification and plays a major role during development as well as in the establishment of cell type-specific chromatin states (Bird, 2002;Kaufman and Rando, 2010). Chromatin remodeling complexes utilize the energy of ATP to disrupt the histone-DNA interactions, and to move, exchange or restructure nucleosomes. They thus provide the accessibility of the regulatory proteins to DNA or histones during cellular processes (Dawson and Kouzarides, 2012;Winter and Fischle, 2010).

1.1.3 Histone modifications

Histones are decorated with a wide variety of complex and dynamic set of covalent PTMs that regulate the structure and function of chromatin in a context dependent manner and thereby regulate various biological processes (Kouzarides, 2007;Zlatanova et al., 2009). These modifications occur primarily within the N-terminal tails of histones protruding from the surface of the nucleosomes as well as on the globular domains (Cosgrove et al., 2004;Cosgrove and Wolberger, 2005;Kouzarides, 2007). So far, with the usage of mass spectrometry, antibody based and metabolic labeling techniques, more than 70 different sites for PTMs and several types of histone PTMs have been described (Kouzarides, 2007; Berger, 2007; Beck et al., 2006; Su et al.,

2007; Young et al., 2009). These include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, lysine ubiquitination and sumoylation, ADP-ribosylation, glycosylation, carbonylation, and many others (**Figure 1.2**). These modifications have a major influence on chromatin structure by affecting the local environment, facilitating the binding of transcription factors that regulate gene expression, or allowing the interaction with various chromatin remodeling enzymes (Kouzarides, 2007;Berger, 2007).

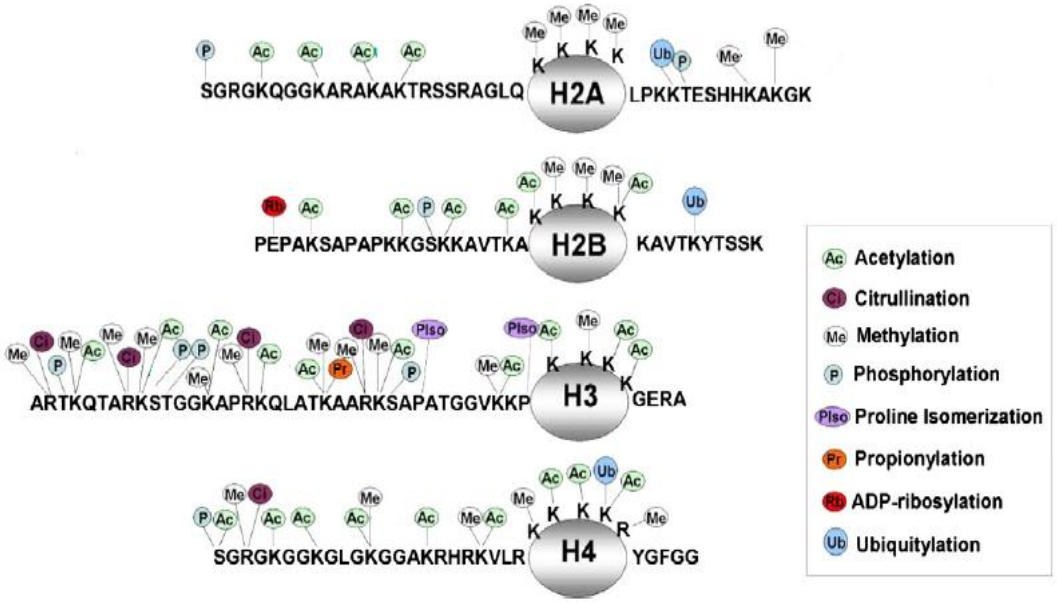

Figure 1.2: Core histone post-translational modifications
Specific amino acid PTM sites (acetylation, ADP-ribosylation, citrullination, methylation, phosphorylation, sumoylation and ubiquitination) that are known to occur on the core histones, H2A, H2B, H3 and H4, are indicated by colored symbols (symbol key in the right inset). Some residues (lysine, arginine) can undergo several, identical or different, forms of PTMs (i.e. methylation or acetylation, dimethylation or trimethylation, etc.), thus increasing the complexity of the histone code. *The figure and the text were adapted and modified with permission from Figure 2 (Redon et al., 2012).*

Given the number of sites, the wide array of possible histone modifications and their involvement in different cellular processes, the 'histone code hypothesis' has been proposed (Strahl and Allis, 2000;Borrelli et al., 2008). The code postulated that these modifications on the

same or different histone tails may be interdependent and can generate various combinations of modifications on any one nucleosome. Further, distinct histone modifications may culminate in specific landscape that can render the interaction sites for different chromatin binding proteins (Jenuwein and Allis, 2001;Strahl and Allis, 2000). For example, many chromatin associated proteins contain bromodomains or chromodomains, which recognize acetylated lysines or methylated lysine residues, respectively (Bottomley, 2004). However, the role of a specific or a combination of histone PTMs may be different depending on the cellular context or gene of interest.

It has been long known that histone modifications exerts profound control over a variety of nuclear processes, including gene transcription, DNA repair, DNA replication and segregation (Dawson and Kouzarides, 2012;Jaskelioff and Peterson, 2003;Peterson, 2003). However, the functional significance of each modifications, particularly, histone acetylation and methylation in other regulatory processes, for example, in facilitating pre-mRNA splicing, alternative splicing, RNA stability or editing, are just beginning to come to light (Hnilicova et al., 2011;Hnilicova and Stanek, 2011;Loomis et al., 2009). Furthermore, aberrations in histone modifications frequently occur in cancers and diseases, the nature of which could be therapeutically exploited (Dawson and Kouzarides, 2012). Therefore, expanding the understanding of histone modifications in various regulatory processes will have a powerful impact on identification and development of new therapeutic targets in diseases and cancers, apart from deciphering their mechanistic role in chromatin architecture and function.

1.1.3.1 Histone acetylation

One of the most extensively studied modifications of histones is acetylation, which occurs at the 'ε'- amino groups of lysine residues within the core histones. Acetylation is a reversible, dynamic process, which is regulated by two classes of antagonizing histone modifying enzymes, lysine acetyl transferases (KATs) and histone deacetylases (HDACs), which add or remove acetyl groups to/from lysine residues within the N-terminal tails of target histones, respectively (Berger, 2007;Walkinshaw et al., 2008) (**Figure 1.3**). The global acetylation level of histones influences the chromatin conformation and affects the accessibility of transcription factors and effector proteins to the DNA, thereby modulates gene expression.

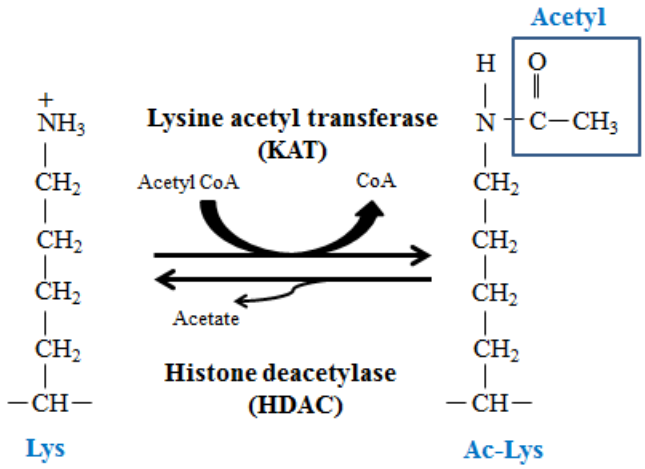

Figure 1.3: Dynamic histone acetylation
KATs and HDACs catalyze the dynamic histone acetylation by adding or removing the acetyl group from the N-terminal tails of histones.

Histone acetylation exerts increased transcriptional activity by two well-defined mechanisms. First, lysine acetylation by KATs neutralizes the positive charge of the histone tails and reduces the affinity of histones for the negatively charged DNA, by weakening histone-DNA or nucleosome-nucleosome interactions as well as by inducing a conformation change (Strahl and Allis, 2000;Jenuwein and Allis, 2001). This results in destabilizing and loosening of nucleosome

and chromatin structure, enables the transcriptional machinery to access the DNA and enhances gene transcription. Conversely, deacetylation by HDACs remove the acetyl group from the histone tails, presumably restores the positive charge to the specific lysine residue, thereby increasing the interaction of histones with negatively charged DNA, and thus reverse the effects of KATs and contribute to altering transcription (Cosgrove and Wolberger, 2005). The role of these enzymes in transcription, their recruitment mechanisms to the regulatory elements of genes as well as their compositions in multiprotein complexes are well documented (De Ruijter et al., 2003;Yang and Seto, 2007). Overall the acetylation levels of histones result from the interplay between histone acetylation and deacetylation reaction catalyzed by KATs and HDACs. Second, histone acetylation can act as recognition docking sites for bromodomain containing effector proteins that can interact with the modified residues (Cosgrove et al., 2004;Cosgrove and Wolberger, 2005). Subsequently, the recruited effector proteins modulate DNA transcription.

One of the hallmarks of transcriptionally active genes is the presence of acetylated histones predominantly at upstream promoter regions (UPR), indicating the level of acetylation corresponds to the rate of transcription (Liu et al., 2005). However, histone acetylation has been observed not only at UPR, but also throughout the gene body regions, suggesting that histone acetylation is involved in transcription elongation (Wang et al., 2009). In human CD4+ T-cells, with ChIP-seq techniques (chromatin immunoprecipitation followed by high-throughput sequencing), it has been reported that acetylation of K9, K18, K27 and K36 of H3 is predominantly found at UPR, while acetylation of K14 and K23 of H3, which also localized at promoters, extended significantly downstream of the promoters to the transcribed units (Wang et al., 2009). Similar patterns were observed with acetylated H4, acetylated H4K5 and H4K8 being

localized at promoters, while acetylated H4K12 was detected throughout the gene body. Furthermore, several KATs and HDACs are also identified in UPR and in the coding regions of transcribed genes (Wang et al., 2009). Although the functional significances of the KATs and HDACs in the transcribed regions are not very well defined, there are several reports indicating mostly their role in transcription regulation. For example, it has been demonstrated in yeast that histone acetylation facilitates the binding of RNA polymerase II (RNAPII) and TBP (TATA-box binding protein) to various promoter regions, indicating that it is a necessary step in transcriptional initiation (Bhaumik and Green, 2002;Qiu et al., 2004). Moreover, loss of histone deacetylase activity caused the transcription initiation from cryptic intragenic promoters (Carrozza et al., 2005). Furthermore, interactions between KATs and components of the transcription elongation machinery have also been reported (Cho et al., 1998;Wery et al., 2004). However, role of histone acetylation in other cellular processes, such as in co-transcriptional splicing mechanisms, RNA processing and editing, have recently emerged, indicating their role in diverse biological processes, yet to be characterized.

1.1.3.2 Histone phosphorylation

Phosphorylation on serine or threonine residues of the N-termini of all core histones has been reported with implications in several biological processes such as mitosis/meiosis, DNA-damage repair, transcriptional induction, apoptosis and heterochromatin formation (Ajiro, 2000;Cerutti and Casas-Mollano, 2009;Johansen and Johansen, 2006;Krishnamoorthy et al., 2006;Sabbattini et al., 2007;Singh et al., 2009). For example, phosphorylation of H2A and H3 are associated with mitotic chromatin condensation and phosphorylated H4 has a role chromatin condensation at the later stages of gametogenesis (Krishnamoorthy et al., 2006;Prigent and Dimitrov, 2003). Among

the core histones, phosphorylation of H3 is very well studied and the conserved phospho-residues of H3 are Thr3, Ser10, Thr11 and Ser28 (Cerutti and Casas-Mollano, 2009;Cosgrove et al., 2004). All of these phospho-marks are contributors to chromatin condensation during mitosis. However, phosphorylated H3Ser10 and Ser28 (H3S10ph and H3S28ph) (referred to as inducible PTMs) are also known to be involved in transcriptional activation of specific genes, the immediate-early genes (IEGs), during interphase in response to various stimuli, such as mitogens, growth factors, stress, UV radiation, and cytokines (Clayton et al., 2000;Cerutti and Casas-Mollano, 2009). Overall, the effect of histone H3 phosphorylation on chromatin is context dependent, and is associated with two different chromatin states; chromatin condensations (mitosis) and accessible chromatin structure (transcription).

Phosphorylation of histone H3 during interphase occurs rapidly after stimulation, is transient and targeted to a small subset of nucleosomes, compared to that observed in mitosis (Thomson et al., 1999). However, this event indicates a direct link between diverse signaling pathways such as MAPK (mitogen-stimulated protein kinases) or ERK (extracellular signal-regulated protein kinase) and activation of gene transcription. In 1991, Mahadevan et al, first coined the term ‘nucleosomal response’ which described the rapid phosphorylation of histone H3 with concurrent activation of two IEGs of interest, *c-Fos* and *c-Jun* (Mahadevan et al., 1991). These and other IEGs are transiently activated in response to extracellular stimuli and intracellular signaling cascades, encode for transcription factors which have been implicated in cellular transformation, differentiation, diseases and cancer. Although H3S28ph is less well characterized than H3S10ph, this can also be induced with mitogens, stress or UV irradiation (Zhong et al.,

2001a;Zhong et al., 2001b;Zhong et al., 2003). However, whether Thr3 and Thr11 are also inducible and whether these modifications are linked to one another is not known yet.

Aurora B is the major kinase for the mitotic H3 phosphorylation, although several kinases have been reported to be associated with inducible H3 phosphorylation, including MAPKs, ERKs, p38 kinase, and c-Jun N-terminal kinases (JNKs), mitogen- and stress-induced kinases (MSKs), and, cAMP-dependent protein kinases (PKAs) (Clayton and Mahadevan, 2003;Soloaga et al., 2003;Thomson et al., 1999;Zhong et al., 2000). It is likely that the responses may be cell type- or stimulus-specific and that many more kinases yet to be identified are involved.

1.1.3.3 Histone methylation

Methylation of histones can occur in the lysine and arginine residues on the N-terminal tails as well as on the histone fold domains of histone H3 and H4. The 'ε'-amino group of lysine residues is methylated in the states of mono-, di- or trimethylation by lysine methyltransferases (KMTs) whereas arginine residues are mono- or dimethylated in a symmetrical or asymmetrical manner by protein arginine methyltransferases (Agger et al., 2008;Zhang and Reinberg, 2001). Histone methylation is now considered as a dynamic modification with enzymatic conversion of methylated arginine residues to citrulline and with the discovery of histone demetylases that can remove the mono- and dimethylated groups (Cuthbert et al., 2004;Schneider and Shilatifard, 2006;Wang et al., 2004). Furthermore, the Jumonji family of histone demethylases is also identified, which is not only able to demethylate specific H3 lysines (trimethylated residues), but also can demethylate the methylated arginine residues (Agger et al., 2008). The functional consequences of the different degree of methylation results in various biological outcomes

including transcriptional activation, elongation or repression, imprinting, DNA replication and DNA-damage repair. For example, methylation of H3 at K4 and R17 is linked to transcriptional activation, whereas methylation of H3 at K9 or K27 and H4 at K20 is associated with condensed chromatin and transcriptional repression (Bauer et al., 2002;Lachner et al., 2001;Santos-Rosa et al., 2002;Schotta et al., 2004). Although histone arginine methylation has been less reported, methylation of H3 at R17 is recently demonstrated and suggested to be an activating step in mammalian gene transcription (Bauer et al., 2002).

1.1.3.4 Other modifications of histones

Histones are found to be ubiquitinated on lysine residues through a series of enzymatic reactions with diverse biological functions (Shukla et al., 2009). Ubiquitination involves the covalent linkage of ubiquitin monomers to histones as either a single addition (monoubiquitination) or as a polyubiquitin chain (Pickart and Fushman, 2004). Monoubiquitination of H2B (H2BK120), catalyzed by the mammalian RAD6/RNF20 complex, is a mark linked to transcriptional stimulation, elongation, and nucleosome remodeling (Pavri et al., 2006;Xiao et al., 2005;Zhu et al., 2005). While the dynamic regulation of H2B ubiquitination is essential for gene transcription, ubiquitination of H2A is considered as a repressive mark (Shukla et al., 2009;Stock et al., 2007). Furthermore, the tails of H1 and H3 can also undergo ubiquitination (Chen et al., 1998;Pham and Sauer, 2000).

The small ubiquitin like modifier (SUMO) is a large modification (10Ka in mass) which is covalently linked, and is found on all core histones (Shiio and Eisenman, 2003). This modification contributes to the repressive chromatin environment for transcription (Shiio and

Eisenman, 2003). For example, sumoylation of histone H4 has been reported to associate with transcriptional repression.

By mass spectrometry, multiple biotinylation sites have been identified on histones H3, H4 and H2B (Zempleni et al., 2009). Two enzymes, biotinidase and holocarboxylase synthetase, can independently catalyze the biotinylation reaction (Hymes et al., 1995;Narang et al., 2004). The enzymes that catalyze debiotinylation are largely unknown, although there is evidence that biotinidase may catalyze both biotinylation and debiotinylation of histones (Ballard et al., 2002). However, this modification is not detected in native histone preparations using multiple approaches (Healy et al., 2009). Biotinylated H4 has been detected *in vitro* in pericentromeric heterochromatin associated with gene silencing (Hassan and Zempleni, 2006;Zempleni et al., 2009). As this is not a natural modification of histones, the impact in gene regulation remains unclear (Healy et al., 2009).

Histones can also be reversibly modified by ADP-ribosylation on the arginine and glutamate residues in either mono- or poly-ribosylated forms. Distinct enzymes are responsible for this modification including mono-ADP-ribosyltransferases and poly (ADP-ribose) polymerases (Kouzarides, 2007). ADP-ribosylation of nucleosomes is involved in the epigenetic regulation of higher order structural organization of chromatin (Rouleau et al., 2010).

1.2 Overview of mitosis: Role of histone modifications

Mitosis is fundamental to life and is the key event of cell cycle regulation. The progress of mitosis is a highly regulated and complex process, which results in the division of duplicated sets

of chromosomes and two genetically identical daughter cells. Mitosis is accompanied by dramatic changes in chromatin organization and nuclear architecture. Proper mitotic chromosome structure is essential for faithful chromosome segregation. Errors in this process can cause genomic instability, a condition which is frequently associated with tumorigenesis and cancer (Janssen and Medema, 2012;Kastan and Bartek, 2004;Lengauer et al., 1998). Mitosis has emerged as an important target for cancer therapy although it has the potential to induce tumorigenesis and cancer. In fact, abrogation of mitosis can induce mitotic arrest, which in turn, often can lead to apoptosis in cancer cells (Nigg, 2001).

Mitosis is an elaborate process, divided into distinct phases, which is defined largely by the organization and behavior of the spindle and the chromosomes (**Figure 1.4**) (Jackson et al., 2007;Ruchaud et al., 2007).

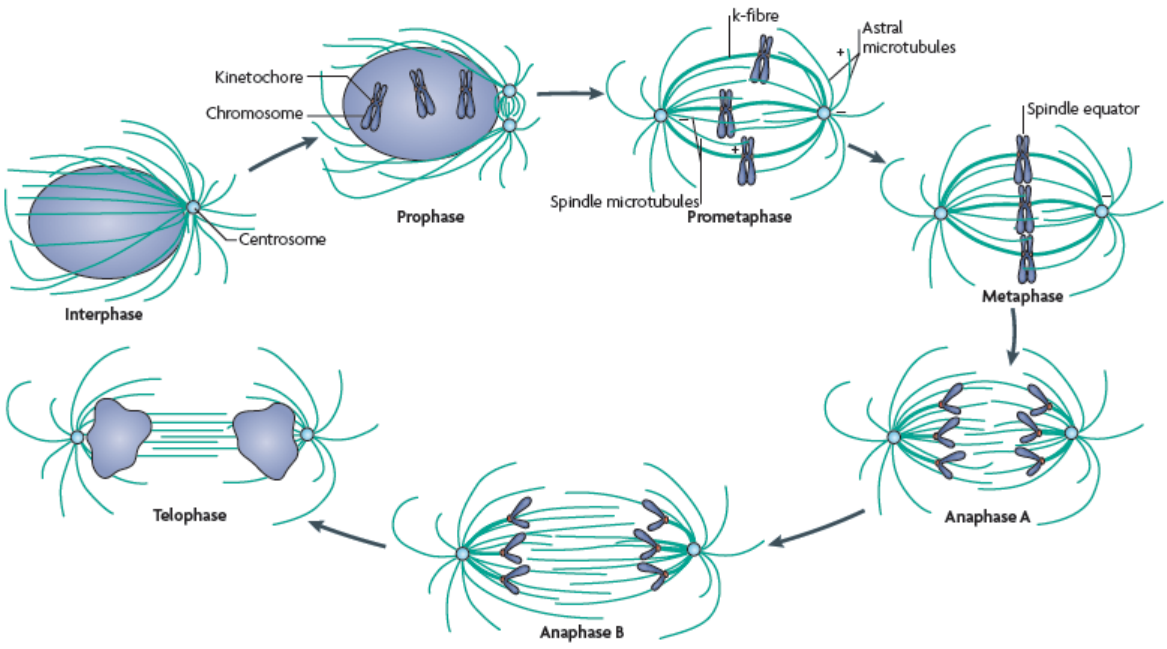

Figure 1.4: Phases of mitosis
The progression of mitosis through the individual phases is shown. *The figure was reproduced with permission from Figure 1 (Walczak et al., 2010).*

The onset of mitosis is marked by the breakdown of nuclear envelope and the condensation of chromatin into chromosomes during prophase (Jackson et al., 2007;Ruchaud et al., 2007;Walczak et al., 2010). The mitotic spindle begins to develop and centrosomes enlarge and start to separate while moving to opposite poles. In prometaphase, the kinetochore (k)-fibers (bundles of stabilized microtubules) connects the spindle microtubules and the kinetochores on the chromosomes, such that the chromosomes can start aligning at the equatorial plane. The chromosomes continue to condense. During metaphase, the chromosomes, each with two sister chromatids are aligned at the equatorial plane, with the centrosomes at opposite ends and the spindle fibers attached to the centromeres. In anaphase, each chromosome's sister chromatids separate and move towards the opposite poles and towards the centromeres, pulled by spindle fibers attached to the kinetochore regions (anaphase A). The two spindle poles separate during anaphase B. Finally, in telophase, the nuclear membrane reforms around the chromosomes grouped at either pole of the cell, the chromosomes decondense and the spindle fibers disappear. Cytokinesis divides the cytoplasm of the cell so that the two daughter nuclei are segregated into individual cells, which enter interphase and begin the process again (Jackson et al., 2007;Ruchaud et al., 2007;Walczak et al., 2010).

During mitosis, chromatin condensation is accompanied by global phosphorylation of histone H3 (Wang and Higgins, 2013;Xu et al., 2009). This phosphorylation is a crucial step in the higher order chromatin condensation and compaction, which are essential for subsequent chromosome congression and segregation. Mitotic phosphorylation of histone H3 exhibits highly coordinated spatiotemporal distribution that occurs at several residues, including Ser10 and Ser28 as well as at Thr3 and Thr11 (Garcia et al., 2005;Bonenfant et al., 2007;Zhou et al., 2008). Among these,

H3S10ph and H3S28ph are well studied and are considered as markers for chromosomal condensation and segregation during mitosis (Goto et al., 1999;Goto et al., 2002). Phosphorylation at these two sites is mediated by aurora B kinase and initiates at late G2 phase, reaches its maximal level during metaphase, followed by a rapid decrease at the transition to anaphase (Hendzel et al., 1997;McManus and Hendzel, 2006). This characteristic pattern is consistent with the chromosomal condensation, indicating a potential role of site specific H3 phosphorylation in this process (Giet and Glover, 2001). However, H3S10ph increases faster and decreases slower than H3S28ph during the course of mitosis, indicating the involvement of differential spatiotemporal regulation for the two marks (McManus and Hendzel, 2006). Further, their relative abundance in mitosis appears to be different, the level of H3S28ph was not as abundant as that of H3S10ph, although both phosphorylation marks occurred along the same chromosomes during mitosis (Goto et al., 1999;Goto et al., 2002). Whether both of the phosphorylation marks can simultaneously occur on the same H3 tail within the same nucleosome on the mitotic chromosomes, is not known yet.

In contrast to histone phosphorylation, histone acetylation in mitotic progression has been less studied. During mitosis, an overall reduction in acetylation levels of histones has been reported which correlate with the decreased activities of KATs and HDACs (Kruhlak et al., 2001;Bonenfant et al., 2007;Valls et al., 2005). The histone acetylation begins to decrease at the early stages of mitosis, is absent in metaphase and anaphase, then gradually increases in the late mitotic phase, telophase (Kruhlak et al., 2001;Chen et al., 2005a;Nishiyama et al., 2006;Valls et al., 2005). However, some residues on histones H3 and H4 can still remain acetylated on mitotic chromosomes, which may serve as markers for transcriptional memory (Valls et al.,

2005;Kruhlak et al., 2001). Furthermore, the decrease in the activities of KAT and HDACs is not because of enzymatic inactivation, rather it is due to their exclusion from the mitotic chromosomes (Kruhlak et al., 2001). Although the re-localization of chromatin binding proteins is an integral part of the mitotic chromosome condensation, this process has not been studied in detail. However, from the observation that condensed mitotic chromatin remains fully accessible to transcription factors and structural proteins, it appears that the displacement of these proteins from mitotic chromatin is not due to changes in the chromatin structure (Chen et al., 2005a). More likely alterations in their associated proteins or in the proteins themselves play a major role in their displacement from mitotic chromatin. Along with the chromatin associated proteins, displacement of some transcription factors such as Sp1 and Sp3, and RNAPII also takes place from mitotic chromosomes, which correlates with the steep fall in transcriptional activities in mitosis (He and Davie, 2006;Kruhlak et al., 2001;Delcuve et al., 2008). As cells proceed towards the end of mitosis, chromosomes decondense, and, chromatin associated proteins, transcription factors, and RNAPII are reloaded onto chromatin, with the consequence of re-establishment of transcription and gene expression (Kruhlak et al., 2001;Martinez-Balbas et al., 1995;Prasanth et al., 2003;Zaidi et al., 2003). Thus, a mechanism has been proposed involving histone acetylation and the re-association of KATs and HDACs in late mitosis, which could re-establish the chromosome territories and/or compartments (Kruhlak et al., 2001). A role for histone acetylation during mitosis has been further suggested by the impact of HDAC inhibitors (Marks et al., 2001;Wong et al., 2005), which effects mitotic progression with associated defects in chromosome condensation, segregation, and kinetochore assembly (Robbins et al., 2005;Shin et al., 2003;Warrener et al., 2003). Other histone PTMs have been also reported to play role in mitosis, including histone methylation and ubiquitination (Xu et al., 2009). For example, histone

methylation has been considered as a stable and transmissible epigenetic mark. However, a change in the H3K9me3 level has been detected during mitosis and is thought to have a role in mitotic chromatin compaction and segregation (McManus et al., 2006).

1.3 Regulators of dynamic histone acetylation: Interplay between KATs and HDACs

1.3.1 Lysine acetyltransferases (KATs)

KATs are classified into several evolutionary conserved superfamilies including GNAT (Gcn5-related N-acetyltransferases), E1A-associated protein of 300 kDa (p300)/CREB (cAMP-responsive element binding protein)-binding protein (CBP), and the MYST proteins (Lee and Workman, 2007). The GNAT superfamily is the largest group of KATs, which includes Gcn5 (KAT2A), PCAF (KAT2B) and ELP3 (KAT9). These proteins are known as transcriptional activators with more than 70% sequence homology (Roth et al., 2001). They share similar carboxyl terminal ends, which contain the HAT (histone acetyl transferase) domain and bromodomains. The substrates of this family are H3K9, 14, 18 and 36 (Furdas et al., 2012;Vernarecci et al., 2010). The p300/CBP family contains a large catalytic HAT domain, a bromodomain and three cysteine-histidine rich domains, required for protein-protein interactions (Santos-Rosa and Caldas, 2005). Histone residues H2AK5, H2BK12, H2BK15, H3K14, H3K18, H3K23, H4K5 and H4K8 are specifically acetylated by one or both of these KATs (Peterson and Laniel, 2004). Members of the MYST family includes Tip60 (Tat-interactive protein 60 kDa) (KAT5), MOZ (monocytic leukemia zinc finger protein) (KAT6A), MORF (MOZ-related factor) (KAT6B), HBO1 (histone acetyltransferase bound to ORC) (KAT7), and HMOF (KAT8) (Lee and Workman, 2007). This family has a unique catalytic mechanism, different from that shared by other families of KATs. They contain a conserved 370 residue MYST domain that acetylates

target lysine residues through an acetyl-cysteine intermediate. They also contain a cysteine-rich zinc binding domain within the MYST HAT domain and a chromodomain (recognizes methylated residues) (Santos-Rosa and Caldas, 2005). MYST HATs are involved in the acetylation of histone residues H2AK5, H3K14, H4K5, H4K8, H4K12 and H4K16 (Peterson and Laniel, 2004). In addition to their interaction with the primary substrate histones, KATs can acetylate non-histone proteins, including p53, c-Myc, c-Fos, α-tubulin, cohesin, and NF-κB (Barlev et al., 2001;Terret et al., 2009;Wort et al., 2009).

KATs are recruited to the targeted gene promoters through interaction with sequence-specific DNA binding factors or transcription activators (An et al., 2002). However, for the proper recruitment by DNA binding factors as well as for their substrate specificity on nucleosomes, KATs are assembled into the multiprotein complexes (Lee and Workman, 2007). The catalytic activities of KATs depend largely on the context of the subunits of these complexes. Recently several KATs (e.g. Tip60, PCAF, HBO1) have been also identified in the coding region of active genes (Govind et al., 2007;Obrdlik et al., 2008;Saksouk et al., 2009), suggesting their role in increased acetylation, transcription elongation, recruitment of chromatin remodelers as well as in co-transcriptional histone eviction.

1.3.2 Histone deacetylases (HDACs)

HDACs are enzymes that catalyze the removal of acetyl groups from the lysine 'ε'- amino groups in histones and many other proteins. While histones represent the primary target for the physiological function of HDACs, it is noteworthy that HDACs predate the evolution of histone proteins, indicating that their primary substrates were non-histone proteins (Gregoretti et al.,

2004). Some of the non-histone substrates of HDACs include Sp1, p53, E2F, tubulin, and YY1 (Hubbert et al., 2002; Marks et al., 2000; Martinez-Balbas et al., 2000; Yao et al., 2001). Thus the name of the enzyme does not reflect its broad range of substrates. Researchers in the field have been reluctant to rename the enzyme as lysine deacetylases (KDACs), which would be in line with the new nomenclature for the enzymes that add acetyl groups onto the lysines of histones and other substrates, the KATs, which were previously named HATs (Allis et al., 2007).

To date, 18 different mammalian HDACs have been identified and are divided into four classes based on their structure and sequence similarity to yeast counterparts (De Ruijter et al., 2003;Gregoretti et al., 2004) (**Figure 1.5**).

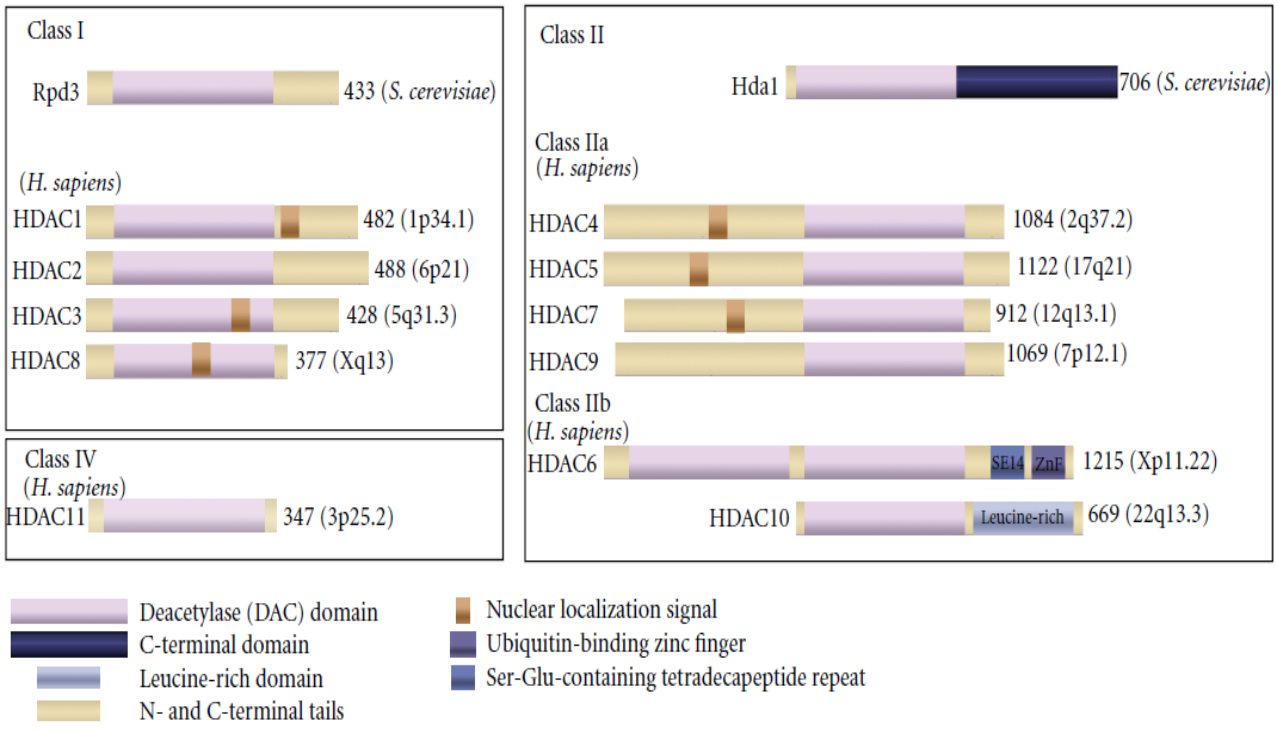

Figure 1.5: Schematic representation of the classical class I, II, and IV HDACs
HDAC enzymes have been separated into four classes based on their homology to yeast proteins. Class I enzymes are structurally similar to the yeast, reduced potassium dependency-3 (RPD3) and consisting of HDAC1, 2, 3, and 8. Class II HDACs are related to the yeast, histone deacetylase-1 (HDA1). This class is subdivided into two classes-class IIa (HDAC4, 5, 7, 9) and

class IIb (HDAC6, 10). The HDACs have a conserved deacetylase (DAC) domain. The number of amino acid residues of the longest isoform of each HDAC is shown on the right. *The figure and the text were adapted from Figure 2 (Rodd et.al, 2012).*

HDACs from the classical family are dependent on Zn^{2+} for deacetylase activity and constitute classes I, II and IV. Class I HDACs, closely related to yeast RPD3, comprised of HDAC1, HDAC2, HDAC3 and HDAC8. The class I HDACs are found primarily in the nucleus of most cell types but these enzymes are also present in the cytoplasm and the endoplasmic reticulum, where the HDAC has a role in the activation of the unfolded protein response (Kahali et al., 2012). Class II HDACs, related to yeast HDA1, which have tissue-specific expression and functions, are divided into subclass IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and subclass IIb (HDAC6 and HDAC10). Class IIa HDACs are found in complex with class I HDACs (e.g. HDAC3 with HDAC4). It also appears that most of the enzymatic activity on histones is from the class I HDACs (Lahm et al., 2007). Class III HDACs consist of seven sirtuins, which require the NAD+ cofactor for activity. Class IV contains only HDAC11.

1.4 Class I histone deacetylase complexes

1.4.1 HDAC1 and 2 corepressor complexes

HDAC1 (482 amino acid residues) and HDAC2 (488 amino acid residues) are homologous with 75% identity in DNA sequences and 82% identity in protein sequences (Tsai and Seto, 2002). This gene pair has been suggested to have evolved by independent gene duplication event with different chromosomal locations, 1p34 and 6q21, respectively (De Ruijter et al., 2003;Gregoretti et al., 2004). HDAC1 was the first mammalian HDACs to be identified, which was isolated, cloned and characterized by Schreiber and colleagues in 1996 by using HDAC inhibitor trapoxin A affinity matrix (Tauton et al., 1996). Ed Seto group isolated HDAC2 by yeast two hybrid

screening with YY1 transcription factor as bait (Yang et al., 1996b).

HDAC1 and HDAC2 form homo- and heterodimers between each other (Brunmeir et al., 2009;Luo et al., 2009;Taplick et al., 2001), which presumably allow them to act together or separately. The dimer is a requirement for HDAC activity (Luo et al., 2009). The two proteins interact through the N-terminus. Dissociation of the dimer with a HDAC1 N-terminal peptide (first 67 amino acids) inhibits HDAC activity (Luo et al., 2009). Viruses have capitalized on this mechanism to inhibit HDAC activity. The adenoviral protein GAM1 inhibits HDAC1 activity by binding to the N-terminal region of HDAC1, which likely dissociates the dimer (Chiocca et al., 2002). Both HDACs have to be catalytically active to form a functional homo- or heterodimer. Combining a catalytically inactive HDAC1 (H141A) with wild type HDAC1 resulted in an inactive mutant-wild type dimer (Luo et al., 2009). PTMs also impact the activity of the dimer, with acetylation of HDAC1 at K432 inhibiting the activity of the dimer (either a HDAC1 homodimer or a HDAC1 and 2 heterodimer). Interestingly, the corresponding amino acid in HDAC2 is R433, which cannot be acetylated (Luo et al., 2009). The HDAC involved in deacetylating acetylated HDAC1 and thus regulating the activity of the dimer, has yet to be identified.

HDAC1 and 2 heterodimer levels seem to depend on the cell types, because it was shown that 80% to 90% of HDAC1 and 2 proteins were associated with each other in the nucleus of human breast cancer MCF7 cells (He et al., 2005), whereas 40% to 60% of HDAC1 and 2 proteins were found to be free from each other in mouse embryonic fibroblasts (Yamaguchi et al., 2010). Moreover, a genome wide mapping study in primary human CD4+ T- cells revealed a

differential distribution of HDAC1 and 2 along regulatory and coding regions (Wang et al., 2009). Conversely, HDAC1 and 2 were both associated with regulatory and coding regions in MCF7 cells (He et al., 2005;Sun et al., 2007). However, whether they localized to the same complexes in these regions is not known. HDAC1 and 2 relative expression levels also vary with cell types. For example, T-lymphocyte Jurkat cells express negligible levels of HDAC2 compared to HDAC1 levels (Hassig et al., 1998), and throughout the adult brain HDAC2 is preferentially expressed in neurons, whereas HDAC1 is more abundant in glial cells (Guan et al., 2009;MacDonald and Roskams, 2008). It is likely that, at least in cells expressing markedly different relative levels of HDAC1 and 2, homodimer formation would prevail over heterodimer formation.

HDAC1 and 2 are found in multiprotein corepressor complexes Sin3, nucleosome-remodeling and deacetylase repressor (NuRD) and CoREST, which are recruited to chromatin regulatory regions by transcription factors (for example, Sp1, Sp3, p53, NF-κB and YY1) and have very diverse, often cell-specific roles (**Figure 1.6**) (De Ruijter et al., 2003;Yang and Seto, 2008). Although it is generally assumed that both HDACs can be paired within the same complex, it has been demonstrated only in studies using exogenously expressed, tagged HDAC1 and not in studies characterizing endogenous HDAC corepressor complexes (Luo et al., 2009).

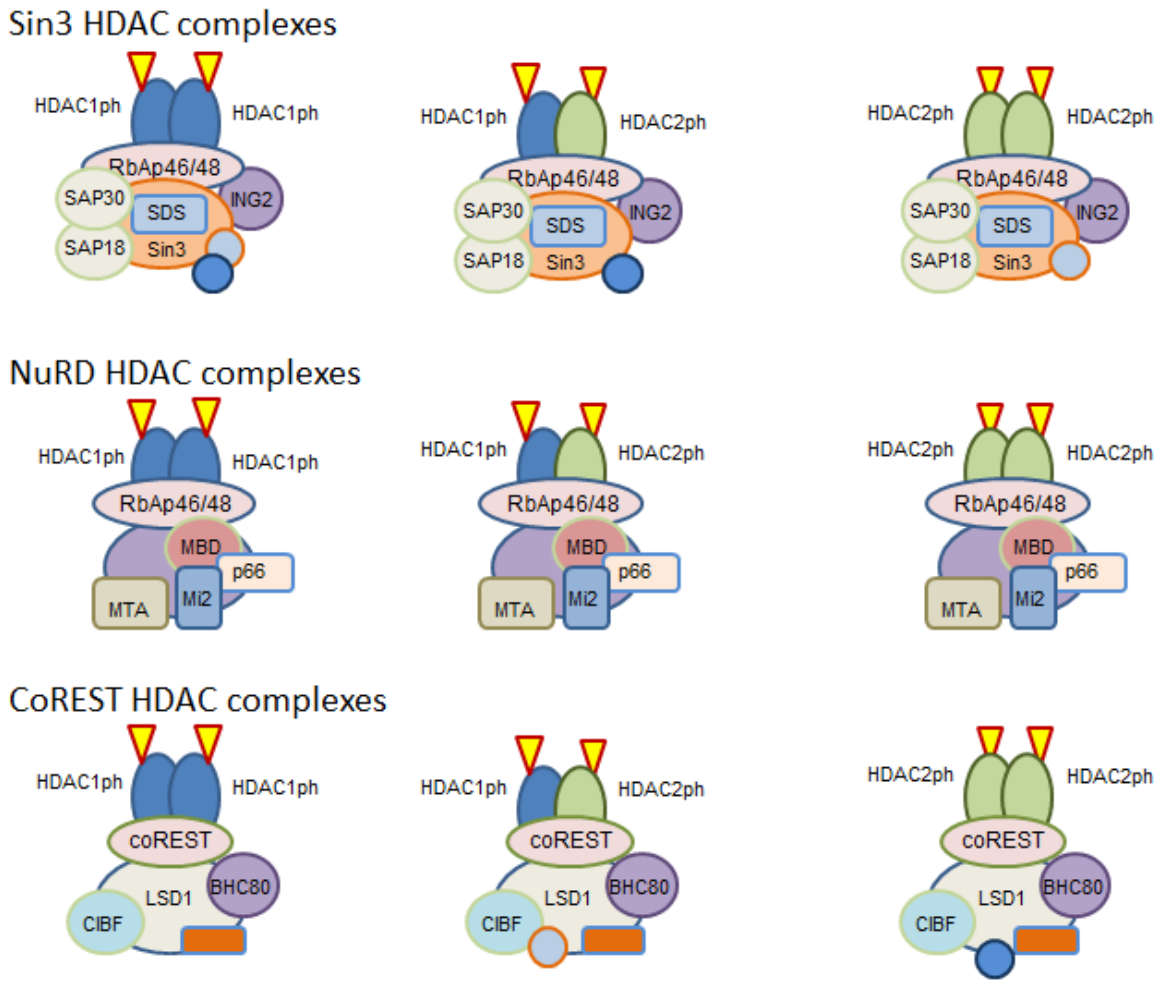

Figure 1.6: HDAC1 and 2 multiprotein complexes
Multiprotein complexes containing HDAC1 and 2 homo- or heterodimers are shown. HDAC1 and 2 are shown as phosphorylated, which is a requirement for multiprotein complex formation. Phosphorylation is indicated by red-outlined yellow triangles. *The figure and the text were adapted from Figure 1 (Delcuve et al., 2012).*

The Sin3 core complex contains Sin3A or Sin3B, HDAC1 and 2, SAP18, SAP30/L and retinoblastoma-associated proteins (RbAps) RbAp46/48 and serves as a platform for the addition of other modules with enzymatic functions such as nucleosome remodeling, DNA methylation, histone methylation (KDM5) and N-acetylglucosamine transferase activity (Hayakawa and Nakayama, 2011;Silverstein and Ekwall, 2005). Other proteins associated with the Sin3 core complex include SAP130, SAP25, SUDS3, BRMS1/L, ARID4A/B and ING1/2 (inhibitor of

growth 1/2) (Hurst, 2012). HDAC-complexes containing either Sin3A or Sin3B have distinct roles (Hayakawa and Nakayama, 2011). Sin3A complex is involved in regulation of cell cycle, whereas Sin3B complex has a role in cell cycle exit (Telles and Seto, 2012). Further, Sin3B complex is associated with the coding region of transcribed genes (Jelinic et al., 2011).

The NuRD complex has lysine deacetylase and ATP-dependent chromatin remodeling activities, which are carried out by HDAC1 and 2, and, the helicase-like ATPases Mi-2, Mi-2α and/or β, respectively. The other known components of NuRD are structural and/or regulatory proteins RbAp46/48 and, in some instances p66α or p66β, the methyl-CpG-binding domain-containing proteins (MBD2 or MBD3), with only MBD2 being able to recognize methylated DNA and the three members of the metastasis-associated protein family (MTA1, MTA2 or MTA3), with different MTA proteins allowing distinct downstream responses to the activation of different signaling pathways (Denslow and Wade, 2007;Hayakawa and Nakayama, 2011). Lysine-specific demethylase 1 (KDM1/ LSD1) has also been identified as a component of NuRD (Wang et al., 2009a).

CoREST HDAC complex (also known as the BRAF-HDAC complex or BHC) consists of the HDAC1 and 2, RCOR1/CoREST, KDM1/LSD1 (H3K4 and H3K9 demethylase), HMG20B/BRAF35 and PHF21A/BHC80 (Lakowski et al., 2006). The CoREST complex acts as a corepressor in terminally differentiating non-neuronal cells by recruiting KDM1/LSD1 to demethylate H3K4me2, and, the methyltransferase G9a to methylate H3K9 at the RE1 (repressor element-1) sites of target genes (Delcuve et al., 2012). However, in embryonic stem cells and neuroral stem cells, this complex acts as a coactivator by recruiting an H3K4 methyltransferase

to the RE1 sites of target genes. CoREST also forms larger complexes by association with ZNF217, a Krüppel-like zinc finger protein and with other complexes such as the chromatin remodeling complex SWI/SNF (SWItch/Sucrose Non Fermentable) or the C-terminal binding protein (CtBP) complex (Battaglia et al., 2010;Hayakawa and Nakayama, 2011).

HDAC1 and 2 are components of the Nanog and Oct4 associated deacetylase (NODE) complex, a NuRD-related repression complex, also comprising MTA1 or MTA2, p66α or p66β, but not the histone-binding proteins RbAp46/48 or Mi-2. NODE is involved in the control of embryonic stem cell fate by repressing Nanog and Oct4 target genes (Liang et al., 2008).

HDAC1 and 2 are present in numerous complexes; many of which remain to be characterized. HDAC1 is present in a complex, referred to as the SHMP complex, that contains Sin3B, Mrg15 and PHF12 (also known as Pf1) (Jelinic et al., 2011). The SHMP complex is present along the gene body of transcribed genes.

A novel HDAC complex, mitotic deacetylase complex (MiDAC), is specific to mitotic cells and includes HDAC1 and 2, either one of the related ELM-SANT proteins MIDEAS or TRERF1, and DNTTIP1 (terminal deoxynucleotidyl transferase (TdT)-interacting protein), although the authors who published these findings suggested that the MiDAC complex has a TdT-independent function in cell division (Bantscheff et al., 2011). Whether the putative histone acetylase CDYL is also a MiDAC component is presently unclear (Bantscheff et al., 2011). The discussion above illustrates that the HDAC1 and 2 homo- or heterodimers can exist with different proteins. The

combination of these proteins likely determines the overall activity, substrate specificity and genomic location of the HDAC1 and 2 containing complexes.

1.4.2 Phosphorylation dependent regulation and targeting of HDAC1 and 2 containing complexes

HDAC1 and 2 undergo several PTMs e.g. phosphorylation, acetylation, ubiquitination and sumoylation, which regulate their biological and functional activities depending on the extent and nature of PTMs (Brandl et al., 2009). Among the different modifications of HDAC1 and 2, phosphorylation is very well studied. Phosphorylation of HDAC1 and 2 is a prerequisite for forming the corepressor complexes (**Figure 1.6**). The enzymatic activity of the HDAC1 and 2 dimers is considerably enhanced within these multiprotein complexes. HDAC1 is phosphorylated by protein kinase CK2 at Ser393, Ser421 and Ser423, whereas HDAC2 is phosphorylated at Ser394, Ser422 and Ser424 (Brandl et al., 2009;Segre and Chiocca, 2011;Sun et al., 2007;Tsai and Seto, 2002). *In vitro,* HDAC2 is phosphorylated by CK2, whereas HDAC1 can be phosphorylated by CK2, PKA and protein kinase G (Tsai and Seto, 2002). This difference constitutes more evidence that, although they share a high degree of homology and often occur together in corepressor complexes, HDAC1 and 2 have distinct and separately regulated functions. Mutations in any of these phosphorylation sites (Serine to Alanine mutations at Ser393, Ser422 and Ser424 sites) prevent the associations of the HDAC1 and 2 into the Sin3, NuRD or CoREST complexes (Pflum et al., 2001;Sun et al., 2007;Tsai and Seto, 2002). Further, the enzymatic activities of the mutant HDACs are low.

It is to be noted that HDAC phosphorylation is dynamic and dependent on the balance of opposing activities of involved kinases and phosphatases. Treatment of cultured cells with the protein phosphatase inhibitor, okadaic acid, resulted in HDAC1 and 2 hyperphosphorylation concomitant with the dissociation of HDAC1 and 2 heterodimers, as well as the dissociation of HDAC1 and 2 from Sin3A or YY1 (Galasinski et al., 2002). On the other hand, the HDAC1 and 2 interactions with RbAp46/48 were not disrupted. In view of the above described results from several groups (Pflum et al., 2001;Sun et al., 2007;Tsai and Seto, 2002); however, it appears that the observed dissociation of the HDAC-corepressor complexes subsequent to okadaic acid treatment was not due to the hyperphosphorylation of HDAC1 and 2, but rather to the hyperphosphorylation of other unidentified factors.

The corepressor complexes with phosphorylated HDAC1 and 2 are directed to regulatory regions of transcribed genes by a number of transcription factors (e.g., Sp1, Sp3, p53, NF-κB and YY1) (De Ruijter et al., 2003;Sun et al., 2007). On the other hand, un- or monophosphorylated HDAC2 is associated with coding regions of transcribed genes (Sun et al., 2007) (**Figure 1.7**). Although unmodified and monophosphorylated HDAC2 are more abundant than highly phosphorylated HDAC2, it is the highly phosphorylated form that is preferentially cross-linked to chromatin, with formaldehyde or cisplatin (Sun et al., 2002a). Thus, under conditions typically used in ChIP, highly phosphorylated HDAC2, but not un- or monophosphorylated HDAC2 is preferentially cross-linked to nuclear DNA *in situ* with formaldehyde. Through the use of a dual cross-linking ChIP assay, however, all isoforms of HDAC1 and 2 could be mapped along the regulatory and coding regions of transcribed genes, with the un- or monophosphorylated HDAC2 being associated with the coding region (Sun et al., 2007).

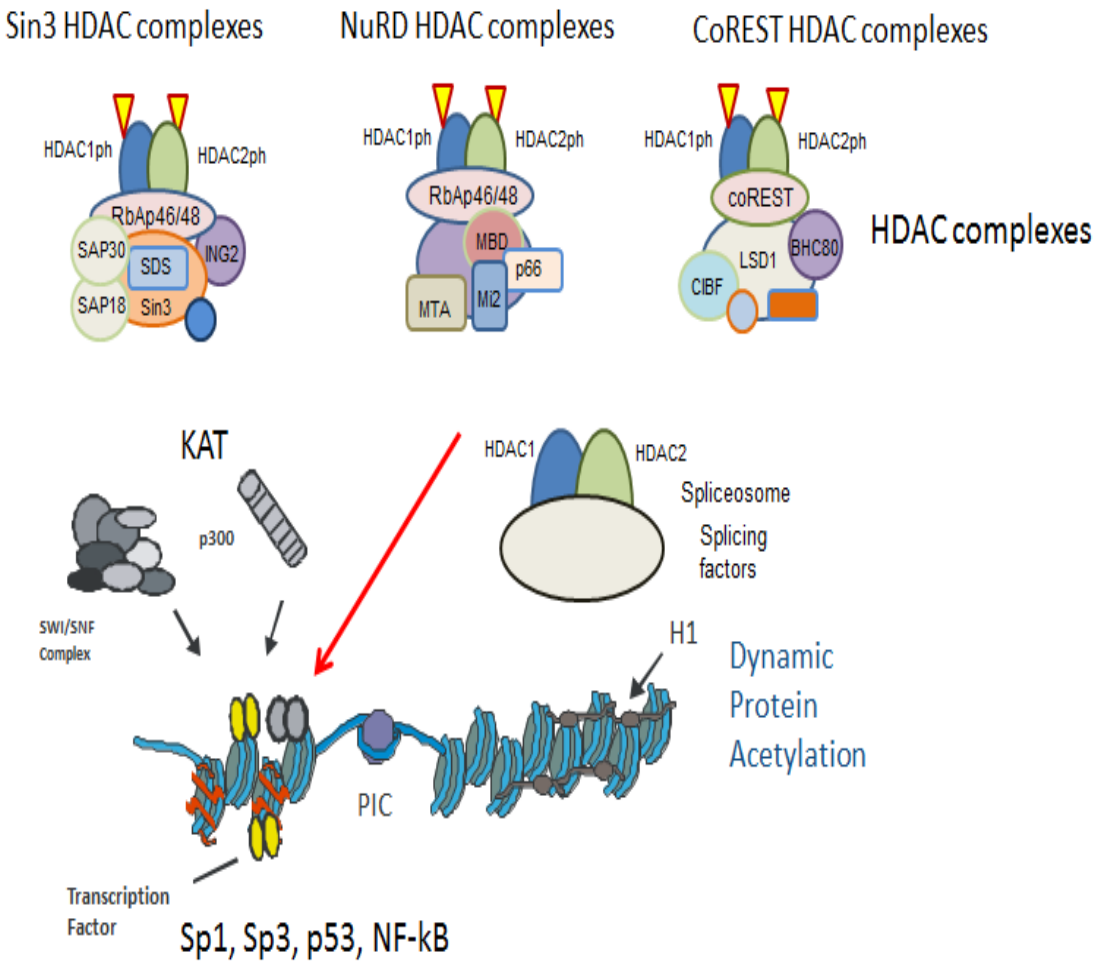

Figure 1.7: Recruitment of HDAC1 and 2 complexes to regulatory regions and the gene body of transcriptionally active genes
Protein kinase CK2-mediated phosphorylation (indicated by red-outlined yellow triangles) is required to form the Sin3, NuRD and CoREST HDAC-complexes. The drawing shows the ATPase chromatin remodeling complex SWI/SNF and the KAT p300/CBP recruited to the upstream promoter region by transcription factors. HDAC and splicing factors are recruited to the gene body by RNAPII. Histones and non-histone proteins associated with the regulatory region and body of the gene are dynamically acetylated. PIC is the pre-initiation complex. HDAC1ph and HDAC2ph are phosphorylated HDAC1 and 2. *The figure and text were adapted from Figure 1 (Delcuve et al., 2013).*

1.4.3 HDAC3 complexes

HDAC3 is in the multiprotein corepressor complexes SMRT (silencing mediator of retinoid and thyroid hormone receptor) and NCoR (nuclear receptor corepressor) (De Ruijter et al., 2003),

which consist of HDAC3, transducin β-like 1 (TBL1), TBL-related 1 (TBLR1) (a WD40 repeat-containing protein) and G protein pathway suppressor 2 (GPS2) (Hayakawa and Nakayama, 2011;Perissi et al., 2010). NCoR and SMRT also interact with class IIa HDACs, which exhibit no deacetylase activity of their own but are believed to recruit NCoR/SMRT HDAC3 activity to distinct promoters through their associated factors, such as myocyte enhancer factor 2 (MEF2) (Fischle et al., 2002). NCoR, but not SMRT, interacts with zinc finger and BTB domain-containing 33 (ZBTB33 or Kaiso), which is a protein that binds to methylated DNA. NCoR and SMRT are regulated by different kinase pathways and play different roles in development. Although NCoR binds preferentially to the thyroid hormone receptor, SMRT prefers the retinoic acid receptor (RAR) (Hayakawa and Nakayama, 2011;Perissi et al., 2010). It is noteworthy to indicate that repression by NCoR/SMRT is an integral phase of the cyclical process that is the transcriptional activation of genes controlled by liganded receptors. NCoR/SMRT repression is necessary to prime chromatin for subsequent transcription initiation (Metivier et al., 2003). Besides its role in transcriptional control, the HDAC3-NCoR/SMRT complex is critical to the maintenance of heterochromatin content and genomic stability (Bhaskara et al., 2010). Within the SMRT and NCoR complexes HDAC3 is stabilized. The free HDAC3 is in contrast unstable, with the rate of free HDAC3 degradation being inversely correlated with the levels of SMRT and NCoR complexes (Guo et al., 2012).

The crystal structure of HDAC3 was recently published and yielded a surprise in that D-myo-inositol-(1,4,5,6)- tetraisophosphate [Ins(1,4,5,6) P4] was identified as a major component of the multiprotein complex required for the interaction between HDAC3 and SMRT (Watson et al., 2012). The nuclear enzyme inositol polyphosphate multikinase (IPMK) catalyzes the synthesis of

Ins(1,3,4,5) P4, Ins(1,4,5,6) P4 and Ins(1,3,4,5,6) P5 and has a role in growth factor- and nutrient-signaling (Chakraborty et al., 2011). As Wortmannin inhibits IPMK activity, this inhibitor may have an impact on the integrity of HDAC3 complexes. The identification of Ins (1, 4, 5, 6) P4 as a 'molecular glue' of the HDAC3 complex represents new therapeutic strategies in the regulation of this HDAC complex.

The HDAC3 complex binds to multiple proteins, including transcription factors and proteins regulating apoptosis. The pro-apoptotic kinase HIPK2 (homeodomain-interacting protein kinase 2) is a master regulator of redox-regulating genes. HIPK2 binds to HDAC3 when HIPK2 is sumoylated (de la Vega et al., 2012). Reactive oxygen-induced de-sumoylation of HIPK2 prevented the association with HDAC3, resulting in HIPK2 acetylation. In the acetylated state HIPK2 diminished the activity of the kinase to repress the transcription of redox-regulated enzymes and protected the cells from ROS (reactive oxygen species)-induced cell death.

HDAC3 recruitment to regulatory regions of the genome follows a circadian rhythm in mouse liver. This temporal recruitment of HDAC3 is a consequence of the circadian nuclear receptor Rev-erbα rather than a change in HDAC3 levels throughout the day/night cycle (Feng et al., 2011). Through the activity of Rev-erbα, HDAC3 regulates the temporal expression of genes regulating lipid metabolism. Similar to HDAC2, HDAC3 is a regulator of memory formation (McQuown and Wood, 2011).

1.5 HDAC inhibitors

HDAC inhibitors are generally considered as a group of structurally diverse compounds that act as chromatin modifiers. The active site of Zn^{2+}-dependent HDACs consists of a tubular pocket with two adjacent histidine residues, two aspartic acid residues, one tyrosine residue and a Zn^{2+} ion at the bottom of the pocket, all forming a charge-relay system (Finnin et al., 1999). The class I, II and IV HDAC inhibitors have a bidentate chelator, which binds to catalytic Zn^{2+} (Bressi et al., 2010). Crystal structures of TSA (trichostatin A) and SAHA (suberoylanilide hydroxamic acid) with the HDAC homolog HDLP (HDAC-related protein) from the hyperthermophilic bacterium *Aquifex aeolicus* and those with HDAC8 show that, the Zn^{2+}-chelating group and a linker spanning the length of the tubular pocket are connected to a cap that blocks the active site by interacting with the external surface of HDACs. Depending on their chemical Zn^{2+}-binding group, HDAC inhibitors belong to different classes including hydroxamic acids (TSA and SAHA), carboxylic acids (valproic acid, VPA, butyrate), benzamides (MS-275) and cyclic tetrapeptides (apicidin, depsipeptide), epoxyketones (trapoxins) and hybrid molecules (Smith and Workman, 2009) (**Figure 1.8**). These inhibitors induce a dose-dependent inhibition of either class I or class II HDACs, or both. For example, SAHA and TSA are the pan-inhibitors that effects the activity of HDAC1-9 with roughly equivalent potency (Khan et al., 2008). Other inhibitors such as MS-275 and apicidin, are more selective than SAHA or TSA and primarily inhibits HDAC1, 2 and 3 (Khan et al., 2008).

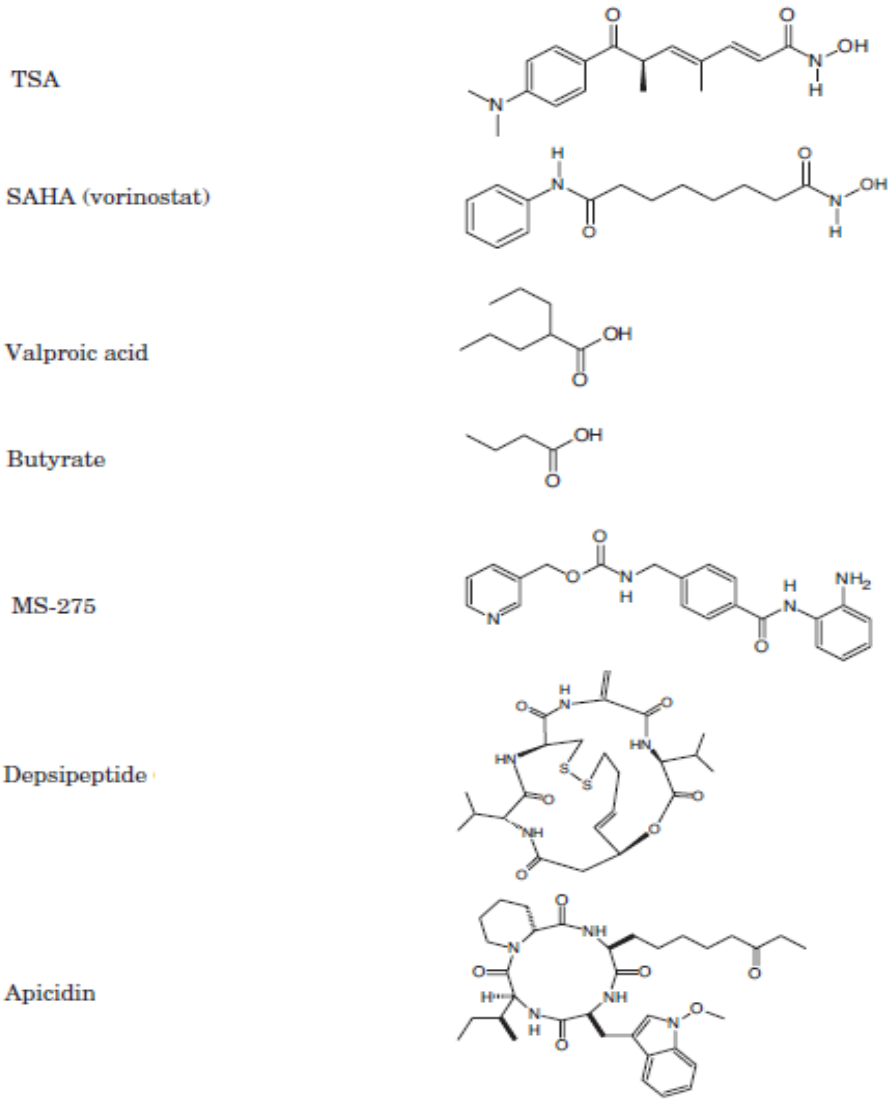

Figure 1.8: Structures of common HDAC inhibitors
The figure was modified with permission from Table 2 (Grayson et al., 2010).

A central theme in the literature on HDAC inhibitors is their isoform selectivity or, rather, their perceived lack of isoform selectivity. HDAC inhibitors have generally been considered as pan-inhibitors, inhibiting all HDACs isoforms or class I specific inhibitors. This view has recently been dispelled, by a study revealing no targeting of class IIa HDACs by most HDAC inhibitors (Bradner et al., 2010). Although it is not known which HDAC isoform's inhibition is responsible

for the therapeutic or toxic effects observed in clinical trials, it has generally been assumed that the development of isoform-selective inhibitors would result in preferable clinical outcomes. This theory is unproven to date (Wagner et al., 2010;Witt et al., 2009). However, researchers who have performed conventional assays have analyzed the affinities of HDAC inhibitors for different HDACs by using purified HDACs, whereas HDAC activity is mostly associated with multiprotein complexes, the role and composition of which are often cell type-specific. This fact was taken into consideration in a pioneering study in which the investigators carried out the chemoproteomic profiling of 16 HDAC inhibitors with different chemical structures across six human cell lines and six mouse tissues (Bantscheff et al., 2011). In that study, a nonselective HDAC inhibitor bound to sepharose beads was added to cell lysates under conditions that preserved the integrity of protein complexes. In a competition assay, the mixture was spiked with a range of concentrations of a free inhibitor interfering with the capture of HDAC complexes by the immobilized inhibitor. Captured proteins were analyzed by quantitative mass spectrometry, and target complexes were reconstituted by matching half-maximal inhibitory concentration values. This initial complex identification was further confirmed by quantitative immunoprecipitation experiments. Although the results collected in this study confirmed that class IIa HDACs were not targeted by any of the studied inhibitors, they mostly conflicted with the isoform selectivity data previously obtained in assays using purified HDACs (Bertrand, 2010;Bradner et al., 2010). This is not surprising, in view of a previous kinetic study suggesting that the *in vitro* mode of action of the HDAC inhibitor TSA depended on whether the assay conditions preserved HDAC complexes or resulted in their dissociation (Sekhavat et al., 2007). Incidentally, it was also shown that TSA did not disrupt HDAC1 and 2 interactions with Sin3A (Sekhavat et al., 2007). However, it was shown that TSA and SAHA, but not less bulky

inhibitors such as sodium butyrate or VPA, dissociated ING2 from the Sin3 complex, thus disrupting the ING2-mediated recruitment of Sin3 to chromatin (Smith et al., 2010). Bantscheff et al. found that some inhibitors had different affinities for different complexes (Bantscheff et al., 2011). In particular, inhibitors from the benzamide class displayed a higher affinity for the HDAC3-NCoR complex than for NuRD and CoREST complexes, whereas they did not target the Sin3 complex. The affinity of VPA, an inhibitor from the carboxylic acid class with moderate potency for class I HDACs, was highest for the CoREST complex, decreased gradually for the NuRD and NCoR complexes and was lowest for the Sin3 complex (Bantscheff et al., 2011). The different affinities of HDAC inhibitors detected for different complexes are in agreement with the previous observation that proteins in close proximity to the HDAC active site could interact with the cap of HDAC inhibitors, leading to the suggestion that HDAC-associated proteins could specify inhibitor selectivity (Salisbury and Cravatt, 2007). The methodology used by Bantscheff et al. (Bantscheff et al., 2011) in combination with the elucidation of the genome wide distribution of the different HDAC complexes in cancer and normal tissues might provide the means to reverse the expression of crucial genes and their protein products in cancer cells.

1.5.1 HDAC inhibitors as a therapeutic target

The maintenance of the balance of acetylation within histones and non-histone proteins, catalyzed by KATs and HDACs, is regarded as an important epigenetic layer of gene expression. The implication of lysine acetylation in fundamental processes like transcription, DNA repair, recombination, cell differentiation, neuronal plasticity, vascular remodeling, inflammation or metabolic cascades indicates this covalent modification act as a master regulator in cellular biology (Barnes, 2009;Feinberg, 2007;Johnsson et al., 2009;Keenen and de, I, 2009;Selvi and

Kundu, 2009). As a corollary, aberrant regulation of acetylation are evident in a number of diseases and cancers. Hence, KAT-HDAC interplay is considered as an important target for therapeutic modalities (Dekker and Haisma, 2009;Ellis et al., 2009). This led to the development of KAT and HDAC inhibitors (and other chromatin modifying agents) as therapeutic targets, although the HDAC inhibitors are relatively widely used in diseases and cancer treatments (Kouraklis and Theocharis, 2006).

The mechanisms of action of HDAC inhibitors are complex and not fully characterized. HDAC inhibitors have multiple cell type-specific biological effects *in vivo* and *in vitro* including growth arrest, cellular homeostasis, cell cycle progression, cell differentiation, migration, angiogenesis, and apoptosis (Deroanne et al., 2002;Donadelli et al., 2003;Fandy et al., 2005;Hu and Colburn, 2005). These compounds can be used as mono-therapeutic agents or in combination with other therapies. Several lines of evidence have implicated HDACs in diseases, malignancies and tumorigenesis, providing the rationale for development of HDAC inhibitors as therapeutic modality. First, the differential expression patterns of HDACs in tumor versus normal cells are reported, although structural mutations in HDACs linked with cancers or diseases are rare. Cancer cells often exhibit increased expression of HDACs (Lane and Chabner, 2009). For example, HDAC1 is overexpressed in prostate, gastric, colon and breast cancers and an overexpression of HDAC2 has been documented in colon, cervical and gastric cancers (Choi et al., 2001;Halkidou et al., 2004;Huang et al., 2005;Song et al., 2005;Wilson et al., 2006;Zhang et al., 2005;Zhu et al., 2004). Second, aberrant recruitment of HDACs to the regulatory region of various genes, through the interaction with fusion oncoproteins, can lead to down regulation of their expression. As an example, in case of acute promyelocytic leukemia (APL), chromosomal

translocations of t (15; 17) and t (11; 17) results in production of a chimeric protein, PML-RAR-α. This protein causes transcriptional repression of RAR-α target genes by recruiting the HDAC containing corepressor complexes to promoters (Lin et al., 2001b). Third, a decrease in the global level of histone H4 acetylation is considered as a hallmark of cancers (Fraga et al., 2005). In several cancer cell lines, a loss of acetylated Lys16 (H4K16) and trimethylated Lys20 (H4K20) has been reported.

1.6 Roles of class I HDACs in regulation of gene expression

1.6.1 Transcriptional regulation and HDACs

Inhibition of HDAC activity results in transcriptional reprogramming, which is believed to contribute largely to the therapeutic benefits of HDAC inhibitors in cancers, cardiovascular diseases, neurodegenerative disorders and pulmonary diseases (Haberland et al., 2009). Inhibition of HDAC enzymatic activity affects the expression of only 5% to 20% of genes, however, with equal numbers of genes being up- and down regulated (Smith and Workman, 2009). Only a fraction of these changes are direct effects of HDAC inhibitors, and others are downstream effects, necessitating new protein synthesis. Only some of the direct effects can be inferred as direct consequences of inhibition of histone deacetylation. The rest of them are the results of other mechanisms, such as the inhibition of transcription factor deacetylation, resulting in an altered affinity for DNA binding sites on target gene regulatory regions, an altered interaction with other factors or an altered half-life (Glozak et al., 2005). For example, HDAC inhibitors often induce the expression of *CDKN1A (p21 or Cip1/Waf1)*. Alternatively, pan-HDAC inhibitors, which inhibit class I and II HDACs, reduced or prevented the epidermal growth factor (EGF) or TPA (phorbol ester) mediated induction of *c-Jun* and *c-Fos* genes in

mouse fibroblasts (Hazzalin and Mahadevan, 2005). Further inhibition of the KAT (p300/CBP) attenuated the induction of these IEGs, whereas inhibition of HDAC and p300/CBP activities abolished the induction of these genes (Crump et al., 2011). The authors concluded that dynamic histone acetylation at the UPR is required for induction of these genes to take place (Crump et al., 2011). This dynamic histone acetylation of UPR nucleosomes was occurring before and after induction, although the steady state of acetylation increased after induction was due to the increased recruitment of p300/CBP to the UPR. However, the EGF mediated increased activity of the sphingosine kinase 2, which is bound to the Sin3 (or NuRD) HDAC1 and 2 complexes, and the resulting sphingosine-1-phosphate inhibition of HDAC1 and 2, may also contribute to the increased steady state of acetylated histones (Hait et al., 2009).

1.6.1.1 Immediate-early gene (IEG) expression and nucleosomal response

IEGs or inducible genes are members of a class of genes that are rapidly induced, usually in a transient manner, in response to a wide variety of stimuli (e.g. growth factors, nutrients, mitogens, phorbol esters and environmental stimuli etc.) without *de novo* synthesis of proteins (Thomson et al., 1999). In the absence of stimulation, their basal expression level is found to be very low. These genes are expressed following the activation of one of the two MAPK signaling cascades: the RAS-MAPK pathway, which is activated by growth factors, phorbol esters, and mitogens and results in activation of ERK1/2, and the p38 MAPK pathway, which is activated by stressors such as UV irradiation (McKay and Morrison, 2007;Yang et al., 2003) (**Figure 1.9**). The nuclear kinases, MSK1/2 are activated by RAS-MAPK-ERK1/2 and p38 stress kinase pathways (Soloaga et al., 2003). The substrates of MSK1/2 include a number of transcription factors, such as CREB and NF-κB, which regulate IEG expression. The target of MSK1/2 also

includes histone H3, which is phosphorylated at Ser10 and Ser28 by MSKs at the UPR of IEGs (Chadee et al., 1999;Clayton et al., 2000;Clayton and Mahadevan, 2003;Mahadevan et al., 1991;Soloaga et al., 2003;Thomson et al., 2001).

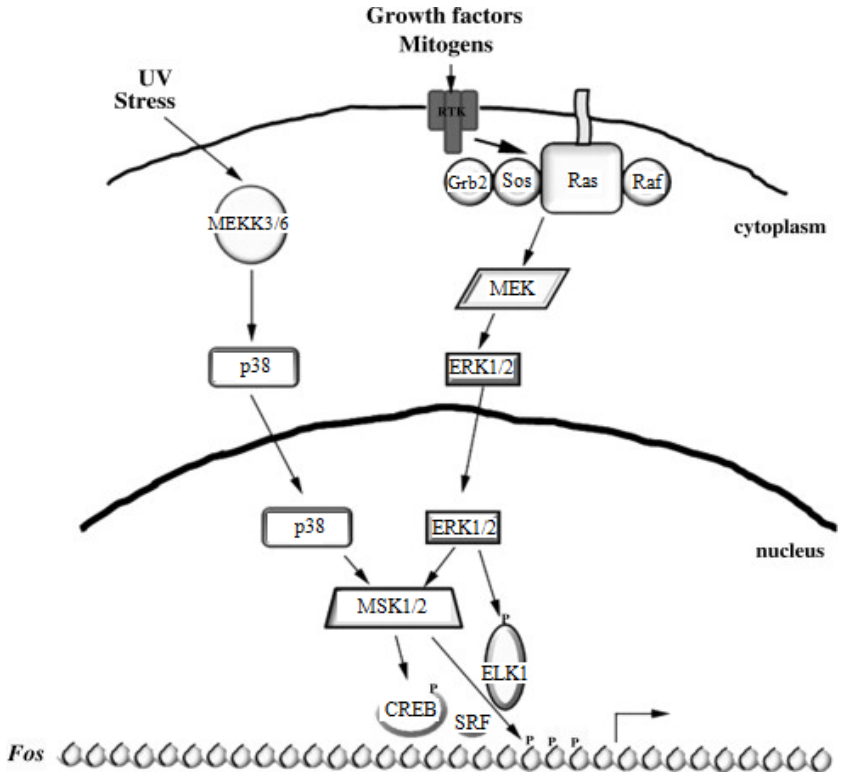

Figure 1.9: The MAPK signaling pathway
Activation of the RAS-MAPK or p38 MAPK pathway by different external stimuli leads to phosphorylation of regulatory factors, including CREB and Elk1, phosphorylation of histone H3 at Ser10 and Ser28, and immediate early gene *FOS* activation. Additional abbreviations used: MAPK/ERK1/2 kinase, MEK; SOS, Son of sevenless. *The figure and the text were reproduced with permission from Figure 1 (Healy et al., 2013).*

H3S10ph or H3S28ph is recognized as binding sites for 14-3-3 (14-3-3s are phosphoserine- or phosphothreonine binding proteins, and can act as a scaffolding protein, leading to multiprotein complex formation on the target phosphoproteins), which in turn, mediates the recruitment of other chromatin remodeling complexes and allows for the accessibility of transcription factors to the transcription machinery (Drobic et al., 2010) (**Figure 1.10**).

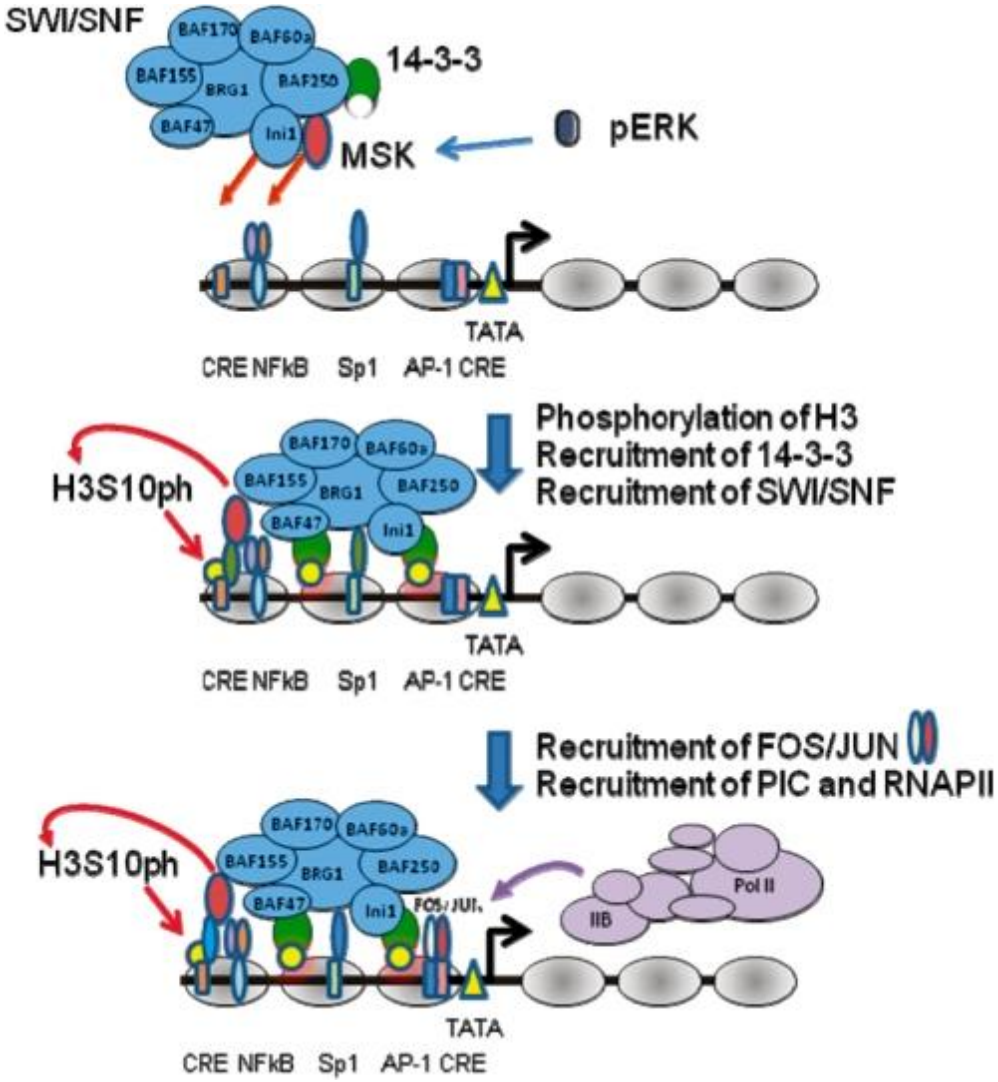

Figure 1.10: Schematic model representing the role of MSK1 and 14-3-3 in IEG remodeling and induction in response to MAPK signaling .

MSK shown as a large red oval, and 14-3-3 as a green partial oval are in complex with the SWI/SNF chromatin remodeling complex. The grey ovals along the DNA represent nucleosomes; the NF-κB transcription factor (p65/p50 dimer) is shown as a purple/orange pair of ovals binding to the NF-κB binding site (blue oval). In response to the activation of the ERK and p38 MAPK pathways, the MSK1 multiprotein complex is recruited to the regulatory regions of IEGs by transcription factors such as Elk1, NF-κB or C/EBPβ. MSK1 phosphorylates either H3Ser10 or H3Ser28, with H3S10ph being shown (yellow circles). H3S10ph and H3S28ph recruit 14-3-3 proteins, which mediate the recruitment of the SWI/SNF complex, with BRG1 being the ATPase subunit. The ensuing remodeling of promoter nucleosomes allows the access of transcription factors such as AP-1 to the promoter target sequences. The pre-initiation complex is recruited at the TATA box (yellow triangle), and initiation of transcription follows as is made apparent by the presence of initiation form of RNAPII (RNAPIIS5ph). *The figure and the text were reproduced with permission from figure 7 (Drobic et al., 2010).*

Consequently, the pre-initiation complex (PIC) is recruited to the promoter, transcription begins and the transcription cycle proceeds. In this way, the extracellular signaling pathways, via the activation of MSK1/2, leads to chromatin modification (rapid enrichment of H3S10ph or H3S28ph), a mechanistic regulation of IEG, known as mentioned previously, as the nucleosomal response (Dunn and Davie, 2005;Lim et al., 2004;Soloaga et al., 2003;Strelkov and Davie, 2002).

1.6.1.2 Examples of transcriptional reprograming of inducible genes by histone deacetylases inhibitors

The *p21* (*Cip1*/*Waf1* or *CDKN1A*) gene, which encodes for the protein product cyclin-dependent kinase inhibitor p21, mediates cell cycle arrest, differentiation or apoptosis, is considered as a model gene. Its transcription is directly upregulated in different cell types by different HDAC inhibitors, thus contributing to the antitumor effect of HDAC inhibitors. In parallel with transcriptional activation, a reorganization of chromatin, including histone hyperacetylation, takes place in both the proximal and distal promoter regions (Gui et al., 2004). *p21* is regulated by a variety of factors, including p53. HDAC inhibitor mediated transcriptional activation is independent of p53, and this can, consequently, occur in tumor cells lacking a functional p53. In a recent study researchers demonstrated that the nucleosomal response to the stimulation of the MAPK signaling pathway was required for *p21* induction by the HDAC inhibitor, TSA. As part of the nucleosomal response, histone H3 in the *p21* proximal promoter region was phosphorylated on Ser10 by MSK1. It was shown that this phosphorylation event was crucial to the acetylation of neighboring lysine 14. The phosphoacetylation mark was recognized by the 14-3-3 ζ protein, reader of phosphoserine marks, and was thus protected from removal by

protein phosphatase PP2A (Simboeck et al., 2010). Presumably, 14-3-3 also acts as a scaffold for the recruitment of chromatin remodeler, leading to initiation of transcription (Drobic et al., 2010). Additionally, treatment with the HDAC inhibitor depsipeptide, can induce *p21* expression by inducing acetylation of p53, protecting it from ubiquitination-induced degradation and allowing the recruitment of the KAT p300 to the p53-responsive *p21* promoter (Zhao et al., 2006). The *p21* gene can generate several alternate variants (Chen et al., 2011;Radhakrishnan et al., 2006). The impact of HDAC inhibitors on the genesis of these variants remains to be determined. Some HDAC inhibitors alter pre-mRNA splicing by changing the expression of splicing factors, which are components of the spliceosome. As an example, butyrate, but not TSA, increases the expression of splicing protein, SFRS2 (serine/arginine-rich splicing factor 2), a factor required for the expression of *p21* (Edmond et al., 2011).

The induction of the *c-Fos* and *c-Jun* IEGs following the activation of the MAPK pathway is also dependent on MSK-mediated phosphorylation of histone H3 in the UPR. However, the outcome of HDAC inhibition by TSA on these genes was opposite to that of on *p21* and, contrary to the common belief, that histone hyperacetylation is linked to transcription activation. Treatment with TSA resulted in rapid enhancement of H3 acetylation at the UPR of these genes, but transcription was inhibited (Hazzalin and Mahadevan, 2005). Furthermore, it was shown that continuous dynamic turnover of acetylation was characteristic of genes carrying the active methylation mark on H3K4, but not of genes carrying the repressive methylation mark on H3K9. The authors concluded that acetylation turnover rather than stably enhanced acetylation was crucial to the induction of the *c-Fos* and *c-Jun* genes (Hazzalin and Mahadevan, 2005). A similar cyclical process that entails alternating activating and repressive epigenetic events during the

hormone dependent activation of genes has been described (Metivier et al., 2003). Nonetheless, other scenarios are possible; for example, the transcription activation of *c-Fos* and *c-Jun* could require the deacetylation of a non-histone protein associated with their regulatory region. Investigators in several studies have suggested a role for deacetylation of transcription factors or other proteins in gene induction (Smith, 2008;Zupkovitz et al., 2006). A proposal for the role of HDACs in the basal transcription from the mouse mammary tumor virus (MMTV) promoter and some other TATA/Inr-containing core promoters is that deacetylation of protein components of the pre-initiation complex would allow the recruitment of RNAPII (Lee et al., 2011).

1.6.2 HDACs and regulation of RNA

1.6.2.1 Pre- mRNA splicing, microRNAs and HDACs

Several studies have reported a relationship among histone PTMs; transcription elongation and splicing regulation (will be discussed in sections 1.8 and 1.9). Alternative splicing of pre-mRNA gives rise to mature mRNA isoforms coding for functionally different proteins. This alternative splicing plays essential roles in differentiation and development as well as in diseases. Following the treatment of cells with the pan-HDAC inhibitor, sodium butyrate (5mM) for 15 h, splicing-sensitive exon-arrays detected the changes in the splicing patterns of approximately 700 genes, with many of these genes being involved in cell signaling, differentiation and cell cycle regulation (Hnilicova et al., 2011). Furthermore, studies show that HDAC1 and 2 are bound to proteins involved in pre-mRNA splicing. HDAC2 is associated with the spliceosome (Rappsilber et al., 2002;Zhou et al., 2002). However, the nature and function of HDAC complexes in splicing regulation is not well defined. It remains to be demonstrated whether HDAC1 and 2 associated with proteins involved in splicing, are in an unphosphorylated or phosphorylated state. Our

studies provided evidence that the unphosphorylated form of HDAC2 was recruited to the gene body of transcribed genes (Sun et al., 2007), suggesting that unmodified HDAC2 is bound to splicing factors.

HDAC inhibitors have both short- and long term impacts on pre-mRNA splicing. Short term impacts include changes in elongation rates affecting splicing site selection and altered activity of splicing factors, as a consequence of increased histone acetylation (Delcuve et al., 2013). In the longer term (24-48 h), the effects of HDAC inhibitors on altering the transcriptional activity of genes coding for splicing factors and/or the stability of splicing factors, may change the abundance of critical splicing factors and splicing events the factor are involved in. Serine/arginine-rich splicing factor 1 (SRSF1, also known as splicing factor 2/alternative splicing factor, SF2/ASF) levels impact the alternative splicing of *MCL1*, *BCL2L1 (Bcl-X)*, *CASP2* and *CASP9* (Anczukow et al., 2012;Moore et al., 2010). Reduction of SRSF1 levels results in G2 cell cycle arrest and apoptosis.

HDAC inhibitors also have a profound impact on the expression of microRNAs (miRNAs), altering the expression of about 40% of the expressed miRNAs (Scott et al., 2006). miRNAs are short, noncoding RNAs (ncRNAs) of about 23 nucleotides in length, that regulate gene expression at the post-transcriptional level by binding to the 3′ UTRs of target mRNAs, leading to their degradation or translation repression. Although the biogenesis of miRNAs is well understood, little is known of the regulation of miRNA expression, but there is increasing evidence that miRNA expression is widely misregulated in tumors, with tumor suppressor miRNAs targeting growth-inducing genes being down regulated and oncogenic miRNAs

targeting growth-inhibiting genes being up regulated (Sato et al., 2011). Similarly, misregulation of miRNA expression is characteristic of metastasis (Lujambio and Esteller, 2009). The expression of miRNAs is deregulated in cancer cells (Lopez-Camarillo et al., 2012). Pan-HDAC inhibitors, including SAHA, TSA and LAQ824, up- and down regulate the expression of miRNAs in colon carcinoma, lymphoma and breast cancer cells (Izzotti et al., 2012). In turn, the altered expression of the miRNAs impacts the expression of many proteins. One of the challenges in the miRNA field is to identify the mRNA targets and the resulting changes in protein translation. Novel bioinformatic programs, which use proteomic data following changes in miRNA expression, will be important tools in understanding the impact of miRNA levels on protein expression (Reczko et al., 2012).

The microprocessor complex processes primary transcripts into pre-miRNAs. This complex consists of Drosha (a RNase III enzyme), DGCR8 (a double-stranded RNA-binding protein), DEAD-box (Glu-Asp-Ala-Glu) helicases (DDX5 and DDX17) and HDAC1, 2 and 3 (Wada et al., 2012). Similar to pre-mRNA splicing, miRNA processing occurs co-transcriptionally at the site of transcription. The role of the HDACs, particularly HDAC1, was reported to increase the affinity of DGCR8 for pre-miRNAs by deacetylating acetylated DGCR8, leading to the increased expression of some miRNAs. These observations suggest that class I HDAC inhibitors will decrease the expression of some miRNAs.

1.6.2.2 RNA stability and HDACs

HDAC inhibitors can alter the stability of mRNA. Pan-HDAC inhibitors, TSA and butyrate, stabilized *CDKN1A* (*p21 or Cip1/Waf1*) mRNA, in HepG2, a liver hepatocellular carcinoma cell

line. RNA-binding proteins associating with the 3′ UTR of the *CDKN1A* mRNA are thought to be involved in HDAC inhibitor-mediated mRNA stabilization. Alternatively, the HDAC inhibitors may alter the expression of miRNAs that target the *CDKN1A* mRNA 3′ UTR (Hirsch et al., 2010). Further, studies have reported that HDACs are also involved in destabilization of mRNAs (Krishnan et al., 2010;Scott et al., 2008). For example, the expression of claudin-1 (a major constituent in tight junctions, frequently deregulated in colon cancers), is decreased in response to TSA or butyrate treatment, through the regulation of mRNA stability by its 3′ UTR (Krishnan et al., 2010).

1.7 Pre-mRNA splicing

Pre-mRNA splicing is a complex regulatory process that plays a major role in gene expression regime and proteome diversity. It is the co-transcriptional process of intron removal and exon joining, carried out by the spliceosome (Goldstrohm et al., 2001). Aberrant regulations of splicing mechanisms have been implicated in a number of diseases and cancers (Faustino and Cooper, 2003). As such, targeting splicing as a therapeutic intervention has emerged as a rapidly moving field of biomedical research.

1.7.1 Regulation of splicing

1.7.1.1 Regulatory elements of splicing

The splicing mechanism is highly complex. It requires an interaction network among the pre-mRNA, core regulatory elements, *cis*- and *trans*-regulatory elements, small ribonucleoproteins (snRNPs) and splicing factors (Black, 2003;Wahl et al., 2009). The core regulatory elements involved in splicing reaction include the 5′ and 3′ ends of introns (referred to as 5′ and 3′ splice

sites, SS, respectively), a branch point sequence located upstream of the 3′ SS, a poly-pyrimidine tract, [Y]n, [Y denotes a pyrimidine (U or C)] located between the branch point and 3′ SS. The 5′ SS, also known as the splice donor site, contains invariant GU nucleotide sequence and the 3′ SS, the splice acceptor, terminates the intron with the normally invariant AG nucleotide sequence. These elements are the interaction sites with different components of splicing machinery and participate in the biochemical splicing reactions. These elements are estimated to contain about half the information required for splicing (Lim and Burge, 2001). The remaining is largely derived from the *cis*-regulatory elements.

The *cis*-regulatory elements are classified into two groups, splicing enhancers and silencers, which play an important role in the recognition of the 5′ SS and 3′ SS regions (Douglas and Wood, 2011;Hernandez-Lopez and Graham, 2012). These elements function by recruiting sequence specific RNA binding factors that either activate or repress the usage of adjacent splice sites. Depending on the localization within the genome, splice enhancers and silencers are further sub-classified into exonic or intronic splice enhancers (ESE and ISE, respectively), and exonic or intronic splice silencers (ESS and ISS, respectively). Interplay between these elements is considered the major determinant of the splicing outcomes, providing the widespread means of regulation of alternative splicing.

The *trans*-elements of splicing include the multicomponent complex, referred to as spliceosome as well as other splicing accessory proteins like RNA helicases, SR (serine/ariginine rich) proteins, other RNA binding proteins, hnRNPs (heterogeneous nuclear ribonucleoproteins), that function in splicing regulation (Wahl et al., 2009). For example, multiple members of the

DEAD-box helicase family (e.g. DDX5 and DDX17) are thought to play a role in facilitating the unwinding of RNA duplexes within snRNPs and spliceosomes, thus control the molecular rearrangements that take place during the spliceosome cycle (Silverman et al., 2003). The members of the SR family proteins mostly promote splicing by participating in protein-protein interactions during spliceosome assembly, as well as play a key role in splice site selection, while the hnRNPs are usually considered as the splicing repressors (Wang and Burge, 2008).

1.7.1.2 The chemical reaction of splicing

Pre-mRNA splicing reaction proceeds via two sequential *trans*-esterification reactions (Black, 2003). In the first step, the 2′-hydroxyl group of the branch point adenosine attacks the phosphate at the 5′ SS. This causes the cleavage of phosphodiester bond at the 5′ SS and yields two intermediates: the 5′ exon with a free 3′ hydroxyl, and intron-3′ exon in a branched or lariat structure containing a 2′-5′ phosphodiester bond. In the second step, the free 3′ hydroxyl group of the first exon attacks the 5′ phosphate of a second exon at the 3′ SS leading to the cleavage at 3′ SS. As the intron is released, the exons are ligated via a 3′-5′ phosphodiester bond. This generates the two products of splicing reaction: the spliced mRNA (ligated exons) and the intron in lariat form (**Figure 1.11**).

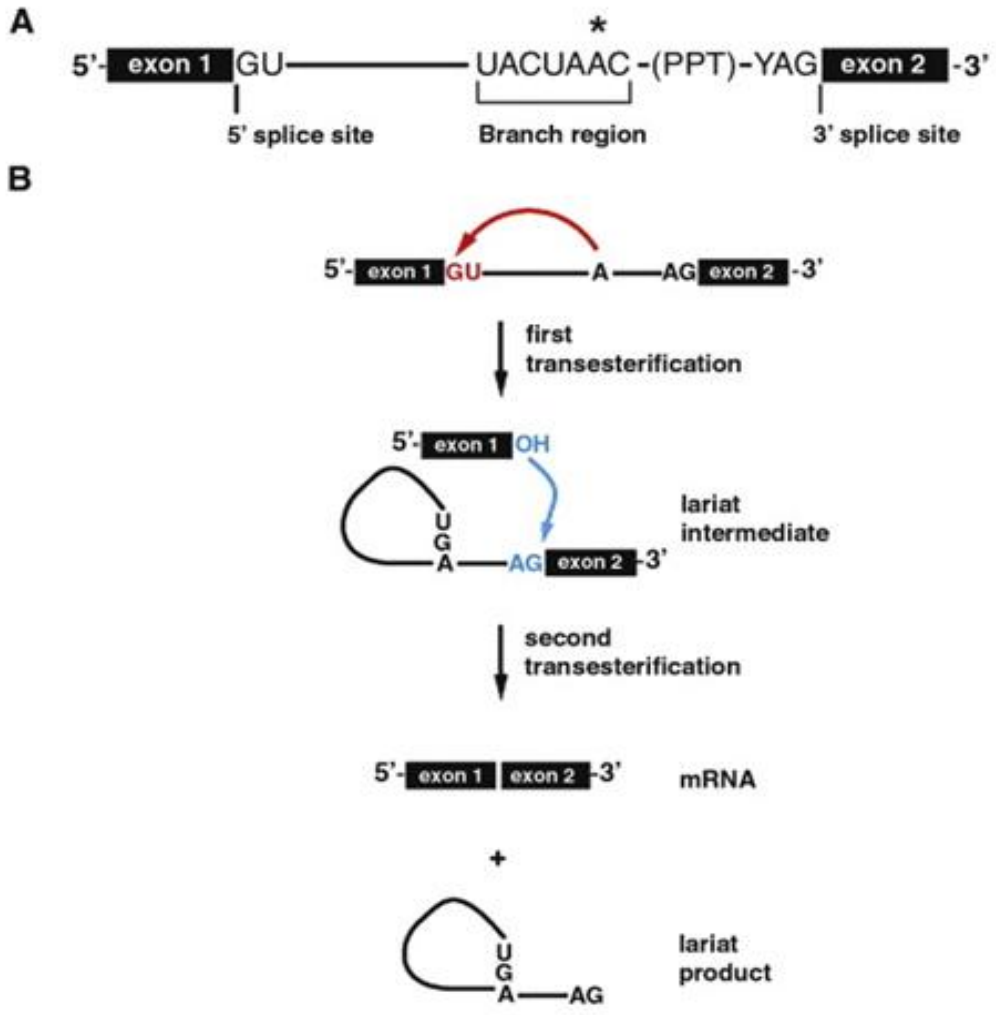

Figure 1.11: Splicing reaction
A. Intron structure highlighting conserved sequences at the 5′ and 3′ splice sites, the optimal branch sequence, and polypyrimidine tract. The preferred branch adenosine is indicated (*). B. Sequential *trans*-esterification reactions catalyzed by the spliceosome. *The figure and the text were reproduced with permission from Figure 1 (Ritchie et al., 2009).*

1.7.1.3 Major effector of splicing: the spliceosome

The splicing process is catalyzed by the spliceosome, which consists of uridine rich U1, U2, U4, U5, and U6 snRNPs and a large number (~100-200) of non-snRNP proteins (Valadkhan and Jaladat, 2010) (**Figure 1.12**). Each snRNP contains a small nuclear RNA (snRNA) and several proteins. The spliceosome is highly dynamic in nature and undergoes a large number of changes

in composition or conformation during the splicing reactions (Brow, 2002;Matlin and Moore, 2007). Spliceosomal assembly is initiated by the formation of the E complex, in which the U1 snRNP and heterodimeric U2AF (U2 snRNP auxiliary factor) recognize the 5′ and 3′ SS, respectively, and the branch point binding protein (BBP) SF1 (splicing factor 1) binds to the branch site, in an ATP-independent manner (Black, 2003;Graveley, 2000;Wahl et al., 2009). Recruitment of the U2 snRNP to the branch point (in an ATP-dependent manner), results in the formation of the A complex. Subsequent recruitment of the U4/U6.U5 tri-snRNP forms the B complex, which is catalytically inactive. This process also requires the involvement of some SR proteins, such as SRSF1 and SC35. A series of structural and compositional rearrangements, such as dissociation of U1 and U4 snRNPs from the spliceosome, gives rise to the activated spliceosome (**Figure 1.12**). The activated spliceosome carries out the first catalytic step of splicing and generates the C complex. Additional structural rearrangements occur prior to the second catalytic step of splicing. After the second step, the spliceosome is disassembled with the release of U2, U5 and U6 snRNPs, which are to be recycled for additional rounds of splicing with the generation of the splicing products, the spliced mRNA and the lariat intron (Wahl et al., 2009).

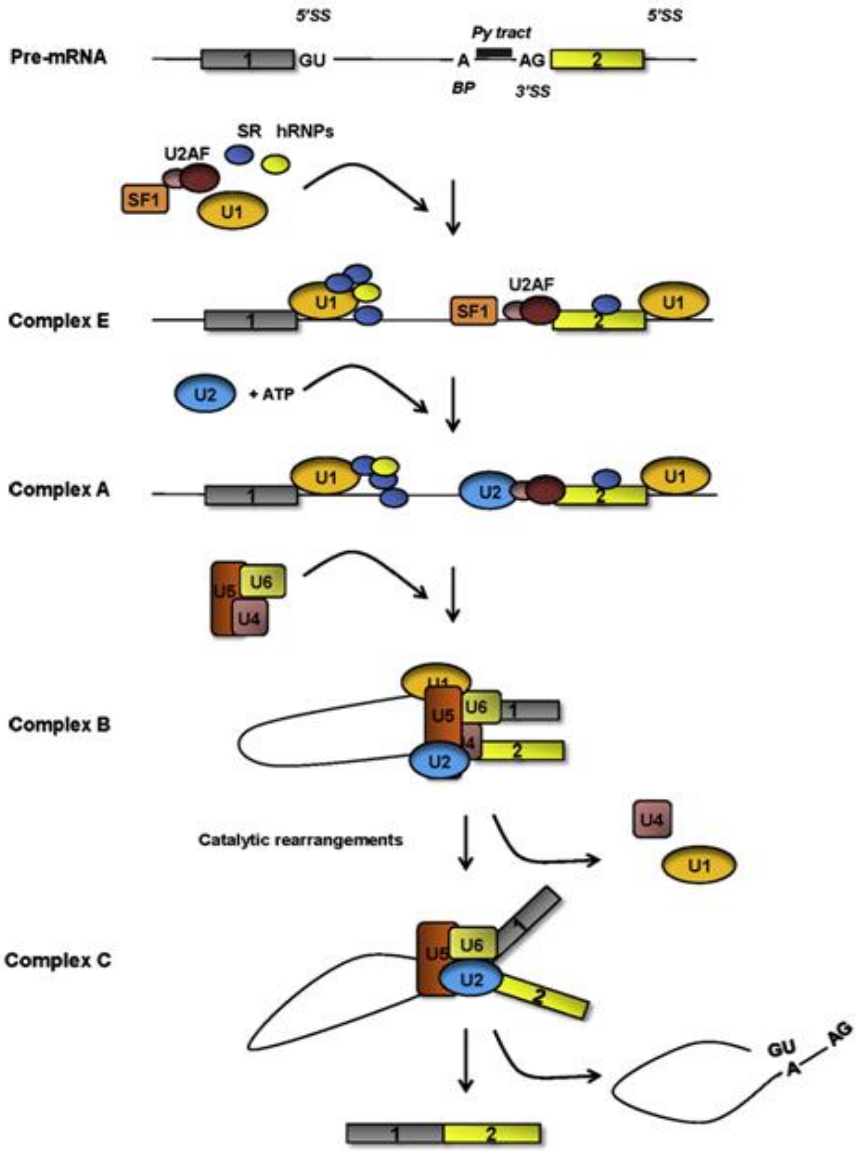

Figure 1.12: Schematic representation of the spliceosome assembly and the splicing of a pre-mRNA

The spliceosome is composed of a core of five small ribonucleoprotein particles (snRNPs), U1, U2, U4, U5, and U6, and ~200 additional proteins. The first step in spliceosome assembly is the formation of complex E (the commitment complex). The 5′ splice site (GU, 5′ SS) is bound by the U1 snRNP, and the splicing factors SF1 and U2AF cooperatively recognize the branch point sequence (BP), the Py, and the 3′ splice site (AG, 3′ SS). In an ATP-dependent manner, the pairing of the U2 snRNP with the branch point results in the pre-spliceosomal complex A. Subsequent steps lead to the binding of the U4/U6.U5 tri-snRNP and the formation of the complex B. The catalytic complex C, which performs two *trans*-esterification reactions at the splice sites, is formed after rearrangements that detach the U1 and U4 snRNPs. These reactions result in the ligation of the exons and the excision of the intron, which is removed as the lariat RNA. *The figure and the text were reproduced with permission from Figure 1 (Montes et al., 2012).*

1.7.2 Types of pre-mRNA splicing: constitutive and alternative splicing

Splicing reactions are of two types: constitutive and alternative splicing. Constitutive splicing involves the removal of all pre-mRNA introns and joining together of every exons of a gene to generate a mature RNA. However, most genes in higher eukaryotes contain more than one intron or exon, which provides the possibility to remove various combinations of introns or exons from a single pre-mRNA through alternative splicing (Hastings and Krainer, 2001) (**Figure 1.13**).

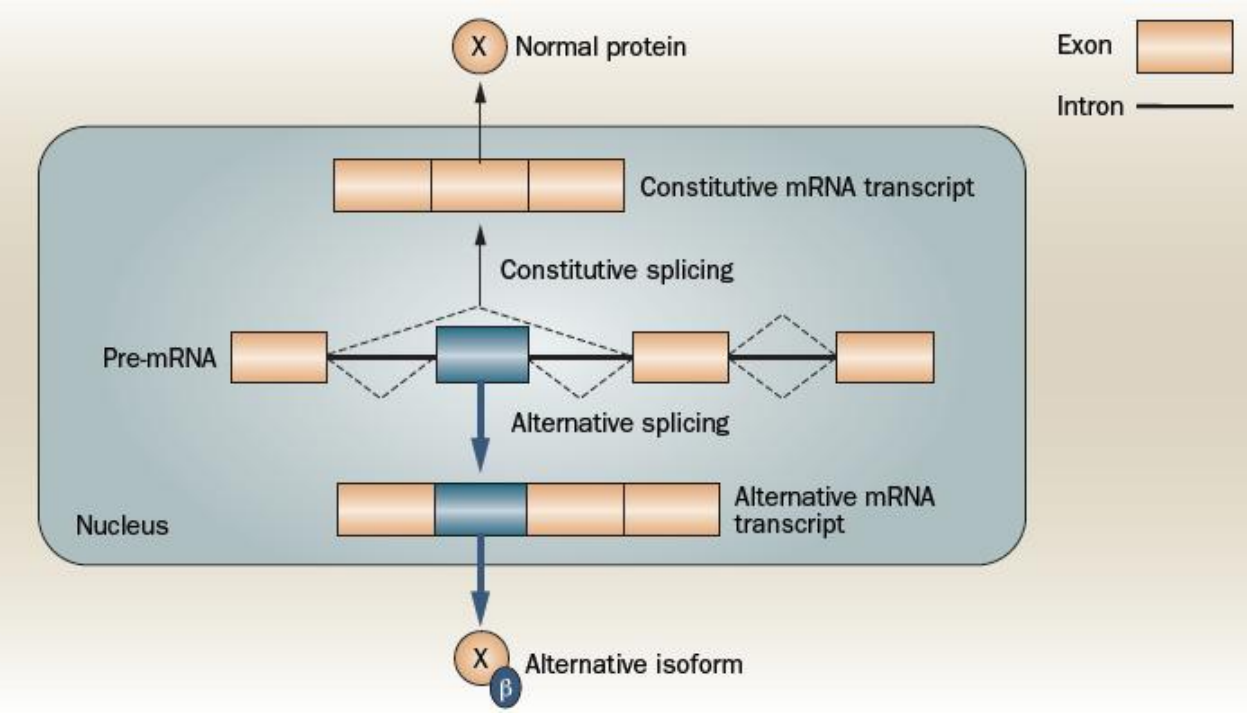

Figure 1.13: Types of pre-mRNA splicing
During pre-mRNA splicing, introns are removed and exons ligated together to produce a spliced mRNA transcript, which is exported to the cytoplasm for translation into a protein, X. Alternative pre-mRNA splicing involves rearrangement of the exons and introns to generate an alternative mRNA transcript, which is translated into a different protein isoform, Xβ. The alternative splicing event shown here is inclusion of a cassette exon (shown in blue). Splicing events are represented by broken lines linking exons. Abbreviation: mRNA, messenger RNA. *The figure and the text were reproduced with permission from Figure 1 (Rajan et al., 2009).*

Alternative splicing is a widespread mean of generating proteome diversity as well as a versatile mechanism of regulation of gene expression. Based on unbiased transcriptome analysis, it is estimated that up to 95% of genes undergo alternative splicing (Pan et al., 2008;Wang et al., 2008). Many genes are expressed as multiple splice variants ranging in numbers from two to several thousands, that encode proteins with diverse and antagonistic biological functions (Black, 2000;Modrek et al., 2001). Alternative splicing events mostly affect the coding sequences by altering the reading frames or by non-sense mediated decay of the mRNA products (Thanaraj et al., 2003).

From a single gene, multiple isoforms of functional variability can be generated through alternative splicing, via one or more mechanisms: exon skipping or exon inclusion, intron retention, alternative splice site selection, alternative promoter usage, and alternative polyadenylation events (McManus and Graveley, 2011) (**Figure 1.14**). However, the splicing events can also contribute to the proteome complexity and stability through coupling with other post-transcriptional mechanisms such as non-sense mediated mRNA decay or miRNA induced mRNA degradation (Chang et al., 2007;Valencia-Sanchez et al., 2006). To ensure the specific splicing outcomes occur accurately and efficiently, the involvement of core splicing signals at the 5′ and 3′ SSs and at the branch point, as well as other splicing regulatory elements, are pivotal (Matlin et al., 2005;Wang and Burge, 2008). As a result, alternative splicing requires a complex interplay between splicing factors (positive or negative) that function through the cognate enhancers and silencers (Matlin et al., 2005). Furthermore, alternative splicing is often regulated in a cell type and developmental stage specific manner, determined by the presence of particular combinations of splicing regulators in time and space (Wang et al., 2008).

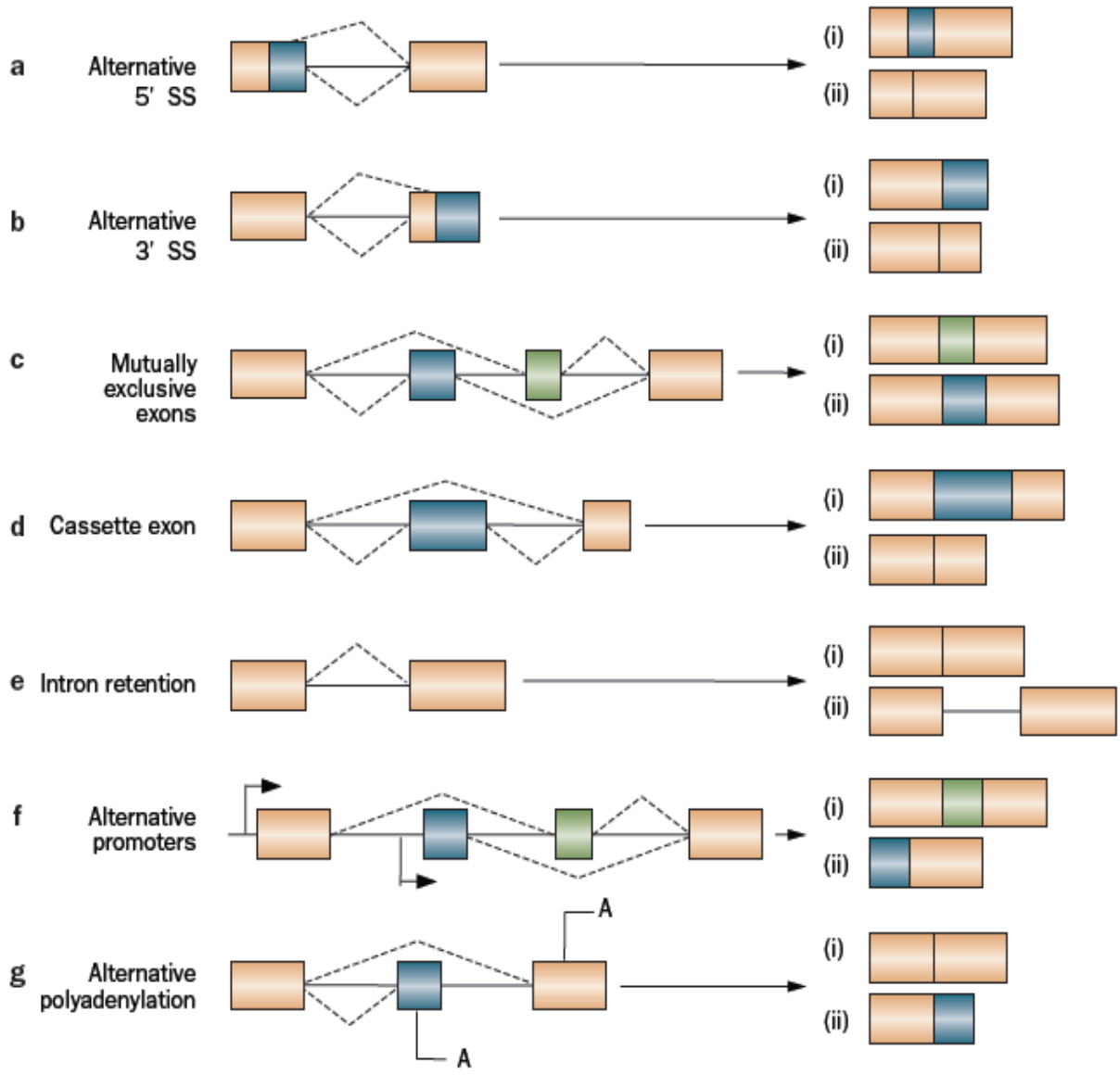

Figure 1.14: Alternative splicing events

Binary outcomes (i) or (ii) of elementary alternative splicing events. Competing 5′ a. and 3′ b. SS represent exon-modification events. c. A mutually exclusive exon is selected from two or more exons in the pre-mRNA. d. Discrete cassette exons are independently included or excluded, and can be subdivided into 'skipped' or 'cryptic' types depending on whether the constitutive event includes or excludes the exon, respectively. e. Failure to recognize intron-flanking SS results in intron retention. Alternative splicing can occur in conjunction with f. alternative promoters or g. polyadenylation signals. Splicing events are represented by broken lines linking exons. Abbreviations: A, polyadenylation site, SS, splice site. *The figure and the text were reproduced with permission from Figure 2 (Rajan et al., 2009).*

1.7.3 Role of SR proteins in splicing regulation

Serine/arginine-rich (SR) proteins constitute a highly conserved and structurally related family of pre-mRNA splicing factors. Apart from their well-defined roles in constitutive and alternative splicing, these proteins have been implicated in a number of cellular processes including transcription elongation, genomic stability, chromatin binding, mRNA stability, mRNA export, mRNA translation, and many more (Lin et al., 2008;Loomis et al., 2009;Huang and Steitz, 2005;Michlewski et al., 2008;Sanford et al., 2004;Zhang and Krainer, 2004). It is conceivable that deregulation in any of these functions can lead to diseases and cancers. Therefore, studying SR protein can be considered as a gateway to understand a wide range of cellular activities in gene expression regulation.

1.7.3.1 General features of SR proteins

SR proteins are predominantly localized in nuclear speckles, which act as a storage and/or assembly sites for many splicing and transcription components (Mintz and Spector, 2000;Shopland and Lawrence, 2000;Sleeman and Lamond, 1999). The SR proteins are distinct from the other RNA binding proteins for some unique structural features. These proteins contain the signature 'RS domain' (Arg/Ser-rich domain), a protein domain composed of several repeats of the arginine-serine dipeptide at the C-terminal, and one or two RRM (RNA recognition motif) at the N-terminal that provides the RNA binding specificity (Kohtz et al., 1994;Wu and Maniatis, 1993). With the RS domain, SR proteins interact with a number of other RS domain containing proteins that facilitate the recruitment of spliceosome. RS domain can also directly interact with pre-mRNA *via* the branch point and 5′ SS, providing another way to facilitate spliceosomal assembly (Shen et al., 2004;Shen and Green, 2004). Further, RS domains can function as nuclear

localization signals (NLS), thereby targeting the SR proteins primarily in speckles (Caceres et al., 1997;Hedley et al., 1995).

During the course of their discovery, several names have been given to the SR proteins. Recently a new nomenclature has been designated for 'core' SR protein family members, which includes twelve well characterized SR proteins (Manley and Krainer, 2010). According to the new nomenclature that has started to be adopted by the scientific community, the prototype member of SR family, SF2/ASF is now denoted as SRSF1, and other SR proteins such as SC35 and SRp20 are denoted as SRSF2 and SRSF3, respectively (**Figure 1.15**).

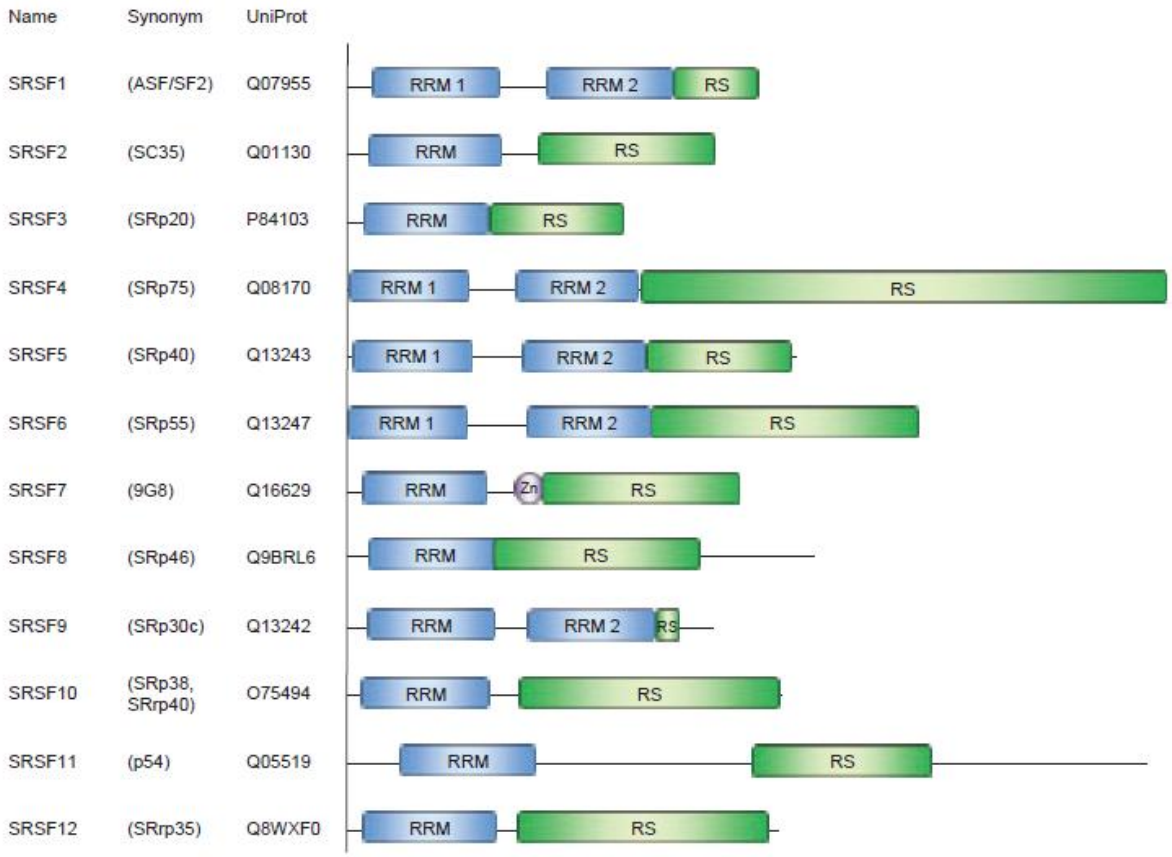

Figure 1.15: SR proteins
Schematic representation of the 12 human SR proteins as defined by Manley and Krainer (Manley and Krainer, 2010). Zn, zinc finger. *The figure and the text were reproduced with permission from Figure 1 (Twyffels et al., 2011).*

The relative abundance of SR proteins that vary among different cell types can modulate the splicing outcomes depending on the cellular contexts (Hanamura et al., 1998). For example, overexpression of SRSF1 has been reported in various tumors. The upregulation of SRSF1 resulted in unusual accumulation of alternatively spliced transcripts including the oncogenic isoform 2 of ribosomal protein S6 kinase (S6K1) (Karni et al., 2007). Conversely, several auto regulatory mechanisms acting at the post-transcriptional level can maintain homeostatic levels of SR proteins. SRSF1 can be down regulated at the post-transcriptional level through the binding of miRNA at its 3′ UTR, thereby can change the splicing outcomes (Meseguer et al., 2011;Wu et al., 2010).

1.7.3.2 SR proteins and constitutive splicing regulation

SR proteins, SRSF1 and SRSF2, the founding members of the protein family, were first identified for their essential roles in constitutive splicing (Fu and Maniatis, 1990;Ge et al., 1991;Krainer et al., 1991). SR proteins appear to function redundantly to regulate constitutive splicing mechanisms as demonstrated by the ability of any individual SR protein to complement a splicing deficient cytosolic HeLa S100 extract (contains all spilceosomal components except SR proteins) (Krainer et al., 1990). Since their discovery, the activities of these proteins in different steps of the constitutive splicing mechanisms have been well documented. For example, SR proteins promote the assembly of the earliest detectable pre-spliceosomal complex E by favoring the recruitment and the stabilization of the U1 snRNP to the 5′ SS and U2AF65 to the polypyrimidine tract (Staknis and Reed, 1994). These activities, most likely, involve interactions between the RS domains contained in each of these proteins. SR proteins are also required for the transition of the pre-spliceome complex A to spliceosome complex B, as their depletion

inhibit the spliceosome assembly (Roscigno and Garcia-Blanco, 1995). Moreover, in the pre- and mature spliceosomes, SR proteins help to establish the communication between the 5′ and 3′ SS with the concurrent binding of U1 snRNP and U2 snRNP at the 5′ and 3′ SSs, respectively (Cho et al. 2011; Fu and Maniatis 1992; Kohtz et al. 1994; Roscigno and GarciaBlanco 1995). In addition, they are involved in the later stages of spliceosome assembly by modulating the recruitment of the U4/U6.U5 tri-snRNP to the pre-mRNA (Roscigno and Garcia-Blanco, 1995). Although the mechanism is not well understood, the RS domain-containing proteins present in the U4/U6.U5 tri-snRNP suggest that protein-protein interaction network among the RS domains of the different partners may be involved (Hastings and Krainer, 2001).

1.7.3.3 SR proteins and alternative splicing regulation

SR proteins are well characterized for controlling alternative splicing regulation, which primarily relies on their ability to interact with RNA regulatory sequences. The widely known functions are to promote splice site selection by binding to ESEs, recruiting the spliceosomal proteins and stabilizing protein-RNA interactions (Long and Caceres, 2009). ESEs are usually bound by one or multiple members of SR protein family (Black, 2003;Blencowe, 2000;Graveley, 2000). Binding of SR proteins facilitate the recruitment of spliceosome complex to adjacent introns. These proteins need to be phosphorylated for efficient splice site recognition and to be de-phosphorylated for splicing catalysis (Mermoud et al., 1992;Mermoud et al., 1994).

One model for splicing activation by SR proteins proposed that the ESE bound SR protein can interact with another splicing protein with the RS domain, such as U1snRNP and U2AF65, and thus recruit them at the 5′ or 3′ SS, a process known as exon definition (Graveley, 2000;Ibrahim

et al., 2005). An alternative model suggested that RS domains of SR proteins interact with pre-mRNA within the functional spliceosome complex. Irrespective of the models of RS domain activation mode, SR proteins promote the recruitment of spliceosome complexes to the splice sites (Shen et al., 2004;Shen and Green, 2004). Thus, SR proteins bound to ESE act as general activators of exon definition. Studies have shown that splicing activation is also dependent on the number of SR proteins bound to ESEs as well as the number of Arg-Ser repeats within the RS domains of SR proteins, which apparently determine the activation potential of these proteins (Graveley et al., 1998).

In some instances, SR proteins binding within the intronic sequences, can act as negative regulator of splicing. This is best exemplified during adenovirus infection, where the binding of SRSF1 to an intronic repressor element in the adenovirus pre-mRNA, located upstream of 3′ SS branch point sequence, represses the splicing (Kanopka et al., 1996). SRSF1 bound to the repressor element, prevents the recruitment of U2snRNP as well as the use of 3′ SS. Other studies provided further evidence that SR proteins bound to intron sequences can affect the assembly of spliceosome complex, and thereby repress splicing mechanisms (Ibrahim et al., 2005). Depending on how they interact with pre-mRNA, SR proteins can function differently. SR protein binding to an exon promotes its inclusion, but its binding to an intron has the opposite effect (Dembowski et al., 2012;Erkelenz et al., 2013).

A major challenge to deciphering the detailed mechanisms of the splicing outcomes regulated by SR proteins is how these proteins mediate the protein-protein or protein-RNA interaction network in the spliceosome complexes. It is possible to predict the potential binding sites for SR

proteins using genome wide *in vivo* CLIP-seq (UV cross-linking and immunoprecipitation followed by high-throughput sequencing) analysis or *in vitro* binding assays for consensus motifs (Sanford et al., 2009;Anko et al., 2012;Cartegni et al., 2003). However, the interaction of SR proteins with RNA can be influenced by a number of factors, including pre-mRNA secondary structure, competition with other SR or RNA binding proteins, small nucleolar RNAs (snoRNAs) (Kishore and Stamm, 2006;Lin and Fu, 2007). Therefore, the RNA binding specificity of SR proteins, with respect to their binding landscapes in transcriptome, requires further detailed investigations for better understanding of splicing regulatory mechanism.

1.8 Epigenetic control of alternative splicing

In recent years, substantial evidences have been accumulated suggesting a major role of epigenetic mechanisms in the alternative splicing regulation. Some of the key players of this regulatory network involve: (1) nucleosome occupancy or positioning and exon-intron architecture, (2) DNA methylation, (3) PTMs of histones, (4) chromatin-splicing adaptor complexes, and (5) chromatin remodeling complexes. Nucleosomes and five methyl cytosine (5meC) are non-uniformly distributed along the body of transcribed genes. Nucleosomes and 5meC are enriched on exons relative to introns, and nucleosomes exhibit preferential positioning at exon-intron and intron-exon boundaries (Chodavarapu et al., 2010;Hodges et al., 2009;Shukla and Oberdoerffer, 2012;Dhami et al., 2010;Schwartz et al., 2009;Spies et al., 2009;Tilgner et al., 2009). The phenomenon of nucleosomal positioning is evolutionary conserved and independent of transcription, GC content or DNA sequences (Andersson et al., 2009;Nahkuri et al., 2009;Schwartz et al., 2009). However, alternatively spliced exons are relatively less enriched in nucleosomes in comparison to the constitutive exons, correlating nucleosome positioning with

alternative splicing outcomes (Schwartz et al., 2009). Nucleosomes are suggested to function as 'speed bumps' to impose barrier to RNAPII elongation and thereby affects splicing, which supports the kinetic model of splicing regulation (Carrillo et al., 2011;Schwartz and Ast, 2010).

In addition to nucleosomes, genome wide mapping of histone PTMs reveal a non-random distribution pattern with relative enrichment of some specific modifications in exons, attributing their role in splicing (Andersson et al., 2009;Kolasinska-Zwierz et al., 2009;Schwartz et al., 2009). Trimethylation of H3K36 (H3K36me3), monomethylation of H3K79, H4K20 and H2BK5 are enriched on exons (Andersson et al., 2009;Dhami et al., 2010;Schwartz et al., 2009). Some of these modifications have been linked to the splicing regulation via change in chromatin configuration and RNAPII elongation behavior. Histone PTMs can also interact with the splicing factors such as SRSF1 and splicing machinery with the help of chromatin-splicing adaptor complexes which act as a scaffold, providing another mechanism of splicing regulation (Luco and Misteli, 2011;Luco et al., 2011). Furthermore, chromatin remodeling complexes, such as SWI/SNF and CHD1 (chromodomain helicase DNA binding protein 1), have been reported to modulate splicing with consequences in splicing efficacy and spliceosome assembly, as well as by regulating the rate of RNAPII elongation (Luco and Misteli, 2011;Luco et al., 2011). These complexes interact with several members of spliceosome complex and splicing proteins and are recruited by specific histone PTMs. For example, H3K4me3 can create a binding site for CHD1 which associates with snRNPs, and facilitates their recruitment (Sims, III et al., 2007). These studies illustrate the interplay between epigenetic mechanisms and splicing regulation.

The MED23 subunit of Mediator interacts with hnRNP L. Mediator is a multiprotein complex that acts as an integrator of various signaling pathways and is a critical component of the RNAPII transcription initiation apparatus (Conaway and Conaway, 2011;Spaeth et al., 2011). MED23 interacts with the splicing machinery and regulates alternative splicing of several hnRNP L targets (Huang et al., 2012).

There is emerging evidence that pre-mRNA splicing itself influences chromatin organization (Kim et al., 2011), providing a means of two-way communication between chromatin organization and the splicing regulation. Studies show that splicing mechanisms are required to establish and to maintain epigenetic marks such as H3K36me3 (de Almeida et al., 2011;Kim et al., 2011). H3K36me3 is considered as a major mark of exons and transcriptional activation (Edmunds et al., 2008;Luco et al., 2010). Genome wide mapping with high throughput sequencing reveals the increased accumulation of H3K36me3 mark toward the 3′ end of genes and in intron-containing genes, the enrichment of H3K36me3 mark is higher than intronless genes (Barski et al., 2007;de Almeida et al., 2011;Kolasinska-Zwierz et al., 2009;Schwartz et al., 2009) This observed phenomenon is irrespective of gene sizes, transcription activities and nucleosomal occupancies. Pharmacological inhibition of splicing or siRNA-mediated knockdown of splicing factor (e.g. SAP130) leads to the reduced level of H3K36me3 with consequences of reduced recruitment of histone methyltransferase, HYPB/SetD2 (KMT3A) (de Almeida et al., 2011). However, activation of splicing or change in the splicing patterns (e.g. shift from exon skipping to inclusion), exhibits opposing effects. Furthermore, blockage of splicing by mutating the SS (e.g. deletion of 3′ SS) or by use of splicing inhibitor such as spliceostatin A cause the relative shift of H3K36me3 mark, which is a generalized observation

on thousands of genes containing introns, but not on intronless genes (Kim et al., 2011). Tom Misteli and colleagues reported a connection between H3K36me3 and pre-mRNA splicing (Luco et al., 2010). MRG15, which binds to the polypyrimidine tract-binding protein, binds to H3K36me3. SetD2 (KMT3A), the enzyme catalyzing H3K36me3, binds to the elongating, phosphorylated RNAPII (Sun et al., 2005). Knocking down SetD2 did not impact elongation but altered pre-mRNA splicing (Edmunds et al., 2008;Luco et al., 2010). MRG15 can form complexes with HDAC2 and KAT5, but whether either of these enzymes are co-loaded onto the body of transcribed genes remains to be shown (Doyon et al., 2004). The splicing inhibitor, meayamycin, reduced H3K36me3 levels without altering elongation rates or chromatin-associated RNA (de Almeida et al., 2011).

However, in regulation of alternative splicing, the effect of H3K36me3 is not consistent. For neural cell adhesion molecule (*NCAM*) and fibroblast growth factor receptor 2 (*FGFR2*) genes, increased accumulation of H3K36me3 has been correlated with exon skipping (Luco et al., 2010). However, in *CD45* and *YPEL5* genes, it is reported that H3K36me3 has no role in regulating the splicing outcomes, rather this histone PTM is involved in exon definition (Huff et al., 2010). Reduced levels of H3K36me3 are also observed in alternative exons of these genes than in constitutive ones. This is in contrast with a recent report that alternative exons have higher levels of H3K36me3 relative to constitutive exons (for example, *CD44* gene) (de Almeida et al., 2011). However, this may not be a pre-requisite or a generalized mechanism for all genes. Depending on cellular contexts or particular splicing events, H3K36me3 may exhibits increased or decreased levels in alternative exons. All of these studies point towards the fact that deposition and maintenance of H3K36me3 is dependent on splicing and localized changes in this epigenetic

mark can modulate the splicing activity. However, the detailed mechanism of regulation still requires further validation.

HP1γ (heterochromatin protein 1) is recruited to the coding region of transcribed genes by the elongating form of RNAPII (Kwon and Workman, 2011a; Kwon and Workman, 2011b). HP1γ recruits the histone chaperone complex FACT and has a similar distribution as H3K36me3 (Kwon et al., 2010;Kwon and Workman, 2011b). A recent study demonstrated that H3K9me3 and HP1γ, which binds to H3K9me3, was present at greater levels on the variant exons of the *CD44* gene (Saint-Andre et al., 2011). HP1γ played a role in the alternative splicing of *CD44* pre-mRNA by slowing down the elongating RNAPII, allowing the inclusion of the variant exons (**Figure 1.16**). Interestingly HP1γ was also bound to the *CD44* pre-mRNA in the variant region, thus linking the pre-mRNA to the *CD44* chromatin (Saint-Andre et al., 2011).

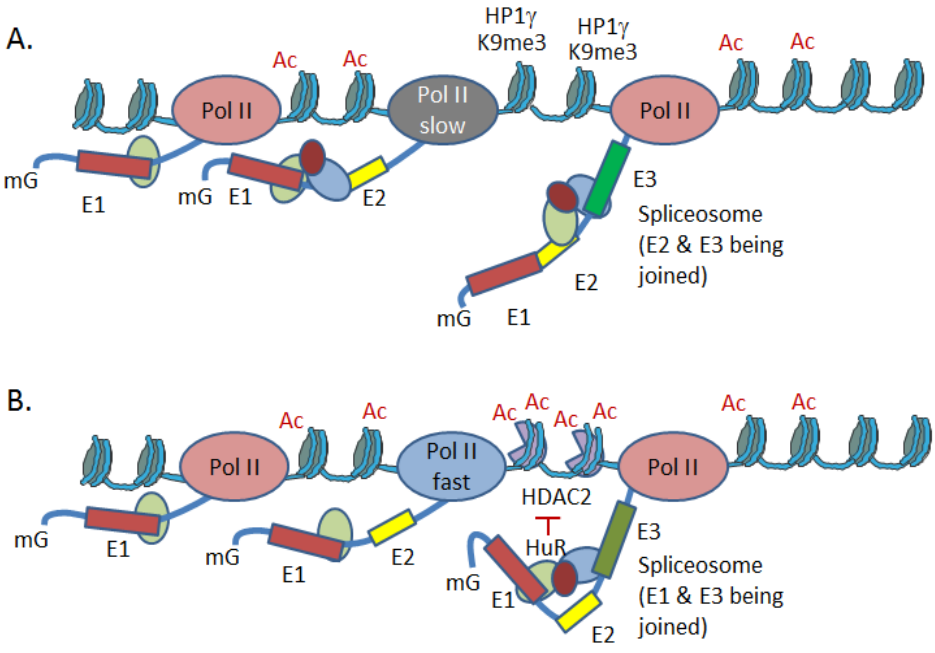

Figure 1.16: Histone PTMs alter nucleosome structure facilitating or hindering elongation
A. Nucleosomes with H3K9me3 recruit HP1γ. HP1γ retards the movement of RNAPII, allowing splicing events for the inclusion of variant exon (E2) to take place. B. HuR inhibits HDAC2

resulting in increased acetylation of nucleosomes. The highly acetylated (Ac) nucleosome will stay in the atypical form established by elongation. The atypical nucleosome will facilitate passage of RNAP II, preventing the formation of spliceosome and the inclusion of alternate exons. In this situation exon 1 (E1) and exon 3 (E3) are spliced together, while alternate exon 2 (E2) is excluded. *The figure and the text were adapted from Figure 1 (Khan et al., 2012).*

There is also a growing list of readers that bridge a gap between pre-mRNA splicing components and histone PTMs along the body of the transcribed gene (Luco et al., 2011). SRSF1 potentially links U1-70K snRNP, involved in early spliceosome assembly, to histone H3 (Hnilicova et al., 2011;Luco et al., 2011).

1.9 Histone deacetylases: modulator of alternative splicing

A role for histone acetylation regulated by KATs and HDACs, in RNA splicing and processing is currently gaining momentum, which adds an additional regulatory layer to splicing mechanisms. As such, KATs and HDACs are identified in spliceosome complexes (Rappsilber et al., 2002;Zhou et al., 2002). As mentioned previously, the DEAD-box RNA helicases, for example DDX5 and DDX17, that have been shown to be involved in the splicing of pre-mRNAs, are also associated with class I HDACs, HDAC1, 2 and 3 (Mooney et al., 2010;Wilson et al., 2004). Also, an *in vitro* study revealed that KATs and HDACs are required for spliceosomal rearrangements and assembly (Kuhn et al., 2009). These reports indicate that HDACs must have a key role in splicing.

Multiple studies have demonstrated the association of histone acetylation with alternative splicing regulation (Gunderson et al., 2011;Hnilicova et al., 2011;Zhou et al., 2011;Kuhn et al., 2009). A recent study by Zhou et al. has suggested that a family of RNA binding protein, HuR proteins, can enhance the localized histone acetylation in regions at the alternative exons

of *NF1* and *FAS* genes, involving the functional interaction with the activity of histone deacetylases, in particular, HDAC2 (Zhou et al., 2011). From a mechanistic point of view, HuR proteins are co-transcriptionally recruited to the target genes, directly interact with HDAC2 and inhibit its activity. This localized change in chromatin structure leads to the increase in elongation rate and decreases inclusion of alternative exons in mature mRNA (**Figure 1.16**). During transcription the nucleosome structure is perturbed, forming atypical structures (Czarnota et al., 1997;Locklear et al., 1990). Typically the cysteine residue at position 110 of histone H3 is buried in the interior of the nucleosome. However, the transcribed unfolded nucleosome has its H3 cysteine exposed, offering a tag to isolate and study transcribed nucleosomes (Chen-Cleland et al., 1993;Sun et al., 2002b). The atypical nucleosome requires elongation to expose the nucleosome's cysteinyl-thiol, and histone acetylation will maintain this unfolded structure (Walia et al., 1998). It is conceivable that through histone acetylation maintaining the unfolded nucleosome structure, subsequent rounds of transcription elongation are facilitated (**Figure 1.16**).

Towards understanding of the mechanism as to how changes in histone PTMs alter the splicing of several genes, the current literature supports the kinetic model of transcriptional elongation or processivity, where a change in RNAPII elongation rate acts as a sensor to decide the alternative splicing outcomes. For genes with alternative exons that have weak SSs, a slowly moving RNAPII results in the inclusion of the exons in the RNA. For example, depolarization of neuronal cell membrane induced enhanced acetylation of H3K9 surrounding the exon 18 (E18) of *NCAM* gene (Schor et al., 2009). However, the increased acetylation status was not detectable in the promoter region of the gene and was localized only in intragenic gene body region. This

relaxed chromatin structure leads to an increased rate of RNAPII elongation, preventing the inclusion of this exon in mature mRNA (**Figure 1.16**). Furthermore, removal of depolarization signal can reverse the effect of acetylation and splicing outcomes. Also, the physiological response of membrane depolarization can be functionally mimicked by HDAC inhibitor, TSA treatment, with E18 exon skipping as well as open chromatin structure (Schor et al., 2009). This phenomenon is also observed for other genes such as fibronectin (Hnilicova et al., 2011). HDAC inhibitor mediated alternative splicing of this gene (exon 25 or EDB) has been attributed with increased RNAPII processivity, which is correlated with increased histone H4 acetylation as well as with reduced association of one of the major splicing regulatory protein, SRSF5, along the alternative exon of the gene. In addition, siRNA-mediated down regulation of HDAC1, but not HDAC2, had similar effect as HDAC inhibitor in the splicing pattern change of this gene, suggesting HDAC1 is primarily involved in the splicing regulation of this gene (Hnilicova et al., 2011). However, it is still not clearly understood how RNAPII slows down at some exons and whether variation in RNAPII kinetics at the alternatively spliced exons occurs at all genes or only in a subset of genes. One possibility is the change in the dynamics of phosphorylation status of RNAPII CTD from elongating Ser2 phosphorylation to Ser5 phosphorylation form, facilitates RNAPII slow down by promoting recruitment of specific phosphatases or kinases (Munoz et al., 2010). However, it is also possible that a change in the phosphorylation cycle of RNAPII may not be the major player. Combinatorial effect of chromatin structure, histone PTMs and properties of template DNA sequences, which can act as recruiters for other proteins, may play key roles in determining the elongation rate, which needs to be studied in further detail. Furthermore, it is yet to be demonstrated whether change in RNAPII kinetics along a gene is a generalized mechanism to determine the splicing outcome in response to HDAC inhibitor

treatment. In support of this, it has been shown recently in yeast (*Saccharomyces cerevisiae*, intron-containing genes, *DBP2* and *ECM33*), that the genetic deletion of multiple histone deacetylases Hos3 and Hos2, but not the single deletion of HDAC (either Hos2 or Hos3), caused an enhanced acetylation of H3K9 and H3K14 throughout the body of the genes, with a very slight change in the distribution pattern of RNAPII (Gunderson et al., 2011). However, an aberrant regulation in the co-transcriptional spliceosome complex assembly is observed. Due to the small change in RNAPII occupancy in *DBP2* and *ECM33* genes with HDACs deletion, it is unlikely that changes in RNAPII elongation can affect the assembly of spliceosome complex. Presumably, histone acetylation dynamics influences the dynamics of co-transcriptional spliceosome assembly and can affect splicing without altering the transcriptional elongation rate in yeast, indicating a direct role of chromatin structure in spliceosome complex recruitment.

1.10 *Myeloid cell leukemia-1* (*MCL1*): Model gene to study alternative splicing regulation

Apoptosis or programmed cell death plays important roles in the development and maintenance of tissue homeostasis and in the pathogenesis of many diseases and cancers. A large number of apoptotic factors have been shown to be regulated by alternative splicing mechanisms, including the Bcl-2 (B-cell lymphoma 2) protein family (Akgul et al., 2004). Bcl-2 family members are important mediators of cell fate decisions. Alternative splicing is one of the major mechanisms that generate the proteomic complexity and functional diversity of the Bcl-2 family members. Bcl-2 family proteins are characterized by the presence of BH (Bcl-2 homology) domains and, include anti-apoptotic and pro-apoptotic members (Akgul et al., 2004). The balance between relative levels of these antagonistic proteins is critical for cell fate (Youle and Strasser, 2008). The pro-apoptotic Bcl-2 proteins are further divided into two subgroups: one group containing

proteins with multiple BH domains (BH1, BH2, BH3) such as Bak (Bcl-2 homologous antagonist killer) and Bax (Bcl-2-associated X protein) and the second group containing proteins including Noxa, Puma, Bim and Bid. The latter group is called as BH3-only proteins as they contain only BH3 domains. The anti-apoptotic Bcl-2 proteins are structurally very similar to the founding member Bcl-2, which includes several proteins such as MCL1, Bcl-XL, Bcl-w and Bcl-A1 (Adams and Cory, 2001).

MCL1 (*Myeloid cell leukemia-1*), the second member of the Bcl-2 family discovered, was first identified as an IEG, during myeloblastic leukemia cell differentiation upon stimulation with a phorbol ester (TPA), in a screening assay (Kozopas et al., 1993). MCL1 shares sequence homology with Bcl-2 in its BH1-3 domains. Like Bcl-2, this gene undergoes alternative splicing, gives rise to two different *MCL1* mRNAs encoding long and short splice variants with opposite functions (Bae et al., 2000;Bingle et al., 2000). The relative amount of these isoforms can determine the fate of *MCL1* expressing cells (Bae et al., 2000). Since its discovery, *MCL1* has been reported as a survival gene and now is considered as an important regulator of cell survival and cancer progression (Akgul, 2009). The better understandings of the mechanisms regulating *MCL1* or its alternative splicing can provide useful insights to fine tuning of the therapeutic control of diseases and cancers.

1.10.1 Structure and regulation of *MCL1*

The gene *MCL1* is located on chromosome 1q21 and the encoded protein product is approximately 40kDa, which is larger than its prototypical family member, Bcl-2 (26kDa) (Michels et al., 2005;Zhang et al., 2002). MCL1 has some unique structural characteristics

among the Bcl-2 family. The C-terminal domain contains the BH1-3 domains but lacks the BH4 domain, present in other anti-apoptotic Bcl-2 family members (Kozopas et al., 1993) (**Figure 1.17**).

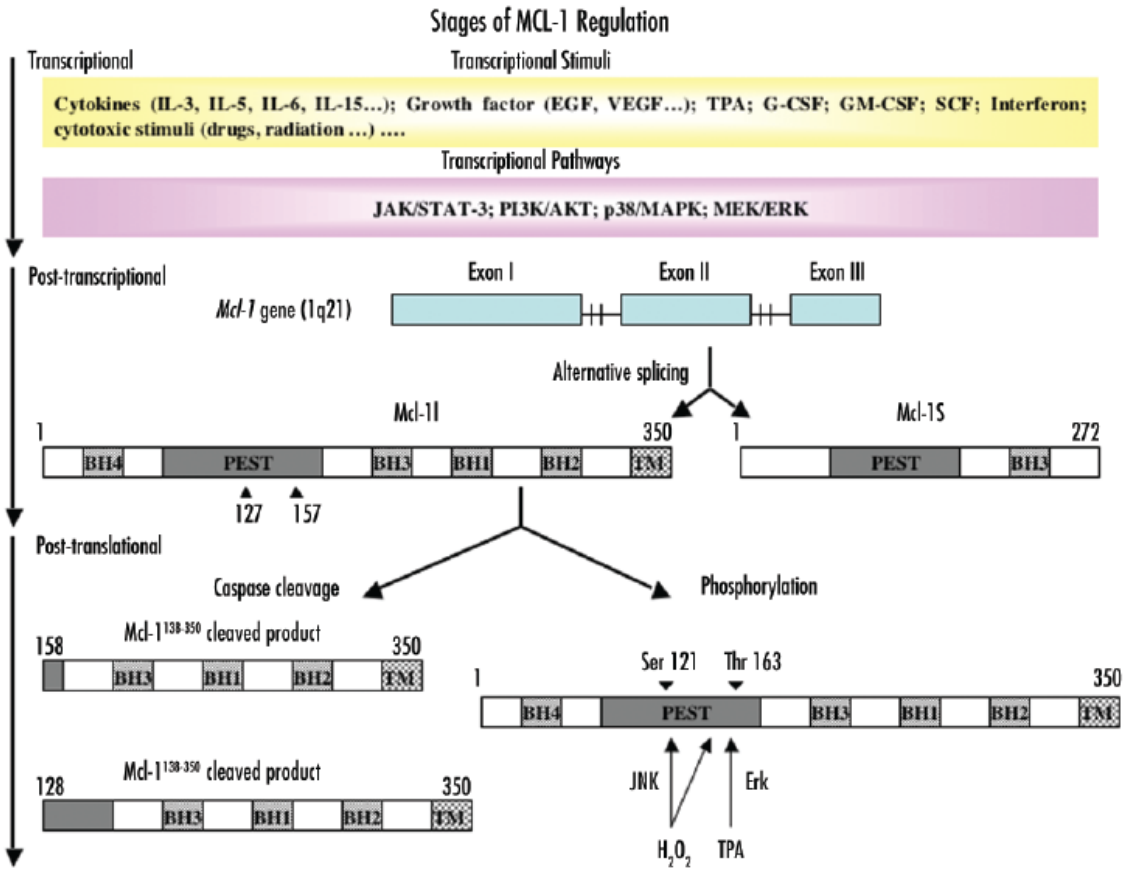

Figure 1.17: Transcriptional, post-transcriptional and post-translational regulation of *MCL1*

A wide variety of extra-cellular stimuli (including pro- or anti-survival) regulate *MCL1* expression in a cell-specific manner *via* activation of one (or more) transcription signaling pathways. A variety of factors promote *MCL1* transcription. *MCL1* also undergoes alternative splicing: the joining of 3 exons encodes for the full length 350 amino acids anti-apoptotic MCL1L protein, whereas joining of the first and the third exons, without the second, encodes for a 272 amino acids protein named MCL1S. In contrast to MCL1L, MCL1S is a pro-apoptotic protein. Finally, at the post-translational level, MCL1L can either be cleaved by caspases or phosphorylated. MCL1 has two cleavage sites (Asp127 and Asp157) and two phosphorylated sites (Ser121 and Thr163). All sites are located in the PEST sequences. Interestingly, the MCL1L 128-350 cleaved product (28kDa) has pro-apoptotic function whereas the phosphorylated form of MCL1 has antagonist function. Unique phosphorylation of Thr163 *via* ERK signaling in response to TPA slows MCL1 turnover and promotes cell survival, whereas H_2O_2 promotes Ser121 and Thr163 phosphorylation via JNK signaling and induces apoptosis. *The figure and text were reproduced with permission from Figure 1 (Le et al., 2004).*

The BH1-3 domain forms a hydrophobic cleft, which plays role in the interaction of MCL1 with other Bcl-2 family proteins (Gross et al., 1999;Petros et al., 2004). Additionally, the C-terminal domain also contain a transmembrane domain (TM), allowing MCL1 to interact with membrane (Yang et al., 1995). The N-terminal domain of MCL1 is longer than other Bcl-2 family members, which contains two PEST sequences (polypeptide sequences enriched in proline [P], glutamic acid [E], serine [S] and threonine [T]) (Kozopas et al., 1993). The PEST sequences are involved in the proteosomal degradation of MCL1 and may account for its relatively short half-life ranging from 30 min to 120 min, depending on the cellular context (Rechsteiner and Rogers, 1996;Zhuang and Brady, 2006). The rapid turnover of MCL1 (both isoforms) protein indicates that it plays a crucial role in survival or apoptotic control in response to rapidly changing environmental cues (Yang et al., 1996a). MCL1 is also distinct from Bcl-2 (localized in outer mitochondrial membrane) with its wide intracellular localizations in nuclear envelope, endoplasmic reticulum and mitochondrial membrane (Yang et al., 1995).

The transcriptional regulation of *MCL1* is cell type dependent and modulated by several extra cellular stimuli, signaling pathways and transcription factors (Craig, 2002;Townsend et al., 1998;Yang et al., 1996a). A growing list of stimuli have been reported to induce the transcriptional upregulation of *MCL1*, which includes cytokines (interleukins, IL-3, IL-6 and IL-15); growth factors (VEGF, EGF); colony stimulating factors (G-CSF, GM-CSF, SCF); interferon (IFN) and phorbol ester , TPA (Craig, 2002). The promoter region of *MCL1* contains a variety of transcription factor binding sites, including Sp1, NF-κB, cAMP-response elements, consensus STAT response elements, serum response factors (SRF), and many others (Akgul et al., 2000;Akgul, 2009). Upon stimulation by a wide array of extracellular stimuli, and, with the

activation of the downstream signaling pathways (e.g. MEK/ERK, JAK/STAT, PI3K/ARK and p38/MARK cascades), these transcription factors can bind to the *MCL1* promoter and subsequently regulate the gene expression (Akgul, 2009). For example, induction of *MCL1* expression by TPA involves the activation of MEK/ERK signaling pathway. This pathway drives the activation of transcription factor complex containing SRF and Elk-1 at the *MCL1* promoter, and thereby results in the up-regulation of the gene (Boros et al., 2009;Townsend et al., 1998;Vickers et al., 2004). Conversely, *MCL1* can be also down regulated in a number of conditions, which includes withdrawal of growth factors or UV exposure (Chao et al., 1998; Nijhawan et al., 2003) (**Figure 1.17**).

MCL1 can be further regulated at the post-transcriptional, translational and post-translational levels. At the post-transcriptional level, *MCL1* can be regulated through alternative splicing, resulting in two spliced isoforms of antagonistic functions or by miRNAs through miR29b binding in 3′ UTR of *MCL1* mRNA (Bae et al., 2000;Bingle et al., 2000;Mott et al., 2007). Translational regulation includes the rapid protein turnover through proteosomal pathways and caspase-mediated cleavage of the protein, which results in the removal of a large part of the N-terminus of MCL1 (Herrant et al., 2004;Warr et al., 2005;Weng et al., 2005;Zhong et al., 2005). Several phosphorylation sites present in MCL1 can be differentially phosphorylated in response to signaling pathways. This adds another layer of complexity in the regulatory mechanism of *MCL1* gene (Domina et al., 2000;Maurer et al., 2006) (**Figure 1.17**).

1.10.2 Alternative splicing of *MCL1*

MCL1 gene consists of three exons and two introns. The alternative splicing of *MCL1* leads to two distinct *MCL1* mRNA species either including or lacking exon 2 and encoding the long (*MCL1L*), and the short (*MCL1S*) isoforms, respectively (Bae et al., 2000;Bingle et al., 2000). *MCL1L* is the major isoform while *MCL1S* expression is very low in normal mature cells (Bae et al., 2000;Bingle et al., 2000;Legartova et al., 2009). The *MCL1L* encodes for the full length anti-apoptotic protein containing 350 amino acids (MCL1L), whereas the *MCL1S* encodes for the pro-apoptotic protein comprised of 272 amino acids (MCL1S) (Bae et al., 2000;Bingle et al., 2000). Structurally, MCL1S does not have the TM or BH1-2 domains, and therefore resembles the BH3-only protein pro-apoptotic Bcl-2 family members (**Figure 1.17**). However, the two isoforms harbor the PEST domains that are responsible for high turnover (Bae et al., 2000;Bingle et al., 2000). Overexpression of MCL1S resulted in cell death indicating that MCL1S possesses an opposite function to that of its other counterpart, MCL1L (Bingle et al., 2000). The two splice variants can form heterodimers, and can antagonize each other. The fate of *MCL1* expressing cells could be regulated by the relative ratio of the two isoforms (Bae et al., 2000). Of note, it would be interesting to determine if the splicing machinery could be manipulated to increase the expression of pro-apoptotic MCL1S in cells where *MCL1* overexpression provides the apoptosis resistance phenotype (Gojo et al., 2002;Lin et al., 2001a;Zhang et al., 2002). More recently, another splice variant of *MCL1*, *MCL-1ES* (extra short) has been identified (Kim et al., 2009). The MCL-1ES is generated by the exclusion of a large region of exon 1 that removes the PEST domain but it retains the BH3 domain. Similar to MCL1S, MCL-1ES can also interact with MCL1L and can induce apoptosis. The apoptotic effect of MCL-1ES is increased with the overexpression of MCL1L, although the mechanism remains elusive as yet (Kim et al., 2009).

Several *cis*-regulatory elements and *trans*-acting factors have been reported to be involved in the regulation of *MCL1* splicing (Moore et al., 2010). Using genome wide siRNA screening, the alternative splicing regulators of *MCL1* have been identified, including the splicing factors, hnRNPs and SR proteins (e.g. SRSF1 and SRSF3), and core spliceosomal assembly proteins such as U2 SnRNP (e.g. SF3B1 and SF3B4). Despite this, relatively very little is known about the splicing switch or factors involved in the exclusion or inclusion of the alternate exon of *MCL1*. A recent study has reported that *MCL1* splicing can be regulated by another splicing factor, SRSF5 in breast cancer cells (Gautrey and Tyson-Capper, 2012).

Targeting *MCL1* splicing can be an attractive therapeutic intervention as it has the potential to modulate the cell fate depending on the expression levels of the splice variants. Various pharmacological agents and splice switching oligonucleotides have been reported to up regulate *MCL1S* isoform. For example, EGCG (epigallocatechin gallate) (green tea extract) and ibuprofen synergistically induced the cell death in prostate cancer lines with the concomitant increase in the level of pro-apoptotic *MCL1S* isoform (Kim, 2008). Other studies have used anti-sense oligonucleotides to alter the splicing of *MCL1*, which are targeted to specifically shift the *MCL1* pre-mRNA from the anti-apoptotic *MCL1L* to pro-apoptotic *MCL1S* (Akgul et al., 2000;Mercatante et al., 2001a;Mercatante et al., 2001b). As an example, morpholino oligonucleotides (chemically modified anti-sense oligonucleotides that specifically binds to target sequences, and blocks splicing mechanisms) efficiently induce apoptosis in a number of cancer cell lines by switching the balance towards the short *MCL1* splice variant (Shieh et al., 2009).

1.10.3 *MCL1* as a therapeutic target

Overexpression of *MCL1* has been documented in a variety of human cancers including colon, breast, lung, ovarian, prostate, renal, melanoma, leukemia, as well as in other malignancies (Placzek et al., 2010;Sieghart et al., 2006;Thallinger et al., 2004). *MCL1* overexpression appears to be an important genomic change in diverse cancers, and plays a significant role to drive the cancer cells not only to survive, but also to be resistant to conventional chemotherapy (Hussain et al., 2007;Nguyen et al., 2007;Paoluzzi et al., 2008). In lines, the down regulation of *MCL1* is often sufficient to promote cell death or apoptosis in cancer cells (Andersson et al., 2004;Chetoui et al., 2008;Wei et al., 2008). For example, cancer cell lines that are resistant to ABT-737, a small molecule inhibitor of Bcl-2, are reported to have the increased expression of the *MCL1L*, the dominant splice variant of *MCL1* (Tahir et al., 2007). The down regulation of *MCL1L*, led the cells to be sensitized to ABT-737 (Chen et al., 2005b;Tahir et al., 2007). Taken together, these studies indicate the potential of *MCL1* as a therapeutic agent in targeting cancers and malignancies.

The regulation of *MCL1* occurs at many levels and is multifactorial in nature. Several approaches have been exploited to target *MCL1* based on three main mechanisms (Quinn et al., 2011). One of the approaches is to reduce the level of MCL1 in target cells by decreasing its expression (transcription inhibition or RNA interference) or by induction of its rapid degradation. The second approach is to decrease the pro-survival function of *MCL1* by disrupting its interactions with its binding partners, such as Bak, via a mimic (e.g. BH3 mimetics), which can interfere with the interaction. A third approach might be to direct *MCL1* splicing mechanisms towards the production of pro-apoptotic *MCL1S* through the use of pharmacological inhibitors and anti-sense

oligonucleotide approaches. This would induce apoptosis and/or render tumor cells more sensitive to chemotherapeutics. However, none of the approaches appears to be the sole strategy to target *MCL1*. Studies have suggested that the potentials for targeting *MCL1* are mainly context-based and can be used as an adjunct therapy as well. For example, in U937 cells (human macrophage cell line), use of an HDAC inhibitor and cyclin-dependent kinase inhibitor was more effective to induce apoptosis than either of the compound alone (Rosato et al., 2005). Again, HDAC inhibitors, alone or in combination with other therapeutics, induced apoptosis in cancer cells by down regulating the expression of *MCL1* (Maggio et al., 2004;Ryu et al., 2006). The underlying mechanism is yet to be defined.

1.11 Rationale, hypotheses and study objectives

The central theme that threads the studies presented in this thesis is that class I HDACs, HDAC1 and HDAC2, can form HDAC1 and 2 homo- or heterodimers while interacting with a wide variety of protein complexes, and thereby intervene in a multitude of biological processes including, but not limited to, transcription regulation and alternative splicing. The activity, stability or targeting of HDACs are dynamically regulated by PTMs such as phosphorylation and by the interactions with other proteins. By deacetylating histones, transcription factors, epigenetic modifiers and many other non-histone proteins, HDACs act at multiple levels to regulate gene expression. Aberrant regulation of gene expression is at the basis of many diseases and cancers. Therefore, targeting HDACs with HDAC inhibitors is widely used as a therapeutic intervention. Moreover, the use of these inhibitors can help deciphering the molecular mechanisms underlying the biological processes regulated by HDACs.

We have investigated the HDAC1 and 2 complexes in three different aspects that contribute to the main theme of the thesis. In the first study (**Chapter 2**), we explored the distributions of HDAC1 and 2, their steady state levels of phosphorylation and the nature of HDAC1 and 2 multiprotein complexes during mitosis. The phosphorylation state of HDAC1 and 2 influences the activity and stability of the complexes in which they are incorporated into homo- or heterodimer configurations, and are thereby involved in various biological functions. HDAC1 and/or 2 dimer formation is a pre-requisite for the catalytic activity of the proteins. Furthermore, the HDAC complexes are dynamic in nature and their compositions fluctuate as a function of intracellular stimuli. It was previously reported that HDAC2 becomes highly phosphorylated during mitosis, although, the protein kinase involved remains to be identified (Galasinski et al., 2002). It was also demonstrated that when HDAC1 and 2 were in a highly phosphorylated state induced by okadaic acid, a phosphatase inhibitor, they disassembled from each other and from corepressor complexes (Galasinski et al., 2002). Whether similar events occur during mitosis are currently not known and needs to be investigated to explore the functional diversity of HDAC1 and 2 complexes. Our lab has previously demonstrated that the protein kinase CK2 is associated with the HDAC corepressor complexes (Sun et al., 2002a). We, thus, sought to examine the role of CK2 in regulating the mitotic-increased phosphorylation of HDAC1 and 2. We hypothesized that CK2 acts as the regulator of increased steady state levels of phosphorylated HDAC1 and 2 during mitosis, and, modulates their incorporations into corepressor complexes in distinct configurations as either HDAC1 or HDAC2 homodimers. These may attribute to diversify the biological functions of these complexes, by targeting different cellular substrates.

Both KATs and HDACs are shown to be displaced from condensed mitotic chromosomes and to be spatially reorganized as proceeding through the phases of mitosis, despite maintaining their catalytic activities (He and Davie, 2006;Kruhlak et al., 2001). These enzymes as well as other transcription associated proteins such as Sp1 and Sp3, are displaced from or re-associate with chromatin in prophase and telophase, respectively (He and Davie, 2006;Kruhlak et al., 2001). Furthermore, studies have reported that these proteins re-enter into newly formed daughter nuclei in an orderly manner for the re-establishment of gene expression programs (He and Davie, 2006;Kruhlak et al., 2001). However, the factors that promote the dissociation of HDAC1 and 2 from mitotic chromosomes have not been yet determined. In addition, the order of re-entry of these enzymes compared to other transcription regulatory proteins is currently unknown. Thus, in the second study (**Chapter 3**), we continued to investigate the HDAC1 and 2 complexes during mitosis, with the goals to examine their spatial distribution patterns, to uncover the factors leading to HDAC1 and 2 dissociation from mitotic chromosomes, and to determine the sequential order of re-entry of these enzymes into the daughter nuclei, which is critical to re-establish gene expression.

Dynamic histone acetylation plays a pivotal role in IEG expression. Our previous studies of *Trefoil factor 1* (*TFF1*) gene have demonstrated that un- or monomodified, and phosphorylated HDAC2 complexes were differentially distributed within the gene. The unmodified HDAC2 was associated with the coding region, whereas the corepressor bound, CK2-phosphorylated HDAC2 was recruited to promoter region of *TFF1*gene (Sun et al., 2007). Whether similar distributions occur in other IEGs is not known. Further, HDAC inhibitors have been reported to alter the expression of several IEGs, although the mechanism is not very well described (Hazzalin and

Mahadevan, 2005). Thus, we aimed to investigate the transcriptional roles of HDAC complexes in regulation of IEG, *FOS-like antigen-1* (*FOSL1*) with two HDAC inhibitors, TSA (pan inhibitor) and apicidin (class I specific HDAC inhibitor), in the third study (**Chapter 4**). *FOSL1*, a member of the FOS family of transcription factors, is overexpressed in a variety of human cancers (Chiappetta et al., 2007;Young and Colburn, 2006). The induction of IEGs due to various signaling pathways such as MAPK, ERK or p38, is accompanied by MSK-mediated H3S10ph or H3S28ph at the regulatory regions (referred to as nucleosome response) of the genes (Clayton et al., 2000;Clayton and Mahadevan, 2003;Drobic et al., 2010). MSK-induced nucleosome response is required for the induction of the genes to occur. Further, dynamic acetylation of histones occurs independently of MSK-mediated H3 phosphorylation. Inhibition of HDAC activity affects the expression of a small proportion of genes including the IEGs, which can be either up-or down regulated. Transcriptional reprogramming by the HDAC inhibitors is considered to contribute to their therapeutic benefits. Although the attenuation of mitogen- or stress-induced expression of IEGs by HDAC inhibitors is well documented, the mechanistic role of HDACs involved in transcriptional regulation of IEGs, in particular, for *FOSL1* has not been reported. In addition, whether the nucleosome response pathway, one of the major regulators of IEG induction, is intact and responsive in these genes with treatment with HDAC inhibitors remains elusive. We hypothesized that HDACs are co-transcriptionally involved in *FOSL1* gene induction independent of the nucleosome response, and regulate the transcriptional initiation of *FOSL1* gene, downstream of the nucleosomal response pathway.

In the concluding study (**Chapter 5**), we further continued to explore the role of HDAC1 and 2 complexes associated in gene body regions with focus in the regulation of splicing, which is an

important regulatory mechanism of gene expression. Our lab and other groups have previously reported that HDACs are targeting to the transcribed regions of active genes (Sun et al., 2007;Wang et al., 2009). Yet, it remains to be determined how they are directed to gene body and in which protein complexes they reside in. Towards understanding of their functions in transcribed regions, a relatively new research field has emerged, revealing the role of histone modifications and HDAC inhibitors in impinging transcription elongation and alterative splicing. We thus attempted to decipher the molecular details of the role of HDAC1 and 2 complexes in alternative splicing mechanisms using an IEG, *MCL1*, as a model gene of study. Alternative splicing of *MCL1* generates two protein products with antagonistic functions: an anti-apoptotic long isoform MCL1L and, a pro-apoptotic short isoform MCL1S, respectively. The balance of the expression of these isoforms can determine the cell fate. We hypothesized that transcription dependent accumulation of HDAC1 and 2 along the coding region of active genes, functions in concert with a number of splicing proteins to regulate the alternative splicing mechanism, by modulating the chromatin structure and environment.

1.11.1 General hypothesis

Class I HDACs, HDAC1 and HDAC2, are distributed along the regulatory and transcribed regions of active genes as part of multiprotein complexes in either homo- or heterodimer configurations. The co-transcriptional distributions of HDAC1 and 2 over the gene body regions affect the regulatory mechanisms of transcription and alternative splicing, by modulating the chromatin structure and function.

1.11.2 Thesis objectives

The objectives are:

A. To characterize the distribution and nature of HDAC1 and 2 multiprotein complexes during mitosis
B. To investigate the steady state levels of phosphorylated HDAC1 and 2, and to identify the kinase responsible for phosphorylation of HDAC1 and 2 during mitosis
C. To identify the factors that mediate the displacement of HDAC1 and 2 from mitotic chromosomes
D. To examine the transcriptional role of HDAC complexes in the induction of IEG, *FOSL1*
E. To elucidate the molecular mechanisms and functions of HDAC1 and 2 complexes in the gene body, particularly in alternative splicing regulation of the IEG, *MCL1* gene.

Chapter 2: Protein Kinase CK2 Regulates the Dimerization of HDAC1 and 2 during Mitosis

2.1 Abstract

HDAC1 and 2 are components of corepressor complexes that are involved in chromatin remodeling and regulation of gene expression by regulating dynamic protein acetylation. HDAC1 and 2 form homo- and heterodimers, and their activities are dependent upon dimer formation. Phosphorylation of HDAC1 and 2 in interphase cells is required for the formation of HDAC-corepressor complexes. In this study, we show that during mitosis, HDAC2 and, to a lesser extent, HDAC1 phosphorylation levels dramatically increase. When HDAC1 and 2 are displaced from the chromosomes during metaphase, they dissociate from each other, but each enzyme remains in association with components of the HDAC-corepressor complexes Sin3, NuRD, and CoREST as homodimers. Enzyme inhibition studies and mutational analyses demonstrated that protein kinase CK2-catalyzed phosphorylation of HDAC1 and 2 is crucial for the dissociation of these two enzymes. These results suggest that corepressor complexes, including HDAC1 or 2 homodimers, might target different cellular proteins during mitosis.

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Dilshad H. Khan has generated 80% of the data presented, drafted the manuscript and assisted in the revisions and editing of the paper.

2.2 Introduction

KATs and HDACs have important roles in the control of gene expression by remodeling chromatin through their regulation of dynamic acetylation of histones, transcription factors and chromatin modifying enzymes. Class I HDAC1 and 2 have roles in the regulation of gene transcription and pre-mRNA splicing (Hnilicova and Stanek, 2011;Zhou et al., 2011;Delcuve et al., 2012). They are highly homologous proteins with respect to DNA (75% identity) and protein sequences (85% identity) (Tsai and Seto, 2002). Although they have undergone little functional divergence and co-exist in multiprotein complexes, HDAC1 and 2 also have specific and distinct roles (Brunmeir et al., 2009;Guan et al., 2009;Jurkin et al., 2011). Both HDAC1 and HDAC2 are dysregulated in disease states and are overexpressed in cancer cells, while HDAC2 is under expressed in chronic obstructive pulmonary disease (Marshall et al., 2010;Upadhyay et al., 2008). HDAC1 and 2 are phosphorylated, a modification that is required for these enzymes to be assembled into the multiprotein Sin3, NuRD and CoREST corepressor complexes (Pflum et al., 2001;Tsai and Seto, 2002;Sun et al., 2002;Sun et al., 2007;Segre and Chiocca, 2011). In these complexes HDAC1 and 2 exist as heterodimers, although it is possible that these enzymes are present as homodimers. Regardless of the configuration, dimer formation of HDAC1 and 2 is a requirement for catalytic activity (Luo et al., 2009). Further, HDAC1 and 2 activities are augmented by phosphorylation, with the non-phosphorylated HDAC1 and 2 showing low activity (Sun et al., 2007;Pflum et al., 2001;Tsai and Seto, 2002). HDAC1 and 2 can be phosphorylated at multiple serines in the C-terminal portion of the protein by protein kinase CK2 (HDAC1 at Ser393, Ser421 and Ser423; HDAC2 at Ser394, Ser422 and Ser424). Mutation in any of the three phosphorylation sites is sufficient to disrupt the interaction of HDAC1 and 2 with RbAp48 and other binding partners of the corepressor complexes (Pflum et al., 2001;Tsai and Seto, 2002;Sun et al., 2002;Sun et al., 2007;Segre and Chiocca, 2011). However, these

mutations have no major effect on the binding of HDAC2 with HDAC1 (Tsai and Seto, 2002). The corepressor complexes containing HDAC1 and 2 are directed to regulatory regions of transcribed genes by a number of transcription factors such as Sp1 and Sp3 (De Ruijter et al., 2003;Sun et al., 2007). While the HDAC1 and 2 corepressor complexes containing phosphorylated HDAC2 are recruited to regulatory regions of transcribed genes, the un- or monophosphorylated HDAC2 is directed to coding regions of transcribed genes (Sun et al., 2007).

During mitosis both KATs and HDACs are displaced from mitotic chromosomes; however, these enzymes maintain their activities in the cell (Kruhlak et al., 2001;He and Davie, 2006). HDAC inhibitors do not induce histone hyperacetylation in mitotic HeLa cells (Patzlaff et al., 2010), which is consistent with the observation that although HDACs are catalytically active during mitosis, the enzymes are not located on the chromatin substrate. Furthermore during mitosis, HDAC2 becomes highly phosphorylated, but the responsible protein kinase remains to be identified (Galasinski et al., 2002). Evidence was also presented that, when HDAC1 and 2 were in an highly phosphorylated state induced by okadaic acid, they dissociated from each other and from corepressor complexes (Galasinski et al., 2002). Whether similar events occur in mitosis is currently not known.

In this study, we investigated the distribution of HDAC1 and 2, their phosphorylation state, and the state of the HDAC1 and 2 corepressor complexes during mitosis using high resolution microscopy and biochemical approaches. Our results demonstrate an important role for protein kinase CK2 in catalyzing the phosphorylation of HDAC2 during mitosis, an event which results

in the dissociation of HDAC1 from HDAC2, but not the dissociation of HDAC1 or 2 from the HDAC-corepressor complexes.

2.3 Materials and Methods

2.3.1 Cell culture

HeLa, HEK 293, MCF7 and Flp-In 293 cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1.0 % D-glucose, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B, at 37°C in a humidified atmosphere containing 5% CO_2.

2.3.2 Mitotic HDAC1 and 2 sample preparation

Whole protein extracts were prepared as described in Winter et al., (2008) from HeLa cells treated with taxol (Sigma T7402), nocodazole (M1404 Sigma) (1.0 μM each) or DMSO either in the presence (+) or absence (-) of inhibitors (phosphatase inhibitors: 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 10 mM sodium butyrate). Phosphatase inhibitors were omitted for samples used in calf intestinal phosphatase (CIP) assays.

2.3.3 Calf intestine phosphatase (CIP) assay

Protein extract (150 μg) from phosphatase inhibitors free samples was supplemented with adequate volume of 10× NEBuffer2 (New England Biolabs) and incubated with 10 units of CIP (New England Biolabs) at 37°C for 2 h. Phosphatase inhibitor-containing or phosphatase inhibitor-free control samples were treated only with buffer. Equal volumes of CIP treated and

untreated samples were analyzed by SDS-PAGE with antibodies specific for HDAC1 and 2 (produced in Christian Seiser's lab), and Cdc27. The Cdc27 antibody was a gift from Dr. Jan-Michael Peters (Research Institute of Molecular Pathology, Vienna, Austria). For two-dimensional SDS-PAGE analyses, 50 µg of protein extract and 3-4 units of CIP were used.

2.3.4 Double thymidine block and mitotic block

60-70% confluent HeLa cells were treated with thymidine (Sigma) at a final concentration of 2.0 mM for 19 h. After the incubation period, cells were washed three times with DMEM and incubated with fresh serum-rich medium for 10 h before the addition of thymidine (2.0 mM) again. Cells were incubated for 16-17 h and washed as described. Protein extracts and Fluorescence-activated cell sorting (FACS) samples were prepared 6, 7, 8, 10 and 12 h after second release. For mitotic block, protein samples were prepared after 20 h treatment with nocodazole or taxol. In the immunoprecipitation experiments, cells treated with 1.0 µM of nocodazole for 16 h time period were used to get the mitotic protein extracts.

2.3.5 Two-dimensional gel electrophoresis

Fifty µg of proteins of total cell lysate from untreated and nocodazole treated HeLa cells were loaded on isoelectric focusing strips (pH 3-10 and 4-7) and electrophoresed according to manufacturer's instructions (Biorad). The second dimension electrophoresis was done on SDS-7.5% PAGE, and immunoblotting was done with HDAC1, HDAC2 and HDAC2 S394 phospho (Abcam) antibodies.

2.3.6 Indirect immunofluorescence

Indirect immunolocalization of HDAC1 and 2 during mitosis was performed as described previously (He and Davie, 2006). Mouse monoclonal antibody against HDAC2 (1:250, Millipore) and rabbit polyclonal antibody against HDAC1 (1:5000, Affinity BioReagents) were used. Alexa Fluor 488 donkey anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, OR), and Alexa Fluor 594 donkey anti-rabbit or anti-mouse IgG (Molecular Probes, Eugene, OR), were used as secondary antibodies. DNA was counterstained with 4´, 6-diamidino 2-phenylindole (DAPI). Control experiments including epitope-peptide-blocking or primary antibody-omission demonstrated the specificity of the antibodies used. Digital images were captured with Zeiss Axio Imager Z1 microscope and AxioCam HRm camera. The images were captured with 100 slices at stepwise of 200 nm. The deconvolution analysis of stack images was done with the Axio Vision software (Carl Zeiss).

2.3.7 Immunoprecipitation and immunoblotting

HeLa cells were lysed in IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 0.5% NP-40) containing phosphatase and protease inhibitors, and immunoprecipitations were done as described earlier (Sun et al., 2002). Briefly, 500 μg of total cell extracts were incubated with 3.0 μg of different antibodies overnight at 4°C. Thirty μL of protein G Sepharose beads (Pierce) were added in the following day and incubated for 3-4 h at 4°C. The beads were then washed three times with ice-cold IP buffer. For each cell lysate, an immunoprecipitation with isotype specific non-related IgG was also performed as negative control to check for nonspecific immunoprecipitation. Also, an immunodepleted fraction corresponding to each immunoprecipitation reaction was included in immunoblotting analysis to test the

immunoprecipitation efficiency. Immunoblot analysis was carried out as described previously (Samuel et al., 1998). Rabbit polyclonal antibodies against human HDAC1 (Affinity BioReagents), HDAC2 (Affinity BioReagents), Sin3A (Affinity BioReagents), CoREST (Abcam), V5 (Abcam), mouse monoclonal antibodies against HDAC1 (Millipore), HDAC2 (Millipore), RbAp48 (Abcam) and V5 (Invitrogen) were used. For quantification purposes, RbAp48 antibody immunoprecipitates from control and nocodazole treated cell lysates were immunoblotted with anti-HDAC1 or anti-HDAC2 antibodies. The corresponding immunoblot membranes were imaged with Fluorchem 9900 imaging system (Alpha Innotech). The densitometry values of the bands of input and immunodepleted fractions were quantified and were normalized to the background levels. The unbound fractions were determined as relative to input (% of input). The average values of three independent experiments were used to calculate the bound fractions by subtracting the unbound fractions from 100%.

2.3.8 HDAC activity assay

HDAC activity assay was performed with the Fluor-de-Lys® HDAC fluorometric activity assay kit (Enzo life sciences) following the manufacturer's instruction. Briefly, HDAC1 and 2 complexes were immunoprecipitated from 100 µg of HeLa cell lysates (cycling and mitotic) with 1.0 µg of rabbit polyclonal anti-HDAC1 or anti-HDAC2 antibodies. The beads with antibody-HDAC complex were washed 3 times with the IP buffer and once with the HDAC activity assay buffer, before used for the HDAC activity assay. For the assay, the beads were incubated with or without 1.0 µM TSA, before the addition of 150 µM Fluor de Lys® Substrate. The reactions were then incubated at room temperature for 30 min while shaking on a rocker. After that, the developer I solution containing 1.0 µM TSA were added and the reactions were incubated for

another 15 min to stop the reactions. The fluorescence signal was measured using fluorometric plate reader (Spectra MAX GEMINI XS, Molecular devices).

2.3.9 Treatment conditions for CK2 inhibitors

Cells were treated with 100 µM of 4, 5, 6, 7-tetrabromobenzotriazole (TBB) (Calbiochem) or 50 µM of quinalizarin (EMD Biosciences) for 12 h before the addition of nocodazole (1.0 µM) and incubation for 16 h. The cell lysates were analyzed by immunoprecipitation and immunoblotting assays with anti-HDAC1 and anti-HDAC2 antibodies.

2.3.10 Flp-In 293-HDAC2 (WT)-V5 and Flp-In 293-HDAC2 (M3A)-V5 stable cell lines construction

Flp-In 293 host cell lines were purchased from Invitrogen. Plasmid HDAC2-WT and HDAC2-triple 3A mutant (S/A mutations) were constructed by using plasmid FLAG-HDAC2 and FLAG-HDAC2-M3A as templates, as described previously (Sun et al., 2007), and with the following primers:

forward:.5′-ACCATGGCGTACAGTCAAGGAGGAGGCAA-3′

reverse: 5′-AGGGTTGCTGAGTTGTTCTGACTTTC-3′

The constructs were cloned in pcDNA5/FRT expression vector. Stable Flp-In 293 cell lines expressing the wild type HDAC2 and M3A mutant HDAC2 established according to the manufacturer's instructions (Invitrogen).

2.3.11 Plasmids and transfections

Plasmid HDAC1-MYC was previously described (Taplick et al., 2001) and plasmids HDAC1-FLAG and FLAG-HDAC2 were kind gifts from Dr. Edward Seto. Plasmids FLAG-HDAC2-S394A and FLAG-HDAC2-S422A/S424A were constructed using the GENEART site-directed mutagenesis kit (Invitrogen). Mutagenesis PCR was carried out using FLAG-HDAC2 as template and primer pairs S394A for FLAG-HDAC2-S394A plasmid (mutated.sites underlined):

forward: 5′-GATGCTGTTCATGAAGACGCTGGAGATGAGGATGGAGAAG-3′

reverse:.5′-CTTCTCCATCCTCATCTCCAGCGTCTTCATGAACAGCATC-3′.

For FLAG-HDAC2-S422A/S424A plasmid, the following primer pair was used:

forward: 5′-GCTTGCGATGAAGAGTTTGCAGATGCTGAGGATGAAGGTGAAG-3′

reverse: 5′-CTTCACCTTCATCCTCAGCATCTGCAAACTCTTCATCGCAAGC-3′

All the plasmid constructs were verified by DNA sequencing. Four µg of HDAC1-MYC and HDAC1-FLAG plasmids were co-transfected into HEK 293 cells using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. FLAG-HDAC2 or FLAG-HDAC2-S394A or FLAG-HDAC2-S22/424A plasmids were transfected individually into HEK 293 cells. Approximately 32 h after transfection, the cells were treated with 1.0 µM of nocodazole for 16 h. After the treatment period, cells were harvested by mitotic shake-off. Cell lysates were prepared for immunoprecipitation with antibodies against anti-FLAG (Sigma), anti-MYC (Sigma) and anti-HDAC1 (Affinity Bioreagents).

2.4 Results

2.4.1 Increased phosphorylation of HDAC1 and 2 during mitosis

It has been previously reported that HDAC2 is hyperphosphorylated in nocodazole treated cells, which arrest in prometaphase (Galasinski et al., 2002). To further explore the levels of HDAC1 and 2 phosphorylation throughout the cell cycle, HeLa cells were synchronized in the G1/S phase of the cell cycle by using a double thymidine block. Following release from the block, cells were collected at various time points. At each time point, the cell extracts were prepared and analyzed by immunoblotting for HDAC1 and 2. The level of phosphorylated HDAC2 increased at 10 and 12 h post release of the block at which time cells had entered mitosis (**Figure 2.1A**). Consistent with previous observations, phosphorylated HDAC2 (HDAC2*) had a reduced mobility in SDS-PAGE (Galasinski et al., 2002;Sun et al., 2002). Cells blocked in mitosis by treatment with taxol or nocodazole (microtubule inhibitors) also had increased levels of phosphorylated HDAC2 (HDAC2*) as well as phosphorylated Cdc27, a component of the anaphase-promoting complex that is phosphorylated during mitosis. HDAC1 of mitotic cells had a retarded band that typically migrated very close to the parent band (**Figure 2.1A**).

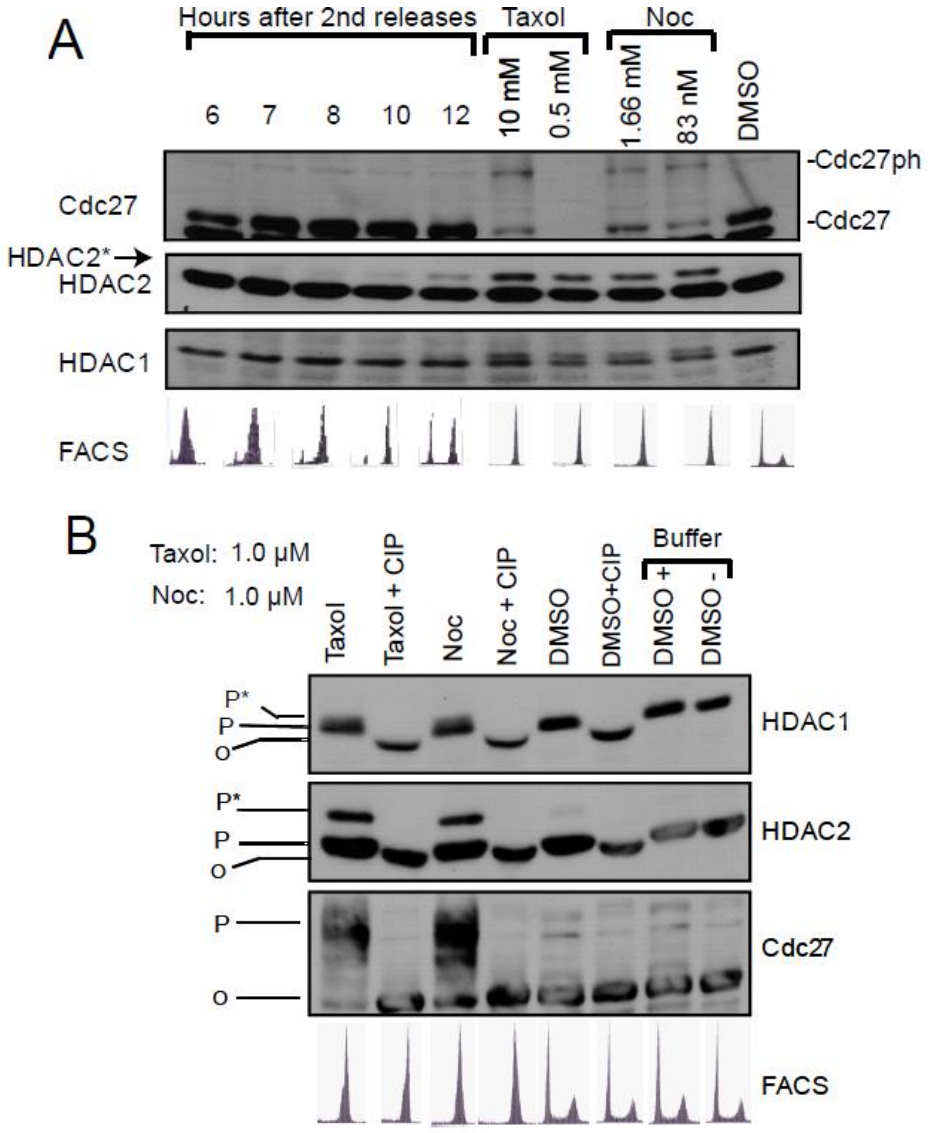

Figure 2.1: Mitotic phosphorylation of HDAC1 and 2
Whole cell protein extracts were prepared in the presence of protein phosphatase inhibitors except for samples used in the CIP assay. For thymidine double block, protein and FACS samples were prepared 6, 7, 8, 10, and 12 h after second release. For mitotic block, protein samples were prepared after 20 h treatment with taxol or nocodazole (Noc). A and B. Protein samples were analyzed by immunoblotting using the indicated antibodies. The state of phosphorylation is indicated as *O*, *P*, or *P**, representing unmodified, phosphorylated, and hyperphosphorylated isoforms, respectively.

To confirm that the slower migrating bands observed for HDAC1, 2 and Cdc27 corresponded to the phosphorylated forms of these proteins, the cell lysate was treated with CIP prior to SDS-

PAGE (**Figure 2.1B**). CIP treatment increased the mobility of HDAC1 and 2 in mitotic cell extracts as well as cycling cell extracts. The retarded bands seen for HDAC1, 2 and Cdc27 in mitotic extracts were not observed following treatment with CIP. These data indicate that the differences in migration patterns of these proteins between the control and mitotic extract were mostly due to phosphorylation and not due to other post-translational modifications.

Further analyses of HDAC1 and 2 modifications in cycling and mitotic HeLa cells were performed by resolving the cell lysates by two-dimensional PAGE followed by immunoblotting. In cycling HeLa cells, only one HDAC2 isoform was detected with the anti-HDAC2 antibody (**Figure 2.2A**). The same HDAC2 isoform was also detected with anti-HDAC2 S394 phospho antibody in a two-dimensional PAGE pattern. However, treatment of the cycling HeLa cell lysate with CIP resulted in detection of HDAC2 with the anti-HDAC2 antibody but not with the anti-HDAC2 S394 phospho antibody (**Figure 2.2A**). We thus conclude that in HeLa cycling cells most HDAC2 is in a monophosphorylated state.

In mitotic cells, three isoforms of HDAC2 were detected with anti-HDAC2 antibody, which were also detected with the anti-HDAC2 S394 phospho antibody (**Figures 2.2B** and **C**, respectively). Treatment of the mitotic cell lysate with CIP resulted in the detection of only one HDAC2 form with the anti-HDAC2 antibody, the non-phosphorylated isoform (**Figure 2.2B**). Based upon these results we have assigned the three isoforms as the mono-, di- and tri-phosphorylated HDAC2. These data demonstrate the dramatic increase in the steady state level of HDAC2 phosphorylation in the mitotic HeLa cells.

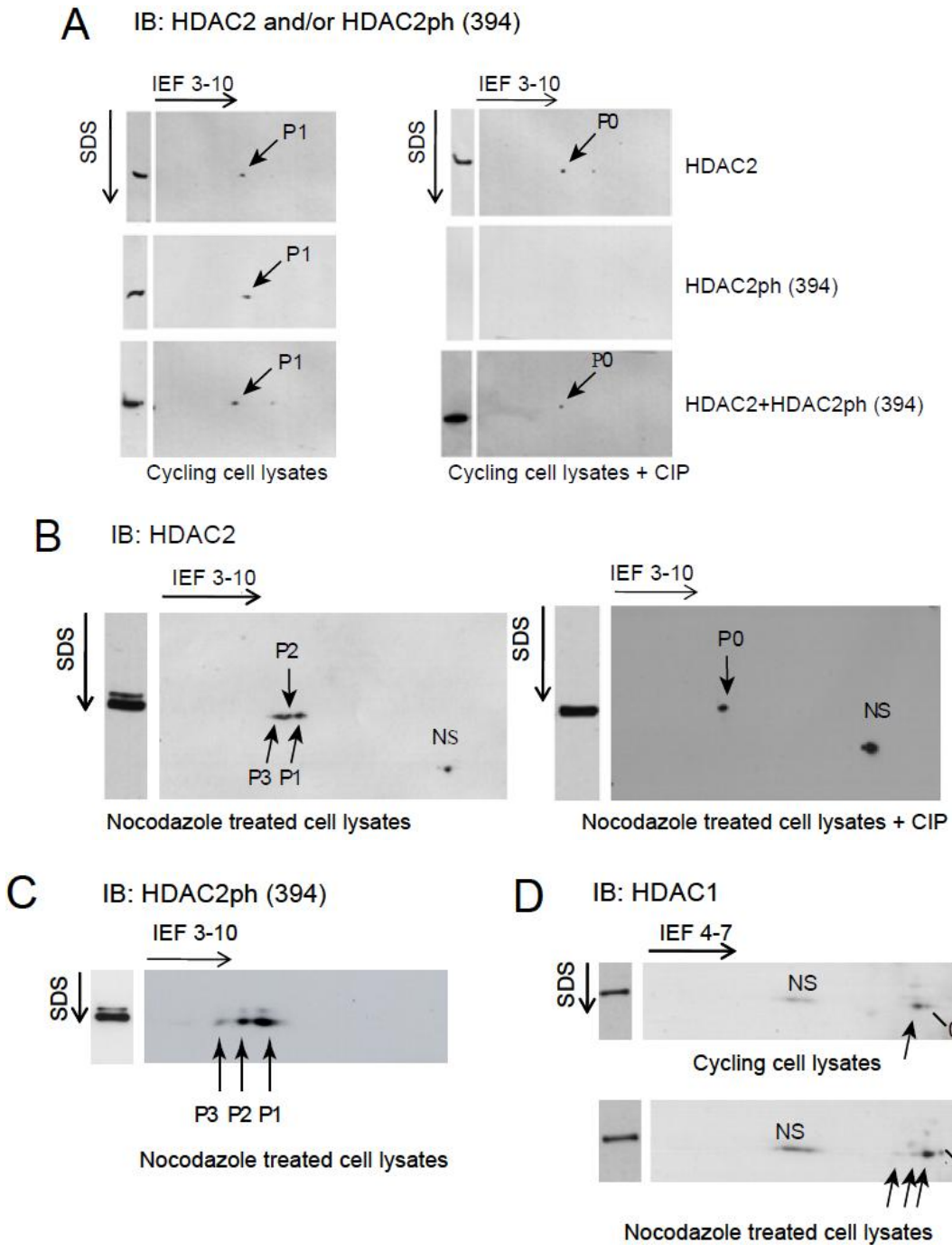

Figure 2.2: HDAC2 is highly phosphorylated in mitotic HeLa cells
Fifty µg of whole cell lysates from cycling and nocodazole treated HeLa cells were separated by isoelectric focusing (IEF) and then by SDS-PAGE and subsequently immunoblotted (IB) with anti-HDAC2 (A and B), anti-HDAC2ph (S394) (A and C), or anti-HDAC1 (D) antibodies. Cycling and nocodazole arrested samples treated with CIP were also analyzed (A and B). Shown are unmodified (P0), monophosphorylated (P1), diphosphorylated (P2), and triphosphorylated (P3) protein and nonspecific protein (NS) that was not reproducibly observed with antibodies against HDAC2.

In addition, we provide evidence that the steady state of HDAC1 in HeLa cycling cells is the monophosphorylated state (**Figure 2.2D**). Mitotic cells had increased levels of multiple phosphorylated isoforms of HDAC1; however, the increase in the steady state of the di- and tri-phosphorylated forms of HDAC1 in mitotic cells was not as pronounced as for HDAC2, in agreement with results shown in **Figure 2.1**.

2.4.2 HDAC1 or 2 corepressor complexes during mitosis

Previous reports showed that HDAC1 and 2 dissociate from condensed chromosomes during mitosis (Kruhlak et al., 2001). To determine whether HDAC1 and 2 remained as heterodimers in mitosis as observed in the cycling cells, we studied the distributions of HDAC1 and 2 in cycle-asynchronized cell population of HeLa cells by fluorescence microscopy after indirect immunofluorescence labeling of cells grown and fixed on cover slips (**Figure 2.3**). The cell cycle phases were determined by DAPI staining. In interphase stage there was partial co-localization of HDAC1 and 2 (see merge in **Figure 2.3A**). However, in metaphase cells, there was no co-localization of the two enzymes. We repeated the above analyses with human breast cancer cell line MCF7, to evaluate the generality of these observations. As observed in HeLa cells, HDAC1 and 2 were co-localized in interphase MCF7 cells, but not in prometaphase stage (**Figure 2.3B**). We have previously reported that in cycling MCF7 cells Sp1 and Sp3 occupied different nuclear sites than those of HDAC1 and 2 (He et al., 2005).

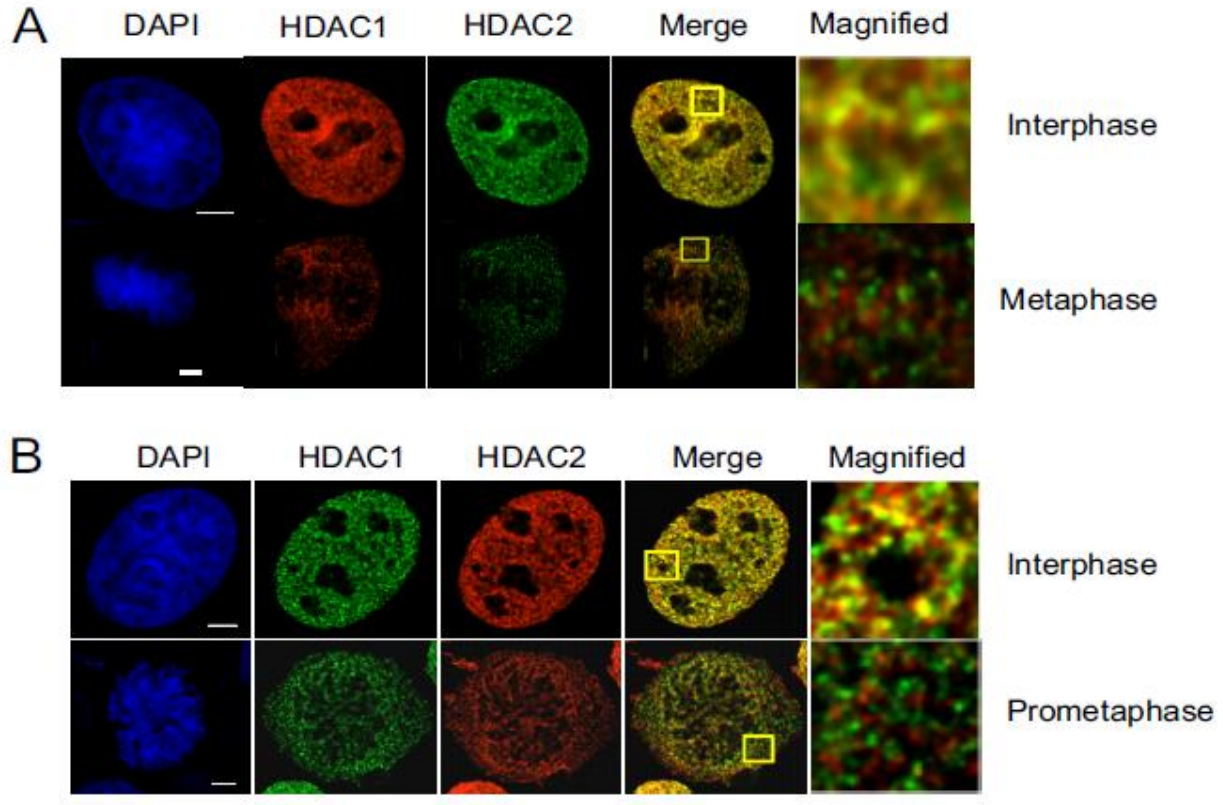

Figure 2.3: HDAC1 and 2 are located at distinct foci during mitosis
A. HeLa cells were subjected to indirect immunofluorescence labeling with HDAC1 and 2 antibodies and co-stained with DAPI for identification of cell cycle stages. Yellow signals in the merged image signify co-localization. Bar, 5μm. B. MCF7 cells were digitally imaged as described in A.

The dissociation of HDAC1 from HDAC2 during mitosis was further studied in co-immunoprecipitation assays with cell lysates from cycling and mitotic HeLa cells. To isolate HeLa cells arrested in prometaphase, cells were cultured in presence of nocodazole for 16 h, followed by shake-off of mitotic cells from the tissue culture plates. Cycling and prometaphase-arrested cell lysates were incubated with anti-HDAC2 or anti-HDAC1 antibodies under low stringency conditions, and the immunoprecipitated fractions were analyzed by immunoblotting with anti-HDAC1 or anti-HDAC2 antibodies, respectively, to test if these proteins interacted with each other. Consistent with previous studies using MCF7 cells, HDAC1 and 2 were co-

immunoprecipitated from cycling cell lysates (**Figures 2.4A** and **B**) (He et al., 2005). However, co-association of these two enzymes was not observed in the lysates of nocodazole treated (prometaphase-arrested) cells. These observations show that HDAC1 and 2 do not form heterodimers during mitosis.

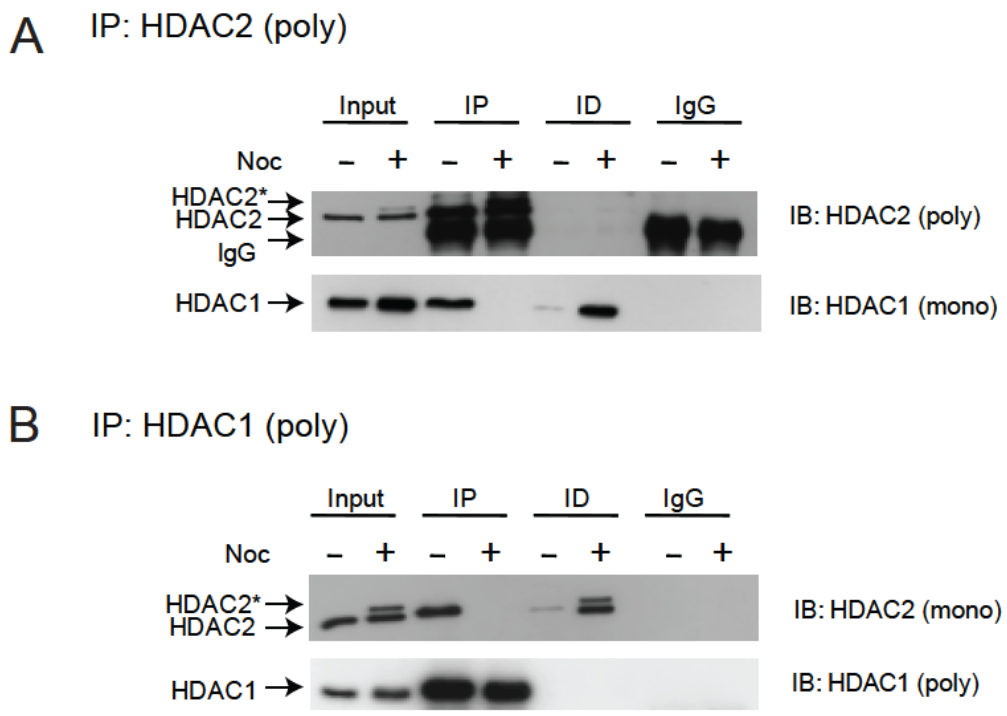

Figure 2.4: Mitotic phosphorylation results in the dissociation of HDAC1 and 2
Total cell lysates (500µg) from cycling and nocodazole (Noc) treated HeLa cells were incubated with 3.0µg of rabbit polyclonal anti-HDAC2 (A) or anti-HDAC1 (B) (poly) antibodies. The co-immunoprecipitations were checked by immunoblotting with mouse monoclonal anti-HDAC1 and anti-HDAC2 (mono), or rabbit polyclonal anti-HDAC1 and anti-HDAC2 (poly) antibodies. Immunoprecipitated and immunodepleted fractions are indicated as IP and ID, respectively. The phosphorylated form of HDAC2, which has reduced mobility in SDS-PAGE, is shown as HDAC2*.

HDAC1 and 2 exist in large multiprotein complexes, Sin3A, NuRD and CoREST. We investigated whether the mitotic increased phosphorylation of HDAC2 resulted in dissolution of the complexes as previously observed in cells treated with the phosphatase inhibitor, okadaic

acid (Galasinski et al., 2002). To this end, the HDAC1 and 2 immunoprecipitated fractions from cycling and mitotic extracts of HeLa cells were analyzed for the presence of Sin3A, RbAp48 and CoREST. Sin3A, RbAp48 and CoREST proteins were co-immunoprecipitated with HDAC1 and 2 in HeLa cycling as well as nocodazole arrested prometaphase cells (**Figures 2.5A** and **B**, respectively).

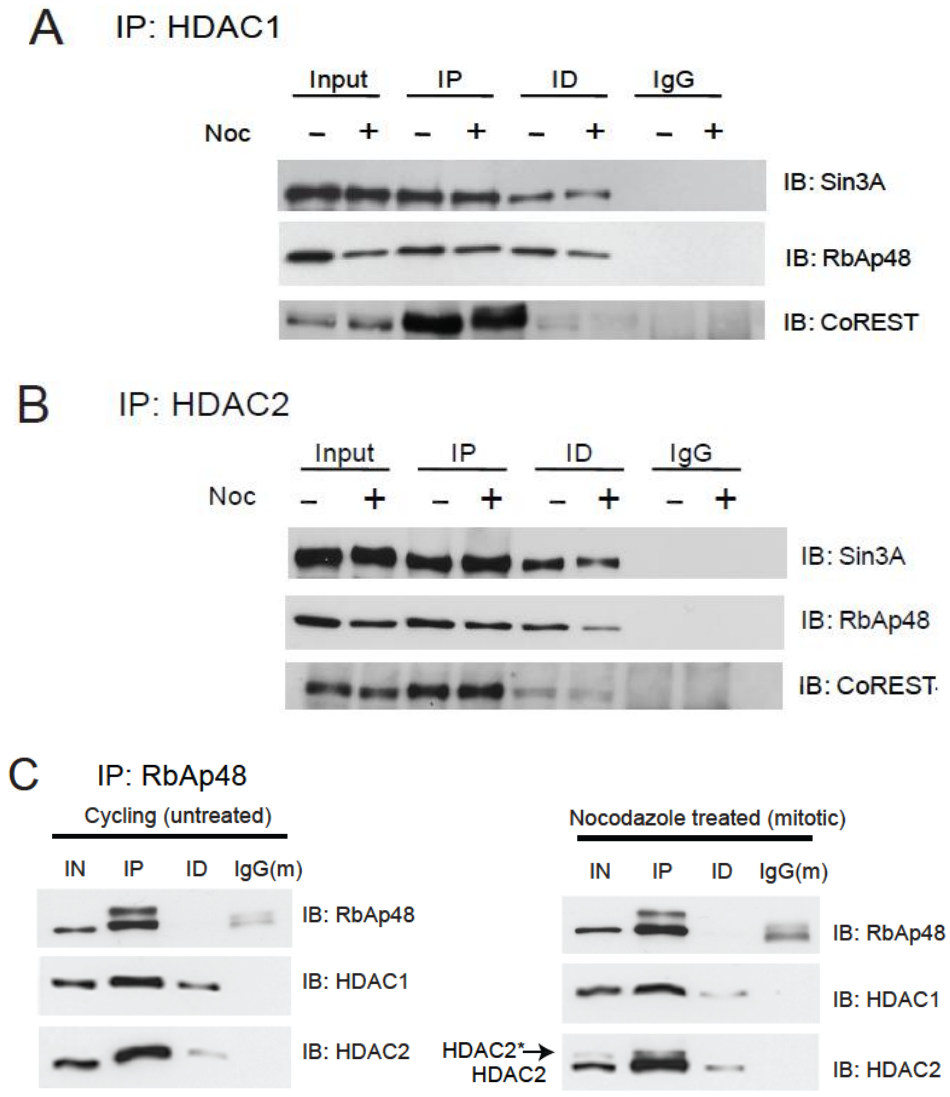

Figure 2.5: HDAC1 and 2 maintain the interactions with corepressor complex proteins during mitosis

A and B. Total cell lysates (500µg) from cycling and nocodazole (Noc) treated HeLa cells were incubated with 3.0µg of rabbit anti-HDAC1 (A) or anti-HDAC2 (B) antibodies. The immunoprecipitated fractions were checked with antibodies against Sin3A, RbAp48, or CoREST. Isotype-specific non-related rabbit IgG was used as negative control. IP and ID,

represent immunoprecipitated and immunodepleted fractions, respectively. C. Total cellular lysates (500µg) from cycling (untreated) and nocodazole treated (mitotic) HeLa cells were incubated with 3.0µg of mouse anti-RbAp48 antibody. The immunoprecipitated fractions were analyzed by anti HDAC1 and anti-HDAC2 antibodies. Isotype specific non-related mouse IgG was used as negative control. IN, IP, and ID represent input, immunoprecipitated, and immunodepleted fractions, respectively. The slower migrating band in the RbAp48 immunoprecipitated fraction may be phosphorylated RbAp48, but this has not been validated. The representative immunoblots are shown from one of three independent experiments, which are used for quantifications as mentioned under 'Materials and methods'.

We, reproducibly, observed an increase of RbAp48 with HDAC1 in mitotic versus cycling cells, while the amount of HDAC2 with RbAp48 did not change (**Figure 2.5C**). Quantification of the amount of RbAp48 in cycling versus mitotic HeLa cells demonstrated an increase in RbAp48 association with HDAC1 (27 ± 3% in cycling versus 54 ± 2% in mitotic, n = 3) and, to a lesser extent with HDAC2 (64 ± 1% in cycling and 66 ± 1% in mitotic, n = 3), in mitotic cells. Together, these results suggest that mitotic cells harbor corepressor complexes containing homodimers of either HDAC1 or HDAC2.

To determine whether the HDAC1 and 2 complexes were enzymatically active, we immunoprecipitated HDAC1 and 2 complexes from cycling and mitotic HeLa cells (nocodazole treated) and assayed the HDAC complexes for HDAC activity (**Figure 2.6A**). As shown in **Figure 2.6B**, the HDAC1 and HDAC2 immunoprecipitates from cycling cells had both HDAC1 and HDAC2. In mitotic cells (nocodazole treated) only HDAC1 or HDAC2 was immunoprecipitated with their respective antibodies. The immunoprecipitated HDAC1 and 2 complexes from cycling and mitotic cells had HDAC activity, which was inhibited by TSA. Control immunoprecipitates lacked HDAC activity.

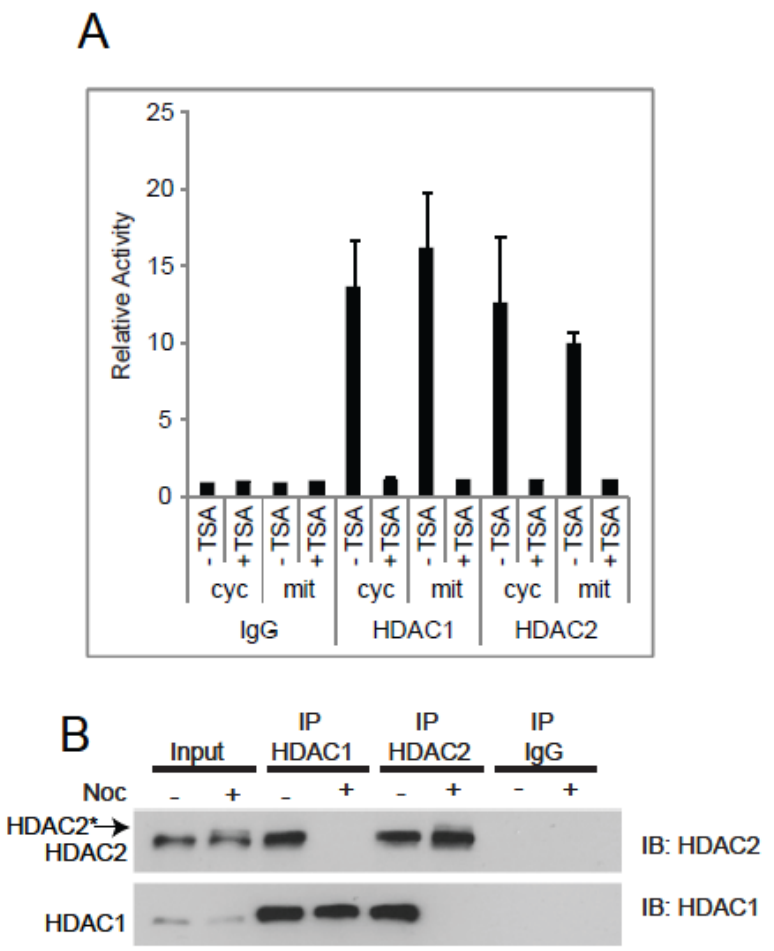

Figure 2.6: HDAC1 and 2 complexes are catalytically active during mitosis
A. HDAC1 or HDAC2 complexes were isolated by immunoprecipitation with anti-HDAC1 or anti-HDAC2 antibodies from cycling (cyc) and nocodazole treated HeLa cells (mit). HDAC activity in the immunoprecipitated fractions was measured by fluorometric activity assay kit. The activity of the HDAC1 or 2 complexes represents the average of three independent experiments and is relative to the activity from a non-related IgG control in cycling cells (without TSA treatment, which is set to 1 in all experiments) in arbitrary units. Error bars represent S.D. B. Representative immunoblot analysis (IB) from three independent experiments of HDAC1 and HDAC2 complexes in cycling (Noc-) and mitotic HeLa cells (Noc+) for corresponding HDAC activity assay shows that HDAC1 and HDAC2 did not interact with each other during mitosis. HDAC2*, phosphorylated form of HDAC2.

2.4.3 Increased mitotic phosphorylation of HDAC2 and protein kinase CK2

As mentioned previously, in cycling cells, protein kinase CK2 phosphorylates HDAC2 at Ser394, Ser422 and Ser424. We therefore investigated whether CK2 was involved in the increased phosphorylation of HDAC2 during mitosis. For this, HeLa cells were incubated with

the CK2 inhibitor TBB before the addition of nocodazole, and the protein extracts were analyzed by immunoblotting. We found that the slow migrating HDAC2 band (HDAC2*) disappeared in extracts from mitotic cells pre-treated with TBB (**Figure 2.7A**, left panel, compare lanes 1 and 2). Similar results were obtained when the experiments were repeated with another CK2 inhibitor, quinalizarin (**Figure 2.7A**, left panel). Pre-treatment with quinalizarin, prevented the appearance of the slow migrating HDAC2 band in the mitotic cell lysate. Further, two-dimensional immunoblotting analyses of the HDAC2 phosphorylation state following pre-treatment of nocodazole incubated cells with the CK2 inhibitors (TBB or quinalizarin) detected one form of HDAC2 under these conditions, the same form as in cycling cells (**Figure 2.7A**, right panel).

To investigate our observations in further detail, we established Flp-In 293 cell lines stably expressing HDAC2 wild-type (WT) or a triple mutant HDAC2 (M3A)-V5-tagged proteins, where the three CK2 phospho-sites mutated from serine to alanine (Ser to Ala mutation at Ser394, Ser422 and Ser424). Treatment of these stable cell lines with nocodazole or okadaic acid resulted in the appearance of a slower migrating HDAC2 band in the cellular extract for the wild type constructs but not for the mutants (**Figure 2.7B**). Alanine substitutions for Ser394, Ser422 and Ser424 abolished the slow migrating HDAC2 band in mitotic extracts, implying the role for phosphorylation of these residues in altering the mobility and conformation of this protein in SDS-PAGE. This observation provides evidence that the reduced mobility of HDAC2 is due to CK2-mediated phosphorylation at one or more of the Ser394, Ser422 and Ser424 phospho-sites. Taken together, our data demonstrate that protein kinase CK2 plays a key role in the enhanced phosphorylation of HDAC2 during mitosis.

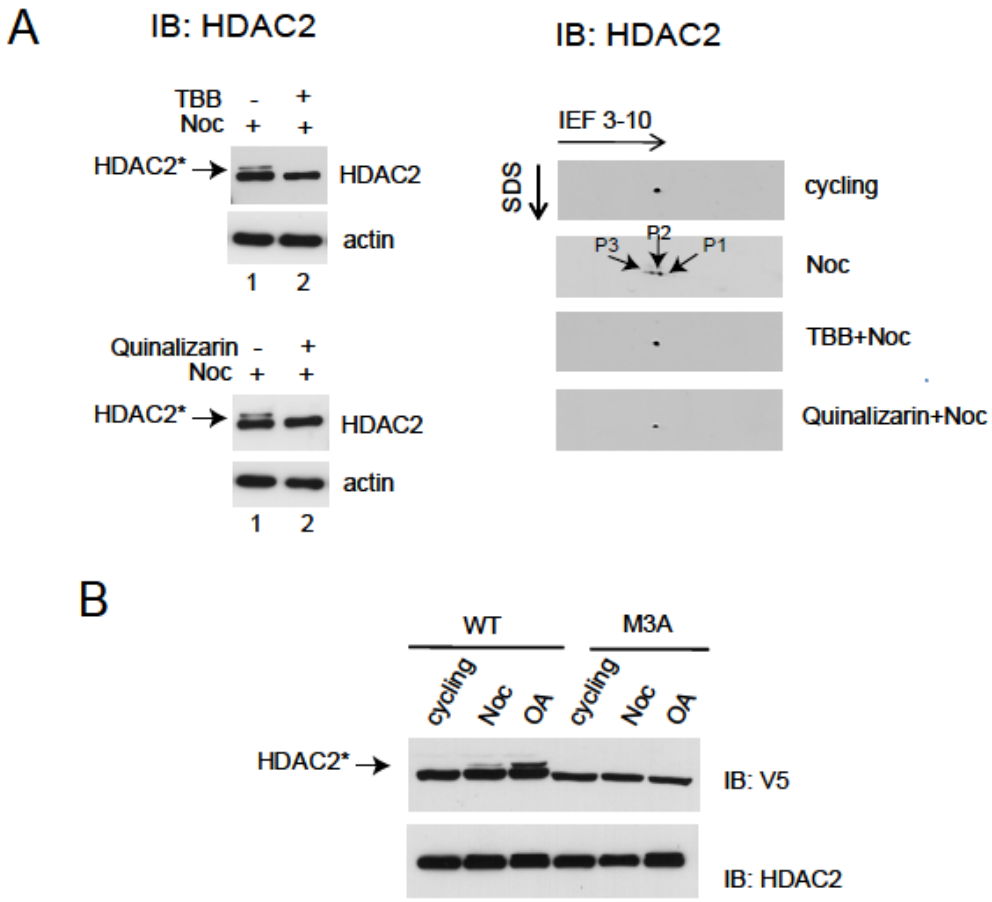

Figure 2.7: CK2 mediated increased phosphorylation of HDAC2 during mitosis
A. whole cell lysates from TBB-nocodazole (Noc) or quinalizarin-nocodazole treated HeLa cells were analyzed by SDS-PAGE (left) and two-dimensional gel electrophoresis (right) and subsequently immunoblotted (IB) with anti-HDAC2 antibody to check for phosphorylation levels. β-Actin levels in extracts were also examined on an immunoblot to demonstrate that there was equal loading of proteins from each of the lysates. B. Immunoblot analysis of V5 and HDAC2 from cellular extracts of Flp-In 293 cells stably expressing HDAC2-WT-V5 (WT) or HDAC2-M3A-V5 proteins (M3A), treated with nocodazole (Noc) or okadaic acid (OA). HDAC2*, phosphorylated form of HDAC2.

2.4.4 Protein kinase CK2 and separation of HDAC1 and 2 during mitosis

Since protein kinase CK2 is involved in the enhanced mitotic phosphorylation of HDAC2, we next sought to determine whether the CK2-mediated phosphorylation of HDAC2 had a role in the disruption of the HDAC1 and 2 heterodimers during mitosis. HDAC1 and 2 were

immunoprecipitated from lysates of cells incubated with nocodazole or TBB and nocodazole. Immunoblotting analysis on the immunoprecipitated fractions with anti-HDAC2 or anti-HDAC1 antibodies demonstrated that HDAC2 was not associated with HDAC1 in nocodazole incubated cells, in agreement with results shown in **Figure 2.4**. However, this interaction was preserved when the cells were incubated with the CK2 inhibitor TBB before nocodazole treatment (**Figure 2.8A**, lanes 3 and 4).

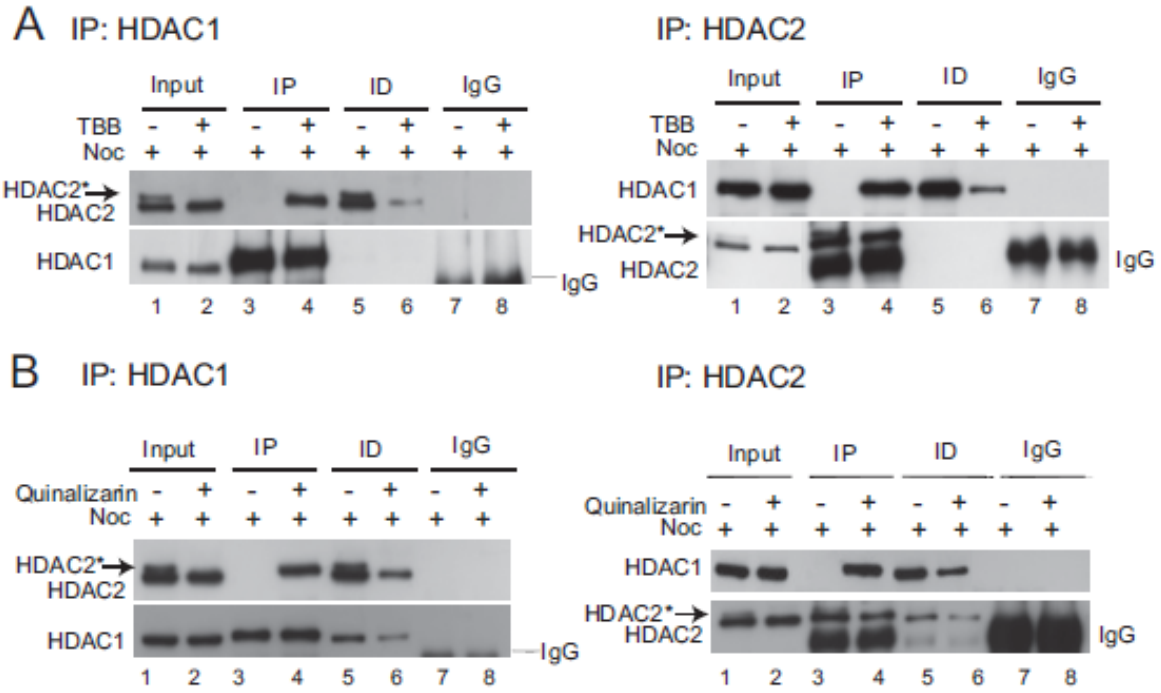

Figure 2.8: Inhibition of protein kinase CK2 activity prevents dissociation of HDAC1 and 2 during mitosis

A and B. Total cell lysates (500 µg) from nocodazole (Noc) and TBB-nocodazole treated (A) or from nocodazole and quinalizarin-nocodazole treated (B) HeLa cells were immunoprecipitated with 3.0 µg rabbit polyclonal anti-HDAC1 or anti-HDAC2 or control isotype-specific non-related rabbit IgG antibodies. The co-immunoprecipitates were checked by immunoblotting with mouse monoclonal anti-HDAC2 and rabbit polyclonal anti-HDAC1 antibodies (left panels of A and B) or mouse monoclonal anti-HDAC1 and rabbit polyclonal anti-HDAC2 antibodies (right panels of A and B). HDAC2*, phosphorylated form of HDAC2. IP and ID represent immunoprecipitated and immunodepleted fractions, respectively.

To check for the reproducibility of our findings, we repeated the experiment with another CK2 inhibitor, quinalizarin. Co-immunoprecipitation followed by immunoblotting analyses showed that HDAC1 or HDAC2 immuno-complexes from quinalizarin pretreated-nocodazole incubated cell extracts contained both HDAC1 and 2, while fractions immunoprecipitated by anti-HDAC1 or anti-HDAC2 antibodies from nocodazole treated cells had either HDAC1 or HDAC2 (**Figure 2.8B**).

We surmised that if CK2-mediated phosphorylation was sufficient to dissociate HDAC1 from HDAC2 during mitosis, then mutating all CK2-phosphorylation serines on HDAC2 would prevent HDAC1 and 2 dimer dissociation during mitosis. To test this idea, we prepared cell lysates from Flp-In 293-HDAC2 (WT)-V5 and triple mutant HDAC2 (M3A)-V5 cell lines and did immunoprecipitation with an antibody against the V5 tag. In lysates from cycling Flp-In 293-HDAC2 (WT)-V5 cells, HDAC1 and 2 were co-immunoprecipitated with the HDAC2-V5 (**Figure 2.9A**, left panel). In nocodazole incubated cells, the HDAC2 (WT)-V5 did not associate with HDAC1 (**Figure 2.9A**, right panel). However, the triple mutant HDAC2 (M3A)-V5 co-immunoprecipitated with HDAC1 and HDAC2 in cycling and nocodazole treated cells (**Figure 2.9A**).

To determine the relative contribution of the three HDAC2 phosphorylation sites to the dissociation of HDAC2/HDAC1 during mitosis, we constructed two FLAG-HDAC2 plasmids, FLAG-HDAC2-S394A and FLAG-HDAC2-S422/424A, where the S394 residue and S422/424 residues were mutated to alanine, respectively. HEK 293 cells were transfected with FLAG-HDAC2 wild type (WT), FLAG-HDAC2-S394A or FLAG-HDAC2-S424/424A plasmids.

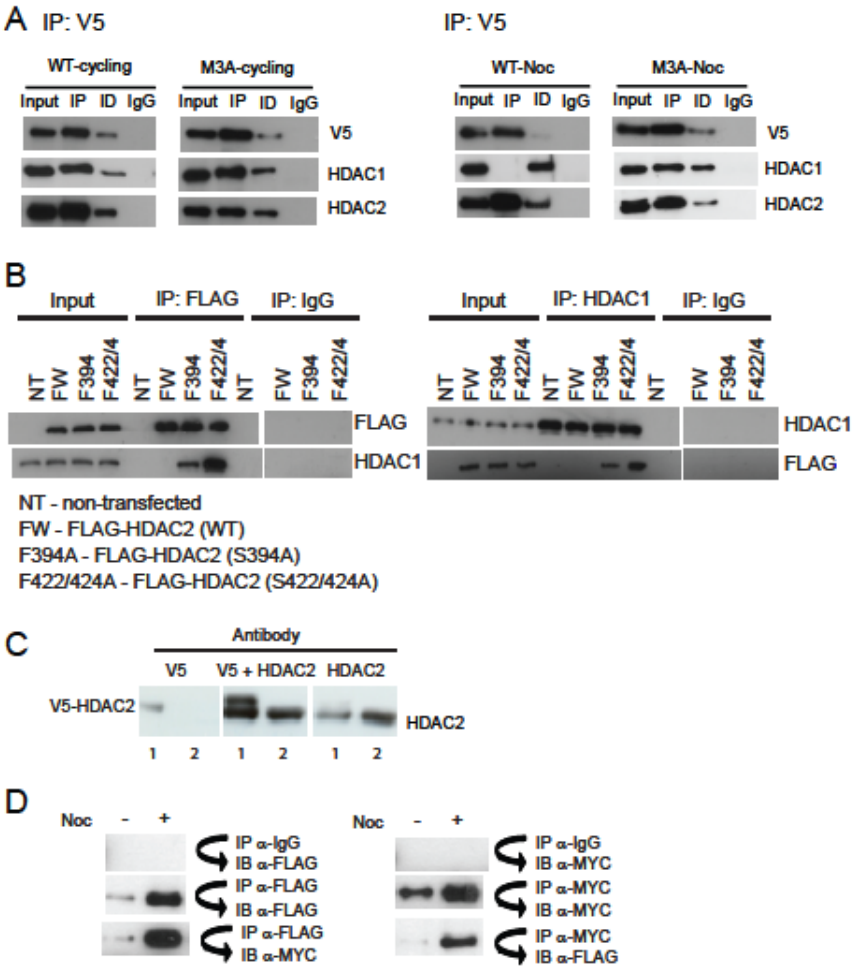

Figure 2.9: Formation of HDAC1 or 2 homodimers in mitosis

A. HDAC2-WT-V5 and HDAC2-M3A-V5 mutant proteins were immunoprecipitated with anti-V5 antibody from cycling (left) and nocodazole (Noc) treated (right) Flp-In 293 cells stably expressing HDAC2 WT or HDAC2 M3A mutant V5-tagged proteins. The immunoprecipitated fractions were analyzed for the presence of indicated proteins. B. HEK 293 cells were transfected individually with the indicated FLAG-HDAC2 plasmids. Thirty-two h after transfection, cells were treated with nocodazole for 16 h. Following this period, immunoprecipitations were carried out with anti-FLAG (left) or anti-HDAC1 (right) antibodies. The co-immunoprecipitates were analyzed by immunoblotting with anti-HDAC1 and anti-FLAG antibodies. Isotype-specific non related rabbit IgG was used as negative control. C. 40 µg of total cellular lysates from Flp-In 293-HDAC2 (WT)-V5 stable cells (lanes 1) and HeLa cells (lanes 2) were separated on a SDS-7.5% PAGE and were analyzed by immunoblotting with anti-V5, anti-V5 and anti-HDAC2 (together), or anti-HDAC2 antibodies. The shifted band corresponds to HDAC2-V5 protein. HeLa cellular lysates were used as a control. D. HEK 293 cells were co-transfected with HDAC1-MYC and HDAC1-FLAG plasmids. Thirty-two h after transfection, cells were left untreated or treated with nocodazole for 16 h. Following this period, immunoprecipitation of HDAC1 complexes was performed with anti-FLAG (left) and anti-MYC (right) antibodies. The immunoprecipitated fractions were analyzed for the presence of the indicated proteins. IB indicates immunoblot.

Following transfection, cell lysates were prepared from the nocodazole treated cells followed by immunoprecipitation with anti-FLAG antibodies and immunoblotting with anti-HDAC1 antibodies. **Figure 2.9B** left panel shows that in nocodazole treated cells FLAG-HDAC2 (WT) did not associate with HDAC1. FLAG-HDAC2 (S422/424A) associated with HDAC1 to a greater level than did FLAG-HDAC2 (S394A) with HDAC1. In the reciprocal experiment, nocodazole treated cell lysates were immunoprecipitated with anti-HDAC1 antibodies and the immunoprecipitated fractions were immunoblotted with anti-FLAG antibodies. **Figure 2.9B** right panel shows that HDAC1 did not co-immunoprecipitate with FLAG-HDAC2 (WT) from lysates of nocodazole treated HeLa cells. In contrast, HDAC1 associated with FLAG-HDAC2 (S422/424A) and to a lesser extent with FLAG-HDAC2 (S394A).

Overall, these data demonstrate that HDAC1 forms a heterodimer with HDAC2 in cycling cells, however, during mitosis, the dramatic increase in the steady state level of HDAC2 phosphorylation mediated by CK2, results in the dissociation of the HDAC1 and 2 heterodimers. Further, the results show that phosphorylation at Ser422, Ser424 and, to a lesser extent, Ser394 contribute to the dissociation of the HDAC1 from HDAC2 during mitosis.

2.4.5 HDAC1 and 2 form homodimers during mitosis

Figure 2.9C shows that V5-HDAC2 migrates slower than the endogenous HDAC2 and can be distinguished from untagged HDAC2. Thus **Figure 2.9A** right panel shows that HDAC2-V5 co-immunoprecipitates with untagged HDAC2 in nocodazole treated cells, providing evidence that HDAC2 forms homodimers in mitotic cells. To determine whether HDAC1 forms homodimers during mitosis, HEK 293 cells were co-transfected with HDAC1-FLAG and HDAC1-MYC

constructs. Cells were either left untreated or treated with nocodazole. Immunoprecipitations were carried out with anti-FLAG or anti-MYC antibodies and immunoprecipitated fractions were analyzed with anti-FLAG or anti-MYC antibodies. **Figure 2.9D**, left panel, shows that in cycling and nocodazole treated HEK 293 cells HDAC1-FLAG co-immunoprecipitated with HDAC1-MYC. Similar results were obtained in the reciprocal order, where immunoprecipitations were done with anti-MYC antibodies and the immunoprecipitates were analyzed with anti-FLAG antibody (**Figure 2.9D**, right panel). Again HDAC1-MYC co-immunoprecipitated with HDAC1-FLAG from nocodazole treated cell lysates.

2.5 Discussion

During mitosis, protein kinase CK2 activity is stimulated by CDC2 kinase (Escargueil et al., 2000). Further, protein phosphatase PP2A is excluded from the nucleus in early prophase, while CK2 remains nuclear until pro-metaphase (Escargueil and Larsen, 2007). Also, protein phosphatase PP1 activity is low until metaphase and increases at the metaphase-anaphase transition period (Wang et al., 2008). Our studies show that the highly phosphorylated state of HDAC2 is due to the activity of CK2. Interestingly, although both HDACs are substrates for CK2, HDAC2 is more extensively phosphorylated than HDAC1 by CK2 during mitosis in HeLa cells. A similar observation was reported for the differential phosphorylation of HDAC2 versus HDAC1 in mitotic K562 cells (Galasinski et al., 2002).

In interphase HeLa cells, most of the HDAC2 (86.5%, data not shown) is associated with HDAC1. In these cells, we found HDAC1 and 2 to be in a monophosphorylated state. Our data show that phosphorylation at Ser394 of HDAC2 is one of the HDAC2 monophosphorylated

forms. Also, our data show that mono-phosphorylation of HDAC2 at Ser394 is not sufficient to result in the reduced mobility observed for some of the HDAC2 phosphorylated forms. The reduced mobility of HDAC2 observed during mitosis must be due to phosphorylation at Ser422 and/or Ser424 of HDAC2 in a mono-, di- or tri-phosphorylated state.

The elevated phosphorylation level of HDAC2 and to a lesser extent HDAC1 during mitosis in HeLa cells results in dissociation of the HDAC1 and 2 heterodimer; however, the HDAC1 and 2 corepressor complexes remain intact. Previous reports demonstrate that HDACs, although displaced from mitotic chromosomes, are catalytically active (Kruhlak et al., 2001;Chuang et al., 2010). Our results measuring the activity of HDAC1 or 2 containing complexes isolated from mitotic cell lysates concur with this observation. Further, our data show that in mitotic cells the catalytically active HDAC1 and 2 complexes consist of HDAC1 and 2 homodimers, respectively, consistent with the requirement that HDAC1 and 2 form a homodimer or a heterodimer to be catalytically active (Jurkin et al., 2011;Luo et al., 2009).

Current evidence suggests that the extent of phosphorylation of proteins associated with the HDAC1 and 2 multiprotein complexes have impact on the composition and integrity of the complexes. Treatment of K562 cells with okadaic acid to inhibit protein phosphatase activity resulted in the hyperphosphorylation of HDAC2, the dissociation of HDAC1 from HDAC2, and the dissociation of the Sin3 HDAC complex (Galasinski et al., 2002). Under these conditions multiple proteins including those in the multiprotein HDAC complexes likely become highly phosphorylated and contribute to the dissociation of the HDAC1 and 2 complexes. However, during mitosis CK2 mediated phosphorylation of HDAC2 is sufficient to dissociate HDAC1

from HDAC2 but the multiprotein complex remains intact and catalytically active. Further, the CK2-mediated phosphorylation of HDAC1 and 2 during mitosis may promote increased levels of HDAC1 or 2 corepressor complex formation as indicated by the increased association of RbAp48, a component of the Sin3A and NuRD corepressor complexes, with HDAC1 during mitosis.

The significance and the functional role of the formation of HDAC1 or 2 homodimers within the corepressor complexes during mitosis awaits further analysis; however, the HDAC1 or 2 complexes may have an opportunity to deacetylate various proteins during mitosis. Multiple proteins are acetylated during mitosis (Chuang et al., 2010). HDAC inhibitors such as apicidin, increase the acetylation state of the anaphase promoting complex 1 (APC1) and the dynein/dynactin associated and Polo-like kinase 1 (Plk1)-interacting protein NudC (nuclear distribution protein C). The acetylated state of these proteins may govern their function in mitosis and/or cytokinesis (Chuang et al., 2010). HDAC1 and 2 have both distinct and redundant functions (Jurkin et al., 2011;Delcuve et al., 2012). As an example of specific role for HDAC2, it is the HDAC2 homodimer CoREST corepressor complex, not the HDAC1 complex, that is involved in the silencing of genes involved in synaptic plasticity and memory in hippocampus neurons (Guan et al., 2009). It is possible that corepressor complexes with HDAC1 or 2 homodimers are directed to specific substrates during mitosis.

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Chapter 3: Dynamic Distribution of HDAC1 and 2 during Mitosis: Association with F-actin

3.1 Abstract

During mitosis, HDAC2 becomes highly phosphorylated through the action of protein kinase CK2, and HDAC1 and 2 are displaced from mitotic chromosomes. KATs and HDAC1 and 2 corepressor complexes regulate dynamic protein acetylation and gene expression. In this study, we show that HDAC1 and 2 associate with F-actin in mitotic cells. Inhibition of Aurora B or protein kinase CK2 did not prevent the displacement of HDAC1 and 2 from mitotic chromosomes in HeLa cells. Further, proteins of the HDAC1 and 2 corepressor complexes and transcription factors recruiting these corepressors to chromatin, were dissociated from mitotic chromosomes independent of Aurora B activity. HDAC1 and 2 returned to the nuclei of daughter cells during lamin A/C re-assembly and before Sp1, Sp3 and RNAPII. Our results show that HDAC1 and 2 corepressor complexes are removed from the mitotic chromosomes and are available early in the events leading to the re-establishment of the gene expression program in daughter cells.

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Dilshad H. Khan optimized the biochemical conditions used to prepare figures 3.6 to 3.9 in this paper, assisted in the drafting and editing of the manuscript.

3.2 Introduction

In epigenetic programming, DNA replication and mitosis are two critical periods in which the epigenetic program is transmitted from the mother to the daughter cells. At the onset of mitosis in mammalian cells, Aurora B-mediated H3S10ph and H3S28ph occurs concomitantly with the condensation of interphase chromatin into metaphase chromosomes (Hendzel et al., 1997;Perez-Cadahia et al., 2009). RNAPII, chromatin remodeling complexes and most transcription factors are excluded from chromatin and dispersed throughout the cells, while the bulk of transcription is arrested (Zaidi et al., 2010b;Rizkallah et al., 2011). As cells exit mitosis, chromosomes decondense and components of the transcription machinery and regulatory proteins re-enter the newly formed daughter nuclei in a sequential and controlled manner (Martinez-Balbas et al., 1995;Zaidi et al., 2003;Prasanth et al., 2003;He and Davie, 2006;Delcuve et al., 2008). With this spatio-temporal reorganization taking place, reformation of daughter nuclei is a critical time to re-establish or to alter the epigenetic program and gene expression profile.

During mitosis both KATs and HDACs are displaced from mitotic chromosomes (Kruhlak et al., 2001). Consistent with the displacement of the HDACs from the mitotic chromatin, HDAC inhibitors do not induce histone hyperacetylation in mitotic HeLa cells (Patzlaff et al., 2010). However, the HDACs maintain their activities in the mitotic cells (Kruhlak et al., 2001;He and Davie, 2006). As with many chromatin remodeling and transcription factors, HDAC2 becomes highly phosphorylated during mitosis (Galasinski et al., 2002;Olsen et al., 2010). HDAC1 and 2 re-associate with the chromatin in late telophase/early interphase (Kruhlak et al., 2001). HDAC1 and 2 exist in interphase cells as multiprotein corepressor complexes (Sin3, NuRD and CoREST) complexes (Sun et al., 2002;Sun et al., 2007;Segre and Chiocca, 2011). The Sin3 core complex

contains Sin3A or Sin3B, HDAC1 and 2, SAP18, SAP30, RbAp46/48 (Delcuve et al., 2012). The CoREST HDAC complex consists of HDAC1 and 2, CoREST, LSD1 and other proteins (Hayakawa and Nakayama, 2011). The HDAC1 and 2 corepressor complexes are recruited to regulatory regions of transcribed and repressed genes by a number of transcription factors such as Sp1 and Sp3 (De Ruijter et al., 2003;Sun et al., 2007). The HDAC-corepressor complexes and KATs play important roles in the epigenetic regulation of gene expression by remodeling chromatin via their action in catalyzing dynamic acetylation of histones, transcription factors and chromatin modifying enzymes.

The factors promoting the dissociation of HDAC1 and 2 from mitotic chromosomes have not been determined. Furthermore, the timing of these enzymes re-entry into the newly formed daughter nuclei, particularly their chronological order of re-entry compared to other transcription and epigenetic regulatory proteins is currently unknown.

In this study, we explored the factors that may be involved in displacement of HDAC1 and 2 from mitotic chromosomes. Here, we report that during mitosis in HeLa and MCF7 cells, HDAC1 and 2 dissociate from mitotic chromosomes and associate with F-actin. Inhibition of Aurora B-mediated H3 phosphorylation or of CK2-mediated HDAC2 phosphorylation was not sufficient to prevent the displacement of HDAC1 and 2 and several transcription factors, from mitotic chromosomes. HDAC1 and 2 returned to the newly formed nuclei of the daughter cells before Sp3, Sp1 and RNAPII.

3.3 Materials and Methods

3.3.1 Cell culture

MCF7 and HeLa cells were grown in DMEM (Invitrogen) supplemented with 10% FBS, 1.0 % D-glucose, 2 mM L-glutamine, and antibiotic-antimycotic (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2.

3.3.2 Indirect immunolocalization and fluorescence microscopy

Indirect immunolocalization was performed as described previously (He and Davie, 2006). The following antibodies were used: mouse monoclonal antibodies against Sp1 (1:50, Santa Cruz Biotechnology), HDAC2 (1:250, Millipore), lamin A/C (1:50, Novocastra Laboratories), RNAPII (clone CTD4H8) (1:2000, Millipore), cytokeratin 18 (1:250, Zymed Laboratories), α-tubulin (1:2000, Sigma), CENP-A (1:200, Abcam) and rabbit polyclonal antibodies against Sp1 (1:1000, Millipore), Sp3 (1:500, Santa Cruz Biotechnology), HDAC1 (1:5000, Affinity BioReagents), HDAC2 (1:2500, Affinity BioReagents), H3S10ph (1:1000, Santa Cruz Biotechnology), Sin3A (1:50, Affinity BioReagents), CoREST (1:50, Abcam), and rat monoclonal antibodies against H3S28ph (1:1000, Sigma). Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) or anti-rabbit IgG (Invitrogen), Alexa Fluor 568 donkey anti-rabbit IgG (Invitrogen) or anti-mouse IgG (Invitrogen), and Texas Red conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. DNA was counterstained with DAPI and the coverslips were mounted onto glass slides using the Prolong Gold anti-fade reagent (Invitrogen). Control experiments including epitope-peptide-blocking or primary-antibody-omission demonstrated the specificities of the antibodies used. Digital images were captured with Zeiss Axio Imager Z1 microscope and AxioCam HRm camera. The images

were captured either in Apotome mode or the stack images with 100 slices at stepwise of 200 nm. The deconvolution analysis of stack images was done with the AxioVision software (Carl Zeiss, Germany).

3.3.3 Quantitative image analysis

Quantitative image analysis was performed as described previously (He et al., 2005;He and Davie, 2006). Briefly, in a linescan, the merged images were imported into the MetaMorph imaging software (Universal Imaging, Downingtown, PA). The pixel intensity in each channel was then plotted versus the position along a straight line across the image. A superposition of red, green, and blue peaks indicates image overlap. Furthermore, partition coefficient (PC), expressed as the ratio of integrated signal intensities between two daughter cells ($PC = I_x/I_{y,}$ where I_x and I_y are integrated pixel intensities of each of the progeny cells), was determined to indicate the relative protein or DNA distribution between two daughter nuclei following mitosis. The significance of the observed differences was tested by two-tail paired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

3.3.4 Formaldehyde-cross-linked DNA bound protein isolation

Formaldehyde cross-linking was done as described previously (Sun et al., 2002;Spencer and Davie, 2002). Briefly, proteins cross-linked to DNA were isolated by hydroxyapatite column chromatography. DNA-protein cross-links were reversed, and proteins were isolated, dialyzed and analyzed by immunoblotting.

3.3.5 Immunoblotting

Immunoblot analysis was carried out as described previously (Samuel et al., 1998). Rabbit polyclonal antibodies against human HDAC1 (Affinity BioReagents), HDAC2 (Affinity BioReagents), Sp3 (Santa Cruz), H3S10ph (Santa Cruz), Histone H3 (Millipore), and mouse monoclonal MPM-2 (Mitotic protein monoclonal-2) (Millipore) antibodies, were used.

3.3.6 Treatment conditions for CK2 and Aurora B inhibitors

Cells grown on the coverslips were treated with 100 μM of TBB (Calbiochem) for 12 h before the addition of nocodazole (1μM) (Sigma) for another 16 h, and the cells were then analyzed by immunofluorescence microscopy. Cells grown on coverslips were treated with 2 mM thymidine for 24 h, released into fresh media for 1 h, and then treated with or without 2.5 μM ZM447439 (Aurora B inhibitor) (Tocris Bioscience), by changing media every 2 h, for total of 8 h. After the treatment, the cells were subjected to immunostaining procedures.

3.3.7 In vitro peptide pull down assay

Nuclear extracts from logarithmically growing HeLa cells (250-500 μg at 0.25-0.5 μg/μL in PD-buffer) were incubated with differentially modified histone H3 peptides. All histone H3 peptides (residues 1-20) were purchased from 'Peptide Specialty Laboratories GmbH' (Heidelberg, Germany) except the H3S10ph, the H3K9me2/3, the H3S10phK9me2/3, which were a gift from Thomas Jenuwein (Max-Planck Institute of Immunobiology, Freiburg, Germany). Peptides were covalently coupled to agarose beads via free sulfhydryl-groups present at the carbox-terminus (SulfoLink Kit, Pierce Biotechnology) at a concentration of 2.5 μg peptide/μL solid gel. Coupling efficiency was monitored by peptide dot-blotting and Ponceau staining or using

Ellman's reagent. After washing of the binding reactions [3 times with PD-buffer (20 mM Tris-HCl pH 8.0, 135 mM NaCl, 0.5% NP-40, 10% glycerol, supplemented with phosphatase inhibitors: 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 10 mM sodium butyrate), 1 time with radio-immunoprecipitation assay (RIPA) 300 buffer (300 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% NP-40 with phosphatase inhibitors: 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 10 mM sodium butyrate), again 3 times with PD-buffer], bound proteins were eluted by boiling in SDS-sample buffer and analyzed by immunoblotting sequentially with anti-RbAp48 (Millipore), anti-HDAC1 (Millipore), anti-HDAC2 (Millipore), anti-HP1γ (Millipore) and anti-14-3-3 ζ (affinity purified Serum) antibodies.

3.4 Results

3.4.1 Mitotic re-distribution of HDAC1 and 2

The spatial distributions of HDAC1 and 2 in human breast cancer cell line MCF7 (**Figure 3.1**) and cervical cancer cell line HeLa (**Figure 3.2**) were analyzed at different stages of mitosis by fluorescence microscopy after indirect immunofluorescence labeling of cells grown and fixed on cover slips. The cells from each mitotic stage were identified according to their DAPI staining in the cycle-asynchronized cell population. In both cell lines, following disassembly of the nuclear membrane, HDAC1 and 2 immunostaining was dispersed throughout the cell, with HDAC1 and 2 being displaced from the condensed chromosomes in prophase, and re-associating with the newly formed daughter nucleus in telophase. The linescans highlight the displacement of HDAC1 and 2 from the metaphase chromosomes in MCF7 and HeLa cells (**Figures 3.1** and **3.2**).

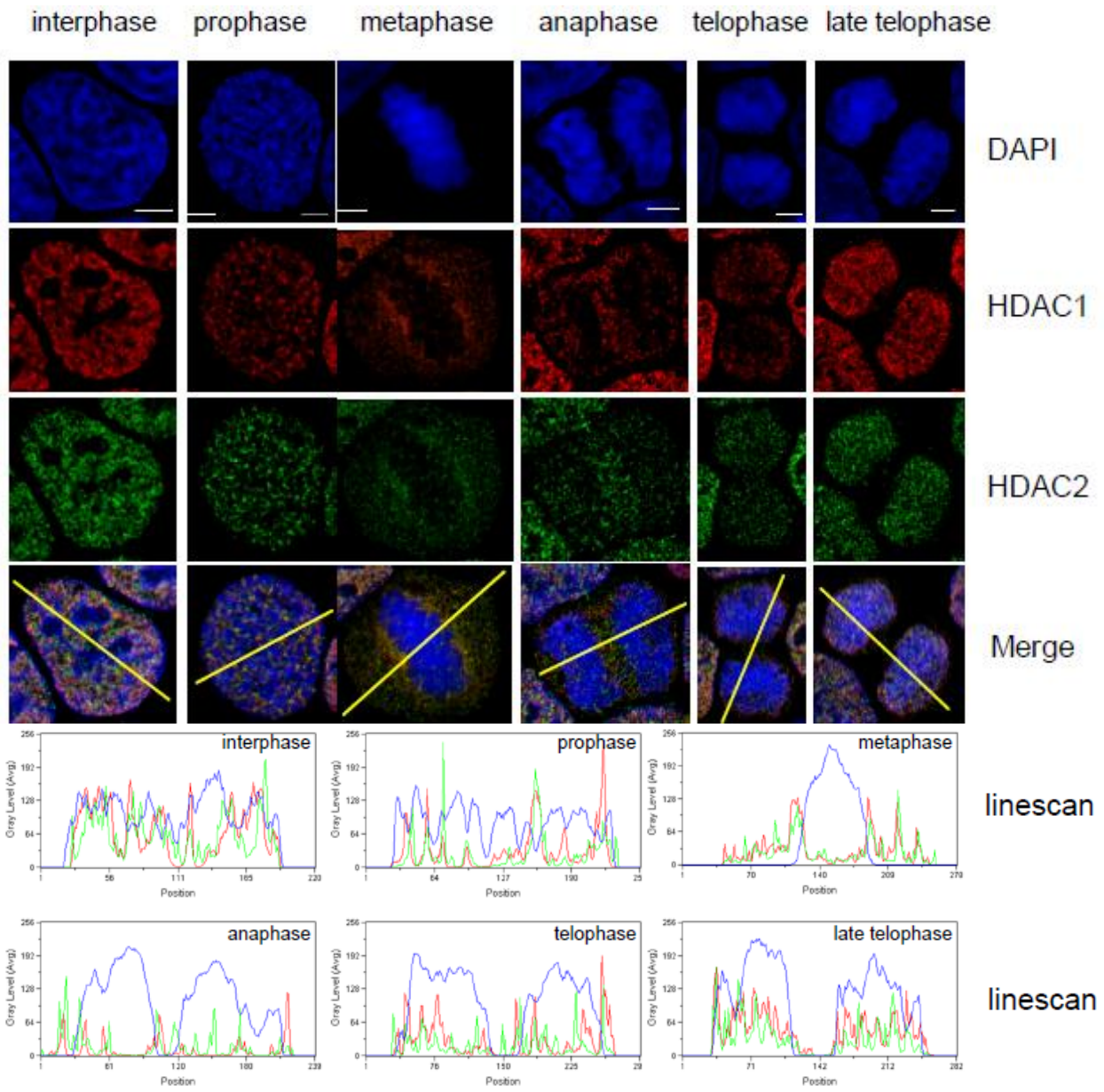

Figure 3.1: Mitotic distribution of HDAC1 and 2 in MCF7 cells
MCF7 cells were subjected to indirect immunofluorescence labeling with HDAC1 (rabbit polyclonal) and HDAC2 (mouse monoclonal) antibodies, and co-stained with DAPI, for identification of different stages of cell cycle. Cells at various mitotic stages were digitally imaged. The linescan analysis was performed with MetaMorph software as described in 'Materials and Methods' section. The blue, red, and green lines show the distribution of the DNA, HDAC1 and HDAC2, respectively. Bar, 5µm.

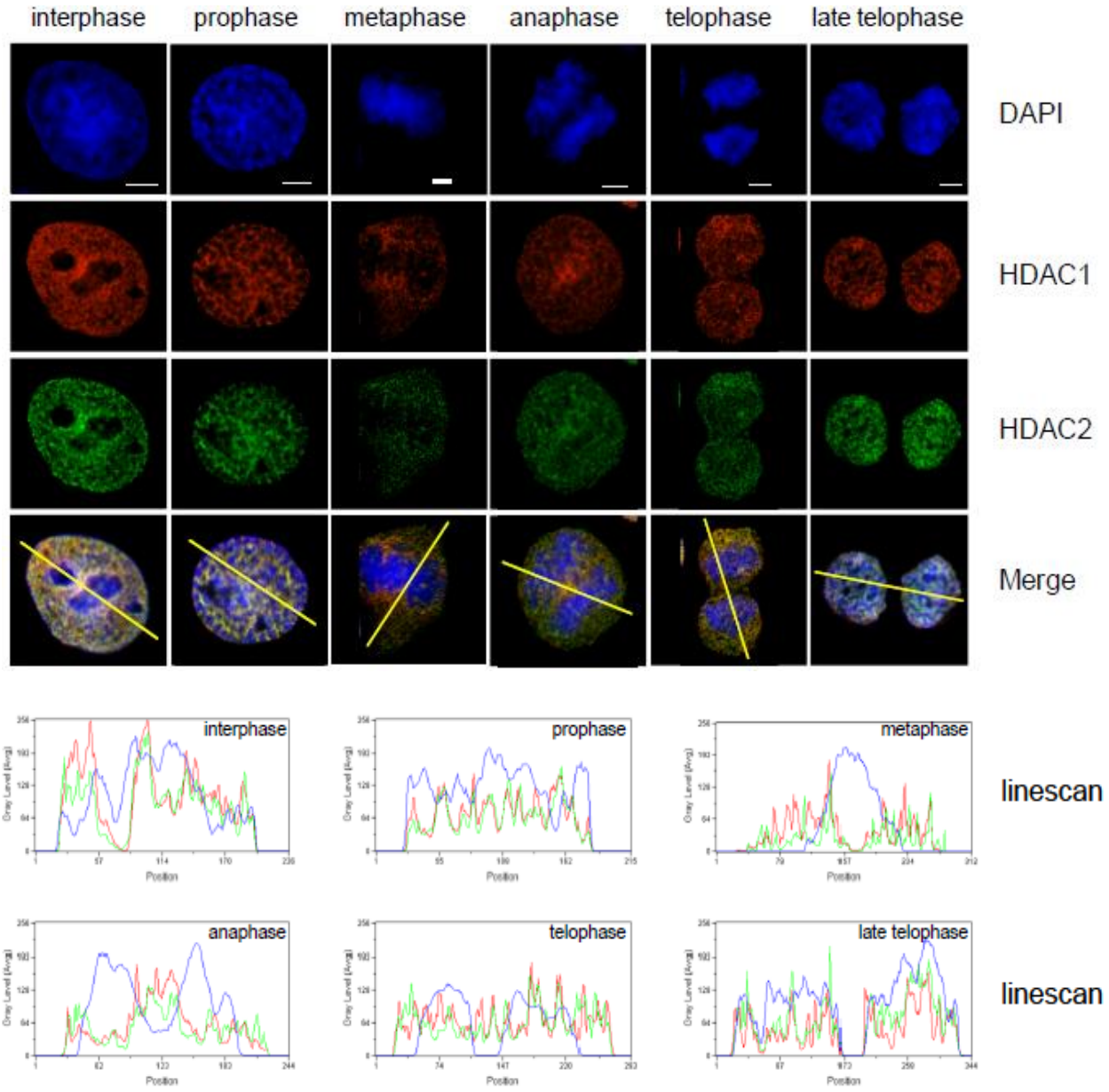

Figure 3.2: Mitotic distribution of HDAC1 and 2 in HeLa cells
HeLa cells at various mitotic stages were digitally imaged and analyzed as described in Figure 3.1.

Formaldehyde cross-linking approach was applied to further investigate whether HDAC1 and 2 were dissociated from mitotic chromosomes. Treatment of the HeLa cycling and mitotic (nocodazole treated and shake-off) cells with formaldehyde was followed by capture of the genomic DNA on hydroxyapatite columns and subsequently the release of the proteins cross-

linked to DNA. The DNA cross-linked proteins were resolved by SDS-PAGE followed by immunoblotting analysis (**Figure 3.3**). Cycling cells, but not mitotic HeLa cells, had HDAC1 and 2, and, Sp3 bound to genomic DNA, providing evidence that these three chromatin associated proteins were displaced from mitotic chromosomes. **Figure 3.3** also shows that the level of mitotic Aurora B-mediated H3S10ph associated with genomic DNA has increased significantly compared to the H3S10ph levels in cycling cells.

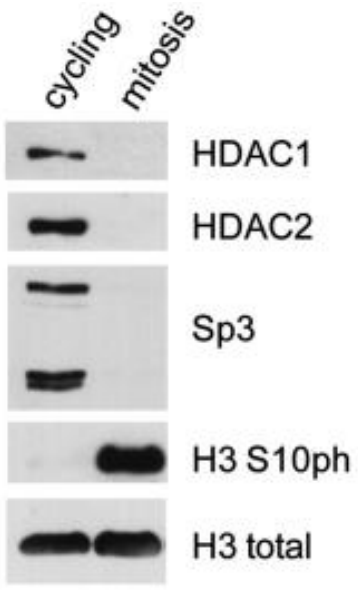

Figure 3. 3: Displacement of HDAC1/ 2 from mitotic chromosomes
From control and nocodazole treated cells, proteins cross-linked to DNA by formaldehyde were isolated and analyzed by inmmunoblotting with antibodies against HDAC1, HDAC2, Sp3, and H3S10ph. H3 was used as a loading control.

We did not observe association of HDAC1 and 2 with centromeres in MCF7 and HeLa mitotic cells using CENP-A antibodies as was previously observed in human lymphoblastoid cells and mouse T cell line VL3-3M2 cells (**Figure S1**) (Craig et al., 2003).

3.4.2 Mitotic association of HDAC1 and 2 with F-actin

Our previous studies demonstrated that Sp1 and Sp3 localized with microfilaments during mitosis (He and Davie, 2006). To address the question of whether HDAC1 and 2 also bound to a scaffold structure in a mitotic cell, we used immunofluorescence microscopy and image deconvolution to determine the spatial distribution of HDAC1 and 2 with various structures. We performed double immunolabeling of HDAC1 and 2 with intermediate filament protein cytokeratin 18, microtubule forming protein, α -tubulin and microfilament F-actin in metaphase MCF7 cells. **Figure 3.4** shows that HDAC1 and 2 did not co-localize with cytokeratin 18 and α-tubulin. Conversely, there was a high degree of co-localization between HDAC1 and 2 and the microfilaments F-actin, with HDAC1 and 2 staining aligned along the actin fibers. These observations suggest that the microfilaments are involved in the organization of HDAC1 and 2 in mitotic cells.

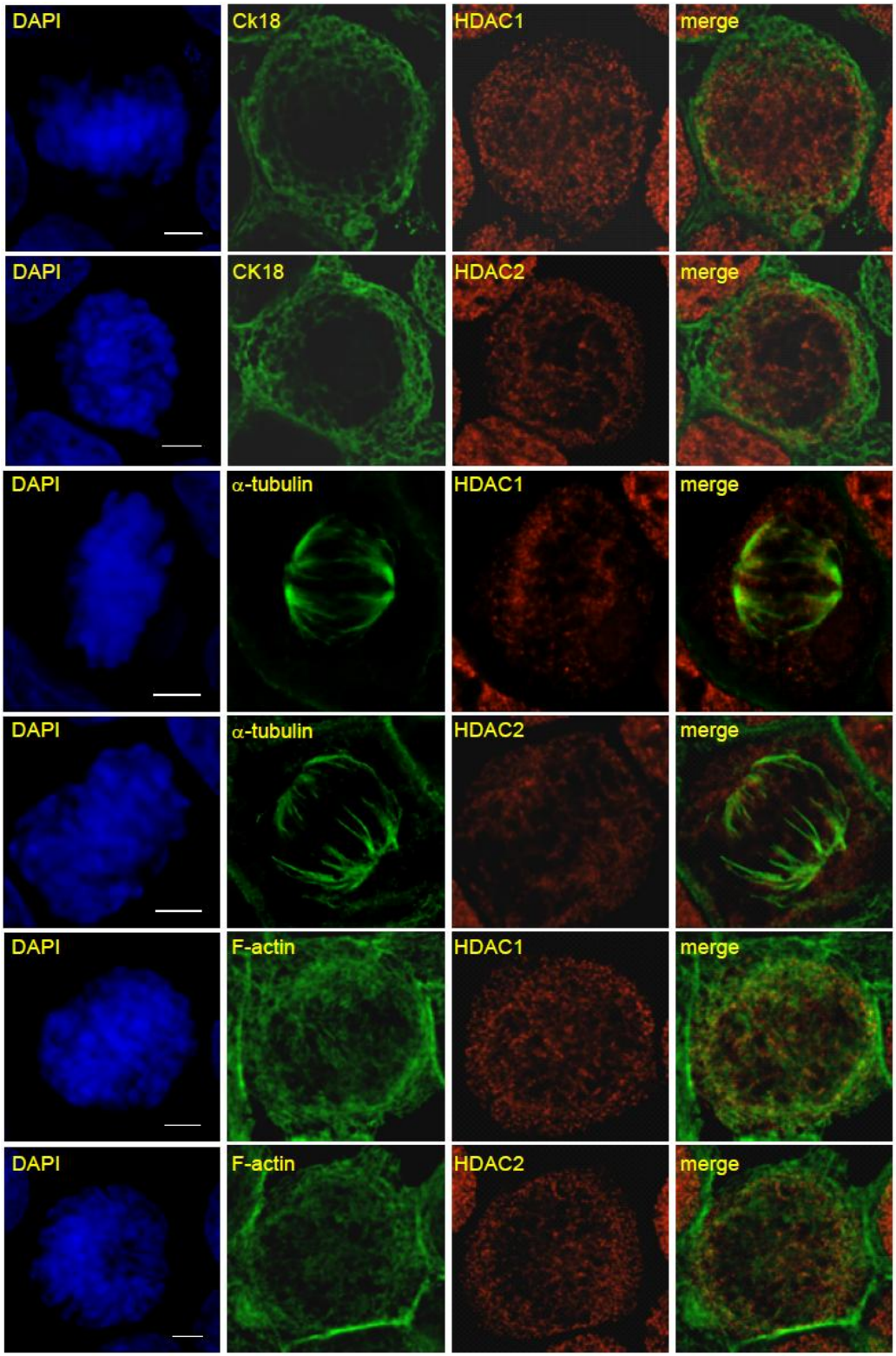

Figure 3.4: Mitotic association of HDAC1 and 2 with F-actin
MCF7 cells were grown, fixed, and immunostained with anti-HDAC1, anti-HDAC2, anti-cytokeratin 18 (CK18), and anti-α-tubulin antibodies. F-actin was labeled by Alexa-Fluor-488

conjugated phalloidin. DNA was stained by DAPI. Spatial distribution was visualized by fluorescence microscopy and image deconvolution as described in 'Materials and Methods' section. Yellow signal in the merge images indicates the co-localization. Bar, 5µm.

3.4.3 Mitotic histone H3 phosphorylation

As the phosphorylation of histone H3 changes profoundly in mitosis, we determined whether H3S10ph with or without other additional PTMs would impact HDAC1 and 2 associations with the N-terminal tail of H3 (amino acids 1-20). Nuclear extracts from HeLa cells were incubated with H3 peptides containing a range of PTMs. Proteins binding to the unmodified and modified H3 peptides were detected in immunoblot experiments. **Figure 3.5** shows that in agreement with previous studies, H3 peptides with S10ph and K14ac bound 14-3-3 ζ, independently of the methylation status of K9 (Winter et al., 2008a; Winter et al., 2008b).

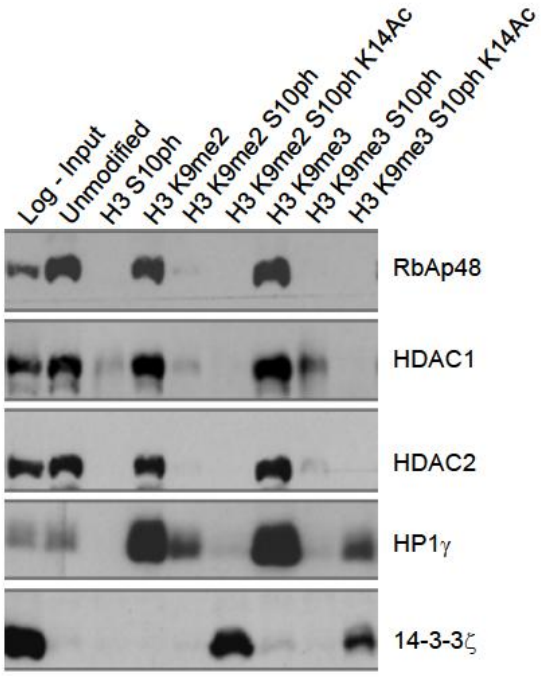

Figure 3.5: HDAC1 and 2 do not bind to histone H3S10ph
Nuclear extracts from HeLa cells were incubated with unmodified and modified histone H3 peptides as indicated in the figure and described in 'Materials and Methods' section. After extensive washing, bound proteins were analyzed by immunoblotting with antibodies against RbAp48, HDAC1, HDAC2, HP1γ, and 14-3-3 ζ.

HP1γ was bound strongly to H3 di- or trimethylated at K9, but this binding was greatly diminished when S10 was phosphorylated. HDAC1 and 2 bound to unmodified and K9me2 or K9me3 modified H3 peptides, but binding was lost when S10 was phosphorylated. These observations suggested that Aurora B-mediated H3S10ph may contribute to the events resulting in the dissociation of HDAC1 and 2 from mitotic chromosomes.

It has been demonstrated that the bulk of H3 phosphorylation starts from the pericentromeric heterochromatin at late G2 phase (Hendzel et al., 1997). To test if H3 phosphorylation has a role in the displacement of HDACs *in situ*, we did double staining with antibodies against HDAC2 and H3S10ph in MCF7 and HeLa cells grown on coverslips. G2 phase cells were determined according to H3S10ph staining in defined nuclear domains and DAPI staining of chromatin in diffuse pattern. We observed that HDAC2 was excluded from condensed pericentromeric heterochromatin, which is associated with H3S10ph during late G2 phase of the cell cycle (**Figure 3.6A**) (Hendzel et al., 1997). This observation is consistent with H3S10ph preventing the binding of HDAC2 to chromatin.

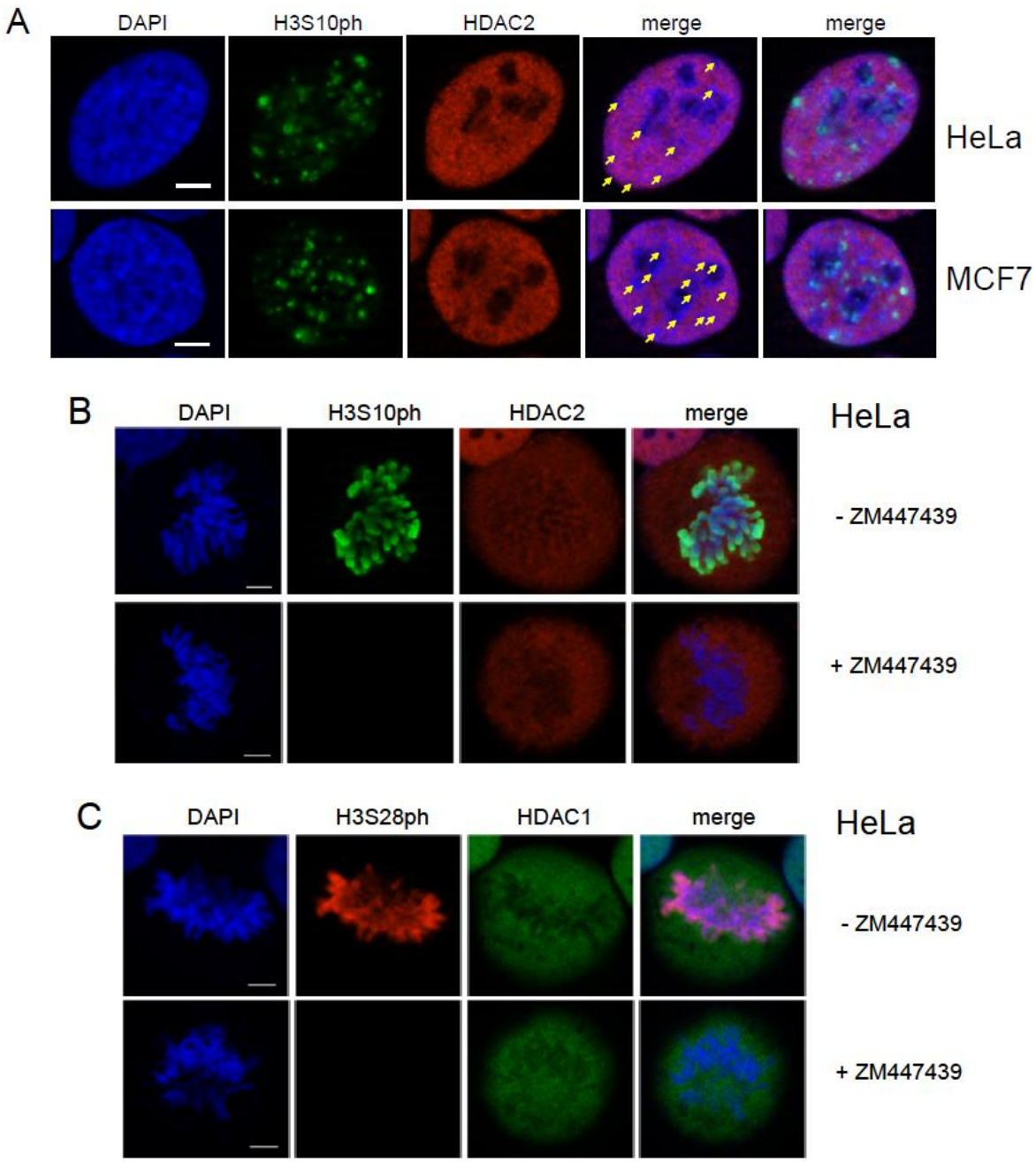

Figure 3.6: Aurora B independent dissociation of HDAC1 and 2 from condensed chromosomes in HeLa cells

A. HeLa and MCF7 cells grown on coverslips were fixed and immunostained with antibodies against H3S10ph and HDAC2, and co-stained with DAPI. The areas indicated by the arrows show the absence of HDAC2 from the heterochromatin which is phosphorylated at ser10 of H3. B, C. HeLa cells grown on coverslips were treated with or without ZM447439 as described in 'Materials and Methods'. After the treatment, the cells were subjected to immunostaining procedures with antibodies against H3S10ph and HDAC2 (B), H3S28ph and HDAC1 (C), and co-stained with DAPI for identification of cell cycle stages. Digital images were captured in Apotome mode. Bar, 5µm.

H3 is phosphorylated by protein kinase Aurora B during mitosis. To explore the role of H3 phosphorylation mediating the displacement of HDAC1 and 2 from mitotic chromosomes, the Aurora B inhibitor, ZM447439, was used to prevent H3 from becoming highly phosphorylated. HeLa cells were synchronized by treatment with thymidine block. Once released from the block, the cells were cultured in the presence or absence of ZM447439 for 8 h. Previous studies have shown that the Aurora B inhibitor, ZM447439, prevents the completion of chromosome condensation; however, the initial stages of chromosome condensation are not blocked (Gadea and Ruderman, 2005). Without ZM447439 treatment, H3S10ph and H3S28ph staining was clearly detected along the condensed chromosomes during mitosis (**Figure 3.6B**). After ZM447439 treatment, H3S10ph and H3S28ph immunostaining was absent during mitosis. In HeLa cells treated with ZM447439, HDAC1 and 2 remained largely displaced from the condensed chromosomes, which lacked H3S10ph (**Figure 3.6B**) and H3S28ph (**Figure 3.6C**). These observations suggest that H3 phosphorylation is not sufficient to displace the HDACs from mitotic chromosomes.

3.4.4 HDAC1 and 2 associated proteins are displaced from mitotic chromosomes, independent of Aurora B activity

HDAC1 and 2 corepressor complexes are recruited to the genome by regulatory proteins, such as transcription factors and RNAPII (Wang et al., 2009). We applied immunofluorescence microscopy of synchronized HeLa cells incubated with and without the Aurora B inhibitor, ZM447439, to determine whether the proteins associated with HDAC1 and 2 were dissociated from the mitotic chromosomes independent of Aurora B activity and H3 phosphorylation. As

shown in **Figure 3.7**, Sin3A and CoREST were displaced from mitotic chromosomes, independent of Aurora B activity.

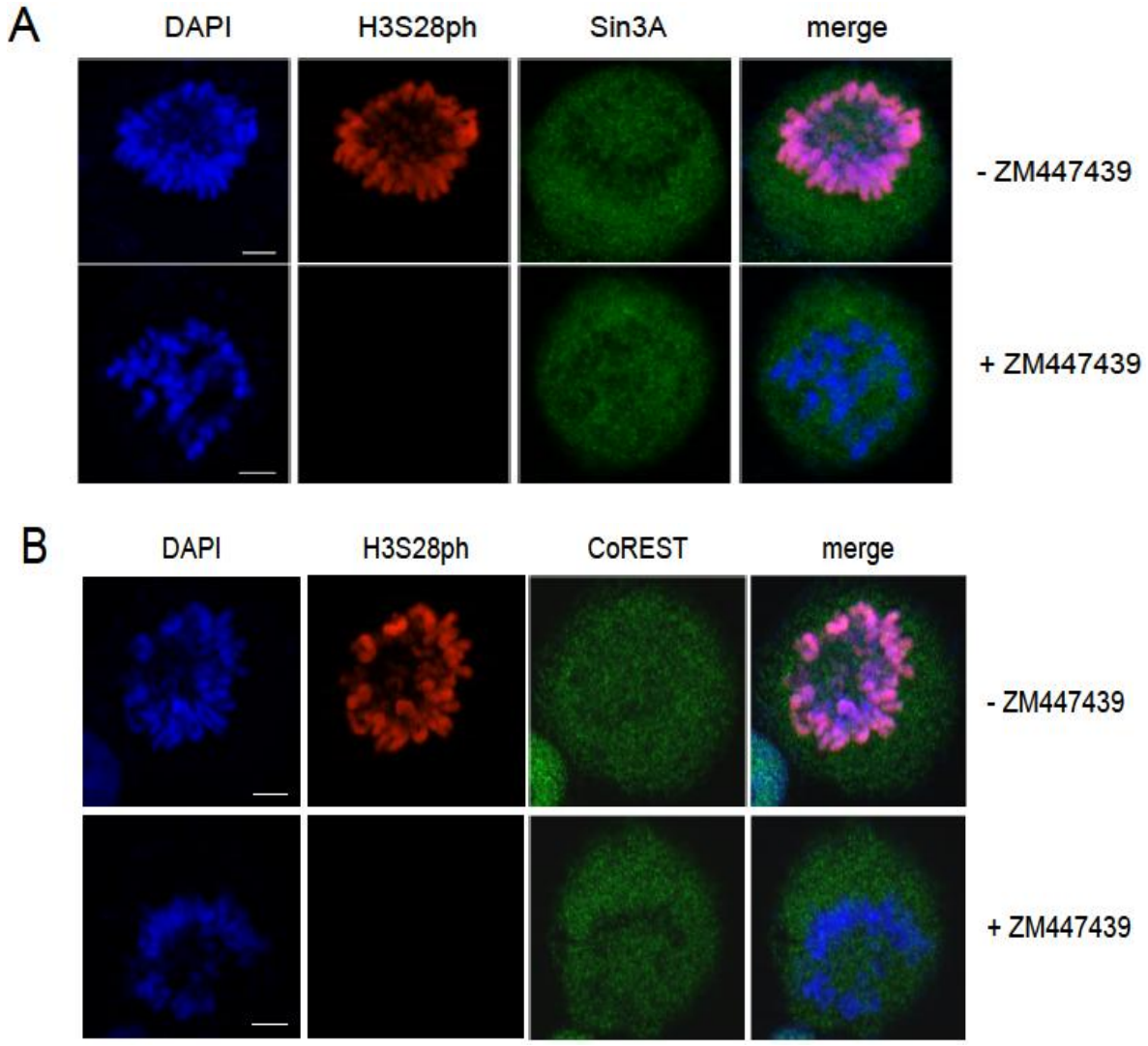

Figure 3.7: Sin3A and CoREST dissociated from condensed chromosomes in HeLa cells treated with the Aurora B inhibitor, ZM447439
HeLa cells grown on coverslips were synchronized and then treated with or without ZM447439. After the treatment, the cells were subjected to immunostaining procedures with antibodies against H3S28ph and Sin3A (A) or CoREST (B), and co-stained with DAPI for identification of cell cycle stages. Digital images were captured in Apotome mode. Bar, 5μm.

We reported previously that Sp1 and Sp3 recruit HDAC1 and 2 complexes to regulatory regions of the genome (Sun et al., 2002). Similar to Sin3A and CoREST, Sp1 and Sp3 were displaced

from the mitotic chromosomes independent of Aurora B activity (**Figure 3.8**). Together these results demonstrate that the proteins associated with and those that recruit HDAC-corepressor complexes are displaced from mitotic chromosomes, an event that does not require the activity of Aurora B.

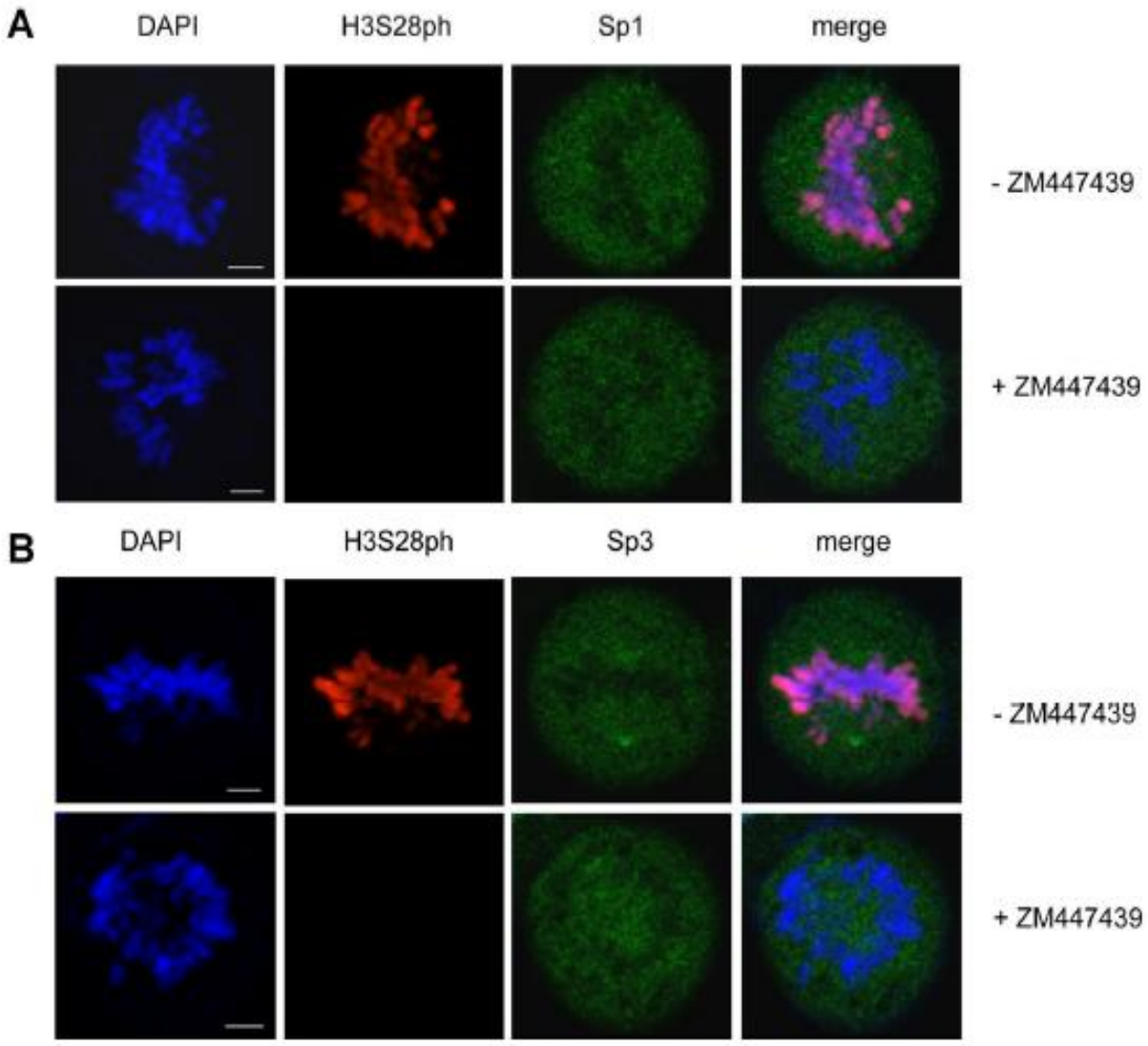

Figure 3.8: Sp1 and Sp3 dissociated from condensed chromosomes in HeLa cells treated with the Aurora B inhibitor, ZM447439
HeLa cells grown on coverslips were synchronized and then treated with or without ZM447439. After the treatment, the cells were subjected to immunostaining procedures with antibodies against H3S28ph and Sp1 (A) or Sp3 (B), and co-stained with DAPI for identification of cell cycle stages. Digital images were captured in Apotome mode. Bar, 5μm.

3.4.5 HDAC1 and 2 associated proteins are displaced from mitotic chromosomes, independent of protein kinase CK2 activity

Galasinski et al reported that nocodazole incubated cells, which were blocked in prometaphase, had elevated levels of highly phosphorylated HDAC2; the phosphorylation of which is catalyzed by protein kinase CK2 (Galasinski et al., 2002;Olsen et al., 2010). We investigated whether inhibition of CK2 with TBB would prevent HDAC2 phosphorylation and the displacement of HDAC1 and 2 from prometaphase chromosomes in HeLa cells. Nocodazole treated HeLa cells had increased levels of phosphorylated HDAC2 (**Figure 3.9A**).

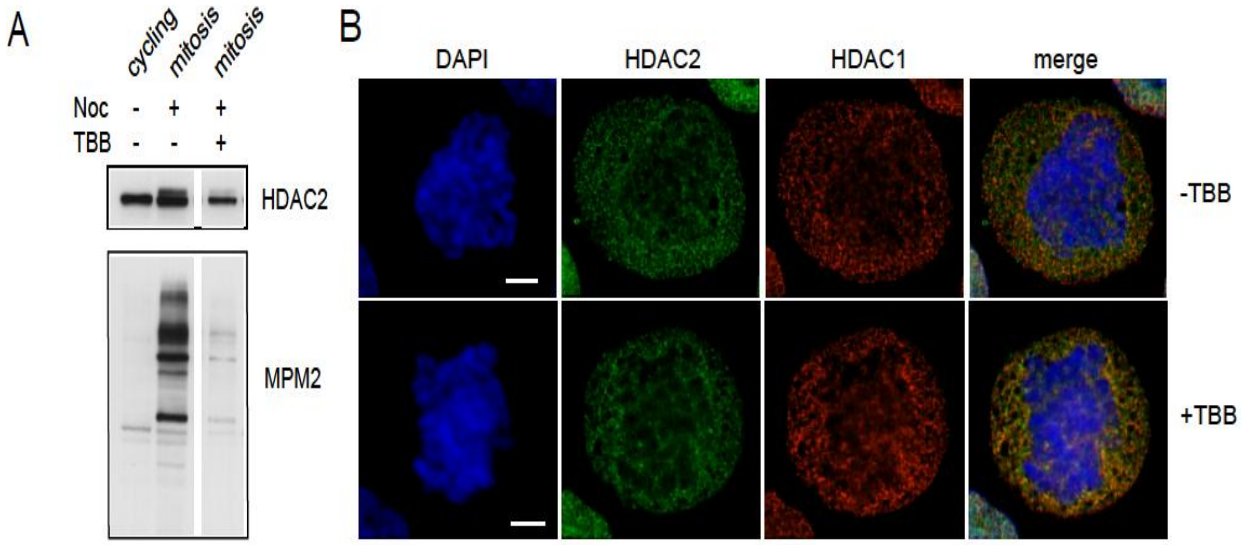

Figure 3.9: HDAC1 and HDAC2 dissociated from condensed chromosomes in HeLa cells treated with the protein kinase CK2 inhibitor, TBB
A. Whole cell lysates from cycling, TBB and/or nocodazole treated cells were separated by SDS-PAGE and subsequently immunoblotted with anti-HDAC2 and MPM-2 antibodies. B. HeLa cells grown on the coverslips were treated with or without TBB before the addition of Nocodazole. The cells were then fixed and immunostained with antibodies against HDAC1 and HDAC2, and co-stained with DAPI for identification of cell cycle stages. Bar, 5µm.

Further an increase in the level of phosphorylated proteins in the prometaphase HeLa cells was detected with the MPM-2 antibody, which detects cell cycle-regulated phosphorylated proteins in mitotic cells (Escargueil et al., 2000). Inhibition of CK2 with TBB reduced the level of highly

phosphorylated HDAC2 and reduced the immunostaining of proteins with the MPM-2 antibody. However, TBB-inhibition of CK2 did not prevent the displacement of HDAC1 and 2 from prometaphase chromosomes (**Figure 3.9B**).

3.4.6 Sequential entry of HDAC1 and 2 into daughter nuclei

The partition of HDAC1 and 2 between daughter nuclei was determined by measuring the fluorescence intensities in two post-mitotic nuclei, and comparing the mitotic partition-coefficient of HDAC1 and 2 with that of the DNA staining with DAPI. We found that HDAC1 and 2 were equally segregated between daughter nuclei after division in MCF7 cells (**Figure 3.10**).

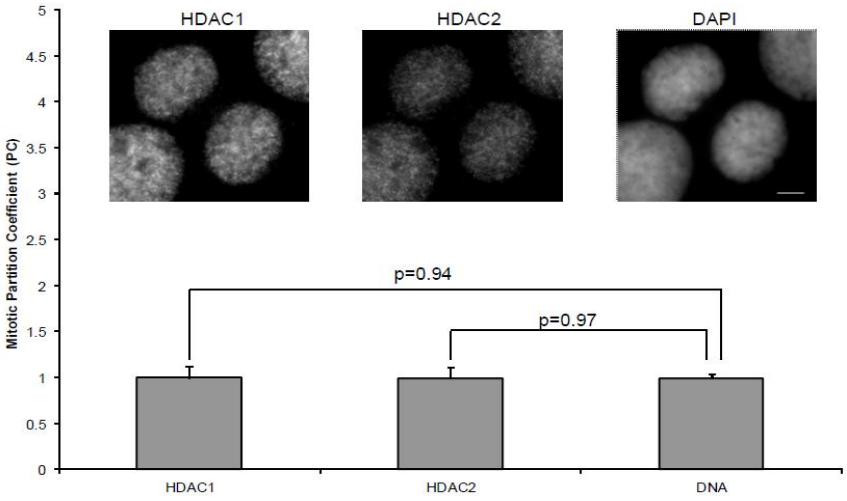

Figure 3.10: Equal partitioning of HDAC1 and 2 between daughter nuclei
To determine the relative levels of HDAC1 and 2 in the telophase cells, a quantitative image analysis was performed. The partition coefficient (PC) describes the ratio of integrated signal intensities between two daughter nuclei.

We had previously reported that transcription factors Sp1, Sp3 and RNAPII re-entered the daughter nucleus after the nuclear envelope and/or lamina assembly with the order of re-entry being Sp3 before Sp1, which was in advance of RNAPII (He and Davie, 2006) . To investigate

the order in which HDAC1 and 2 re-enter post-mitotic nuclei with respect to RNAPII, Sp1, Sp3 and lamin A/C, we performed indirect double-immunofluorescence labeling of asynchronized MCF7 cells, using antibodies against HDAC1, HDAC2, Sp1 or Sp3, RNAPII and lamin A/C. Fluorescence microscopy was used to select and study the post-mitotic nuclei. Among the asynchronized MCF7 cells, we searched for cells that were between telophase and late telophase. **Figure 3.11** shows that HDAC1 and 2 re-entered the daughter nucleus at a time when the nuclear envelope is being assembled. HDAC1 and 2 were located in the daughter nucleus before RNAPII. HDAC2 re-established in the daughter nucleus before Sp1 or Sp3. Together these results show that re-entry into the MCF7 daughter nuclei follows the order HDAC1 and 2, Sp3, Sp1 and RNAPII.

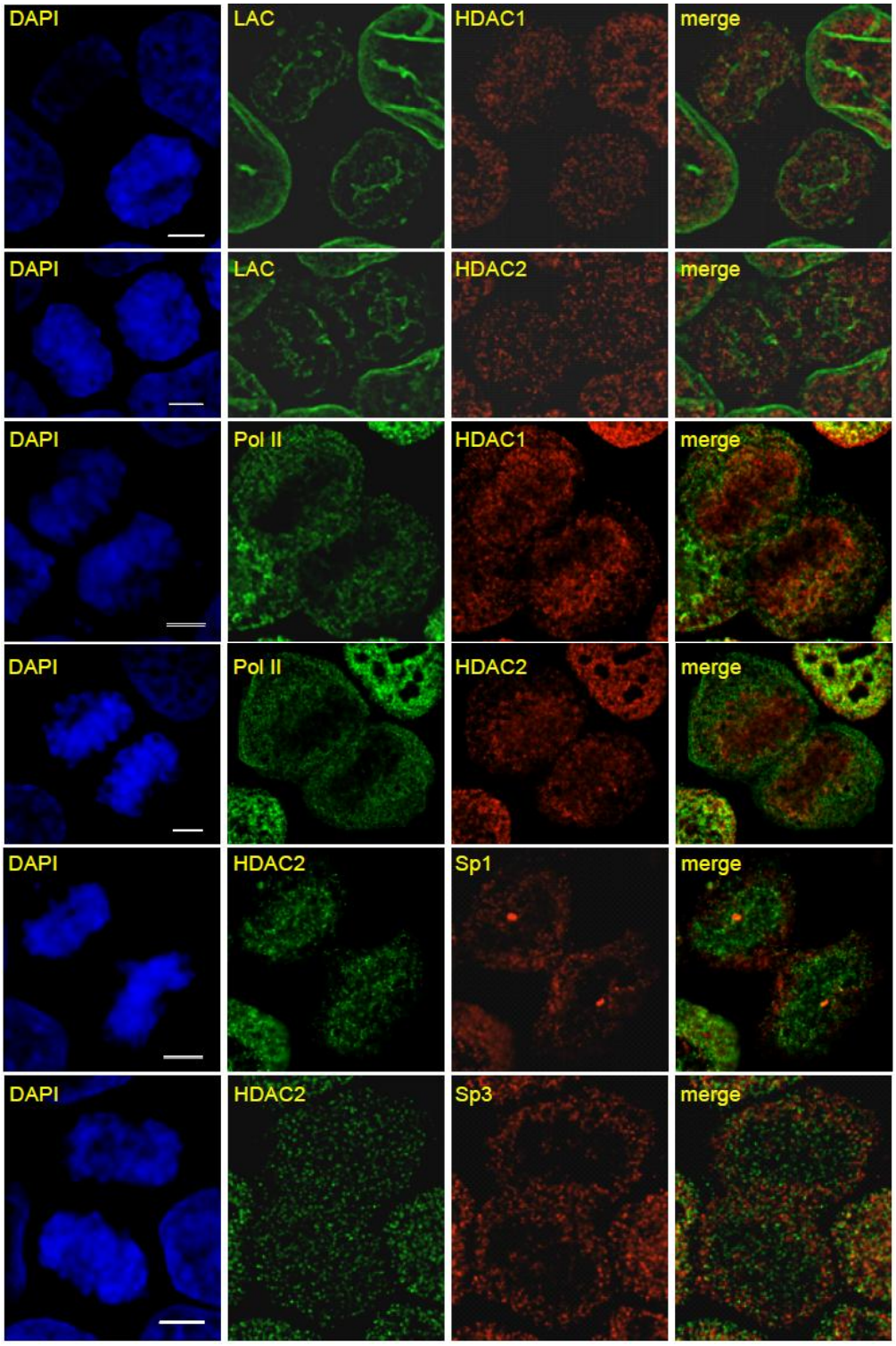

Figure 3.11: Sequential re-entry of HDAC1 and 2 into daughter nuclei
MCF7 cells were grown on coverslips, fixed, and double-labeled with anti-HDAC1 or anti-HDAC2, and anti-lamin A/C (LAC), anti-RNAPII, anti-Sp1 and anti-Sp3 antibodies. DNA was stained by DAPI. Spatial distribution was visualized by fluorescence microscopy in Axio Vision software. Bars, 5μm.

3.5 Discussion

During mitosis, several proteins involved in regulation of gene expression are displaced from the mitotic cells, including transcription factors, KATs, HDACs and RNAPII (Zaidi et al., 2010a;Delcuve et al., 2008). However, some transcription factors and cofactors remain associated with mitotic chromosomes and 'bookmark' the chromosomes (Kadauke and Blobel, 2012;Zaidi et al., 2010a). For those factors/cofactors that are displaced from mitotic chromosomes, this stage of the cell cycle provides an opportunity for a re-setting of the epigenetic program of the daughter cells. As the cells approach the end of mitosis in telophase, HDAC1 and 2 are equally dispersed among the daughter cells. Of the proteins we studied, HDAC1 and 2 re-enter the newly formed nucleus of the daughter cells earlier than transcription factors (Sp3 and Sp1) and RNAPII. The HDAC1 and 2 corepressor complexes would be available as the nucleus re-establishes the gene expression programming, with the HDAC1 and 2 complexes being directed to maintain gene silencing or to regulate the activity of regulatory regions of potentially active genes.

During mitosis, the displacement of HDAC1 and 2 as well as the KATs from chromosomes, results in changes in the steady state of acetylated histones associated with mitotic chromosomes (Kruhlak et al., 2001). Recent studies by Yoshida and colleagues using real-time imaging of H4 acetylation in living cells revealed that histone H4 acetylated at K5 and K8 declined in mitotic cells, while H4 acetylated at K12 remained unaltered (Sasaki et al., 2009;Ito et al., 2011). Interestingly the displaced HDAC1 and 2 retain enzymatic activities in the mitotic cells. We have data demonstrating that HDAC1 and 2 complexes immunoprecipitated from mitotic HeLa cells were catalytically active (not shown). It is conceivable that mitosis presents an opportunity

for the HDAC1 and 2 complexes to deacetylate proteins not available to these nuclear enzymes during interphase.

The events resulting in the displacement of HDAC-complexes from the mitotic chromosomes may be multifactorial. Aurora B catalyzes the global phosphorylation of H3 at Ser10 during mitosis. H3S10ph prevents the association of HDAC1 and 2 with the N-terminal tail of H3. Several studies have demonstrated a role of H3S10ph in displacing proteins, including HP1γ, SRSF1 and SRSF3 from chromatin (Winter et al., 2008b;Loomis et al., 2009). The arrest of transcription and the displacement of the transcription factors and cofactors, which recruit HDAC1 and 2 complexes to specific genomic sites, may be the major factors resulting in displacement of HDAC1 and 2 from mitotic chromosomes. However, the disruption and rearrangement of the nucleoskeleton (also called nuclear matrix) may be a major contributor to the displacement of HDAC1 and 2 complexes from chromatin. The association of HDAC1 and 2 with the nucleoskeleton has been well documented (Hendzel et al., 1991). Of the components of the nucleoskeleton, actin filaments have a role in binding to HDAC-complexes (Andrin and Hendzel, 2004). Nuclear actin has multiple roles in regulating gene expression (Spencer et al., 2011;Obrdlik and Percipalle, 2011;Gieni and Hendzel, 2009). Our observation that HDAC1 and 2 associate with F-actin in mitotic cells, suggest that HDAC1 and 2 are released from the nucleus and chromatin following nucleoskeleton, particularly F-actin reorganization during mitosis (Simon and Wilson, 2011). Once nuclear F-actin and nucleoskeleton is re-organized in the daughter cells, HDAC1 and 2 re-associate with this structure before the resumption of transcription.

3.6 Acknowledgements

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3.8 Supplementary figure

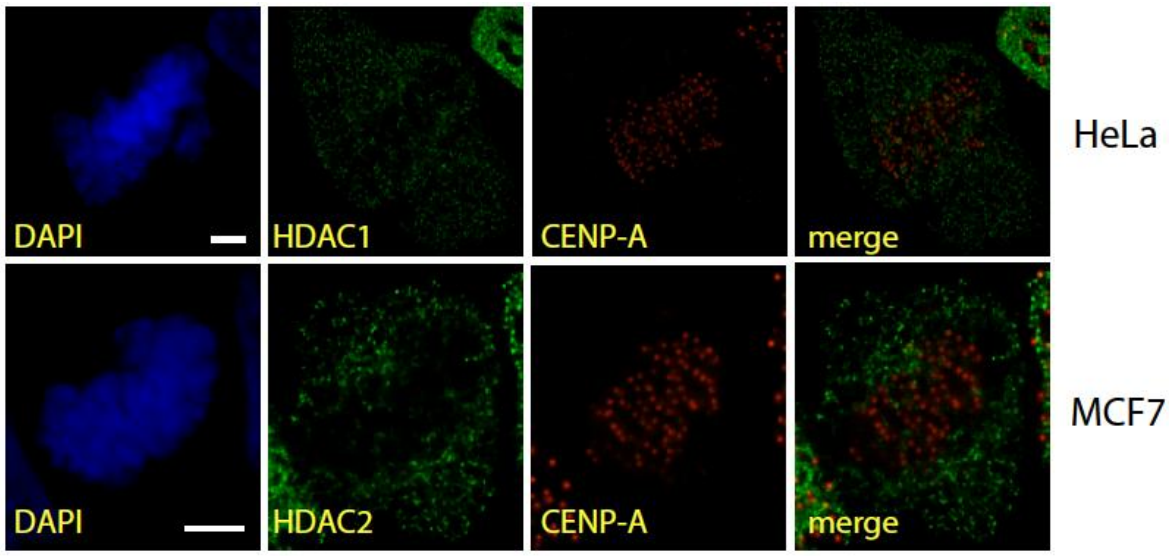

Figure S1: HDAC1 and 2 are not co-localized with CENP-A during mitosis
HeLa and MCF7 cells were grown on coverslips, fixed, and double-labeled with anti-HDAC1, anti-HDAC2, and anti-CENP-A antibodies. DNA was stained by DAPI. Spatial distribution was visualized by fluorescence microscopy in AxioVision software. Bars, 5µm.

Chapter 4: HDAC inhibitors prevent the activation of the IEG *FOSL1*, but do not alter the Nucleosome Response

4.1 Abstract

Dynamic histone acetylation, catalyzed by KATs and HDACs, is critical to IEG expression. Expression of IEGs, such as *FOSL1*, is induced by several signal transduction pathways resulting in activation of the protein kinase MSK and H3S10ph (the nucleosome response) at the upstream promoter and the regulatory region of target genes. HDAC inhibitors prevent *FOSL1* gene induction and the association of HDAC1, 2 and 3 with the gene body. However, HDAC inhibitors did not prevent the nucleosome response. Thus, HDAC inhibitors perturb events downstream of the nucleosome response required for *FOSL1* transcription initiation.

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Dilshad H. Khan *and James R. Davie.*

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Dilshad H. Khan has generated 100% of the data presented, prepared the figures, drafted the materials and methods in the manuscript as well as assisted in writing and editing of the paper.

4.2 Introduction

The nucleosomal response plays a major role in the expression of IEGs, such as *c-Fos*, *c-Jun*, *Fosl1* and *Ptgs2* (Drobic et al., 2010;Clayton et al., 2000). The nucleosomal response results from the stimulation of the MAPK or stress activated pathways and the activation of ERK or p38, respectively. ERK and p38 phosphorylate and activate MSK1/2. Once activated, MSK is recruited to regulatory regions of IEGs to phosphorylate histone H3 at Ser10 or Ser28. The phosphorylated H3 recruits proteins 14-3-3 ε or ζ. The events that follow result in the remodeling of nucleosomes at the regulatory region, recruitment of transcription factors and the initiation of transcription (Drobic et al., 2010;Macdonald et al., 2005).

Important in transcriptional induction of the IEGs is dynamic histone acetylation (Hazzalin and Mahadevan, 2005;Crump et al., 2011). KATs and HDACs recruited to the regulatory regions of IEGs catalyze dynamic protein acetylation of histones and other proteins. These events contribute to the remodeling of nucleosomes located at the regulatory regions. HDAC1 and 2 in association with several other proteins, are present in corepressor complexes Sin3A, NuRD and CoREST. HDAC3 is in corepressor complexes, SMRT and NCoR, with other HDACs, such as HDAC4. The HDAC corepressor complexes are recruited to regulatory regions by transcription factors and other DNA binding proteins. Dynamically acetylated histones are also associated with the coding regions of transcribed genes (Drobic et al., 2010;Spencer and Davie, 2001;Carrozza et al., 2005) and have a role in splicing of pre-mRNA (Delcuve et al., 2012;Khan et al., 2012). In yeast, there is evidence that recruitment of HDACs to the gene body is mediated by RNAPII elongation (Govind et al., 2010;Drouin et al., 2010). Whether a similar mechanism is

in play in recruiting HDACs to the coding region of IEGs in mammalian cells, is currently not known.

Inhibition of HDAC activity impacts the expression of 5-20% of genes, with these genes either being up- or down regulated. Further, there is emerging evidence that inhibition of HDACs impacts the splicing of many genes (Delcuve et al., 2012;Khan et al., 2012;Hnilicova and Stanek, 2011). Among the genes that are up-regulated by HDAC inhibitors is *CDKN1A*. However, for induction to take place the MSK-induced nucleosomal response must be active (Simboeck et al., 2010). In contrast to *CDKN1A*, the mitogen- or stress-induced expression of several IEGs is attenuated by the treatment of cells with HDAC inhibitors before the addition of the mitogen or stress agent (Hazzalin and Mahadevan, 2005). Whether the nucleosomal response was compromised for these IEGs by HDAC inhibitors, is currently not known.

In this study we determined which HDACs are recruited to the regulatory and coding regions of the induced IEG *FOSL1* and the dependence of HDAC recruitment on transcription. Further we determined whether HDAC inhibitors altered the nucleosomal response required for the induced expression of the *FOSL1* gene. We demonstrate that HDAC1, 2 and 3 are recruited to the regulatory and coding regions of the induced *FOSL1* gene, with on-going transcription being required for recruitment of HDAC1, 2 and 3 to the gene body. HDAC inhibitors did not impact the nucleosomal response, but disconnected the nucleosomal response from the events required for the initiation of transcription.

4. 3 Materials and methods

4.3.1 Cell culture and treatments

The human colon carcinoma cell line, HCT116 was cultured at 37°C in a humidified atmosphere containing 5% CO_2 in McCoy's 5A medium (Sigma) supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B. To induce the RAS-MAPK signaling pathway, 80-90% confluent cells were serum starved for 48 h and then treated with a phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (100 nM, Sigma) for various time periods (0, 30, or 60 min). When mentioned, serum starved HCT116 cells were treated with HDAC inhibitor, TSA (250 nM, Sigma) or apicidin (150 nM, Sigma) for 30 min prior to TPA treatment. In transcriptional inhibition studies, serum starved HCT116 cells were treated with TPA (60 min) or pre-treated with DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) (25µg/mL) or actinomycin D (Sigma) (20 µg/mL) for 1 h followed by TPA treatment for 60 min.

4.3.2 RNA isolation and real-time RT-PCR

Total RNA was isolated from either serum starved HCT116 cells treated with TPA (0, 30, or 60 min) alone or cells pretreated with TSA or apicidin for 30 min followed by TPA treatment (0, 30, or 60 min), using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA (400 ng) was used for cDNA synthesis using M-MLV reverse transcriptase (Invitrogen). Real-time PCR (qPCR) reactions were performed on iCycler IQ5 (Biorad). *FOSL1* and *PTGS2* mRNA levels were normalized against a house keeping gene, Cyclophilin E. Fold change was calculated relative to 0 time values.

4.3.3 ChIP assay

ChIP experiments were performed as previously described, with an additional protein-protein cross-linking step (Drobic et al., 2010). Cells were incubated with 1.0 mM DSP (dithiobis[succinimidylpropionate]) (Thermo Fisher Scientific) for 30 min at room temperature according to manufacturer's instruction, prior to formaldehyde cross-linking. Dual cross-linked chromatin was processed to mononucleosomes and chromatin immunoprecipitations were done with antibodies against HDAC1 (Affinity BioReagents), HDAC2 (Affinity BioReagents), HDAC3 (Abcam), acetyl histone-H3 (Millipore), acetyl histone-H4 (Millipore), RNAPII (Millipore), RNAPIIS2ph (Abcam), H3S10ph (Santa Cruz Biotechnology), 14-3-3 ε (Santa Cruz Biotechnology) and 14-3-3 ζ (Santa Cruz Biotechnology). Equal amounts of input and ChIP DNA (1.0 ng) were used for qPCR on iCycler IQ5 (BioRad). The fold enrichment was calculated as previously described (Drobic et al., 2010). The following primers were used:

FOSL1-promoter-F: 5′-GTGCTATTTTGTGGGAGCAG-3′

FOSL1-promoter-R: 5′-TGGTGTAACTTCCTCGCCGC-3′

FOSL1-Ex1-F: 5′-GCATGTTCCGAGACTTCGGG-3′

FOSL1-Ex1-R: 5′-TGCTGGGCTGCCTGCGCTGC-3′

FOSL1-Ex4-F: 5′- CACACCCTCCCTAACTCCTTT-3′

FOSL1-Ex4-R: 5′-TGCTGCTACTCTTGCGATGA-3′

4.3.4 Preparation of cell extract, histone isolation and immunoblotting

Cell extracts were prepared in IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 0.5% NP-40) containing phosphatase and protease inhibitors (Roche). Total cell extracts (20 μg) were resolved by 10% SDS-PAGE and immunochemical staining was performed with

anti-ERK1/2 (Invitrogen) and anti-phospho-p44/42 MAPK (ERK1/2) Thr202/Tyr204 (Cell Signaling Technology), anti-p38 MAPK (Cell Signaling Technology), and, anti phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology) antibodies. Acid extraction of histones was done as described previously (Chadee et al., 1999), and were resolved (5.0 μg of histones) by 15% SDS-PAGE and stained immunochemically with antibodies against anti-H3S10ph (Santa Cruz Biotechnology), anti-H3S28ph (Abcam), and anti-H3 (Millipore).

4.4 Results

4.4.1 Transcription dependent recruitment of class I HDACs to *FOSL1*

We have previously reported that TPA stimulation of the RAS-MAPK pathway in mouse fibroblasts results in the induced expression of the *Fosl1* and *Ptgs2* genes (Drobic et al., 2010). TPA treatment of serum starved human colorectal carcinoma HCT116 cells also resulted in the increased expression of the *FOSL1* and *PTGS2* genes (**Figures 4.1A** and **S1A**). Histone acetylation turnover is important for activation of inducible genes (Crump et al., 2011). To determine the loading of class I HDACs along the *FOSL1* gene, we applied a dual cross-linking ChIP assay (Sun et al., 2007). In response to the TPA-induction of the *FOSL1* gene, there was an accumulation of class I HDACs (HDAC1, 2 and 3) at the UPR and gene body (exons 1 and 4), increasing in parallel with the increased expression of the *FOSL1* gene (**Figures 4.1B** and **C**).

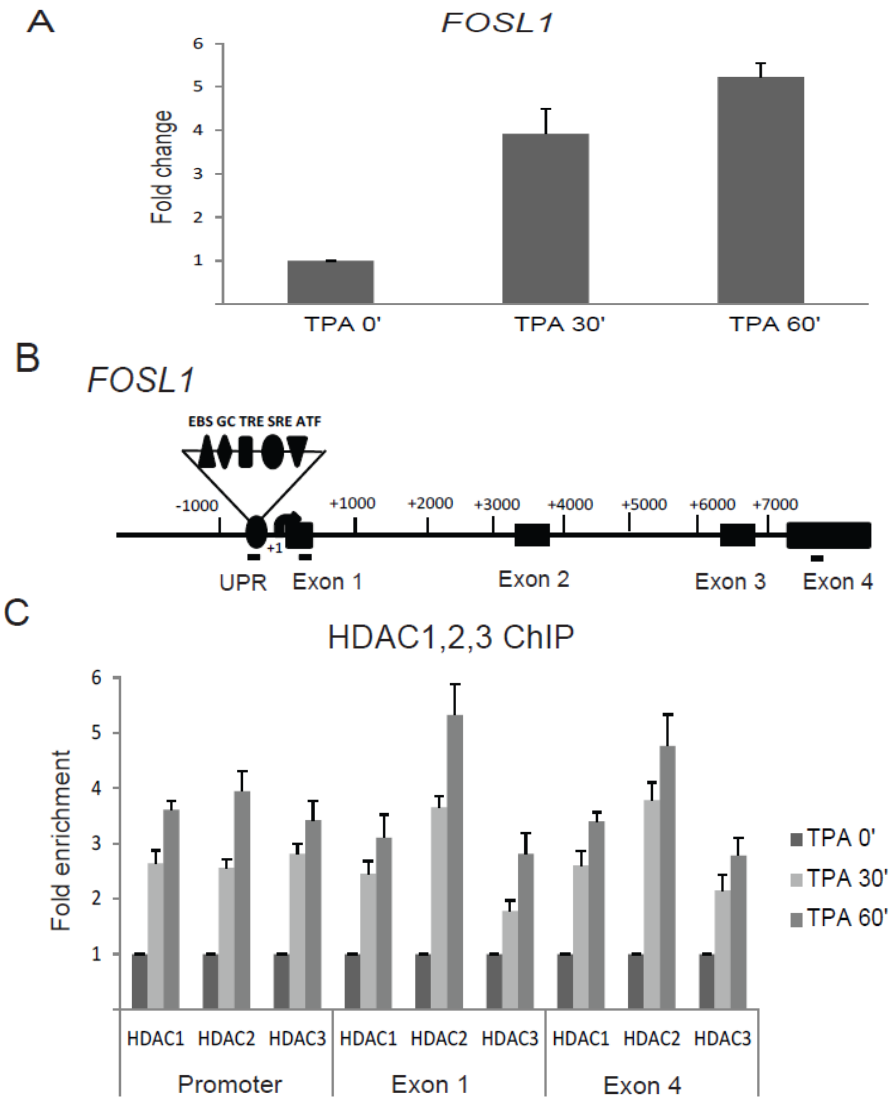

Figure 4.1: TPA induced increased expression of *FOSL1* gene with the accumulation of class I HDACs in gene body region in HCT116 cells

A. Serum starved HCT116 cells were treated with 100 nM TPA for 0, 30 or 60 min. Total mRNA was isolated and quantified by qPCR. Fold changes of *FOSL1* mRNA were normalized to cyclophilin E levels and time 0 values, and are the mean of three independent experiments. The error bars represent standard deviation. B. Schematic representation of *FOSL1* gene showing the positions of the amplicons used in ChIP assays. Exons are indicated by boxes. Transcription factor binding sites in the upstream promoter region (UPR) of the gene are shown in the amplified region. EBS, Ets binding site; GC, GC box; TRE, TPA-responsive element; SRE, serum-responsive element; ATF, activating transcription factor. C. HCT116 cells were serum deprived and were treated with TPA as indicated in figure. Cells were double cross-linked with DSP and formaldehyde and ChIP assays were performed with HDAC1, 2 or 3 antibodies. Equal amounts of input and immunoprecipitated DNA were analyzed by qPCR at the promoter and gene body region (exons 1 and 4) of *FOSL1* gene. The enrichment values are relative to values of time 0, and are the mean of three independent experiments, represented as mean ± standard deviation.

The requirement of on-going transcription in the recruitment of HDACs to the UPR and *FOSL1* gene body was determined. Serum starved HCT116 cells were incubated for 60 min with or without the transcription inhibitors, DRB or actinomycin D, followed by an incubation with TPA for 60 min to induce *FOSL1* gene expression. The results of the dual cross-linking ChIP assays show that both transcription inhibitors prevented the recruitment of class I HDAC to the *FOSL1* UPR and gene body (**Figure 4.2**). These results suggest that TPA-induced transcription of the *FOSL1* is required to recruit class I HDACs to the gene.

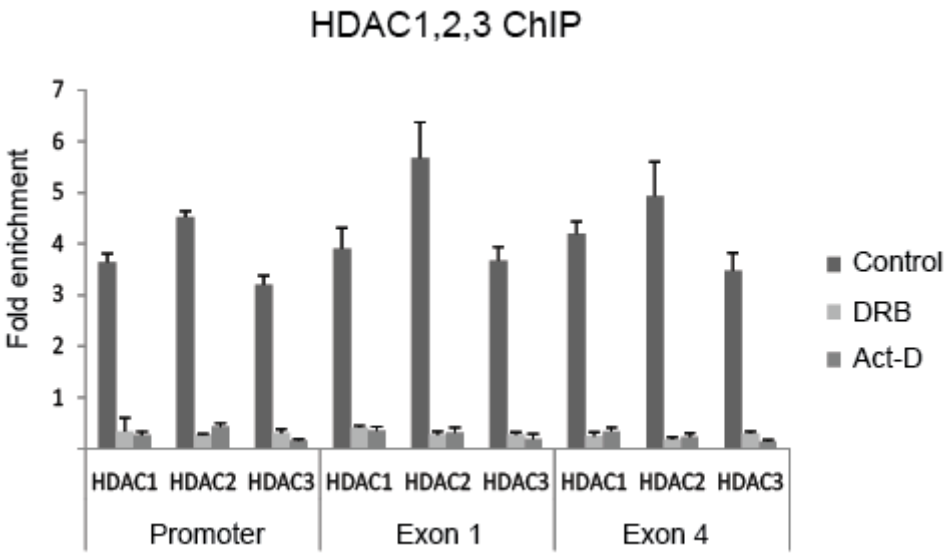

Figure 4.2: Transcription dependent recruitment of class I HDACs in the gene body of *FOSL1* gene
Serum starved HCT116 cells were treated with 100 nM TPA for 60 min (Control) or were treated with DRB (25µg/mL) or actinomycin D (Act-D) (20µg/mL) for 1 h prior to TPA treatment (60 min). Cells were dual cross-linked, and ChIP assays were done with HDAC1, 2 or 3 antibodies. Equal amounts of ChIP and input DNA were used for qPCR analyses at the UPR and gene body region of the *FOSL1* gene. Enrichment values of each treatment were relative to input values, and are the average of three independent experiments. The error bars represent standard deviation.

4.4.2 HDAC inhibitors attenuate the recruitment of HDACs to *FOSL1* gene body

Previous reports have shown that the HDAC inhibitor, TSA attenuated the TPA-induced expression of IEGs in mouse fibroblast (Hazzalin and Mahadevan, 2005). Incubation of TSA or apicidin 30 min before the addition of TPA to serum starved HCT116 cells, prevented the induction of the *FOSL1* and *PTGS2* genes (**Figures 4.3** and **S1B**, **C**). However, TSA or apicidin alone did not induce these genes (**Figure S2**).

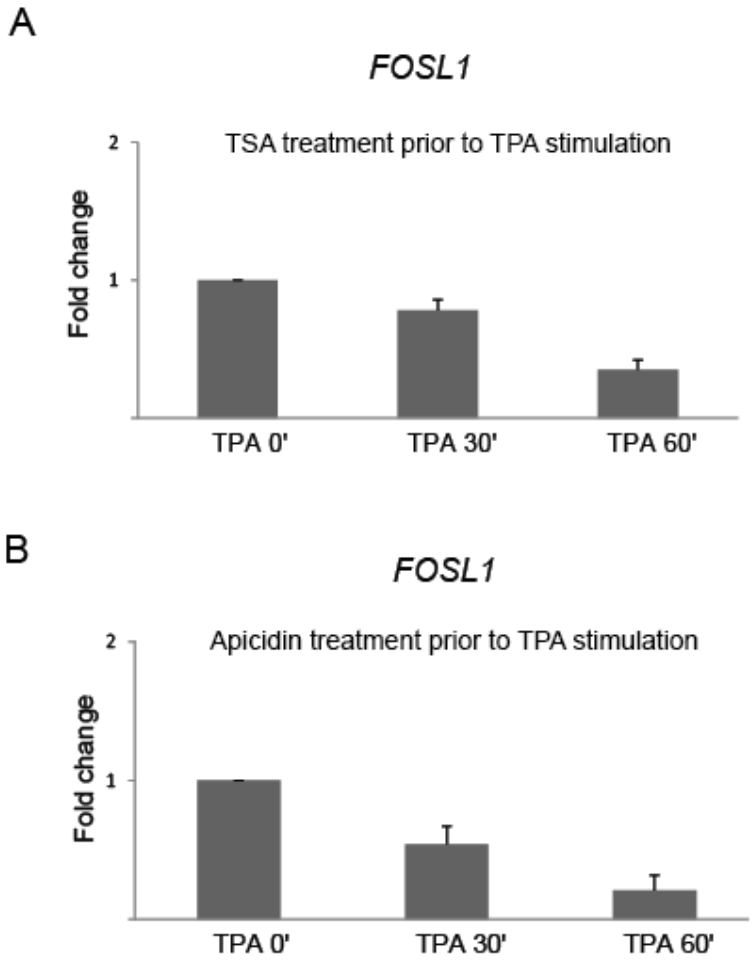

Figure 4.3: Effect of HDAC inhibitors on *FOSL1* expression
HCT116 cells cultured under serum-deprived conditions were treated with 250 nM TSA (A) or with 150 nM apicidin (B) for 30 min before a time course treatment with 100 nM TPA for 0, 30 or 60 min. Total mRNA was isolated and fold change of *FOSL1* mRNA was calculated by normalizing values against cyclophilin E, relative to 0 time values. Fold change values are the mean of three independent experiments, and error bars indicate standard deviation.

Dual cross-linking ChIP assays were applied to determine whether the HDAC inhibitors were affecting the recruitment of RNAPII to the *FOSL1* promoter. TPA induction of the *FOSL1* gene resulted in the accumulation of RNAPII at the promoter at 30 min, followed by a decrease of RNAPII at 60 min (**Figure 4.4A**). The initiation-competent form of RNAPII, RNAPIIS5ph also increased at 30 min followed by a decline at 60 min. TSA or apicidin incubation of HCT116 cells before TPA addition prevented the accumulation of RNAPII at the *FOSL1* promoter and the initiation of transcription as indicated by the lack of elevated levels of RNAPIIS5ph following TPA induction (**Figures 4.4B** and **C**).

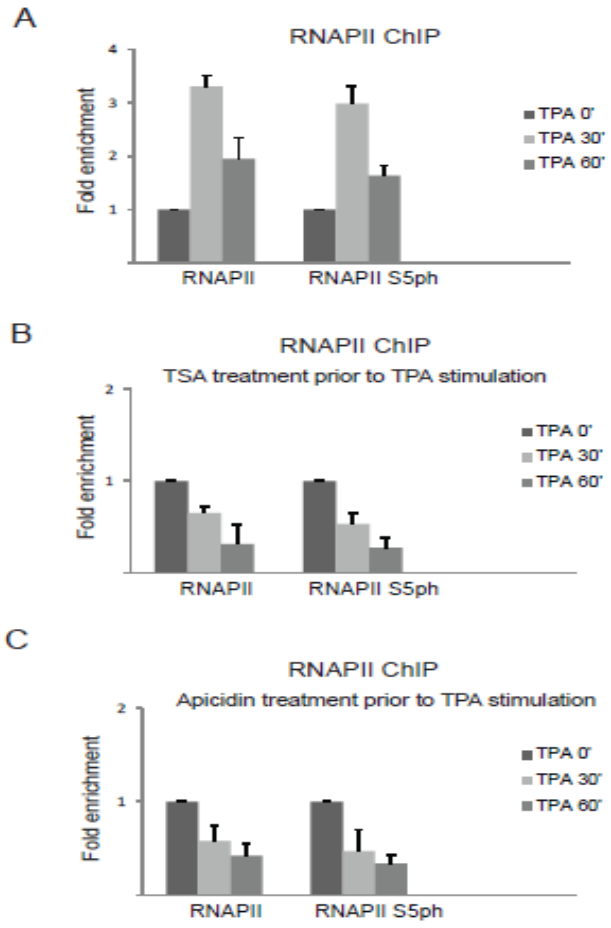

Figure 4.4: HDAC inhibition with TSA or apicidin attenuates the recruitment of RNAPII at regulatory region of *FOSL1* gene
Serum starved HCT116 cells were left untreated (A) or treated with 250 nM TSA (B) or 150 nM apicidin (B) for 30 min prior to TPA stimulation (100 nM) for 0, 30 or 60 min. Dual cross-linked

mononucleosomes were prepared and used in ChIP assays with anti-RNAPII and anti-RNAPIIS5ph antibodies (total and initiation forms of RNAPII, respectively). Equal amounts of input and immunoprecipitated DNA were assessed at the UPR of *FOSL1* gene with qPCR. Enrichment values are the mean of three independent experiments, relative to time 0 values, represented as mean ± standard deviation.

The effect of the HDAC inhibitor on the loading of class I HDACs on the *FOSL1* gene was evaluated by dual cross-linking ChIP assays. **Figure 4.5** shows that both TSA and apicidin treatment prevented the recruitment of HDAC1, 2 and 3 to the gene body, but had minimal impact on the accumulation of class I HDACs to the *FOSL1* UPR.

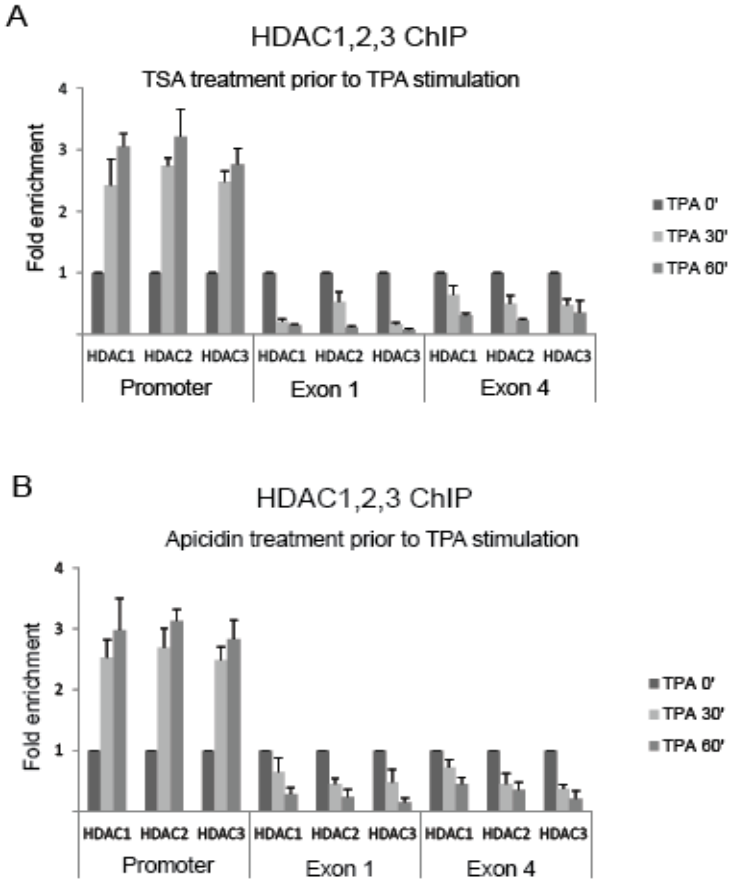

Figure 4.5: HDAC inhibitors restrict the accumulation of class I HDACs in gene body of *FOSL1* gene

HCT116 cells were serum starved and incubated with TSA (250 nM) (A) or apicidin (150 nM) (B) prior to the treatment with TPA for different time points as indicated. Dual cross-linked cells were processed for ChIP assays with HDAC1, HDAC2 and HDAC3 antibodies and were

analyzed by qPCR at promoter and gene body (exons 1 and 4) of *FOSL1* gene. Enrichment values, the average of three independent experiments, are expressed as relative to time 0 values, and error bars indicate standard deviation.

To analyze the changes in histone acetylation levels at the UPR of *FOSL1*, dual cross-linking ChIP assays were performed with antibodies against acetylated H3 (H3acK9/14) and acetylated H4 (H4acK5/8/12/16) in TPA-induced cells pretreated with or without TSA or apicidin. A temporal increase in histone H3 and H4 acetylation was observed in promoter region of *FOSL1* gene, with a greater level of H3 and H4 acetylation being attained in the HDAC inhibitor treated cells (**Figure 4.6**). These observations provide evidence that the increased KAT activity at the *FOSL1* UPR following TPA induction is not prevented by inhibiting the activity of the HDACs residing at the UPR.

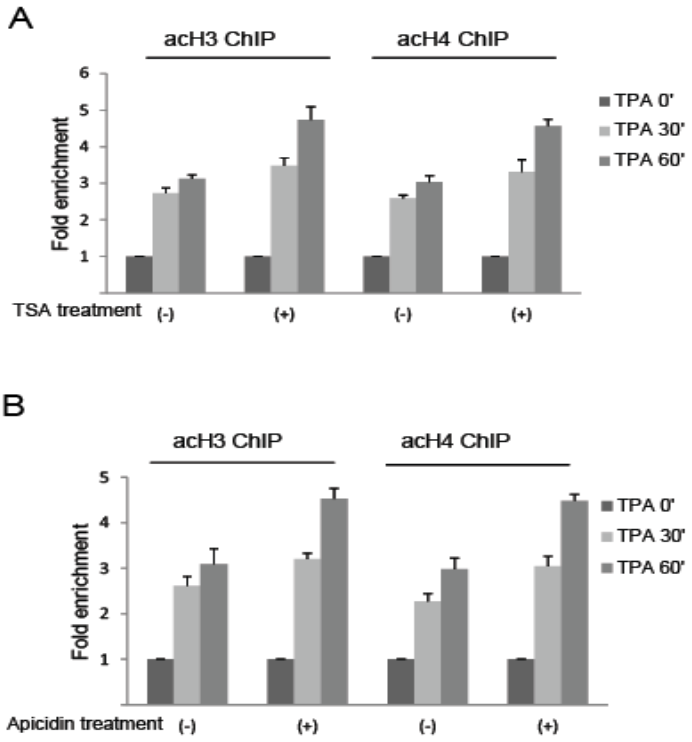

Figure 4.6: Effect of TSA or apicidin on acetylation of histone H3 and H4 at the *FOSL1* UPR

HCT116 cells were serum starved and then incubated with or without HDAC inhibitors, TSA (250 nM) (A) or apicidin (150 nM) (B) for 30 min before being stimulated with TPA (100 nM)

for 0, 30 or 60 min. Cells were double cross-linked, processed to mononucleosomes for ChIP assays with acetylated Histone-H3 and H4 antibodies and analyzed by qPCR at the UPR of *FOSL1* gene. Enrichment values are relative to time 0 values and are the average of three independent experiments. The error bars represent standard deviation.

4.4.3 HDAC inhibitors do not perturb the nucleosome response

TPA induction of the RAS-MAPK pathway leads to the phosphorylation of ERKs which in turn phosphorylate and activate MSK, resulting in the phosphorylation of histone H3 (the nucleosome response) at the regulatory regions of IEGs (Hazzalin and Mahadevan, 2005). As the nucleosome response is required for the induction of *CDKN1A* gene, we determined whether HDAC inhibitors affected the nucleosome response pathway. As ERK activation is a key step in the pathway, we determined the levels of phosphorylated ERK in TPA and TSA/TPA or apicidin/TPA treated serum starved HCT116 cells. **Figure 4.7A** shows that neither TSA nor apicidin affected the phosphorylation and activation of ERK. MSK can be activated by p38 MAPK pathway as well (Clayton et al., 2000); we therefore analyzed the activation and phosphorylation of p38 kinase in our experimental settings. As a control, we treated HCT116 cells with UV to activate p38 kinase. **Figure 4.7B** shows that UV treatment resulted in the phosphorylation of p38. In contrast, separately or in combination of TPA and the HDAC inhibitors (TSA or apicidin) did not activate the p38 kinase pathway.

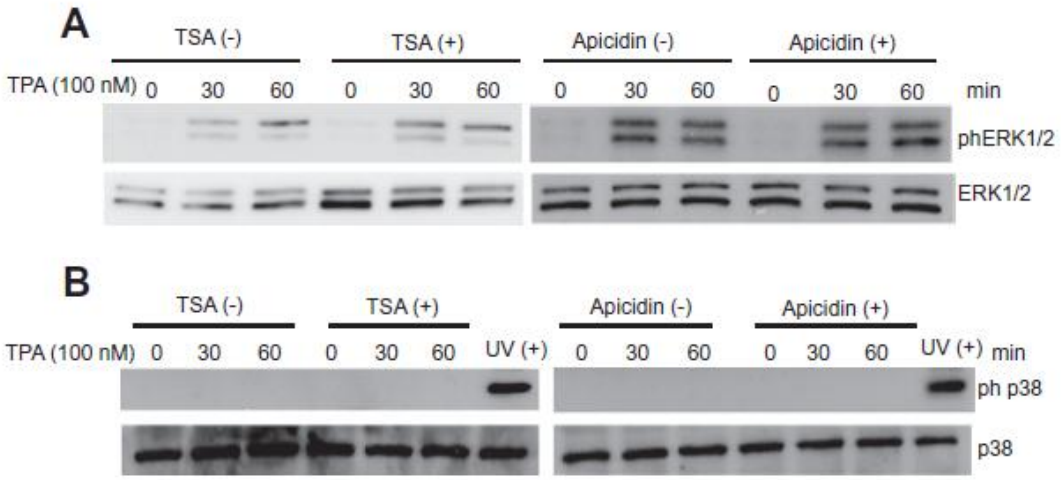

Figure 4.7: Effect of HDAC inhibitors on TPA-induced activation of MAP kinase pathways Serum starved HCT116 cells were pretreated or not with 250 nM TSA (left panel) or with 150 nM apicidin (right panel) for 30 min followed by TPA treatment (100 nM) for indicated time points. Total cellular extracts (20 µg) were analyzed for the induction of phospho-ERK (A) and phospho-p38 (B) by phospho-ERK1/2 and phospho-p38 antibodies. Total ERK1/2 and p38 were used as loading controls and UV treated cell lysates were analyzed as a positive control for phospho-p38.

Next we determined the level of H3S10ph along the regulatory and coding region of *FOSL1* gene by ChIP assay and whether this phosphorylation event was altered by HDAC inhibitors. **Figure 4.8A** shows that TPA-induced H3S10ph occurred at the *FOSL1* UPR but not within the gene body (exon 1 and exon 4). The TPA-induced H3S10ph and the localization of this PTM to the UPR were not prevented by the HDAC inhibitors TSA and apicidin. TSA induced phosphorylation of H3S28, but not of H3S10, has been reported in mouse epidermal JB6 cells (Zhong et al., 2003). In serum starved HCT116 cells, neither of the HDAC inhibitors induced H3S10ph or H3S28ph. Further, neither of the HDAC inhibitors prevented TPA induction of H3S10ph and H3S28ph (**Figure 4.8B**).

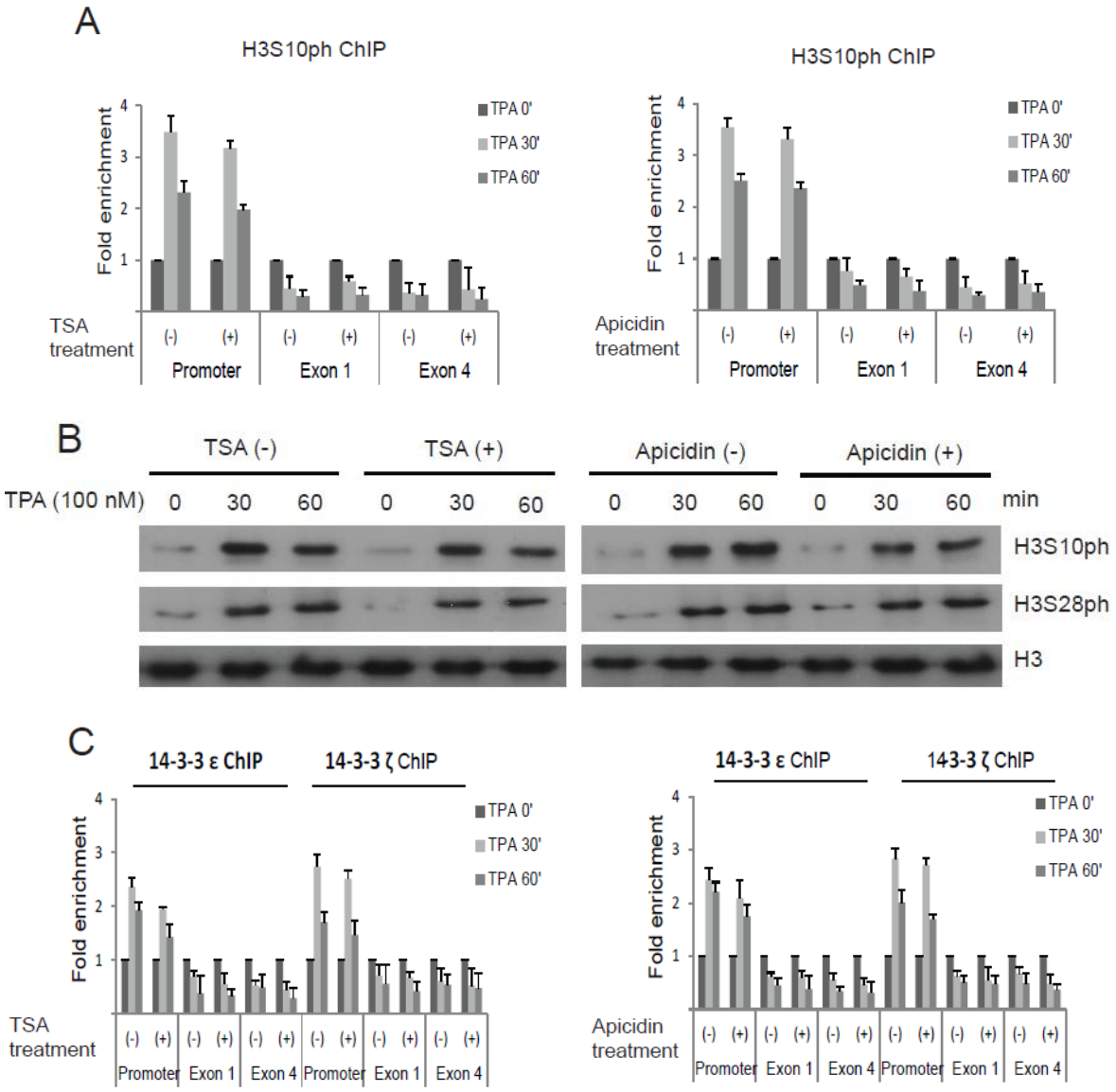

Figure 4.8: TSA or apicidin does not alter the TPA-induced nucleosomal response or the recruitment of chromatin modifiers to the regulatory region of *FOSL1* gene

A. HCT116 cells were cultured in serum depleted conditions and were treated or not with 250 nM TSA (left panel) or with 150 nM apicidin (right panel) for 30 min prior to 100 nM of TPA stimulation for 0, 30 or 60 min. Cells were dual cross-linked, processed to mononucleosomes and ChIP assays were performed with anti-H3S10ph antibody and analyzed by qPCR at the promoter and coding region of *FOSL1* gene. Enrichment values are relative to time 0 values and are the average of three independent experiments. The error bars represent standard deviation. B. Serum starved HCT116 cells were treated or not with TSA (250 nM) (left panel) or with apicidin (150 nM) (right panel) for 30 min before the stimulation with TPA (100 nM) for 0, 30 or 60 min. Acid-soluble nuclear histones (5µg) were resolved on a 15%-SDS-PAGE and immunoblotting were done with anti-H3S10ph, anti-H3S28ph and anti-Histone H3 (loading control) antibodies. C. ChIP assays were done with or without TSA (left panel) or apicidin (right panel) pretreatment followed by TPA induced conditions with 14-3-3 ε and 14-3-3 ζ antibodies as described in A.

Following phosphorylation of H3, a critical event in the induction of IEGs is the recruitment of 14-3-3 ε and 14-3-3 ζ (Winter et al., 2008). We investigated whether HDAC inhibitors prevented the recruitment of the 14-3-3 proteins to the UPR of the *FOSL1* gene following TPA induction. Following TPA induction, the 14-3-3 proteins were recruited to the UPR, but not to the coding region of the *FOSL1* gene (**Figure 4.8C**). Neither TSA nor apicidin prevented the recruitment of the 14-3-3 proteins to the *FOSL1* UPR, indicating that the phospho mark (H3S10ph) was effectively 'read' by 14-3-3 proteins in cells treated with HDAC inhibitors. Together these results show that the HDAC inhibitors do not interfere with the nucleosome response pathway.

4.5 Discussion

Dynamic histone acetylation plays a critical role in the expression of IEGs (Crump et al., 2011). Further dynamic acetylation occurs independently of MSK-catalyzed H3 phosphorylation (Thomson et al., 2001). The dynamic acetylation is catalyzed by KATs (CBP/p300/PCAF) and TSA-sensitive HDACs (Crump et al., 2011). TPA-induction of the IEG genes results in an increased acetylation of histones at the UPR of these genes (Crump et al., 2011;Drobic et al., 2010). For nucleosomes positioned at the *FOSL1* UPR, HDAC inhibitors (TSA and apicidin which are structurally unrelated) did not prevent but enhanced the TPA-induced histone acetylation. However, the HDAC inhibitors prevented initiation of *FOSL1* transcription induced by TPA. Previous reports have shown that HDAC inhibitors alter gene expression, ranging from 5-20% of genes within the genome, with the responsive genes being either up- or down regulated (Glaser et al., 2003;Joseph et al., 2004;Mitsiades et al., 2004). Thus *FOSL1* is with the group of genes which are down-regulated by HDAC inhibitors.

In this report, we demonstrate the transcription dependent recruitment of HDAC1, 2 and 3 to the gene body of the *FOSL1* gene in HCT116 cells. Throughout the study, we applied a dual cross-linking ChIP assay. We previously reported that the use of DSP before formaldehyde was more efficient in monitoring HDACs along genes than using formaldehyde alone or other cross-linker pairs (such as EGS/formaldehyde) (Zeng et al., 2006;Sun et al., 2007). We have also observed the transcription dependent recruitment of HDAC1 and 2 to the induced (TPA or estradiol) *Trefoil factor 1* gene body in human breast cancer cell line MCF7 (data not shown). Thus the transcription dependent recruitment of class I HDACs in mammalian cells to the coding region of transcribed genes appears to be by a similar mechanism as reported in yeast (Govind et al., 2010;Spain and Govind, 2011). The mechanisms by which HDACs are recruited to UPR and gene body regions of *FOSL1* can be distinguished with HDAC inhibitors. The mechanism of HDAC recruitment to the gene body is thought to be via RNAPII which is required to transfer HDAC to the coding region of the gene. As the HDAC inhibitors prevent the initiation of transcription, RNAPII is not present to do this task. For the UPR, the nucleosome response pathway results in nucleosome remodeling of the *FOSL1* UPR, allowing the recruitment of transcription factors which in turn recruit HDACs which are in multiprotein complexes such as Sin3A. HDAC inhibitors do not inhibit these events and thus HDAC recruitment to the UPR is not affected by the HDAC inhibitors.

The activation of MSK and the nucleosome response is required for the HDAC inhibitor-induced expression of *CDKN1A* (Simboeck et al., 2010). For several IEGs, HDAC inhibitors attenuate the TPA-induced expression of IEGs (Hazzalin and Mahadevan, 2005). Our results show that the HDAC inhibitors, TSA or apicidin did not impact the signal transduction pathways resulting in

the activation and phosphorylation of p38 and ERK kinases or the MSK catalyzed nucleosome response events, including H3S10ph and 14-3-3 recruitment to the *FOSL1* UPR. The HDAC inhibitors prevented subsequent events leading to the productive initiation of *FOSL1* transcription. Consistent with our results, others have shown that HDAC inhibitors can attenuate transcriptional initiation by abrogating the binding of RNAPII and basal transcription factors to promoter regions (Yamaguchi et al., 2005;Rascle et al., 2003). These results demonstrate that inhibition of class I HDACs and dynamic protein acetylation can either promote or hinder transcription and likely depends on promoter/UPR context.

4.6 Acknowledgments

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4.8 Supplemental data

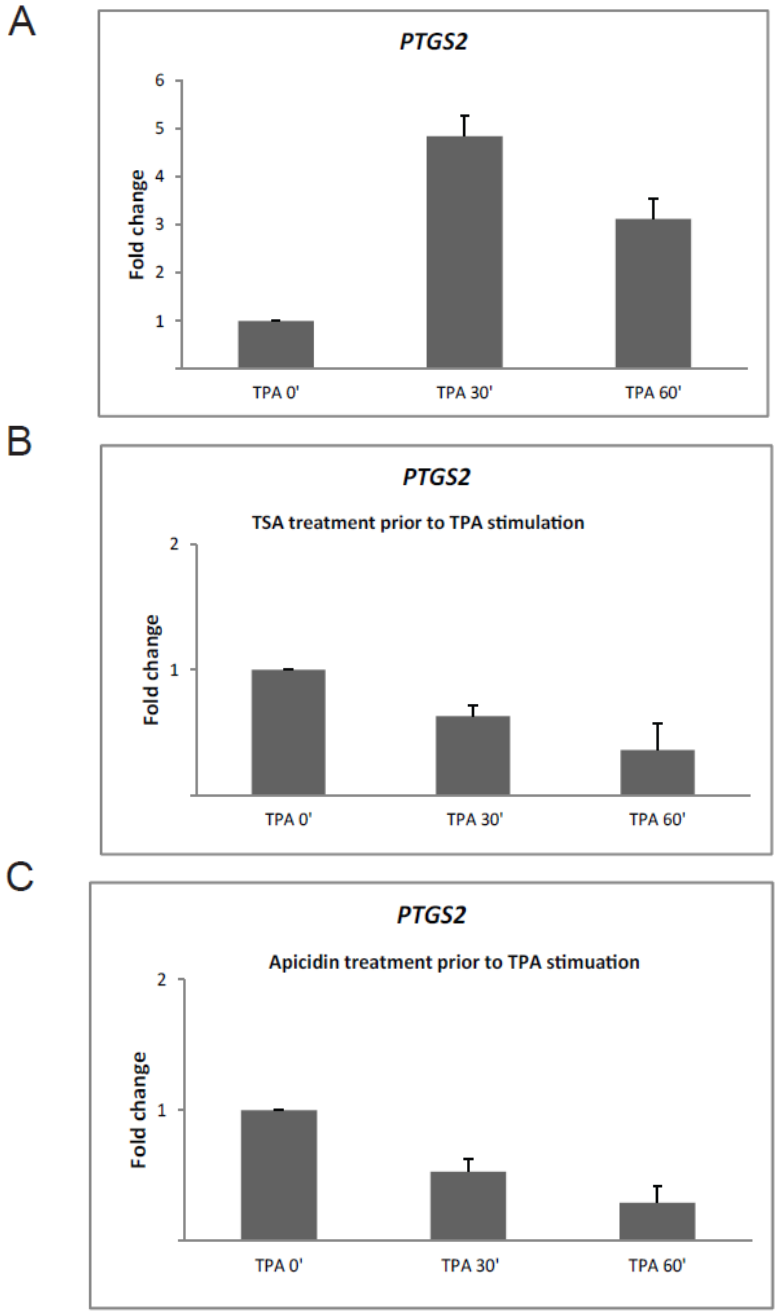

Figure S1: Modulation of *PTGS2* gene expression
A. HCT116 cells were serum starved and treated with 100 nM TPA for 0, 30 or 60 min. Total mRNA was isolated and the *PTGS2* mRNA level was quantified relative to a housekeeping gene, cyclophilin E and are shown as fold increase relative to 0 time values, mean ± standard deviation, (n=3). B, C. HCT116 cells were serum starved and treated with TSA (B) or apicidin (C) followed by TPA treatment as described in Fig 3. The level of *PTGS2* mRNA was quantified using cyclophilin E as a reference and is expressed as fold change relative to 0 time values.

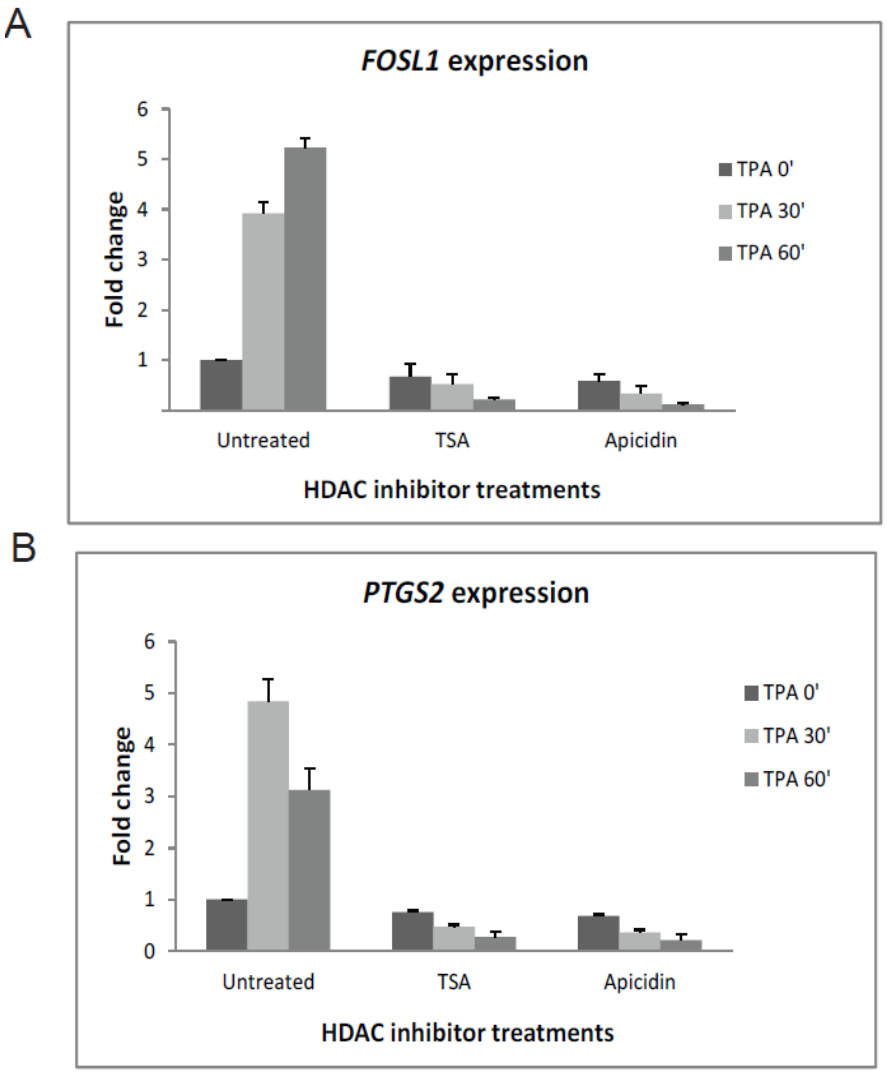

Figure S2: HDAC inhibitors do not induce the transcription of *FOSL1* and *PTGS2* genes
Serum starved HCT116 cells were untreated or treated with 250 nM TSA or 150 nM apicidin for 30 min before stimulating with TPA (100 nM) for 0, 30 or 60 min. The levels of *FOSL1* (A) and *PTGS2* (B) mRNA were quantified using an internal control, cyclophilin E and are expressed as fold change relative to 0 time values (without TPA treatment) in untreated samples (without HDAC inhibitor treatment).

Chapter 5: RNA-Directed Dynamic Histone Acetylation Regulates *MCL1* Alternative Splicing

5.1 Abstract

HDACs and KATs catalyze dynamic acetylation of histones, at regulatory regions and within the body of active genes. Highly phosphorylated forms of HDAC1 and 2 are recruited within corepressor complexes to upstream promoter regions to regulate transcription initiation, while the non-phosphorylated form is present along the body of transcribed genes. The aims of this study were to identify the HDAC1 and 2 complexes associated with the body of transcribed genes and to find out the function of HDAC1 and 2 complexes-mediated dynamic histone acetylation. Mass spectrometry studies revealed that both HDACs were in complexes with RNA splicing factors. In particular, HDAC1 and 2 were associated with the splicing factor SRSF1 along the body of transcribed genes. Enzymatic HDAC inhibition and siRNA-mediated HDAC1 and/or 2 or SRSF1 knockdown experiments showed that these three proteins were involved in the splicing of *MCL1* alternative exon 2. The inhibition of HDAC activity triggered an increased occupancy of KAT2B and a parallel increase in histones H3 and H4 acetylation over *MCL1* exon 2. Moreover, HDAC1 and 2 as well as KAT2B, were associated with the nascent pre-mRNA. Our data indicate that non-phosphorylated HDAC1 and 2 are recruited to the nascent pre-mRNA of transcribed genes by SRSF1 and/or other splicing factors, and act at the RNA level, in concert with KAT2B and perhaps other KATs, to catalyze dynamic histone acetylation and alter the nucleosomal structure over *MCL1* alternative exon 2.

This collaborative work has been submitted for publication as:

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RNA-Directed Dynamic Histone Acetylation Regulates MCL1 Alternative Splicing.

Nucleic Acids Research (in revision)

Dilshad H. Khan has generated 70% of the data presented, prepared the figures, drafted and edited the manuscript.

5.2 Introduction

KATs and HDACs catalyze dynamic acetylation of proteins, including histones, associated with transcribed DNA (Yang and Seto, 2007). KATs often have transcriptional coactivator activity, increasing the level of acetylated histones and enhancing transcription when recruited to a gene promoter by a transcription factor (Lee and Workman, 2007). HDAC1 and 2 are present in large multiprotein corepressor complexes such as Sin3, NuRD, CoREST, which are recruited to regulatory regions by transcription factors (Sun et al., 2002;Sun et al., 2007). Phosphorylation of HDAC1 at Ser393, Ser421 and Ser423 and HDAC2 at Ser394, Ser422 and Ser424 is required for the formation of these corepressor complexes (Tsai and Seto, 2002;Pflum et al., 2001;Sun et al., 2002;Sun et al., 2007). On the other hand, the non-phosphorylated HDAC2 is associated with the body of transcribed genes (Sun et al., 2007). Although the unmodified HDAC2 is more abundant than highly phosphorylated HDAC2, it is the highly phosphorylated form that is preferentially cross-linked to chromatin with formaldehyde or cisplatin (Sun et al., 2002). However, through the use of a dual cross-linking ChIP assay, all isoforms of HDAC1 and 2 could be mapped along regulatory and coding regions of transcribed genes, with the unmodified HDAC2 being associated with the coding region (Sun et al., 2007). Yet, it remains to be determined which proteins the HDAC1 and 2 interact with, when targeted to the body of transcribed genes.

Recent studies suggest a role for HDAC1 and 2 in alternative splicing (Hnilicova et al., 2011;Zhou et al., 2011; Braunschweig et al., 2013). Approximately 95% of human multi-exon genes generate alternatively spliced transcripts, giving rise to mature mRNA isoforms coding for functionally different proteins. Most of these splicing events are regulated in a tissue- and/or developmental stage-specific manner or in response to naturally occurring external stimuli

(Wang et al., 2008;Castle et al., 2008;Kalsotra and Cooper, 2011). Pre-mRNA splicing is a co-transcriptional process, which is regulated by RNAPII elongation rate, as inclusion of an alternative exon occurs only if splicing components have time to interact with the nascent RNA before its 3′ end cleavage and release (Carrillo Oesterreich et al., 2011; Ip et al., 2011).

There is emerging evidence that histone modifications and chromatin structure influence splicing, and *vice versa* pre-mRNA splicing itself influences chromatin organization (Luco and Misteli, 2011; Carrillo Oesterreich et al., 2011; Shukla and Oberdoerffer, 2012;Khan et al., 2012; Braunchweig et al.,2013). Recent studies have demonstrated a correlation between local increase in histone acetylation and exon skipping (Schor et al., 2009;Hnilicova et al., 2011;Zhou et al., 2011). It was found that skipping of *NCAM* exon 18 upon membrane depolarization of neuronal cells was linked to localized increased histone H3 acetylation (H3K9ac) and could be replicated by the HDAC inhibitor TSA (Schor et al., 2009). However, the mechanisms involved were not elucidated. In HeLa cells treated with the pan-HDAC inhibitor, sodium butyrate, splicing-sensitive exon-arrays detected a change in the splicing pattern of approximately 700 genes (Hnilicova et al., 2011). It was found that the levels of SR proteins and other proteins involved in splicing, as well as the acetylation status of splicing factors were not altered by 15 h of HDAC inhibition. In the case of the fibronectin (*FN1*) alternative exon 25, histone H4 acetylation increased rapidly following HDAC inhibition and reached its maximal level after 6-9 h. An increased exclusion of exon 25 could be detected within this time frame, if cells were treated with DRB, a reversible inhibitor of RNAPII, so that analysis would be limited to *de novo* synthesized pre-mRNA (Hnilicova et al., 2011). It was shown that HDAC inhibition decreased the association of one of the SR proteins, SRSF5 with the *FN1* gene, including but not restricted

to exon 25. siRNA-mediated knockdown of HDAC1, but not HDAC2, resulted in exon 25 skipping, suggesting that HDAC1 is primarily involved in the splicing regulation of this gene (Hnilicova et al., 2011), but the mode of action of HDAC1 remains unclear. In neuronal cells, it was suggested that HDAC2 association with the splicing regulator HuR proteins enhanced the localized histone acetylation at the alternative exons of *NF1* and *FAS* genes, an event that was correlated with a localized increased elongation rate and the exclusion of these exons. *In vitro,* the HuR proteins inhibited HDAC2 activity (Zhou et al., 2011). It was proposed that HuR proteins, co-transcriptionally recruited to their target RNA sequences, inhibit HDAC2 activity through a 'reach back' interaction with chromatin (Zhou et al., 2011). However, the association of HDAC2 with the alternative exons of *NF1* and *FAS* genes was only implied and not directly demonstrated.

Thus, a correlation between histone acetylation and skipping of alternative exons was recognized, but the recruitment and distribution along the body of the transcribed genes of HDACs and KATs, the enzymes catalyzing histone acetylation, were not addressed in the above studies. In this study, we found that the SRSF1 associated with HDAC1 and 2. Since this is a SR protein with a major role in alternative splicing (Sanford et al., 2009), we explored the role of HDAC1 and 2 in splicing using *MCL1* gene as a model gene. The *MCL1* gene undergoes alternative splicing of exon 2 and produces a protein that either prevents or supports cell death. The long form MCL1L is an anti-apoptotic protein, while the short form MCL1S is pro-apoptotic (Bae et al., 2000). We have analyzed the effects of HDAC pan- and class I specific inhibitors on the splicing of the *MCL1* gene. We show that HDAC inhibitors enhanced histone acetylation over exon 2, an event that paralleled with exon 2 exclusion. We also show, for the first time, that

HDAC1 and 2 are recruited to the pre-mRNA in a complex with SRSF1, to catalyze, in concert with KATs, dynamic histone acetylation on exon 2.

5.3 Materials and Methods

5.3.1 Cell cultures and treatments

HEK293, Flp-In 293 stable cells expressing HDAC2-WT-V5 or HDAC2-M3A-V5 and MCF7 cells were cultured in DMEM (Gibco), and HCT116 cells were cultured in McCoy's 5A media (Sigma), supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B, and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with HDAC inhibitors, TSA (250 nM) or apicidin (150 nM) for indicated time periods. When indicated, sub-confluent cells (~80-90%) were serum starved for 48 h and then either left untreated or treated with TPA (100 nM, Sigma) for 30 or 60 min. In inhibition studies, serum starved cells were pretreated with either TSA or apicidin for 30 min followed by treatment with TPA (0, 30 or 60 min).

5.3.2 Mass spectrometry

HDAC1 and 2 complexes were immunoprecipitated from HEK293 cells or Flp In 293 stable cells expressing WT-HDAC2-V5 or HDAC2-M3A-V5, with anti-HDAC1, anti-HDAC2 (Affinity BioReagents) or anti-V5 (Abcam) antibodies. Covalent immobilization of the antibodies onto the surface of Dynabeads Protein G (Invitrogen) was used for co-immunoprecipitation of intact protein complexes. The immunoprecipitated fractions were eluted from the Dynabeads with 1% SDS/ 0.1 M $NaHCO_3$. The eluted fractions were vacuum dried and washed with 100 mM NH_4HCO_3 and iodoacetamide. After lyophilization, the fractions were

digested with trypsin for 16 h at 37°C. The nano-liquid chromatography and tandem mass spectrometry were performed on the trypsin-digested samples as described previously (Meng and Wilkins, 2005). The MSDB, version 20060831, database was searched using the Global Proteome Machine (http://www.thegpm.org) search engine, to identify the peptide sequences.

5.3.3 Immunoprecipitation and immunoblotting

Cells were harvested and lysed in cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 0.5% NP-40) containing phosphatase and protease inhibitors (Roche), and immunoprecipitations were done as described earlier (Sun et al., 2002). In brief, 500 μg total cellular or nuclear extracts were incubated with 3.0 μg of anti-HDAC1 (Affinity BioReagents), anti-HDAC2 (Affinity BioReagents), anti-SRSF1 (Santa Cruz Biotechnology), anti-acetyl lysine (Cell signaling Technology), anti-H3 (Millipore), or anti-RNAPIIS2ph (Abcam) antibodies overnight at 4ºC. Forty μL of protein A/G UltraLink resin were added and incubated for 3 h at 4ºC. The beads were then washed four times with ice-cold lysis buffer. Immunoprecipitation with isotype specific non-related IgG was performed as negative control. One third of the fractions immunoprecipitated by anti-HDAC1 or anti-HDAC2 antibodies and one half of the fractions immunoprecipitated by anti-RNAPIIS2ph or anti-SRSF1 antibodies were analyzed by immunoblotting, while equivalent volumes of lysate (Input) and immunodepleted fractions were analyzed, corresponding to 20 μg (for anti-HDAC1 or anti-HDAC2 antibodies) or 25 μg (for anti-RNAPIIS2ph or anti-SRSF1 antibodies) of lysate proteins. When mentioned, cellular extracts were treated with RNase A (at a final concentration of 400 μg/ mL for 30 min at 37°C) before immunoprecipitation reactions. Immunochemical staining was performed with rabbit polyclonal antibodies against human HDAC1, HDAC2, HDAC2 S394 phospho (Abcam), Sin3A

(Affinity BioReagents), CoREST (RCOR1) (Abcam), or mouse monoclonal antibodies against HDAC2 (Millipore) or RbAp48 (RBPP4) (Abcam).

5.3.4 HDAC activity assay

HDAC activity assay was performed with the Fluor-de-Lys® HDAC fluorometric activity assay kit (Enzo life sciences) following the manufacturer's instructions. SRSF1 complex was immunoprecipitated from 500 µg of HCT116 cell lysates with 5 µg of mouse monoclonal anti-SRSF1 antibody. As a negative control, immunoprecipitation with isotype specific non-related IgG was performed. Forty µL of protein A/G UltraLink resin (Pierce) were added and incubated for 3 h at 4ºC. The beads were washed 3 times with IP buffer (50 mM Tris.Cl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) and twice with the HDAC activity assay buffer before used for the HDAC activity assay. For the assay, the beads treated or not with 1.0 µM TSA were incubated with 150 µM Fluor de Lys® Substrate for 30 min at room temperature with rocking. After that, the developer I solution containing 1.0 µM TSA was added and aliquots were incubated for another 30 min to stop the reactions. For boiling control, after immunoprecipitation, the beads were boiled for 15 min and the assay was performed as mentioned above. The fluorescence signal was measured using fluorometric plate reader (Spectra MAX GEMINI XS, Molecular devices).

5.3.5 ChIP and re-ChIP assays

ChIP and re-ChIP experiments performed as previously described with an additional protein-protein crosslinking step with DSP (Thermo Fisher Scientific) (Drobic et al., 2010). Cells were incubated with 1.0 mM DSP for 30 min at room temperature according to manufacturer's

instruction (Pierce), followed by crosslinking with formaldehyde for 10 min. Dual cross-linked chromatin was processed to mononucleosomes and immunoprecipitations were done with anti-RNAPIIS2ph (Abcam), anti-HDAC2 (Affinity BioReagents), anti-SRSF1 (Santa Cruz Biotechnology), anti-H3K14ac (Abcam), anti-H3K9ac (Abcam), anti-acH3 (Millipore), anti-H4K5ac (Millipore), anti-H4K8ac (Millipore), anti-acH4 (Millipore), anti-H3 (Millipore), anti-KAT2B (Abcam) or anti-KAT7 (Santa Cruz Biotechnology) antibodies. Negative control included performing ChIP/reChIP assays with an isotype matched non-related IgG. For RNase A-treated extracts, DSP and formaldehyde cross-linked chromatin was processed to mononucleosomes and was treated with 400 μg/mL of RNase A for 30 min at 37°C. The further processing of chromatin fragments were performed as previously described (Drobic et al., 2010). Input and ChIP/reChIP DNAs were quantified using PicoGreen assay. Equal amounts of input and immunoprecipitated DNA (1.0 ng) or re-ChIP (0.5 ng) DNA were used to perform SYBR Green qPCR on iCycler IQ5 (Biorad). Primers are described in Supplementary Table S1. Enrichment values, calculated as previously described (Drobic et al., 2010), are relative to input DNA and are the mean of three independent experiments. The error bars indicate standard deviation (n = 3).

5.3.6 siRNA-mediated transient knockdown

HCT116 cells were transfected at about 30-40% confluence, with 75 nM of scramble (Non-targeting Pool), human HDAC1, HDAC2 or SF2 (SRSF1) ON-TARGET plus SMARTpool siRNAs (Thermo Scientific-Dharmacon), using Polyplus Interferin siRNA transfection reagent (VWR) according to manufacturer's protocol. Forty-eight h after transfections, cells were

harvested and the knockdown efficiencies were analyzed by immunoblotting and changes in *MCL1* splicing were analyzed by radiolabeled PCR (^{32}P).

5.3.7 RNA isolation, reverse transcription PCR (RT-PCR) and radiolabeled- PCR (^{32}P)

Total RNA was isolated from untreated and treated HCT116 cells using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA (400 ng) was used as template for synthesis of cDNA using M-MLV reverse transcriptase and Oligo dT primers (Invitrogen). Radiolabeled ^{32}P PCR was performed in a final volume of 15 µL containing 7.5 ng of cDNA, in the presence of 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1 unit of Platinum Taq DNA polymerase and 10 nM α^{32}P dCTP (10 mCi/mL). *MCL1* PCR conditions comprised of 5 min incubation at 95°C, followed by 35 cycles of (95°C for 30 sec, 56°C for 45 sec, 72°C for 45 sec) and a final incubation at 72°C for 10 min. Primers are described in Supplementary Table S1. Following PCR amplification, 2.5 µL of radiolabeled PCR products were denatured in 80% formamide buffer (containing 1 mM EDTA pH 7.5, 0.1% xylene xyanol, 0.1% bromophenol blue) and run on a denaturing polyacrylamide gel (6%) at 40W (constant wattage). After electrophoresis, the gels were dried and exposed to a Molecular Imager ^{TM} FX (Biorad) and PCR signals were quantified. Percentages of the short isoform (*MCL1S*) among total transcripts are presented. The Student's *t*-test for paired samples was used for calculation of statistical significance.

5.3.8 RNA-CLIP assay C

Cycling HCT116 cells, treated or not with HDAC inhibitors TSA (250 nM) or apicidin (150 nM) for 2 h, were either incubated with 1.0 mM DSP for 30 min or not, followed by irradiation under ultraviolet (UV) light (400mJ/cm^2) (Stratalinker). Cells were harvested and lysed in ice-cold lysis buffer (20 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.5% NP-40, 0.5% Triton X 100, 0.1% SDS, protease and phopshatase inhibitor cocktail [Roche], 80 U/mL RNasin [Promega]) with sonication. The cellular extracts were treated with a dilute cocktail of RNase A/T1 (Ambion) as previously described (Sanford et al., 2009). Cellular extracts were pre-cleared with protein A/G UltraLink resin (Pierce) and tRNA (Sigma) (at a final concentration of 100 μg/mL) for 2 h at 4°C. The lysate was incubated with anti-HDAC1, anti-HDAC2, anti-SRSF1 or anti-KAT2B antibodies or normal rabbit or mouse IgG on a rotator overnight at 4°C, followed by addition of protein A/G UltraLink resin (Pierce) for 3 h at 4°C. Beads were then washed six times using the lysis buffer. The beads were re-suspended in 200 μL of lysis buffer and then treated with proteinase K (2 mg/mL) for 1 h at 55°C. Immunoprecipitated RNA was then extracted using Trizol (Invitrogen) and precipitated with ethanol. RNA precipitates were resuspended in 20 μL of ultrapure water, treated with RQ DNase (Promega) for 1 h at 37°C. Equal amounts of input and immunoprecipitated RNA were used for cDNA synthesis and reversed transcribed using oligo dT and random hexamers with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's specifications. The resulting cDNA was analyzed by real time PCR with the indicated primer sets in table S1. Results are representative of three independently performed experiments.

5.4. Results

5.4.1 HDAC1 and 2, but not HDAC2 S394 phospho, is in complexes with SRSF1 along the body of transcriptionally active genes

To identify the proteins associated with HDAC1 and 2 along the body of transcribed genes, we established Flp-In 293 stable cell lines expressing V5-tagged versions of wild type HDAC2 (WT) or a triple mutant HDAC2 (M3A), which has the three CK2-phosphorylation sites mutated from serine to alanine, and immunoprecipitated HDAC complexes using antibodies against the V5 tag (Khan et al., 2013). We also isolated native HDAC1 and 2 complexes from HEK293 cells. Proteins co-immunoprecipitated with the HDACs were identified by mass spectrometry. Table 5.1 shows that HDAC1 and HDAC2 were bound to the Sin3, NuRD and CoREST complexes as well as to numerous RNA splicing proteins. Non-phosphorylated HDAC2, however, interacted with RNA splicing factors, but not with components of corepressor complexes, confirming previous results that HDAC2 phosphorylation is required for corepressor complex formation (Tsai and Seto, 2002;Sun et al., 2002;Sun et al., 2007). Since HDAC1 and 2 is associated with splicing factors, we determined whether these interactions were mediated by RNA. Nuclear extracts from Flp-In 293 expressing HDAC2-WT-V5 or HDAC2-M3A-V5 were treated with RNase A prior to immumoprecipitation with anti-V5 antibodies. This RNase A treatment did not affect the association of HDAC2 with RNA splicing factors (data not shown).

Table 5.1: Proteins associated with exogenous wild type (WT) or mutated (M3A) HDAC2, and with endogenous HDAC1 and 2

	Flp-In 293 expressing HDAC2-WT-V5	**Flp-In 293 expressing HDAC2-M3A-V5**	**HEK293**	**HEK293**
	Anti-V5 IP	**Anti-V5 IP**	**Anti-HDAC2 IP**	**Anti-HDAC1 IP**
Corepressor complexes	HDAC1, 2 RBBP4, 6, 7 MTA1, 2, 3 SIN3A CHD3, 4 MBD2, 3 RCOR1 KDM1A		HDAC1, 2 RBBP4, 6, 7 MTA1, 2, 3 SIN3A CHD3, 4 MBD2, 3 RCOR1 KDM1A	HDAC1, 2 RBBP4, 6, 7 MTA1, 2, 3 SIN3A CHD3, 4 MBD2 RCOR1 KDM1A
RNA splicing	SRSF1, 3, 4, 6, 7, 10, 11, 12 HNRNPA2/B1, H, K, M, Q, U RBMX PTBP1 SNRNP200 SNRNP40 SNRPB2 SNRPD1 SNRPD3 SNRPE PRPF4B, 6, 8, 19 RBM22, 25, 39 SF3B1- 4 SFPQ	SRSF1, 3, 4, 6, 7, 10, 11, 12 HNRNPA2/B1, H, K, M, Q, U RBMX PTBP1 SNRNP200 SNRNP40 SNRPB2 SNRPD1 SNRPD3 SNRPE PRPF4B, 6, 8, 19 RBM22, 25, 39 SF3B1- 4, SF3A1, 2 SFPQ	SRSF1, 4, 6, 7, 11, 12 HNRNPA2/B1, H, K, M, Q, U RBMX PTBP1 SNRNP200 SNRNP40 SNRPB2 SNRPD1 SNRPD3 SNRPE PRPF4B, 6, 8, 19 RBM22, 25, 39 SF3B2	SRSF1, 3, 4, 6, 7, 11, 12 HNRNPA2/B1, H, K, L, M, Q, U RBMX PTBP1 SNRNP200 SNRNP40 SNRPB2 SNRPD1 SNRPD3 SNRPE PRPF4B, 6, 8, 19 RBM22, 25, 39 SF3B1,3

Note: Except for HNRNPQ, which is coded for by *SYNCRIP* gene, immunoprecipitated proteins are identified by the genes encoding them.

Among the proteins associated with HDAC1 and 2, we focused our studies on SRSF1. SRSF1 is a sequence-specific RNA binding factor that promotes spliceosome formation by binding to exonic splicing enhancers during pre-mRNA splicing (Cho et al., 2011). Interactions between HDAC1 and 2 and SRSF1 were validated in reciprocal co-immunoprecipitation assays using HCT116 cell lysates (**Figure 5.1A**). SRSF1 was associated with HDAC2, but not with either the

HDAC2 S394 phospho or the Sin3A, RBPP4 (RbAp48) or RCOR1 (CoREST) components of corepressor complexes (**Figure 5.1A**).

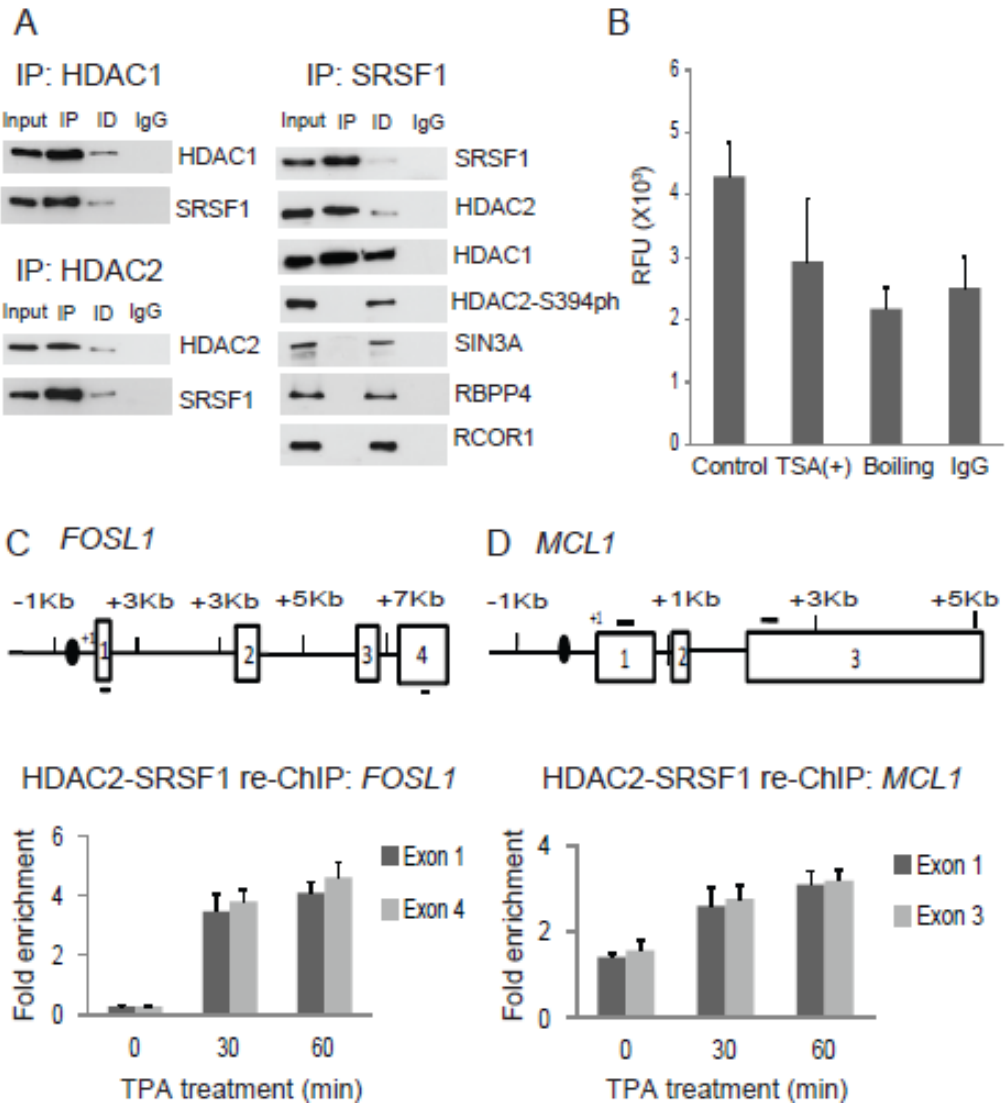

Figure 5.1: SRSF1 forms complexes with HDAC1 and 2, but not with HDAC2 S394 phospho, along the body of transcribed genes

A. HCT116 cell lysates (500 μg) were incubated with anti-HDAC1, anti-HDAC2 or anti-SRSF1 antibodies. Immunoprecipitated (IP) and immunodepleted (ID) fractions were analyzed by immunoblot assays for the presence of indicated proteins, as described in 'Materials and Methods' section. Isotype specific non-related IgGs were used as negative control. B. HDAC activity in anti-SRSF1 immunoprecipitate from HCT116 cell lysate was measured, using a fluorometric activity assay. RFU is relative fluorescent units. C. Schematic representation of *FOSL1* gene, with amplicons generated in ChIP assays shown below map. Open boxes represent exons, and oval represents the upstream promoter element. HDAC2/SRSF1 re-ChIP experiments were performed on dual cross-linked mononucleosomes prepared from serum starved HCT116 cells treated with 100 nM TPA for 0, 30 or 60 min. D. As in C, for *MCL1* gene.

Thus, HDAC2 phosphorylation specifies its incorporation into corepressor complexes at UPR of genes, while lack of phosphorylation directs HDAC2 association with complexes involved in pre-mRNA splicing. Similarly, SRSF1 co-immunoprecipitated with unmodified HDAC2, but not with HDAC2 S394 phospho in MCF7 and HEK293 cells (**Figure S1**).

As splicing regulator HuR proteins interact with HDAC2 and inhibit its activity (Zhou et al., 2011), we measured the HDAC activity associated with SRSF1 complexes. **Figure 5.1B** shows that the HCT116 cell lysate fraction immunoprecipitated by antibodies against SRSF1 had HDAC activity, which was sensitive to the HDAC inhibitor TSA or to boiling. Further, the addition of a recombinant GST-SRSF1 fusion protein to HDAC2 complexes immunoprecipitated from a HCT116 cell lysate with antibodies against HDAC2 did not affect HDAC activity (data not shown). Thus, SRSF1 did not inhibit HDAC activity.

Following transcription initiation, RNAPII pauses until it becomes the elongation-competent form, RNAPIIS2ph. SRSF1 is recruited to RNAPIIS2ph, as soon as the transition to productive elongation occurs (Barboric et al., 2009). In agreement with this result, SRSF1 was shown to accumulate along the body of the *FOS* gene only upon induction (Sapra et al., 2009). Similarly, we found that SRSF1 and HDAC1 and 2, were recruited to the body of the *FOSL1*, *MCL1* (**Figure S2**) and *TFF1* (data not shown) genes upon induction. To determine if HDAC1 or 2 was co-recruited with SRSF1 to the body of transcribed genes, we studied three different genes in two cell lines. Serum starved HCT116 cells were stimulated with the phorbol ester, TPA, to activate the MAPK pathway and induce the expression of IEGs (i.e., *FOSL1*) (Drobic et al., 2010). To determine the co-occupancy of HDAC2/SRSF1 or HDAC1/SRSF1 on *FOSL1* exons 1

and 4, sequential ChIP assays were performed, using the dual cross-linking high resolution ChIP assay sequentially with antibodies against HDAC2 or HDAC1 and SRSF1. Prior to TPA induction, there was no HDAC2/SRSF1 or HDAC1/SRSF1 associated with these exons, but following TPA induction, HDAC2/SRSF1 or HDAC1/SRSF1 loaded onto the *FOSL1* gene (**Figure 5.1C** and **S3A**). The expression of the *MCL1* gene was also increased in response to the MAPK pathway activation (Booy et al., 2011). Accordingly, we observed an increased HDAC1/HDAC2 and SRSF1 co-occupancy on *MCL1* exons 1 and 3, upon TPA induction (**Figure 5.1D** and **S3A**). Likewise, induction of the *TFF1* gene in MCF7 cells by either estrogen or TPA (Pentecost et al., 2005;Espino et al., 2006) resulted in the co-recruitment of HDAC2 and SRSF1 to the body of the gene (**Figure S3C**). Thus, the co-recruitment of HDAC1 or 2 and SRSF1 to the body of transcribed genes was dependent on transcription, and occurred whether splicing was constitutive (*FOSL1* and *TFF1*) or alternative (*MCL1*).

5.4.2 SRSF1 and HDAC1 and 2, regulate alternative splicing of *MCL1*

SRSF1 regulates the alternative splicing of many genes (Sanford et al., 2009), including the splicing of *MCL1*, which undergoes alternative splicing of exon 2 (Moore et al., 2010) (**Figure 5.2A**). Hence, toward the understanding of the mechanisms by which HDAC inhibitors alter pre-mRNA splicing, we selected *MCL1* as a model gene. It was shown that a decline in availability of SRSF1 favored the production of the pro-apoptotic *MCL1* short form (*MCL1S*) transcript (Moore et al., 2010). Similarly we found that knockdown of SRSF1 affected the splicing of *MCL1* transcripts in HCT116 cells. We observed a greater exclusion of alternative exon 2 in HCT116 cycling cells transiently transfected with SRSF1 siRNA compared to cells transfected with scramble (non-targeting) siRNA. Four independent sets of data showed that the increase of

the percentage of *MCL1S* transcripts in SRSF1 knockdown cells had a strong statistical significance (**Figure 5.2B**). A value of 84.5 ± 5.3 % SRSF1 knockdown was determined by immunoblot analysis (**Figure S4A**).

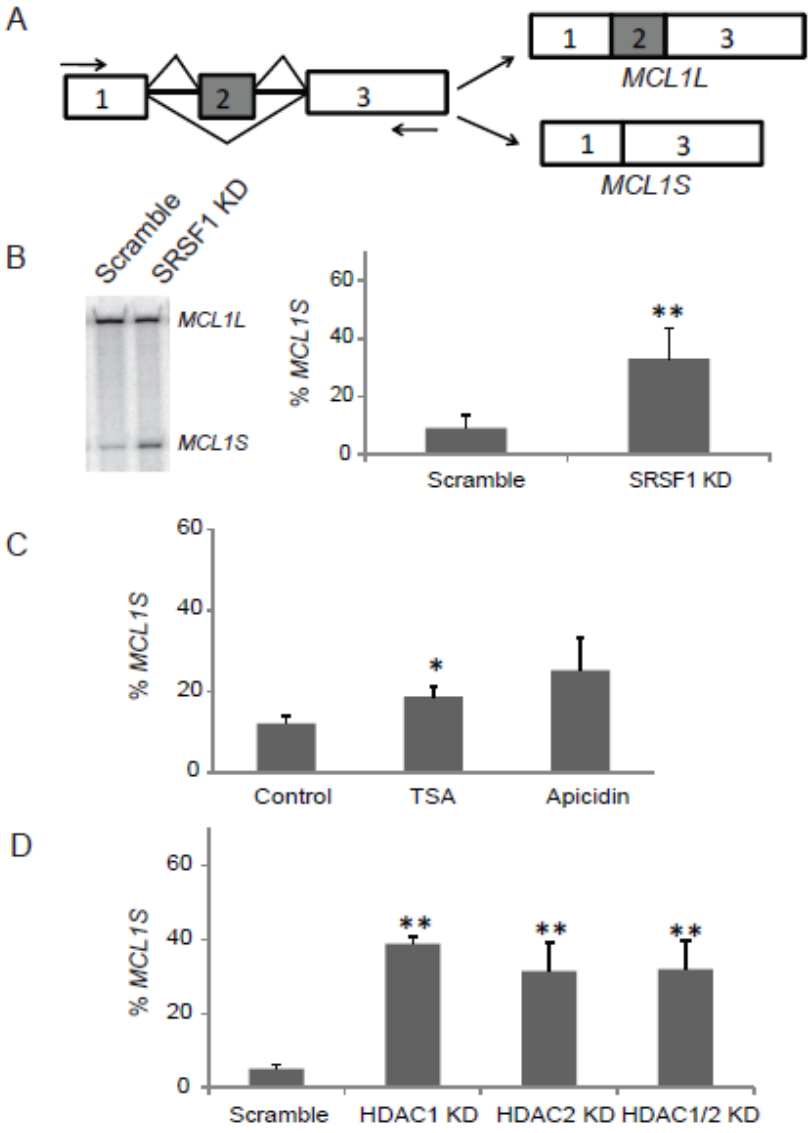

Figure 5.2: SRSF1, and HDAC1 and 2 regulate alternative splicing of *MCL1*
A. Schematic representation of *MCL1* alternative splicing. Arrows above and below map represent primers used in RT-PCR assays. *MCL1* mature mRNAs were visualized and quantified on denaturing polyacrylamide gels after ^{32}P labeling RT-PCR, in HCT116 cycling cells following B. SRSF1 knockdown, C. HDAC inhibitor (250 nM TSA or 150 nM apicidin) 2 h treatment or D. HDAC1, HDAC2 or both knockdown. The average of at least three experiments is shown including SD (error bars), ** indicates $p \leq 0.01$ and * $p \leq 0.05$ of the *t*-test.

Next, we determined the effect of HDAC inhibition on the alternative splicing of *MCL1*. To control for any off-target effect, we used two HDAC inhibitors with different selectivity: TSA or apicidin. TSA, like sodium butyrate, is considered a pan-inhibitor; meaning it inhibits class I and II HDACs. On the other hand, apicidin is a class I HDAC inhibitor, inhibiting specifically HDAC1, 2 and 3 (Bantscheff et al., 2011). To minimize secondary effects of HDAC inhibitors, we limited treatment times to a maximum of 2 h. Moreover, we tested the expression and acetylation levels of SRSF1 following treatment with HDAC inhibitors. In cycling HCT116 cells treated with TSA or apicidin for only 2 h, a shift in the splicing of the *MCL1* was observed in favor of *MCL1S* (**Figures S4B** and **5.2C**). Immunoblot analysis showed that the change in splicing resulting from HDAC inhibition was not due to a reduced expression of SRSF1. Up to 24 h treatment with TSA or apicidin did not affect SRSF1 protein levels (**Figure S4C**). As SRSF1 is modified by acetylation (Choudhary et al., 2009), we probed its acetylation level with or without 2 h TSA or apicidin treatment. Reciprocal co-immunoprecipitation assays showed that the acetylation level of SRSF1 was not affected by HDAC inhibitors, while the histone H3 acetylation level was markedly increased (**Figure S4D**). Thus, we can rule out an indirect effect of TSA or apicidin on *MCL1* splicing through differences in SRSF1 levels or SRSF1 acetylation.

To specifically study the outcome of loss of HDAC1 and 2 activities, we determined the effects of HDAC1, HDAC2 or both (HDAC1 and 2) knockdown on the *MCL1* splicing pattern. A representative immunoblot analysis of HDAC1 and 2 knockdowns in HCT116 cells is shown in **Figure S4E**. HDAC1 knockdown was determined to be 78 ±1% in HDAC1 knockdowns and 76 ±2% in both knockdowns in HCT116 cells, while HDAC2 knockdowns were 84 ± 10 % and 85 ± 12%, in HDAC2 and both knockdown cells, respectively. We show that knockdown of either

HDAC1 or HDAC2 or both led to a strongly significant change in splicing in favor of exon 2 skipping (**Figures S4F** and **5.2D**). The immunoblot in **Figure S4E** shows that the SRSF1 level was not affected by HDAC knockdown. These results indicate that HDAC1 and 2 play a role in splice site selection in association with SRSF1, by a mechanism other than altering expression or acetylation levels of SRSF1.

5.4.3 HDAC inhibitors affect the *MCL1* gene occupancy by RNAPII

The current literature supports the kinetic model where variation in RNAPII elongation rate or processivity modulates the splice site recognition efficiency. For genes with alternative exons that have weak splice sites, a slowly moving RNAPII results in the inclusion of the exons in the RNA (Carrillo Oesterreich et al., 2011) To investigate the effect of HDAC inhibitors on the *MCL1* gene occupancy by RNAPII, we performed dual cross-linking high resolution ChIP assays to assess the positioning of the elongating form of RNAPII, RNAPIIS2ph. The distributions of SRSF1 and HDAC2 were also determined. The location of the amplicons generated in the ChIPs assays is shown in **Figure 5.3A**. In cycling HCT116 control cells, the level of RNAPIIS2ph along the *MCL1* gene was fairly constant; however, in apicidin treated cells, there was a variation in RNAPIIS2ph levels along the gene. Highest levels, associated with exon 1, decreased progressively along the gene to reach a minimum in alternative exon 2, and then started to increase again (**Figure 5.3B**). In agreement with their RNAPII transcription-dependent recruitment to the body of active genes, the distributions of SRSF1 and HDAC2 along the *MCL1* gene were similar to that of RNAPIIS2ph (**Figure 5.3B**).

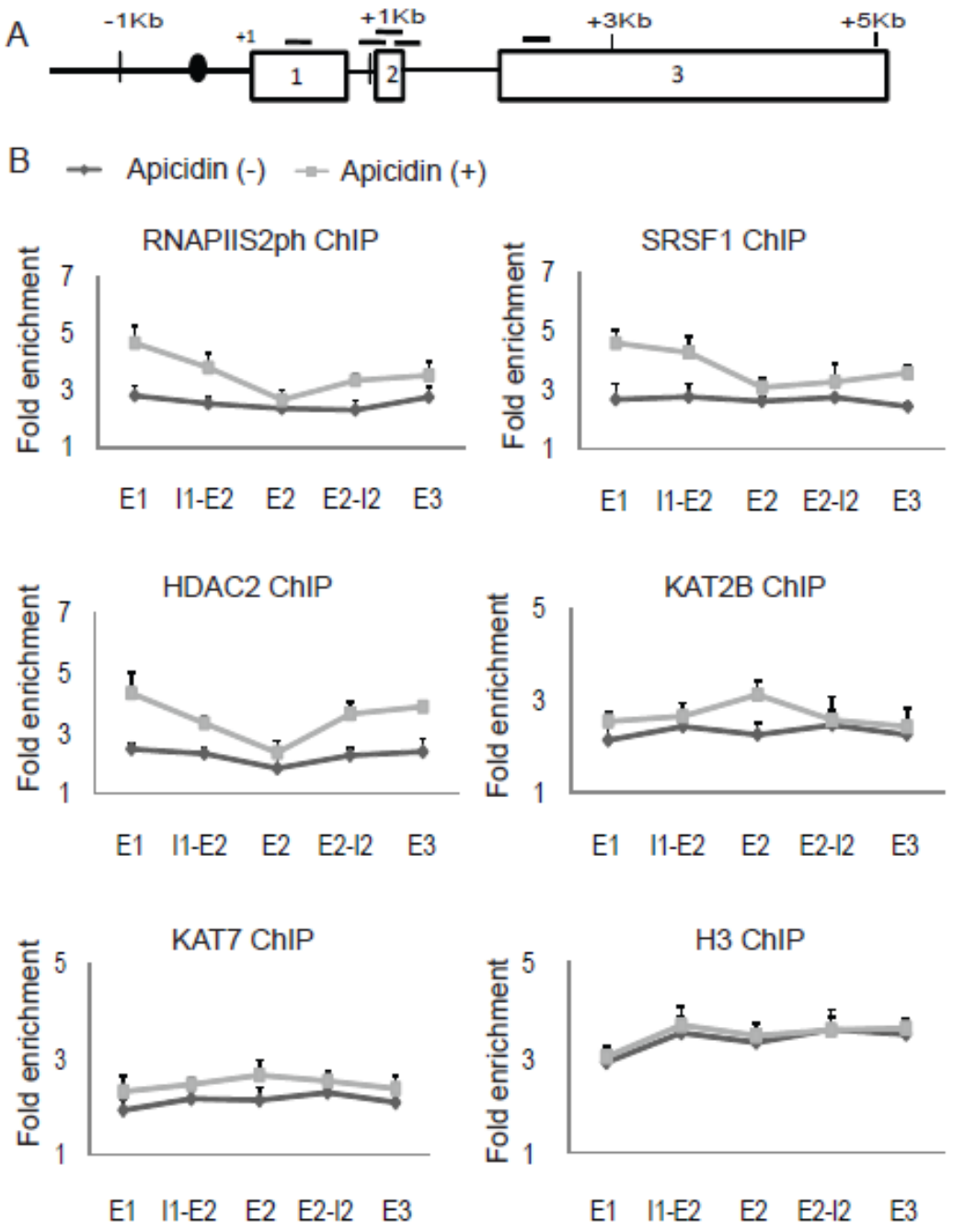

Figure 5.3: HDAC inhibition affects the distribution along the *MCL1* gene of RNAPIIS2ph and associated proteins

A. Map of amplicons generated in ChIP. B. Effect of apicidin on the distribution of RNAPIIS2ph, SRSF1, HDAC2, KAT2B, KAT7 and total H3 along the *MCL1* gene body. ChIP experiments were performed on dual cross-linked mononucleosomes prepared from HCT116 cells treated or not with 150 nM apicidin for 2 h.

As dynamic acetylation results from the balance of opposing activities of HDACs and KATs, we turned our attention to KATs. A genome wide mapping of several KATs revealed that p300 and CBP as well as KAT4 were associated with promoter regions, while KAT2B, KAT5 and KAT8 were located with transcribed regions (Wang et al., 2009). Moreover, KAT2B and KAT7 have been implicated in the elongation phase of transcription (Cho et al., 1998;Obrdlik et al., 2008;Saksouk et al., 2009;Selth et al., 2010). Both KAT2B and KAT7 are H3 and H4 modifying enzymes (Lee and Workman, 2007). Hence, their distribution along the *MCL1* gene body was studied. The distributions of KAT2B and KAT7 were opposed to that of HDAC2, with an increased loading on exon 2 (**Figure 5.3B**). Meanwhile, results of H3 ChIP assays showing a uniform association of H3 along the *MCL1* gene with or without apicidin (**Figure 5.3B**) are consistent with ChIP assays being performed on mononucleosomes obtained by MNase digestion of chromatin.

Next, we tested the effects of HDAC inhibition on the acetylation levels of nucleosomal histones H3 and H4. In apicidin treated cells, levels of H3 and H4 overall acetylation as well as levels of H3K9ac, H3K14ac, H4K5ac and H4K8ac peaked in exon 2, showing a positive correlation with KAT2B and KAT7 levels. In contrast, levels of H3K9ac and H3K14ac in control untreated cells exhibited a reduction in exon 2 (**Figure 5.4**).

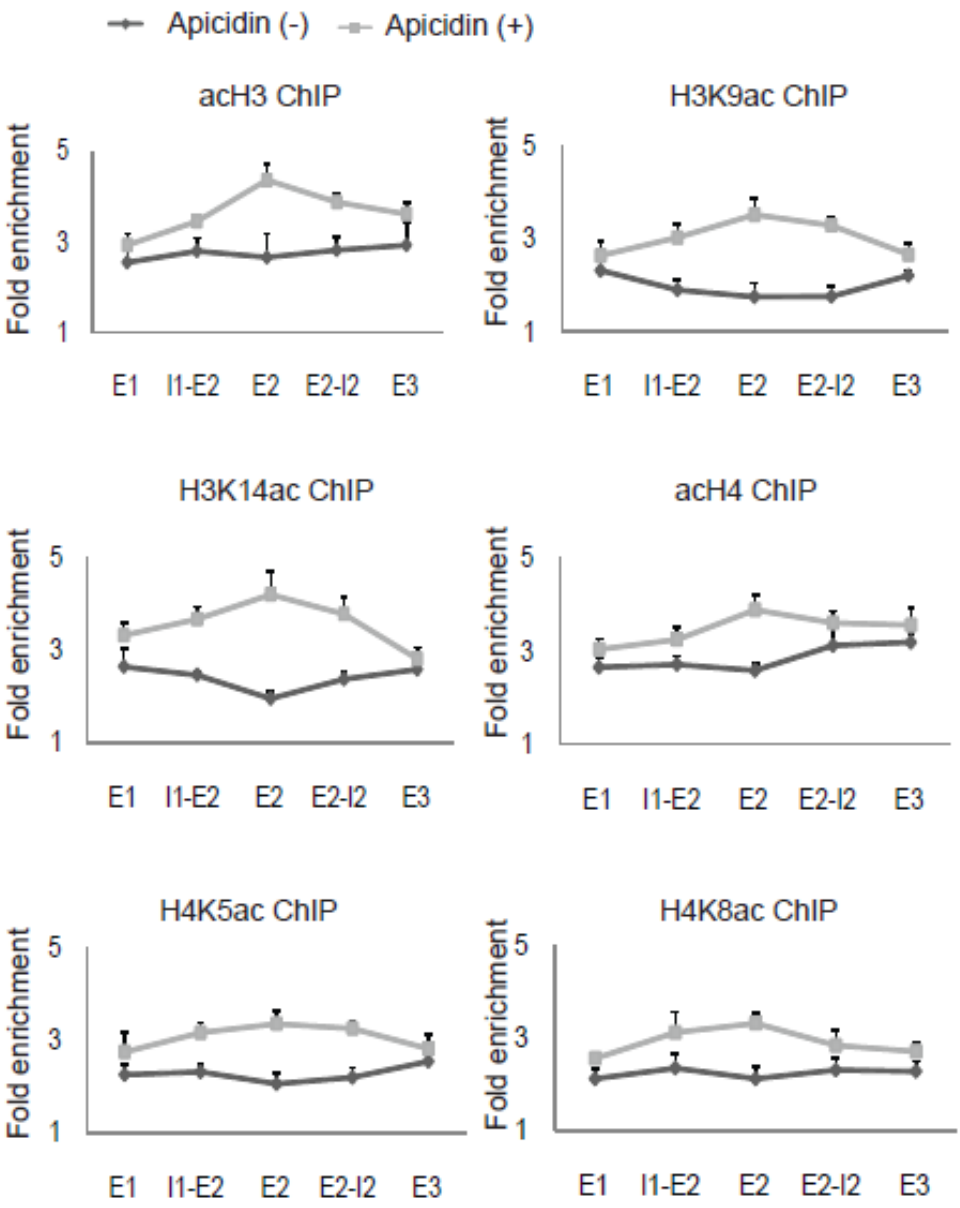

Figure 5.4: HDAC inhibition specifically increases H3 and H4 acetylation on *MCL1* exon 2
ChIP experiments were performed as described in Figure 5.3.

As TSA has a broader specificity than apicidin, we repeated the above ChIP assays to assess the effects of TSA. Comparable results were obtained when HDAC activity was inhibited by TSA (**Figure S5**). Overall, under the influence of HDAC inhibitors, occupancy by RNAPIIS2ph was reduced over the acetylated nucleosome at exon 2, resulting in a change in alternative splicing towards the production of the *MCL1S* transcript. The capture by ChIP of fewer RNAPIIS2ph

molecules over exon 2 than over exons 1 and 3 suggests that RNAPIIS2ph progresses faster over exon 2 than over exons 1 and 3.

5.4.4 Effect of HDAC inhibitors on *MCL1* splicing is amplified upon TPA stimulation of serum starved cells

Considering the obvious consequences of HDAC inhibition on chromatin surrounding exon 2, the effects on splicing were unexpectedly small (**Figure 5.2C**). This discrepancy was also observed previously and was interpreted as an interference from the mRNA synthesized and spliced before HDAC inhibition (Hnilicova et al., 2011). To alleviate this problem, the authors treated the cells with DRB, a reversible inhibitor of RNAPII, prior to HDAC inhibition. In this study, we exploited the transcriptional response of the *MCL1* gene downstream of the MAPK pathway (Booy et al., 2011) to study the effects of HDAC inhibitors on splicing, during a synchronized induction of *MCL1* expression. Serum starved HCT116 cells, pretreated or not for 30 min with apicidin or TSA, were stimulated with TPA. TPA stimulation of *MCL1* expression in control cells resulted in a shift in the splicing pattern towards the short RNA form *MCL1S* (See apicidin (-), TPA 60 min in **Figure 5.5A**; TSA (-), TPA 30 and 60 min in **Figure S6A**). With an increase in transcription initiation at the *MCL1* promoter, a higher density of elongating RNAPII was expected along the body of the gene. Indeed, RNAPIIS2ph ChIP assays revealed an increase in the number of RNAPII molecules along the body of the *MCL1* gene (See apicidin (-), TPA 60 min in **Figure 5.5B**; TSA (-), TPA 30 and 60 min in **Figure S6B**). This increased occupancy along the *MCL1* gene was also observed for SRSF1, HDAC2, KAT2B and KAT7 (**Figures 5.5B** and **S6B**), suggesting that all three proteins were associated with the elongating RNAPII complex.

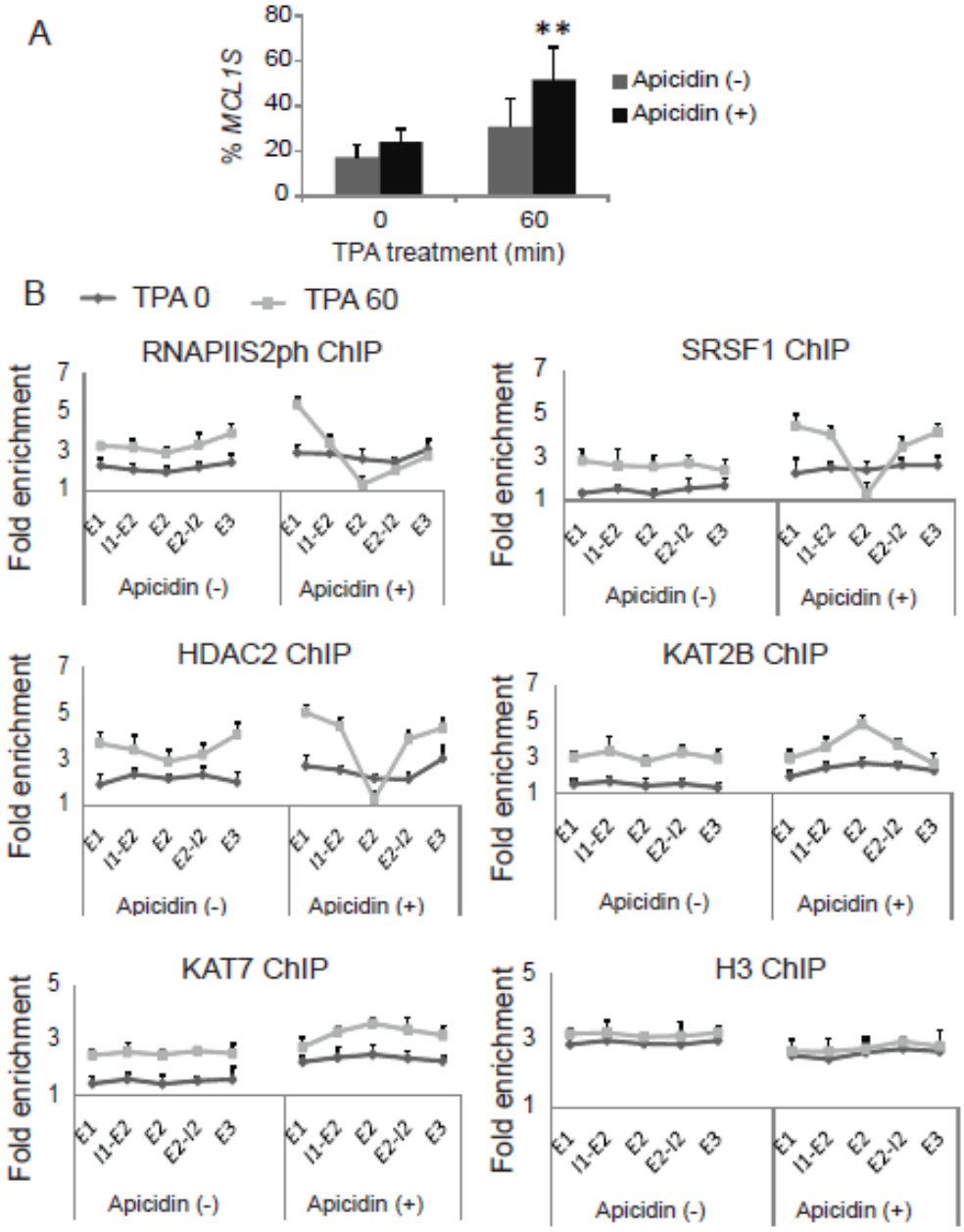

Figure 5.5: HDAC inhibition favors exclusion of alternative exon 2 upon TPA induction of *MCL1* gene

A. *MCL1* alternative exon 2 splicing was analyzed in TPA-stimulated serum starved HCT116 cells treated or not with 150 nM apicidin prior to TPA induction. B. ChIP assays were performed on TPA-stimulated serum starved HCT116 cells treated or not with 150 nM apicidin for 30 min prior to TPA induction, as described in Figure 5.3.

MCL1 TPA stimulation was also accompanied by a global H3 and H4 acetylation (as well as H3K9ac, H3K14ac, H4K5ac and H4K8ac) increase along the body of the *MCL1* gene (**Figures 5.6** and **S7**).

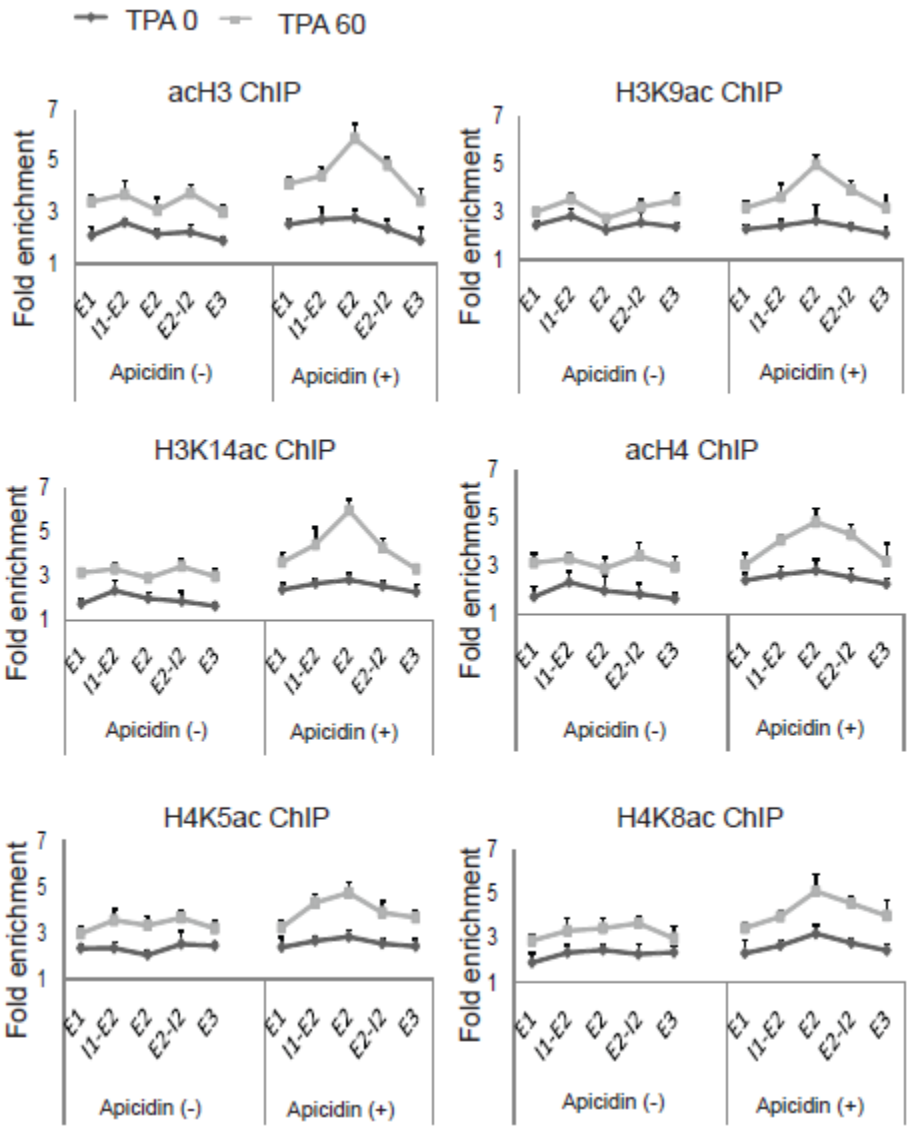

Figure 5.6: HDAC inhibition specifically increases H3 and H4 acetylation over exon 2 upon TPA induction of *MCL1* gene
ChIP experiments were performed as described in Figure 5.5.

Besides interfering with dynamic acetylation of histones, HDAC inhibitors upregulate *MCL1* transcription (Inoue et al., 2008). Thus, we analyzed the *MCL1* splicing pattern in serum starved

HCT116 cells subjected to a 30 min pre-incubation with apicidin or TSA, just prior to TPA induction. Similar to the TPA-induced control cells, apicidin or TSA treated cells had a slightly greater level of *MCL1S* than control cells (See apicidin (+), TPA 0 min in **Figure 5.5A**; TSA (+), TPA 0 min in **Figure S6A**). Apicidin or TSA treated cells also exhibited an increased occupancy of RNAPIIS2ph, SRSF1 and HDAC2 along the body of the *MCL1* gene compared to untreated cells, reflecting the HDAC inhibitor induced upregulation of transcription initiation. However, at the same time, KAT2B and KAT7 started to associate specifically with exon 2. Moreover, a parallel exon 2-specific H3 and H4 acetylation increase was observed (**Figures 5.6** and **S7**). So, after 30 min of inhibition of HDAC activity in serum-starved HCT116 cells, KAT2B and KAT7 have started to target the nucleosome positioned on the alternative exon.

Following TPA stimulation, apicidin and TSA had a profound impact on splicing, resulting in a marked increase of *MCL1S* transcript (See apicidin (+), TPA 60 min in Figure 5A; TSA (+), TPA 30 and 60 min in Figure S6A). In the meantime, while RNAPIIS2ph on exon 1 increased as a consequence of TPA induction, the occupancy over exon 2 dropped dramatically, suggesting a sharp acceleration of the elongation process over exon 2. At exon 3, RNAPIIS2ph occupancy was similar to those on exon 1 (See apicidin (+), TPA 60 min in **Figure 5.5B**; TSA (+), TPA 30 and 60 min in **Figure S6B**). Moreover, the distribution of SRSF1 and HDAC2 on the body of the *MCL1* gene was consistent with these proteins being associated with RNAPIIS2ph. On the other hand, KAT2B and KAT7 levels rose sharply at exon 2 (**Figures 5.5B** and **S6B**), as did overall H3 and H4 acetylation, as well as H3K9ac, H3K14ac, H4K5ac, H4K8ac (**Figures 5.6** and **S7**). It should be noted that changes at exon 2 were gradual changes occurring over time. This is particularly noticeable in the TSA inhibition experiment (**Figures S6B** and **S7**). As a control, the

total H3 distribution along the body of the *MCL1* gene was analyzed. It was unaffected by TPA induction with or without apicidin or TSA treatment (**Figures 5.5B** and **S6B**).

To recapitulate the above results, the TPA induction of *MCL1* expression was accompanied by an overall increase in density of RNAPIIS2ph and a slight increase in alternative exon 2 exclusion. On the other hand, the TPA induction of *MCL1* expression with HDAC inhibition led to gradual important local changes on exon 2 chromatin, resulting in exclusion of exon 2 from the pre-mRNA. Increased acetylation of H3 and H4 on nucleosome residing on exon 2 and targeting of KAT2B to exon 2 preceded the apicidin or TSA induced RNAPIIS2ph reduced occupancy over exon 2 (See apicidin (+), TPA 0 min in **Figures 5.5B** and **5.6**; TSA (+), TPA 0 min in **Figures S6B** and **S7**). These results suggest that the destabilization of the nucleosome over exon 2 leads to an increased elongation rate of this exon.

To test that HDAC inhibitors caused changes in elongation rates along the body of the *MCL1* gene, we applied an elongation assay previously described (Zhou et al., 2011). Briefly, HCT116 cycling cells were treated with the transcription inhibitor DRB to block transcription elongation. After DRB treatment, cells were incubated with bromouridine (BrU) with or without apicidin, so that BrU was incorporated into newly synthesized pre-mRNA transcripts, which were then immunoprecipitated at different time points by anti-BrU antibodies and quantified by real-time RT-PCR. The amplicons generated in the RT-PCR assays covered exon-intron junctions to eliminate the amplification of spliced RNAs (**Figure S8A**). While the accumulation rate of exons 1 and 3 was not affected, a 2.2-fold increase in pre-mRNA accumulation rate occurred for alternative exon 2 (**Figure S8B**, left panel) with cycling cells treated with apicidin [by

comparing the rates of exon 1 (0.68) and exon 2 (1.47)]. Comparable results were obtained when HCT116 cycling cells were treated with TSA (**Figure S8B**, right panel). However, with the serum starved cells, a 3.7-fold increase in the pre-mRNA accumulation rate of alternative exon 2 compared to exons 1 and 3 was observed upon HDAC inhibition by apicidin (**Figure S8C**). The results also were reproduced with TSA treatment. Although our aim was to measure the RNAPII elongation rate from this method as previously described, it is not feasible for this gene, given the short distance between the *MCL1* promoter and exon 2 (transcription rate ranges from 1 to 6 kb/min) (Henriques and Adelman, 2013). Further, transcription rates can be influenced by splicing and *vice versa*, and splicing takes significantly less time than transcription (~30 sec to 5 min) (Brugiolo et al., 2013). Therefore, the observed results cannot be explained by increased RNAPII elongation rate over exon 2. However, our results point towards an interesting possibility that the efficiency of exon 2 splicing (e.g. intron removal) might change or slow down with HDAC inhibitor treatment, which results in the increased accumulation of exon 2 detected by PCR. As such, the increased rate of the accumulation of exon 2-intron 2 pre-mRNA detected in turn might reflect the formation of the 'exon-intron lariat' with HDAC inhibitor treatment. HDAC inhibitors might affect the activity or expression of the RNA debranching enzyme (DBR1) required for hydrolyzing the lariats, resulting in the stabilization of the 'exon-intron lariat' and accumulation of more pre-mRNA.

5.4.5 HDAC1 and 2, and KAT2B are associated with both the pre-mRNA and chromatin

ChIP assays have shown that the association of several splicing factors (SR proteins and HuR) to transcribed genes was sensitive to RNase A treatment, indicating that these proteins bind more strongly to the pre-mRNA than to elongating RNAPII (Zhou et al., 2011;Sapra et al., 2009). To

determine whether this was the case for SRSF1, we performed ChIP assays on dual cross-linked mononucleosomes prepared from lysates of TPA-stimulated serum starved HCT116 cells, which were treated or not with RNase A prior to the immunoprecipitation step. Our results show that RNase treatment of dual cross-linked mononucleosomes reduced the association of SRSF1 with the body of the *MCL1* gene in serum-starved and TPA-stimulated HCT116 cells (**Figure 5.7A**), suggesting that SRSF1 was bound to the pre-mRNA. As HDAC2 is co-recruited with SRSF1 to the body of transcribed genes, we repeated our ChIP assay with antibodies against HDAC2. **Figure 5.7A** shows that HDAC2 association with *MCL1* was markedly reduced upon RNase digestion, suggesting that HDAC2 interacted with the *MCL1* pre-mRNA.

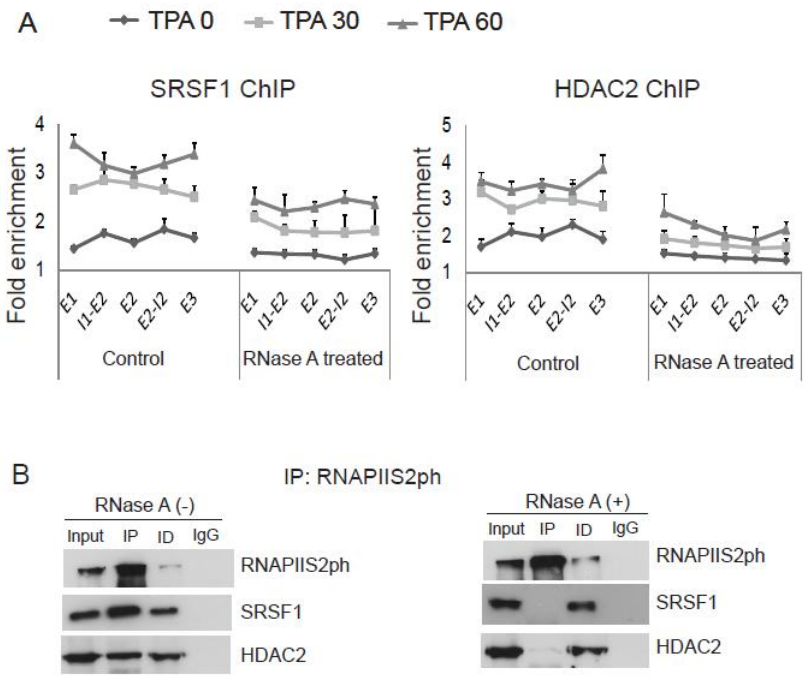

Figure 5.7: SRSF1 and HDAC2 recruitment to *MCL1* gene body and their interactions with RNAPII are RNA-dependent

A. ChIP experiments were performed on dual cross-linked mononucleosomes prepared from lysates of serum starved HCT116 cells stimulated with 100 nM TPA for 0, 30 or 60 min. Lysates were treated or not with RNase A. B. SRSF1 and HDAC2 interactions with RNAPIIS2ph were dependent on RNA. HCT116 cell lysates treated or not with RNase A were incubated with anti-RNAPIIS2ph antibodies. Immunoprecipitated (IP) and immunodepleted (ID) fractions were analyzed by immunoblot assay for the presence of indicated proteins. Isotype specific non-related IgGs were used as negative control.

It was shown that SRSF1 interaction with RNAPIIS2ph was also mediated by the pre-mRNA given that it was sensitive to RNase as opposed to a direct protein-protein interaction, which would be RNase-insensitive. We set out to confirm this finding in HCT116 cells and find out if HDAC2 also formed an RNase-sensitive co-transcriptional complex with RNAPIIS2ph. Thus, we carried out reciprocal co-immunoprecipitation assays using HCT116 cell lysates treated or not with RNase A. **Figure 5.7B** shows that SRSF1 interaction with RNAPIIS2ph was RNA-dependent as SRSF1 did not co-immunoprecipitate with RNAPIIS2ph in the presence of RNase A, and *vice versa* (**Figure S9**). Similar results were obtained for HDAC2 and RNAPIIS2ph interactions (**Figures 5.7B** and **S9**).

To confirm the association of SRSF1 and HDAC2 with the *MCL1* mRNA, we used the CLIP method to analyze their *in situ* direct binding to RNA. We also investigated the interactions of HDAC1 and KAT2B with RNA, as KAT2B had been reported to associate with RNAPII, in complex with hnRNP U and actin (Obrdlik et al., 2008). RNP complexes in cycling HCT116 cells, treated or not for 2 h with apicidin or TSA, were covalently cross-linked by UV exposure only or by DSP and UV exposure, and RNA complexes were immunopurified with anti-SRSF1, anti-HDAC1, anti-HDAC2 or anti-KAT2B antibodies. In UV-treated cells, RT-PCR analysis showed a significant enrichment of *MCL1* exon 1, exon 2 and exon 3 mRNAs in SRSF1 immunoprecipitation versus an irrelevant IgG control, but the enrichments in HDAC1, HDAC2 or KAT2B immunoprecipitations were minimal for all three exon mRNAs (**Figure 5.8**). On the other hand, when RNP complexes were cross-linked by DSP and UV exposure, there was a significant enrichment of *MCL1* exon 1, exon 2 and exon 3 mRNAs in each of the immunoprecipitations versus an irrelevant IgG control. The results were independent of HDAC

activity, as they were not affected by the treatment of cells with HDAC inhibitors apicidin or TSA (**Figure 5.8**). The detection of significant HDAC1, HDAC2 and KAT2B association with the *MCL1* mRNA being dependent on dual cross-linking indicates that the interactions of these proteins with the mRNA are indirect and are mediated by splicing factors including SRSF1.

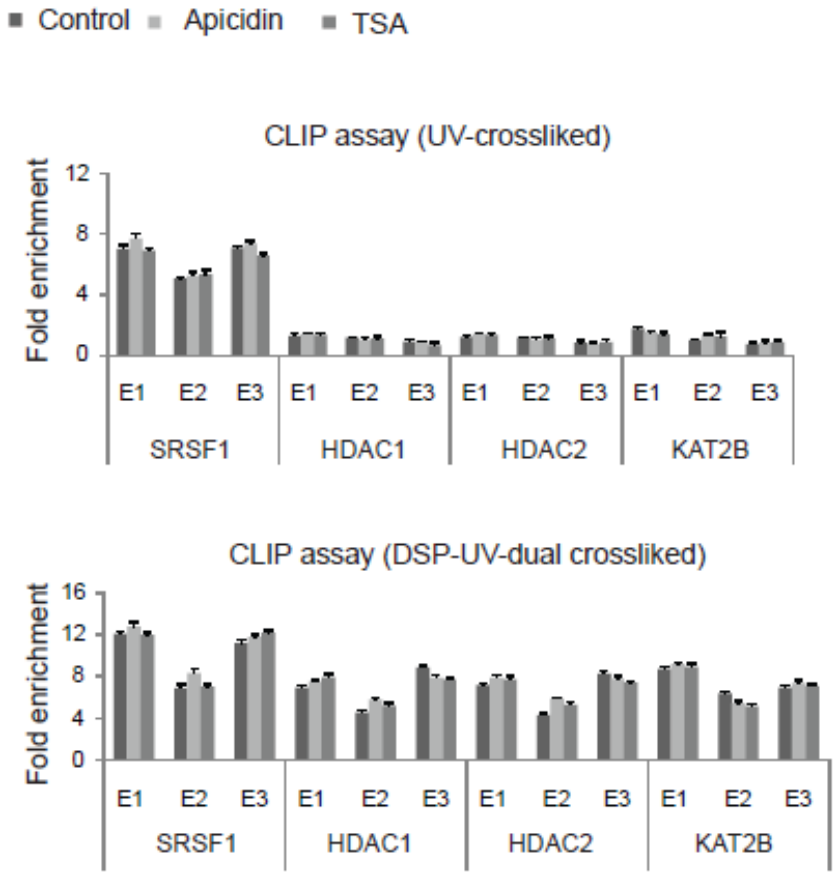

Figure 5.8: SRSF1, HDAC1 and 2, and KAT2B recruitment to *MCL1* gene body is mediated by pre-mRNA
Immunoprecipitations with indicated antibodies were performed on UV light-exposed or dual DSP and UV light cross-linked RNP complexes isolated from HCT116 cycling cells. RT-PCR measurements were normalized to the value obtained with IgG control antibodies.

Together, these results are consistent with the idea that HDAC1, HDAC2 and KAT2B are associated with the pre-mRNA while catalyzing the dynamic acetylation of *MCL1* chromatin.

5.5 Discussion

Nucleosomes are non-uniformly distributed along the body of transcribed genes and are enriched on exons relative to introns. Notably, the average length of human internal exons is very close to the length of DNA within a nucleosome (146 bp). This marked correspondence also applies to six other tested metazoans (Schwartz et al., 2009;Tilgner et al., 2009), suggesting a conserved role for the nucleosome in exon definition and splicing regulation. Our studies demonstrate that the nucleosome positioned over the alternative exon 2 of the *MCL1* gene is highly dynamic with regard to histone acetylation, a state which greatly impacts on splicing decision.

Our mass spectrometry data showed that HDAC1 and 2, more accurately non-phosphorylated HDAC2, is associated with numerous splicing factors. Interestingly mass spectrometry analyses of the spliceosome had identified HDAC2 as a component of this complex (Rappsilber et al., 2002). SR proteins associate with chromatin in a transcription-dependent manner (Sapra et al., 2009), and we show that HDAC1 or 2 are co-recruited with SRSF1 to the body of genes upon transcription induction, regardless of whether these genes are constitutively or alternatively spliced. It was proposed that SRSF1 is recruited to transcribed genes by RNAPII upon stimulation of elongation, which occurs with the LARP7 (P-TEFb)-mediated phosphorylation of the CTD of RNAPII at Ser2 (Barboric et.al, 2009). It is most likely that HDAC1 and 2 are recruited at the same time, in a complex with SRSF1 and other splicing factors. This model is supported by our data showing that HDAC2 interactions with numerous splicing factors are unaffected by RNase treatment. Furthermore, we have data demonstrating that SRSF1 knockdown does not affect the recruitment of HDAC1 or 2 to the body of the *TFF1* gene upon its induction (not shown). Since HDAC1 and 2 are associated with so many splicing proteins, it

is not surprising that their recruitment to the body of a transcribed gene is not affected by the knockdown of one of them. *Vice versa*, HDAC1 and /or HDAC2 knockdown did not affect SRSF1 recruitment to the *TFF1* gene.

SR proteins interact with the nascent pre-mRNA, RNAPIIS2ph and chromatin. SR proteins interactions with RNAPIIS2ph are RNase-sensitive, indicating that they are RNA-mediated. On the other hand, SR proteins interactions with chromatin were reduced by RNase treatment, but not abolished, suggesting that these interactions are partially mediated by RNA (Sapra et al., 2009). Indeed, in the case of SRSF1, interaction with chromatin also occurs *via* the tail of nucleosomal histone H3 (Loomis et al., 2009). In this study, we show for the first time that HDAC1 and HDAC2 also reside on the pre-mRNA. In fact, the association of HDAC2 with the body of the *MCL1* gene was particularly sensitive to RNase, more so than the association of SRSF1. Likewise, HDAC2 association with RNAPIIS2ph was RNA-dependent, indicating that HDAC2 was not bound directly to RNAPIIS2ph. Our results demonstrating that HDAC1 and HDAC2 are associated with the emerging RNA transcripts are supported by our previous ChIP experiments, in which we needed to use dual crosslinking to monitor HDAC1 and HDAC2 along the body of genes; formaldehyde alone worked poorly (Sun et al., 2007). Likewise, a genome wide mapping study of KATs and HDACs applied dual crosslinking (Wang et al., 2009). In agreement with previous results revealing that KAT2B is associated with RNAPII, in complex with hnRNP U and actin (Obrdlik et al., 2008), we show that KAT2B is bound to the pre-mRNA. Interestingly, the remodeler SWI/SNF is also associated with nascent pre-mRNPs (Tyagi et al., 2009). We propose that HDAC1 and 2, in concert with KAT2B, and other KATs, catalyze nucleosomal dynamic acetylation by acting at the RNA level.

Evidence is accumulating to show the importance of nuclear RNA in maintaining the structure of transcribed chromatin as well as the nuclear location of transcribed genes (Caudron-Herger et al., 2011;Caudron-Herger and Rippe, 2012;Guil and Esteller, 2012;Mitchell et al., 2012). It is quite possible that nuclear RNA associated with regulatory and coding regions of transcribed and silent chromatin domains serves as a platform for several chromatin modifying enzymes.

SR proteins regulate alternative splicing by binding to exonic splicing enhancers and enhancing U1 snRNP and U2 snRNP recruitment, thus promoting recognition of exons with suboptimal splice sites (Sapra et al., 2009). In agreement with this mode of action, we and others (Moore et al., 2010) found that SRSF1 knockdown resulted in increased *MCL1* exon 2 exclusion. HDAC inhibition or HDAC1 and/or HDAC2 knockdown also resulted in increased *MCL1* exon 2 exclusion, without affecting SRSF1 levels. *Vice versa*, SRSF1 does not affect HDAC activity, as SRSF1/HADC1 or HDAC2 complexes have a deacetylase activity. Of note, we repeated the i*n vitro* experiment showing that HuR proteins inhibit the HDAC activity in a HDAC2 immunoprecipitate from HeLa nuclear extracts (Zhou et al., 2011), but contrary to what was published, we found no impact of the HuR protein on HDAC activity.

Our model is that the nucleosome positioned on exon 2 has unique characteristics conferring an enhanced susceptibility to loss of HDAC activity and leading to increased acetylation and elongation rate. Hence, the spliceosome assembly is compromised and exon exclusion favored. Indeed, within 30 min of HDAC inhibition, an enhanced recruitment of KAT2B and KAT7 to *MCL1* exon 2 was observed. Clearly, KATs recruitment to exon 2 and increased acetylation of exon 2 nucleosome precedes locally decreased RNAPII occupancy, implying that increased

nucleosomal acetylation contributes to faster elongation. This conclusion is supported by an *in vitro* transcriptional elongation experiments showing that acetylation of H3 and H4 tails overcomes the inhibitory effect of nucleosomes (Protacio et al., 2000). *In situ* experiments have also demonstrated that histone acetylation is required to maintain the unfolded structure of the transcribed nucleosome (Walia et al., 1998). It is assumed that histone acetylation facilitates subsequent rounds of transcription elongation (Khan et al., 2012). Histone acetylation could also recruit chromatin remodelers. For example, the BRM (Brahma) subunit of the human remodeler SWI/SNF has been implicated in the regulation of alternative splicing by regulating the elongation rate (Batsche et al., 2006).

Future studies will determine the mechanism driving the specific recruitment of KAT2B, KAT7 and perhaps other KATs specifically to *MCL1* exon 2 in response to HDAC inhibition. It should be noted that in cycling cells, the steady state levels of H3K9ac and H3K14ac are decreased compared to the rest of the gene body, while the occupancies of RNAPII and associated proteins, including KAT2B known to mediate H3K9 acetylation (Jin et al., 2011), are constant along the gene. This suggests that HDAC activity is locally increased relative to KAT activity on exon 2. As a direct consequence of HDAC inhibition, H3 acetylation on exon 2 increases, and it is possible that KAT2B binds to acetylated H3 and H4 through its bromodomain (Spedale et al., 2012). By binding to the product of its activity, KAT2B occupancy could be self-reinforcing. Alternatively, KAT2B could interact with an acetylated regulatory factor associated with exon 2. The underlying DNA sequence of and surrounding exon 2 may play a key role, acting as recruiters of other proteins either at the DNA or RNA levels. To conclude, our results highlight

the roles of HDACs and KATs in regulating the interface between chromatin organization and alternative splicing.

5.6 Acknowledgments

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5.7 References

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5.8 Supporting information

5.8.1 Supplementary protocol: Elongation assay

A previously described method of 'transcription elongation assay' was used with some modifications (Zhou et al., 2011). Cycling or serum starved HCT116 cells were treated with DRB (Sigma) (50 μM, 6 h) to block transcription. After release from the inhibition, cycling cells were incubated in fresh medium containing 2 mM of BrU (Sigma), with or without 250 nM TSA (Sigma) or 150 nM Apicidin (Sigma) for 2 h or the serum starved cells were incubated in fresh medium containing 2 mM of BrU, with or without 250 nM TSA or 150 nM Apicidin for 30 min prior to TPA treatment (60 min). Following the treatment periods, RNA was isolated at different time points (0, 30, 60 and 90 min) using RNeasy mini kit. For immunoprecipitation, 5 μL of Anti-BrU (Sigma) monoclonal antibody was preincubated with 20 μL of protein G Dynabeads in RSB-100 buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.4% Triton X-100, 80 U/mL RNasin and 25 μg/mL tRNA) for 2 h at 4ºC. After that, the beads were washed four times with RSB-100 buffer and resuspended in 200 μl of RSB-100 buffer. Total RNA (50 μg) was then added and incubated for 3 h at 4ºC with rotation. The beads were washed six times with RSB-100 buffer and eluted by addition of 300 μL RLT buffer (RNeasy mini kit). The

immunoprecipitated RNA were purified using RNeasy mini kit. cDNA was synthesized using the purified RNA (20 ng) with the Superscript III First Strand Kit (Invitrogen) in a total volume of 20 µL and 2 µL of the reversed transcribed product was used for each qPCR reaction. For each sample, Ct value was obtained by subtracting the Ct value of a non-related IgG from the Ct value of anti-BrU antibody. The expression level of cyclophilin E exon 8 was used to further normalize the values. Pre-mRNA expressions were calculated as previously described (Zhou et al., 2011).

5.8.2 Supplementary figures

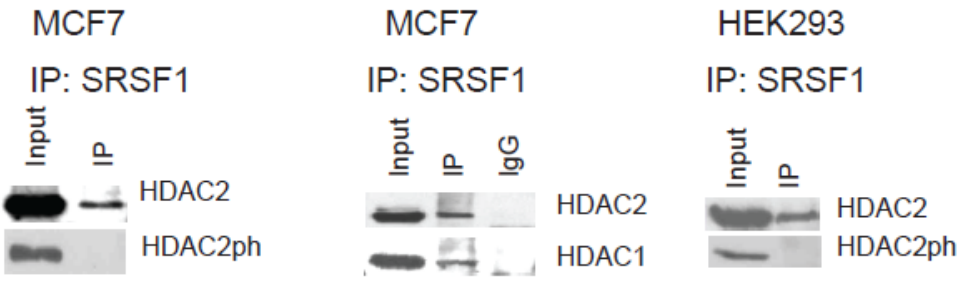

Figure S1: SRSF1 is associated with unmodified HDAC2, but not with HDAC2 S394 phospho in MCF7 and HEK293 cells
MCF7 or HEK293 cell lysates were incubated with anti-SRSF1 antibodies and immunoprecipitated fractions (IP) were probed with anti-HDAC2 or anti-HDAC2 S394 phospho antibodies by immunoblot assay.

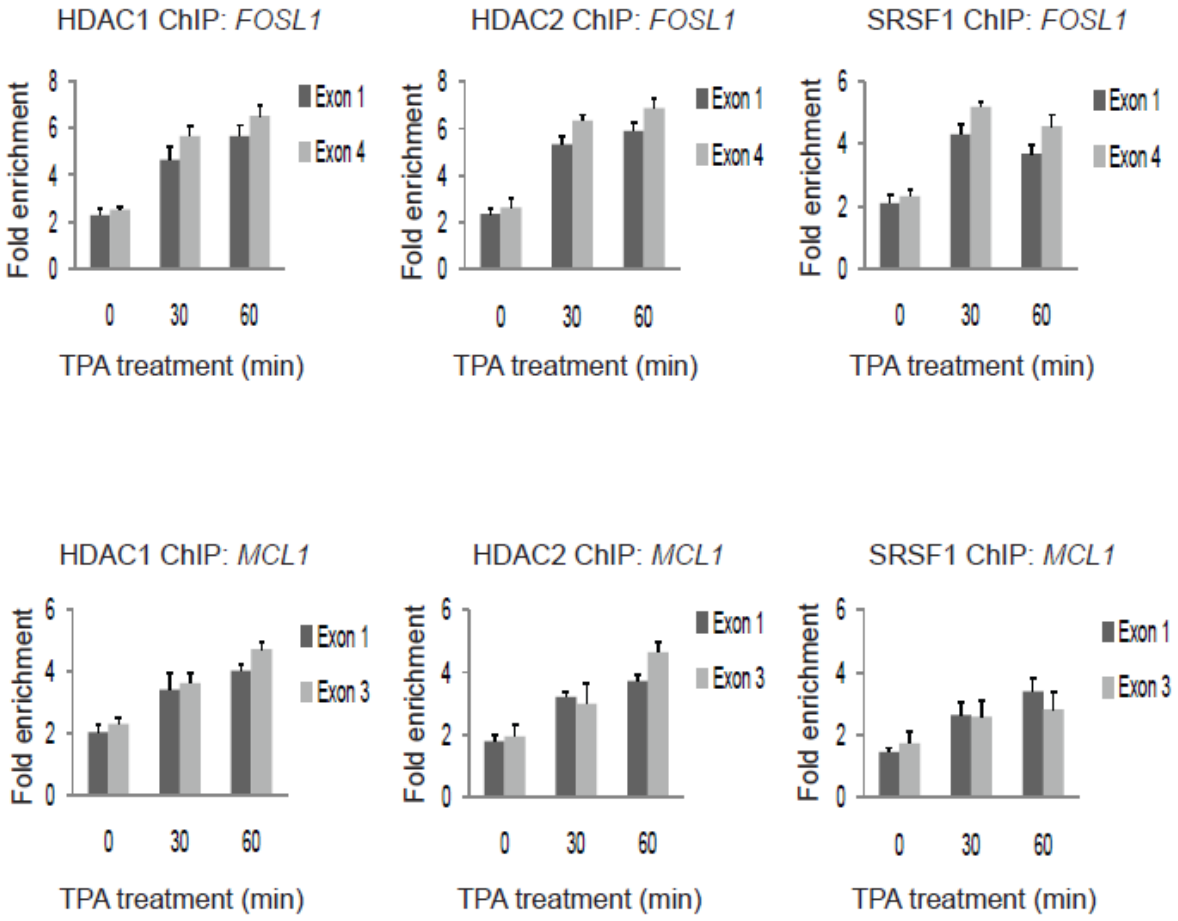

Figure S2: HDAC1, HDAC2 or SRSF1 associates with the body of the *FOSL1* and *MCL1* genes upon induction of transcription

HDAC1, HDAC2 or SRSF1 ChIP experiments were performed on dual cross-linked mononucleosomes prepared from serum starved HCT116 cells treated with 100 nM TPA for 0, 30 or 60 min. Schematic representations of the *FOSL1* and *MCL1* genes, showing amplicons generated in ChIP assays, are shown in Figure 5.1.

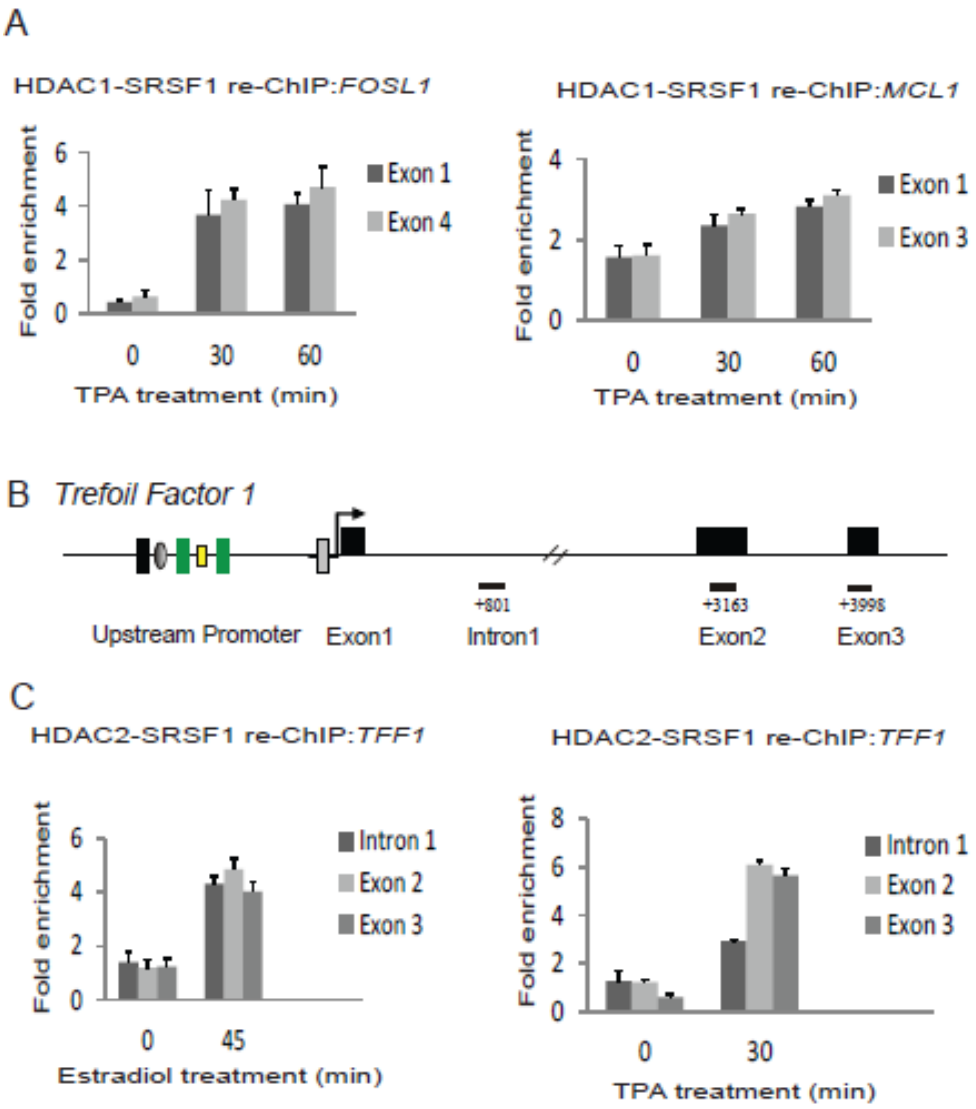

Figure S3: HDAC1 or 2 and SRSF1 complexes are recruited to the body of *FOSL1*, *MCL1* and *TFF1* genes upon induction of transcription

A.HDAC1/SRSF1 re-ChIP assays were as described in Figure 5.1. B. Schematic representation of the *TFF1* gene, showing amplicons generated in re-ChIP assays. C. HDAC2/SRSF1 re-ChIP assays were performed on dual cross-linked mononucleosomes prepared from serum starved MCF7 cells untreated or treated with 100 nM TPA for 30 min or 10 nM estradiol for 45 min.

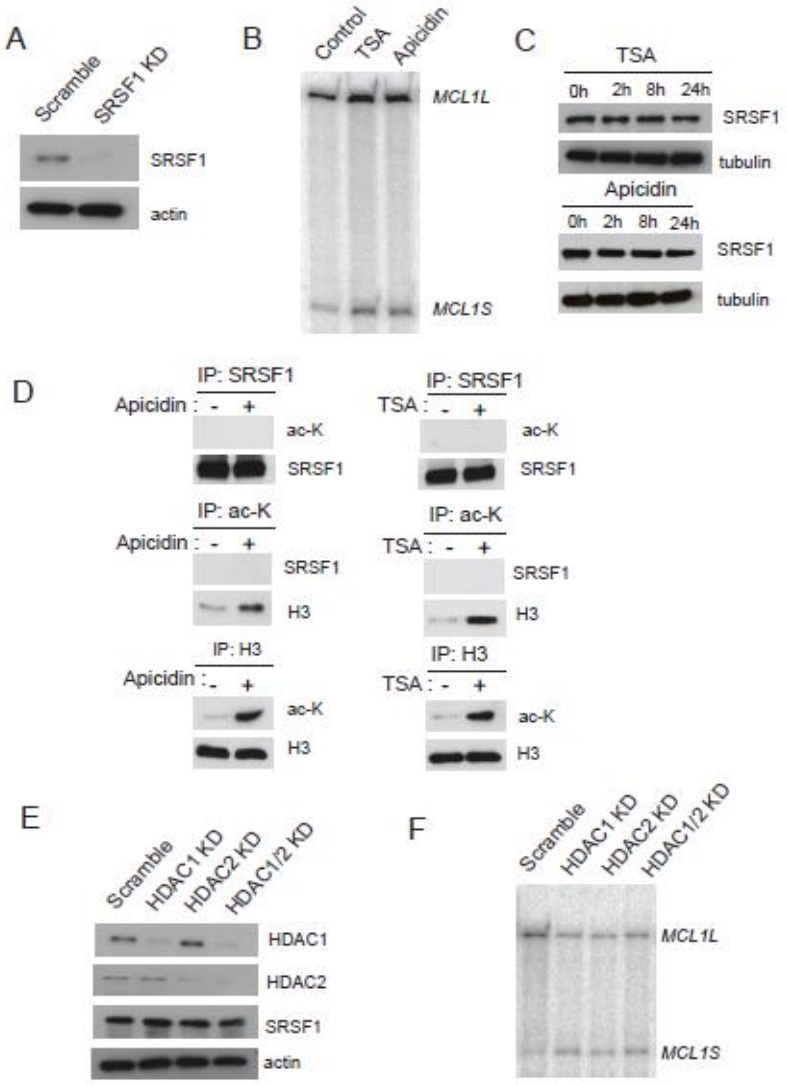

Figure S4: HDAC inhibition or knockdown affects *MCL1* alternative splicing but not SRSF1 level or acetylation level

A.The efficiency of SRSF1 knockdown in HCT116 cycling cells was analyzed by immunoblot analysis. A representative blot is shown. B. Effects HDAC inhibitor (250 nM TSA or 150 nM apicidin) 2 h treatment on *MCL1* alternative exon 2 splicing were analyzed in HCT116 cycling cells by ^{32}P labeling RT-PCR. Amplicons were visualized on a denaturing polyacrylamide gel. C. HDAC inhibitors do not affect SRSF1 level. HCT116 cells were treated with 250 nM TSA or 150 nM apicidin for indicated time periods and level of SRSF1 protein was analyzed by immunoblotting. β-tubulin was used as loading control. D. HDAC inhibitors do not affect SRSF1 acetylation level. Co-immunoprecipitation was carried out with anti-SRSF1, anti-acetyl lysine (ac-K) or anti-H3 antibodies on lysates from HCT116 cells treated with 250 nM TSA or 150 nM apicidin for 2 h. Immunoprecipitated proteins were analyzed on immunoblots using antibodies as indicated. (E) HDAC1, HDAC2 and SRSF1 levels were analyzed by immunoblot analysis in HDAC1, HDAC2 or both knockdown HCT116 cells. (F) Effects of HDAC1, HDAC2 or both knockdown in HCT116 cells on *MCL1* splicing. RNA was amplified by ^{32}P labeling RT-PCR, and amplicons were visualized on a denaturing polyacrylamide gel.

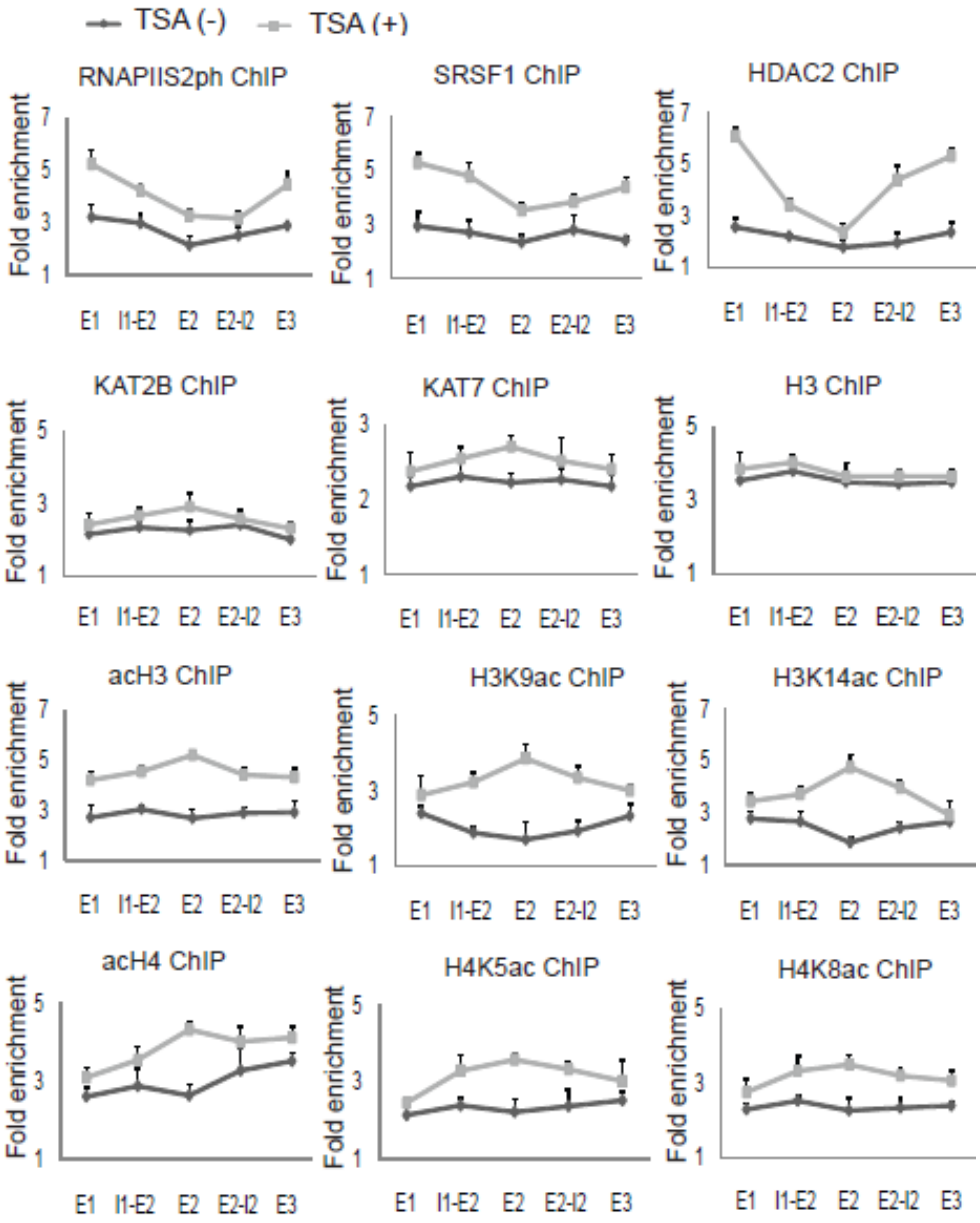

Figure S5: TSA affects RNAPIIS2ph, SRSF1, HDAC2, KAT2B, KAT7, H3 and H4 acetylation distribution along the *MCL1* gene

ChIP experiments were performed as described in Figure 5.3, but on HCT116 cells treated with 250 nM TSA.

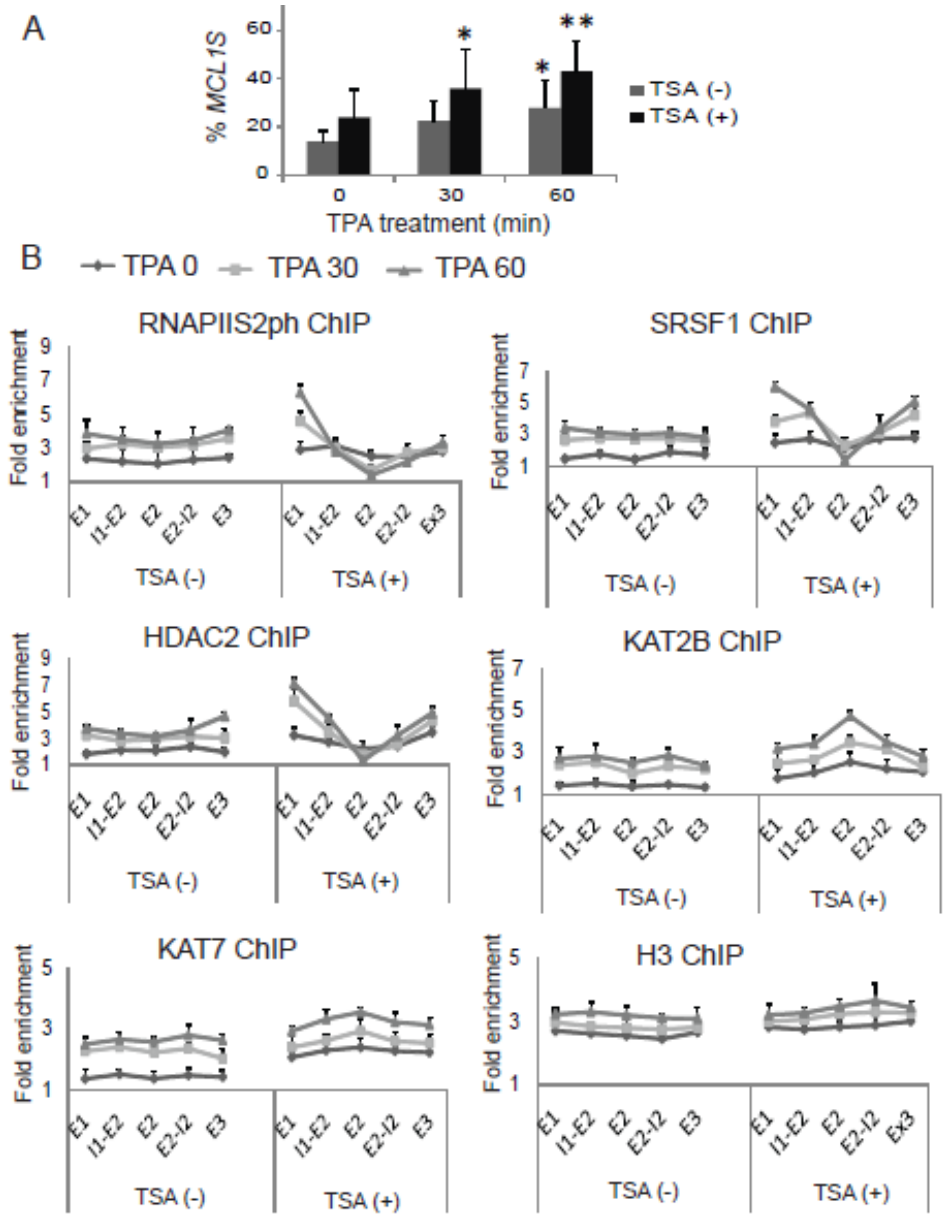

Figure S6: TSA favors exclusion of alternative exon 2 upon TPA induction of *MCL1* gene
A. *MCL1* alternative exon 2 splicing was analyzed in TPA-stimulated serum starved HCT116 cells treated or not with 250 nM TSA prior to TPA induction. B. ChIP assays were performed on TPA-stimulated serum starved HCT116 cells treated or not with 250 nM TSA for 30 min prior to TPA induction, as described in Figure 5.3.

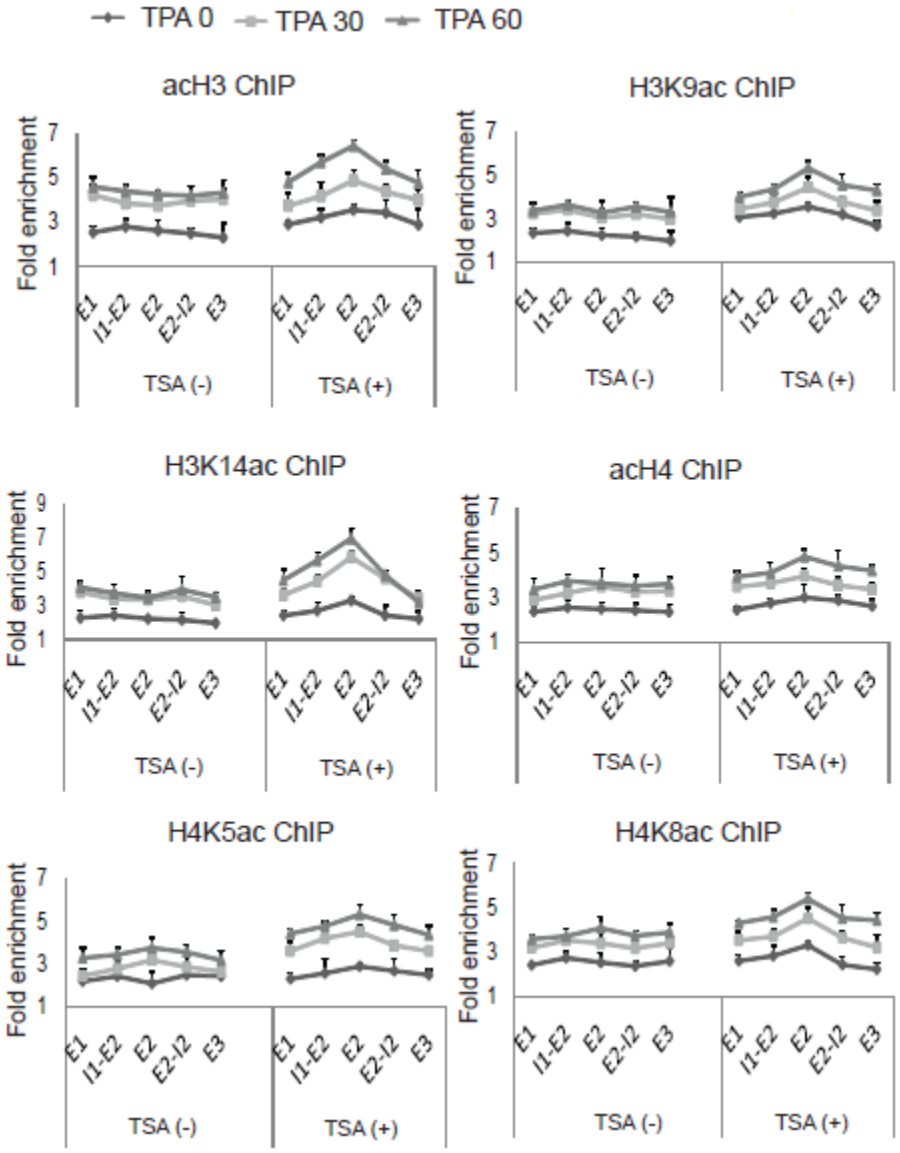

Figure S7: TSA specifically increases H3 and H4 acetylation over exon 2 upon TPA induction of *MCL1* gene

ChIP experiments were performed as described in Figure S6.

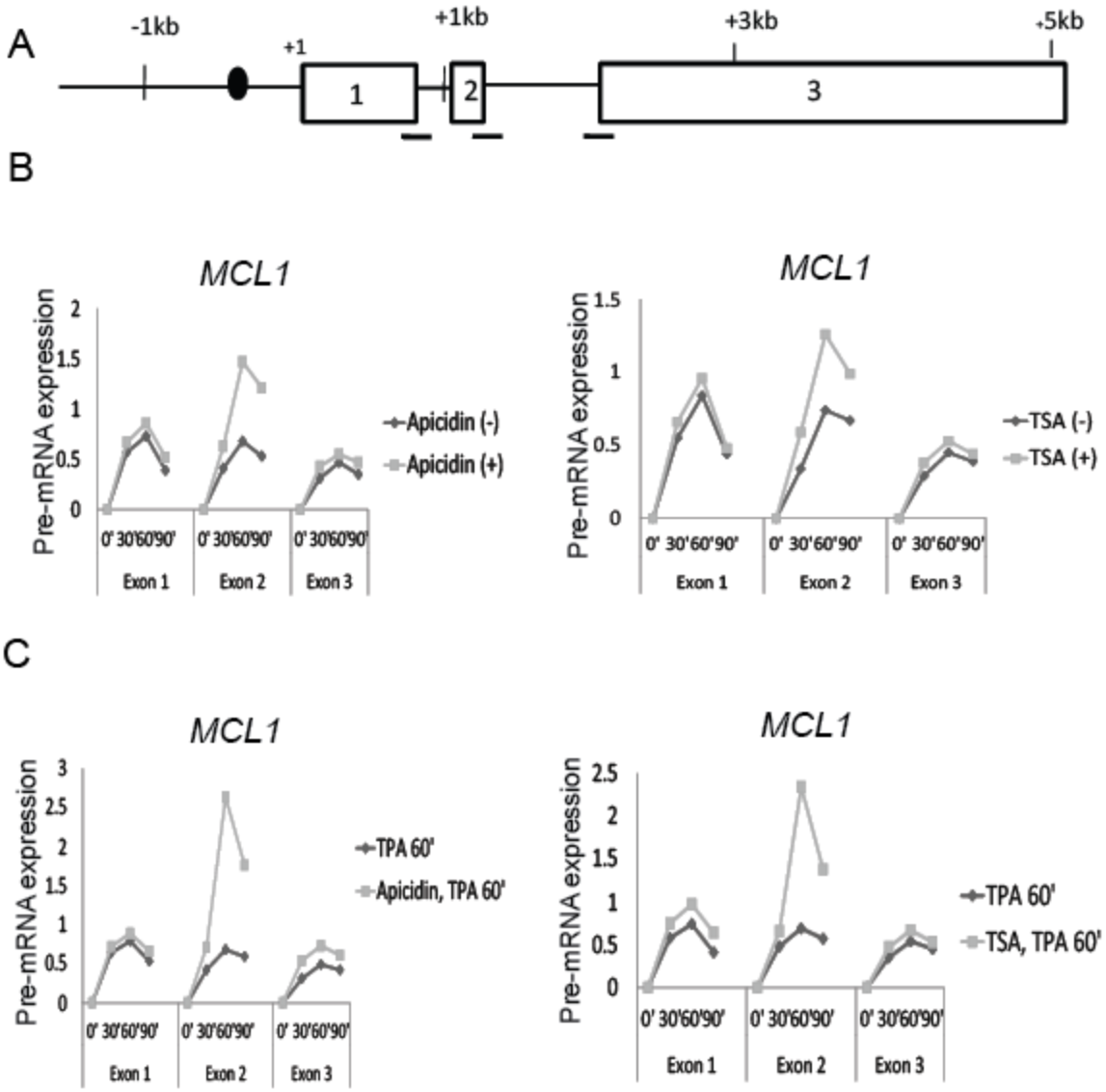

Figure S8: Effect of HDAC inhibitors on accumulation of nascent *MCL1* pre-mRNA at different exons

Cycling HCT116 cells were treated with DRB to block transcription. After release from DRB, expression levels of BrU labeled pre-mRNA as shown in (A) from the control cells treated with or not with apicidin (150 mM) (B, left panel) or TSA (250 nM) (B, right panel) for 2 h were analyzed at indicated times and normalized to the expression level of cyclophilin E exon 8. C. Quantitative RT-PCR were performed as described in A and B but with serum starved HCT116 cells pre-treated or not with apicidin (left panel) or with TSA (30 min) followed by TPA treatment (60 min). The results are average of two independent experiments.

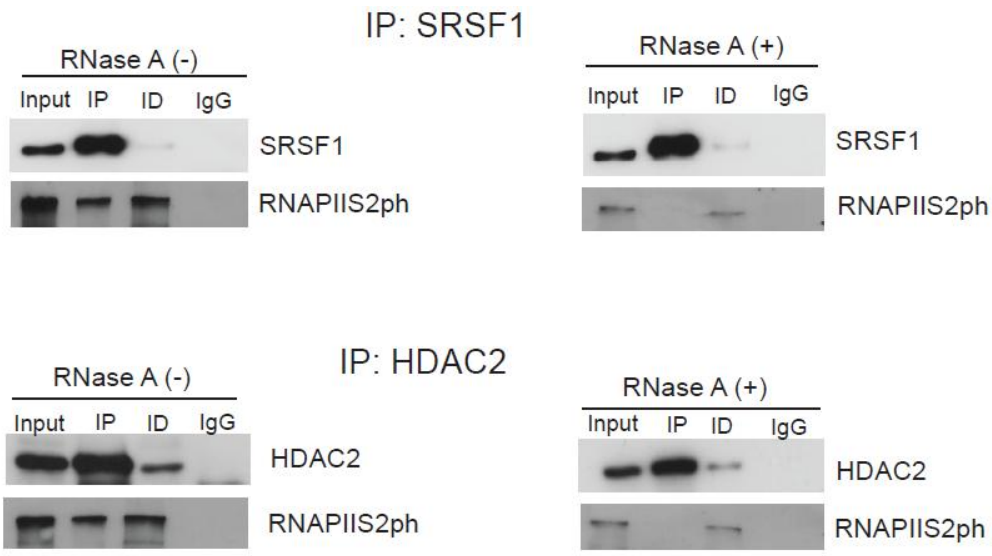

Figure S9: SRSF1 and HDAC2 interactions with RNAPIIS2ph are dependent on RNA HCT116 cell lysates treated or not with RNase A were incubated with anti-SRSF1 or anti-HDAC2 antibodies. Immunoprecipitated (IP) and immunodepleted (ID) fractions were analyzed by immunoblot assay for the presence of indicated proteins. Isotype specific non-related IgGs were used as negative control.

Table S1. Primer sequences used in ChIP, CLIP, splicing and elongation assays

Primers	Sequences (5′ to 3′)	Applications
FOSL1 Ex1-F *FOSL1* Ex1-R	5′-GCATGTTCCGAGACTTCGGG-3′ 5′-TGCTGGGCTGCCTGCGCTGC-3′	ChIP
FOSL1 Ex4-F *FOSL1* Ex4-R	5′- CACACCCTCCCTAACTCCTTT-3′ 5′-TGCTGCTACTCTTGCGATGA-3′	ChIP
MCL1 Ex1-F *MCL1* Ex1-R	5′-GGTCGGGGAATCTGGTAATAA-3′ 5′-CGGTACAACTCGTCCTCCTC-3′	ChIP, CLIP
MCL1 In1-Ex2-F *MCL1* In1-Ex2-R	5′-GTGGGCAGGCGAATCTTGCG-3′ 5′-TCGTTTTTGATGTCCAGTTT-3′	ChIP
MCL1 Ex2-F *MCL1* Ex2-R	5′-TAACAAACTGGGGCAGGATT-3′ 5′-ATGGTTCGATGCAGCTTTCT-3′	ChIP

MCL1 Ex3-F *MCL1* Ex3-R	5′-TGGGTTTGTGGAGTTCTTCC-3′ 5′-CCAGCTCCTACTCCAGCAAC-3′	ChIP
TFF1 In1-F *TFF1* In1-R	5′-AGAATGGATCAACGGTCTGC-3′ 5′-CAAAGTGCAAGTCGCAGATG-3′	ChIP
TFF1 Ex2-F *TFF1* Ex2-R	5′-CCCCGTGAAAGACAGAATTG-3′ 5′-TCGAAACAGCAGCCCTTATT-3′	ChIP
TFF1 Ex3-F *TFF1* Ex3-R	5′-CCTCACTAAAGCATCTCTTTCTCC-3′ 5′-GCAGATCCCTGCAGAAGTGT-3′	ChIP
MCL1 Ex1-F *MCL1* Ex3-R	5′-GAGGAGGAGGAGGACGAGTT-3′ 5′-AACCAGCTCCTACTCCAGCA-3′	Splicing
MCL1 Ex2-In2-F *MCL1* Ex2-In2-R	5′-ACAAAGAGGCTGGGTAAGTT-3′ 5′-TCATAAAAACCTTTAGATAT-3′	ChIP, elongation assay
MCL1 Ex1-In1-F *MCL1* Ex1-In1-R	5′-AGACCTTACGACGGGTTGG-3′ 5′-AAAAAGGAGTGAGGCCTTG-3′	Elongation assay
MCL1 In2-Ex3-F *MCL1* In2-Ex3-R	5′-GAGAGCAGAAACCCATACTTGAA-3′ 5′-ACATTCCTGATGCCACCTTC-3′	Elongation assay
MCL1 Ex1-F *MCL1* Ex1-R	5′-CGAGGCTGCTTTTCTTCG-3′ 5′-GTACCCGTCCAGCTCCTCTT-3′	CLIP
MCL1 Ex2-F *MCL1* Ex2-R	5′-TTCTTTTGGTGCCTTTGTGG-3′ 5′-GTCCCGTTTTGTCCTTACGA-3′	CLIP
MCL1 Ex3-F *MCL1* Ex3-R	5′-AAGTCCCCTCAGGAATTTTCA-3′ 5′-CTGAGGTTTAACACAGCTCACC-3′	CLIP

Chapter 6: Methodologies

The ‘Material and methods’ sections are self-contained in the manuscripts presented in previous chapters. Some of the commonly used and newly developed methods are described in further details for future references.

6.1 Cell culture and related techniques

6.1.1 Cell culture conditions

The cell lines (e.g. HeLa, MCF7, HEK293, Flp-In 293, and HCT116) used in studies were obtained from the American Type Culture Collection (ATCC). Cell lines were cultured in the recommended media, supplemented with 10% FBS (Life technologies), and with 1X antibiotic-antimycotic (Life technologies) (100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B). Cells were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. When cells reached 80-90% confluence, they were trypsinized and passaged to new culture plates containing fresh media.

6.1.2 Passaging of cell lines

To passage cells, the cell culture medium was removed from plate by aspiration and the cells were rinsed twice with sterile pre-warmed 1X PBS. Upon removal of the PBS, cells were detached from the culture plate by incubating with 3 mL of TrypLE express stable trypsin (Invitrogen) per 100 mm plate for 5 min 37ºC. The reaction was neutralized by adding an equal amount of complete medium. Cells were transferred to a 15 mL conical centrifuge tube (VWR) and centrifuged at 250 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in 10 mL of complete medium followed by seeding into new culture plates as per experimental requirements.

6.1.3 Cell freezing, storage and recovery

For long term storage, all the cell lines used in studies were cryopreserved in liquid nitrogen. Cell pellets isolated from an 80-90% confluent 100 mm plate were resuspended in 1.0 mL of

freezing medium (90% v/v FBS and 10% v/v DMSO) and transferred to cryovials (Fisher Scientific). The cryovials were placed in a cryo freezing container in -80ºC freezers overnight prior to be transferred to liquid nitrogen tank. To revive the frozen cells, cryovials were thawed in a 37ºC water bath until the dislodging of the ice pellet and the contents were then transferred into a 100 mm plate. The culture medium was replaced with fresh complete medium when the cells attached to the plate. Cells were passaged a minimum of three times prior to use for experiments.

6.2 Protein-based techniques

6.2.1 Cell extract preparation

Cells were harvested and lysed in appropriated volume of cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, 0.5% NP-40) containing phosphatase and protease inhibitors (Roche), depending on the size of cell pellets. The lysed suspension was left on ice for 15-20 min followed by homogenization with sonication (Fisher Scientific Model 100 Sonic Dismembrator) at setting in between 2 to 3 (3X 10 sec, each with 1 min interval on ice). The resulting cell extract was subjected to centrifugation at 17,000 g for 10 minutes at 4ºC, and the supernatant was saved. The protein concentration of the supernatant was determined using Coomassie Plus (Bradford) Protein Assay Reagent (Fisher Scientific) as per manufacturer's instructions using BSA (bovine serum albumin) as a standard. The cell extracts were generally stored at -20ºC or -80ºC.

6.2.2 Electrophoresis and immunoblotting

Protein samples were denatured by boiling for 5-6 min in SDS-loading buffer [65 mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 2-5% v/v β-mercaptoethanol (BME), and 0.01 mg bromophenol blue] before subjecting to SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was applied to separate proteins based on their molecular weight under denaturing conditions, according to Laemmli's protocol (Laemmli, 1970). Depending on the target protein size, total protein was separated on 7.5%, 8%, 10% or 15% polyacrylamide gels using Mini-Protean® 3 Cell apparatus (Biorad). Gels were run at a constant voltage of 120V for approximately 1.5-2.0 h depending on the desired resolutions. The separated proteins were transferred to 0.45 µm nitrocellulose membranes (Biorad) using the wet transfer apparatus (Biorad) at a constant voltage of 100V for 1 h at 4ºC. After transfer, the membranes were stained with Ponceau S [0.5% (w/v) Panceau S, 1% acetic acid] to determine the efficiency of the transfer and then were baked at 65ºC for 30 min prior to blocking. For immunoblotting with specific protein of interest, the nitrocellulose membranes were blocked with 5% (w/v) non-fat dry milk in 0.05% TTBS (0.05% Tween-20, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1.0-1.5 h at room temperature on a rocking platform (VWR, Model 200). The membranes were then incubated with primary antibodies for 1 h at room temperature or overnight at 4ºC on an orbitron (Boekel Scientific, Model 260200), depending on the optimized experimental conditions for each antibody that were used in the experiments. The following day, the membranes were washed three times in 0.05% TTBS for 30 min (10 min/wash). The membranes were then incubated with horseradish peroxidase-linked anti-IgG secondary antibodies for 1 h at room temperature. After that, the membranes were washed again three times with 0.05% TTBS for 30 min (10 min/wash). Finally, the proteins of interest were visualized on Hyperfilm ECL (Amersham) with Western

Lightning™ Plus-ECL reagent (Perkin Elmer) according to the supplier's instructions.

6.2.3 Immunoprecipitation

For immunoprecipitation experiments, cell extracts from various cell lines were prepared as previously mentioned in section 6.2.1. Cell extracts were then pre-cleared with the addition of 50% slurry of A/G UltraLink resin (Pierce) (30-40 µL of beads/1.0 mL of extract) for 1 h at 4ºC on an orbitron. The beads were then quick spun and the supernatant were transferred to a new tube (referred to as Input). After the pre-clearing step, protein concentration of the extract was determined using Coomassie Plus (Bradford) Protein Assay Reagent. Five hundred µg of total cell extracts were incubated with 3.0-5.0 µg of the antibody of interest (depending on the optimized conditions that were previously determined for each antibody) overnight at 4ºC on an orbitron. A fraction of Input, equivalent to 20-30 µg of cell extract, was saved at -20ºC to use later for checking the immunoprecipitation efficiency. In the next day, 40 µL of protein A/G UltraLink resin were added and incubated for 2-3 h at 4ºC. The beads were then collected by quick centrifugation at 17,000 g in 4ºC. The supernatant, referred to as immunodepleted fraction (ID), was set aside that was equal to volume of Input (previously saved), to check for the efficiency of immunoprecipitation by PAGE. The beads were then washed four times with ice-cold lysis buffer (1.0 mL/wash) by centrifugation (900 g, 3-5 min/wash). After the final wash, the beads were resuspended in 2X loading buffer (4% SDS, 20% glycerol, 0.12 M Tris pH 6.8, and 10% BME, 0.2% bromophenol blue) and the proteins were denatured by boiling at 95ºC for 5-6 min before loading and resolving on a SDS-PAGE gel.

6.2.4 Chromatin immunoprecipitation (ChIP/ re-ChIP)

ChIP and re-ChIP experiments were performed as previously described with an additional protein-protein cross-linking step with DSP (Thermo Fisher Scientific) (**Figure 6.1**) (Drobic et al, 2010).

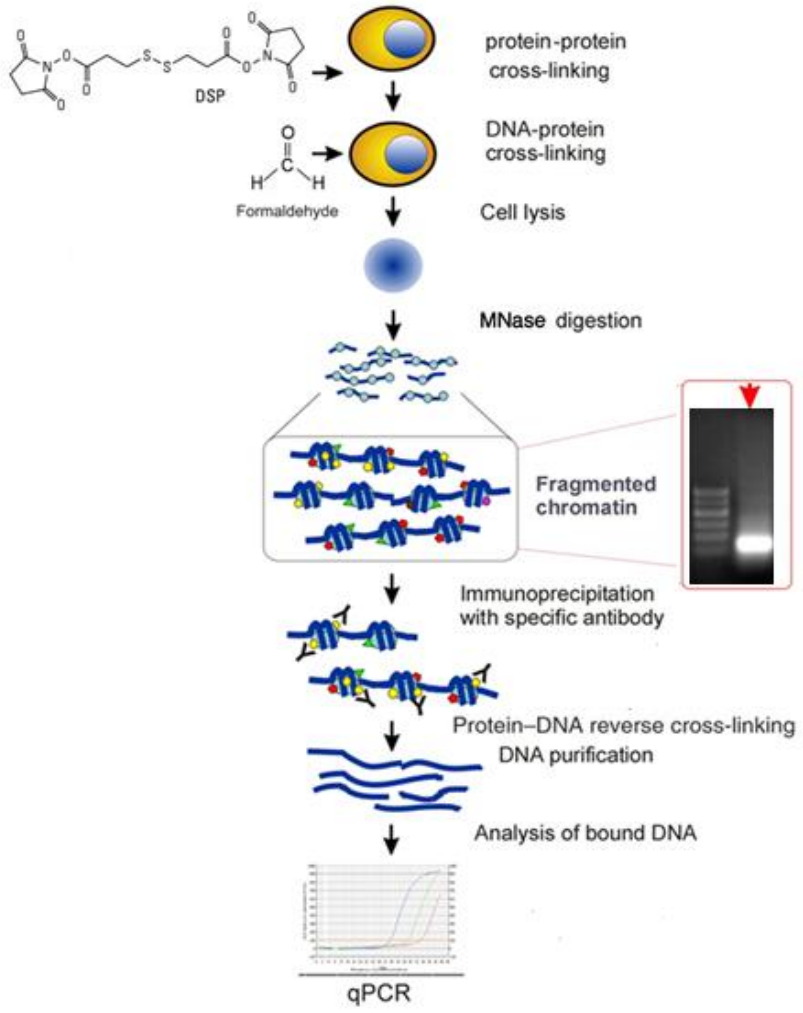

Figure 6.1: Schematic representation of ChIP assay
The figure was adapted and modified with permission from Figure 2 (Collas, 2010).

Cells were treated as per experimental requirements, and then harvested by scraping. Cell pellets were collected in 15 mL centrifuge tube by centrifugation at 250 g for 5 min. Cell pellets were resupended in 5.0 mL of 1.0 mM DSP (prepared in 1X PBS) and incubated at room temperature

for 30 min with gently rocking at a rocking platform. After this, formaldehye (37% stock solution from Fisher) was added drop-wise to the suspended cells at a final concentration of 1% and incubated for additional 10 min at room temperature with shaking. The cross-linking reaction was stopped by the addition of 1.25 M glycine to a final concentration of 125 mM and incubating with shaking for 5 min. The cross-linked cell pellets were collected by centrifugation at 250 g for 5 min. Cell pellets were rinsed twice with 1X PBS and were frozen at -80ºC prior to processing for ChIP. For ChIP assay, the frozen cell pellets were thawed on ice and resuspended in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, supplemented with protease and phosphatase inhibitors) and incubated at 4ºC for 10 min while shaking on an orbitron. The cellular material was then spun at 2000 g for 10 min to obtain the nuclei. These two steps were repeated once. The nuclear pellet was resuspended in an appropriate volume of micrococcal nuclease (MNase) digestion buffer (depending on the nuclear pellet size) (10 mM Tris-HCl pH 7.5, 0.25 M sucrose, 75 mM NaCl supplemented with protease and phosphatase inhibitors) and the A_{260} was measured. To reduce the variability in A_{260} measurement, caution was taken to fully resuspend the nuclei to get an evenly homogenized nuclear material. In order to obtain mononucleosomes (~150 bp of DNA fragments), 2.5U of MNase/A_{260} of nuclear suspension were added in the presence of 3 mM $CaCl_2$ and incubated at 37ºC for 40 min. The DNA fragment size was routinely verified by 1 % agarose gel electrophoresis. The reaction was stopped by the adding EDTA pH 8.0 at a final concentration of 5.0 mM. The nuclear material was released and solubilized by adding 10% SDS at final concentration of 0.5% and incubating with rotation for 1.0-1.5 h at room temperature. The nuclear extract was then homogenized by passing through a syringe for a total of five times. Insoluble material was removed by centrifugation at 2000 g for 10 min and the soluble material was diluted to 0.1% SDS with radio-

RIPA buffer (10 mM Tris-HCl pH 8.0, 1% Triton-X-100, 0.1% SDS, 0.1% sodium deoxycholate, supplemented with protease and phosphatase inhibitors). The extract was then pre-cleared with 50% slurry of protein A/G Plus agarose (Santa Cruz Biotechnology) (60 µL beads/ 1.0 mL of material). The beads were removed by centrifugation at 2000 g for 10 min and the A_{260} was measured. Nuclear material equivalent to 12-15 A_{260} pre-cleared extract was incubated with 1.0-2.0 µg of antibody/A_{260}, overnight at 4ºC, depending on the previously optimized conditions for antibodies of interest. In the following day, 10-15 µL of Magnetic protein G Dynabeads (Invitrogen) (resuspended in RIPA buffer)/A_{260} were added and incubated for 2-3 h at 4ºC. Following this period, the beads were washed sequentially 2X 5 min with each of the following buffers: Low Salt Wash Buffer (0.1% SDS, 1% Triton-X-100, 2 mM EDTA, 20mM Tris-HCl pH 8.1, 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton-X-100, 2mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Wash Buffer (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl, pH 8.1), 1X TE Buffer (10 mM Tris-HCl pH 7.5, 1.0 mM EDTA). Washing steps with 1XTE buffer were done with precaution as the beads would tend to come out with buffer. After the final wash with 1X TE buffer, the immunoprecipitated fractions were eluted by incubating in 150-250 µL of elution buffer (1% SDS, 100 mM $NaHCO_3$) for 30 min with rotation at room temperature.

For re-ChIP assays, after the elution of first ChIP, the samples were diluted 10 times with dilution buffer (15 mM Tris-HCl pH 8.1, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl) and were subjected to another round of the ChIP procedure. After elution, the samples were incubated at 65ºC overnight to reverse the cross-linking. In the next day, the samples were incubated for 30 min at 37ºC with RNase A (final concentration: 0.02 µg/ mL) (Sigma) followed

by 1 h incubation at 55ºC with proteinase K (final concentration: 0.5 μg/mL) (Invitrogen). DNA was then isolated using the QiaQuick PCR purification kit (Qiagen) according to manufacturer's specifications and eluted in 30-40 μL of ultra-pure water (Nuclease free water from Fisher). DNA concentration was determined for Input and ChIP/reChIP DNAs using the PicoGreen dsDNA quantitation assay (Life technologies). Equal amounts of input, ChIP DNA (1.0 ng) or re-ChIP (0.5 ng) DNA were used to perform SYBR green (Invitrogen) qPCR on iCycler IQ5(Biorad). The Enrichment values were calculated as previously described (Drobic et al., 2010;Ciccone et al., 2004).

6.3 Transient transfection of with siRNAs

Before performing siRNA-mediated knockdown experiments, dose curve transfection (gradients of concentration of siRNA and incubation time after transfection) analysis were carried out for each siRNA to determine the optimal conditions necessary for sufficient target knockdown. After optimization of the experimental conditions, HCT116 cells were seeded in 60 mm plates for 24 h prior to transfection. At 30-40% confluence, cells were transfected with 75 nM of scrambled (Non-targeting Pool) (D-001810-10-20), human HDAC1 (L-003493-00-0020), HDAC2 (L-003495-00-0020) or SF2 (SRSF1) (L-018672-01-0020) ON-TARGET plus SMARTpool siRNAs (Thermo Scientific-Dharmacon), using Polyplus Interferin siRNA transfection reagent (VWR) according to the manufacturer's instructions. In a typical siRNA transfection protocol, siRNA was diluted in 400 μL of serum free media at the final concentration (75 nM) and mixed well by pipeting. After this, 18 μL of INTERFERin™ reagent was added to the siRNA/media mixture and immediately mixed very well by brief (~10 sec) vortex and incubating for 10 min at room temperature. During this incubation period, previously seeded cells were rinsed once with

1X PBS and then 4.0 mL of fresh complete media was added. Following the incubation, the transfection mix (siRNA/media/reagent) was added drop-wise directly to the cells and homogenized by gently swirling the plate. Transfection was allowed to proceed for 48 h by incubating the cells at 37ºC in a CO_2 incubator. After 48 h of transfection, cells were harvested by scraping and processed as required for downstream experimental procedures, such as splicing assay and immunoblotting experiments. For some experiments, after 24 h post-transfection with siRNAs, cells were serum-depleted for another 48 h. Following serum starvation, synchronized cells were treated with 100 nM TPA for 60 min. After that, cells were harvested and used for further experiments as mentioned before.

6.4 RNA-based techniques

6.4.1 RNA isolation and cDNA synthesis

Total RNA was purified from cell pellets with RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol in 20-30 µL of ultra-pure water. The concentration and purity of RNA samples was determined by the ratio $A_{260/280}$ using a Nanodrop 2000 (Thermo Fisher Scientific). Total RNA (400 ng) was used as the template for synthesis of cDNA in a volume of 80 µL reaction using M-MLV reverse transcriptase and Oligo dT primers (Invitrogen). A tabular representation of cDNA sample preparation is shown below:

Reagents	Stock concentration	Final concentration	Volume required/ reaction (total 80µL)
RT buffer (first strand synthesis buffer)	5X	1X	16
DNTPs	2.5 Mm	0.2 mM	16
BSA	0.10%	0.01%	8.0
DTT	100 mM	10 mM	8.0
DMSO (autoclaved or filter sterilized)	100%	---	8.0

Oligo dT primer	0.125µg/µL	0.0125µg/µL	8.0
M-MLV Reverse transcriptase	200U/ µL	1.0U/reaction (20U/ µL)	8.0
Total RNA	50 ng/ µL	5 ng/µL	8.0

The reverse transcription reaction was carried out at 37ºC for 90 min. Negative controls with DNase/RNase-free water instead of RNA as a template (no template control) or the reverse transcription reaction without reverse transcriptase enzyme were also performed in parallel.

6.4.2 RNA-CLIP assay (crosslinking and immunoprecipitation of RNA-protein complexes)

Cells were treated as per experimental requirements. The media was removed from the plate and cells were rinsed twice with 1X PBS. Cells were then cross-linked with DSP by adding 1.0 mM DSP (in PBS) into the plate and incubated for 30 min at room temperature with gentle rotation. Following this, DSP solution was removed and the plates were kept on ice and cells were then cross-linked by irradiation under UV light (400mJ/cm^2) (Stratalinker). Cells were then harvested and lysed in appropriate volume (1.0-2.0 mL) of ice-cold lysis buffer (20 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.5% NP-40, 0.5% Triton X 100, 0.1% SDS, protease and phopshatase inhibitor cocktail, 80 U/mL RNasin [Promega]) depending on pellet size. After incubating on ice for 15-20 min, the cell extract was homogenized with sonication at setting 2 (3X10 sec, each with 1 min intervals on ice) and centrifuged at 17,000 g for 10 min at 4ºC. The supernatant was saved and treated with a dilute cocktail of RNase A/T1 (Ambion) as previously described (Sanford et al, 2009). Cell extract was pre-cleared with 50% slurry of protein A/G UltraLink resin (40µL/mL) and yeast tRNA (Sigma) at 100 µg/mL for 2 h at 4ºC with rotation. Cell extract (1.0-2.0 mg) was incubated with 8-15 µg of antibodies of interest depending on the optimized conditions for each antibody, on a rotator overnight at 4ºC, followed by addition of

100-200 µL of 50% slurry of protein A/G UltraLink resin in the next day for 3 h at 4ºC. The beads were collected by quick spin and the ID fraction was saved to check the efficiency of IP as described in section 6.2.3. Beads were then washed six times using the cold lysis buffer (900 g, 5min/wash). After the final wash, the beads were resuspended in 200-400 µL of lysis buffer and then treated with proteinase K (2 mg/mL) (Invitrogen) for 1 h at 55ºC. Immunoprecipitated RNA was then extracted using Trizol (Invitrogen) as per manufacturer's instructions and was precipitated with ethanol. RNA precipitates were resuspended in 20 µL of ultra-pure water, and then treated with RQ DNase (Promega) for 1 h at 37ºC. After DNase digestion, RNA was purified with RNeasy Plus Mini Kit. Equal amounts of input and immunoprecipitated RNA were used for cDNA synthesis and were reversed transcribed using oligo dT and random hexamer primers with SuperScript III reverse transcriptase (Invitrogen) following the manufacturer's specifications. The resulting cDNA was used for qPCR analysis. A schematic representation of CLIP assay is shown In **Figure 6.2**.

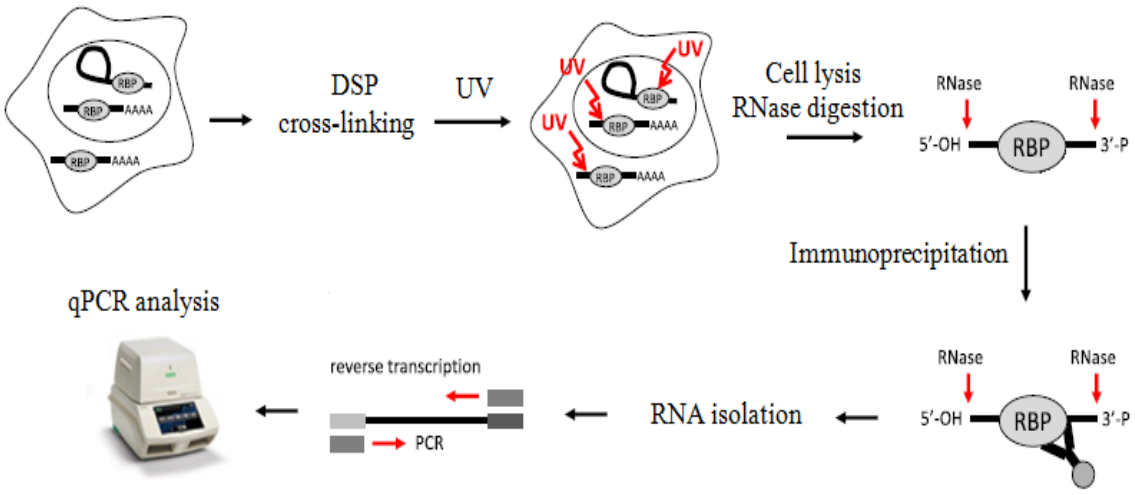

Figure 6.2: Schematic diagram of CLIP assay
The figure was adapted and modified with permission from Figure 1 (Jaskiewicz et al., 2012).

6.5 PCR

6.5.1 Real time PCR (qPCR)

SYBR Green qPCR reactions were carried out on iCycler IQ5 (Biorad). cDNA or ChIP/re-ChIP DNA to use in qPCR was prepared as described in the sections 6.4.1 or 6.2.4, respectively. PCR reactions were set up on ice and performed in 96-well reaction plates (Biorad). Two µL of cDNA (10 ng) or 0.5-1.0 ng of ChIP/re-ChIP DNA was used in a total volume of 50 µL and the master mix for each reaction was prepared according to the following calculations:

Reagents	**Stock concentration**	**Final concentration**	**Volume required/ reaction (total 50 µL)**
PCR buffer(-$MgCl_2$)	10X	1X	5.0
dNTPs	2.5 mM	0.25 Mm	4.0
$MgCl_2$	50 mM	2.5 mM	2.5
DMSO (autoclaved or filter sterilized)	100%	---	2.0
Forward primer	50 µM	0.5 µM	0.5
Reverse primer	50 µM	0.5 µM	0.5
Dye (SYBR green+ Fluorescein) (freshly prepared)	---	1U/500 µL (1:500 dilution)	1.6
Platinum Taq DNA Polymerase	5U/µL	2U/reaction	0.4
Ultra-pure water	---	---	23.5
Template DNA	0.1 ng/µL	0.02 ng/µL	10.0

All the samples were run in triplicates. The three step thermal-cycling program was as follows: 94ºC, 5 min; 40 cycles of [94ºC, 10sec; 55-64ºC (depending on the annealing temperature optimized for individual primer sets), 15 sec and 72ºC, 20 sec].

Chapter 7: Discussion and Future perspectives

The manuscripts presented in the previous chapters of this thesis add substantially to our understanding of the involvement of multi-dimensional HDAC1 and 2 complexes as an important regulator of various cellular processes such as mitosis and splicing, as well as, in fine tuning of transcriptional regulatory mechanisms. HDAC1 and 2 do not function autonomously, but as a component of multiprotein corepressor complexes, in which the associated proteins help HDAC1 and 2, to mediate different cellular functions. Phosphorylation of HDAC1 and 2 is required to form the corepressor complexes, which regulates the catalytic activities of HDAC1 and 2 effectively and/or specifically (Pflum et al., 2001;Sun et al., 2007;Tsai and Seto, 2002). Phosphorylated HDAC1 and 2 containing corepressor complexes are recruited to the regulatory regions by a number of transcription factors, while the un- or monophosphorylated HDAC1 and 2, associate with the coding region of transcribed genes (De Ruijter et al., 2003;Sun et al., 2007). Hence the un- or monomodified HDAC1 and 2 complexes, control dynamic acetylation and gene expression in a mechanism distinct from those by the phosphorylated HDAC1 and 2 complexes.

Following over approximately two decades of research since the initial discovery of HDAC1 and 2, the functional portfolios of HDAC1 and 2 contain several important biological functions, including, but not limited to, transcription, DNA repair, recombination and splicing regulation. Along these lines, several HDAC inhibitors have been developed and are actively used to target HDACs and to elucidate their functions. Despite the widespread interest in HDACs and growing body of studies, many questions remain to be addressed. Thus research on HDAC1 and 2 complexes and associated proteins, their genomic localizations, functions and the underlying regulatory processes, is an area of active investigation which can expand our understanding of the complex regulatory network of gene expression. In this thesis, we attempted to investigate

HDAC complexes from three different, albeit interconnected aspects, which collectively fit to the main theme to delineate the nature and functions of HDAC complexes, and to elucidate the underlying mechanisms of HDAC1 and 2 regulated processes.

7.1 Summary of findings

The first study in this thesis (**Chapter 2**) explored the nature and regulation of HDAC1 and 2 complexes during mitosis, an important mechanism of cell cycle progression. Using biochemical approaches and high resolution microscopy, we demonstrated that when HDAC1 and 2 were displaced from chromosomes in metaphase, they dissociated from each other, but maintained their associations with the components of corepressor complexes such as Sin3A, RbAp48 or CoREST. Further, using two different classes of CK2 inhibitors (TBB and quinalizarin) and site directed mutagenesis approaches for CK2-phospho sites in HDAC2 (non-phosphorylatable forms of Ser394A and Ser422A/Ser424A), we identified the role of protein kinase CK2 in increased phosphorylation of HDAC1 and 2, which, as mentioned above, caused the dissociation of HDAC1 and 2 heterodimers. Taken together, our studies report for the first time that **increased mitotic phosphorylation of HDAC2, and to a lesser extent, HDAC1, was catalyzed by protein kinase CK2**. **Such phosphorylation event modulates the HDAC1 and 2 dimerization ability and promotes their self-association to form either HDAC1 or HDAC2 homodimer containing active corepressor complexes, without affecting their interactions with the binding partners**. These results uncovered the phosphorylation directed regulation and differentiation of HDAC-corepressor complexes. HDAC1 and 2 often coexist in the same corepressor complexes as HDAC1 and 2 heterodimers. As such, this study also revealed a

previously undescribed mechanism of selective regulation of HDAC1 and 2 containing complexes by PTM (e.g. phosphorylation) during mitosis.

Previous studies, including work from our lab, have reported that KATs and HDACs along with other transcription factors are displaced from mitotic chromosomes (He and Davie, 2006;Kruhlak et al., 2001). Factors promoting the dissociation events have not been well identified, although the dynamic phosphorylation of histones and nuclear factors has been suggested to be involved (Egli et al., 2008;Wang and Higgins, 2013). In addition, the spatio-temporal distribution of HDAC1 and 2 during mitosis and the sequential order of their re-entry into the daughter cells, which is required to re-establish the gene expression mechanisms, have not been studied systemically. Thus, in the second study presented in this thesis (**Chapter 3**), we aimed to investigate the factors that could allow the displacement of HDAC1 and 2 from mitotic chromosomes, their temporal distribution, as well as to examine their order of re-entry into daughter nuclei in comparison to other transcription factors such as Sp1, Sp3 and RNAPII.

Using an Aurora B inhibitor, ZM447439, and a CK2 inhibitor, TBB, we found that Aurora B-mediated H3S10ph or CK2-mediated increased phosphorylation of HDAC1 and 2 did not prevent the dissociation of HDAC1 and 2 from mitotic chromosomes in MCF7 and HeLa cells. Rather our study indicates that **HDAC1 and 2 interacting proteins (e.g. Sin3A and CoREST) as well as transcription factors (e.g. Sp1 and Sp3), which recruit the corepressor complexes to chromatin, were displaced from mitotic chromosomes independently of Aurora B or CK2 activity, might be the major factors that promote the displacement of HDAC1 and 2**. As these proteins were displaced from mitotic chromosomes, they were not able to recruit

HDAC1 and 2 into the chromosomes. Our study also provides evidence that **HDAC1 and 2 were temporally associated with microfilament F-actin during mitosis, suggesting F-actin was involved in the reorganization of HDACs**. **Further, HDAC1 and 2 re-entered daughter nuclei earlier than the re-entry of Sp1, Sp3 and RNAPII, at a time when the nuclear envelope was being re-assembled**. Taken together, our study contributed to the further understanding of the regulated process of mitotic partitioning, as well as laid down a basis for future research to investigate other chromatin modifying enzymes, which may be regulated in a similar manner.

In the second aspect of this thesis, we explored the un- or monomodified HDAC1 and 2 complexes based on the works from our lab and other groups (Wang et al., 2009; Sun et al., 2007). Interestingly, we demonstrated that phosphorylated HDAC2 interacting with the corepressor complexes and transcription factors, are primarily recruited to the UPR of an IEG, *TFF1*, and the un- or monophosphorylated HDAC2 is associated with the coding region (He et al., 2005;Sun et al., 2007). How HDACs get recruited to coding regions and whether a similar event occurs in other IEGs is not known. In a similar vein, HDAC inhibitor treatments induced changes in the expression patterns of IEGs, some of which were up- or down regulated (Hazzalin and Mahadevan, 2005;Simboeck et al., 2010). However, the mechanisms of activation or repression of these genes as well as the role of HDACs have not been fully elucidated.

In the next study presented in this thesis (**Chapter 4**), we investigated the role of HDACs in regulation of an IEG, *FOSL1* in colon cancer cell line, HCT116. *FOSL1* is overexpressed in a variety of human cancers, including colon cancers (Chiappetta et al., 2007;Young and Colburn,

2006). Using dual cross-linking ChIP assays, we demonstrated that class I HDACs, HDAC1, 2 and 3 were recruited to UPR and the transcribed region of *FOSL1* gene induced with TPA, in a transcription dependent manner. Inhibition of transcription prevented the loading of the HDACs in the gene body region but not in the regulatory region. In addition, HDAC inhibitors (TSA and apicidin, pan-HDAC inhibitor and class I HDAC inhibitor, respectively) attenuated the TPA-induced expression of this gene. Our study further illustrated that **HDAC inhibitors altered the TPA-induced *FOSL1* expression by preventing transcription initiation and recruitment of RNAPII without affecting the upstream signaling pathway, the MAPK kinase pathway or the chromatin remodeling pathway, the nucleosome response pathway**. **Thus, this study indicates a previously unrecognized mechanistic discontinuity between TPA-induced transcriptional regulation of *FOSL1* gene and the nucleosome response pathway, with HDAC inhibitors**. Overall, the attenuation of TPA-induced transcriptional activation of *FOSL1* gene by HDAC inhibitors using a mechanism independent of the nucleosome response pathway, can lead to future studies concentrating on the characterization of regulation of other IEGs, to explore the generality of this regulatory mechanism.

In the concluding study (**Chapter 5**), we continued to explore the role of HDAC1 and 2 in the coding region of transcribed genes, by studying the HDAC1 and 2 associated protein complexes and their functional ties to the underlying biological processes. To this end, we studied the endogenous HDAC1 and 2 complexes by immunoprecipitating HDAC1 and 2 proteins followed by mass spectrometry analysis. Our mass spectrometry results revealed that **HDAC1 and 2 were in complex with a large number of splicing proteins that were involved in spliceosome assembly and, the splicing accessory proteins such as SR proteins**. Although HDAC2 was

previously identified in the spliceosome complex by large-scale proteomic analyses, no further studies were carried out to validate the findings in detail (Rappsilber et al., 2002;Zhou et al., 2002). Among the proteins associated with HDAC1 and 2, we focused our studies on the SR protein, SRSF1, which is an important regulator of both constitutive and alternative splicing. By co-immunoprecipitation studies, we showed that HDAC1 and 2 physically interacted with SRSF1. Further, HDAC1 and 2 were associated with SRSF1 along the body of the transcribed genes of interest, *TFF1*, *FOSL1* and *MCL1*, in two different cell lines, HCT116 and MCF7. *TFF1* and *FOSL1* are constitutively spliced and *MCL1* is an alternatively spliced gene. **Thus our study illustrated that HDAC1 or 2 and SRSF1 were co-occupied in the gene body of transcribed genes, regardless of the splicing regulatory mechanisms**. **We further provided evidence that the (HDAC1 and/or HDAC2-SRSF1) complex was distinct from the well characterized HDAC1 and 2 corepressor complexes and was catalytically active**. **In addition, we demonstrated that enzymatic inhibition of HDAC1 and 2 by two unrelated HDAC inhibitors, or siRNA-mediated knockdown of HDAC1 and/or HDAC2 as well as SRSF1, altered the splicing pattern of *MCL1* towards the short isoform, *MCL1S*, transcript**. As mentioned before, alternative splicing of *MCL1* generates two isoforms by exon skipping mechanism, *MCL1L* (long form that contain all three exons, 1-3) and *MCL1S* (short form, which consist of exon 1 and 3) transcripts. This indicates that splicing of *MCL1* alternative exon (exon 2), was regulated by these proteins. However, inhibition of HDAC activity did not alter the expression level or the acetylation level of SRSF1, suggesting the change of splicing was not due to any secondary effect on SRSF1, the major regulator of *MCL1* splicing (Moore et al., 2010). Towards understanding the mechanism of this splicing switch event, our results showed that **with HDAC inhibitor treatment, a local increase in the histone acetylation level of H3 and**

H4 at the alternative exon (exon 2) was observed, a balance that was regulated by HDAC1 and 2 and KAT2B, and/or KAT7. **The increased acetylation level resulted in localized increased in RNAPII elongation, which altered *MCL1* splicing, in particular, towards the increased exclusion of exon 2, in the mature transcript**. **Most significantly, our studies provided evidence for the first time that, similar to SRSF1 and KAT2B, HDAC1 and 2 resided on nascent pre-mRNA, while regulating the dynamic acetylation of *MCL1* chromatin**. **As such, our results suggest a new mechanism where RNA-directed dynamic histone acetylation regulates the alternative splicing of *MCL1*, thereby adding another layer of complexity to its splicing regulatory network**. Thus, this work has provided a framework for further investigations on the correlation among histone acetylation, transcription elongation and splicing proteins, which could be a common mechanism of epigenetic regulation of alternative spliced genes, affected by inhibition of HDAC activity.

7.2 Insights and perspectives

7.2.1 Insights and perspectives from the study-1

7.2.1.1 Protein kinase CK2-catalyzed increased mitotic phosphorylation of HDAC1 and 2

Our study provides the first report that HDAC1 and 2 are the substrates of protein kinase CK2 during mitosis. Although previous studies have observed the increased phosphorylation of HDAC1 and 2 in mitosis, the kinase responsible for this event was not determined (Galasinski et al., 2002). Intriguingly, CK2-mediated phosphorylation of HDAC2 was more robust than HDAC1, which might be temporally and spatially regulated during mitosis. Further, our study also suggests that the observed reduced mobility of HDAC2 in mitosis must be due to the phosphorylation at Ser422 and/or Ser424 of HDAC2 (corresponds to Ser421 and Ser423 in

HDAC1), in addition to Ser394. Through mass spectrometry analyses, additional phosphorylation sites have been identified in HDAC1 at Tyr221 and at Tyr304 in HDAC2 (Olsen et al., 2006;Rush et al., 2005). Furthermore, a recent study has reported Ser411 as a substrate for CK2 (Adenuga et al., 2010). It will be interesting to investigate if this phospho-site (e.g. Ser411) can be a mitotic substrate for CK2 or if any additional kinase can target the other phospho-sites (e.g. Tyr221 and Tyr304) during mitosis. For this, the commonly used techniques for detecting phosphorylation, such as the use of radioactive phosphate, phosphorylation site-specific antibodies, and the gel mobility shift assays can be employed. In this regard, the dynamics of phosphorylation should be considered, and the phosphatase(s) dephosphorylating HDAC1 and 2 during mitosis need to be identified for better understanding of the molecular mechanisms underlying the regulation of HDAC-corepressor complexes. Of note, HDACs have been reported to be in a complex with PP1. Further, HDACs have been shown as the *in vitro* substrates of PP1 and λ-phosphatase (Canettieri et al., 2003;Galasinski et al., 2002;Brush et al., 2004). However the mitotic phosphatase specific to HDAC1 or 2 has not been reported. Nonetheless, our data established an important, previously unrecognized role for CK2 in mitosis by targeting the HDAC1 and 2 complexes.

7.2.1.2 Protein kinase CK2-mediated homodimerization of HDAC1 and 2 in corepressor complexes during mitosis

HDAC1 and 2 do not function in isolation; it is their interactions with one another and with other proteins that forms the multiprotein corepressor complexes which regulate their cellular functions (Pflum et al., 2001;Sun et al., 2007;Tsai and Seto, 2002). We have previously shown that the protein kinase CK2 is an important regulator of HDAC1 and 2 corepressor functions, by

phosphorylating HDAC1 and 2 and promoting their incorporation in the complexes (Sun et al., 2002a). In the current work, we revealed a mitotic role for CK2 in selective regulation of the HDAC1 and 2 corepressor complexes. We further demonstrated that the functional consequences of the CK2-catalyzed increased phosphorylation during mitosis abrogated the HDAC1 and 2 interactions, and, rendered HDAC1 and 2 to self-associate (forms homodimers of either HDAC1 or 2) in the corepressor complexes, without altering their physical interaction capabilities with other binding partners or their catalytic activities. More significantly, our studies identified a physiological context where HDAC1 and 2 could reside in the same complexes in homodimer configurations, which are primarily known to exist as heterodimers in these complexes. Further, multiple CK2-phosphorylation sites (phosphorylation at Ser394 and to a greater extent at Ser422/Ser424) function in a cooperative manner to mediate the homodimerization of HDAC1 and 2, suggesting a certain grade of redundancy among the sites. The effects of CK2-mediated phosphorylation of HDAC1 and 2 during mitosis represents a newly recognized mechanism, where a specific phosphorylation event serves as a regulatory switch to selectively determine the composition of the HDAC1 and 2 corepressor complexes itself, which may have functionally divergent fates and targets. Future research will be required to determine the functions of these complexes in mitosis.

A possible explanation for our finding of CK2-mediated dissociation of HDAC1 and 2 heterodimer could be related to the extent of phosphorylation of proteins interacting with HDAC1 and 2 that affects the composition and the integrity of these complexes. For the previous report of phosphatase inhibitor mediated increased phosphorylation resulting in the disassembly of HDAC1 and 2 heterodimers and the multiprotein complexes (Galasinski et al., 2002), it can be

reasoned that multiple proteins including those in HDAC-complexes are likely become highly phosphorylated and contributed to the dissociation of HDAC1 and 2 complexes. However, during mitosis, CK2-catalyzed phosphorylation of HDAC2 was sufficient to dissociate HDAC1 from HDAC2, but the corepressor complexes maintained their integrity and were catalytically active. The level of phosphorylation during mitosis might not exceed the threshold level that could result in the dissolution of the complexes, as reported previously in case of phosphatase inhibitor induced robust phosphorylation of HDAC1 and 2 (e.g. higher than physiological levels of phosphorylation) leading to the dissociation of the complexes. This suggests that there must be a necessary threshold of HDAC1 and 2 phosphorylation needed to be surpassed in order to observe a dissociation of the corepressor complexes. Besides, increase in phosphorylated HDAC1 and 2 during mitosis may facilitate increased levels of HDAC1 or HDAC2 containing complex formation in a way that is currently not known and warrants further studies. Our findings of increased association of RbAp48, a component of Sin3A and NuRD complex, with HDAC1 in mitotic cells, provided evidence to support this idea. However, we did not observe any changes in the expression levels of HDAC1, 2 or RbAp48, in control versus mitotic cells (data not shown). This indicates that increased association of RbAp48 with HDAC1 in mitosis was unlikely to be related to any alteration in their expression levels. However, we cannot rule out the possibility that additional mechanisms may exist that can act separately or in concert. One such mechanism may involve the conformational and physicochemical changes induced upon CK2-mediated increased phosphorylation affecting HDAC1 and 2 interactions. Conceivably, more work will need to be done to fully delineate the mechanism of by which CK2-catalyzed increased phosphorylation disrupts the HDAC1 and 2 heterodimers in mitosis. Although the crystal structure of HDAC1 has not yet been resolved, the N-terminal dimerization

domain of HDLP shares strong sequence homology to that of HDAC1 and 2 (Finnin et al., 1999). Furthermore, a recent study has reported the crystal structure of HDAC2 (Bressi et al., 2010b). Therefore, further experiments with computational models such as homology modeling, are required to expand our understanding of the interplay between phosphorylation and structural changes in HDAC1 and 2, and to shed lights on the mechanisms of specificity or promiscuity of HDAC1 and 2 interactions. These may also provide clues about their novel cellular targets as well as functions, and could ultimately reveal opportunities for exploiting HDAC1 and 2 complexes in mitotic regulation.

7.2.1.3 Role of HDAC1 and 2 corepressor complexes during mitosis

We have identified distinct HDAC-corepressor complexes in mitosis which contains either HDAC1 or HDAC2 homodimers but not HDAC1 and 2 heterodimers, although the functional significances of these complexes have yet to be determined. During mitosis, the change in the compositions of corepressor complexes (from heterodimers to homodimers) occurs soon after the break down of nuclear envelope. We, thus, speculate that the HDACs might have an opportunity to deacetylate different cellular factors or non-histone proteins that are acetylated in mitosis, but were not available to these enzymes due to their cytoplasmic localizations in interphase (**Figure 7.1**). Conceivably homodimers of HDAC1 and 2 may alter the ability of HDAC1 and 2 complexes to access specific substrates by changing the topology of the enzymes in the corepressor complexes, or may affect an as yet unknown HDAC1 and 2-corepressor function, or may mediate distinct forms of regulation during mitosis.

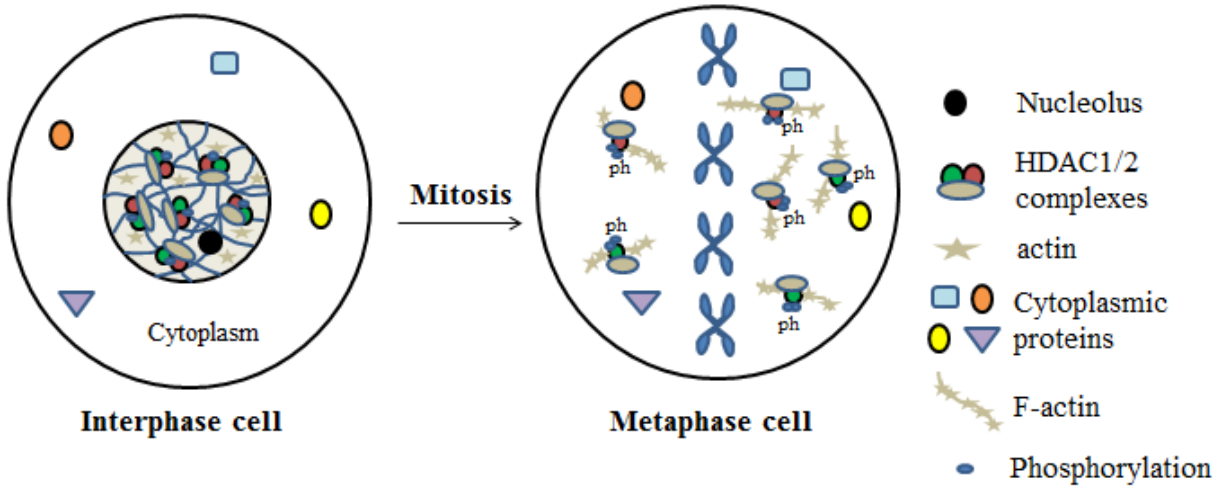

Figure 7.1: HDAC1 and 2 complexes in interphase and mitosis
During mitosis, protein kinase CK2-mediated increased phosphorylation (indicated as ph) of HDAC1 and 2 complexes results in the dissociation of HDAC1 and 2 heterodimers. However, HDAC1 and 2 maintain the interactions with corepressor complex proteins. HDAC1 and 2 homodimer containing complexes may have altered substrate specificities and can be directed to target different cellular proteins.

Studies have indicated that a large number of proteins are acetylated in mitosis (Chuang et al., 2010). For example, HDAC inhibitors have reported to increase the acetylation levels of APC1 and NudC proteins. The acetylated state of these proteins may govern their functions in mitosis and/or in cytokinesis (Chuang et al., 2010). Testing and validation of these proteins as the substrates of HDAC1 and 2 may provide a wealth of new insights towards uncovering the role of HDAC1 and 2 corepressor complexes in mitosis. Further, to potentially identify the proteins that may be associated with HDAC1 and 2 in a mitotic context, a proteomic approach of immunoprecipitation of HDAC1 or HDAC2 from mitotic cells could be coupled to mass spectrometry analysis. In addition to this, the composition of the HDAC1 and 2 interactomes can be studied under different conditions, for example, from control cells or cell-cycle synchronizing cells, which can also shed lights on the roles played by HDAC-complexes during mitosis.

A recent study reported a MiDAC complex in K562 cells containing HDAC1 and 2, and ELM-SANT domain proteins MIDEAS and DNTTIP1 (a DNA binding protein) but not Sin3A, NuRD or CoREST proteins (Bantscheff et al., 2011). Although this study does not rule out the possibility of the existence of alternative complexes with additional interacting proteins, the absence of Sin3A, NuRD or CoREST in MiDAC complex, contrasts with our observations of HDAC1 and 2 complexes isolated from the mitotic HeLa cells by immunoprecipitation, which contained these corepressor complex protein components. However, the role of HDAC-complexes, and their relative cellular activities are often cell type-specific (Delcuve et al., 2012;Delcuve et al., 2013). Therefore, the apparent disparity of the results may be in part attributed to the different cellular backgrounds and the steady state levels of phosphorylation within the cells. As mentioned before, our studies were primarily focused on HeLa (cervical cancer cell line) and to some extent in HEK293 (embryonic kidney cell line) cells while the MiDAC complex was described in K562 (chronic myeloid leukemia cell line) cells. Of note, the configuration of HDAC1 and 2 (e.g. homo- or heterodimers) in this complex was not determined. Taken together, these indicate that HDAC1 and 2 can reside in several multiprotein complexes, depending on cellular and physiological contexts. Nevertheless, we have identified a potentially novel role for CK2 in cell cycle progression, acting by regulating the composition of corepressor complexes, warranting further study in additional cell types. The comprehensive knowledge of the multiprotein complexes involving HDAC1 and 2 on various cellular backgrounds, will provide useful insights into the roles of these complexes, as well as how they may be regulated. Overall, it is our hope that the work presented here, will aid in the formulation of concrete hypotheses about the regulation of cell cycle events involving HDAC complexes and

will serve as a launching pad for future investigations to explore the multifaceted roles and dynamic nature of HDAC complexes in cellular processes.

7.2.2 Insights and perspectives from the study-2

7.2.2.1 Mitotic re-localization and partitioning of HDAC1 and 2 complexes

At the onset of mitosis, while chromosome condensation takes place, several proteins including transcription factors, RNAPII, KATs and HDACs are displaced from the mitotic chromosomes (He and Davie, 2006;Kruhlak et al., 2001;Delcuve et al., 2008). As cells proceed to the end of mitosis in telophase, HDAC1 and 2 are equally dispersed between the daughter cells. The equal partitioning of these proteins provides a mechanism for maintenance of cellular levels and activities of HDAC1 and 2 after mitosis. In addition, our results show that HDAC1 and 2 re-entered into daughter nuclei during the re-assembly of lamin and nuclear envelope and, earlier than transcription factors (e.g. Sp1 and Sp3) and RNAPII re-entry. It is thus conceivable that displacement and re-entry of HDAC1 and 2 complexes are temporally regulated and HDAC1 and 2 would be available early in the events that lead to the re-establishment of the gene expression program in daughter cells.

The chromatin condensation during mitosis is tightly correlated with increased global phosphorylation of histone H3, H3S10ph or H3S28ph (Wang and Higgins, 2013;Xu et al., 2009). Several studies have demonstrated a role of H3S10ph in displacing proteins, including HP1γ, SRSF1 and, SRSF3 from chromatin (Loomis et al., 2009;Winter et al., 2008). However, our study revealed that Aurora B-mediated phosphorylation of H3S10ph was not sufficient enough to displace HDAC1 and 2 from mitotic chromosomes in HeLa cells. Furthermore, it was previously

reported that the mitotic displacement and inactivation of some of the transcription factors can occur through their phosphorylation (Caelles et al., 1995;Dovat et al., 2002;Segil et al., 1991). Thus, we investigated if CK2-mediated increased mitotic phosphorylation of HDAC1 and 2 could promote the displacement and found that inhibition of CK2 did not prevent the displacement of HDAC1 and 2 from mitotic chromosomes. These results suggest the possibility that other factors play key roles in the exclusion of HDAC1 and 2 from mitotic chromosomes.

Towards uncovering of the factors which might allow the dissociation of HDACs, we found that proteins interacting with HDAC1 and 2 in multiprotein complexes such as Sin3A or CoREST and transcription factors (e.g. Sp1 and Sp3) were also excluded from mitotic chromosomes. As these proteins are the recruiters of HDAC1 and 2 complexes, the absence of these proteins on mitotic chromosomes can account for the displacement of HDAC1 and 2. However, other factors may be involved in this process including the disruption and rearrangement of the nuclear matrix. Of the components of the nuclear matrix, actin filaments have a role in binding to HDAC complexes (Andrin and Hendzel, 2004). Our observation that HDAC1 and 2 associated with F-actin in mitotic cells suggesting HDAC1 and 2 were released from the chromatin following nuclear matrix, in particular, F-actin reorganization during mitosis (Simon and Wilson, 2011). It is plausible that, once the nuclear F-actin and nuclear matrix are reorganized in the daughter cells, HDAC1 and 2 re-assemble with this structure before the resumption of transcription. Thus, the nuclear matrix might have an architectural role in the nuclear reorganization of HDAC1 and 2.

Although we were not able to decipher the detailed molecular mechanism how HDAC1 and 2 were excluded from mitotic chromosome, our results indicate that this is a multifactorial process in nature. Future experiments can be directed to investigate the mechanisms how the recruiters of HDACs, such as transcription factors or HDAC-associated proteins, get displaced from mitotic chromosomes. These studies may provide further clues to understand the mechanisms of HDAC1 and 2 dissociation from chromosomes during mitosis. However, it is worth noting that mitotic partitioning of transcription factors and chromatin modifying enzymes vary among various cancer lines and primary cell lines (He and Davie, 2006;Kruhlak et al., 2001;Delcuve et al., 2008). Thus it will be of considerable interest to study the extent of this phenomenon in other cell lines and in primary cells as well. Furthermore, very little is known about the role of ncRNAs present within the nuclear matrix. It is plausible that the ncRNAs play architectural roles in mitotic reorganization of these enzymes. This awaits future investigations.

7.2.3 Insights and perspectives from the study-3

7.2.3.1 Class I HDACs are localized to gene body of *FOSL1* in a transcription dependent manner

Our study extends the previous observation that HDAC inhibitors prevented the induction of *c-Fos* and *c-Jun* IEGs in mouse fibroblasts (Hazzalin and Mahadevan, 2005). With the use of dual cross-linking ChIP assays, we provided the first evidence that class I HDACs were recruited to the UPR as well as the gene body of *FOSL1* with TPA-induced transcription activation in HCT116 cells. In a similar manner, we observed a similar trend of recruitment of HDAC1 and 2 in a transcription dependent manner to another IEG, *TFF1* (induced with TPA or estradiol) in MCF7 cells (data not shown). Thus the transcription dependent recruitment of class I HDACs to

transcribed genes likely follows a similar mechanism as reported in yeast (Govind et al., 2010;Spain and Govind, 2011). The mechanism that governs the distribution of these HDACs to the UPR or the gene body region is apparently discernible with HDAC inhibitors. HDACs are recruited to the transcribed regions of active genes by interacting with RNAPII. As the HDAC inhibitors prevent the transcriptional activation of *FOSL1*, RNAPII would not be present to recruit the HDACs. However, at the UPR, the nucleosome response pathway remodeled the *FOSL1* promoter, thus allowing the transcription factors to recruit HDAC-corepressor complexes. HDAC inhibitors do not affect these events and thus the localization of HDACs to UPR was not altered.

7.2.3.2 Uncoupled mechanisms of the nucleosomal response and *FOSL1* gene transcription in response to HDAC inhibitors

The nucleosome response pathway is required for the induction of IEGs in response to various stimuli, such as growth factors or the phorbol ester, TPA. TPA-induced establishment of MSK mediates the rapid phosphorylation of H3 molecules at the regulatory region of IEG, which in turn is involved in UPR remodeling (e.g. recruitment of 14-3-3 and other chromatin remodeling complexes) required for the transcription initiation of the gene (Drobic et al., 2010). In our study, we demonstrated that pre-treatment of cells with HDAC inhibitors (TSA and apicidin) repressed the TPA-induced activation of *FOSL1*. However, HDAC inhibitors did not affect the upstream ERK1/2 signal transduction pathway or the nucleosome response event including H3S10ph level or the recruitment of 14-3-3 proteins to the UPR. This indicates that HDAC inhibitors impact the downstream steps of gene activation, leading to the abortive transcription initiation. Accordingly, our study highlight that HDAC inhibitors promotes the mechanical uncoupling

between the nucleosome response event and transcription activation of *FOSL1* gene, thus adding another layer of fine-tuned mechanism of transcription regulation. Subsequently, HDAC activity was required for transcriptional activation of this gene that involved a mechanism independent of the well-defined mechanistically linked regulatory mechanism of the nucleosome response and IEG induction. Interestingly, in response to the treatment with HDAC inhibitors, *FOSL1* UPR exhibited increased level of acetylation, without transcription repression. This indicates that HDAC inhibitors induced a general deacetylation of UPR nucleosomes and the increased KAT activity at UPR was not prevented by the treatment. It is unlikely that changes in chromatin remodeling or acetylation level are responsible for the repressive effects of HDAC inhibitors on *FOSL1* promoter as suggested previously for MMTV promoter (Bartsch et al., 1996;Bresnick et al., 1990). Rather HDAC inhibitors might impact the downstream steps of gene activation which leads to transcriptional repression. However, for further confirmation nucleosome remodeling studies might be required.

7.2.3.3 Involvement of other HDAC isoforms in transcription regulation of *FOSL1* gene

Our work focused on to study the effect of two different classes of HDAC inhibitors, TSA and apicidin, that target class I, II HDACs and class I HDACs, respectively. Therefore, the involvement of other HDAC isoforms or possible secondary effects that resulted in transcriptional repression cannot be ruled out. Thus, it is pertinent to verify that the effects we observed upon treatment with HDAC inhibitors are attributed to impedance of class I HDAC activity. Although we attempted to include class II HDACs in our analyses, we could not obtain good quality class II specific HDAC antibodies amenable to ChIP analyses, from commercially available sources. However, future studies can be directed to use of other class specific HDAC

inhibitors to imply for the generality of the observed results or to use specific knockdown of HDACs by siRNA-mediated approaches. In a similar manner, overexpression studies of individual HDACs can be performed. If a particular HDAC is involved in *FOSL1* transcriptional induction, an increase in *FOSL1* activity might be expected upon overexpression. However, overexpression of an individual HDAC isoform can be challenging as the HDAC of interest may already be present at the gene and the presence of more HDAC protein would be redundant. Furthermore, HDACs function in multiprotein complexes in association with other proteins and, as such, the overexpression of a HDAC alone may not be sufficient to achieve a promoter-context specific effect. In any event, knockdown studies of particular HDACs is more feasible to delineate the roles of these HDACs in *FOSL1* transcription.

7.2.3.4 Molecular details of HDAC inhibitors mediated attenuation of TPA-induced transcriptional repression of *FOSL1*

Towards understanding the mechanism of HDAC inhibitor mediated TPA-induced transcription repression of *FOSL1*, our observation that HDAC inhibitor prevented the binding of RNAPII at UPR suggests that HDAC activity is required independently of the ERK1/2 signaling and the nucleosome response pathway, for subsequent RNAPII recruitment and transcription initiation. Although we did not investigate further for the molecular details of the mechanism, it can be envisioned that any of steps of transcription initiation can be affected with HDAC inhibitors (Svejstrup, 2004). Transcription initiation begins with the formation of PIC on promoter. RNAPII can locate and utilize a promoter through the PIC. A series of protein-protein interactions results in the recruitment of RNAPII and general transcription factors (GTFs) and formation of PIC. It is likely that HDAC inhibitors could target and modulate the activity of the

members of PIC complex, such as TBP-associated factors (TAFs), GTFs (e.g. TFIIB, TFIID, TFIIE, TFIIF and TFIIH), cellular factors or other non-histone proteins that are involved in the necessary steps of transcription initiation, leading to abrogated recruitment of RNAPII and transcription repression. Furthermore, transcription elongation factors such as DSIF (DRB sensitivity inducing factor) or NELF (negative elongation factor), which interact with elongating RNAPII, can be modulated by HDAC inhibitors (Ping and Rana, 2001). It is unlikely that the modulation of the elongation factors is involved as transcription initiation step was abrogated for *FOSL1* gene induction with HDAC inhibitors. Further experiments are obviously required to delineate the precise regulatory mechanism.

In recent years, IEGs came to light as important targets for cancer research due to their involvement in regulation of various oncogenic responses (Healy et al., 2013). Currently, little is known about HDACs targets in IEG expression. Here, our study provided insights into how the *FOSL1* could be regulated by HDAC inhibitors, which could be applicable to other IEGs which are down regulated with HDAC inhibitors as the *FOSL1* gene. Thus, our studies have provided the basis for future research to explore the regulatory mechanisms to therapeutically target these genes with HDAC inhibitors.

7.2.4 Insights and perspectives from the study-4

7.2.4.1 HDAC1 and 2 associate with a large number of splicing proteins

Our mass spectrometry analyses from the HDAC1 and 2-immunoprecipitated fractions demonstrated that HDAC1 and 2 were associated with Sin3A, NuRD and CoREST complexes, as well as with numerous splicing proteins of diverse functions such as spliceosome assembly

proteins, splicing accessory proteins, SR proteins, and RNA helicases. Consistent with our data, it has previously reported that HDAC2 is associated with the spliceosome complex (Rappsilber et al., 2002;Zhou et al., 2002). Here, we validated our mass spectrometry results by co-immunoprecipitation studies and found that HDAC1 and 2 were associated with SRSF1, a splicing factor, which functions in both constitutive and alternative splicing. We further analyzed HDAC1 and 2 interactions with some other splicing proteins that were identified in our mass spectrometry results, such as SRSF3, SRSF7, PRPF4B, DDX5, and DDX17 (unpublished data). Our results illustrated that splicing machinery physically interacts with HDAC1 and 2. Furthermore, in the hunt for the binding partners associated with HDAC1 and 2 in coding region of transcribed genes, our work has provided a list of candidate proteins (e.g. splicing proteins). These proteins can be of interest for future studies to expand the knowledge of the role of HDAC1 and 2 complexes in fine tuning the splicing mechanisms.

7.2.4.2 HDAC1 and 2 are in complex with SRSF1 in the coding region of transcribed genes

Of the splicing proteins identified, we concentrated on SRSF1 for subsequent studies. SRSF1 is one of the major regulators of splicing events, plays important roles in both constitutive and alternative splicing, and is often overexpressed in many cancers. Further, the SRSF1 antibody which was available to use, performed quite well in all our stringent optimization criteria for immunoblotting, immunoprecipitation, ChIP and CLIP assays. As mentioned earlier, the interactions of SRSF1 with HDAC1 and 2 were validated by immunoprecipitation and immunoblotting assays, although our low-stringency co-immunoprecipitation studies do not discriminate the nature of the association of SRSF1 with HDAC1 and 2. The interaction of SRSF1 and HDAC1 and 2, may be direct or indirect, through interaction with another protein.

However, our sequential co-immunoprecipitation results from DSP cross-linked MCF7 cell extracts demonstrated that SRSF1 was in a complex that contained both HDAC1 and HDAC2 (heterodimers of HDAC1 and 2) (data not shown). Interestingly, SRSF1 did not interact with HDAC-corepressor complex proteins Sin3A, CoREST, RbAp48 or with HDAC2 S394 phospho form. As previously mentioned, phosphorylation of HDAC1 and 2 is required for its incorporation into corepressor complexes and subsequent recruitment to regulatory regions of genes (Sun et al., 2007). Further, the activity of (HDAC1 and/or HDAC2-SRSF1) complex was inhibited by TSA. Thus these results indicate that SRSF1, and, HDAC1 and 2, were in a complex, distinct from the well-defined corepressor complexes and was catalytically active.

The recruitment of HDAC1, 2 and SRSF1 to the body of active genes require on-going transcription (Sapra et al., 2009;Khan and Davie, 2013). Here, we further extended this observation that HDAC1 or 2 was co-recruited with SRSF1 to the coding region of transcribed genes (e.g. *FOSL1, TFF1* and *MCL1*) in a transcription dependent manner, regardless of the nature of underlying splicing regulatory mechanisms of these genes. It is tempting to speculate that other splicing proteins interacting with HDAC1 and 2 can be recruited in a similar manner, a mechanism which awaits further investigations.

7.2.4.3 Regulation of alternative splicing of *MCL1* by SRSF1, HDAC1 and HDAC2

Previous studies have demonstrated that SRSF1 is a major regulator of *MCL1* splicing and the down regulation of SRSF1 favored the production of the short form, *MCL1S* over the long form, *MCL1L* (Moore et al., 2010). We were able to reproduce this result in our study system (HCT116), by siRNA-mediated knockdown of SRSF1. In addition, with two HDAC inhibitors,

TSA and apicidin, we found that HDAC inhibitors also altered the splicing of *MCL1* towards the short isoform. However, the change in *MCL1* splicing was significantly greater in the synchronized cells (serum starved, TPA-induced cells, pretreated with or without HDAC inhibitors for 30 min) than those of the asynchronized ones (cycling cells treated with or without HDAC inhibitors for 2 h). This may be related to the heterogeneity of cell population and the interference from the mRNAs that are synthesized and spliced before HDAC inhibition, as suggested previously (Hnilicova et al., 2011). In contrary to other studies which investigated the effect of HDAC inhibitors on splicing by a long treatment period (Hnilicova et al., 2011), our aim was to study the immediate effects with a short time period of treatment and also to minimize any secondary effects due to inhibitor treatments, such as cell cycle arrest or apoptosis. As a complimentary approach to inhibitor studies, we showed that the knockdown of HDAC1 and/or HDAC2 also had similar but more profound effects on *MCL1* splicing, favoring the short isoform by exon skipping mechanism. However, knockdown of HDAC3, the other member of class I HDAC, did not impact the alternative splicing of *MCL1* (unpublished data). Taken together, our results illustrated that HDAC1 and 2 play key roles in regulation of *MCL1* alternative splicing. Follow up studies can be undertaken in the near future to assuage any remaining off-target issues associated with siRNA treatments by designing 'rescue' experiments, involving the expression of siRNA-resistant HDAC1 and 2 (e.g. introduction of silent mutations in siRNA target sequences) or catalytically dead HDAC1 and 2 (e.g. HDAC1 H141A). Rescue of the splicing switch event will conclusively demonstrate that the phenomenon is due to knockdown of the HDAC1 and/or 2 and not due to some off-target activity of the siRNAs.

7.2.4.4 HDAC inhibitor mediated regulation of alternative splicing of *MCL1*

Towards understanding of the mechanism of how HDAC inhibitors regulate splicing of *MCL1*, we have showed by ChIP assays with RNAPIIS2ph (elongation form of RNAPII) antibodies that the rate of RNAPII elongation was increased at the alternative exon of *MCL1* (exon 2). A significantly reduced occupancy of RNAPIIS2ph at exon 2 was observed with HDAC inhibitors treatment, particularly in the synchronized cells, which is indicative of a faster elongation rate over that region (Zhou et al., 2011). However, to analyze a more direct measurement of changes in transcription rates along the body of the *MCL1* gene HDAC inhibitors, we applied an 'elongation assay' protocol previously described (Zhou et al., 2011) to monitor the accumulation of BrU-labeled nascent *MCL1* pre-mRNAs with or without HDAC inhibitor treatment. While the nascent pre-mRNA accumulation rate of exon 1(exon 1-intron 1 junction) and exon 3 (intron 2-exon 3 junction) was not affected by HDAC inhibitor treatments, a 2.2-fold increase in pre-mRNA accumulation occurred for alternative exon 2 (exon 2-intron 2 junction) for cycling cells treated with or without HDAC inhibitors and a 3.7-fold increase was observed for serum starved TPA-induced cells pretreated or not with HDAC inhibitors. Although our aim was to measure the RNAPII elongation rate, it is not feasible for a gene like *MCL1*, which is rather short and is transcribed within minutes; given the fact that transcription rate ranges from 1 to 6 kb/min (Henriques and Adelman, 2013). Further, transcription rates can be influenced by splicing and *vice versa*, and splicing takes significantly less time than transcription (~30 sec to 5 min) (Brugiolo et al., 2013). Therefore, the observed results cannot be explained simply by increased RNAPII elongation rate over exon 2. However, our results point towards an interesting possibility that the efficiency of exon 2 splicing (e.g. intron removal) might change or slow down with HDAC inhibitor treatment. As such, an increased rate of the accumulation of exon 2-intron

2 pre-mRNA detected in our studies, in turn, might reflect the formation of the 'exon-intron lariat' with HDAC inhibitor treatment. It is fascinating to envision that HDAC inhibitors might affect the activity or expression of the RNA debranching enzyme (DBR1) required for hydrolyzing the lariats, leading to stabilization of the 'exon-intron lariat' and accumulation of more pre-mRNA. Clearly more work will be needed to determine the functional role of HDACs in lariat formation or RNA stability; however, our studies provide a basis for further investigation to explore this 'novel' function of HDACs.

In addition to RNAPIIS2ph, we studied the distributions of HDAC1, 2 and SRSF1 along the *MCL1* gene body. In agreement with their RNAPII transcription-dependent recruitment to the gene body of transcribed genes, the distribution profiles of HDAC1, 2 and SRSF1 along *MCL1* gene were similar to that of RNAPIIS2ph. Previous studies reported that SR proteins interact with the nascent pre-mRNA, RNAPIIS2ph and chromatin (Sapra et al., 2009). SR proteins interactions with RNAPIIS2ph were RNase-sensitive, while their interactions with chromatin were reduced by RNase treatment, but not abolished. We successfully reproduced these results with SRSF1 in *MCL1* gene. Intriguingly, our results show that the association of HDAC2 with the gene body of *MCL1* gene was sensitive to RNase, more so than the association of SRSF1. The reduced association of HDAC2 and SRSF1 along *MCL1* gene with RNase treatment suggests that large portions of HDAC2 and SRSF1 were associated with *MCL1*, and mediated through their associations with the emerging RNA or another RNase sensitive protein. The residual signal indicates their interactions were also mediated by chromatin which was preserved by protein-protein and protein-DNA interactions at a given time and space and/or by an incomplete digestion of the cross-linked RNA by RNase. Furthermore, by co-

immunoprecipitation studies we demonstrated that the interaction of HDAC2 with RNAPIIS2ph was RNase sensitive, indicating that HDAC2 was not bound directly to RNAPIIS2ph. Taken together, our studies indicate that the association of HDAC1 and 2 with RNAPIIS2ph is RNA dependent, and their distribution in *MCL1* gene body was mediated by RNA and chromatin.

HDAC inhibitors also resulted in a change in the histone acetylation levels pronounced over the region of exon 2, the level of which was maintained by the recruitment of HDAC1, 2 and the KAT2B/7 at exon 2. Although it is conceivable that KATs-HDACs interplay was the regulator of the dynamic acetylation level at this region, we are the first to show by dual cross-linking ChIP assays that HDAC1, 2 and the KAT2B/7 were recruited to this region. Other KATs or HDACs might play roles in regulating the dynamic acetylation levels; we explored this possibility by performing ChIP assays with KAT5 or HDAC3 antibodies. However, we were not able to detect any enrichment for these proteins at *MCL1* gene body region (unpublished data). However, KAT5 has been reported within the gene body of active genes (by genome wide sequencing) and we previously reported that HDAC3 was associated with the coding region of *FOSL1* gene (Wang et al., 2009;Khan and Davie, 2013). Thus depending on the gene-contexts, the association of KATs or HDACs varies over the transcribed regions of genes. This is also consistent with our finding that HDAC3 knockdown did not affect the splicing pattern of *MCL1* (unpublished data). The increased acetylation of exon 2 nucleosome preceded by local increased in elongation rates, implying that increased nucleosomal acetylation contributed to faster elongation of RNAPII. Previous studies from our lab demonstrated that histone acetylation would maintain the 'unfolded' conformation of the nucleosome and/or chromatin, facilitating the passage of RNAPII through this region (Walia et al., 1998). Further, HDAC inhibitors did not alter the expression

level or the acetylation level of SRSF1. HDAC inhibitor mediated change in alternative splicing of *MCL1* was unlikely due to any secondary effect on SRSF1 protein, rather inhibition of HDACs resulted in localized increased in the level of acetylation at exon 2 region, which itself leads to increased transcription elongation over this region. Hence, the spliceosome assembly was compromised and exon exclusion was favored. Apart from this, histone acetylation could also recruit a subset of chromatin remodeling proteins, such as SWI/SNF complex, which can play role in modulating splicing mechanism. In line with this, the BRM subunit of the human remodeler SWI/SNF has been implicated in the regulation of alternative splicing by regulating the elongation rate (Batsche et al., 2006).

Future studies focusing on determining the 'unique characteristics' of the nucleosome positioned on exon 2 of *MCL1* will provide a comprehensive description of the molecular basis of splicing regulation associated with HDAC inhibition. Furthermore, many issues regarding the mechanisms of histone acetylation mediated *MCL1* splicing regulation still remain open questions for further investigations, e.g. how do KAT2B/7 and perhaps other KATs get specifically recruited to *MCL1* exon 2 leading to increased acetylation, or why do HDAC inhibitors have a specific effect on one exon of *MCL1*? We envision that KAT2B binds to acetylated H3 and H4 through its bromodomain, as a consequence of the increased H3 acetylation on exon 2 with HDAC inhibition (Spedale et al., 2012). KAT2B occupancy could be self-reinforcing by binding to its specific target. Alternatively, KAT2B could interact with an acetylated regulatory factor or non-histone proteins associated with exon 2. The underlying DNA sequence of, and surrounding, exon 2 may play a key role, acting as target for recruitment of other proteins. Addressing these questions will not only clarify the means and contexts by which

the KAT-HDAC interplay is required to alter the splicing of *MCL1,* but will also will unearth many new and tantalizing questions for further research. As such, including KAT inhibitors in the studies (Crump et al., 2011) can be useful to provide new insights.

From the genome wide mapping of H3K4me3 mark (ChIP-seq), an active marker of transcription, it was found that the level of this PTM was significantly enriched at *MCL1* alternative exon 2 relative to the other two exons (unpublished data from our lab). This result positively correlated with that of our dual cross-linking ChIP results with H3K14ac and H3K9ac antibodies on *MCL1* gene, where we detected an increased acetylation levels at exon 2 with HDAC inhibitor treatment. These indicate that there may be 'crosstalk' between these two marks, whereby one PTM influences the establishment or maintenance of another (Crump et al., 2011;Maltby et al., 2012). For example, KAT2B may mediate dynamic acetylation through direct or indirect recognition of H3K4me3, which may serve as a binding platform or enhance KAT activity. Alternatively, KAT7 can be recruited to H3K4me3 to induce histone acetylation, which recognizes H3K4me3 via its ING4 subunit (Hung et al., 2009). H3K4me3 might 'bookmark' the hyperacetylated alternative exon and could possibly explain why exon 2 was preferentially acetylated following HDAC inhibitor treatment. Likely a specific knockdown of H3K4 methyltransferase, SetD1 will be required for confirmation of this idea.

Our present study mostly focused on histone H3 and H4 acetylation; however studies are emerging showing that other histone modifications (e.g. H3K36me3, catalyzed by SetD2) can influence pre-mRNA splicing. Knocking down of SetD2 did not impact elongation but altered the splicing (Edmunds et al., 2008;Luco et al., 2010). Pre-mRNA splicing also regulates SetD2

recruitment and H3K36me3 levels along the body of transcribed genes (de Almeida et al., 2011;Kim et al., 2011). In addition, ubiquitinated H2B has been reported to be associated with coding region and there are several proteins, such as histone chaperone FACT, which interacts with HP1γ and RNF20, is responsible for ubiquitinating H2B (Davie and Murphy, 1990;Davie and Murphy, 1994;Kwon et al., 2010;Zhang and Yu, 2011). Thus it will be of interest to investigate these modifications and the corresponding modifying enzymes in future studies. Furthermore, it is plausible that a number of alternatively spliced genes can be regulated in a similar manner to that of *MCL1* in response to HDAC inhibitors. RNA-seq and ChIP-seq techniques can be employed to identify such genes and to determine any 'signature features' that can provide clues to selectively distinguish these genes.

7.2.4.5 RNA-directed dynamic histone acetylation and *MCL1* alternative splicing

As previously mentioned HDAC1 and 2 interactions with *MCL1* gene was mediated by both RNA and chromatin. We further extended this observation and reported for the first time that HDAC1 and 2 were associated with *MCL1* mRNA by dual cross-linking (UV followed by DSP cross-linking) CLIP assay. Consistent with a previous report, we have found that KAT2B was also bound to mRNA (Obrdlik et al., 2008). The HDAC1, 2-immunoprecipitated RNP complexes from dual cross-linked cycling or apicidin or TSA treated cells, showed that all three exons of *MCL1* were bound by HDAC1 and 2. However, the *MCL1* exon 2 was less enriched in the immunoprecipitated fractions in comparison to the other two exons. This enrichment pattern might correlate with the relative level of *MCL1* isoforms present within the cells at a given time. Depending on the relative expression of *MCL1* isoforms, the steady state levels of the individual exons may vary. It is likely that HDAC1 and 2 interactions with each exon might reflect the

steady state levels of the exons. Our dual cross-linking CLIP assays correlates with this idea where we found that the HDAC1 and 2 were associated more with exon 1 and exon 3 than with exon 2, suggesting the steady state levels of exon 1 and exon 3 were relatively high. However, with HDAC inhibitor treatments, the interaction of HDAC1 and 2 were slightly changed with the exons compared to the control. This reflects the moderate change of *MCL1* splicing with HDAC inhibitors, that resulted in an increased level of both isoforms, but more so of the short isoform by exon skipping (exon 2) mechanism. The change in splicing pattern would alter the steady state levels of all exons. This could therefore account, at least in part, for the observed binding patterns of HDAC1 and 2 with the exons with HDAC inhibitors treatments relative to control. In addition to HDAC1 and 2, SRSF1 and KAT2B also followed similar binding patterns with *MCL1* mRNAs. Thus splicing behavior of *MCL1* was partly reflected in the differential binding of splicing modulators (e.g. SRSF1 and KATs/HDACs) in the exons. However, by comparing HDAC1 and 2 associations with *MCL1* mRNA from the nuclear (pre-processed RNAs) or cytoplasmic RNA fractions (mature RNAs) may help to explain the RNA binding profiles of these proteins more clearly. Furthermore, the change in *MCL1* splicing was robust in synchronized cells; it will be of interest to study this phenomenon using synchronized cell populations.

The intriguing finding of the association of HDAC1 and 2 with RNA transcripts are further supported by our previous ChIP assays, in which we needed to use dual cross-linking approach to monitor HDAC1 and 2 along the gene body, use of formaldehyde alone worked inefficiently (Sun et al., 2007). In line with this notion, a recent study of genome wide mapping of KATs and HDACs applied dual cross-linking approach (Wang et al., 2009). Interestingly, the chromatin

remodeler SWI/SNF is also associated with nascent pre-mRNPs (Tyagi et al., 2009). Thus we proposed a model whereby the dynamic acetylation of *MCL1* chromatin, maintained by HDAC1 and 2 and KAT2B/7, acts at RNA level to modulate alternative splicing. Recently accumulating evidence suggest that nuclear RNA play important role in maintaining the structure of transcribed chromatin as well as the nuclear location of transcribed genes (Caudron-Herger and Rippe, 2012;Guil and Esteller, 2012;Mitchell et al., 2012). It is plausible that nuclear RNA associated with regulatory and coding regions of transcribed genes may serve as a binding platform for several chromatin modifying enzymes. Along these lines, our recently developed method of dual cross-linking CLIP assay can turn out to be a useful tool which will be applied to validate other chromatin associated proteins. Alternative splicing of several genes can be regulated by similar mechanisms. Thus it would be integral to examine the extent and involvement of HDACs in alternative splicing regulation of other genes and to decipher the mechanism of their involvement in other cancer and normal cell lines.

It has been long known that histone modifications regulate transcription, but it has recently emerged that these modifications play a significant role in pre-mRNA splicing. Therefore, we hope our studies presented herein will expand the understanding of how specific chromatin features exert regulatory functions on splicing and will lead to a new frontier for splicing research. Furthermore, putting splicing in the context of chromatin will provide many new opportunities to discover and understand yet unknown mechanisms of gene regulation.

7.3 Conclusions and significance

The studies provided in this thesis represent the steps towards unraveling the dynamic nature and functions of HDAC1 and 2 complexes in mitosis and in transcribed chromatin. We demonstrated that CK2-mediated increased phosphorylation could direct HDAC1 and 2 to be incorporated into corepressor complexes in homodimer configuration during mitosis, which is an intriguing observation given that HDAC1 and 2 are well defined to exist as heterodimers in corepressor complexes. These distinct complexes might play roles in targeting different cellular proteins. Again, the exclusion of HDAC1 and 2 from mitotic chromosomes was not due to aurora B-mediated H3S10ph, was rather largely due to the absence of the HDAC1 and 2 recruiters such as Sp1 and Sp3. Further, we provided evidence that un- or monophosphorylated HDAC1 and 2 complexes are recruited to gene body of IEGs in transcription depended manner and HDAC inhibitor mediated transcriptional repression of *FOSL1* was independent of the nucleosome response pathway, which is required for IEG expression and is correlated with gene activation. The depth of our study is further underscored by the identification of splicing proteins, in particular SRSF1, interacting with HDAC1 and 2 complexes in the coding regions of *FOSL1*, *TFF1* and *MCL1*, regardless of the underlying differences in their splicing mechanisms. In addition, we reported that HDAC1 and 2 were involved in the alternative splicing of *MCL1*, mediated by the RNA-directed changes in the histone acetylation level, leading to the increased elongation rate of RNAPII and subsequent changes in splicing. While we have found clear relationships between histone acetylation dynamics and alternative splicing, we just started to explore what we believe could be an exciting new area at the interface of chromatin and splicing. Clearly much remains to be learned, but to conclude, we can state that these studies have broaden our understanding of how the multi-faceted HDAC1 and 2 complexes can be regulated depending on their associated PTM (e.g. phosphorylation), and functions in various cellular

processes, including, but not limited to, transcriptional regulation and alternative splicing (**Figure 7.2**).

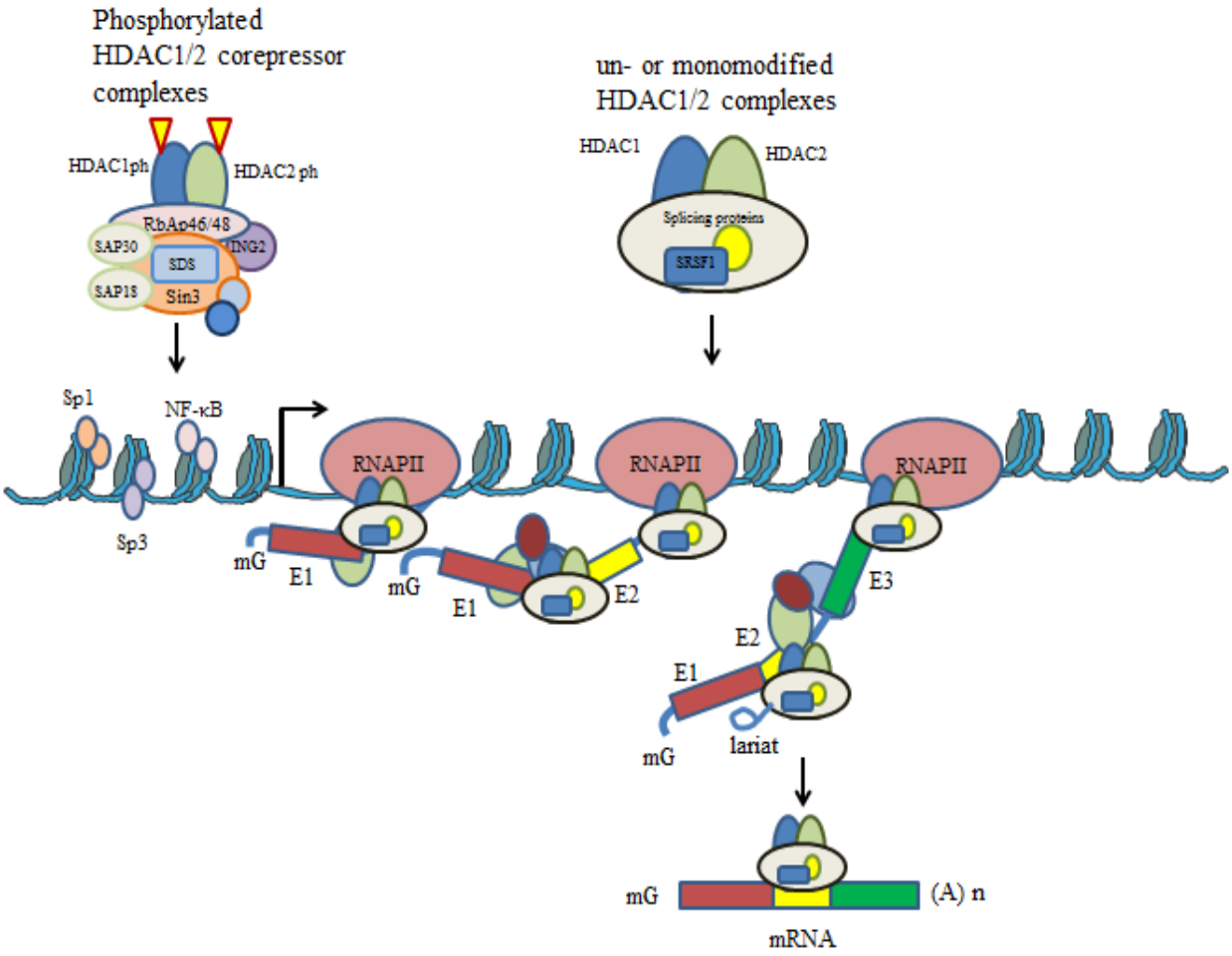

Figure 7.2: HDAC1 and 2 complexes in regulatory region and gene body of transcribed genes

Phosphorylated HDAC1 and 2 corepressor complexes are recruited to the regulatory regions by a variety of transcription factors (e.g. Sp1, Sp3 and NF-κB). Un- or monomodified HDAC1 and 2 complexes are recruited in RNAPII-transcription dependent manner with a number of splicing proteins (e.g. SRSF1) in gene body of transcriptionally active genes. RNAPII, chromatin and RNA-bound HDAC1 and 2 complexes are involved in many steps of transcription and splicing regulatory mechanisms as indicated. E1, E2 and E3 represent exons 1, 2 and 3.

In the near future, we can look forward to a frontier in the current research field as ‘consanguinity’ of chromatin and pre-mRNA splicing. A comprehensive understanding of the epigenetic regulators, splicing code, and interplay between epigenetics and pre-mRNA splicing will provide new insights into the complex network of gene regulation. Furthermore, it is our hope that this work will be opening up new and promising avenues of research that will lead in a more distant future, to develop better therapeutic strategies, involving modulation of chromatin and/or splicing to treat diseases that are the consequences of aberrant splicing.

Chapter 8: References

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