Dissection of in vivo functions of HDAC-6 and HDAC-1

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Preface/Research goals

Protein post-translational modifications play important roles in almost all biological processes. Those modifications extend the biological complexity based on primary amino acid sequence and have dramatic impact on protein's structure and functions. Specially, acetylatlion on histone N-terminal tails has been extensively studied and shown to be involved in the regulation of chromatin structure and functions. The acetylatlion not only directly influences the packaging of the chromatin but also provides new interaction platforms and signals for other chromatin-interacting proteins, complexes, and activities. Moreover, increasing numbers of nonhistone proteins have been shown to be regulated by acetylation/deacetylation. To understand *in vivo* role of histone and protein acetylation, we started to investigate two histone deacetylases, HDAC-6 and HDAC-1. For this a variety of techniques were used including gene targeting in mice.

In the introduction part the general definition and contents of epigenetics are presented. Then, various histone modifications and their *in vivo* functions will be summarized. Specially, the acetylation/deacetylation of histones and nonhistone substrates and their regulation by histone acetyltransferases (HATs) and histone deacetylases (HDACs) are discussed. Finally, the clinical aspects of protein acetylation are considered.

The result section can be divided into four parts. First, we identified HDAC-6 as a novo tubulin deacetylase *in vitro* and *in vivo*. Second, mainly based on experiments based on HDAC-6, we analyzed the structural basis of the histone and tubulin deacetylation reaction and proposed a model for general protein deacetylation reaction. In the third part, we generated HDAC-6 deficient mice and cells and have begun to characterize them. Finally, we presented ongoing results on the generation of HDAC-1 floxed mice and HDAC-1 deficient cells.

Part I: Introduction

1.1 Epigenetic regulation of mammalian genome

1.1.1 Epigenetics and mammalian genome

To maintain a stable/reliable inheritance of the genetic information is the basic dogma for life. On the other hand, gene activation/repression is a dynamic process that allows cells to respond rapidly to physiological signals. To achieve these goals, epigenetics is used as a bridge between faithful digital DNA replication and dynamic signaling networks. Therefore, epigenetics is defined as heritable phenomena that regulate gene expression without involving changes to the DNA code (Pennisi, 2001). Epigenetics translates the genetic information thereby sets up the signaling networks. On the other hand, signaling networks also need epigenetics to transduce the dynamic signals to refine the integrity and the stability of the whole system. Moreover, epigenetics provides the important learning function which helps either the short-term evolution (e.g. development and differentiation from embryos to organisms) or the long-term evolution (e.g. from mono-cellular organism to multi-cellular organisms).

Chromatin structure plays central role in epigenetic regulation. Chromatin not only is the hardware where genetic information is stored but also provides the complex signaling regulation on the maintenance, translation, and evolution of the genome. Regulation of higher order chromatin structure is directly coupled with regulation of the expression and integrity of the genetic information of eukaryotes and is likely to be a major force in the origin and evolution of genes, chromosomes, genomes, and organisms. DNA methylation and histone modification are the major contributors to chromatin modification, which combined with ATP-dependent chromatin remodeling, are the principal epigenetic mechanisms by which tissue-specific gene expression and global gene silencing are established and maintained (Li, 2002). DNA methylation is the rewritable signal on mammalian genome DNA and it was proposed in 1975 that this mechanism might be responsible for the stable maintenance of a particular gene expression pattern through mitotic cell division. Since then, ample evidences have been obtained to support this concept, and DNA methylation is now recognized to be a chief contributor to establish and maintain gene expression states (Jaenisch and Bird, 2003). Specifically, DNA methylation establishes a silent chromatin state by collaborating with proteins that modify nucleosome.

Functions of epigenetics

The main functions of epigenetics include regulation of transcription and cellular memory. The temporal and spatial regulation of gene expression is one of the most fundamental processes in biology and it encompasses many layers of complexity and intricate mechanisms. The evolution of epigenetic regulation is the main characteristic of the evolution of eukaryotic organisms, which increases biological complex dramatically independent of the increasing of genetic information. For example, it is difficult to explain the dramatic phenotypic differences between worms and humans if

only based on increased gene numbers (i.e., worms, 19K and humans, 30K). But with the discovery of multi-layer of epigenetic regulation, it can at least partially be rationalized by differences in the complexity of the regulatory code and not merely gene content. The discovery of multiple covalent modifications of the regulatory apparatus suggests that organisms have evolved various mechanisms to maximize the usage of a relatively limited number of genes and transcription factors (Freiman and Tjian, 2003). While many components of the transcriptional machinery are conserved through evolution, it can be hypothesized that some modification networks may be specific to individual organisms, resulting in different gene expression outcomes depending on the species. By utilizing multiple distinct mechanisms to modify and control the transcriptional machinery, organisms have evolved much greater potential for directing diverse expression profiles by a finite number of transcription factors. Taking advantage of multiple covalent modifications of transcription factors organisms have effectively gained the ability to utilize the same regulatory factor in different ways and thus expand their range of gene expression patterns. Regulation by modification not only enhances the functional potential of each individual transcription factor but also provides an effective means of greatly amplifying the functional plasticity of the transcriptional machinery required for combinatorial diversity. This quantum increase in the repertoire of regulatory events ultimately provides the rich tapestry of molecular interactions necessary to direct the diverse arrays of gene expression programs that define complex organisms.

Another important function of epigenetics is cellular memory. The identity of a cell is defined by its characteristic pattern of gene expression and silencing, so remembering who it is consists of maintaining that pattern of gene expression through the traumas of DNA replication, chromatin assembly, and the radical DNA repackaging that accompanies mitosis. Cytosine DNA methylation was the first epigenetic mark correctly identified and its inheritance mechanism at least superficially understood. The association of DNA methylation maintenance enzymatic activity with DNA replication foci guarantied the faithful copy of DNA methylation pattern in daughter cells. DNA methylation directs chromatin structure and gene transcription regulation, therefore set up the cellular memory. It has also been suggested recently that nucleosome and chromatin structure can transmit the epigenetic information from one cell generation to the next and has the potential to act as the cell's memory bank (Turner, 2002). This information storage function resides primarily in the amino-terminal tails of the four core histones, which are exposed on the nucleosome surface and are subject to a variety of enzyme-catalyzed, posttranslational modifications of selected amino acids, including lysine acetylation, lysine and arginine methylation, serine phosphorylation, and attachment of the small peptide ubiquitin (Spotswood and Turner, 2002). These modifications are not just a means of reorganizing nucleosome structure, but provide a rich source of epigenetic information. It has been suggested that specific tail modifications, or combinations therefore, constitute a code that defines actual or potential transcriptional states (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Richards and Elgin, 2002; Spotswood and Turner, 2002). The code is set by histone modifying enzymes of defined specificity and read by nonhistone proteins that bind in a modification-sensitive manner. In order to realize its full information carrying potential, the code must use *combinations* of modifications. This requires not only proteins that can read such combined modifications, but also mechanisms by which they can be put in place and maintained. Recent papers have provided new insights into how specific combinations of tail modification might be generated and revealed mechanisms by which the modification of one residue can influence modifications of other residues. Moreover, histone modifications such as methylation and acetylation have the potential to self-perpetuate through binding of histone acetyltransferases and histone methyltransferases to specifically modified tail domains. Together with DNA methylation, the histone code can specify a stable transcriptional state and serve as an epigenetic code.

Epigenetic regulation might also play important roles in the evolution from non-vertebrate eukaryotes to vertebrates (Bird, 1995). It is generally considered that the accumulation of genes during the evolution of more-complex organisms has been gradual. But genome statistics suggest that genome content, in particular gene number, increased suddenly at two periods of macroevolutionary change (the origin of eukaryotes and the origin of vertebrates), but otherwise remained relatively constant. It was suggested that this jumping gap of the biological complexity might come from the evolutionary emergence of regulation mechanisms which reduce the transcriptional background noise coming from the increased genetic complexity. Noise reduction at the prokaryote/eukaryote boundary might be achieved by appearing of the nuclear envelope and histones, whereas DNA methylation serves as a novel noise-reduction mechanism in vertebrates.

Clinical epigenetics

In carcinogenesis, the epigenetic inactivation of genes is a driving force as important as the inactivation of genes by mutations. DNA methylation and chromatin structure abnormals are frequent in cancer cells and difficult to distinguish from mutations, leading to the burgeoning field of cancer epigenetics. Epigenetic transcriptional repression has been demonstrated in a wide variety of tumor types and occurs in tumor suppressor genes, cell-cycle genes, DNA repair genes and genes involved in invasion and metastasis. For many of these genes, it has been shown that their re-expression in tumor cells can lead to suppression of cell growth or altered sensitivity to existing anticancer therapies. The treatment of inherited diseases that affect the epigenetic state represents an interesting challenge. As compounds have been identified that can readily reverse epigenetic silencing (e.g. inhibitors for HDACs and DNA methyltransferases), there is increasing interest in epigenetic regulation of gene expression as a basis for new approaches to cancer treatment. Many of these compounds are small molecules that have pharmacological properties that enable easy delivery to tumors. This is in sharp contrast with the challenge of delivering gene therapy to reverse genetic inactivation silencing caused by gene mutation in tumors.

The crosstalk between these different mechanisms of epigenetic regulation (DNA methylation, histone methylation and acetylation, chromatin remodeling) is essential for the appropriate control of gene transcription. The different epigenetic layers engaging in this complex crosstalk means that drug development strategies must consider what are the crucial target(s) for an effective epigenetic drug and how these drugs might be used in combination. For instance, to date, DNMT inhibitors, such as 2'-deoxy-5-azacytidine, appear to be the most active compounds for inducing re-expression of epigenetically silenced genes in tumor cell models. However, HDAC inhibitors can increase levels of gene expression and have been shown to work together with DNMT inhibitors to induce gene re-expression.

1.1.2 DNA methylation

DNA methylation is one of the most direct modifications on the genome and occurs in bacteria, fungi, plants and animals with widely different pattern and roles. In vertebrates, DNA methylation plays center roles in transcription

regulation and chromatin structure remodeling. Thus 5-mC can be even considered to be the fifth base in eukaryotic genome.

DNA methylation and CpG islands

In humans, approximately 1% of DNA bases are modified postsynthetically by adding a methyl group to carbon-5 of the cytosine pyrimidine ring, predominantly at CpG dinucleotides. In mammalian cells, approximately 70% of the CpG residues are methylated, and are largely dispersed through the genome (in all classes of sequences: exons, introns and regulatory elements, as well as repetitive DNA, transposons and other foreign DNA) but some of them are enriched and located in transcribed regions and intergenic DNA. Those modifications are thought to be involved in the genome stability and silencing, which is similar as in bacterial to protect bacterial genome from invasion by foreign DNA. Therefore, it was suggested that the genome-wide silencing by DNA methylation serves as an important mechanism to reduce transcriptional noise that come from the increased genome and gene numbers in mammalian organisms (Bird, 1995). Exceptions to this generalization are CpG islands, which are predominantly methylation-free and protected from methylation by unknown mechanisms. CpG islands contain high densities of CpG dinucleotides and are found at the promoter regions of about 60% of human genes that are transcribed by RNA polymerase II. In distinct spatial and temporal conditions, CpG islands have regulated methylation and demethylation, which are important for regulation of silencing of the associated genes. Methylation-free CpG islands have a markedly open chromatin structure that is deficient in the linker histone H1 and contains nucleosomes enriched in acetylated forms of histones H3 and H4. On the other hand, densely methylated promoter regions associate with a compacted chromatin structure, and accompanying transcriptional silencing of the affiliated genes. As a mechanism for control of specific gene expression in adult tissues, DNA methylation at CpG islands appears to be mainly restricted to two small classes of genes: inactivation of the X chromosome in females is often associated with widespread methylation of CpG islands on the inactivated X chromosome; in imprinted genes (where either only the paternally or maternally inherited allele of the gene is expressed), inactivation of the non-expressed allele is associated with methylation within their promoters. However, in normal tissues, for the majority of genes associated with CpG islands, the island remains methylation free, regardless of whether the gene is expressed or not. But in various human diseases, such as cancer, the silencing of tumor suppressor genes by abnormal DNA hypermethylation plays an important role.

The extent of DNA methylation changes in an orchestrated way during mammalian development, starting with a wave of demethylation during cleavage, followed by genome-wide *de novo* methylation after implantation (Li, 2002). Demethylation is an active process that strips the male genome of methylation within hours of fertilization; by contrast, the maternal genome is only passively demethylated during subsequent cleavage divisions. The extent of methylation in the genome of the gastrulating embryo is high owing to *de novo* methylation, but it tends to decrease in specific tissues during differentiation. *De novo* methylation occurs rarely during normal postgastrulation development but can be seen frequently during the establishment of cell lines *in vitro* and in cancer.

Dynamic regulation of DNA methylation

Cytosine methylation occurs after DNA synthesis, by enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM). The enzymatic reaction is performed by a family of dedicated enzymes called DNA

methyltransferases (DNMTs). DNMT1 is the main enzyme in mammals, and is responsible for the post-replicate restoration of hemi-methylated sites to full methylation, referred to as maintenance methylation, whereas DNMT3A and DNMT3B are thought to be primarily involved in methylating new sites, a process called *de novo* methylation. Homozygous deletion of DNMT3A and 3B does not alter pre-existing methylation patterns in mouse ES cells, whereas homozygous deletion of DNMT1 causes a roughly 70% reduction of 5-mC content. Genetically inactivation of DNMT1 in mice showed that DNMT1 is required for proper embryonic development, imprinting and X-inactivation (Li et al., 1992, and Lei et al., 1996). DNMT3A and 3B are required for the wave of *de novo* methylation that occurs in the genome following embryo implantation, and establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells (Okano et al., 1999, and Chen et al., 2003). Although numerous putative DNA demethylases have been suggested, none of them have been unambiguously identified yet and whether the DNA demethylation is passive or active process is still debating.

DNA methylation and gene transcription regulation

DNA methylation regulates gene transcription in at least two ways. First, it can directly interfere with the binding of transcription factors, such as c-Myc/Myn, AP-2, E2F and ATF/CREB-like proteins. But this mechanism requires that a CpG dinucleotide presents within the binding site, a criterion clearly not fulfilled by most transcription factors. Also, there are some exceptions in which methylation cannot block the binding of the transcription factors (e.g. Sp1). The second mechanism was evolved after identification of methyl-CpG binding proteins and complexes whose binding are methylation-dependent but not sequence-dependent. Such proteins may compete with transcription factors for their binding sites in methylated DNA or reorganize DNA into tightly packed chromatin structures incompatible with transcription. Four of these proteins, MeCP2, MBD1, MBD2 and MBD4, have related DNA binding domains. A fifth unrelated protein, Kaiso, requires two symmetrically methylated CpGs for binding. Four of these five proteins can repress transcription from methylated promoters in model experiments (the exception is MBD4 which is a DNA repair protein). At least three members of this family (MeCP2, MBD2, and MBD4) have been shown to associate with large protein complexes containing histone deacetylases (HDAC-1 and HDAC-2) and chromatin-remodeling activities (Sin3A and Mi-2). Thus they may mediate transcriptional silencing by deacetylating histones in the vicinity of the promoter, which directs the formation of stable repressive chromatin structures. It has also been suggested that DNA methylation could lead to gene silencing by MBD proteins that recruit histone methylases (HMTs). Gene repression would occur by methylation of the Lys9 residue of histone H3 and the subsequent recruitment of the chromatin silencer HP1, a family of heterochromatic adaptor molecules implicated in both gene silencing and supra-nucleosomal chromatin structure. Studies utilizing a variety of different systems have shown that repression of transcription from some promoters appears to rely more on the former mechanism while others have indicated a more prominent role of the latter mechanism. The exact mode of transcriptional repression *in vivo* most likely results from a combination of these two mechanisms and is also dependent on the CpG density and regulatory element composition of the specific promoters.

DNA methylation and chromatin modifications

Recent studies suggested that DNA methylation pattern may dictate histone acetylation pattern or conversely: purification of a DNMT1 complex that contains the retinoblastoma (Rb) protein, E2F1 and HDAC-1; and yeast two-

hybrid experiments that show that DNMT1 can form a complex with HDAC-2 and the co-repressor proteins DMAP1 and tumor susceptibility gene 101 (TSG101). It has been suggested that loss of function of Rb, a frequent event in several types of tumors, might result in improper regulation of this complex, which results in mislocalization of DNMT1 and the production of aberrant methylation patterns. Moreover, DNMT3a and DNMT3b also recruit HDACs to silence genes, using their PHD-like motif (Fuks et al., 2001; and Bachman et al., 2001). The association of DNMTs with chromatin modification processes is not restricted to HDACs. The observation that mammalian DNMT3a colocalizes with HP1, which binds to methylated histones, suggested a close link between DNMTs and methylated histones. An important implication of these results is that, if changes in histones can indeed influence DNA methylation, then it is possible that the methylation of genes observed in tumors is a result of transcriptional silencing owing to histone modification or chromatin remodeling, rather than of DNMT activity *per se*. On the other hand, those protein-protein interactions might be used to target DNMTs and subsequently DNA methylation to particular genomic regions.

Although many observations argue that DNA methylation is a key signal leading to histone modifications, chromatin remodeling and gene silencing, this signaling can also operate in the opposite direction. For example, disruption of histone methylation in *Neurospora crassa* results in the elimination of DNA methylation (Tamaru and Selker, 2001). It also has been shown to be essential for DNA methylation in *Arabidopsis thaliana* (Jackson et al., 2002). Similarly, increased histone acetylation in cells treated with HDAC inhibitors can also lead to demethylation of DNA.

DNA methylation and carcinogenesis

Since DNA methylation plays an important role in regulating the translation of the genetic information, it is not surprising that defects in the DNA methylation machinery and aberrant DNA methylation pattern have been shown to be involved in human disease and aging. For example, mutations in the *de novo* DNA methyltransferase DNMT3B result in reduced methylation of pericentromeric DNA sequences and cause the ICF (for immunodeficiency, centromeric instability and facial anomalies) syndrome, which shows immunodeficiency, facial abnormalities, mental retardation, and instability of pericentromeric heterchromatin. Recently a numbers of publications showed that Rett syndrome, a relatively frequent form of mental retardation occurring sporadically once every 10,000-20,000 female births, is caused by mutations in MeCP2. Moreover, aberrant DNA methylation has been found as a general event in various human cancers. Moreover, DNA methylation changes have been reported to occur early in carcinogenesis and therefore are potentially good early indicators of existing disease (Larid, 2003).

Cancer-specific DNA methylation changes at individual gene loci have so far focused primarily on hypermethylation of CpG islands and are known to contribute to gene silencing. Pioneering work by Bird and colleagues in the early 1990s (Tate and Bird, 1993) showed that inactivation of gene expression in cancer cell lines was frequently associated with hypermethylation of CpG islands. Furthermore, the abnormal hypermethylation of CpG islands in some tumor suppressor genes (e.g. *BRCA1*, *hMLH1*, *p16INK4a*, *APC*, *VHL*) have been shown in various human tumors (from review see Esteller 2003; Patel et al., 2003; Szyf, 2003), which causes their inactivation. Reactivation of these silenced tumor suppressors by relief of DNA hypermethylation can reverse the tumorgenic phenotypes in tumor cell lines. For example, antisense oligonucleotides directed against the *DNMT1* mRNA have been shown to reduce DNMT1 protein

levels and induce DNA demethylation and reexpression of the *p16* tumor suppressor gene in human tumor cells, and also inhibit tumor growth in mouse models. This DNMT1 antisense molecule has also been used in phase I and II clinical trials.

1.1.3 Chromatin structure

In eukaryotic cells genomic DNA is packaged in chromatin and higher order chromosome structures, which have a major impact on all nucleus processes involving the DNA substrates and provide multi-level of epigenetic regulation. In principle, all reactions that involved DNA can be regulated by altering DNA packaging and hence DNA accessibility. More importantly, chromatin structures in either distinct loci or global chromosomes are developmentally regulated in mammalian nucleus. The dynamic remodeling and accurate replication of chromatin status, therefore, play a critical role in the epigenetic regulation of eukaryotic genomes.

Chromatin regulation operates at both local and global levels. Local effects often occur at the level of single genes. Global effects, however, can involve changes in the properties of enormous chromosomal domains or even entire chromosomes ultimately. Both types of regulation act at the level of the nucleosomes which are the fundamental unit of chromosome folding in eukaryotes. For example, gene expression may be affected by the positioning of individual nucleosomes relative to regulatory elements, by the dynamic packaging of local chromatin structure, or by the folding of the compartmentalization of functional domains within the nucleus. The chromatin structure provides an essential, but not the only, level of regulation for regulating gene expression in that while regulating chromatin is necessary for transcription regulation, it is not sufficient since the functions of sequence-specific activators and repressors, mediator complexes, and general transcription factors are also required to ensure proper regulation.

Higher order chromatin structures

Global regulation of chromosome domains appears to involve the assembly of higher order supernucleosomal structures that control DNA accessibility. Cytogenetically, chromosomes are composed of two types of domains, euchromatin and heterochromatin. While heterochromatin is cytogenetically defined as the portions of chromatin that remain deeply stained and highly condensed during cell division, euchromatic domains define more accessible and transcriptional active portions of the genome. Heterochromatic domains are in general inaccessible to DNA binding factors and are transcriptionally silent. Large blocks of heterochromatin surround functional chromosome structures such as centromeres and telomeres, whereas smaller heterochromatic domains are interspersed throughout the chromosome. The extent of heterochromatin of specific regions may differ in different individuals or tissue types responsible for chromatin remodeling. After several decades of speculation, it has recently become clear that the packaging of DNA into heterochromatin exerts epigenetic control over important biological processes. For example, heterchromatin plays a crucial role in centromere function and is involved in genome stability and aging. Heterochromatin proteins are associated with DNA repeats that surround centromeres and are required for proper sister-chromatid cohesion and chromosome segregation. Heterochromatin also stabilizes repetitive DNA sequences at centromeres, telomeres, and elsewhere in the genome by inhibiting recombination between homologous repeats.

Moreover, it is suggested that heterochromatin recruits ORC (Origin Recognition Complex) to facilitate replication of hard-to-replicate heterochromatic regions. In addition to its role in the maintenance of genome stability, heterochromatin plays an important role in the regulation of gene expression during development and cellular differentiation. Heterochromatin-like structures are involved in the stable inactivation of developmental regulators such as the homeotic gene clusters in *Drosophila* and mammals, and the mating-type genes in fungi. Dosage compensation in female mammals involves the heterochromatic inactivation of one of the two X chromosomes in somatic cells. Several properties of heterochromatin make it particularly suitable for processes that require the stable maintenance of expression states over long periods. First, the heterochromatic state is epigenetically and stably inherited through many cell divisions, which may take place under different developmental conditions and environmental inputs. Second, the mechanisms of assembly of heterochromatin and the spreading of heterochromatin from nucleation sites to surrounding DNA regions allow a precise transition from sequence-specific genetic control to sequence-independent epigenetic control.

Heterochromatic structures can be nucleated by specific cis-acting sequences, called silencers, which are recognized by DNA binding proteins (Fig.1.1.1). Alternatively, repetitive DNA elements such as transposons in the genome are believed to serve as signals for heterochromatin formation. Transcripts generated by repetitive DNA are processed into snRNAs by a mechanism requiring components of the RNAi machinery such as RNA-dependent RNA polymerase (RdRP), Dicer, and Argonaute proteins. Then heterochromatin assembly spreads to nearby sequences in a manner that requires the physical coupling of histone-modifying activities and structural proteins such as Sir3, Sir4, and Swi6/HP1. On the other side, to protect active regions from the repressive effects of nearby heterochromatin, specialized DNA elements known as boundary elements have been shown to mark the borders between adjacent chromatin domains and to serve as barriers against the effects of silencers and enhancers from the neighboring regions. Studies in organisms ranging from yeast to mammals suggest strongly that histones and their posttranslational modifications play a pivotal role in the assembly of heterochromatin. In nearly all organisms, the heterochromatic state associates with hypoacetylation of histones. In addition to histone hypoacetylation, in fission yeast, *Drosophila*, and mammals methylation of histone H3 lysine 9 correlates with heterochromatin assembly. DNA methylation is also believed to contribute to the stability of silenced chromatin states in higher eukaryotes with complex genomes. Evidences from plants and fungi suggest the existence of feedback mechanisms between DNA and histone methylation, such that one promotes maintenance of the other. The interdependence of these epigenetic markers suggests that DNA methylation and chromatin-mediated epigenetic mechanisms act in concert to maintain a silenced chromatin state.

Nucleosomes and local chromatin remodeling

Since the discovery of the basic principles of chromatin organization, which involve the wrapping of DNA around histone octamers to form nucleosomes and the folding of the nucleosomal fiber into higher-order structures (Woodcock and Dimitrov, 2001), the question of how such extensive packaging can be compatible with reactions that involve 'reading' the DNA has stimulated extensive research. It soon became apparent that many aspects of chromatin structure could be explained by interactions between nucleosomal histones, DNA, neighboring nucleosomes and non-histone proteins. The solving of the crystal structure of a single nucleosome (Fig.1.1.2) suggested the N-terminal tails

of the core histones, which reach out from the rather compact nucleosomal core particle, are involved in these interactions and play important roles in the regulation of chromatin structure and functions. The tails are subject to

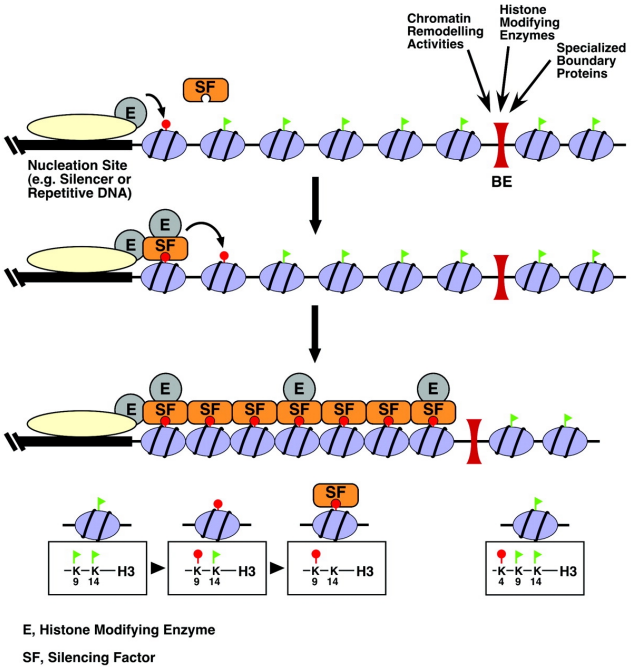


Fig. 1.1.1.Model for formation of silenced chromatin domains.

After the recruitment to a specific heterochromatin nucleation site by proteins that directly bind DNA or are targeted by way of RNAs, histone-modifying enzymes (E) such as deacetylases and methyltransferases modify histone tails to create a binding site for silencing factors (SF). After this nucleation step, self-association of silencing factors (such as Swi6/HP1 or Sir3) is hypothesized to provide an interface for their interaction with histone-modifying enzymes, which then modify adjacent histones, creating another binding site for silencing factors. Sequential rounds of modification and binding result in the stepwise spreading of silencing complexes along nucleosomal DNA for several kilobases (spreading). Spreading of silencing complexes is blocked by the presence of boundary elements (BE). The modifications associated with the amino terminus of histone H3 in fission yeast heterochromatin (bottom left) and euchromatin (bottom right) are illustrated as an example. Deacetylation and methylation of H3 Lys9are followed by deacetylation of H3 Lys14 and create a binding site for the Swi6 silencing factor. From Grewal and Moazed, 2003.

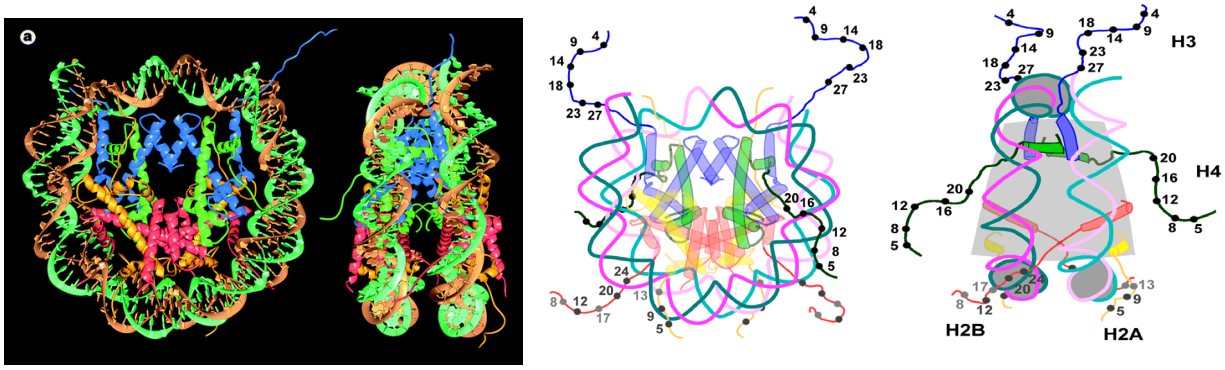


Fig .1.1.2 picture of a nucleosome. From Luger et al., 1997.

several of enzyme-catalyzed, posttranslational modifications of selected amino acids, including lysine acetylation, lysine and arginine methylation, serine phosphorylation, and attachment of the small peptide ubiquitin. These modifications provide a rich source of epigenetic information and their specific pattern and combinations have been proposed to constitute a "histone code" which defines transcriptional status. (see details in next chapter).

Chromatin-modifying complexes

"The structure determines the function". As a complex system, the basic units of the biology organisms are macromolecules which contain huge amount of flexible information. For example, proteins, themselves, have very complicated structures: primary, secondary, etc. All of them provide the basic structure for the activity of the protein such as enzymatic activity, binding activity, interaction activities. To achieve the flexible regulation, reversible covalent modifications on macromolecules play the central roles.

Alterations of chromatin structure can be achieved by two main mechanisms: the post-translational modification of histones in their amino-terminal tails by acetylation, phosphorylation, methylation and ubiquitination; or remodeling of nucleosomes via the ATP-dependent chromatin remodeling complexes such as Swi/Snf. Posttranslational histone modifications may affect chromatin structure by altering histone–DNA interaction. The modifications (or combinations of modifications) may also act as binding platforms/recognition motifs for interaction with other proteins that may provide further enzyme activity or recruit additional regulatory proteins, as proposed by the "histone code" hypothesis. So far, two types of specific protein domains, the bromodomain that binds to acetyl-lysine and the chromodomain that binds to methyl-lysine have been identified.

These various chromatin-modifying complexes can be classified based on their substrates. The enzymes which modify histone tails will be mainly discussed in this review. More and more biochemical evidences indicate that different chromatin modifications together with DNA methylation are either mutually reinforcing or mutually inhibitory. The resulting feedback loops may function to ensure functional polarization of chromatin domains, stably committing them to either transcriptional activation or silencing. Similar as a chemical equivalent, the intermediate state of the chromatin, which is uncommitted to neither active nor repression, is very unstable and has a very short life. The repression or active state is relative stable and has strong buffer capacity, which comes from the complex cooperations between different regulation mechanisms, against the alteration of environmental noise. But once the environment input is stronger than the energy bury, the active or repression state can be quickly shifted and kept at relative stable state again (Fig.1.1. 3).

ATP-dependent remodeling complexes

In eukaryotic cells, every reactions involving DNA need the opening of the chromatin structure and unveil the genomic DNA from the nucleosome octamers. Therefore different enzymes and binding factors are able to contact their substrate. ATP-dependent remodeling complexes use ATP hydrolysis to increase the accessibility of nucleosomal DNA, which is also a fundamental requirement for several steps in transcription. These complexes can be divided into three main classes based on the identity of their catalytic ATPase subunit. These ATPase subunits display homology

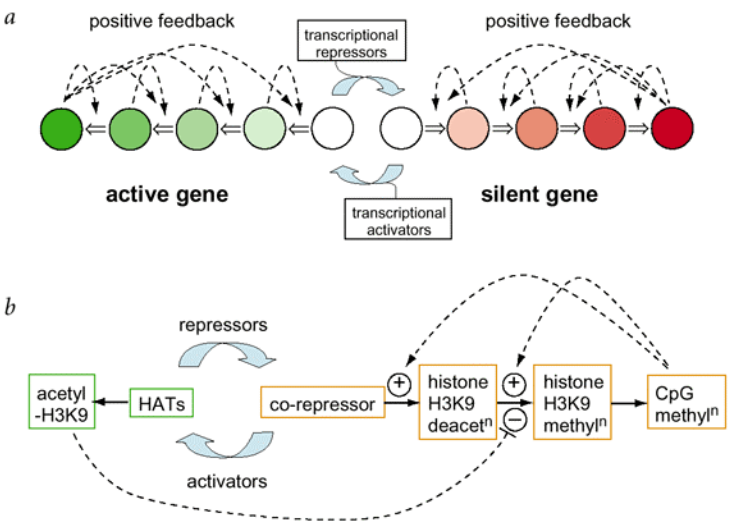


Fig.1.1.3 Polarization of active and silent chromatin states owing to feedback loops between intermediate states

A, Different chromatin states. Each circle represents a particular DNA or histone modification status (examples are shown in *b*). The effect of positive feedback is to polarize domains at either the active or the inactive extremity. Broken lines indicate positive feedback loops between products and reactions that interconvert intermediate states. Negative interactions between the silent and active states of the gene occur (see *b*) but are not shown. The likelihood that a chromatin domain will occupy a particular state is indicated by the color intensity, with dark shades representing more probable states. *B*, Potential examples of the polarization scheme shown in *A*. Both positive feedback of CpG methylation on Lys9 of histone H3 (H3K9) mediated by MeCP2 (ref. 185) and stimulation of histone deacetylation by CpG methylation through methyl-CpG binding proteins have both been reported. HATs, histone acetyltransferases. From Jaenisch and Bird, 2003

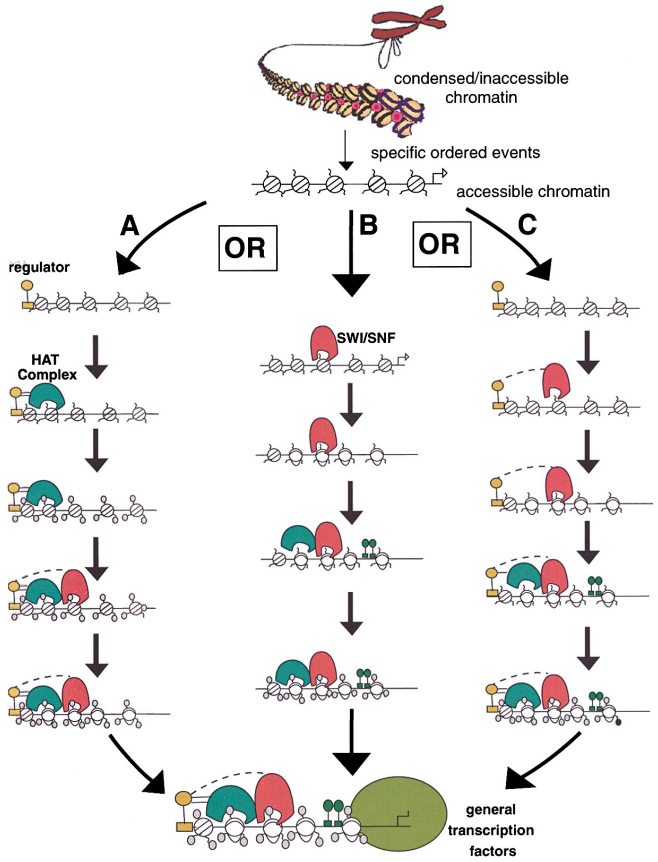


Fig.1.1.4. Models Depicting Different Orders of Action by Regulators and Chromatin-Remodeling Complexes.

Regulators, HAT complexes, and ATP-dependent remodeling complexes can act in different orders (pathway A, B, or C) and still give the same end result: a template competent for transcription. Although not shown, it is also possible that binding by the general transcription factors precedes the action and recruitment of HAT complexes and ATP-dependent remodelers.

only within the ATPase domain and contain different additional domains. The central ATPase subunits are able to alter chromatin structure in the absence of the remaining subunits (Kingston and Narlikar, 1999; Langst and Becker, 2001; Wang and Zhang, 2001). The contributions of each other components for the activity and specificity of *in vivo* whole complexes are extensively analyzed by biochemical and genetic methods and are still not well known. It has been suggested that the additional subunits in the SWI/SNF and Mi-2 based complexes might be involved in modulation of substrate binding and/or enzymatic activity.

Using a variety of different assays, ATP-dependent remodeling complexes have been shown to have different activities such as: creation of altered DNase I cleavage pattern; altered restriction enzyme access; creation of remodeled dinucleosome species; histone octamer transfer; creation of altered translational positions of histone octamer; assembly and spacing of nucleosomes; generation of cruciforms from inverted repeats of DNA; generation of topological changes in closed circular arrays (Narlikar et al., 2002). Different ATP-dependent remodeling complexes show similarity and differences in certain kinds of activities. For example, it is supposed that SWI/SNF and ISWI-based complexes might expose nucleosomal DNA by different mechanisms.

In vivo, at the simplest level, all ATP-dependent remodeling complexes help regulate transcription by regulating the accessment of nucleosomal DNA to various transcription factors. However, the challenges of exposing DNA differ according to local nucleosome organization. At promoters where multiple closely spaced nucleosomes occlude different factor binding sites, DNA has to be made accessible at several sites without significantly altering the transitional position of the histone octamers. This is different from the task of creating DNA access in regions of low nucleosome density, where there is sufficient space for moving the nucleosomes and DNA can be exposed by creating nucleosome-free regions. Exposing DNA from densely packed nucleosomes also differs from the essential task, often the responsibility of ATP-dependent remodelers, of creating appropriately spaced nucleosomes throughout the genome following replication. In theory ATP-dependent remodeling can be accomplished by sliding mechanisms, or instead by mechanisms that do not require transitional movement of the histone octamer. It appears that nature has evolved different classes of ATP-dependent remodelers that use different mechanisms, each suited to a specific biological task. For example, the proposed ability of SWI/SNF-based complexes to expose nucleosomal DNA without requiring sliding of the histone octamer would allow it to function at promoters, such as the mouse mammary tumor virus promoter (Deroo and Archer, 2001), which have closely spaced nucleosomes. On the other hand, the ability to transitionally reposition nucleosomes would allow ISWI-based complexes to establish specifically spaced nucleosomal structures at various other promoters. Finally, the coming genetic loss-of-function and gain-of-function models in cells and whole animals will provide more detailed *in vivo* functions.

Cooperative regulation by different chromatin regulatory complexes

ATP-dependent remodeling complexes and histone modifiers not only can individually contribute to transcription by increasing transcription factor access to DNA but also can kinetically and/or thermodynamically modulate each other's activities in the context of activation or repression on different genes. For example, to remodel and open the local chromatin structure need both ATP-dependent remodeling and histone-modifying complexes work together. ATP-dependent remodeling complexes use energy to modify chromatin structure in a noncovalent manner, while histone-

modifying complexes add or remove covalent modifications from histone tails. Analogous to the effects of sequence-specific activators, these two types of complexes could help each other in multiple ways. Direct physical interactions between the ATP-dependent remodelers and chromatin modifiers could increase their affinity for the chromatin template. Such direct interactions could also affect the activities of each complex. Moreover, alteration of the chromatin template by one complex could make it a better substrate for the other complex.

Even for a simple eukaryotic gene, during the transcription regulation, distinct multiprotein complexes are needed to modulate higher-order chromatin structure, to bind to promoters, to bind to enhancers, to communicate between activators/repressors and sites of transcription initiation, to modify nucleosomal structure, and to generate transcripts. A major challenge is to determine how all of these complexes work together to ensure proper regulation. *In vivo*, to achieve the tightly regulated gene expression, chromatin-modifying and –remodeling complexes must interact with other components of the transcription machinery in a spatially and temporally coordinated manner (Fig.1.1.4). Genes that start in a highly condensed state are expected to require chromatin decondensation early in the process of regulation. When transcription of a gene is altered, a specific event, frequently the binding of a gene-specific activator or repressor to accessible chromatin, triggers a cascade of reactions. These reactions result in a appropriately remodeled chromatin template , which is bound by regulatory factors and the general transcription machinery. There is no a priori requirement that ATP-dependent remodeling, covalent modification of histones, or binding by regulatory factors and the transcription machinery occur in any specific order; the sole requirement is that the appropriate end stage, e.g., a properly structured template with a functional preinitiation complex poised for transcription, need to be attained in a timely manner. While the functional interactions between the numerous complexes that regulate transcription require further intense study, the emerging picture suggests that these complexes are able to act in many different orders and can assist each other's function. Thus, multiple pathways are available for regulation, allowing multiple options to be considered during the evolution of a specific pathway for regulation of an individual gene.

1.2 Histone post-translational modifications and epigenetic code

1.2.1. Histone code

In eukaryotes, genomic DNA is assembled with histones to form the nucleosomes, in which 147 base pairs of DNA are wrapped around an octameric complex composed of two molecules of each of the four histones H2A, H2B, H3, and H4. Structural analysis of this histone octamer showed that it is made up of a tetramer of $(H3/H4)_2$ with a heterodimer of H2A and H2B bound to either side forming a "tuna-can"-like structure with a diameter of 6.6nm and a height of 5.6 nm (Arents et al., 1991). Crystallographic analysis of the nucleosomal particle has been resolved to a resolution of 2.8A by luger and coworkers (Luger et al., 1999 and Fig.1.1.2) and has shown that DNA is wrapped around the histone octamer with 1.75 turns of a left-handed superhelix. The inner core of the nucleosomal structure is made up of only 75% of the protein mass with the remaining N-termini protruding out of the two DNA gyres, which are therefore accessible for protein-protein interactions. They form contacts with adjacent nucleosomes and can act as platform for nonhistone proteins such as HP1 or NuRD, which may influence chromatin compaction. Although the histone amino-terminal

domains are not needed to maintain the structural integrity of the nucleosome, they have roles in higher-order chromatin structure and in interactions with nonhistone chromosomal proteins. For example, the H3 and H4 tails are dominant players in chromatin fiber folding and intermolecular fiber-fiber interaction. Acetylation of these tails disrupts folding of the fiber and interactions with nonhistone chromosomal proteins. Genetic and biochemical studies also showed the importance of the histone tails in chromatin structure and gene expression. Although tailless histones are able to form nucleosomes in vitro, the N-termini of histones H3 and H4 were shown to be essential for repression of the silent mating type loci in yeast (Kayne et al., 1988; Thompson et al., 1994). Enhancer-dependent activation of other yeast genes also required these N-terminal sequences (Durrin et al., 1991; Mann and Grunstein, 1992). A simultaneous deletion of the H3 and H4 N-terminal tail in yeast is lethal (Ling et al., 1996). More detailed point mutation analysis showed that certain point mutations in the N-terminus of the H4 tail could inhibit transcriptional silencing at telomeric heterochromatin, as well as the silent mating type (Hecht et al., 1995).

Although histones are some of the most evolutionarily conserved proteins, they are also among the most variable in terms of posttranslational modifications (Fig.1.2.1). The N-terminal tails of histones are heavily posttranslationally modified and house the sites for acetylation, methylation, phosphorylation, glycosylation, and ADP-ribosylation whereas ubiquitination occurs on the carboxy-terminal region of H2A and H2B. Some of the modified isoforms are generated immediately after translation, such as histone H4 acetylated on lysine 5 and 12, whereas others occur only later in response to specific external signals, such as phosphorylation and acetylation within the H3 tail. As our understanding of the higher-order structures increases, it becomes apparent that modifications of the tails are key to the modulation of overall chromatin structure, thereby contributing to a tight regulation of gene expression. The correlation between distinct modification patterns and physiological functions raised the concept of "histone code", which posits that the totality of modifications, both in kind and number, dictates a particular biological outcome. Moreover, the spatial and temporal arrangement of those modification events provides the specificity and complexity of a code. Thus, specific and interlinked modifications may dictate specific genomic states, such as gene activation, repression, DNA repair, recombination, chromosome segregation, and so on. In particular, histone H3 appears to be critical: known markers occur at Lys-4 (methylation), Lys-9 (methylation), Ser-10 (phosphorylation), Lys-14 (acetylation) and Arg-17 (methylation). In fact, around K9/S10/K14 in histone H3 there appear to be specific patterns for inactivity and activity. An inactive state is characterized by histone deacetylation at Lys-14, which precedes methylation at Lys-9. The enzymes that carry out these modifications are genetically linked in *Schizosaccharomyces pombe*, and deacetylation at Lys-14 precedes methylation at Lys-9. In contrast, acetylation at Lys-14 is preceded by, and dependent upon, phosphorylation at Ser-10. This has been shown *in vitro* for the Gcn5 acetyltransferase, and *in vivo* the Snf1 histone kinase and Gcn5 are a linked pair of enzymes that operate in this sequence. In addition, another transcriptional `on' state within histone H4 consists of methylation at Arg-3 preceding and promoting p300-mediated acetylation at Lys-8 and Lys-12. The fact that histone modifications not only affect each other on the same histone in a *cis* mechanism (Cheung et al., 2000; Clayton et al., 2000; Lo et al., 2000; Nakayama and Takami 2001; Rea et al., 2000), but recent results also demonstrate *trans* effects between histones, further provides the stability for "histone code". Specifically, ubiquitination of histone H2B enhances methylation of histone H3 and is involved in gene silencing at telomeres (Sun and Allis, 2002). It appears that acetylation is used in conjunction with phosphorylation in both histone and transcription factor substrates (e.g. p53). In both cases, phosphorylation appears to occur prior to, and appears to promote, acetylation. In the case of histones, both *in vitro* and *in vivo* experiments showed phosphorylation of histone H3 at serine 10 results in a higher level of

enzymatic activity by the Gcn5 HAT on the neighboring lysine 14. This may result from stabilization of binding to the phosphorylated substrate.

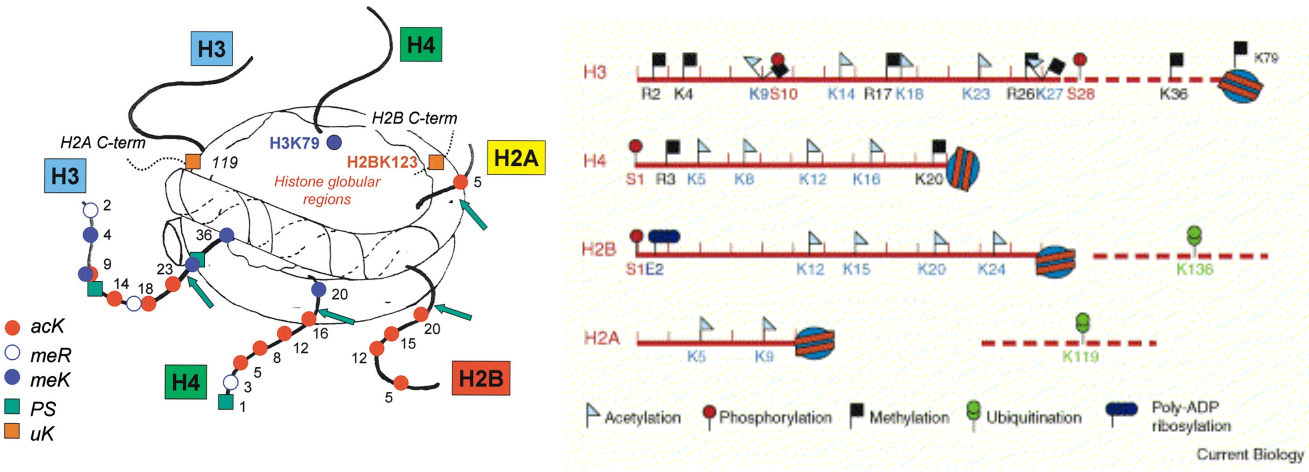


Figure 1.2.1. Histone Modifications on the Nucleosome Core Particle

A: The nucleosome core particle showing 6 of the 8 core histone N-terminal tail domains and 2 C-terminal tails. Sites of posttranslational modification are indicated by colored symbols that are defined in the key (lower left); acK, acetyl lysine; meR, methyl arginine; meK, methyl lysine; PS, phosphoryl serine; and uK, ubiquitinated lysine. Residue numbers are shown for each modification. Note that H3 lysine 9 can be *either* acetylated or methylated. The C-terminal tail domains of one H2A molecule and one H2B molecule are shown (dashed lines) with sites of ubiquitination at H2A lysine 119 (most common in mammals) and H2B lysine 123 (most common in yeast). Modifications are shown on only one of the two copies of histones H3 and H4 and only one tail is shown for H2A and H2B. Sites marked by green arrows are susceptible to cutting by trypsin in intact nucleosomes. Note that the cartoon is a compendium of data from various organisms, some of which may lack particular modifications (e.g., there is no H3meK9 in *S. cerevisiae*). Adapted from Spotswood and Turner (2002). *B*: Amino-terminal tails are depicted as solid red lines; carboxy-terminal tails of H2A and H2B, as well as the part of the H3 tail located inside the DNA, are drawn as dotted red lines. From Imhof, 2003,

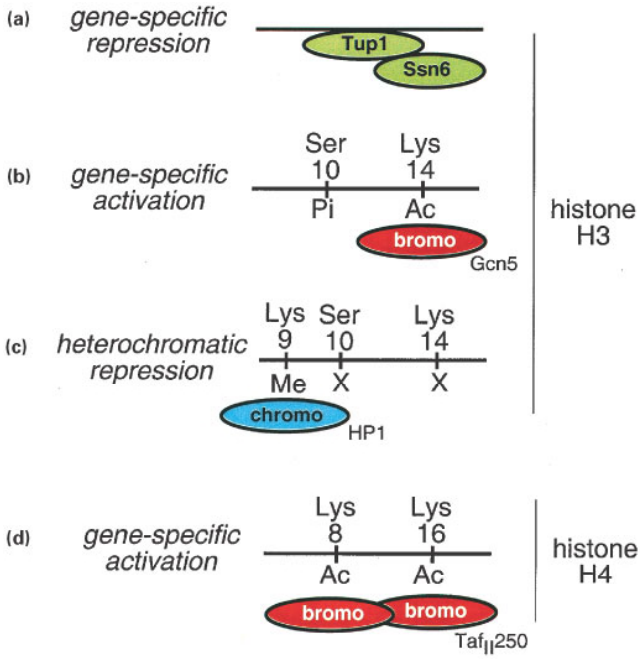


Fig 1.2.2. Interactions of specific proteins with modified histone tails. *A:* The heterodimeric repressor Tup1/Ssn6 binds to the unmodified histone H3 tail to repress specific genes. *B:* The bromodomain of the Gcn5 acetyltransferase binds with high affinity to histone H3 acetylated at lysine 14 to induce specific genes. *C:* The chromodomain of the heterochromatic protein HP1 binds with high affinity to histone H3 methylated at lysine 9 to silence transcription. *D:* The double bromodomains of $Taf_{II}250$ binds with high affinity to doubly acetylated lysine 8/16 within the histone H4 tail.

The function of histone code may be to cause specific changes in activity at the affected loci (see details in later chapters). These changes could be direct physical alteration of histone-DNA contacts, or within a higher order structure. A second possibility is that the modifications create altered surfaces on nucleosomes for interaction with effector proteins that are the actual agents of altered activity. Examples include the bromodomain, present in HATs, which has been shown to specifically interact with acetylated lysine. The second example is the chromodomain, present in numerous histone methyltransferases and other proteins, which in some cases binds to the Lys-9-methylated histone H3 tail. Suvar3-9 methylates Lys-9 during gene silencing, and this leads to increased association of the chromodomain-containing protein HP1, long known to be important in heterochromatic silencing. These data predict the existence of other domains for interaction with the other known, and yet uncharacterized, histone modifications. So, as a heritable code, the modifications on the histone tails not only passively reflect but also can actively influence the distinct genomic and physiological status by the "code reading" system. In other words, the code reader and code maker form a positive feedback to ensure the faithful regulation and memory.

1.2.2 Histone acetylation/deacetylation and gene transcription regulation

The acetylation of histones occurs at the ε-amino group of specific lysines within the N-terminus and is one of the predominant modifications on histone tails. Since Vincent Allfrey and co-workers observed a link between reversible acetylation of lysine residues within the N-terminal domains of the core histones and RNA synthesis some fourty years ago (Allfrey et al., 1964), accumulated literatures support a general model in which histone acetylation contributes to the formation of a transcriptionally competent environment by 'opening' chromatin and allowing general transcription factors to gain access to DNA template. As a consequence, the transcription machinery is able to access promoters and hence initiates transcription more frequently. In addition, the unfolding of chromosomal domains facilitates the process of transcription elongation itself. Conversely, histone deacetylation mainly contributes to a 'closed' chromatin state and transcriptional repression (Fig.1.2.3.). Condensed heterchromatin regions are generally poorly acetylated, whereas euchromatin regions containing transcriptionally poised or active genes are associated with acetylated histones. Chromatin loop domains containing poised--a transcriptionally inactivated gene that is in a DNase I sensitive domain--or expressed genes are sensitive to DNase I digestion, suggesting a decondensed chromatin structure. RNA polymerase II and I expressed and poised genes are bound to highly acetylated histones, with the highly acetylated histones associated with expressed ribosomal genes being in a non-nucleosomal form. Highly acetylated histones are not limited to the coding region, however, but are also found along the entire loop domain. By recently developed chromatin immunopreciption (CHIP) technique, the distribution of histone acetylation and its correlation with gene activity and chromatin structure were mapped more in details in yeast (Suka et al., 2001). An important conclusion from such studies in yeast was that the ground state of chromatin, if be transcribed or not, is characterized by intermediate levels of H3 and H4 acetylation, a state brought about by a mix of untargeted HAT and HDAC activities (Vogelauer et al., 2000). In this context, site-specific acetylation/deacetylation leads to locally restricted activation or repression of transcription, respectively. However, this overall level of flexibility appears to be a specific feature of the highly active yeast genome. In differentiated, higher eukaryotic cells, most of the genome consists of hypoacetylated, inactive chromatin, which may be considered the ‘ground state’. Activation of housekeeping and cell-type-specific genes

involves the acetylation of histones across broad chromatin domains. While broad acetylation of histone H3/H4 leads to partial decondensation of chromosomal domains, this opening is not tightly correlated with active transcription per se, but rather marks regions of transcriptional competence. It is known that a domain that has been rendered 'permissive' by broad acetylation will never be found close to repressive heterochromatic structures in nuclei (Schubeler et al., 2000). Nevertheless, the causal relationship between residence in euchromatin and histone acetylation has not yet been established whereas locally targeted histone acetylation/deacetylation of histones at promoter region correlates with transcription activation.

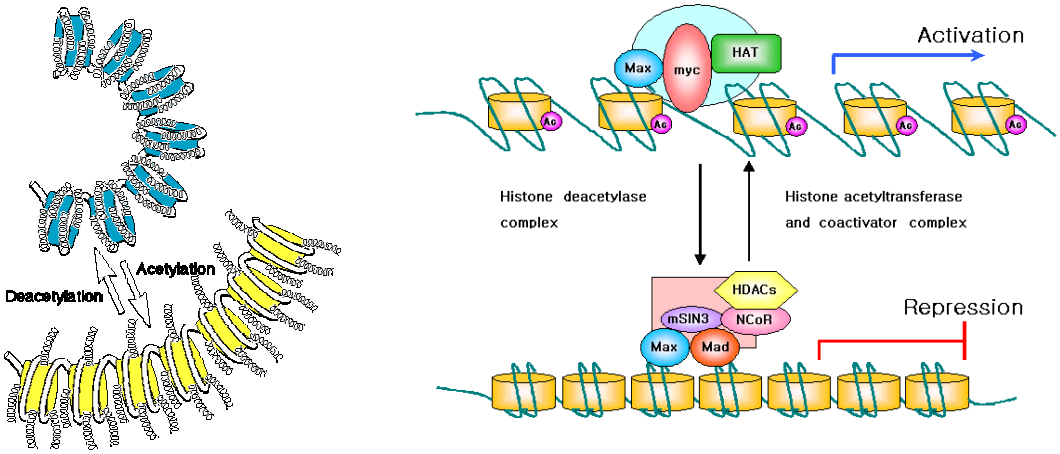


Fig.1.2.3. Histone acetylation/deacetylation is dynamically regulated by HATs and HDACs and associates with chromatin structure and gene transcription regulation.

The mechanisms by which histone acetylation regulates chromatin structure and thereby transcription has been analyzed in last 30 years. The "direct" model proposes that acetylation results in charge neutralization of conserved, often invariant, lysine residues located in N-terminal tails of the histones. Changes in the charge of the histone tails are hypothesized to weaken interactions between the histone tails and the negatively charged DNA backbone. However, the data on if whether is the cause for enhanced transcription factor binding on acetylated nucleosomes are still controversial. Moreover, since the study of mononucleosomes is of limited value as nucleosomes are usually embedded in a chromatin fiber *in vivo*, it is more physiological to consider the function of the tails within a nucleosomal array. In the second model, the hypothesis that acetylation could modulate the higher-order structure of chromatin has recently gained considerable attention. Acetylation can also alter histone:histone interactions between neighboring nucleosomes as well as interactions between histones and regulatory proteins. Any of these changes can affect the structure of individual nucleosomes as well as higher-order folding, leading to a more open and permissive chromatin environment for transcription. *In vitro*, histone acetylation has been shown to enhance the accessibility of DNA to restriction enzymes and transcription factors (Lee et al., 1993; Anderson et al., 2001; Sewack et al., 2001). Acetylation of histones H3 and H4 counteracts the tendency of nucleosomal fibers to fold into highly compact structures *in vitro* by disrupting internucleosomal interactions made via the histone tails (Tse et al., 1998). The third model, "indirect" model, which does not exclude the first two, is commonly known as the "histone code" and proposes that covalent modification of histones provides an epigenetic marker for gene expression. In the case of acetylation, this means that acetyl-lysines, normally together with other modifications, on histone tails which are exposed to the environment outside of the chromatin polymer, provide an attractive signaling platform that may mediate critical interactions with proteins or other complexes that themselves function to remodel chromatin. For example, specific acetylation patterns displayed by the histone tails

may also function to recruit further modulators of chromatin structure. These include other covalent modifications such as phosphorylation, as well as the rearrangement of histones/nucleosomes relative to the DNA by nucleosome remodeling complexes. Moreover, bromodomains, an extensive family of evolutionarily conserved protein modules originally found in proteins associated with chromatin and in nearly all nuclear HATs, have been recently discovered to function as acetyl-lysine binding domains. Such an interaction supports the hypothesis that bromodomains can contribute to highly specific histone acetylation by tethering transcriptional HATs to specific chromosomal sites, and to the assembly and activity of multi-protein complexes of chromatin remodeling such as SAGA and NuA4. Overall, the direct effect of acetylation per se on nucleosome structure appears rather modest (Wang et al., 2000). The dramatic changes in promoter structure that accompany transcriptional activation are, therefore, presumably not the direct result of acetylation, but due to the synergistic actions of several factors such as other covalent modifications and nucleosome remodeling factors.

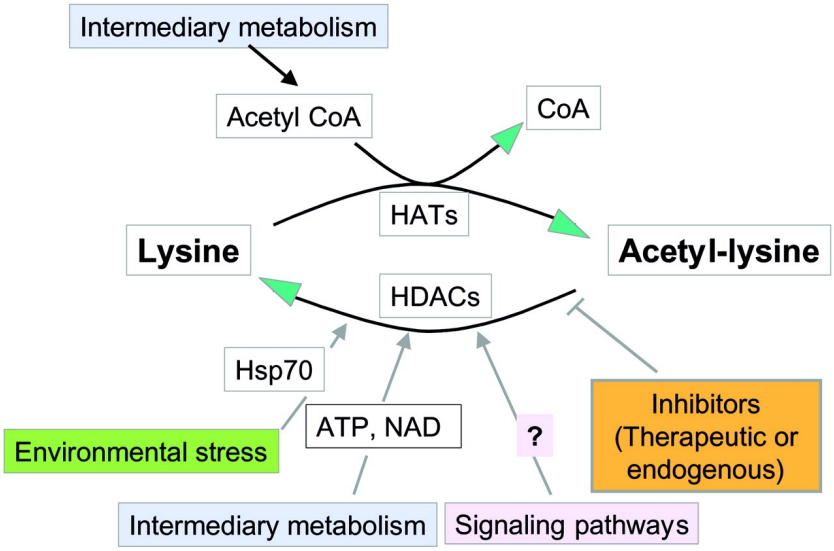


Figure 1.2.4. Environmental and metabolic factors that can influence patterns of histone acetylation.

The identification of the enzymes responsible for the addition and the removal of histone acetylation provides direct mechanistic insights into distinguishing whether change in histone acetylation is a cause or an effect of increased transcription. Genetic studies in combination with ChIP studies suggested that this steady-state level of acetylation is maintained by the opposing actions of histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes (Reid et al., 2000; Vogelauer et al., 2000). It is believed that targeting of HAT and HDAC complexes to promoter regions then creates specific patterns of hyper- and hypoacetylation in a background of global acetylation that correlate with transcription activation and repression, respectively. Indeed, ChIP studies using antibodies to specifically acetylated histone tails have shown that increased acetylation in promoter proximal regions of specific genes correlates with recruitment of HAT complexes and increased gene expression (Kuo et al., 2000). Hypoacetylation at specific promoters has analogously been correlated with recruitment of HDAC complexes to repressed genes (Khochbin et al., 2001). The discovery that certain HAT complexes contained general transcription factors and that certain HDAC complexes

contained known transcriptional repressors further strengthened the correlations between hyperacetylation and activation and between hypoacetylation and repression.

Besides the targeted site-specific histone acetylation, the function and regulation of global genome-wide acetylation are largely unknown. For example, recent measurements of bulk acetylation levels in yeast suggest that as many as 13 lysines per octamer are acetylated. Such high overall acetylation levels are difficult to reconcile with the targeting of HATs to a few specialized sites. Perhaps all HATs can function in both targeted and nontargeted manners. Alternatively, particular HATs may be dedicated to general (non-promoter-specific) acetylation events that govern more global patterns of acetylation. Although we may not yet understand the mechanisms involved, both targeted and nontargeted histone acetylation events appear important to gene regulation. Indeed, recent studies argue for a multistep pathway of gene activation, wherein general histone acetylation by nontargeted HATs occurs first, followed by promoter proximal hyperacetylation of specific residues in particular histones by targeted activities. On the other side, HDACs may also achieve transcription regulation through diverse local and genome-wide mechanisms. The achievement of substrate specificity of each HDAC is a very complex and multi-level regulation. *In vivo*, HDACs mediate gene-specific transcription repression when recruited to distinct genomic loci by a particular DNA-binding protein. Moreover, besides this targeted recruitment, HDACs also deacetylate large regions of chromatin, including promoters and coding regions that do not contain specific DNA-binding sites, in a process termed 'global deacetylation' (Kasten et al. 1997).

1.2.3. Histone methylation

The major methylation sites within histone tails are the basic amino side chains of lysine and arginine residues. Unlike acetylation, which is normally mono-covalent, methylated lysines can be found in mono-, di-, trimethylated forms. Arginines can be either mono- or dimethylated (which can be asymmetric or symmetric). *In vivo* studies have indicated that mammals have different ratios of methylated species of lysine and arginine, depending on the cell type or tissue source. The methylation status of a given histone tail, on a given gene, can change during the transcription activation or repression by recruiting methylases to promoters as coactivators. Unlike histone acetylation, histone methylation does not alter the overall charge of the nucleosome. Therefore, it is suggested histone methylation does it job mainly by serving as "markers" or binding sites that facilitate downstream events resulting in altered gene expression (Sims et al., 2003). Indeed, distinct histone lysine methylation has proven to `mark' chromatin for downstream events that result in the `activation' or `repression' of specific genes, and large chromosomal regions.

Histone arginine methylation is involved in gene activation and methylases are recruited to promoters as coactivators, whereas recent studies of the mechanisms of specific histone lysine methylation have revealed a complex process that controls aspects of short- and long-term transcriptional regulation, in addition to the propagation of bulk chromosome structure and stability. The discussion below will mainly focus on lysine methylation.

To date, there are five lysines within histone H3 (K4, K9, K27, K36 and K79) and one lysine within histone H4 (K20) that have been shown to be methylated by specific histone lysine methyltransferases (HKMTs) . Conceptually, it is useful

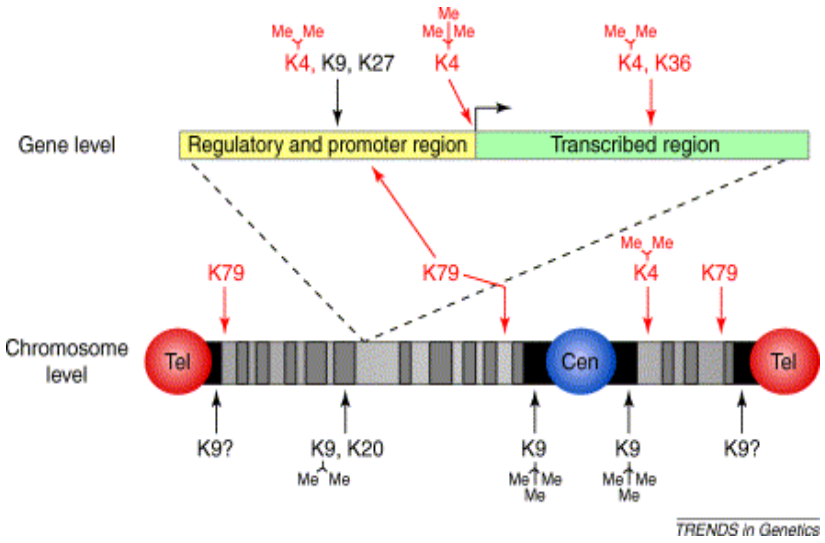


Figure1.2.5. The distinct regulatory levels of chromatin formed by histone lysine methylation.
The methylation of histone lysines known to regulate individual genes (i.e. `gene level') regulation is shown in the upper panel. The `chromosome level' designation represents methylation of various large chromatin domains and elements involved in basic chromosome structure. Methylation sites that function in either transcriptional activation or repression are shown in red and black, respectively; the bars represent di- or tri-methylation. The telomere (Tel) and centromere (Cen) are indicated. Dark gray boxes depict condensed regions of chromatin (heterochromatin). Light gray boxes depict euchromatic regions. From Sims et al., 2003.

to consider two levels of regulation carried out by HKMTs: the regulation of specific gene expression (`gene level') and the organization of bulk chromosomal regions to maintain the basic structure of chromosomes (`chromosome level') (Fig.1.2.5.). All known HKMTs contain a conserved methyltransferase domain termed a SET (Su(var)3–9, Enhancer-of-zeste, Trithorax) domain, which contains the methyltransferase activity. Comparison of the SET domains of previously characterized proteins has enabled their classification into four subfamilies: SUV39; SET1; SET2, and RIZ subfamilies (Kouzarides, 2002). Although the data are still limited, increasing *in vivo* analysis suggested the important roles of HKMTs in development and differentiation. Moreover, there appear to be many connections between the deregulation of SET-domain proteins and cancer (reviewed by Schneider et al., 2002).

The first identified and best-characterized HKMTs selectively methylate H3-K9 (reviewed in Lachner et al., 2004). There are numerous reports demonstrating the silencing effects correlated with, or resulting from, H3-K9 methylation, including the inactive X chromosomes (Xi) of female mice and humans (Heard et al., 2001; Boggs et al., 2002; Mermoud et al., 2002; Peters et al., 2002), and developmentally regulated genes (Litt et al., 2001). Highly condensed centromeric (heterochromatic) regions of chromosomes correspond with a specific degree of methyl addition to H3-K9. Recent reports suggest that H3-K9 and H3-K27 methylation might be important histone methylation events that participate in the X chromosome inactivation process. H4-K20 methylation has been shown to be associated with silent chromatin and prevent acetylation on H4-K16, a mark for active chromatin (Nishioka et al., 2002). Methylation of H3-K9, H4-K20 and possibly H3-K27 are, so far, the major covalent histone modifications that correlate with large-scale, or chromosome level, repression.

Epigenetic silencing through heterochromatin appears to require the histone-binding protein heterochromatin-associated protein 1 (HP1) in a process that is fundamentally conserved from *Schizosaccharomyces pombe* to humans but not in *Saccharomyces cerevisiae* (reviewed in Sims et al., 2003). Acetylation of H3-K9 prevents H3-K9 methylation; thus, step one in this model, involves the deacetylation of H3-K9 by specific HDACs, and the subsequent methylation by a histone lysine acetyltransferase (HKMT), such as Suv39H1. In step two, HP1 selectively recognizes methylated H3-K9. Step

three involves the propagation of heterochromatin through HP1 recruitment of Suv39H1 via protein association. Once nearby H3-K9 sites are methylated, additional heterochromatin-associated protein 1 (HP1) molecules recognize this mark through its chromodomain. HP1 then recruits Suv39H1 through protein interactions recruiting further H3-K9 methylation activity. The processes that establish heterochromatin formation are probably complex and might not only require HP1 recruitment, as experiments designed to artificially recruit HP1 to chromatin domains in *Drosophila* was not dependent on SU(VAR)3–9 dosage (Li et al., 2003). The chromoshadow domain of HP1, a protein domain found associated with chromodomains, contributes to HP1 binding to chromatin. The chromoshadow domain of HP1 appears to play a role in directing it to euchromatic regions, as opposed to the chromodomain, which recruits HP1 to heterochromatic areas (Cheutin et al., 2003). H3 methylation in X chromosome inactivation occurs through a process that does not seem to involve HP1. Several reports have shown that H3-K9 methylation correlates with X inactivation (Heard et al., 2001; Boggs et al., 2002; Mermoud et al., 2002; Peters, et al., 2002). A recent study using highly specific antibodies in mouse embryos demonstrated that the inactive X chromosome (Xi) is also marked by tri-methyl H3-K27 and this modification might participate in the initiation of Xi (Plath et al., 2003). It is suggested that H3-K27 and H3-K9 methylation signify disparate aspects of Xi, such as establishment and maintenance, respectively, where the detailed mechanisms remains to be clarified.

The basic epigenetic mechanisms governing chromosome level and gene level silencing appear to be similar, in that a methylated H3 tail residue can serve as a docking site for a repressor protein that ultimately recruits a core repressor complex. In contrast to constitutive heterochromatin, DNA-sequence-specific repression probably requires an additional agent of specificity, perhaps contributed by a sequence-specific DNA-binding protein. For example, the chromodomain-containing Polycomb (PC) protein has been shown to bind specifically methylated H3-K27 (Cao et al., 2002; Kuzmichev et al., 2002; Czermin et al., 2002) and this results in the site-specific formation of an active Polycomb repressor complex. PC binds its cognate modified histone tail (H3-K27) with a similar degree of specificity as the recognition of H3-K9 by HP1, although PC and HP1 do not bind to the same methyl residue. Moreover, it is suggested that different processes mediate the downstream events that silence genes in heterochromatin and euchromatin, even though both regions are marked by H3-K9 methylation and HP1 recruitment. For example, recruitment of HP1 at the reporter gene occurred at very specific promoter regions, and did not facilitate HP1 spreading that can contribute to heterochromatin formation (Ayyanatha et al., 2003).

Transcriptionally competent euchromatin is methylated at three positions, H3-K4, H3-K36 and H3-K79. H3-K4 methylation is generally associated with transcriptionally active chromatin: di-methyl H3-K4 appears to be a global epigenetic mark in euchromatic regions and tri-methylation of H3-K4 correlates with active transcription (Ng et al., 2003; Santos-Rosa et al., 2002). It appears that two mechanistic levels exist regarding the establishment of competent euchromatin and the degree of methylation of H3-K4 plays a central role. The methylation of H3-K4 specifically impairs Suv39h1-mediated methylation at H3-K9, thereby thwarting a major pathway of heterochromatin formation (Nishioka et al., 2002). Moreover, binding of the histone deacetylase NuRD repression complex to the H3 N-terminal tail is precluded by methylation at lysine 4, but not lysine 9 (Nishioka et al., 2002; Zegerman et al., 2002). Aside from H3-K4, methylation of H3-K79 appears to be involved in preventing the spread of heterochromatin. H3-K79 methylation prevents Sir association, halting silencing; in a feedback loop, Sir binding blocks H3-K79 methylation by Dot1, leading to hypomethylated H3-K79 (Ng et al., 2003; Leeuwen, et al., 2002). Therefore, both H3-K4 and H3-K79 participate in

establishing euchromatic regions by preventing the spreading of heterochromatic regions. Once large-scale permissive chromatin is established, separate HKMT-mediated processes appear to participate in transcriptional activation at specific loci. In addition to establishing large regions of permissive chromatin where transcription initiation complexes can be formed, the role of histone lysine methylation in transcriptional elongation has been appreciated recently (Hampsey and Reinberg, 2003).

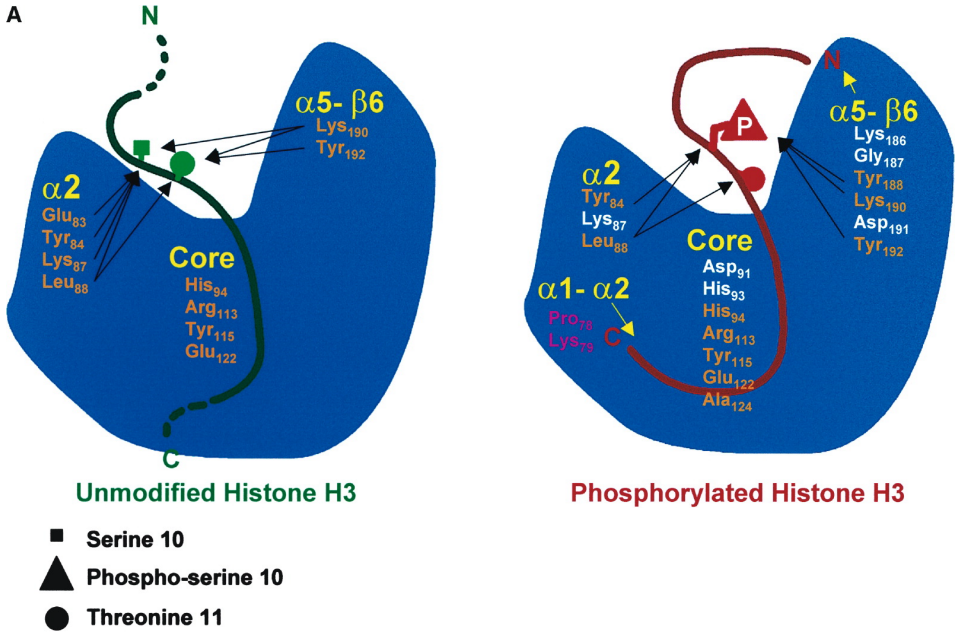
1.2.4. Histone phosphorylation

On the histone H3 tail serines 10 and 28 are both proceeded by the same three amino acids (alanine-arginine-lysine) and both these phosphorylatable motifs are very highly conserved through evolution, being identical in yeast and man. It is now becoming clear that histone phosphorylation is an important regulatory mechanism both to condense chromosomes during mitosis and meiosis, and to regulate transcriptional activation upon stimuli, both of which can utilize these two serine residues.

Several studies have indicated that histone H3 phosphorylations on S10 and S28 correlate with mitotic chromosome condensation (reviewed by Pascreau et al., 2003). The role of histone phosphorylation during cell cycle was clearly established in *Tetrahymena*, where mitosis is restricted to the germ line micronuclei, and transcription is limited to the amitotic macronuclei. A mutation of histone H3 serine 10 to alanine caused abnormal condensation, segregation and loss of chromosomes, demonstrating that this phosphorylation has a critical role in micronuclear mitotic cell division (Wei et al., 1999). Two classes of kinases, previously known to be required for chromosome condensation, were shown to phosphorylated histone H3 serine 10 during cell cycle. In *Aspergillus nidulans*, the NIMA kinase phosphorylates serine 10 *in vitro*, and is required for H3 phosphorylation *in vivo* (De Souza et al., 2000). In yeast and *C. elegans*, the Ip11/Aurora kinase family, and the opposing G1c7/PP1 phosphatases, were shown to establish the level of mitotic serine 10 phosphorylation by mutation analysis (Hsu et al., 2000). More recently serine 28 has been identified as a second site of phosphorylation in mammals that also occurs during chromosome condensation at early mitosis (Goto et al., 1999), where Aurora B is also implicated in mitotic serine 28 phosphorylation. It is not yet clear how phosphorylation actually generates the condensed chromatin structure.

Histone phosphorylation also has a role in transcriptional activation. Here the phosphorylation is transient, affects a minute fraction of nucleosomes and is associated with active genes, whereas, mitotic phosphorylation is extensively observed through condensed chromosomes. In mammalian cells, phosphorylation of H3 Ser-10 has been linked to transcriptional activation of mitogen-stimulated immediate-early response genes, such as c-fos and c-jun, as well as camp-dependent protein kinase A-responsive genes. When cells receive extracellular stimuli, intracellular signaling pathways are activated which rapidly induce immediate-early (IE) genes. Phosphorylation of H3 concomitant with IE gene induction has been demonstrated, and the MAP kinase cascades that deliver this response have been elucidated. In yeast, certain genes regulated by the acetyltransferase Gcn5 are also regulated by phosphorylation of serine 10 on histone H3 (Lo et al., 2000). In *D. melanogaster*, the heat shock genes are regulated by phosphorylation of the same residue (Nowak and Corces, 2000). Also, the mammalian circadian clock may be controlled in part by light-induced phosphorylation of the histone H3 serine 10 in neuronal cells of the hypothalamus (Crosio et al., 2000). The kinases that

carry out histone H3 phosphorylation of serine 10 in the mammalian growth factor response have been identified. One study identified Rsk-2 (Sassone-Corsi et al., 1999) and a second study identified Msk1 (Thomson et al., 1999) as mitogen-stimulated, histone H3 serine 10 kinases. Interestingly, mutations in Rsk2 causing a loss of histone H3 phosphorylation may be the direct cause of Coffin-Lowry syndrome in humans, a profound genetic disease (Sassone-Corsi et al., 1999). A transcriptionally linked histone kinase also has been identified in *S. cerevisiae* as the previously known Snf1 kinase. The identity of these histone kinases as previously known transcription-associated factors suggests that they may be recruited to specific promoters as coactivators, much like the HATs and Swi/Snf complexes discussed above.



B

Unmodified Histone H3	Q T A **R K** S **T** G G K(14) A P R K Q L A S K
Phosphorylated Histone H3	**Q T A** R K **SPi** T **G G K(14) A P** R K Q L A **S K**

Figure1.2.6. Summary of tGCN5/CoA/H3 Interactions. (From Clements, 2003) (A) General features of the tGCN5/CoA/H3p19 complex and tGCN5/CoA/H3p19P*i* complex phosphorylated at serine 10. The tGCN5 HAT domain is shown in blue, the H3p19 peptide is shown in green, and the H3p19P*i* peptide is shown in red. Disordered regions are shown as dashed lines. Ser10, phospho-Ser10, and Thr11 are represented with a rectangle (■), a triangle (▲), and a circle (), respectively. The tGCN5 residues and corresponding domains/secondary structure elements that interact with the N-terminal regions of the histone-derived peptides (residues 7–11 and residues 5–11 for nonphosphorylated H3 and phosphorylated H3, respectively) are shown. The tGCN5 residues that specifically contact either Ser10 or Thr11 of histone H3 are shown in orange. The residues in white only contact other H3 residues from the N terminus of the peptides through Thr11. Additionally, the tGCN5 residues (within the α1-β2 loop) that interact with the H3 residues at the C terminus of the phosphorylated H3-derived peptide (amino acids 22–23) are shown in purple. (B) Residues of H3-derived peptides that make interactions with tGCN5/CoA. For the unmodified histone H3 peptide, residues in bold type represent amino acids that have enhanced van der Waals interactions with tGCN5/CoA relative to the tGCN5/CoA/H3p11 complex. Residues in aqua represent additional hydrogen bonds in the H3p19 complex. For the phosphorylated histone H3 peptide, residues in bold type represent amino acids that have enhanced van der Waals interactions with tGCN5/CoA relative to the tGCN5/CoA/H3p19 complex. Residues in aqua represent additional hydrogen bonds relative to the tGCN5/CoA/H3p19 complex.

Although a clear link between mitogen- or stress-inducible histone H3 phosphorylation and gene transcription is established, its precise molecular functions remain unclear. One broad class of function would be that phosphorylation mediates changes in nucleosome and chromatin structure by disrupting/altering histone–DNA charge interactions facilitating easier access to the underlying DNA sequences by transcription factors. A second function, more aligned with the histone code hypothesis, is that the phosphoacetyl epitope on histone H3 at Ser10 (as well as on Ser6 of HMG-14) serves as a binding motif for recruitment of coactivator complexes such as HATs or chromatin remodeling complexes, in the same way as is already established for phosphorylated transcription factors at upstream promoter sequences. For example, it has been shown that inducible genes such as c-fos and c-myc may exhibit enhanced acetylation upon activation, which is further supported by the finding that phosphorylation on H3 Ser10 could stimulate the activity of Gcn5 on H3 K9 and K14 (Clements et al., 2003).

From the increasing number of chromatin immunoprecipitation (ChIP) experiments with a variety of modification-specific histone antibodies, the picture of inducible H3 phosphorylation, acetylation and phosphoacetylation, targeted to (IE)/inducible gene chromatin, is emerging as one of a complex and dynamic pattern of multiple modifications. Present indications are that phosphorylation occurs at Ser10, and lysines 9 and 14 are both acetylated, probably simultaneously. Recently, Clements et. al. (2003) resolved the structure of Tetrahymena GCN5 (tGCN5) and coenzyme A (CoA) bound to unmodified and ser10-phosphorylated 19 residue histone H3 peptides (H3p19 and H3p19pi, respectively). Comparison with the tGCN5/CoA/H3p19P*i* structure reveals that phospho-Ser10 and Thr11 mediate significant histone-protein interactions, and nucleate additional interactions distal to the phosphorylation site. Functional studies show that histone H3 Thr11 is necessary for optimal transcription at yGcn5-dependent promoters requiring Ser10 phosphorylation. Together with *in vivo* ChIP results, a synergistic coupled modification model is suggested. (Fig. 1.2.6.)

1.2.5 Histone ubiquitination

Uibiquitin is a small, 76 amino acid long polypeptide, which is attached as a polymer to the ε-amino group of lysine residues in polypeptides targeted for proteasomal degradation. However, ubiquitination of histones has a different effect. Histone ubiquitination represents the most bulky structural change to histones. For example, Histone H2A ubiquitination was the first protein found to be posttranslationally modified by covalent ligation to ubiquitin, which is largely mono-ubiquitination, and affects about 5-15% of this histone in most eukaryotic cells. Ubiquitinated H2A and H2B (uH2A and uH2B) are the most abundant ubiquitin conjugates in eukaryotes and have been identified in many eukaryotic organisms except *Schizosaccharomyces pomber* and *Arabidopsis thaliana* (reviewed in Jason et al., 2002 and Zhang, 2003)

The lysine residues to which ubiquitin is conjugated in H2A and H2B are highly conserved, suggesting an important function of this modification. Ubiquitin is linked to the ε-amino group of lysine 119 of H2A by an isopeptide bond, forming a bifurcated structure. Lysine 119 is found at the beginning of the C-terminal tail of uH2A in the trypsin accessible region of the H2A. The crystal structure of the 2.8 Å nucleosome showed that this region is located on the face of the histone octamer (Fig.1.2.7.A). Three years after the identification of ubiquitin in H2A, ubiquitinated H2B was also found to occur naturally. In this case, the isopeptide bond linking ubiquitin and mammalian H2B is formed between the C-terminal

glycine of ubiquitin and lysine 120 of H2B, whereas lysine 123 is modified in yeast. This lysine residue lies within the histone fold region of H2B that is inaccessible to trypsin. About 1.5% of cellular H2B is mono-ubiquitinated. Polyubiquitination also have been detected on histone H2A, H2B, and H2A.Z in preparations of bovine thymus, chicken erythrocytes, *Tetrahymena* macronuclei and micronuclei, trout testis, trout liver and trout hepatocellular carcinoma. Recently, ubiquitination of H3 was also reported to occur *in vivo* in elongating spermatids of rat testes but could not be detected in mouse (Chen, et al., 1998). Moreover, in *Drosophila* embryos, the ubiquitin-conjugating activity of TAFII 250 is involved in the ubiquitination of linker histone H1 (Pham and Sauer, 2000). The sites of H3 and H1 ubiquitination are presently unknown.

The same as general protein ubiquitination reaction, E1, E2, and E3 are necessary for the ubiquitination on the histones. E1 is found in almost all compartments of the eukaryotic cell, including nucleus and it also has been found to be associated with condensed chromosomes during mitosis. Many E2 isoenzymes have been identified, of which only a subset is involved in histone ubiquitination. In *Saccharomyces cerevisiae*, Rad6p/Ubc2p and Cdc34p/Ubc3p are homologous to mammalian reticulocyte $E2_{20KD}$ and $E2_{32kD}$ isoenzymes, which can ubiquitinate histone H2B *in vitro* without any need of E3 ligase. However, only Rad6 is indispensable for H2B ubiquitination *in vivo* (Robzyk et al. 2000). Recent studies suggest that a Rad6-associated RING finger protein Bre1 is likely to be the E3 ligase involved in H2B ubiquitination, because mutation in the RING domain of Bre1 abolished H2B ubiquitination *in vivo* (Hwang et al. 2003; Wood et al. 2003). In contrast to the situation concerning H2B ubiquitination, the physiological E2 and E3 enzymes involved in H2A ubiquitination have not been identified. The best *in vivo* mamalian model for histone ubiquitination is spermatogenesis in mouse. The levels of ubiquitinated histones have been found to vary at different stages and to different extents during spermatogenesis in vertebrate species such as the rooster, trout, rat, and mouse. In mouse spermatogenesis, H2A ubiquitination occurs during the post-meiotic period where protamines replace histones. When a mouse homologue of the yeast E2 enzyme RAD6, mHR6B, is inactivated by gene targeting, male mice are sterile. This effect was thought to be brought about by abnormalities in histone displacement during spermatogenesis. But it was subsequently shown that the overall pattern of histone ubiquitination was not affected during spermatogenesis in HR6B knockout mice, suggesting that the ubiquitin-conjugating activity of HR6B affects other aspects of male fertility and another E2 enzyme must be responsible for global H2A ubiquitination.

Like acetylation and phosphorylation, histone ubiquitination is a reversible modification. Therefore, the steady-state histone ubiquitination levels are determined by the availability of free ubiquitin and enzymatic activities involved in both adding and removing the ubiquitin moiety from histones. Berger and colleagues (Henry et al. 2003) report that Ubp8, a component of the SAGA (Spt–Ada–Gcn5–Acetyltransferase) complex, is a histone H2B ubiquitin protease. Surprisingly, they demonstrate that, unlike other reversible histone modifications in which addition or removal of a group from a histone molecule results in opposing transcriptional effects, sequential ubiquitination and deubiquitination are both involved in transcriptional activation. They provide evidence suggesting that the effect of ubiquitination and deubiquitination signals is likely mediated through histone methylation. These findings establish that sequential ubiquitination and deubiquitination of histones as well as cooperation among different histone modifications play an important role in transcriptional regulation (Fig.1.2.7.B).

Although the data on the role of histone ubiquitination on transcription activation, so far, are still controversial and

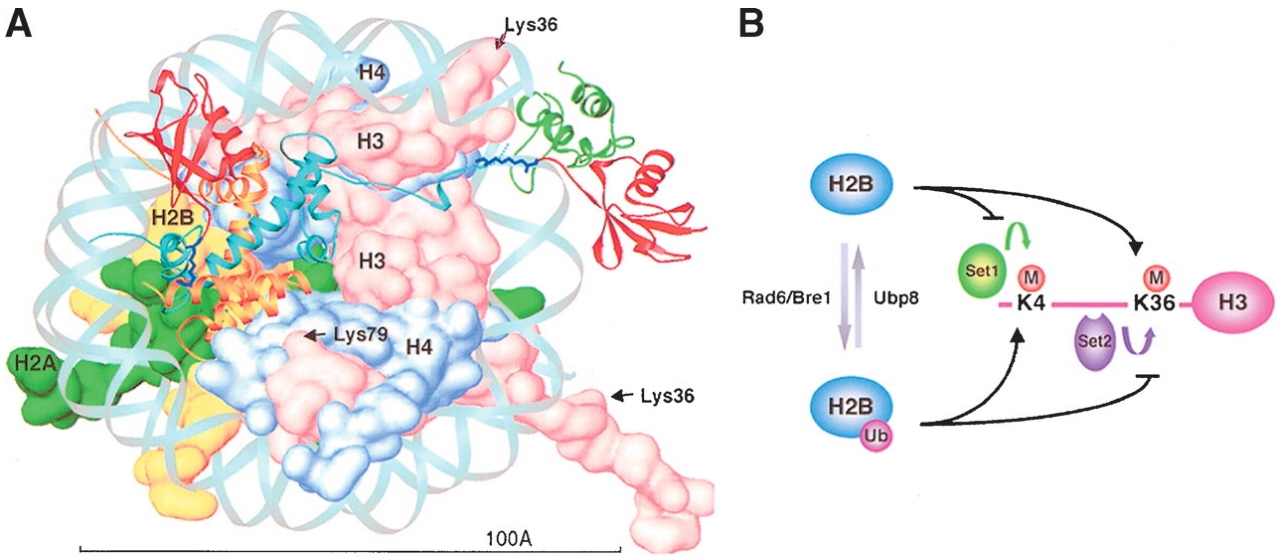


Figure.1.2.7. *A:* Proposed nucleosome structure with ubiquitinated histones. Modeled ubiquitin molecules (red) covalently attached to Lys 119 of histone H2A (cyan) and Lys 120 of histone H2B (gold) are shown as ribbon diagrams. For clarity, only one histone H2A and one H2B are shown in detail. The other H2A (green) and H2B (yellow) are shown in a surface representation together with H3 (pink) and H4 (light blue). The modeled isopeptide bond between the acceptor lysines and Gly 76 of ubiquitin are shown in a stick model (blue). The globular domain of histone H1 is shown in a green ribbon diagram. Location of Lys 36 and Lys 79 of H3 is indicated. The structure is modeled based on previous publications (Vijay-Kumar et al. 1987; Cerf et al. 1994; Goytisolo et al. 1996; Luger et al. 1997). *B:* Diagram depicting the differential effects of H2B ubiquitination and deubiquitination on H3-K4 and H3-K36 methylation. Enzymes (Rad6/Bre1, Ubp8, Set1, and Set2) that participate in this interplay are indicated. From Zhang, 2003.

debating, it was suggested that histone ubiquitination most likely regulates gene transcription in a positive and negative fashion, depending on its genomic and gene location. There are at least three possible explanations of how histone ubiquitination affects transcription. First, histone ubiquitination may affect higher-order chromatin folding, thereby resulting in greater access of the underlying DNA to the transcription machinery. Second, ubiquitination may function as a signal for the recruitment of regulatory molecules that, in turn, affect transcription. The third possibility is that histone ubiquitination affects transcription through its impact on other histone modifications. Although the first two possibilities have not been ruled out, recent studies have given the most support for the third possibility. Substantial evidence indicates that histone ubiquitination is functionally linked to histone methylation (Fig1.2.7.). In a seminal study, both the Allis and Shilatifard groups discovered, independently, that Set1-mediated H3-K4 methylation requires functional RAD6 and intact H3-K12 (Dover et al. 2002; Sun and Allis 2002). However, deletion of the *SET1* gene does not affect H2B-K123 ubiquitination, suggesting a unidirectional regulatory pathway in which H2B ubiquitination is upstream of H3-K4 methylation (Sun and Allis 2002). Subsequent studies indicated that H3-K79 methylation is also dependent on Rad6-mediated H2BK123 ubiquitination. Interestingly, H3-K36 methylation does not appear to have such a requirement (Briggs et al., 2002; Ng et al., 2002). Berger and colleagues (Henry et al., 2003) proposed an explanation based on the observation that H2B ubiquitination has an opposite effect on H3-K4 and H3-K36 methylation. Whereas H2B ubiquitination facilitates H3-K4 methylation (Dover et al., 2002; Sun and Allis 2002), it down-regulates H3-K36 methylation (Henry et al., 2003). Conversely, whereas H2B deubiquitination reduces H3-K4 methylation levels, it dramatically increases H3-K36 methylation levels (Henry et al., 2003).

1.3. Histone acetyltransferases (HATs)

Histone acetylation is carried out by a class of enzymes known as histone acetyltransferases (HATs), which catalyze the transfer of an acetyl group from acetyl-CoA to the lysine ε-amino groups on the N-terminal tails of histones. Based on their suspected cellular origin and functions, HAT activities may be grouped into two general classes: Cytoplasmic, B-type HATs (e.g. Hat1 and Hat2) likely catalyze acetylation events linked to the transport of newly synthesized histones from the cytoplasm to the nucleus for deposition onto newly replicated DNA; Conversely, nuclear, A-type HATs acetylate nucleosomal histones within chromatin in the nucleus, thereby likely catalyze transcription-related acetylation events.

The nucleic histone acetyltransferases are further classified into four families (Fig.1.3.1): 1. The GNAT (GCN5-related N-terminal acetyltransferases)-MYST family whose members have sequence motifs shared with enzymes that acetylate non-histone proteins and small molecules; 2. p300/CBP HATs in animals implicated in regulating genes required for cell cycle control, differentiation and apoptosis; 3. The general transcription factor HATs, which include TAF250, the largest of the TATA binding protein-associated factors (TAFs) within the transcription factor complex TFIID; These three families are widespread in eukaryotic genomes, and homologous proteins are also involved in non-HAT reactions in prokaryotes and Archaea. Mammals have a fourth HAT family that includes nuclear receptor coactivators such as steroid receptor coactivator (SRC-1) and ACTR (SRC-3), a thyroid hormone and retinoic acid coactivator that are not represented in plants, fungi or lower animals. The most consistent functional characteristic of the HATs is that they are transcriptional coactivators (i.e. they do not bind directly to DNA but are recruited by DNA-binding activators). The fact that HATs are coactivators rather than DNA-binding moieties underscores the need for flexibility, regulating and alternative strategies in regulating chromatin and the basal transcriptional machinery. All HATs act in a site-specific and histone-specific manner, and specificity may differ *in vivo* and *in vitro*; such diversity then may help to explain why there are so many HATs. Remarkably, some HATs are associated with other HATs and coactivators, suggesting a layer of complexity that is not yet understood.

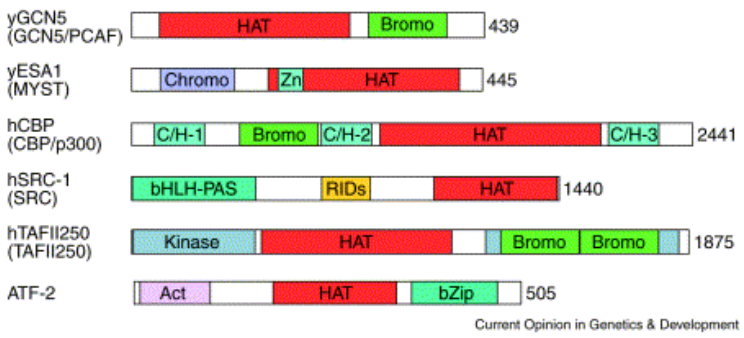


Fig. 1.3.1.Different HAT families and their associated domains. A representative member from each HAT family is shown with the family designation indicated in parenthesis. Act, transcriptional activation domain; bHLH-PAS, basic helix-loop-helix and PAS A and PAS B domains; Bromo, bromodomain; bZip, basic zipper DNA-binding domain; C/H, Cys/His-rich regions (1, 2 and 3); Chromo, chromodomain; HAT, histone acetyltransferase domain; RIDs, LXXLL receptor interacting motifs; Zn, zinc-binding domain. From Marmorstein and Roth, 2001.

The different HAT complexes have different subunit compositions and different histone specificities (Table 1.3.1). Correspondingly, these complexes appear to be involved in distinct biological functions (Roth et al., 2001). For example, genetic and biochemical studies implicate the Spt3, 7, and 8 proteins, which are unique to the SAGA complex, in stabilizing TBP binding to the TATA box, suggesting that SAGA might function as a coactivator at the site of initiation in addition to its acetylation activity. In contrast, Spt16, a subunit of NuA3, and its mammalian homologs have been implicated in transcriptional elongation and replication, events that might require more large-scale acetylation over several kb. A distinct role for NuA4 is suggested based on its HAT, EsaI, which, unlike GCN5, is essential in yeast and is homologous to MOF, the HAT implicated in 2-fold upregulation of the *Drosophila* male X chromosome. Mice that are homozygous for deletion of the HAT proteins p300, CBP, PCAF, or GCN5 exhibit distinct developmental defects, further suggesting differences in function of these highly related HAT subunits.

The complexity of the histone code influences recruitment of HATs to promoters. *In vivo* ChIP experiments show that phosphorylation of histone H3 serine 10 enhances targeting of Gcn5 to the yeast INO1 promoter. In mammals, methylation of histone H3 lysine 9 by SUV39H1 inhibits serine 10 phosphorylation possibly because this methylation may inhibit HAT targeting. Recruitment of HAT complexes will probably be influenced further by patterns of histone modifications. Once targeted, SAGA acetylates histone H3 in the vicinity of the promoter, which further stabilizes its binding and that of a targeted SWI/SNF chromatin-remodeling complex. This requires the bromodomains of Gcn5 and Swi2, respectively, which bind to acetyl-lysine. Because bromodomains are found in other chromatin-modifying complexes and TFIID, acetylation might stabilize promoter interactions of several complexes involved in transcription activation.

HAT	Organisms known to contain the HAT	Known transcription-related functions/effects	HAT activity demonstrated in vitro[b]	Histone specificity of recombinant enzyme in vitro[a,b]	Known native HAT complexes and nucleosomal histone specificities in vitro
GNAT superfamily					
Hat1	Various (yeast to humans)	None (histone deposition-related B-type HAT)	Yes	**H4**	Yeast HAT-B, HAT-A3 (no nucleosome acetylation)
Gcn5	Various (yeast to humans)	Coactivator (adaptor)	Yes	**H3**/H4	Yeast ADA, SAGA (**H3**/H2B); human GCN5 complex, STAGA, TFTC (**H3**)
PCAF	Humans, mice	Coactivator	Yes	**H3**/H4	Human PCAF complex (**H3**/weak H4)
Elp3	Yeast	Transcript elongation	Yes	ND*	Elongator, polymerase II holoenzyme (**H3**/weak H4)
Hpa2	Yeast	Unknown	Yes	**H3**/H4	
MYST family					
Sas2	Yeast	Silencing	ND		
Sas3	Yeast	Silencing	Yes	**H3**/**H4**/H2A	NuA3[c] (**H3**)
Esa1	Yeast	Cell cycle progression	Yes	**H4**/H3/H2A	NuA4 (**H4**/H2A)
MOF	*Drosophila*	Dosage compensation	Yes	**H4**/H3/H2A	MSL complex (**H4**)
Tip60	Humans	HIV Tat interaction	Yes	**H4**/H3/H2A	Tip60 complex
MOZ	Humans	Leukemogenesis, upon chromosomal translocation	ND		
MORF	Humans	Unknown (strong homology to MOZ)	Yes	**H4**/H3/H2A	
HBO1	Humans	ORC interaction	Yes*	ND*	HBO1 complex
p300/CBP	Various	Global coactivator	Yes	**H2A/H2B/H3/H4**	
Nuclear receptor coactivators	multicellular	Nuclear receptor coactivators (transcriptional response to hormone signals)			
SRC-1	Humans, mice		Yes	**H3**/H4	
ACTR	Humans, mice		Yes	**H3**/**H4**	
TIF2	Humans, mice		ND		
$TAF_{II}250$	Various (yeast to humans)	TBP-associated factor	Yes	**H3**/H4	TFIID
TFIIIC		RNA polymerase III transcription initiation			TFIIIC (**H2A/H3/H4**)
TFIIIC220	Humans		Yes*	ND	
TFIIIC110	Humans		Yes	ND	
TFIIIC90	Humans		Yes	**H3**	

[a] Histones that are the primary in vitro substrates for a given HAT are bold; other histones listed are acetylated weakly or in a secondary manner.

[b] Asterisks indicate proteins for which HAT activity has been suggested indirectly or demonstrated in an incomplete manner. Elp3 can acetylate all four histones but has only been tested with them individually in in-gel assays. The HAT function of HBO1 has primarily been shown by the in vitro free histone **H3**/H4-acetylating activity of a purified human complex containing it, although recombinant GST-HBO1 (and the complex) did weakly acetylate nucleosomes. Finally, TFIIIC220 was identified as a HAT only in in-gel assays, and its activity has not been confirmed by recombinant protein studies as of this writing. ND, not determined.

TABLE 1.3.1. Summary of known and putative HATs From Sterner and Berger, 2000

Loss or misdirection of HATs has been linked to embryonic aberrations in mice and to human cancers. Disruption of the genes encoding p300, CBP, and Gcn5 results in embryonic death in mice but the range of abnormalities in these HAT mutants is quite distinct. Loss of p300 causes proliferative defects and improper development of fetal heart tissue (Yao, et al, 1998). In increased incidence of haematological malignancies and abnormal skeletal patterning was reported in CBP but not in p300 heterozygous null mice (Tanaka et al., 1997; Kung et al., 2000). Loss of Gcn5 has no effect on proliferation but does result in rampant apoptosis and mesodermal defects whereas mice lacking PCAF are developmentally normal without a distinct phenotype (Xu et al., 2000; Yamauchi et al., 2000). More recently, knock-in experiment with acetyltransferase deficient mutants showed differential roles of CBP and p300 during myogenesis (Roth et al, 2003) in that p300 but not CBP acetyltransferase activity is required for myogenesis in the mouse and in ES cells. All together these results indicate that particular HATs have very specific functions during development. Misdirection of HAT activities as a result of chromosomal translocations has been shown to associate with multiple human leukemias (Borrow et al., 1996; Carapeti et al., 1999; Redner et al., 1999). Gain-of-function and loss-of-function HAT alleles are associated with other cancers, indicating that some HATs may serve as oncogenes whereas others may serve as tumor suppressors (Anzick et al., 1997;). Many additional connections are sure to come to light in coming years as more tumors are evaluated for changes in HAT expression or activity.

1.4 Histone deacetylases (HDACs) and HDAC inhibitors

1.4.1 Histone deacetylases, from yeast to human

Although the correlation between histone acetylation and gene transcription regulation has been suggested since the mid-1960s, it was not until the early 1990s that the role of HDACs in this regulation came to prominence. The initial observations that implicated a role for HDACs in transcriptional regulation came from a screen to identify small molecules that could return spindle-like transformed NIH3T3 cells to the normal fibroblast-like morphology. An epoxyketone-containing cyclic tetrapeptide, trapoxin, was identified without the knowledge of what proteins this molecule was acting on (Itazaki et al., 1990). Later, it was discovered that cells treated with trapoxin had hyperacetylated histones and that this molecule inhibited histone deacetylation (Kijima et al., 1993). It was not until 1996, however, that the protein target for trapoxin was identified with the purification and cloning of the first histone deacetylase (Taunton et al., 1996). To date, 18 HDACs have been identified in humans, and their activities have been implicated in transcription, cell cycle progression, gene silencing, differentiation, DNA replication, and the DNA damage response (reviewed in Thiagalingam et al., 2003) (Fig.1.4.1).

Major groups of HDACs include the RPD3/HDA1 superfamily, the Silent Information Regulator 2 (SIR2) family and the HD2 family. RPD3/HDA1-like HDACs are found in all eukaryotic genomes and are further divided into two classes: Class I HDACs (HDAC-1, -2, -3, and -8) are similar to yeast RPD3 protein; Class II HDACs (HDAC-4, -5, -6, -7, -9 and -10) are homologous to yeast HDA1 protein. Interestingly, homologous proteins that have acetate utilization and acetylpolyamine aminohydrolase activities are also present in bacteria and Archaea, organisms that lack histones. The SIR2 family of HDACs is distinctive in that it has no structural similarity to other HDACs and requires NAD as a cofactor. In *S.cerevisiae*, *SIR2* is known to play roles in repression of silent mating type loci, repression of rRNA gene recombination, and repression of protein-coding genes inserted near telomeres or within rRNA gene arrays. Mutations

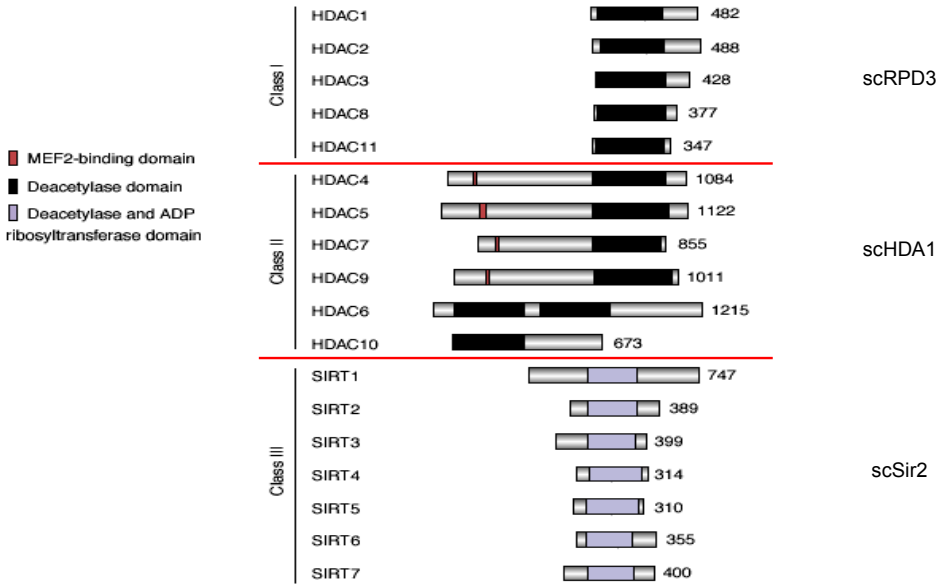


Fig.1.4.1. Human Histone deacetylase (HDAC) families.

in *SIR2* also affect aging and longevity in *S.cerevisiae* . SIR2-related proteins form a large family with members present in all kingdoms of life, including bacteria. The fourth family, the HD2-type HDACs, were first identified in maize and appear to be present only in plants (Lusser et al., 1997) but not in animals or fungi, and is distantly related to cis-trans isomerases found in insects, *S. cerevisiae* and parasitic apicomplexans. There are four HD2 type HDACs, HDT1, HDT2, HDT3, and HDT4, were identified in the Arabidopsis. The first two of these proteins have been analyzed in a recent paper showing that antisense silencing of HTD1 results in aborted seed development (Wu et al., 2000). Beside the complexity from still increasing numbers of HDACs, splice variants of these HDACs, proteins which associate with HDACs either alone or in multiprotein complexes, and posttranslational modifications such as phosphorylation and sumoylation all play a role in the regulating the specificity of HDAC activity.

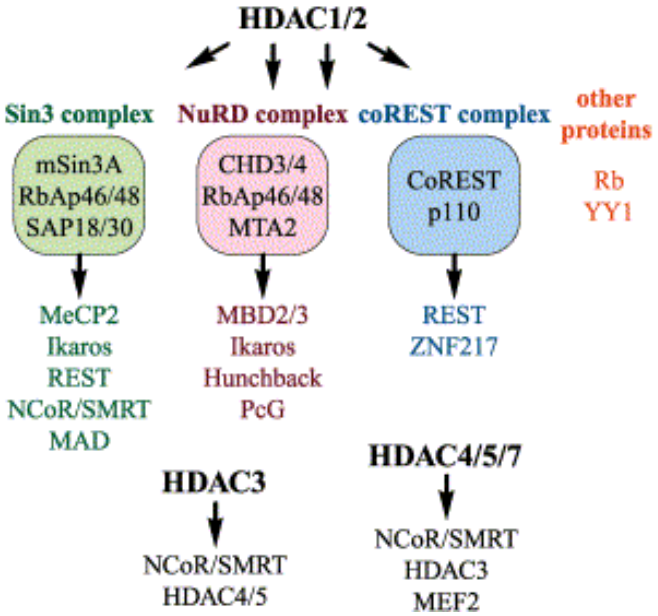


Fig.1.4.2. HDAC containing complexes and interaction partners.

HDACs exist in cells as part of large molecular weight complexes that are recruited to specific promoters via their interactions with sequence-specific DNA binding proteins (Fig.1.4.2). These include the nuclear hormone receptors, the E box binding factors, and the methylcytosine binding protein MeCP2, etc. Class I HDAC-1 and HDAC-2 are found in the SIN3 and NURD/Mi2 complexes. HDAC-3 is associated with corepressor N-CoR and SMRT mediating transcriptional repression by the thyroid hormone receptor and oncoprotein v-ErbA. Class II HDACs 4, 5, 7 can interact with N-CoR, SMRT, and BcoR, an additional corepressor that mediates repression by BCL-6.

1.4.2 Hdac core and deacetylation reaction

The crystal structure of a bacterial HDAC homologue, HDLP (histone-deacetylase-like protein), together with a HDAC inhibitor, trichostain A (TSA), was resolved in 1999 (Finnin et al. 1999). HDLP's catalytic domain is closely related to mammalian hdac domains in all class I and II HDACs, in which the key amino acids in the catalytic core deduced from biochemical and genetic data, are also highly conserved. HDLP has a single domain structure related to the open α/ β class of folds. It contains a central eight-stranded parallel β sheet, with four α helices packed on either face. Eight additional α helices and large loops in the β sheet further extend the structure and result in the formation of a deep, narrow pocket with an adjacent internal cavity. HDLP requires Zn^{2+} for activity; this cation is positioned near the bottom of the pocket and is coordinated by several histidine and aspartate residues. The channel leading to the active site is surrounded by hydrophobic residues, which is presumably where the aliphatic chain of the acetyl-lysine residue is nestled. TSA is one of most potent HDAC inhibitors. It contains a cap group, an aliphatic chain, and a terminal hydroxamic acid functional group. The cocrystallization of TSA with HDLP provided the first explanation for the mechanism of inhibition. TSA binds perfectly to the pocket in the HDLP. The hydroxamic acid coordinates the zinc cation in a bidentate fashion and hydrogen bonds with some of the active site residues. Fitting snugly into the channel, the aliphatic chain makes several van der Waals contacts with the channel residues. The cap group contacts residues on the rim of the pocket and possibly mimics the amino acids adjacent to the acetylated lysine residue in the histone. The binding of TSA causes a conformational change in a tyrosine residue on this rim (tyrosine 91) and thereby allows tighter packing of the cap group.

The proposed mechanism (Fig.1.4.3) for the deacetylation reaction is similar to that seen in metallo- and serine proteases. The carbonyl oxygen of the N-acetyl amide bond is thought to coordinate to the zinc cation and to thereby position it closely to a bound water molecule and activate it for a nucleophilic attack by the water. The nucleophilicity of the water molecule, in turn, could be enhanced by an interaction with the negatively charged histidine-aspartate pair, and it is proposed to be oriented by coordination to the zinc ion. Attack of the water molecule on the carbonyl carbon would produce an oxy-anion intermediate, which is possibly stabilized by the zinc ion and by hydrogen bonding to a nearby tyrosine. The collapse of this intermediate would result in cleavage of the carbon-nitrogen bond, with the nitrogen accepting a proton from the histidine residue, and would thereby produce the observed acetate and lysine products. It is supposed that zinc ion either coordinates the water molecule or stabilizes the oxy-anion intermediate, whereas the other process is carried out by interactions with the amino acid residues in the active site of the enzyme.

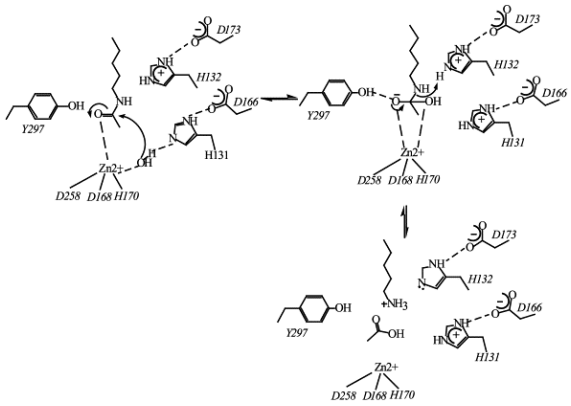


Fig .1.4.3 Proposed Mechanism for HDAC Deacetylation

The HDLP-mediated deacetylation of an acetylated lysine residue is depicted above. A bound water molecule is activated by both the zinc cation and a histidine-aspartate charged relay system to attack the amide bond. The oxy-anion intermediate is stabilized by the zinc cation and an adjacent tyrosine residue. As this intermediate collapses, the carbon-nitrogen bond breaks, and the nitrogen accepts a proton from an activated histidine residue. From Grozinger et al. 2002.

The structure data not only confirmed the published results from biochemical studies but also provide clues for HDAC inhibitor design. The homologous histidine for H132 in HDLP was used as general mutation strategy for mammalian HDACs. Also from proposed catalytic mechanism, H132 mutation might be used to stabilize the catalytic immediate which comprises mutated enzyme and substrates. It might be used as an enzymatic “trap” to fish the acetylated substrates for individual HDACs *in vivo* and *in vitro*. Moreover, comparing the core amino acids in HDLP with mammalian HDACs, there is a striking divergence in the region of tyrosine 91 and this tyrosine residue itself is very poorly conserved among the human HDACs. This is particularly interesting in that tyrosine 91 is positioned on the rim of

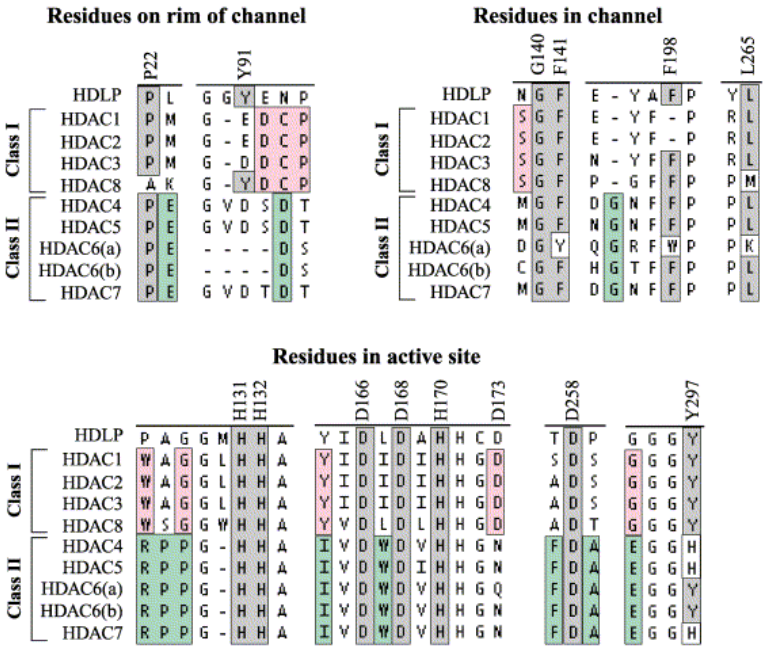


Fig.1.4.4. Partial Sequence Comparison of the HDAC Family Members

The catalytic domain of HDLP was aligned with those of HDACs 1–8, and the regions surrounding the active site were analyzed. The residues of HDLP directly interacting with TSA are labeled and are colored in gray when they are conserved among the other HDACs. Adjacent residues that differ between the class I and class II HDACs are boxed. From Grozinger et al. 2002

the channel and interacts directly with the cap group of TSA, and it is the only residue that shifts its conformation upon TSA binding. Thus, the considerable diversity in the region of the protein might be used to provide specificity for each HDAC members and suggests that it will be possible to develop specific inhibitors by altering the structure of the cap group (Fig.1.4.4).

Besides the catalytic core, another conserved ER motif was found in both eukaryotic HATs and HDACs but not HDLP (Adachi, 2002). By CD spectra analysis and computer prediction, this ER motif has similar secondary structure in both Eas1 and Rpd3. The ER motif in Rpd3 is predicted to be between β8 and α8 and near the active center. In Esa1 the ER motif is located close to the active center and may form a surface of the cleft. Mutation analysis showed the ER motif is required for both HDAC and HAT activities of Esa1 and Rpd3, respectly. In a close look, the ER motif is divided into three regions: ER1, ER2, and ER3. The residues of ER1 and ER2 are involved in catalytic reactions and might be used for recognition of the histone substrates, whereas the residues of ER3 are apparently involved in roles other than catalytic reactions e.g. interaction with DNA. Although the detailed analysis of this ER motif in mammalian HDACs is not done yet, it can be presumed that it may also confer the selective substrate recognition and target for HDAC inhibitors.

Although *in vitro* biochemical data suggested possible mechanism for deacetylation reaction, the detailed in vivo mechanism might be more complicate. The studies on the catalytic reaction of HDAC-6, which contains two hadc domains, suggested that those two domains might have different roles in the catalytic reaction but both are essential for the activity (see result part II). The catalytic inactive mutation in one of the two hdac domains is sufficient to abolish the activity of HDAC-6, even in the presence of another fully normal hdac domain. Furthermore, increase or decrease the distance between two hadc domains in HDAC-6 also affects the enzymatic activity on both tubulin and histone peptide substrates. All of these data suggested the spatial organization of two domains is critical for the HDAC-6 activity. Further tertiary structure analysis of HDAC-6-tubulin/histone complex is necessary to confirm this hypothesis. It is possible that not only HDAC-6 but also other HDACs use the similar catalytic mechanism, in which two hdac domains cooperate to deacetylate *in vivo* substrates. In all identified HDAC-containing complexes there are always more than one HDAC enzyme inside. For example, HDAC-1 and HDAC-2 were both found in Sin3, NuRD, and coREST complexes, in which a "core complex," consisting of HDAC-1/2 and the histone binding proteins RbAp46/48, has been described. Enzymatic activity associated with class II HDACs (e.g. HDAC-4, -5, and -7) is dependent on a multi-protein complex containing HDAC-3 and SMRT/N-CoR (Fischle et al., 2002). So, to efficiently deacetylate *in vivo* acetylated substrates, two hadc domains, either from one enzyme (e.g. HDAC-6) or distinct combinations of different HDACs (e.g. HDAC-1 and HDAC-2) might be necessary. To test this possibility various experiments are going on.

1.4.3. Class I HDACs

The class I HDACs, HDAC-1, HDAC-2, HDAC-3, and HDAC-8, all share homology to the yeast RPD3 gene, are around 400-500 amino acids long, generally localize to the nucleus, and are ubiquitously expressed in many human/mouse cell lines and tissues. All four members have a deacetylase catalytic domain, and HDAC-1 and HDAC-2 have a C-terminal Rb binding motif adjacent to a basic region. All four members have been shown to be sensitive to histone deacetylase-specific inhibitors. Interestingly, the messenger RNAs of HDAC-1, -2, and -3 but not HDAC-8 are upregulated in

response to trichostatin A treatment, suggesting that HDAC inhibitors may trigger an autoregulatory loop that results in a compensatory feedback pathway.

It is now becoming clear that these HDACs are parts of large protein complexes *in vivo* that direct gene-specific regulation of transcription, hormone signaling, the cell cycle, differentiation, and DNA repair. Class I HDACs have been shown to associate with the silencing mediator for the retinoid and thyroid hormone receptor complex (SMRT), the CoREST complex, as well as the Sin3 and Mi-2/NuRD corepressor complexes. HDAC-1 and -2 are part of the core complex along with RbAp46/48. The Sin3 complex consists of this core complex in addition to SAP18 and 30, which aid in stabilizing the protein interactions; and mSin3A, which serves as the scaffold for the assembly of the complex The NuRD complex contains the core complex along with MTA2, CHD3, and CHD4, all of which contain DNA helicase/ATPase domains. HDAC-1 and -2 are also found in the CoREST complex, but unlike the other complexes, neither RbAp46 nor RbAp48 is present, whereas the remaining components are proteins homologous to MTA1 and 2, called CoREST and p110, respectively. Recently, HDAC-3 was shown to form a complex with N-CoR (nuclear receptor corepressor), and this corepressor complex inhibits JNK activation through an integral subunit, GPS2. By contrast, no proteins that partnered with HDAC-8 have yet been reported, and nor has the isolation of an endogenous HDAC-8 complex. Members of the class I HDACs have also been found in association with Rb, DNA methyltransferase 1, TGIF/Smads, glucocorticoid receptor, and Sp1.

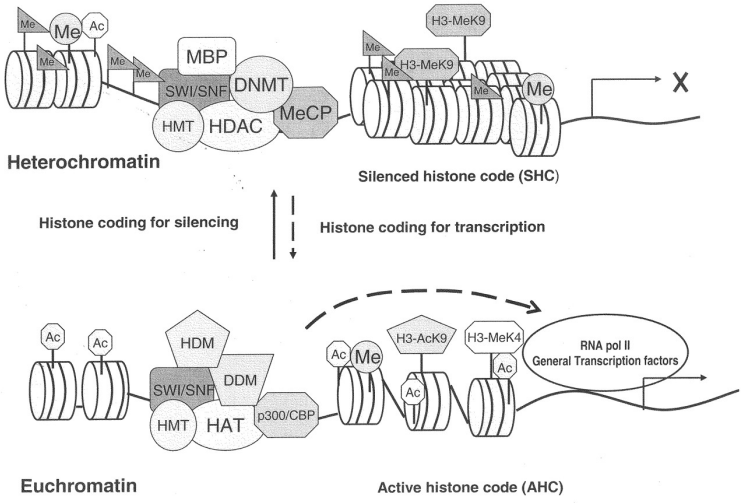


Fig.1.4.5. The role of HDACs in the histone code. The molecular details of the various modifications of the epigenome in relation to the heterochromin and euchromatin can be found in the text. Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; MBP, Methyl Binding Protein; MeCP, methyl-CpG binding protein; H3-meK4, histone H3 methylated at lysine 4; H3-meK9, histone H3 methylated at lysine 9; H3-AcK9, histone H3 acetylated at lysine 9; DNMT, DNA methyl transferase; SWI/SNF, chromatin remodeling multiprotein complex with ATPase activity; Me in a *triangle* denotes DNA CpG methylation; Me in a *circle* denotes histone methylation; Ac, histone acetylation; HMT, histone methyltransferase; p300/CBP, CREB binding protein; DDM, DNA demethylase; HDM, histone demethylase. From Thiagalingam et al., 2003.

Besides synergy among themselves, HDAC-1, HDAC-2, and HDAC-3 cooperate with other chromatin and transcriptional regulators to repress transcription (Fig.1.4.5.). First, these deacetylases collaborate with ATP-dependent chromatin remodelers. Whereas NuRD possesses intrinsic chromatin-remodeling activity required for deacetylation of nucleosomal histones, the Sin3 and N-CoR/SMRT complexes associate with components of the hSWI/SNF complexes.

Second, HDAC-1 and HDAC-2 associate with DNA methyltransferases and transcriptional repressors recruit both HDACs and DNMTs to repress transcription. This link was further strengthened by the discoveries that NuRD and MeCP1 form a chromatographically stable complex and that the chromatin-remodeling complex NoRC targets HDAC-2 and DNMTs for repression of RNA polymerase I-dependent transcription. Third, HDAC-1 and HDAC-2 associate with HMTs. Fourth, HDAC-1 interacts with topoisomerase II, an enzyme that is essential for chromosome condensation and may be involved in gene silencing. Finally, HDAC-1, HDAC-2, and HDAC-3 cooperate with many other transcriptional corepressors.

Recent work has implicated posttranslational modifications of HDACs in regulating HDAC activity and association potential. Galasinski *et al.* (2002) have shown that phosphorylated HDAC-1 and -2 had a small increase in activity relative to that observed in the nonphosphorylated HDACs and that this increase was reversed upon phosphatase treatment. These investigators went on to show that phosphorylation disrupted HDAC-1 and -2 complex formation as well as the interaction between HDAC-1 and mSin3 and YY1 but not RbAp46/48. Though HDAC1 has been shown to be phosphorylated by CK2, cAMP-dependent protein kinase, and protein kinase G *in vitro*, HDAC-2 is uniquely phosphorylated by CK2. This HDAC2 phosphorylation promotes enzymatic activity and regulates complex formation, but has no effect on transcriptional repression. David et al., (2002) have proposed another mechanism of regulation. They demonstrated that HDAC1 is a substrate for SUMO-1 (small ubiquitin-related modifier) modification and that mutations in the target residues reduced transcriptional repression without affecting the ability of HDAC-1 to associate with mSin3. These observations suggest that SUMO-1 modification regulates the biological effects of HDAC-1 by potentiating its histone deacetylase activity.

1.4.4. Class II HDACs

Class II HDACs are homologous to yeast HDA1 and can be subdivided into two subclasses, IIa (HDAC-4, -5, -7, and –9 and its splicing variant MITR) and IIb (HDAC-6 and HDAC-10), based on sequence homology and domain organization. Class II HDACs have been shown several different characteristics comparing with Class I HDACs: Whereas most of Class I HDACs are ubiquitously expressed, the class II HDACs show tissue- and cell-specific expression, with the highest levels being found in heart, brain, thymus, and skeletal muscle. Class II HDACs shuttle between the nucleus and cytoplasm while class I HDACs are mostly localized in the nucleus. They are twice as large (~1,000 amino acids) as the class I members; most have a C-terminal catalytic domain, except for HDAC-6, which has a second catalytic domain in the N-terminal. In addition, HDAC-10 has a N-terminal catalytic domain and a C-terminal pseudorepeat that shares homology with the catalytic domain.

HDAC-4, -5, -7 and -9 contain a highly conserved C-terminal catalytic domain (~420 amino acids) homologous to yHDA1 and an N-terminal domain with no similarity to other proteins. MITR, a splice variant of HDAC-9, consists of only its N-terminus without hdac domain. Class IIa HDACs have also been shown to be part of larger multiprotein complexes. HDAC-4 and -5 associate with HDAC-3 and form a complex with N-CoR and SMRT. The association with HDAC-3 has been shown to be regulated by 14-3-3 protein. A recent study also demonstrated that the catalytic domain of HDAC-4 interacts with HDAC-3 through N-CoR/SMRT. The authors of this study suggest that class II HDACs regulate

transcription by bridging the SMRT/N-CoR-HDAC3 complex and select transcription factors independently of HDAC activity. A common N-terminal extension in HDAC-4, -5, and -7 allows them to interact with the MEF2 family of transcription factors once they translocate from the cytoplasm to the nucleus. These interactions play an important role in activating muscle-specific genes and differentiation in both smooth and skeletal muscle. Class II HDACs have also been reported to interact with the C-terminal binding protein (CtBP) and to repress MEF2-mediated transcription.

Class IIb HDACs are characterized by duplicated HDAC domains, although this duplication is only partial in the case of HDAC-10. Class IIb HDACs also show some degree of tissue-specific gene expression: HDAC-6 is predominantly expressed in testis, and HDAC-10 is expressed in liver, spleen and kidney. Different splice variants are observed for HDAC-6 and HDAC-10, as shown for HDAC-3, -7 and -9, suggesting an additional level of regulation by RNA processing. The enzymatic activities of HDAC-6 and HDAC-10 are more resistant to trapoxin and sodium butyrate than those of class I and class IIa HDACs. Deletion of the second incomplete catalytic domain of HDAC-10 restores its sensitivity to both sodium butyrate and trapoxin, suggesting that the two HDAC domains functionally interact. Class IIa HDACs are predominantly localized in the cytoplasm but also able to shuttle between nucleus and cytoplasm. The cytoplasmic location of both HDAC-6 and HDAC-10, unlike that of class IIa HDACs, is insensitive to leptomycin B. The subcellular localization of HDAC-6 is dependent on a strong nuclear export signal (NES1) in the N-terminus of the protein. Several putative export sequences were identified in HDAC-10, but it is not known whether they function as true export signals.

The *in vivo* roles of class II HDACs are most still unclear. Recently, HDAC-7 was shown highly expressed in CD4+CD8+ double-positive thymocytes. HDAC-7 represses the expression of Nur 77, an immediate-early gene coding for an orphan steroid nuclear receptor responsive to TCR activation, by interacting with MEF2D in the nucleus and plays an important role in the regulation of Nur77 expression and apoptosis in response to T cell activation (Dequiedt et al., 2003). The role of class II HDACs in cardiac hypertrophy and heart failure has also been elucidated recently by Olson's group (Zhang et al., 2002). They showed that class II HDACs, which repress MEF2 activity, are substrates for a stress-responsive kinase specific for conserved serines that regulate MEF2-HDAC interactions. Signal-resistant HDAC mutants lacking these phosphorylation sites are refractory to hypertrophic signaling and inhibit cardiomyocyte hypertrophy. Conversely, mutant mice lacking one of the class II HDACs, HDAC-9, are sensitized to hypertrophic signals and exhibit stress-dependent cardiomegaly. Although the *in vitro* experiments suggest HDAC-4 and -5 might be involved in the development of skeletal muscle, their *in vivo* functions are still being investigated in mouse models.

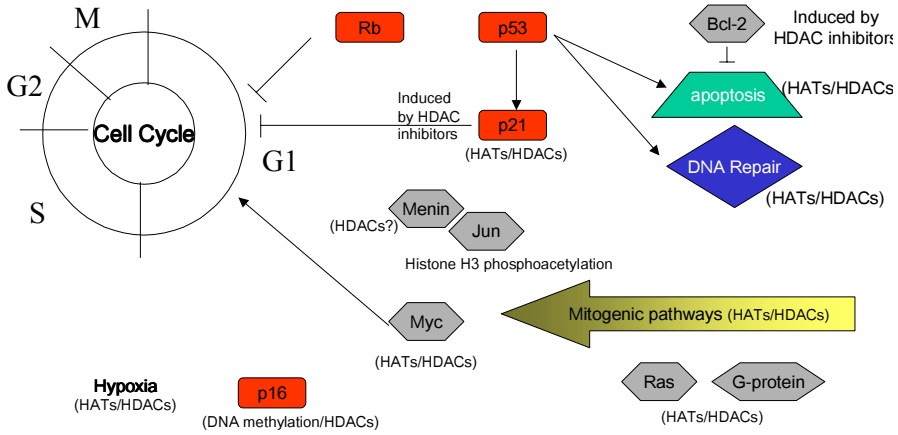
1.4.5 HDAC inhibitors and clinical aspects of HDACs

HDAC as pharmacological target

Aberrant regulation of gene expression is a hallmark of many forms of cancer. Histone acetylation/deacetylation is tightly coordinated with transcription regulation, differentiation, and cell cycle progression in mammalian cells. Aberrant histone acetylation patterns can alter differentiation pathways or disrupt cell cycle checkpoints relevant to the onset or maintenance of cancerous stages (Fig.1.4.6.). More and more *in vivo* evidences have shown the deregulation of histone acetylation machinery (mutations in a HAT and/or aberrant recruitment of HDACs) during cancer development and

genetic diseases. For example, inappropriate repression of genes required for cell differentiation has been linked to several forms of cancer and, in particular, to acute leukemia. In a frequent form of AML, the translocation t(8;21) results in the AML1–ETO fusion protein, in which the transactivation domain of transcription factor AML1 is replaced by the almost complete ETO protein. The translocation partner ETO has been reported to interact with N-CoR, SMRT, mSin3 and HDACs. Thus, the fusion protein recruits corepressor complexes containing HDAC activity instead of recruiting coactivators. This results in a differentiation block, contributing to tumor development. This is also supported by the finding that inhibitors of corepressor-associated HDAC activity are capable of overcoming the differentiation block in cells containing the PLZF–RAR fusion protein. In non-Hodgkin's lymphoma, translocations and point mutations frequently lead to overexpression of the BCL-6 oncogene product, which has been implicated in the control of B-cell proliferation. Because BCL-6 is a transcription factor that interacts with the corepressors N-CoR and SMRT, aberrant repression, as occurs in acute leukemias, could also be involved in the pathogenesis of non-Hodgkin's lymphoma. The overexpression and/or inactivation of histone modifying proteins have also been correlated to the altered repression in cancer.

Fig.1.4.6. The roles of HAT/HDACs and the targets of HDAC inhibitors.



The reversal of aberrant gene repression could thus benefit a wide range of malignancies. Any components of the repression complex; DNA, transcription factor, cofactor, or modifying enzyme might be a therapeutic target. While protein-protein and protein-DNA interactions are considered very difficult to inhibit pharmacologically, enzymes regulating histone modifications represent attractive chemotherapeutic targets. In particular, histone deacetylase inhibitors induce differentiation, cell cycle arrest, or apoptosis in transformed cell lines, processes most like involving transcription activation. Moreover, normal cells were found to be less sensitive to effects caused by inhibition of HDACs (Kim et al., 1999). Importantly, several HDAC inhibitors have demonstrated tumor growth suppression *in vivo*. Several experiments employing rodent models for cancer have shown that HDAC inhibitors significantly reduce the growth of tumors and metastases *in vivo* . Notably, several of the compounds tested lacked considerable side effects at doses where tumor growth was inhibited markedly. The effects of HDAC inhibitors in animal model systems clearly provide

evidence that they preferentially affect tumor cells, rather than causing general toxicity to individual organs or to the whole organism. Thus, clinical studies for a variety of cancer forms appear to be well justified.

The clinical benefits of HDAC inhibition and their implications for re-differentiation therapy are currently being investigated in several locations. At least four HDAC inhibitors (phenylbutyrate, SAHA (hydroxamic acid-based HDAC inhibitor), pyroxamide, and the cyclic tetrapeptide FR901228) are in clinical trials as cancer therapeutics. Phenyl butyrate was administered orally to treat solid tumors and by continuous infusion in the myelodysplastic syndrome (MDS). A few patients in each trial showed evidence of stable disease or mild improvement. A trial of depsipeptide achieved therapeutic levels and one patient with cutaneous T cell lymphoma achieved a complete response. Based on the encouraging early clinical outcomes, numerous programs have been established for the discovery of potent and potentially paralog-selective HDAC inhibitors.

Reversal of transcriptional repression by HDAC inhibition addresses a novel molecular target. Thus, combinations with established therapies can be expected to increase therapeutic efficiency. Combinations of HDAC inhibitors with retinoic acid have been shown to affect overlapping signaling pathways and to cause synergistic anti-tumor effects by potentiating and/or restoring retinoid-induced differentiation of transformed cells from APL patients (He et al., 2001). This finding suggests that a combination therapy could help to extend the therapeutic range of HDAC inhibitors to other tumors for which differentiation-inducing agents are available. Furthermore, the combination of established cytotoxic principles with HDAC inhibition would be expected to show additive or synergistic effects.

Classes of HDAC inhibitors

Both naturally occurring and synthetic HDAC inhibitors have been characterized (Johnstone, 2002; Kelly et al., 2002). HDAC inhibitors are structurally diverse, ranging from simple compounds (i.e., butyrate) to more complex agents such as hydroxamic acids (i.e., suberoylanilide hydroxamic acid [SAHA]), cyclic teratpeptides (i.e., depsipeptide), and benzamides (i.e MS-275).

Aliphatic HDAC inhibitors (short-chain fatty acids). In 1977, sodium butyrate was reported to alter the histone acetylation level of cells and was subsequently identified as a HDAC inhibitor. Several other small aliphatic acids, such as sodium phenylbutyrate and valproic acid have since been described as HDAC inhibitors with IC_{50} values in the millimolar range. Presumably, these compounds inhibit HDAC activity by ligating the active site Zn^{2+} ion with carboxylate, although no direct evidence of this mechanism has been reported. Butyrate is of limited clinical benefit for the treatment of cancer because of its non-specificity, low activity, and short serum-half-life.

Trichostain A and analogous hydroxamic acid HDAC inhibitors. TSA, a fungal metabolite, was first identified as a nanomolar inhibitor of HDAC in 1990 and has since been the subject of much study. Various Cell lines treated with TSA show increased levels of histone acetylation, elevated levels of p21 and gelsolin, and undergo growth arrest, differentiation, and/or apoptosis. TSA can also inhibit the tumor growth as well as angiogenesis in different animal models (Kim et al. 2001; Vigushin et al., 2001). SAHA, developed as a terminal differentiation-inducing agent, inhibits HDAC-1 and HDAC-3 *in vitro*, and causes increased histone H4 acetylatlion. Subsequently, growth inhibition and/or

reversion of transformed phenotype in several cell lines were observed. SAHA has demonstrated efficiency in a transgenic mouse model of acute promyelocytic leukemia (He et al., 2001), in CWR22 human prostate xenografts in athymic mice (Butler et al., 2000) and in the N-methyl-N-nitrosourea-induced rat mammary tumor model (Cohen et al., 1999). More recently, SAHA was shown to dramatically improved the motor impairment in a mouse model of Huntington's disease (Hockly et al., 2003). Hydroxamic acids contain a functional group that interacts with critical HDAC zinc atom, a 5-6 carbon aliphatic chain which mimics a lysine side chain, and a hydrophobic cap moiety which interacts with the edge of the catalytic pocket and may play a role in HDAC selectivity (Grozinger and Schreiber, 2002). In contrast, HDAC inhibitors with lower potency such as phenylbutyrate and valproic acid possess an acyl group to contact the catalytic zinc ion but cannot make significant contact with the catalytic pocket due to their very short side chains.

Cyclic tetrapeptides. Trapoxin (TPX) is a fungal product that can induce morphological reversion of transformed NIH3T3 fibroblasts. Removing an epoxide group in trapoxin completely abolished the inhibitory activity, which suggests that trapoxin binds covalently to the histone deacetylase via the epoxide group. Trichostatin A reversibly inhibits HDACs, whereas trapoxin causes inhibition by irreversible binding to the HDAC. However, they have been shown to induce nearly identical biological effects on the cell cycle and differentiation (Yoshida and Horinouchi, 1999; Yoshida, et al., 2001).

Benzamides (e.g. MS-275). Saito, Suzuki and co-workers (Saito et al., 1999; Suzuki et al., 1999) described a series of benzamides, from which MS-275 was identified as a micromolar inhibitor of HDAC. It was suggested that 2-sustituted amides interact with the HDAC active site $Zn2^{+}$ ion. Oral administration of MS-275 to nude mice bearing human tumor xenografts resulted in the inhibition of tumor growth with minimal weight loss. MS-275 is reported to be in phase II clinical trials.

The mechanisms of anti-tumor effects of HDAC inhibitors

Although HDAC inhibitors have been shown effective in many different cell lines, the mechanisms are still unclear. The simplest explanation of these effects is that the inhibition of the HDAC activity primarily target transcription to induce their biological effects. In the variety of cell lines studied, synthesis of $p21^{WAF1}$, a cyclin-dependent kinase inhibitor, has been found to be induced most consistently. This could explain the G_1 arrest and G_2/M block that is frequently observed in cells treated with HDAC inhibitors. Other genes exhibiting altered expression patterns are mainly factors regulating apoptosis and the cell cycle, molecules with immunological functions, and factors relevant to tumor development. Thus, changes in cell-cycle regulation and cell survival, in addition to enhanced immune surveillance, might contribute to the successful treatment with HDAC inhibitors seen *in vivo*. Stringent causal links between altered gene expression and observed effects on proliferation or cell survival have, however, not been established in many experimental systems employed so far.

The effect of HDAC inhibitors might be not only related to direct effects on gene expression. For example, histone acetylation in heterochromatin is tightly regulated during S phase, and disruption of this process triggers cell cycle arrest within G_2/M. The HDAC inhibitor-associated G_2 checkpoint may be related to HDAC inhibitor-mediated hyperacetylation of the centromere, allowing release of heterochromatin proteins resulting in abnormal chromosomal segregation and

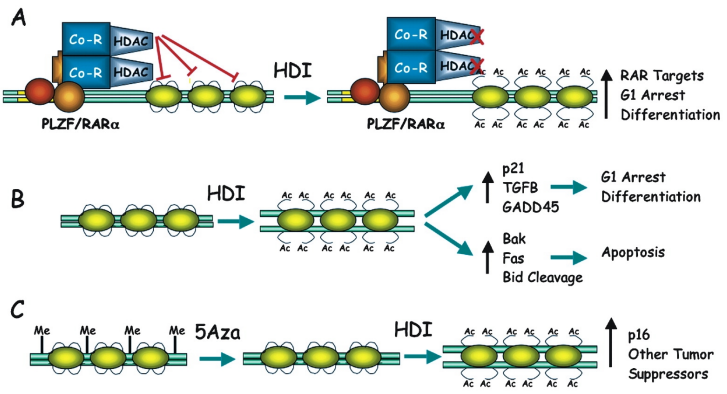


Fig.1.4.7. Transcriptional models of the anticancer actions of HDAC inhibitors.
A: HDAC inhibitors Reverse the repressive effects of fusion transcription factors on target genes.
B: HDAC inhibitors induce p21WAF1/CIP1 to cause G_1 arrest and differentiation. Proapoptotic genes may be induced as well.
C: HDAC inhibitors in combination with 5-Aza reactivate silenced tumor suppressor genes.
From Johnstone and Licht, 2003.

nuclear fragmentation (Taddei et al., 2001). In cancer cells, the HDAC inhibitor-associated G_2 checkpoint can be lost, leading to aberrant mitoses and apoptosis (Qiu et al., 2000). Moreover, as the number of non-histone substrates of acetylation/deacetylation is increasing, they also must be considered as targets of HDAC inhibitors. For example, the acetylation and deacetylation of p53 have been extensively studied. HDAC inhibitors could modulate the function of p53, thereby activate the intrinsic apoptotic pathways. A recent study using dominant-negative p53 suggested that HDAC inhibitor-mediated apoptosis required p53 (Henderson et al., 2003). Also tubulin acetylation has been identified as substrates of HDAC-6. Overexpression of HDAC-6 could promoter the chemotactic cell movement, which might play important roles in tumor metastasis and homeostasis.

Selectivity and specificity of HDAC inhibitors

Although more and more HDAC inhibitors have been shown to have pharmacological activity and are being explored in clinical trials, the selectivity and specificity are still the most important issues that might block the further development. First of all, most of known HDAC inhibitors do not show selectivity of specific HDAC but normally rather inhibit all the HDACs. Currently several HDAC inhibitors are in clinic trials (Kelly et al., 2002) for anti-tumor therapy, however, most of these have not been tested for selectivity versus individual purified HDACs. Actually, many, if not all of these compounds inhibit multiple HDACs. Since different HDACs express in different tissues and/or have different substrates and regulation, the non-selectivity of HDAC inhibitors makes the targeting unclear and may produce possible side-effects. Moreover, even one specific HDAC might well have different substrates therefore being involved in different functions. For example, HDAC-6 is involved in at least three different, possibly also independent, biological pathways: use histone as substrates to mediate gene silencing; use tubulin as substrates to regulation the microtubule dynamics; involved in protein ubiquitination and traffic regulation. Therefore, development of new generation of HDAC inhibitors needs both target/substrate specificity and pathway specificity.

Attempts have been tried to compare the distinct inhibition profile of known inhibitors and to screen HDAC-specific inhibitors. Furumai et al.(2001) showed that CHAP has a preference for HDAC-1 and HDAC-4 over HDAC-6. Recently Hu et al. (2003) also showed that MS-27-275 preferentially inhibited HDAC-1 (IC_{50} value of approximately 0.3 microM) versus HDAC-3 (IC_{50} value of approximately 8 microM) and had no inhibitory activity toward HDAC-8 (IC_{50} value >100 microM).) whereas TSA is potent for all of three HDACs. They also identified several novel HDAC inhibitors that preferentially inhibit HDAC-1 or HDAC-8 using purified recombinant human HDACs. Interestingly, a selective HDAC-6 inhibitor, tubacin, has been identified and seems to specifically increase tubulin acetylation while it has not effect on histone acetylation, TSA-inducible gene expression and cell cycle progression, at least in certain cell types (Haggarty et

al., 2003). In contrast, FK901228, a HDAC inhibitor undergoing phase I clinical trial for cancer, showed strong inhibition for HDAC-1 and HDAC-3 but weak activity toward HDAC-4 and -6 (Furumai et al., 2002).

Genomic approaches are also used to identify the target genes of HDAC inhibitors. Gene profiling studies indicate that HDAC inhibitors modulate only 4%~12% of genes and surprisingly, a similar proportion of genes are activated and repressed, although often with different kinetics. Many of the same genes are regulated by structurally diverse HDAC inhibitors, suggesting that certain loci are in a highly plastic state and the inhibitors can converge on common targets. In colon cancer cells, TSA and butyrate regulated a similar set of genes with different kinetics, correlating with the more rapid induction of histone acetylation by TSA (Mariadason et al., 2000). SAHA and TSA induced a similar set of genes in breast cancer cells while MS-275, which had no effect on tubulin acetylation, regulated a different set of genes, underscoring the possible importance of nonhistone targets of HDAC inhibitors (Glaser et al., 2003).

1.4.6 The intrinsic substrate specificity of HATs and HDACs

Early biochemical and immunocytological studies suggested that histone acetylation is remarkably nonrandom. Particular histones and particular sites within the histones become acetylated under distinct physiological conditions. To set up and maintain this specific acetylation patterns needs multi-level regulations of the specificity. First of all, the transcription factors and other sequence-specific DNA-binding and/or chromatin-interacting factors target HATs and HDACs to certain locus. Moreover, the components in HAT/HDAC complexes can modulate the activity and substrate specificity. Also, posttranslational modifications such as phosphorylation and sumoylation play a role in regulating the specificity of HDAC activity. Finally, the intrinsic substrate specificity of HATs and HDACs might play critical roles. One well-known example of this specificity is the Lys5/Lys12 pattern of H4 di-acetylation that occurs during histone synthesis and deposition in many organisms. Another is the acetylation of H4 at Lys16 that is associated with dosage compensation on the Drosophila male X chromosome. Such observations gave the idea that specific acetylation patterns might directly reflect the substrate specificity of individual HATs and HDACs targeted for particular processes.

Determination of the *in vitro* and *in vivo* substrate specificity of individual HATs in the last few years supports this idea. For example, yeast Hat1 catalyzes Lys5- and/or Lys 12-specific acetylation of H4, consistent with the pattern of H4 di-acetylation associated with histone deposition. Recombinant MOF or native MOF complexes selectively acetylate Lys16 in H4, consistent with the *in vivo* pattern of Lys16 H4 acetylation associated with dosage compensation. Interestingly, the yeast NuA4 complex, which contains the MOF homologue Esa1p as a catalytic subunit, exhibits a different specificity, with a strong preference for acetylation of H4 and H2A *in vitro*. Also, in contrast to recombinant MOF, NuA4 is able to acetylate all four lysines in H4 (K5, K8, K12, and K16). Although the substrate specificities of many HATs are still being defined *in vitro* and *in vivo*, the nonrandom nature of HAT substrate and site specificity observed so far may help to explain why there are so many HATs. Understanding the precise functions of discrete patterns of acetylation, and determining to what extent specific HATs work together or in isolation to create specific histone codes remain a significant challenge for future studies.

On the other side, the substrate specificity of HDACs has not been investigated well, but this might be the next key issue in searching the targets for HDAC inhibitors. The specific recruitment of HDACs onto the certain genomic region

can explain the region specific general deacetylation but cannot be used to explain the specific deacetylation of certain specific sites of histone tails. Although few *in vitro* studies on recombinant or immunoprecipitated mammalian HDACs showed that they could deacetylate all the acetylation sites on purified core histones, it is still very likely that *in vivo* each HDACs might have intrinsic difference on certain histone acetylation sites. This hypothesis is also supported by the sequence differences in each hdac catalytic domains of different HDACs (see chapter 1.4.2.).

Yeast, because of its small size and the availability of its genome sequence, provides the best model for recent genome-wide approaches for the comprehensive determination of the functions of HDACs and HATs. Even in a simple organism such as yeast, there are numerous HATs and HDACs. For example, yeast cells contain as many as 10 different HDACs that include a related family containing Rpd3, Hda1, Hos1, Hos2 and Hos3, Hst1p, 2p, 3p, and 4p, which are related to the NAD-dependent HDAC, Sir2p. Recent studies from expression microarrays, acetylation microarrays, and binding microarrays on yeast HDACs provide the insight of diversities between individual HDACs. First of all, the expression microarray was used to identify genes with altered transcription from yeast strains that have different HDAC mutants (Bernstein et al., 1999). Bioinformatic analysis of expression profiles of rpd3, sir2, and hda1 mutants, suggested that each HDAC influences distinct cellular processes. For example, HDA1 is involved in the regulation of carbon metabolite and carbohydrate utilization and transport; in addition to mediating silencing at mating loci, rDNA repeats, and telomeres, SIR2 appears to repress amino acid biosynthesis genes; RPD3 might directly or indirectly influences expression of cell cycle-regulated genes. To correlate the expression data with acetylation pattern and overcome the possible artifacts due to indirect effects, Robyr et al. (2002) used histone acetylation arrays to identify the intergenic regions at which acetylation is increased when yeast HDACs were deleted. The acetylation microarrays have shown that Rpd3 and Hda1 are principal deacetylases in yeast, affecting numerous promoters throughout the genome but with little overlap between promoters, which suggests existing specific and complex targeting mechanisms. Hos1 and Hos3 are required primarily for deacetylating the ribosomal DNA locus, but it is not known whether they are repressors of rDNA transcription. Hos2 differs from the other deacetylase as recent data show that it deacetylates the coding region of active genes and functions as a general activator of transcription. Moreover, using a battery of antibodies that recognize specific acetylated lysine resides that are representative substrates for a particular HDAC helps to identify the intrinsic substrate specificity of each HDACs. For instance, Rpd3 preferentially deacetylates histone H4 acetylated at lysines 5, 8, or 12, whereas the Hda1 complex only deacetylates Lys residues in histone H3 and H2B, but not the H4 or H2A (Wu et al. 2001). Finally, the localization of Rpd3 to specific chromosomal loci was checked by the binding array (Kurdistani et al., 2002) and was used to delineate the direct effects of a deletion of RPD3 from those that are indirect and the define the role of various members of the Rpd3 complex in its binding to chromatin. Together, these findings have revealed the 'division of labour' among the five different HDACs, as each deacetylase regulates the acetylation state of a selected group of genes or regions of chromatin (Fig.1.4.8.).

On the other hand, from data of acetylation arrays, HDACs might be classified into global deacetylases and targeted deacetylases. Or similar as DNA DMNTs, the deacetylases also can be divided into "maintenance deacetylase", which act in a global and untargeted manner and might be involved in the maintenance of global acetylation pattern, and "*de novo* deacetylases" that dynamically control the acetylation pattern on specific loci by targeted specific recruitment. For example, deletion of yeast RPD3 or HDA1 results in increased acetylation of their target promoters and nucleosomes in a 4.25-kb region that includes the acid phosphatase PHO5 promoter and coding regions, to which neither Rpd3 nor

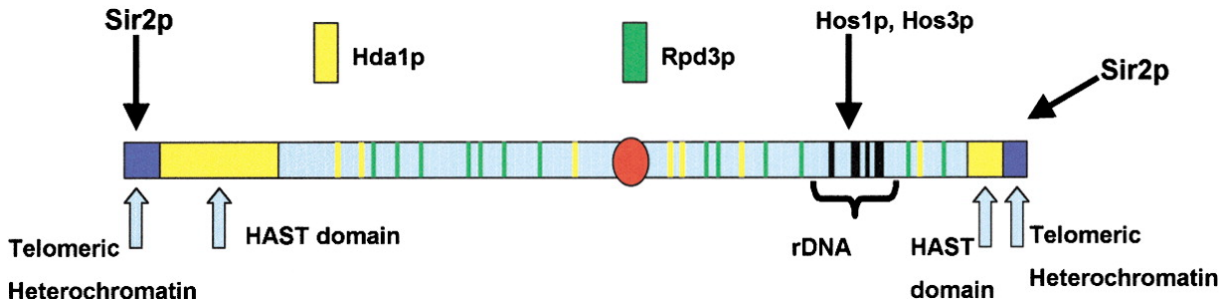


Fig.1.4.8. Division of Labor among HDACs. Schematic representation of an idealized yeast chromosome emphasizing the division of labor among five different HDACs. Differently colored bars represent intergenic regions that are hyperacetylated in cells lacking the indicated HDAC. Not shown are the ribosomal gene promoters that are hyperacetylated after inactivation of Hos2p. Red oval indicates the centromer. From Peterson 2002.

Hda1 is seemingly targeted (Vogelauer et al., 2000). The binding array (Kurdistani et al., 2002) also showed that at most loci, Rpd3 targeting occurs independent of Ume6, the only known recruiter of Rpd3 in yeast. It also has been suggested that yeast Esa1 might have a role in global acetylation (Reid et al., 2000). The enzymatic activities of these HDACs and HATs over large chromosomal domains might account for the rapid turnover of acetylation in yeast, indicating that the state of histone acetylation is in a continual genome-wide flux (Waterborg, 2001). Interestingly, it has been reported that HATs and HDACs might interact as parts of a single multiprotein complex in mammalian cells (Yamagoe et al., 2003). The presence of HAT-HDAC complexes with opposing acetylation and deacetylation activities would be consistent with the roles of these enzymes in establishing dynamic global acetylation patterns. The determinant for global acetylation/deacetylation is not clear yet. But possibly, it may come from some forms of translated epigenetic code.

In mammalian systems, there are even much more HATs and HDACs than in yeast. Therefore, to dissect the specificity of each HAT/HDAC is the most difficult and important key issue to understand the *in vivo* functions and to help the pharmacological targeting. Ideally, as in yeast, the genome-wide analysis of HDAC function should include a combination of gene expression profiles (where changes in gene expression have been analyzed in HDAC mutants), enzyme activity/acetylation maps, and enzyme binding maps (where the binding sites for each HDACs are determined).

Moreover, there are still something might be misleading within published array data. First of all, the *in vivo* functional deacetylase/acetyltransferase activity units are HAT/HDAC containing complexes but not individual enzymes. In many HAT/HDAC complexes there are more than one HAT/HDAC whereas same HDAC/HAT can form distinct complexes by changing the components of the complexes. All of these make the specificity of individual HDAC/HAT mutation on genomic acetylatlion more and more complex. For example, where HDAC-1 was found to associate with HDAC-2 in many HDAC-containing complexes, the functional consequences of inactivation of both HDAC-1 and HDAC-2 cannot be simply explained by the combination effect from individual inactivation of HDAC-1 or HDAC-2. In what extent of the overlapping phenotype between HDAC-1 and HDAC-2 inactivations is very interesting. Second, genetic analysis of HDAC functions in *Drosophila* and *C. elegans* suggested that loss of function, point mutations, and different gene dosage might lead to different physiological phenotypes. For example, point mutation H141-A in human HDAC-1 catalytic domain leads to the loss of the activity, whereas it does not affect the interaction with mSin3A and RbAp48 (Hassig et al., 1998). Therefore this mutant can serve as a dominant-negative mutant to toxicify the HDAC-1 containing complexes. Whereas in the loss of function case, in the absence of HDAC-1, other HDACs might be able to partially

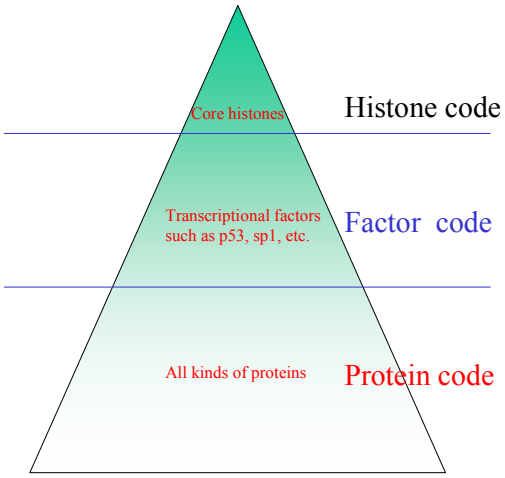
compensate the role of HDAC-1 in HDAC-1 containing complexes. So it is still interesting to check the expression, acetylation, and binding arrays in those kinds of loss of complex function mutants and compare the result with simple loss of deacetylase.

1.5. Protein code and non-histone substrates of HATs/HDACs

1.5.1 Evolution of protein code----more than histones

Modification on the primary protein sequences extends the range of possible molecular structures beyond the limits imposed by the 20 encoded amino acids, and, if reversible, gives a means of control and signaling. There are many different kinds of protein posttranslational modifications on protein including phosphorylation, acetylation, methylation, ubiquitination, etc. The general effects of those modifications on protein structure and function are generally depending on two ways: the covalent modification can modulate the protein structure by changing the charges and/or spatial organization; modified amino acids can serve as new interaction targets to regulate the protein-protein and/or protein-DNA/RNA interaction. For example, protein phosphorylation and dephosphorylation have been extensively investigated and play the central roles in growth control and cellular signaling. Many metabolic and regulatory proteins can be phosphorylated and dephosphorylated, which lead to the changing between active and inactive status of the protein. Many phosphorylation-dependent interactions have also been identified with the discovery of various phospho-binding domains.

Fig.1.5.1. Evolution of protein code.



Many eukaryotic as well as prokaryotic proteins can be acetylated, both co- and posttranslationally. At least for eukaryotic proteins, acetylation is the most common covalent modification out of over 200 types that have been reported. Amino-terminal acetylation is one of the most common protein modifications in eukaryotes, occurring on approximately 85% of eukaryotic proteins, but is rare for prokaryotic proteins. It occurs co-translationally on the bulk of acetylated eukaryotic proteins, posttranslationally on prokaryotic ribosomal proteins and on processed eukaryotic regulatory peptides. Furthermore, ε-lysine acetylation occurs post-translationally on histones, high mobility group

(HMG) proteins, transcription factors, nuclear receptors, and α-tubulin. Unlike the situation for histones and other proteins with acetylated ε-lysine residues, amino-terminal modifications are irreversible. The biological significance of amino-terminal modification varies; some proteins require acetylation for activity or stability whereas others that are acetylated do not absolutely require the modification. The important feature of acetylation of ε-lysine residues is that it is reversible, which makes acetylation as a potential signaling. Besides histones, which are most studied acetylated proteins, more and more proteins are identified to be regulated by ε-lysine acetylation/deacetylation. Acetylation affects many protein functions, including enzymatic activity, stability, DNA binding, protein-protein interaction, and peptide-receptor recognition, and occurs on numerous and diverse proteins.

The evolution from "histone code" to "factor code" started after the identification of non-histone substrates by histone deacetylases and acetyltransferases in chromatin complexes. Although HATs and HDACs are, by definition, able to work on histones, they are capable of acetylating/deacetylating other proteins as well. Several nonhistone substrates have been identified *in vitro*, particularly for p300/CBP. These include transcriptional activators (e.g. E2F1, p53, EKLF, GATA1, HMGi, TCF, HNF-4, MyoD), other coactivators (ACTR), basal transcription factors (TFIIEβ, TFIIE), structural proteins (tubulin), polyamines, and one family of proteins involved in nuclear import (importin α family). In addition, PCAF can autoacetylate, facilitating intramolecular interactions with its bromodomain that may be important to the regulation of its HAT activity. A screen of a large set of proteins involved in different cellular processes resulted in the identification of two nuclear import proteins, Rch1 and importin-α7, as substrates for the acetyltransferase CBP. Both p300 and CBP can mediate acetylation of Rch1 and importin-α7 *in vivo*, most likely in the nucleus. The acetylated residue, ε-Lys22, lies within the binding site in Rch1 for the other nuclear import factor, importin-β, and acetylation of the site promotes interaction with importin-β *in vitro*. Thus, it is possible that nuclear import may be regulated by acetylation, mediated by the p300/CBP HATs.(Bannister et al. 2000). Comparison of the sites acetylated by PCAF and p300/CBP within these proteins and histone reveals, at best, a weak consensus recognition motif, GKXXP. Thus, it is not yet possible to identify putative acetylation sites within a protein simply by sequence analysis. The acetylation of these proteins can alter their ability to bind DNA (E2F1, p53, EKLF, GATA1, HNF-4, Sp1), to interact with other proteins (TCF, ACTR, HNF-4), or to remain in the nucleus (HNF-4). Regulation of such activity through reversible acetylation/deacetylation has led to the intriguing idea that acetylation/deacetylation cascades may transduce signals within cells, as many kinase/phosphotase cascades involved in signal transduction. Following the identification of acetylated proteins other than in chromatin complexes, the concept of "protein code" emerged. Similar as histone code, different modifications on any proteins could interact with each other and therefore provide the "epi-proteomic regulation". The major difference between "protein code" and signal cascade is it is depending on the synergistic effect of the different modifications and contains more complex control.

1.5.2 Factor code----acetylated non-histone substrates in transcription regulation

Numerous histone acetyltransferases (HATs) and histone deacetylases (HDACs) are intimately associated with various components of the core transcriptional machinery. For example, the TAF1 subunit of TFIID has been shown to contain HAT activity, suggesting that it may function to acetylate transcriptional components in addition to histones (Mizzen et al., 1996). Accordingly, the addition and removal of acetyl groups to and from transcription factors may play roles in transcriptional regulation as important as histone acetylation and deacetylation. For example, the HAT-containing

coactivator p300 has been documented to acetylate the p53 tumor suppressor protein thereby stimulating its function as an activator (Barlev et al., 2001; Gu and Roeder, 1997). Thanos and colleagues have also demonstrated that the acetylation state of HMGI(Y) is critical for regulating the assembly and disassembly of an enhancesome at the IFN-β gene, further underscoring the importance of nonhistone acetylation in transcriptional regulation (Munshi et al., 2001). Recently, Bereshchenko et al. (2002) reported the ability of p300 to acetylate the transcriptional repressor BCL6. In this case, acetylation inactivates the ability of BCL6 to recruit HDACs and function as a potent repressor of transcription. Together, these studies illuminate the ability of HATs to either stimulate or inactivate the function of nonhistone regulatory proteins.

Acetylation, together with other modifications (ubiquitination, sumoylation, methylation), of lysine residues within histones, histone-like proteins, and non-histone proteins (such as transcription factors) has recently emerged as a major mechanism used by the cell to overcome repressed chromatin states. As the substrates of acetylation/deacetylation extend from histone tails to transcription factors, several new names, such as "factor code", "factor acetyltransferase", came out. In this new concept, the combined acetylation/deacetylation of both histone tails and transcription factors contributes to more controlled transcription regulation. First of all, on the specific promoter, recruitment of HATs or HDACs can regulate both histone acetylation and factor acetylation thereby both the targets of transcription factors and themselves. Moreover, both acetylation of histones and factors provides the interaction of plat form for bromodomain, which links both acetylation events. Finally, which is the most important, a combinatorial network of multiple modifications can regulate gene expression either in a synergistic or antagonistic fashion.

Lysine modification as a key switcher for transcription regulation

Since ubiquitination, sumoylation, and acetylation can all occur on lysine residues, transcription factors can potentially undergo a cascade of modifications that modulate their function. This obviously complicates the contribution of each individual modification, and it will be important to sort out the order and dynamics of multiple modification events on endogenous regulatory proteins. For example, the major site of Sp3 sumoylation is identical to the major site of acetylation, and both of these modifications have been demonstrated to modulate activation (Braun et al., 2001). Presumably, in such cases one modification may preclude the other or alternatively may be responsible for enhancement of a second modification at a nearby residue. In addition, since most proteins contain many lysine residues, transcription factors may undergo multiple modifications simultaneously or in sequential order, pointing to the possibility of generating complex networks of regulatory events. Sorting out such a molecular switch board, both biochemically and genetically, poses a formidable but necessary task if we are to understand how tissue-specific and gene-selective transcriptional regulation is achieved.

While the modifications described here all target lysine residues, they are inherently quite different in the manner they alter target proteins. While acetylation entails the addition of a relatively small, uncharged acetyl group, addition of ubiquitin and SUMO involves attaching relatively large polypeptides that can significantly add to the mass of target proteins. Not surprisingly, the functional outcomes resulting from these diverse covalent changes can be very different. One can imagine that such biochemically distinct changes mediate important differences in the function of modified proteins. For example, some types of alterations may induce conformational changes while other modifications may

trigger charge surface changes. The net outcomes, however, would be to disrupt or reorganize important protein-protein and protein-DNA contacts. Documenting the biophysical changes in modified proteins should help illuminate the function of these diverse modifications.

Acetylation of p53

p53 is thought of as the cellular gatekeeper in eukaryotic cells. To this, it serves as a critical 'node' of the cellular circuitry (Vogelstein et al., 2000). The activity of p53 as a sequence-specific transcription factor is highly regulated by post-translational modifications, protein-protein interactions and protein stability. For example, the acetylation levels of p53 are significantly enhanced *in vivo* in response to almost every kind of stress, which correlates well with its activation and stabilization induced by stress (Ito et al., 2001). Acetylation of p53 is catalyzed by p300/CBP and PCAF and localizes in multiple lysines (K320, K370, K371, K372, K381, and K382) on the carboxy-terminal regulatory domain (Gu & Roeder, 1997; Appella et al., 2001). Numerous studies indicate that the carboxyl terminus of p53 acts as a critical regulator of p53 and negatively modulates its transcriptional activation. The acetylation of p53 can dramatically stimulate its sequence-specific DNA-binding activity *in vitro*, possibly as a result of an acetylation-induced conformational change. These acetylation sites are essential for ubiquitination and subsequent degradation of p53 by MDM2. They may also have a significant impact on protein-protein interactions between p53 and transcriptional co-activators such as p300/CBP and PCAF. In deed, p53 acetylation is critical for both the efficient recruitment of these complexes to promoter regions and the activation of p53 target genes *in vivo* (Barlev et al., 2001). On the reverse side, p53 was shown to be deacetylated by either HDAC-1 complex (Luo et al., 2000)or SIRT1 (Vaziri et al., 2001; Langley et al., 2002), which can attenuate its transcriptional activity. The deacetylation of p53 may provide a quick acting mechanism to stop p53 function when transcriptional activation of target genes is no longer needed. Deacetylation could also serve as an important step in MDM2-mediated p53 degradation. Moreover, recent studies on eukaryotic Sir2 proteins suggest that the SIRT1-mediated p53 deacetylation may provide a link between p53 and mammalian longevity (Guarente, 2000). Finally, as the case in histone tails, the relationship between phosphorylation and acetylation applies here as well. It has been shown that phosphorylation of p53 precedes and promotes acetylation (Chao et al., 2000, Sakaguchi et al., 1998). In contrast to histones, the phosphorylation is quite distant from the acetylation sites, since acetylation occurs at the C terminus at lysine residues 320, 373, and 382, while phosphorylation occurs at the N terminus within the activation domain, at residues 33, 37 (Sakaguchi et al., 1998). Thus, in the p53 case, phosphorylation is not likely to be promoting acetylation by improving enzyme interaction with the substrate active site, but rather may be helping to recruit acetylation complexes through direct interaction, which would then result in the acetyltransferase gaining access to both p53 and histones.

1.5.3 Tubulin acetylation/deacetylation

Microtubules are cylindrical cytoskeletal structures that are found in all eukaryotic cells types and participate in a great variety of cellular processes, including mitosis, ciliary and flagellar motility, intracellular transport of vesicles and organelles, and possibly in determining morphology of certain cells. The ability of microtubules to quickly polymerize and depolymerize, a process known as dynamic instability, places regulation of microtubule dynamics at the center of active research. Tubulin proteins, the building blocks of microtubules, are subject to several types of evolutionarily

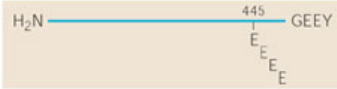
conserved post-translational modifications, including detyrosination, acetylation, generation of Δ2-tubulin, phosphorylation, polyglutamylation, and polyglycylation (reviewed in Westermann and Weber, 2003; MacRae, 1997). Most of these modifications are reversible and all, except acetylation, occur at the highly variable carboxyl termini of tubulin α and β subunits (Table.1.5.1.).

Tubulin acetylation occurs in the primitive eukaryotes *Giardia lamblia*, *Tritrichomonas mobilensis* and *Trypanosoma bruce*, and therefore arose early during eukaryotic evolution. It has since been observed in vertebrates, insects, nematodes and plants, in all of which the acetyl group is attached to the ε-amino group of lysine 40 (LeDizet and Piperno, 1987). Acetylation is mostly associated with stable microtubular structures such as axonemes, and it occurs after microtubule assembly. On the basis of the electron-crystallographic structure, it has been predicted that the modified residue points towards the lumen of the microtubule (Nogales, 1999). Generally, acetylation can happen quickly - almost immediately - and acetylated tubulin therefore does not necessarily demarcate old microtubules. Some correlations have been found between α-tubulin acetylation and microtubule stability. Acetylated microtubules commonly resist drug-induced disassembly but not cold-induced disassembly, although in some cells a subset of acetylated microtubules is cold-resistant. It is still unclear, however, how the intracellular spatial organization of acetylated microtubules is determined.

A partial purification of a tubulin acetyltransferase activity from Chlamydomonas flagella has been reported (Maruta et al., 1986), but the enzyme that is responsible has not been identified. By contrast, two enzymes that catalyze the opposing reaction —deacetylation — have been recently described (Hubbert et al., 2002; Matsuyama et al., 2003 Zhang et al., 2003; North et al., 2003). HDAC-6 interacts with beta-tubulin directly and colocalizes with microtubules. It can deacetylate tubulin and microtubules both *in vitro* and *in vivo*, and suppression of HDAC-6 function by pharmacological inhibitors or by small interfering RNA *in vivo* leads to a marked increase in tubulin acetylation. Moreover, HDAC-6 deficient cells and mice showed tubulin hyperacetylation. Conversely, overexpression of HDAC-6 greatly decreased the level of tubulin acetylation. A second histone deacetylase, SIRT2, which is NAD^+ dependent, has also been identified as a tubulin deacetylase (North et al., 2003), and shows preferential activity towards a tubulin peptide substrate in comparison to a histone peptide substrate. SIRT2 co-immunoprecipitates with HDAC-6 from cell extracts, which raises the possibility that these enzymes cooperate to deacetylate tubulin *in vivo*.

The *in vivo* role of acetylated microtubules in cells remains an important unanswered question. *In vivo*, overexpression of a non-acetylatable α-tubulin variant in *Chlamydomonas* (Kozminski et al., 1993), or complete elimination of tubulin acetylation by site-directed mutagenesis of the usually acetylated lysine residue to arginine in *Tetrahymena* (Gaertig et al., 1995), had no observable phenotype. Also, disruption of the HDAC-6 gene in embryonic stem cells, which led to highly increased tubulin acetylation levels, did not significantly affect cell proliferation or differentiation (Zhang et al., 2003). Although these results indicate that tubulin acetylation/deacetylation is not generally essential for cell survival, it has been reported to affect specialized functions. For example, a role for tubulin acetylation in cell motility has been proposed on the basis that HDAC-6 overexpression increased the chemotactic movement of NIH-3T3 cells (Hubbert et al., 2002), whereas inhibition of HDAC-6 inhibited cell migration (Haggarty et al., 2003). Clearly, knockout and overexpression of HDAC-6 mouse models will be very useful for the identification of the functions of tubulin acetylation *in vivo*.

Table 1 | **Overview of the various tubulin modifications and their proposed functions**

α-tubulin	Modification	Comments	Enzymes	Proposed functions
	Acetylation/deacetylation	Only α-tubulin; marker for stable microtubules	HDAC6, SIRT2	Regulation of cell motility, binding of MAPs to microtubules
	Tyrosination/detyrosination	Reversible; enzyme TTL cloned	TTL	Crosstalk to intermediate filaments; differentiation
	Generation of Δ2-tubulin	Only α-tubulin; marker for stable microtubules	?	Removing tubulin from tyrosination cycle; marking microtubules for polyglycylation?
	Polyglutamylation	α- and β-tubulin; multiple glutamylation sites possible; up to 20 side-chain residues	Nek (Cf)	Centriole maturation and stability; flagellar and ciliary motility; regulation of interaction with MAPs
	Polyglycylation	α- and β-tubulin; multiple glycylation sites possible; up to 30–40 side-chain residues	?	Essential in *Tetrahymena* for: axonemal organization, ciliary motility, cytokinesis (severing of microtubules)
	Palmitoylation	Demonstrated for budding yeast α-tubulin on residue 376	?	Positioning of astral microtubules in budding yeast; interaction with cell cortex?
	Phosphorylation	Better established for β-tubulin on Ser441/444	?	Neuronal differentiation?

Ac, acetate; Cf, *Crithidia fasciculata*; E, glutamic acid; G, glycine; Nek, NIMA (never in mitosis gene A)-related kinase; HDAC6, histone deacetylase 6; MAP, microtubule-associated protein; P, phosphate; SIRT, Sir2 homologue; TTL, tubulin tyrosine ligase; Y, tyrosine.

Table.1.5.1. Overview of the various tubulin modifications and their proposed functions. From Westermann and Weber, 2003

Part II: Results

2.1 HDAC-6 interacts with and deacetylates tubulin and microtubules *in vi*vo.

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HDAC-6 interacts with and deacetylates tubulin and microtubules *in vivo*

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Microtubules are cylindrical cytoskeletal structures found in almost all eukaryotic cell types which are involved in a great variety of cellular processes. Reversible acetylation on the ε-amino group of α-tubulin Lys40 marks stabilized microtubule structures and may contribute to regulating microtubule dynamics. Yet, the enzymes catalysing this acetylation/deacetylation have remained unidentified until recently. Here we report that β-tubulin interacts with histone deacetylase-6 (HDAC-6) in a yeast two-hybrid assay and *in vitro*. We find that HDAC-6 is a microtubule-associated protein capable of deacetylating α-tubulin *in vivo* and *in vitro*. HDAC-6's microtubule binding and deacetylation functions both depend on the hdac domains. Overexpression of HDAC-6 in mammalian cells leads to tubulin hypoacetylation. In contrast, inhibition of HDAC-6 function by two independent mechanisms—pharmacological (HDAC inhibitors) or genetic (targeted inactivation of HDAC-6 in embryonic stem cells)—leads to hyperacetylation of tubulin and microtubules. Taken together, our data provide evidence that HDAC-6 might act as a dual deacetylase for tubulin and histones, and suggest the possibility that acetylated non-histone proteins might represent novel targets for pharmacological therapy by HDAC inhibitors.

Keywords: chromatin/cytoskeleton/deacetylase/HDAC (histone deacetylase)/tubulin

Introduction

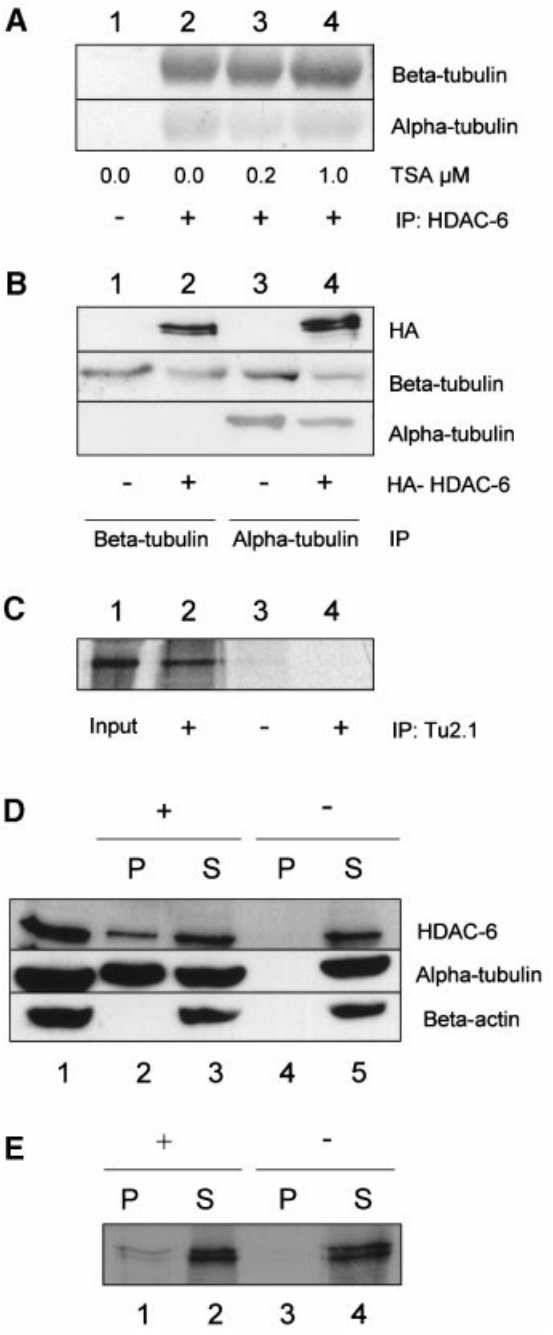
In eukaryotes, the DNA in the cell nucleus is bound by histones and other chromosomal proteins to form a highly organized and compact structure called chromatin. A large body of evidence has demonstrated that chromatin plays a critical role in regulating gene expression. A number of post-translational protein modifications such as acetylation, methylation, ubiquitylation or phosphorylation of histones and other nuclear proteins create a code that orchestrates the organization and function of chromatin (Jenuwein and Allis, 2001). These modifications are interdependent and are under the control of signalling pathways yet to be fully deciphered. Histone acetylation has been shown to affect nucleosome stability and generally is considered to create a chromatin environment conducive to gene activation (Cheung *et al*., 2000). In addition to histones, many other proteins are also acetylated (reviewed by Polevoda and Sherman, 2002). For example, several transcription factors such as p53, E2F1 or c-Jun have been found to be acetylated. In this case, acetylation/deacetylation regulates, for example, the ability of these factors to bind to DNA (E2F1 or p53) or to interact with other proteins (c-Jun). Likewise, some cytoplasmic proteins such as α-tubulin are also known to be acetylated (Piperno *et al*., 1987). Acetylated tubulin is one of the charateristics of stabilized microtubules. The acetylases and deacetylases for non-histone substrates are mostly unknown.

The enzymes responsible for adding or removing acetyl groups on histones were only identified a few years ago. Acetylation of histones is catalysed by proteins that had been studied initially in other contexts, e.g. as transcriptional coactivators, and which are now called histone acetyltransferases (HATs) (Brown *et al*., 2000; Marmorstein and Roth, 2001). Several of these HATs, however, are also able to acetylate non-histone proteins, and the list of new substrates continues to grow. Conversely, histone deacetylases (HDACs) are proteins that catalyse the removal of acetyl groups from histones and thereby lead to gene repression (Narlikar *et al*., 2002). Similarly to the HATs, HDACs may also deacetylate non-histone proteins. In cells, HDACs are often part of large multiprotein complexes that are recruited to promoter sequences through their interaction with specific DNA-binding transcription factors. HDACs have attracted a lot of attention not only for their role in transcriptional control but also because their pharmacological inhibition was found to have pleiotropic effects, such as induction of cell differentiation, arrest of cell growth (Richon *et al*., 2000) or prevention of tumour development in animal models. In addition, HDAC activity was found to be elevated in a number of tumours, potentially leading to deregulation of tumour suppressor genes (Marks *et al*., 2001). Thus, HDACs are considered as valuable targets for cancer treatment, and encouraging results have already been obtained with haematological malignancies (reviewed in Melnick and Licht, 2002). So far, >10 proteins with HDAC activity have been identified in mammalian cells. Based on sequence conservation, they fall into three classes. Class I has homology to the yeast global transcriptional regulator Rpd3 and comprises HDAC-1, HDAC-2, HDAC-3 and HDAC-8. Class II is composed of large proteins such as HDAC-4, HDAC-5, HDAC-6, HDAC-7, HDAC-9 and HDAC-10, all of which show homology to yeast Hda1. Finally, class III proteins have catalytic domains similar to that found in the yeast NAD^+-dependent deacetylase Sir2 (Gray and Ekstrom,

2001; Khochbin *et al*., 2001; Kuzmichev and Reinberg, 2001).

Among the class II members, HDAC-6 is unique in that it has two hdac domains and also a C-terminal zinc finger domain that binds ubiquitin (Seigneurin-Berny *et al*., 2001). HDAC-10 appears to be the closest relative of HDAC-6, but it has only an incomplete second hdac domain (Fischer *et al*., 2002; Guardiola and Yao, 2002; Kao *et al*., 2002; Tong *et al*., 2002). Although several of the class II HDACs have been shown to play a role in transcriptional repression, some like HDAC-4, -5 and -6 have been found to shuttle between the nucleus and the cytoplasm (McKinsey *et al*., 2000a; Verdel *et al*., 2000). For HDAC-4 and -5, this is regulated by association with 14-3-3 proteins (Grozinger and Schreiber, 2000; McKinsey *et al*., 2000b) and, for HDAC-6, which is predominantly cytoplasmic, partial translocation into the nucleus was observed when cell proliferation was inhibited (Verdel *et al*., 2000). In addition, HDAC-6 has not been not found in any known HDAC-containing repression complexes. Thus, HDAC-6 might be functionally distinct from other known HDACs, and in particular might deacetylate substrates other than histones.

Here, we report the identification of β-tubulin as a protein interacting with HDAC-6. We show by a variety of assays that HDAC-6 is a microtubule-associated protein that can deacetylate α-tubulin *in vitro*. Pharmacological inhibition of HDAC-6 activity in culture cells leads to an increase in tubulin acetylation, and transient HDAC-6 overexpression reduces acetylation. Furthermore, using disruption of the HDAC-6 gene in embryonic stem (ES) cells, we provide compelling *in vivo* evidence that HDAC-6 regulates the status of tubulin acetylation. In contrast, global acetylation of histone H3 and H4 is not altered in these cells. Moreover, HDAC-6 was found to be a non-essential protein in ES cells.



Results

Identification of β-tubulin as a protein interacting specifically with HDAC-6

We sought to identify proteins that interact specifically with HDAC-6 and which might help explain the cellular role and regulation of this enzyme. To this end, we set up a

Fig. 1. HDAC-6 interacts with β-tubulin and microtubules *in vivo* and *in vitro*. (**A**) Co-immunoprecipitation assay. NIH-3T3 cells were treated for 6 h with TSA at the indicated concentration in order to inhibit HDAC activity. After cell extract preparation, HDAC-6 was immunoprecipitated with an anti-mouse HDAC-6-specific antibody (lanes 2–4) or with a control antibody (lane 1). The precipitate was analysed by SDS–PAGE and blotted onto nitrocellulose membranes. The filters were probed with antibodies against β-tubulin (TU 2.1, upper panel) or α-tubulin (DM1A, lower panel). (**B**) Co-immunoprecipitation assay in 293T cells. In this case, cells were transfected with an expression vector encoding HA-mHDAC-6 (lanes 2 and 4) or an empty expression vector (lanes 1 and 3). Two days after transfection, cell extracts were prepared and β- or α-tubulin was immunoprecipitated with specific antibodies (lanes 1 and 2 with TU2.1, lanes 3 and 4 with DM1A). The precipitates were analysed by western blotting: the filters were probed with antibodies against HA (upper panel), β-tubulin (TU2.1, middle panel) or α-tubulin (DM1A, lower panel). (**C**) *In vitro* interaction between HDAC-6 and tubulin. Purified bovine tubulin (Cytoskeleton, Inc.) was incubated with *in vitro* translated ^{35}S-labelled mHDAC-6 (lanes 2 and 3) or with a control reticulocyte lysate (lane 4). β-tubulin was immunoprecipitated with the TU2.1 antibody (lanes 2 and 4), and the presence of HDAC-6 protein was detected by fluorography. Lane 1 contains 10% of the ^{35}S-labelled mHDAC6 input. (**D**) HDAC-6 is part of the microtubule-associated proteins in NIH-3T3 cells. Microtubules were purified from NIH-3T3 cell lysate (lane 1) in the presence (lanes 2 and 3) or absence (lanes 4 and 5) of paclitaxel (taxol) and GTP. Microtubules were then pelleted by centrifugation. The proteins present in the pellet (lanes 2 and 4) and supernatant fractions (lanes 3 and 5) were analysed by western blotting using antibodies to detect mHDAC-6 (upper panel), α-tubulin (middle panel) and β-actin (lower panel). (**E**) Interaction between HDAC-6 and microtubules *in vitro*. Purified bovine tubulin (Cytoskeleton, Inc.) was assembled into microtubules in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of paclitaxel (taxol) and GTP. Subsequently, *in vitro* translated ^{35}S-labelled mHDAC6 was added and binding was allowed to proceed. Microtubules were then pelleted by centrifugation. The proteins present in the pellet (lanes 1 and 3) and supernatant (lanes 2 and 4) fractions were analysed by SDS–PAGE, and the presence of HDAC-6 was detected by fluorography.

yeast two-hybrid screen (Fields and Song, 1989; Gyuris *et al*., 1993) and prepared a LexA–HDAC-6 fusion protein, which was used as bait for screening an activation domain-tagged cDNA library derived from the human HeLa cell line. For the screen, we used either wild-type HDAC-6 or a version of this protein in which the histidine at positions 216 and 611 in each hdac domain had been mutated to alanine (HDAC-6-H216A/H611A). We reasoned that this double mutation might lead to stabilization of the HDAC–substrate interaction (Finnin *et al*., 1999) and thus might be advantageous for the identification of specific interactors. In transient transfection assays, these mutations in both hdac domains reduced the repression activity observed upon artificial recruitment of HDAC-6 to the promoter of a reporter plasmid, indicating that the enzyme is functionally inactivated (data not shown). From a screen of ~2 × 10^6 colonies, 11 cDNA clones were identified that allowed growth of the yeast strain on selective medium. Four of these cDNAs were found to be multiple isolates of β-tubulin 2 or of its C-terminal portion (amino acids 323–446). However, in this assay, β-tubulin was found to interact equally with the wild-type or the mutated HDAC-6 bait (data not shown and see below). In addition, several clones encoding ubiquitin were also isolated in this screen, confirming recent results (Seigneurin-Berny *et al*., 2001). When retransformed into the parental yeast strain, containing the LexA–HDAC-6 fusion, these tubulin cDNAs all allowed growth on selective medium and also efficiently stimulated expression of a LexA-lacZ reporter in an HDAC-6-dependent manner (data not shown).

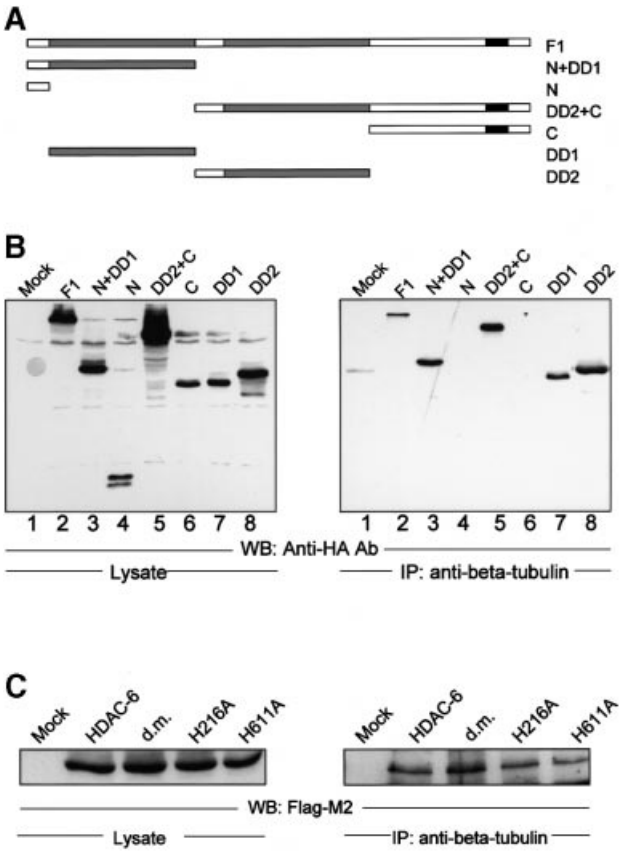


Fig. 2. HDAC-6 interacts with β-tubulin via its HDAC domains. (**A**) Schematic representation of the N-terminally HA-tagged HDAC-6 deletion constructs used. (**B**) Co-immunoprecipitation assay. 293T cells were transfected with the HDAC-6 expression vectors (lanes 2–8), and cellular extracts were prepared. HDAC-6 expression was measured by western blot with the anti-HA antibody (left panel). Association with β-tubulin was measured by performing an immunoprecipitation with an anti-β-tubulin antibody, followed by analysis of the precipitate by western blotting with the anti-HA antibody (right panel). (**C**) 293T cells were transfected with Flag-tagged HDAC-6 constructs and cell extracts were subjected to immunoprecipitation by anti-β-tubulin antibody. In the mutants proteins, the histidine at position 216 or 611 were mutated to alanine. d. m., double mutant protein.

To test whether HDAC-6 and β-tubulin can also interact in mammalian cells, a co-immunoprecipitation assay was performed. First, cell extracts were prepared from NIH-3T3 cells and were immunoprecipitated with an antibody against HDAC-6 (Verdel *et al*., 2000). The precipitated material was separated by SDS–PAGE and the presence of tubulin was detected by western blotting with specific antibodies. As shown in Figure 1A, immunoprecipitation of HDAC-6 led to co-precipitation of β-tubulin and also of α-tubulin (lanes 2–4). Notably, treatment of the cells with increasing amounts of trichostatin A (TSA), a specific HDAC inhibitor, did not influence the interaction between HDAC-6 and tubulin (Figure 1A, lanes 3 and 4). Next, HEK 293T cells were transfected with a construct encoding an HDAC-6 protein tagged at the N-terminus with the haemagglutinin (HA) epitope. Subsequently, cell extracts were prepared and immunoprecipitated with antibodies against β- (Figure 1B, lanes 1 and 2) or α-tubulin (Figure 1B, lanes 3 and 4). In each case, HDAC-6 was found to co-immunoprecipitate with tubulin (Figure 1B, lanes 2 and 4). When β-tubulin was immunoprecipitated with antibody TU2.1, only HDAC-6, but not α-tubulin, was found in the precipitate (lane 2), indicating that this antibody disrupts the α/β-tubulin heterodimer. In contrast, immunoprecipitation of α-tubulin with antibody DM1A led to co-immunoprecipitation of HDAC-6 and also β-tubulin (lane 4). These observations suggest that the interaction between α-tubulin and HDAC-6 might be mediated by β-tubulin.

HDAC-6 interacts with purified tubulin and microtubules in vitro

To test whether HDAC-6 interacts with tubulins directly, we used *in vitro* translated, radioactively labelled HDAC-6 and purified bovine tubulins for an *in vitro* interaction assay. For this, tubulin was pre-incubated with HDAC-6 and subsequently immunoprecipitated with an anti-β-tubulin antibody. The co-precipitation of HDAC-6 was detected by fluorography. As shown in Figure 1C, HDAC-6 bound purified tubulin (lane 2), whereas in the sample immunoprecipitated with a control antibody (lane 3), no HDAC-6 was detected.

Next, to test whether interaction could also be observed with the polymerized form of tubulin, microtubules were purified from NIH-3T3 cells (Tian *et al*., 2000) and the pellet fraction, which contains microtubules and their associated proteins (MAPs), was tested for the presence of HDAC-6. As seen in Figure 1D, HDAC-6 was found to associate with the polymerized microtubules (lane 2). In contrast, actin, a common potential contaminant, was not present in the pellet, showing the specificity of the MAP purification and therefore of the HDAC-6 interaction

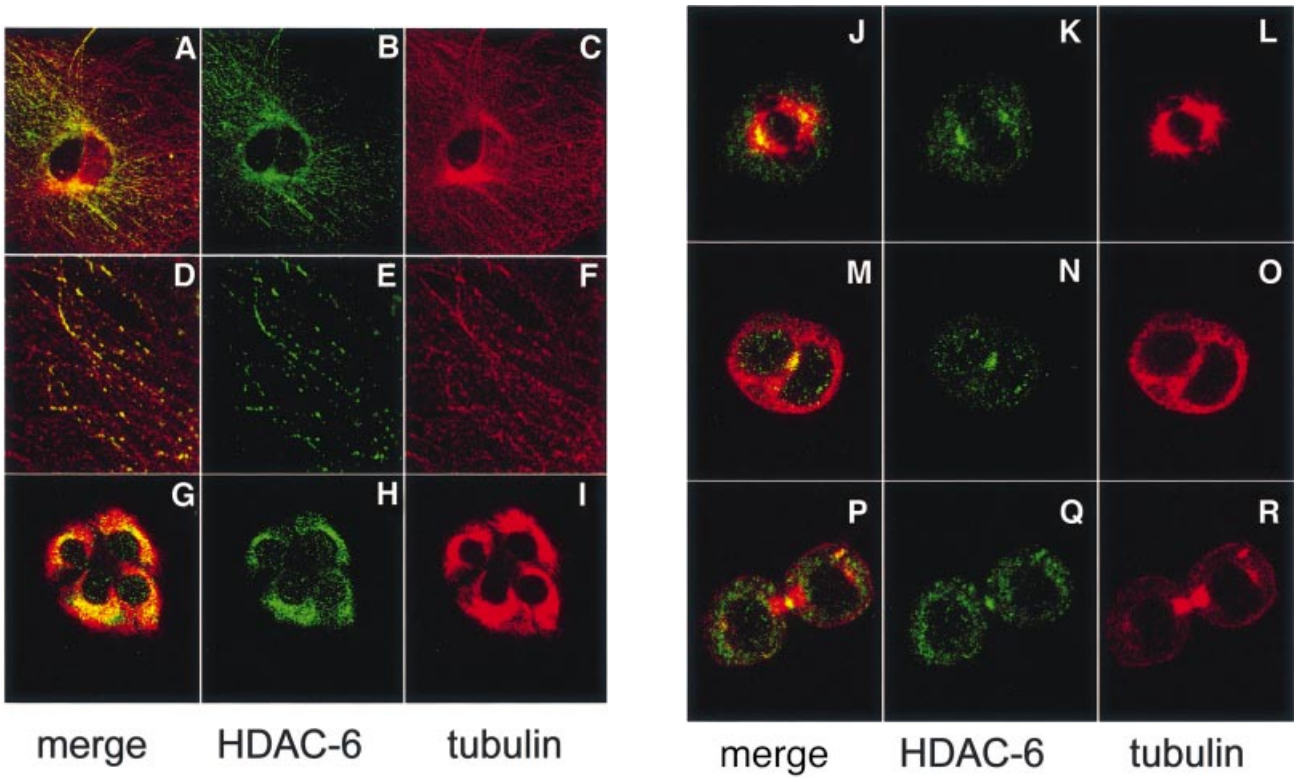


Fig. 3. Partial co-localization of HDAC-6 and microtubules in NIH-3T3 cells. (**A–F**) Exponentially growing NIH-3T3 cells were fixed with cold methanol and double-stained for endogenous HDAC-6 (green) and β-tubulin (red). Analysis was performed by confocal microscopy, and individual stainings were merged digitally. In (**G–I**), cells were treated with 10 μM paclitaxel for 4 h to stabilize microtubules, and HDAC-6 and tubulin were immunolocalized as above. In (J–R), mitotic cells were examined: (**J–L**) metaphase; (**M–O**) anaphase; (**P–R**) telophase.

with tubulin. When GTP and the microtubules stabilizer paclitaxel (taxol) were omitted from the purification, no HDAC-6 was found in the pellet (lane 4), further demonstrating that the interaction is specific.

Finally, microtubules were assembled *in vitro* from purified dimeric tubulin, and radiolabelled HDAC-6 was added to the reaction mixture. Subsequently, polymerized microtubules were separated from tubulin by centrifugation. Again, HDAC-6 was found in the pellet fraction only when microtubules were stabilized by the addition of paclitaxel (Figure 1E, lane 1). Thus, HDAC-6 appears to interact with β-tubulin and also with microtubules.

Domain(s) of HDAC-6 required for interaction with β-tubulin

To define which domain(s) in HDAC-6 is required for interaction with β-tubulin, a series of HA-tagged HDAC-6 deletion mutants (Figure 2A; Seigneurin-Berny *et al*., 2001) were transfected into HEK 293T cells and association with tubulin was detected by a co-immunoprecipitation assay, as in Figure 1B. As shown in Figure 2B, the full-length protein (lane 2) and deletion mutants containing at least one hdac domain (lanes 3, 5, 7 and 8) were highly enriched in immunoprecipitates. In contrast, neither the N- nor the C-terminal portion of HDAC-6, which lack a hdac domain, was co-precipitated. These results demonstrate that the hdac domain is both necessary and sufficient for tubulin binding.

We then made use of HDAC-6 mutants that have point mutations in either or both of the hdac domains (Grozinger *et al*., 1999). Interestingly, both the single and double mutants could be co-precipitated as efficiently as the wild-type enzyme (Figure 2C). This indicates that the binding between HDAC-6 and β-tubulin, while being mediated by the hdac domain, does not depend on its catalytically active centre.

Partial co-localization between HDAC-6 and microtubules in NIH-3T3 cells

The biochemical interaction between HDAC-6 and β-tubulin or microtubules prompted us to test whether some HDAC-6 co-localizes in mammalian cells with the microtubule network. For this, NIH-3T3 cells were fixed and microtubules as well as HDAC-6 were revealed by immunostaining followed by confocal microscopy. As seen in Figure 3A–F, HDAC-6 shows a predominantly cytoplasmic distribution with a distinct punctuated pattern and an accumulation in particular around the nucleus, in agreement with earlier reports (Verdel *et al*., 2000). This distribution partly coincides with the microtubule network and, when examined at high magnification, a fraction of the microtubules appear decorated by HDAC-6 (Figure 3D–F). Furthermore, treatment of the cells with the microtubule stabilizing agent paclitaxel (taxol) led to reorganization of the microtubules and to concomitant changes in the distribution of HDAC-6 (Figure 3G–I). In contrast, the localization of HDAC-6 remained unaffected

in cells treated with latrunculin B, a drug inducing the disassembly of the actin cytoskeleton (data not shown). This pattern of HDAC-6 localization strongly suggests that a major and perhaps primary role of this deacetylase is associated with the microtubule network and not with the control of histone acetylation. We next examined the localization of HDAC-6 during cell mitosis and found that it does not co-localize with DNA (see Supplementary figure S1 available at *The EMBO Journal* Online) but with the mitotic spindle in metaphase (Figure 3J–L) and also with γ-tubulin, a centrosomal marker (Supplementary figure S1). Interestingly, during cytokinesis, HDAC-6 is enriched in the centre of the midbody (Figure 3M–R). Together, these data suggest that HDAC-6 might play a role in mitosis by affecting microtubules.

HDAC-6 inhibition leads to increased tubulin acetylation in mammalian cells

The cytoplasmic localization of HDAC-6 together with its interaction with microtubules suggested the possibility that this protein might deacetylate tubulin and/or microtubules. To examine this, we first treated cells with the deacetylase inhibitors TSA, which inhibits all known HDACs, or sodium butyrate, which inhibits HDACs with the exception of HDAC-6 (Guardiola and Yao, 2002). Cell lysates were then prepared and used to measure the level of tubulin acetylation with an antibody that specifically recognizes acetylated tubulin, TU6-11 (LeDizet and Piperno, 1991). As shown in Figure 4A, treatment of 293T or NIH-3T3 cells with TSA led to an increase in tubulin acetylation, already after 30 min. In contrast, treatment of the cells with sodium butyrate failed to influence the level of tubulin acetylation, suggesting that HDAC-6 might indeed be involved. The total amount of α-tubulin, measured by antibody DM1A, was found to be unaffected by either inhibitor. Histone acetylation was, as expected, increased significantly by both inhibitors (Supplementary figure S2). In agreement with these observations, immunostaining of cells treated with TSA showed a strong increase in tubulin acetylation, whereas trapoxin B (TPX) or butyrate did not change the level of tubulin acetylation (Figure 4B). To demonstrate a link between HDAC-6 expression and tubulin deacetylation more directly, 3T3 cells were transiently transfected with expression vectors encoding different HDAC-6 proteins, and tubulin acetylation was monitored by immunostaining. As shown in Figure 4C, in cells transfected with wild-type HDAC-6, tubulin acetylation became undetectable, while it was clearly visible in untransfected cells. In contrast, expression of the different HDAC-6 mutants failed to alter tubulin acetylation. Interestingly, mutants that encode a single hdac domain (e.g. N + DD1) and had been found to still interact well with β-tubulin (see Figure 2) also failed to deacetylate tubulin. This suggests that HDAC-6 enzymatic activity requires the presence of two intact hdac domains (see also below).

HDAC-6 deacetylates α-tubulin Lys40 in vitro

Although treatment of cells with TSA led to a rapid increase in tubulin acetylation (see Figure 4A), the data presented so far do not demonstrate unambiguously a direct involvement of HDAC-6 in tubulin deacetylation. To test this, an *in vitro* deacetylation assay was used. For this, two peptides corresponding to amino acids 33–46 of human α-tubulin were synthesized with or without acetylation of the lysine residue at position 40. As shown in Figure 5 (1a–c), a mixture of the two synthetic peptides could be well resolved by HPLC and mass spectrometric analysis. Additional peptide fragmentation allowed unambiguous identification of the peptides even in a complex mixture (1b and 1c). The acetylated peptide was next used for an *in vitro* deacetylation assay performed with HDAC-6 protein. As shown in Figure 5 (2a and b), incubation of the acetylated peptide with HDAC-6 led to efficient peptide deacetylation. For this, the hdac domains were necessary, as the HDAC-6 protein mutated in both hdac domains (HDAC-6-H216A/H611A) completely failed to deacetylate the peptide (3a and b). Likewise, the mock-transfected cell extracts did not show any tubulin deacetylase activity (data not shown). To obtain more quantitative results, a radioactive assay was used. HDAC-1,- 5 and -6, as well as mutated versions of the latter protein were used to deacetylate *in vitro* a radioactively acetylated peptide derived from α-tubulin. As shown in Figure 6A, HDAC-6, but not HDAC-1 or HDAC-5, efficiently deacetylated the peptide. In addition, the HDAC-6 proteins with mutations in the hdac domains were found to be completely inactive. In contrast, when an acetylated peptide derived from histone H4 was used, all proteins except the mutated versions of HDAC-6 and HDAC-5 were capable of efficient deacetylation, in agreement with earlier results (Supplementary figure S3). Similarly to histone deacetylation activity, tubulin deacetylation by HDAC6 was sensitive to TSA but not to either TPX or butyrate (Figure 6B). Furthermore, the deletion constructs from Figure 2 were assayed for TDAC activity (Figure 6C). Consistent with our previous *in vivo* data (Figure 4C), only the full-length wild-type HDAC-6 could deacetylate tubulin, whereas the deletions were not active for tubulin deacetylation even if they contained an intact hdac domain and still bound tubulin.

HDAC-6 controls tubulin acetylation in vivo

To test directly the role of HDAC-6 in the control of tubulin acetylation *in vivo*, we made use of mouse ES cells in which the HDAC-6 gene is flanked by lox P sites. These cells are derived from line E14, which has a male genotype. Since the HDAC-6 gene is on the X chromosome, treatment of a targeted ES cell clone with Cre recombinase would lead to a knockout allele. Therefore, a targeted ES cell clone, in which exons 7–10 in the first hdac domain are flanked by loxP sites and contain a floxed Neo^R TK cassette (see Figure 7A; details to be published elsewhere) was used for transient transfection of a Cre recombinase expression vector. After selection against thymidine kinase (tk) resistance, clone 126 was identified as a floxed clone having the tk-Neo cassette deleted but retaining all HDAC-6 exons, while clone 124 was found to be knockout (Figure 7A and B). Cells from these two clones were used to examine the status of tubulin and histone acetylation *in vivo*. Cells were grown under non-differentiating conditions and cell extracts were examined by western blotting. As seen in Figure 7C, the knockout cells indeed completely lack HDAC-6 protein, but have normal levels of HDAC-5 or β-catenin. The absence of HDAC-6 resulted in elevated α-tubulin acetylation

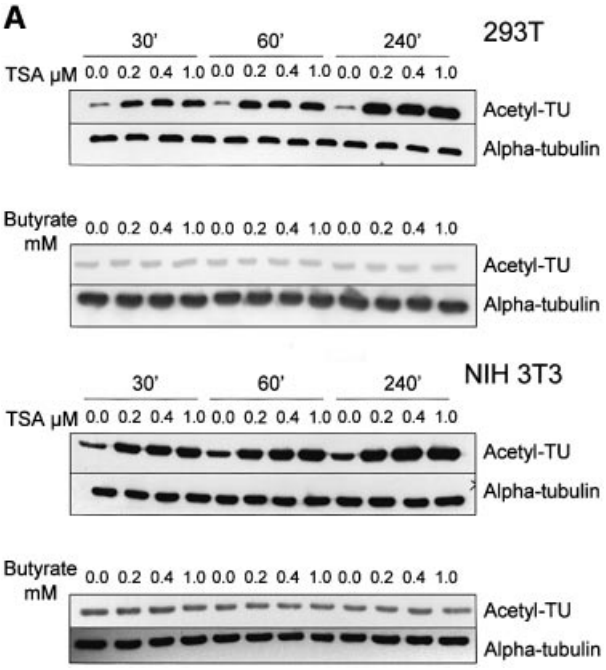
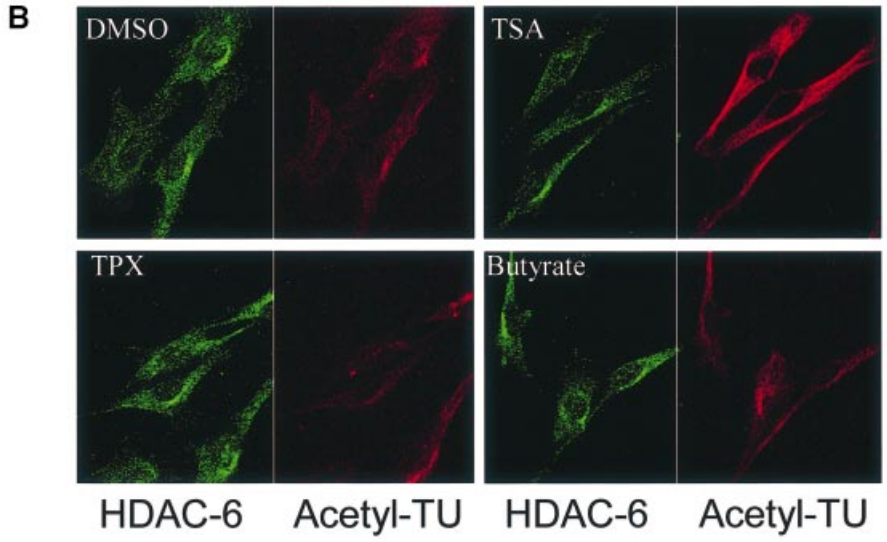
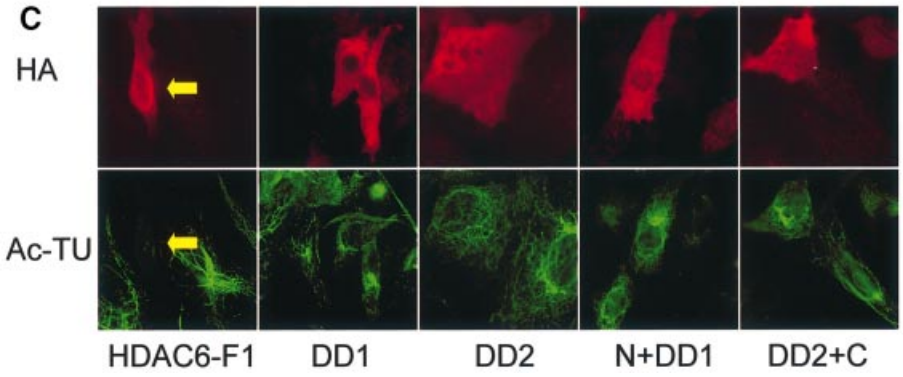


Fig. 4. Pharmacological inhibition of HDAC-6 activity leads to increased tubulin acetylation *in vivo*. (**A**) Exponentially growing 293T or NIH-3T3 cells were treated for 30 min, 1 h or 4 h with different concentrations of the HDAC inhibitors TSA or sodium butyrate, as indicated. Cell extracts were prepared and analysed by SDS–PAGE followed by western blotting. The membranes were probed with antibodies recognizing α-tubulin, either indiscriminately (DM1A) or only when acetylated (TU6-11). (**B**) NIH-3T3 cells were treated for 4 h with the indicated chemicals and then fixed. Immunostaining was used to visualize the level of HDAC-6 (green) and of tubulin acetylation (red). (**C**) Balb/c 3T3 cells were transfected with the indicated expression vectors encoding HDAC-6 proteins tagged at the N-terminus with the HA epitope. Expression of HDAC-6 deletions was monitored with an anti-HA antibody (red), and the state of tubulin acetylation was detected with the TU6-11 antibody (green).





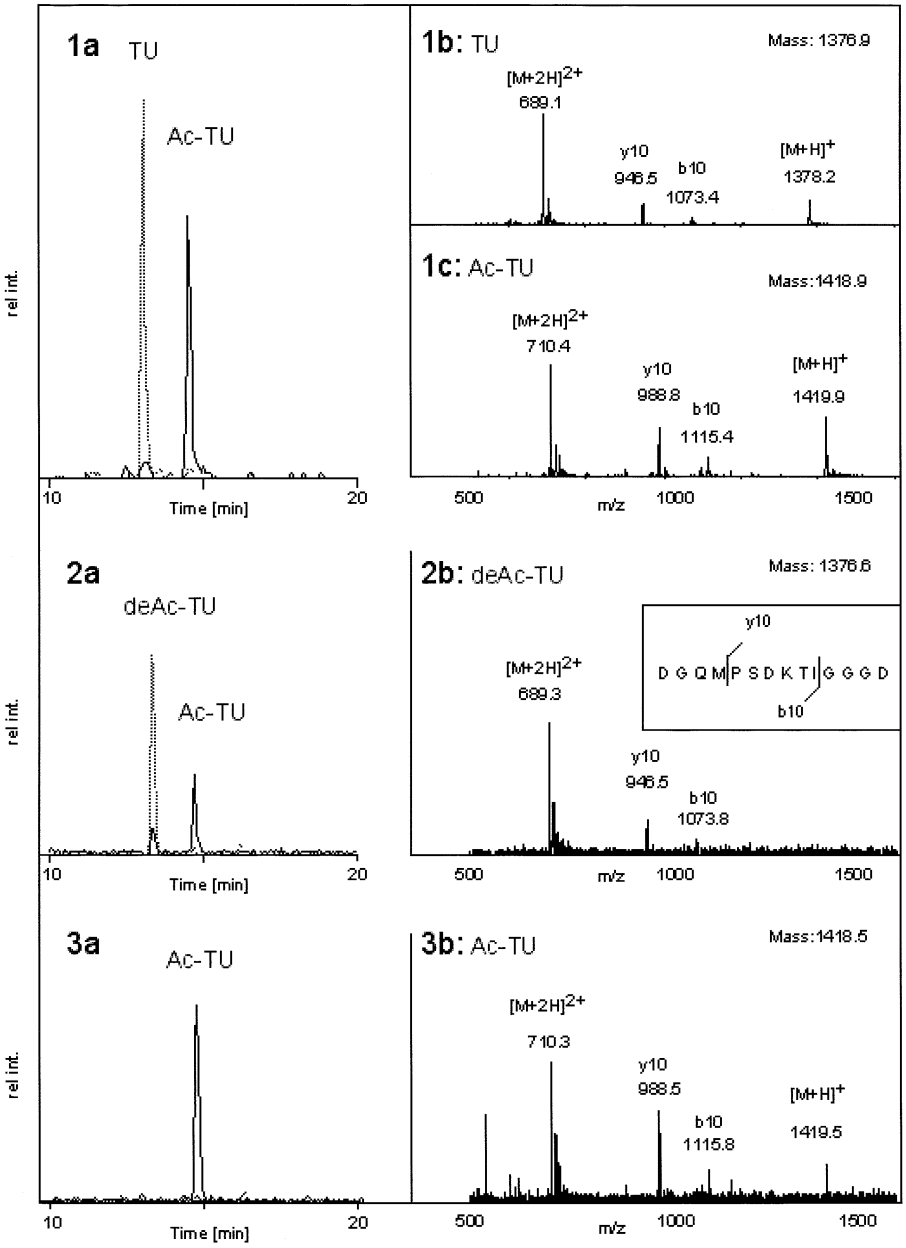


Fig. 5. *In vitro* deacetylation of a tubulin-derived peptide by HDAC-6. A peptide derived from α-tubulin was chemically synthesized as an unacetylated version (TU) or acetylated on Lys40 (Ac-TU). (**1a**) HPLC separation of a 1:1 mixture of the two peptides. The extracted ion current (XIC) of 689 (grey) and 710 (black) is shown, corresponding to the doubly charged ions of TU and Ac-TU, respectively. (**1b**) MS of TU with a mass of 1376.9 Da. The Ac-TU has a mass of 1418.9 Da as shown in (**1c**). Both peptides are partially fragmenting, generating the y10 as well as the b10 ion (insert 2b). (**2a**) The acetylated peptide was incubated with HDAC-6 and the reaction mix was analysed as described for the standard peptide mixture. The deacetylated TU peptide formed had a mass of 1376.6 Da as shown in (**2b**). The specific fragment ions y10 and b10 are indicated and explained in the inset of (2b). (**3a**) Analysis of the products of the incubation of the acetylated peptide with the doubly mutated protein HDAC-6-H216A/H611A shows only the acetylated peptide. This peptide had a mass of 1418.5 Da, and the two specific fragment ions y10 and b10 could be detected again (**3b**).

without change in the protein level of α- or β-tubulin. Strikingly, the global acetylation level of histone H3 or H4 did not appear to be affected by the complete absence of this HDAC. Furthermore, the increase in tubulin acetylation was confirmed further by immunostaining of the ES cells (Figure 7D). Wild-type or HDAC-6-deficient ES cells did not show any obvious morphological difference and both could differentiate *in vitro* equally well into embryoid bodies (Supplementary figure S4), indicating that in ES cells at least, HDAC-6 is not essential. In culture, the ES cells lacking HDAC-6 proliferated marginally more slowly than the floxed cells (Figure 7E), and in a colony formation assay they formed equivalent numbers of colonies, which were, however, somewhat smaller than those from the control cells (Figure 7F).

Discussion

HDAC-6 is a tubulin deacetylase

Post-translational modifications play a critical role in the regulation of protein structure and function. In particular, phosphorylation/dephosphorylation by protein kinases and

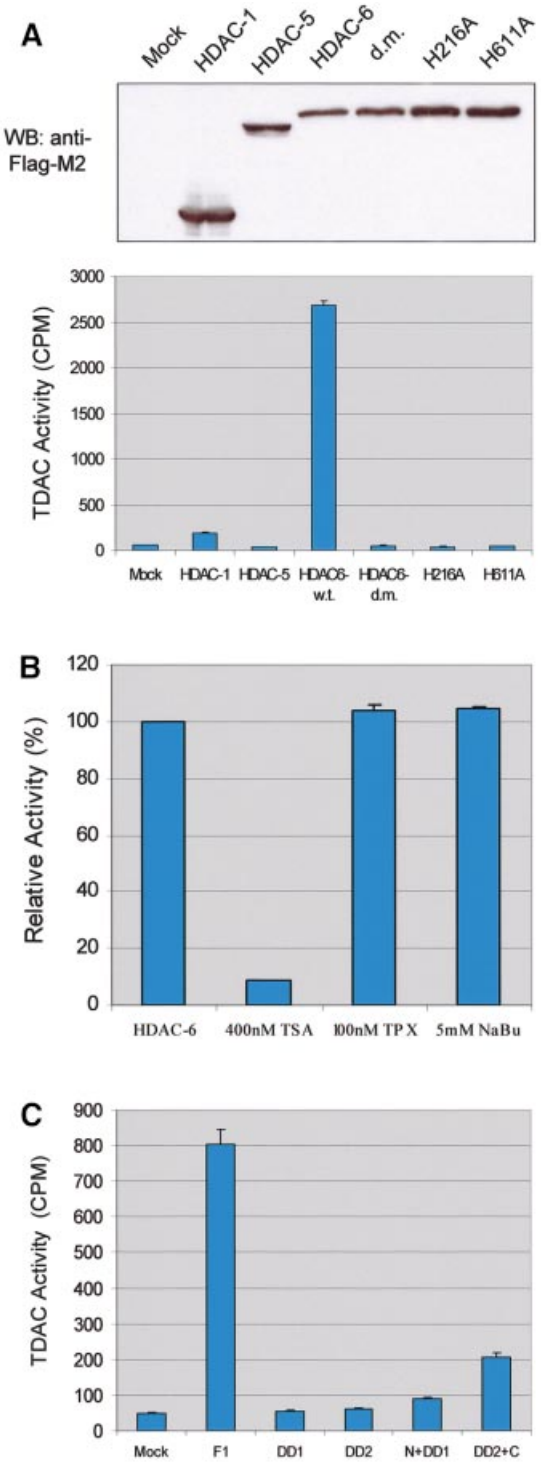


Fig. 6. Tubulin deacetylase activity is specific to HDAC-6. (**A**) 293T cells were transfected with expression vectors encoding FLAG-tagged versions of the indicated proteins. Two days later, cell extracts were prepared and immunoprecipitated with the anti-FLAG antibody M2. Upper panel: expression levels of the different HDACs were verified by western blotting using the anti-FLAG antibody. Lower panel: tubulin deacetylase activity (TDAC) was assayed by incubating an [^{3}H]acetyl-labelled peptide from human α-tubulin (amino acids 33–46) and measuring the release of radioactivity. (**B**) TDAC activity was assayed as above, with immunoprecipitated HDAC-6 alone or in the presence of TSA, TPX or sodium butyrate (NaBu), as indicated. (**C**) HDAC-6 deletion constructs were transfected into 293T cells, and immunoprecipitated material was used for TDAC assay, as in (A).

phosphatases form the mechanistic basis for signal transduction cascades transmitting signals from the cell membrane to the nucleus. So far, protein acetylation/deacetylation has been discovered predominantly in histone tails and thereby implicated in regulation of chromatin structure and gene transcription. However, the list of non-histone proteins that are acetylated is growing continually, and there the role of aceytylation awaits to be understood. In this report, we have identified β-tubulin as a protein interacting specifically with HDAC-6. We found that, within HDAC-6, the hdac domains are necessary and sufficient for interaction with the C-terminal portion of β-tubulin. HDAC-6 was known to be a predominantly cytoplasmic protein (Verdel *et al*., 2000) and, by immunostaining of cells, we observed some co-localization of HDAC-6 with the microtubule network. Furthermore, treatment of cells with agents stabilizing (taxol) or disrupting (nocodazole; data not shown) microtubules led to significant redistribution of HDAC-6, indicating that association of these two proteins is not fortuitous. In contrast, disruption of the actin microfilament network by latrunculin B had no effect on the localization of HDAC-6 (data not shown). We found that treatment of cells with the HDAC inhibitor TSA led to an increase in tubulin acetylation, detectable both in cell extracts and by immunostaining of cells. In contrast, HDAC inhibitors such as TPX and sodium butyrate, which are known not to affect HDAC-6 activity, failed to alter the pattern of tubulin acetylation in cells. Furthermore, transient expression of HDAC-6 was found to reduce dramatically the level of tubulin acetylation in 3T3 cells. For this, the presence of both hdac domains was needed. By *in vitro* experiments, we could show that HDAC-6, but not HDAC-1 or HDAC-5, deacetylates Lys40 of α-tubulin. Finally, we took advantage of ES cells in which the HDAC-6 gene had been disrupted by gene targeting. In these cells, which completely lack HDAC-6, tubulin acetylation was found to be dramatically increased while global acetylation of histone H3 and H4 was unaffected. In addition, *in vivo*, during development and differentiation of the mouse mammary gland, expression of HDAC-6 showed an inverse pattern to that of acetyl-tubulin (N.Li, unpublished result). Together, these data suggest that the primary function of HDAC-6 is to deacetylate α-tubulin, and thereby to influence the stability of the microtubule network. Consistent with this, Hubbert *et al*. (2002) very recently found that 3T3 cells stably overexpressing HDAC-6 have an increased motility, probably due to the deacetylation of microtubules. The denomination HDAC therefore appears to be a misnomer that merely reflects the initial identification of HDAC-6 through its homology with 'true' HDACs such as HDAC-1.

Mechanism of deacetylation by HDAC-6

The interaction between HDAC-6 and β-tubulin might be used as one way of regulating the substrate specificity of HDAC-6. By binding to β-tubulin, HDAC-6 is recruited to either tubulin dimers or microtubules and can deacetylate Lys40 of α-tubulin. The binding between HDAC-6 and tubulin can be uncoupled from the deacetylation reaction since a point mutation in the active centre abolished the enzymatic activity but did not impair the interaction with β-tubulin. Recently a conserved ER (Esa1-Rpd3) motif

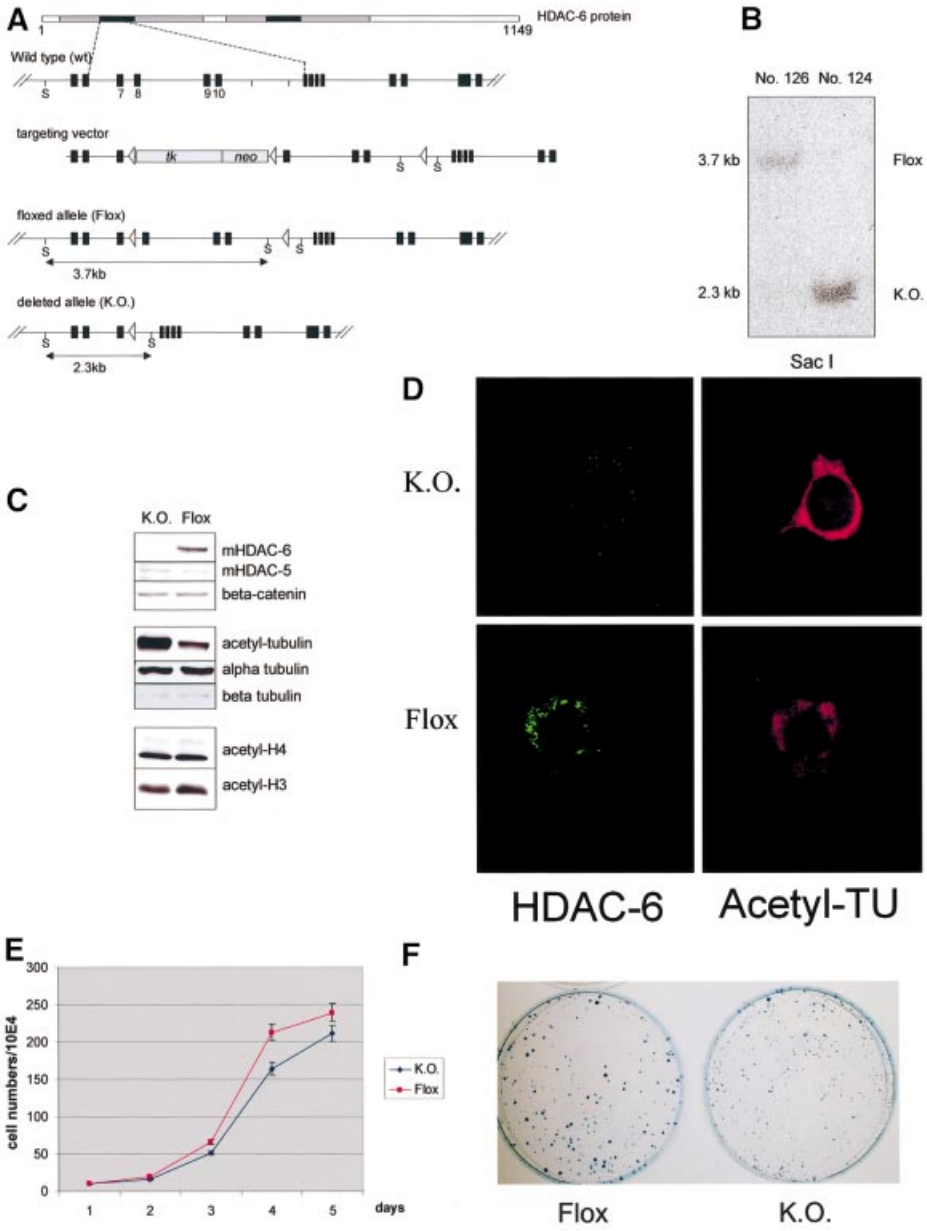


Fig. 7. Increased tubulin acetylation, but not histone acetylation in HDAC-6-deficient ES cells. (**A**) Scheme of the floxed mouse HDAC-6 locus and of the deleted (KO) allele (details to be published elsewhere). The position of *Sac*I (S) restriction sites is indicated, as well as the size of the resulting fragments. At the top, a scheme of the HDAC-6 protein is presented showing the two hdac domains (grey) and their core region (black). Cre-mediated recombination between loxP sites 1 and 2 leads to the floxed allele. Cre-mediated recombination between loxP sites 1 and 3 leads to the knockout allele; in this case, exons 7–10 of HDAC-6 are removed and no functional HDAC-6 is made. (**B**) Southern blot analysis of DNA from a floxed ES cell clone (No. 126) as well as from a knockout clone (No. 124). The position of the floxed or knockout allele is indicated. (**C**) Analysis of protein expression in floxed or knockout ES cells. Protein extracts were prepared from ES cell clones No. 126 and 124 and analysed by SDS–PAGE followed by western blotting with the indicated antibodies. (**D**) Immunostaining of HDAC-6 (green) and acetylated tubulin (red) in floxed or knockout ES cells, as indicated. (**E**) Growth curves of HDAC-6 floxed and knockout ES cell lines. Equal numbers of cells (1×10^5) were seeded in triplicate and aliquots were counted daily during a period of 5 days. (**F**) Colony formation assay with floxed and HDAC-6 knockout ES cells. A total of 2×10^3 cells were seeded and cultivated for 8 days in triplicate. Cells were then fixed and stained with Methylene Blue. One representative plate of each genotype is shown.

was identified near the active centre of the hdac domain and also in the HAT domain (Adachi *et al*., 2002). Mutation and structure analysis of this motif in both Esa1 and Rpd3 suggested that it might be involved in the process of substrate (histone) binding. Our observations also suggest that the tubulin recognition domain is distinct from the active centre of HDAC-6. Further detailed mutational analysis of the hdac domain will be required to verify this hypothesis.

The catalytic mechanism for the hdac domain has been proposed by Finnin *et al*. (1999) after resolving the structure of an HDAC core domain from a hyperthermophilic bacterium. Except for HDAC-6 and HDAC-10, all HDACs have only one catalytic domain and are inhibited similarly by, for example, TSA and TPX. In contrast, HDAC-6 has two catalytic domains and HDAC-10 has one complete and one incomplete catalytic domain (Fischer *et al*., 2002; Guardiola and Yao, 2002; Tong *et al*., 2002).

Both of these enzymes are resistant to inhibition by TPX and sodium butyrate. Here we found that *in vitro* and *in vivo* deacetylation of tubulin or histone by HDAC-6 requires the presence of two intact hdac domains. A single mutation in one hdac domain was sufficient to eliminate the entire activity, irrespective of the substrate. *In vivo*, the deacetylases often are found in large repression complexes in heterodimer or homodimer forms. This suggests that *in vivo*, the active deacetylase complexes may need more than one hdac domain, and mutation in one of the hdac domains may be sufficient to impair the activity of the whole complex. Additional experiments will be needed to test this hypothesis further.

HDAC-6, a dual- or multi-specificity deacetylase?

HDAC-6 was identified by searching for mammalian homologues of yeast histone deacetylase HDA1 (Grozinger *et al*., 1999; Verdel and Khochbin, 1999). *In vitro*, HDAC-6 acts, as shown here, on a tubulin peptide and also on microtubules (Hubbert *et al*., 2002) as well as on a histone H4-derived peptide substrate (Supplementary figure S3) or on purified core histones (Grozinger *et al*., 1999). Although in HDAC-6-deficient ES cells the acetylation of histone H3 and H4 did not change significantly (Figure 7), it cannot be ruled out that in certain cell types, or possibly in response to specific signals, HDAC-6 participates in deacetylating histones, and potentially other nuclear proteins, *in vivo*. We observed that, in transient transfection assays, artificial recruitment of HDAC-6 to promoter DNA represses the transcription of reporter plasmids, suggesting that the presence of HDAC-6 in the nucleus does have an impact on gene activity (data not shown). In addition, Verdel *et al*. (2000) showed that the subcellular localization of HDAC-6 is controlled by specific signals. A potent nuclear export signal at the N-terminus is essential for maintaining HDAC-6 in the cytoplasm: block of CRM1 activity by leptomycin B led to redistribution and nuclear localization of HDAC-6. Also, arrest of proliferation and subsequent differentiation of a mouse melanoma cell line was found to be associated with translocation of a fraction of the HDAC-6 protein into the nucleus (Verdel *et al*., 2000). Other class II HDACs also shuttle in and out of the nucleus to deacetylate histones, and their subcellular distribution is influenced in part by their interaction with 14-3-3 proteins (Grozinger and Schreiber, 2000; McKinsey *et al*., 2000b). Thus, the substrate specificity of HDAC-6 may be controlled at several levels: first, regulated subcellular localization determines the availability or unavailability of the potential targets; secondly, targeting to the substrate (tubulin) is achieved by selective interaction with the hdac domain; and finally, the active centre provides the direct substrate specificity.

HDAC-6 is not essential in ES cells

Here, we found that disruption of the HDAC-6 gene did not obviously alter the morphology of ES cells (data not shown). Yet, proliferation of HDAC-6-deficient ES cells was slightly slower than control cells, at least under the non-differentiating culture conditions used. Moreover, the ES cells lacking HDAC-6 could differentiate *in vitro* into embryoid bodies as efficiently as wild-type cells. Interestingly, when tested in a colony formation assay, knockout ES cells formed similar numbers of colonies, which, however, were smaller than colonies from wild-type cells (Figure 7F); at present, it is not clear whether this is a direct consequence of the tubulin hyperacetylation. In the case of HDAC-1-deficient ES cells, it was also found that *in vitro* differentiation was normal. However, in this case, proliferation was significantly impaired and this was correlated with an increase in expression of the cyclin-dependent kinase inhibitors p21 and p27 (Lagger *et al*., 2002). Also, acetylation of histone H3 and H4 was elevated in the mutant cells. In addition, HDAC-1 was found to be essential for early embryonic development: mutant embryos die before E10.5, probably as a consequence of the proliferation defect. Clearly, definition of the precise biological role(s) of the different HDACs in higher eukaryotes will require analysis of their function by gene targeting in the mouse, and these experiments are in progress.

The dramatically increased level of tubulin acetylation in HDAC-6-deficient ES cells, without a major observable phenotype, raises the question of the *in vivo* role of acetylated microtubules and of the contribution of the tubulin deacetylase/acetylase to cell function. Acetylated tubulin is not required for cell survival since it is missing from some eukaryotic cells even though the Lys40 in α-tubulin is highly conserved. Moreover, attempts have been made to determine the *in vivo* function of acetylated tubulin by either dominantly overexpressing, in *Chlamydomonas*, a tubulin mutant in which Lys40 was replaced by another amino acid (Kozminski *et al*., 1993), or by exchanging, in *Tetrahymena thermophila*, the wild-type tubulin gene with a mutated version (Gaertig *et al*., 1995). In both cases, the mutants had no detectable tubulin acetylation but were indistinguishable from the wild-type cells. Preliminary experiments suggested that in 3T3 cells overexpressing HDAC-6, taxol still could stabilize the microtubule structure in the absence of tubulin acetylation (C.Caron, unpublished results). These data suggest that tubulin acetylation might be redundant for those organisms or that it is an effect secondary to tubulin stabilization. Clearly, important questions remain open about the role of tubulin acetylation in multicellular organisms.

Non-histone substrates as targets of HDAC inhibitors

HDAC inhibitors have been shown to be potent inducers of growth arrest, differentiation and apoptotic cell death of transformed cells *in vitro* and *in vivo*. The overexpression of several HDACs and the HDAC-dependent aberrant transcriptional repression of tumour suppressor genes have been implicated as an important oncogenic mechanism in different types of cancers such as myeloid leukaemia and lymphoma (Melnick and Licht, 2002). Recent genome-wide studies in yeast have demonstrated that each HDAC has a distinct specificity, both on the histone tails and in the chromatin context (Peterson, 2002). In addition, the finding that HDACs also have non-histone substrates, e.g. tubulin, as shown here and by Hubbert *et al*. (2002), suggests that some of the effects ascribed to HDAC inhibition may reflect the function of these newly identified substrates. Therefore, it will be desirable to develop inhibitors that are HDAC specific and possibly also

substrate specific. Tubulin-binding agents constitute an important class of antimitotics and are widely used for the treatment of solid tumours and haematopoietic malignancies. For example, docetaxel and paclitaxel act to promote tubulin polymerization and the formation of stable microtubules, which are resistant to disassembly by physiological stimuli: cells exposed to these agents exhibit an accumulation of disorganized microtubule arrays. Since, as shown here, inhibition of HDAC-6 leads to tubulin hyperacetylation, which is a marker for stable microtubules, it might be very interesting to investigate the collaboration of these two different kinds of chemicals.

Materials and methods

Co-immunoprecipitation

For co-immunoprecipitation, ~500 μg of extracts from either NIH-3T3 cells or HEK 293T cells transfected by Fugene (Roche) were incubated overnight with the primary antibody at 4°C with gentle agitation. Rabbit IgG or mouse IgG was used as control. After this, 20 μl of protein A–Sepharose slurry were added and samples were incubated for 1 h at 4°C with gentle agitation. Beads were washed three times with IP buffer (20 mM HEPES pH 7.4, 0.5 mM EDTA, 150 mM NaCl and 0.1% Triton X-100) and subsequently resuspended and boiled in 20 μl of loading buffer for SDS–PAGE.

Immunofluoresence and immunoblotting

For tubulin and HDAC-6 staining, cells were fixed in methanol at –20°C for 10 min and stained with mHDAC-6 polyclonal antibody (Verdel *et al*., 2000), TU2.1 for α-tubulin or TU6-11 for acetylated tubulin (Sigma). The DNA was counterstained by propidium iodide (PI) or 4′,6-diamidino-2-phenylindole (DAPI).

Protein lysates were resolved by electrophoresis on 8 or 12% SDS–polyacrylamide gels and subsequently blotted to nitrocellulose membranes (Bio-Rad). Antibodies used were: TU2.1, TU6-11, Flag-M2 and DM1A (Sigma), mHDAC-5 and mHDAC-6 (Verdel *et al*., 2000), acetylated H3 and acetylated H4 (Upstate).

Translation in vitro and binding assay

Mouse HDAC-6 was translated *in vitro* by using plasmid pcDNA3.1-HA-mHDAC-6 F1 (Seigneurin-Berny *et al*., 2001) and the TNT T7 Coupled Reticulocyte Lysate system (Promega). [^{35}S]methionine was included to label translated protein. For the binding assay, 5 μl of the final translation reaction mixture was incubated with 5 μg of purified bovine tubulins (Cytoskeleton Inc., Denver, CO) in PIPES buffer [80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT), 5% glycerol, 1% Triton X-100] at 4°C for 4 h. Tubulins were then immunoprecipitated with anti-β-tubulin antibody TU2.1 (Sigma). Fluorography of ^{35}S was performed to detect co-precipitated HDAC-6.

Microtubules and MAP preparation

Microtubules were assembled and purified from NIH-3T3 cells as described previously (Tian *et al*., 2000). Briefly, NIH3-T3 cells were swollen in 9 vols of ice-cold swelling solution and homogenized in 2 vols of swelling solution containing Complete® proteinase inhibitor mixture (Roche). After adding PIPES pH 6.8 to 0.1 M, the homogenate was centrifuged in a TLS-55 rotor (Beckman) at 50 000 r.p.m. for 90 min at 2°C. Paclitaxel and GTP were added to the supernatant at a final concentration of 20 μM and 0.5 mM, respectively. The cytosolic extract was warmed briefly to 37°C (3–5 min) and then chilled for 15 min on ice. The cytosolic extract was transferred to a chilled centrifuge tube underlaid with ice-cold PEG buffer containing 10% (w/v) sucrose, 20 μM paclitaxel, and 0.5 mM GTP. Microtubules were pelleted through the sucrose cushion by centrifugation at 29 000 r.p.m. in a TLS-55 rotor for 30 min at 2°C. Microtuble proteins were washed once by PEG buffer with paclitaxel and GTP, and analysed by SDS–PAGE and immunoblotting.

In vitro microtubule binding assays

Taxol-stabilized microtubules were assembled from dimeric tubulin (Cytoskeleton Inc.) in G-PEM buffer (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP) as instructed by the manufacturer. The microtubule binding experiments were performed as described (Tian *et al*., 2000). Briefly, 50 μl of *in vitro* translated, [^{35}S]methionine-labelled mouse HDAC-6 were diluted in 200 μl of PB (80 mM K-PIPES pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$) containing Complete® proteinase inhibitor mixture (Roche). The mixture was spun at 50 000 *g* for 1 h at 4°C in a TLS-55 rotor. A 100 μl aliquot of supernatant was incubated with or without 50 μg of microtubules for 30 min at 37°C in the presence of 10 μM paclitaxel and 1 mM GTP. The microtubule and mHDAC-6 mixtures were layered over 2.5 ml of 15% sucrose in PB buffer in the presence of 10 μM paclitaxel and 1 mM GTP, and spun for 30 min at 30 000 *g*. Both supernatants (above the sucrose) and pellets were analysed for the presence of mHDAC-6 by autoradiography. The gels were then stained with Coomassie Blue to confirm that an equal amount of microtubules was loaded on the gel in each sample.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Supplementary Material

Legends for the Supplementary Figures S1-S4

Figure S1 HDAC-6 does not colocalize with DNA but with gama-tubulin in metaphase cell.

Exponentially growing NIH3T3 cells were fixed with cold methanol and double-stained for endogenous HDAC-6 (green) and either gama-tubulin (red) or propidium iodide. Analysis was done by confocal microscopy and individual stainings were merged digitally.

Figure S2 Both Butyrate and TSA efficiently increase histone acetylation in NIH3T3 cells.

Exponentially growing 293T or NIH3T3 cells were treated for 4 hrs with different concentrations of the HDAC inhibitors Trichostatin A (TSA) or sodium butyrate, as indicated. Histones were prepared by acid extraction and analysed by SDS-PAGE followed by western blotting. The membranes were probed with antibodies recognizing acetylated histone H3.

Figure S3 Mutation of the hdac domain in HDAC-6 abolishes the HDAC activity.

The HDAC-6 enzymes were prepared as in Fig 6B. Histone deacetylase activity (HDAC) was assayed by incubating an Acetyl-(^{3}H)- labelled peptide from human Histone H4 and measuring the release of radioactivity.

Figure S4 ES cells lacking HDAC-6 can differentiate into normal embryoid body.

Embryoid bodies were generated from HDAC-6 floxed or null ES cell lines in the absence of leukemia inhibitory factor (LIF). The embryoid bodies for each genotypes were photographed.

Supplementary Materials and Methods

Yeast two-hybrid

The classic LexA two-hybrid system was used to hunt the proteins which interact with HDAC-6. The whole-length human HDAC-6 cDNA was cloned into pJG202 plasmid and transformed into yeast SKY 48. Then it was mated with yeast SKY 473 which contains pre-transformed HeLa cDNA library in pJG4-5. Primary mated yeast clones (2×10^6) were screened on medium lacking leucine, and from this beta-tubulin 2 was isolated independently four times. To confirm the interaction, the isolated plasmids were retransformed into the parental yeast strain, containing the LexA-HDAC-6 fusion to test the growth on selective medium and stimulated expression of a LexA-LacZ reporter.

Deacetylation of Lys40 on tubulin peptide by HDAC-6

Preparation of the HDACs: Flag-tagged human HDAC-6 cDNA was cloned into pJG4-5 expression vector (Grozinger et al., 1999). The H216 and H611 were mutated to A in HDAC-6 d.m. expression vector. The plasmids were transfected into 293T cells by Fugene (Roche). 2 days after transfection, the cells were collected by NP-40 lysis buffer (50 mM Tris-Cl, 120 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin).

300 µl extracts were immunoprecipitated by 40 µl Flag-M2 agarose beads (Sigma) for overnight at 4 degree. Then the beads were washed three times with wash buffer (50mM Tris.HCl, pH7.4, 150mM NaCl).

TDAC assay: The peptide corresponding to amino acid 33-46 of human alpha tubulin was chemical acetylated by H^3-acetic acid (PerkinElmer). TDAC assays were performed in a 300-µl volume containing immunoprecipitated HDACs, 10 mM Tris, pH 8, 10 mM NaCl, 10% glycerol, 1mM PMSF, and 50,000 cpm [^{3}H] peptide. Inhibitor-treated reactions were incubated with 400 nM TSA (Sigma), 100 nM TPX-B (Dr. Yoshida), or 5 mM NaB (Sigma) for 20 min on ice before the addition of substrate. Reactions were incubated for 2 h at 37 °C. 75 µl of stop solution (1 M HCl, 0.16 M CH_3COOH) was added,

and 900 µl of ethyl acetate was added to extract the released tritiated acetate. 400 µl was counted in a liquid scintillation counter.

Cold TDAC assay and LC-MS: 250ng of acetylated TU peptide (Neosystem), which has the acetylation of the Lys40, was added to each reaction instead of [^{3}H] peptide in a final volume of 200µl. The reactions were incubated at 37 degree for 2 hrs with shaking. Then the reaction was centrifuged to pellet the agarose beads and the supernatant was analysed by LC-MS as in (Krieg et al., 1998).

Targeting of the mHDAC-6 gene in ES cells

The backbone of the targeting vector was subcloned from a BAC clone containing the mHDAC-6 gene (Means et al., 2000). The exons encoding the core of the first hdac domain were floxed by a single loxP site and a cassette expressing the neomycin resistance gene and thymidine kinase gene flanked by two loxP sites. E14 ES cells were electroporated with the targeting vector and correctly targeted ES clones were identified by PCR and Southern blot analysis. The targeted ES cell clone No 223 was used for transient transfection (electroporation) with the Cre-expressing plasmid pCMV-Cre in order to mediate recombination between the loxP sites. Gancyclovir was used to negatively select against clones having retained the tk-neo cassette and clones containing a deleted or a floxed mutant allele were identified by PCR and Southern blotting. Further details are available on request.

Fig. S 1 Zhang et al.

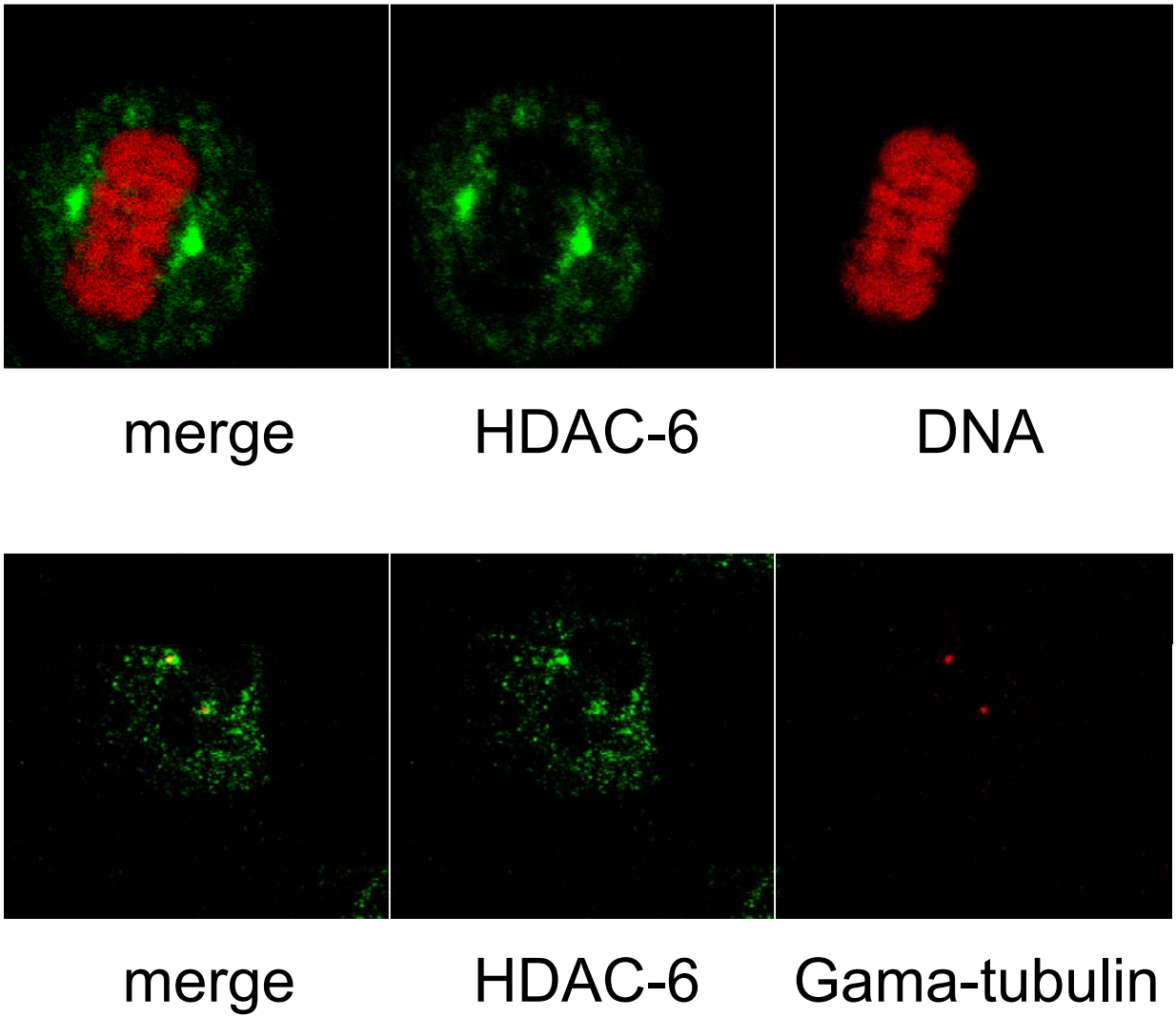


Fig. S 2 Zhang et al.

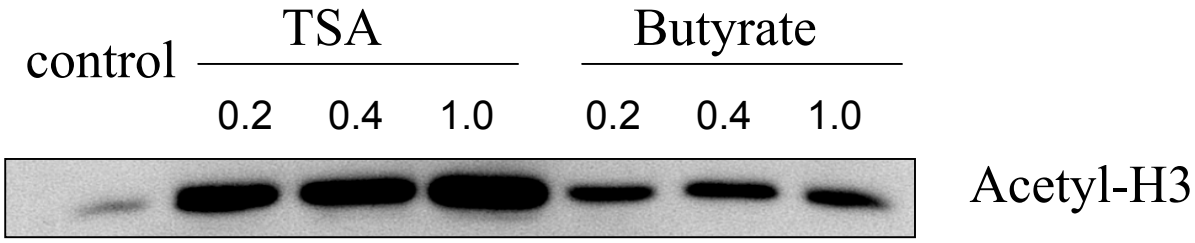
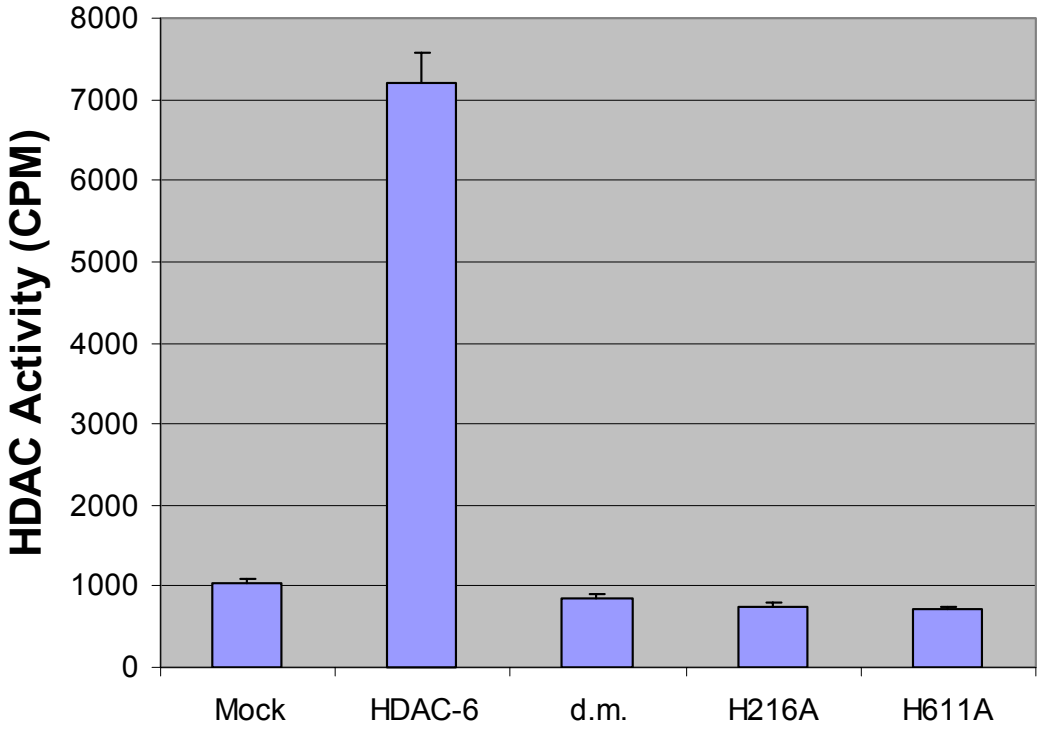


Fig. S 3 Zhang et al.



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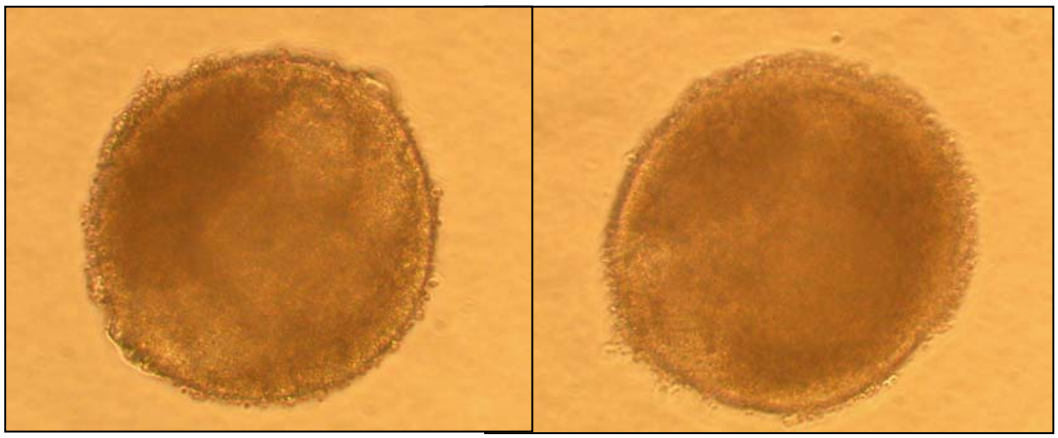


Flox K.O.

2.2 Dissection of the protein deacetylation reaction

2.2.1 abstract/Introduction

Protein acetylation, especially histone acetylation, is one of the most important posttranslational modifications and is involved in the regulation of protein's structure and functions. The acetylation and deacetylation on histone tails are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) respectly. Among the HDACs, HDAC-6 was recently identified as a dual-, even multi-, substrate deacetylase that can deacetylate both acetylated histone tails and α-tubulin Lys40 *in vitro* and *in vivo*. Since HDAC-6 has a unique structure that contains two intact hdac catalytic domains like in many known HDAC-containing repression complexes, it serves as an important model to dissect the general *in vivo* deacetylation reaction. In this report, we show that both hdac domains are necessary for the intact deacetylase activity of HDAC-6, but each might have a different role in selectively recognizing substrates. The spatial arrangement of those two domains in HDAC-6 is essential in that alteration of the linker region between two domains severely affects the catalytic activity. Moreover, the effect of spatial modulation is different for tubulin substrate and histone substrate, which suggested that the substrate specificity of HDAC-containing complexes might be partially determined by the spatial arrangement of the hdac domains. Artificial chimeric HDACs, made by replacing the hdac domains in HDAC-6 with ones from other class II HDACs, showed *de novo* deacetylase activity. Taken together, our results demonstrate for the first time that spatial arrangement of hdac domains is critical for *in vivo* deacetylation reaction and may provide a useful model for the clinical targeting of HDAC inhibitors.

2.2.2 Results

To investigate if the two hdac domains in HDAC-6 might have different roles in deacetylation reaction, we made several mutant constructs, which contain mutations in catalytic core or presumed substrate recognition motif (Fig2.2.1 A). The mutations were either only in one hdac domain or in both domains. The expression levels of each mutant in transient-transfected cells were similar as shown by western blot with HA-antibody (Fig2.2.1.B). Those materials were used for immunoprecipitation by HA-antibody and subsequently for HDAC (histone deacetylase) or TDAC (tubulin deacetylase) activity assays. The chemically acetyl-labeled tubulin peptide and histone H4 peptide were used in the assays (Zhang et al., 2003). As shown in Fig2.2.1 C, double mutations in both hdac domains completely abolished the HDAC and TDAC activities of HDAC-6. Surprisingly, mutating each of the hdac catalytic cores destroyed the whole activity on both histone and tubulin peptide substrates, which suggested the cooperation of those two hadc catalytic cores on both domains is critical for the deacetylation reaction mediated by HDAC-6. Interestingly, the mutations in presumed substrate recognition regions led to somewhat different effects on enzymatic activities. The mutation in second substrate recognition site completely inactivated the catalytic activity on both substrates, whereas the mutation in the first hdac domain only partially inactivated the activity. Moreover, mutation in first recognition motif had a stronger effect on HDAC activity than on TDAC activity. This suggests that in the deacetylation reaction those two different substrates might interact and be recognized differently by HDAC-6's first catalytic domain. Taken together, two intact hadc domains on HDAC-6 are both important for the deacetylation activity whereas they might have different roles in the substrate recognition and provide substrate specificity.

Since both hdac domains are important for the deacetylation reaction, in which sometimes substrate may have only one acetylated lysine residue, such as in α-tubulin, whereas histone tails may have several acetylated lysines, how substrate aligns with those two domains is unclear. To exploit the spatial arrangement of HDAC-6's two catalytic domains we modulated the linker region between those two domains by either deletions or insertions. As shown in Fig2.2.2.A, first of all, the fragments from EGFP protein with various length, from 5 amino acids to full length of EGFP (239 amino acids), were inserted between two hdac domains. To shorten the distance between two hdac domains, the linker region was deleted by 5 amino acids, 15 amino acids, or 68 amino acids, respectively. All of the constructs were equally expressed in transient-transfected 293T cells (Fig.2.2.2.B) and subsequently used for immunoprecipitation and activity assays. Surprisingly, the slightly modulation of the linker length, either increase or decrease only 5 amino acids, while HDAC-6 protein has 1150 amino acids in total, could dramatically affect the catalytic activity (Fig2.2.2.C). Along with the increasing length of the insertions, both HDAC and TDAC activities also decreased. On the other hand, deletion of 5 amino acids in the linker region could also partially suppress the activities on both tubulin and histone peptide substrates, whereas the complete deletion of the linker region showed the most dramatic inactivation. Interestingly, the downregulation of the activity is selective for the substrates in that the repression is more significant on tubulin substrate than that on histone peptide, which is similar as the mutation analysis of the hdac domains (fig.2.2.1.C). Taken all together, our data suggested that HDAC-6's two active catalytic domain might use selective conformation to recognize and catalyze different substrates.

Subsequently, we analyzed the linker region in details. Secondary structure predicated by PSI (McGuffin, et al., 2000) showed that the linker region contains two helical regions (Fig2.2.3). In mutation "-5a", in which 5 amino acids were deleted, the first helix was shortened, whereas in "+5a" the first helix was separated by an extral coil. These structure predictions suggested the modulation of the linker region indeed altered the secondary structure and possibly changed the length of the linker region. Further structure analysis of the linker and the whole protein is necessary to confirm this. In addtion, we also made two point mutants in the linker region without change the primary length. In first mutant, "LALET", the sequence of the five amino acids in the linker was mixed. In the second mutant, "AAAAA", the central five amino acids were changed to alanines. Surprisingly, although the predicted structure did not changed significantly, the enzymatic activity of those two mutants also decreased, especially for TDAC activity. The "AAAAA" mutant showed similar HDAC activity as wild type, whereas "LALET" had lower HDAC activity comparing with wild type. This suggests that the fine structure of the linker region is important for the catalytic activity, which might be due to the fine modulation of the spatial arrangement of two catalytic hdac domains.

Finally, we tested if the artificial HDACs, which were made from different combinations of several class II HDACs, may be selectively active. HDAC-6 is active on both tubulin and histone substrates. On the other hand, in our hands and from published data, HDAC-4 and HDAC-5 are both inactive on tubulin and histone substrates by themselves and their *in vivo* activity on histones might be due to the interaction with HDAC-3. It was supposed that HDAC-3 collaborates with HDAC-4/-5 , in which HDAC-4/5's hdac domain might recognize histone substrates and recruit HDAC-3 to deacetylate the histone tails by direct interaction. To make the chimeric HDACs, we replaced the first and/or the second hdac domain in HDAC-6 by the hdac domains from either HDAC-4 or HDAC-5 (Fig2.2.4.A). Since the distance between two hdac domains is important for the activity (Fig.2.2.2), in chimeric HDACs, we used the linker region from HDAC-6 and

kept the distance between two catalytic centers the same as in the wild type HDAC-6 protein. After transfection into 293T cells, the expression levels were checked by western blot and the extracts were subsequently used for immunoprecipitation and HDAC/TDAC assays. As shown in Fig.2.2.4.C, replacement of second hdac domain of HDAC-6 by domains from either HDAC-4 or HDAC-5 abolished activity of HDAC-6 on both tubulin and histone substrates. On the other hand, the protein expression level of chimeric HDACs, which have first hadc domain from either HDAC-4 or HDAC-5, was significantly lower than wild type HDAC-6 and chimeric HDACs , which have second domain from HDAC-4/-5, because of unknown reason(s). After normalized by protein expression level, Both TDAC and HDAC assays showed that artificial combination of the inactive hdac domains from HDAC-4/-5 and the part of HDAC-6 protein, which lacks the first hadc domain and is also inactive, could raise the novo activity. The relative activities normalized to w.t. HDAC-6 on histone substrate were higher than on tubulin peptide. This might be due to the selective recognization and/or enzymatic activity of HDAC-4/-5 on histone but not tubulin peptide.

To better characterize the enzymatic properties of HDAC-6 protein and analyze the structure, we set up a purification protocol from baculovirus system. Active HDAC-6 could be purified nearly 99% pure (Fig2.2.6) using this protocol. And crystallization of the whole protein and parts of HDAC-6 is going on.

2.2.3 Discussion

As reversible and irreversible markers, protein posttranslational modifications play central roles in maintenance of organisms by modulating protein function and/or providing new signals. Acetylation of ε-NH_2 in lysine amino acids has been found in various protein substrates such as histones, tubulin, some transcription factors, etc. Acetylation/deacetylation on histone N-terminal tails, together with other modifications such as methylation, phosphorylation, ubiquitination, forms epigenetic "histone code", which is supposed to be not only markers which reflect certain physiological events but also one of the key determents of chromatin structure and genome regulation. On the other hand, acetylation of tubulins might be involved in dynamics and stabilization of microtubules. The identification of enzymes that directly acetylate and deacetylate protein substrates is the key requirement to understand the *in vivo* functions of protein acetylation. Recently, increasing numbers of acetyltransferases and deacetylases have been discovered for histone substrates. Subsequently, few of them were shown to be able to catalyze non-histone substrates. HDAC-6 was recently identified as a dual-, or multi-, substrate deacetylase, which can deacetylate both histone and tubulin substrates *in vitro* and *in vivo*. HDAC-6 has a unique structure in that it contains two intact hdac catalytic domains whereas all other HDACs contain only one. But, *in vivo* almost all of the HDAC-containing complexes contain at least two hdac domains from either homodimers or heterodimers of HDACs. Taken all together, investigation of deacetylation reaction catalyzed by HDAC-6 and dissection of two hdac domains on different substrates may provide important information to understand general protein deacetylation and to design *de novo* HDAC inhibitors for clinics. In this report, we showed that the deacetylation reaction catalyzed by HDAC-6 indeed needs both hdac domains. In addition, we found that the spatial conformation of those two domains is important for the specific selective activity on different substrates. Moreover, artificial combination of two independently inactive hdac domains could raise deacetylase activity.

Since the two hadc domains in HDAC-6 are well conserved, the direct question would be whether they are functionally different. In the original publication, in which HDAC-6 was first identified, Schreiber's lab (Grozinger et al., 1999) showed that the two domains are equally functional in that inactivation of one domain reduced 50% activity. But, we and other group (Khochbin, personal communication) got different results. First of all, separation of the two domains in HDAC-6 destroyed the activity in that the deletion constructs of HDAC-6 were shown to be inactive (Zhang et al., 2003). In our hands, mutating a single hdac domain is sufficient to inactive the whole HDAC-6 on both histone and tubulin substrates *in vitro*. Transfecting wild type HDAC-6 into cells could significantly induce tubulin hypoacetylation *in vivo* whereas single mutants as well as double mutant could not (data not shown). Moreover, the fact that the spatial arrangement of those two domains is important for the activity also confirmed that both hdac domains are necessary for the activity. The difference between our results and Schreiber's might be due to the differences in enzyme preparation and the activity assays. Our peptide based TDAC assay are much more sensitive than assays using western blot and microtubule substrates. Moreover their HDAC assay also did not shown strong specific deacetylase activity. For example, in our hands, TSA can completely inactivate HDAC-6 whereas in the assays of Grozinger et al, TSA could only block 30% HDAC activity of HDAC-6. In addition, we could not detect any significant HDAC activity on HDAC-4 and HDAC-5, which also has been shown in recently publication from Verdin's group (Fischle et al., 2002), whereas in the HDAC assay of Grozinger et al, HDAC-4 and -5 had significant activity. This suggested that either their enzyme preparation by immunoprecipitation is not clean or their activity assay is not specific.

We have shown that the single hdac domain from HDAC-6 is sufficient to bind beta-tubulin although this interaction is not mediated by catalytic core. Although both hdac domains of HDAC-6 are equally important for the whole activity, it is interesting to investigate if they are functionally the same in deacetylation reaction or not. In hdac catalytic domain, in addition to the conserved catalytic core, an ER (Esa1-Rpd3) motif (Adachi et al., 2002) has been recently identified by searching the similar structural and functional motifs between HATs (e.g. Esa 1) and HDACs (e.g. Rpd3). In the tertiary structure of Esa1, the ER motif is located near the active center. Recent structure analysis of tGCN5/CoA/H3 complex (Clements, 2003) showed this motif is involved in the interaction and recognition with histone tail. In Rpd3, for which the tertiary structure remains unclear, by circular dichroism analysis it was demonstrated that the ER motif contains a similar secondary structure as found in Esa1. Mutation analysis found that the ER motif regions of Esa1 or Rpd3 are required for HAT activity of Esa1 or HDAC activity of Rpd3, respectively. By mutating those putative substrate recognization motifs in HDAC-6, we found that the second hdac domain might be more important for the selective binding of the substrates in that mutation in second hdac domain completely inactivated the activity whereas mutation in first domain retained partial activity. This is the first evidence to show the functional difference between the two hdac domains in HDAC-6. More interestingly, we observed that the mutation in the first putative substrate recognization site had different effect on the HDAC/TDAC activities. TDAC activity is more sensitive to this mutation than HDAC activity. This also suggested that hdac domains selectively interact and recognize different substrates. Further experiments are ongoing to resolve the tertiary structure of HDAC-6 with different substrates.

It has been generally accepted that the *in vivo* functional histone-deacetylating units are HDAC-containing complexes. Interestingly, in all known HDAC-containing complexes, there are normally two HDACs. For example, HDAC-1 is commonly found to work together with HDAC-2. This raises question whether the *in vivo* deacetylation reactions also need two HDACs together. *In vivo* analysis of HDAC-1 function in Drosophila (Mottus, et al., 2000) found that flies have

different phenotypes when they are either completely deficient for HDAC-1 or only have a single mutation, which may toxicify the complexes. It was suggested that in the absence of HDAC-1 other HDACs might be able to compensate it, therefore the HDAC1/2 containing complexes are still functional, at least partially. But, a HDAC-1 point mutant, which still be able to form the complex but deficient for the catalytic activity, will lead to an inactive HDAC-containing complex therefore to different phenotypes other than the null mutant. This evidence also indirectly suggested that the mutation in one of the hdac domains in the HDAC-containing complexes could inactivate the whole complexes. The result on N-CoR/SMRT demonstrated that class II HDACs regulate transcription by bridging the enzymatically active SMRT/N-CoR-HDAC-3 complex and select transcription factors independently of any intrinsic HDAC activity (Fischle, et al., 2002). The catalytic domain of HDAC-4 interacts with HDAC-3 via the transcriptional corepressor N-CoR/SMRT. All experimental conditions leading to the suppression of HDAC-4 binding to SMRT/N-CoR and to HDAC-3 result in the loss of enzymatic activity associated with HDAC-4. *In vitro* reconstitution experiments indicate that HDAC-4 and other class II HDACs are inactive in the context of the SMRT/N-CoR-HDAC-3 complex and do not contribute to its enzymatic activity. At present, it is still not clear if individual HDACs could be active on a monomer form. The classic purification of HDACs could not answer this question because of the co-purification of endogenous HDACs (Li, et al., 2004). Eukaryotic expression and purification of HDACs always co-purify several HDACs at the same time. On the other hand, the expression of HDACs in bacterial, which is deficient for endogenous HDACs, so far, is unsuccessful. So far, HDAC-6 is the only HDAC that has been shown to process catalytic activity independent on other HDACs. Our results showed that HDAC-6's catalytic activity also is dependent on both intact hdac domains. Moreover, artificial tethering parts of HDAC-6 and HDAC-4/5 could raise catalytic activity, which mimics the result from N-CoR/SMRT complex. Therefore, we suggest a possible model for the general deacetylation reaction, in which two hdac domains are required (Fig 2.2.5.A). As in the case of HDAC-6, HDAC-containing complexes might have similar spatial arrangement of hdac domains, but from two different HDAC molecules. Those two hadc domains cooperate to confer the catalytic activity of the whole complexes. The components of the different complexes determine the spatial arrangement of the two core hdac domains and therefore the specific activity of the complexes. Solving the crystal structure of native whole HDAC-containing complexes should help to verify this hypothesis.

The clinical benefits of HDAC inhibition and their implications for re-differentiation therapy are currently being investigated at several locations. As discussed in the Introduction (1.4.5), the most important question now is to investigate the truely selective *in vivo* targets of different HDAC inhibitors. Using *in vitro* enzymatic assays, some HDAC inhibitors were tested for the specificity on individual HDACs. But, *in vivo*, the functional units are HDAC-containing complexes. It will be more directly to test the specificity of inhibitors on different *in vivo* HDAC-containing complexes, which may have different substrate specificity and specific activities even when they contain same HDACs. The ideal HDAC inhibitor should be complex-specific. How to achieve this specificity? We believe that the central difference between each HDAC-containing complexes is the spatial arrangement of the two hdac domains, which finally decides the substrate specificity. Based on this difference one could design inhibitors specific for certain complexes. As shown in Fig 2.2.5.B, the novel HDAC inhibitor will target both HDAC domains in one complex at the same time. The linker between two functional group of this inhibitor is the key determinant for the specificity because its length correlates the distance between two hdac cores in the complex. By varying the length of the linker, different HDAC-containing complexes might be specificially targeted. It can be assumed that this kind of HDAC inhibitors might be much more potent than current inhibitors since it targets two hdac cores at the same time whereas current hdac inhibitors only

target hdac cores individually. Therefore, the local active inhibitor concentration is much high for the proposed novel inhibitor. Finally, chimeric HDAC inhibitors could be designed by combining different hdac inhibitors. For example, one TSA molecule could be linked with another TPX molecule. This new inhibitor might have different characteristics comparing with either TSA or TPX.

2.2.4 Materials and Methods

Plasmids and mutations

Mutations and deletions were generated by Quickchange kit (Strategene). For EGFP insertions, part of the EGPF was PCR-amplified from pEGFP (Clontech) and subsequently cloned into the Xba I site in the linker region of HDAC-6 cDNA. To clone the chimeric HDAC, the hdac domains from either HDAC-4 or -5 were PCR-amplified and used to replace the first or second hadc domain in HDAC-6.

Co-immunoprecipitation

For co-immunoprecipitation, ~500 µg of extracts from HEK 293T cells transfected by Fugene (Roche) were incubated overnight with the primary antibody at 4°C with gentle agitation. Rabbit IgG or mouse IgG was used as control. After this, 20 µl of protein A–Sepharose slurry were added and samples were incubated for 1 hour at 4°C with gentle agitation. Beads were washed three times with IP buffer (20 mM HEPES pH 7.4, 0.5 mM EDTA, 150 mM NaCl and 0.1% Triton X-100) and subsequently resuspended and boiled in 20 µl of loading buffer for SDS–PAGE.

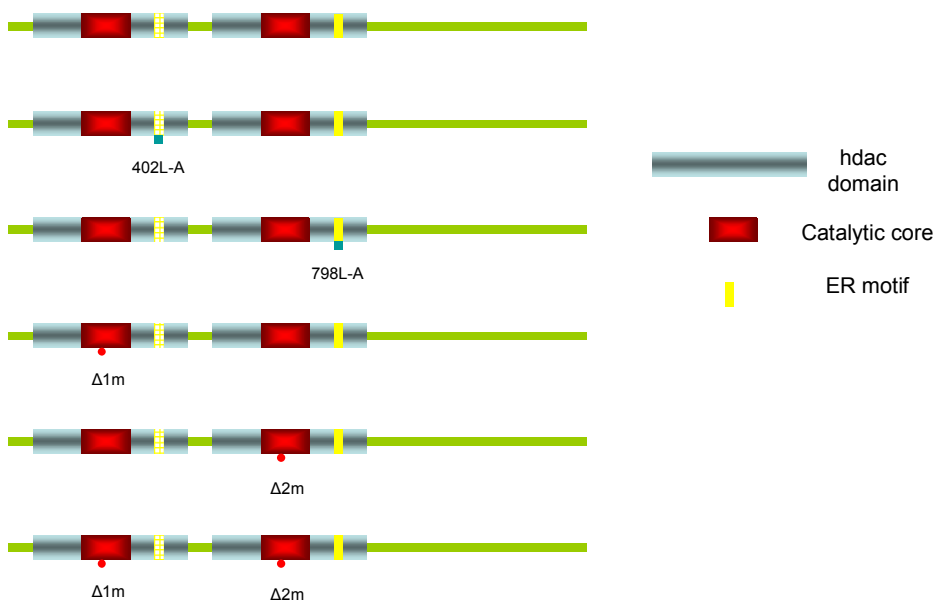
Immunofluoresence and immunoblotting

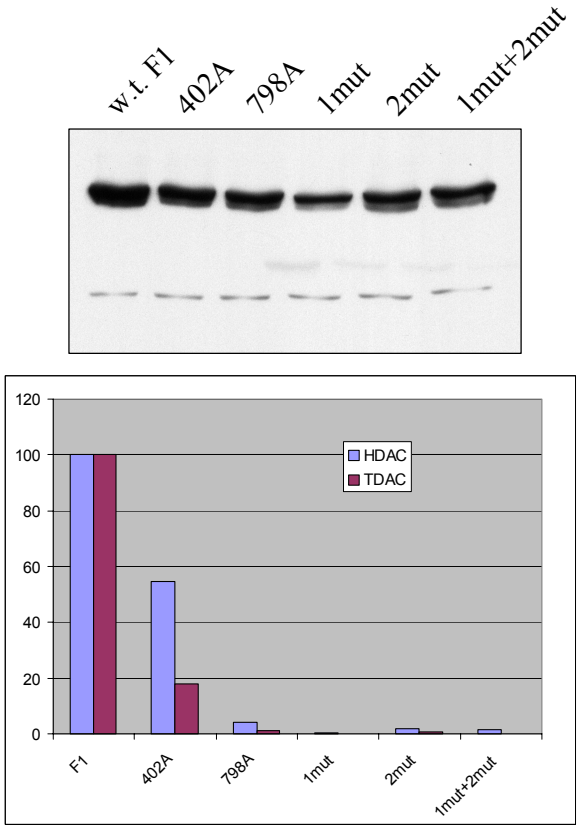
For tubulin and HDAC-6 staining, cells were fixed in methanol at –20°C for 10min and stained with mHDAC-6 polyclonal antibody (Verdel, et al., 2000), TU2.1 for alpha-tubulin or TU6-11 for acetylated tubulin (Sigma). The DNA was counterstained by PI or DAPI. Protein lysates were resolved by electrophoresis on 8% or 12% SDS-PAGE gels and subsequently to nitrocellulose membranes (Bio-Rad). Antibodies used are: Flag-M2 (Sigma), anti-HA (Santacruz), and mHDAC-6 (Verdel, et al., 2000).

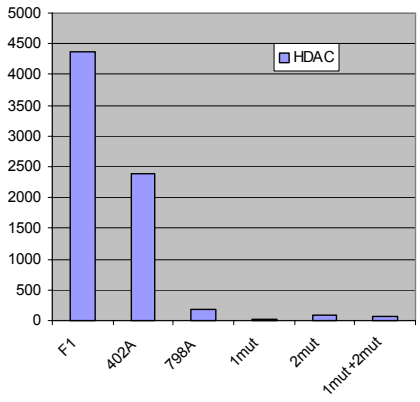
HDAC and TDAC assay

The peptides corresponding to either amino acid 33-46 of human alpha tubulin or histone H4 N-terminus were chemical acetylated by H^3-acetic acid (PerkinElmer). HDAC/TDAC assays were performed in a 300-µl volume containing immunoprecipitated HDACs, 10 mM Tris, pH 8, 10 mM NaCl, 10% glycerol, 1mM PMSF, and 50,000 cpm [^{3}H] peptide. Reactions were incubated for 2 h at 37 °C. 75 µl of stop solution (1 M HCl, 0.16 M CH_3COOH) was added, and 900 µl of ethyl acetate was added to extract the released tritiated acetate. 400 µl was counted in a liquid scintillation counter.

Fig2.2.1 the requirement of two hdac domains for deacetylation by HDAC-6 protein







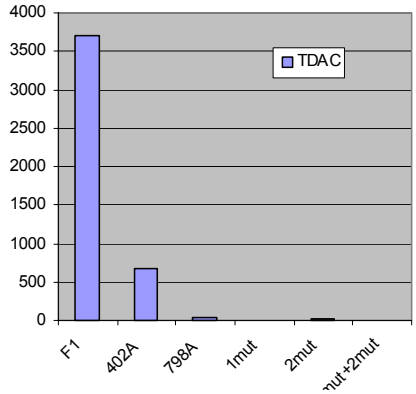


Fig2.2.2 the spatial arrangment of two hdac domains in HDAC-6 is important for the activity

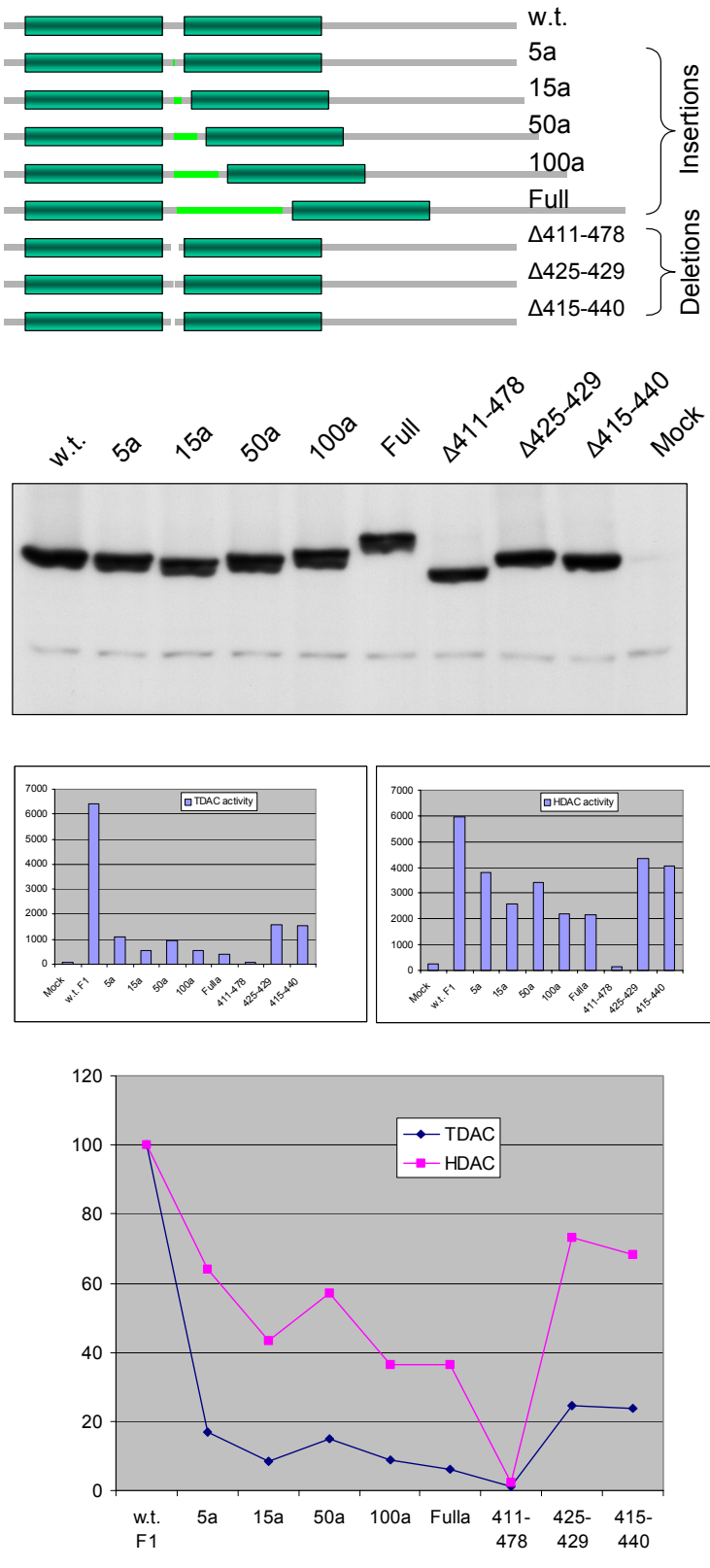
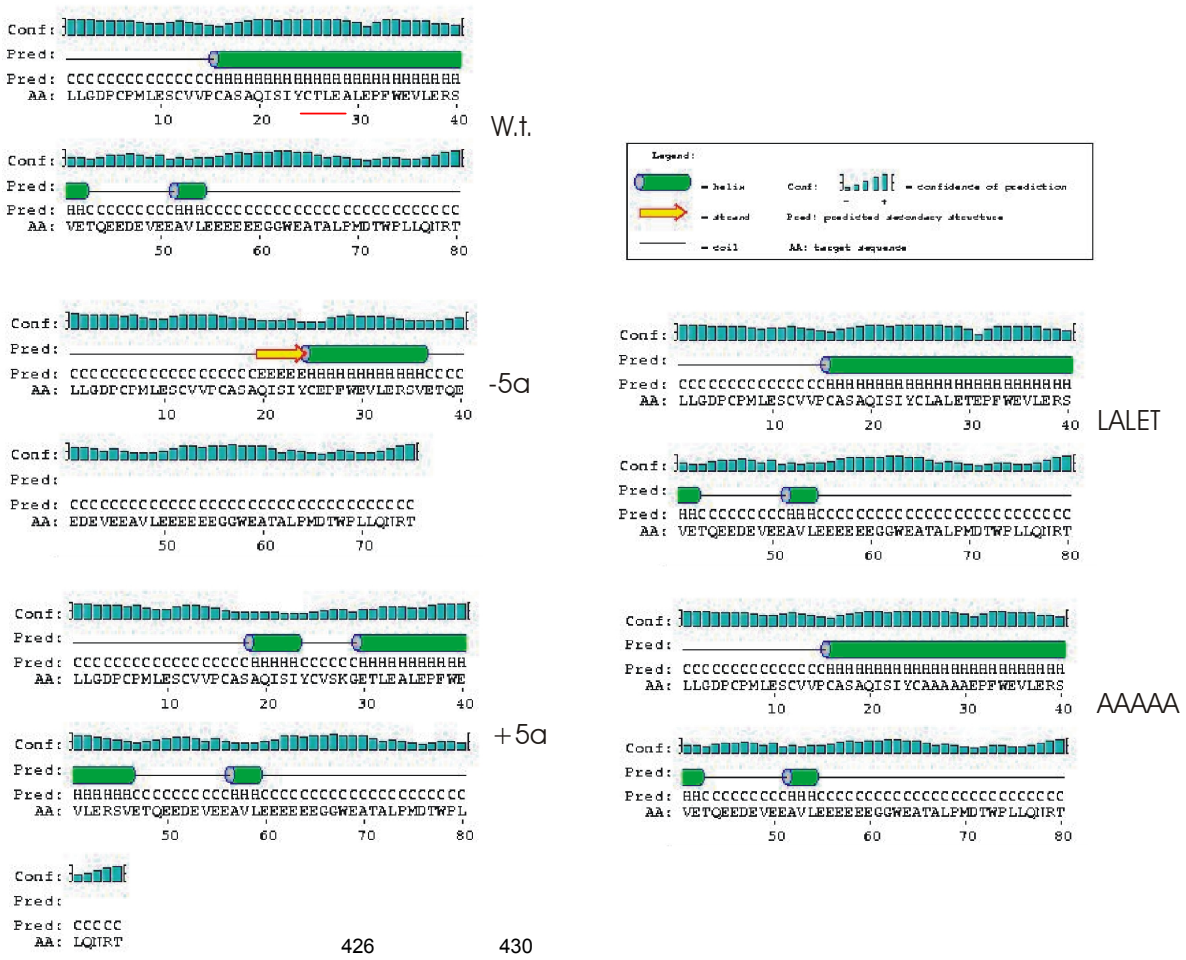


Fig2.2.3 Analysis the linker region of HDAC-6 (predicated by PSI)



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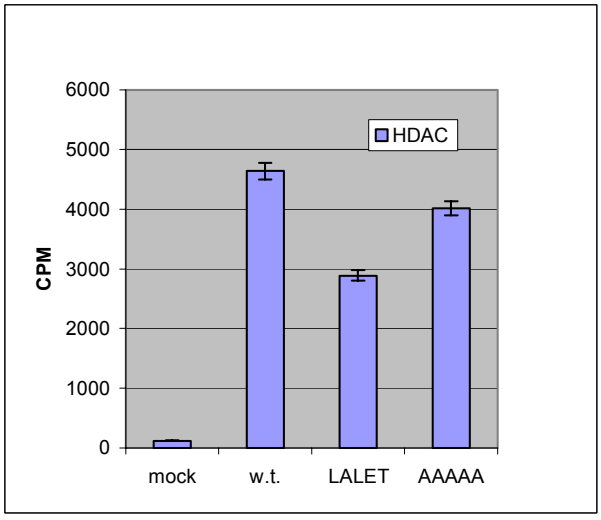
ISIYC L A L E T EPFWE LALET
ISIYC A A A A A EPFWE AAAAA



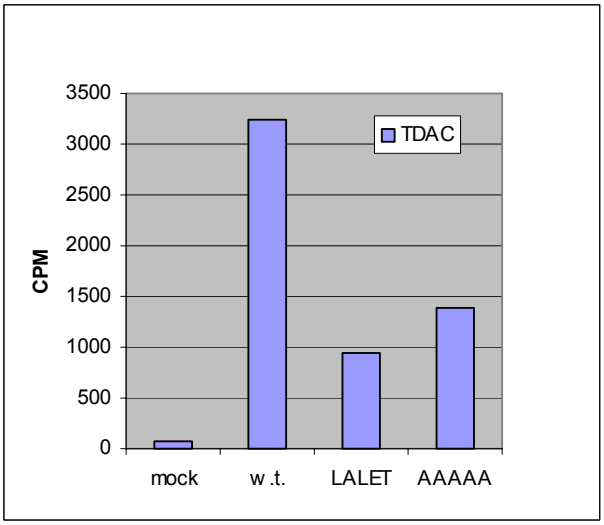
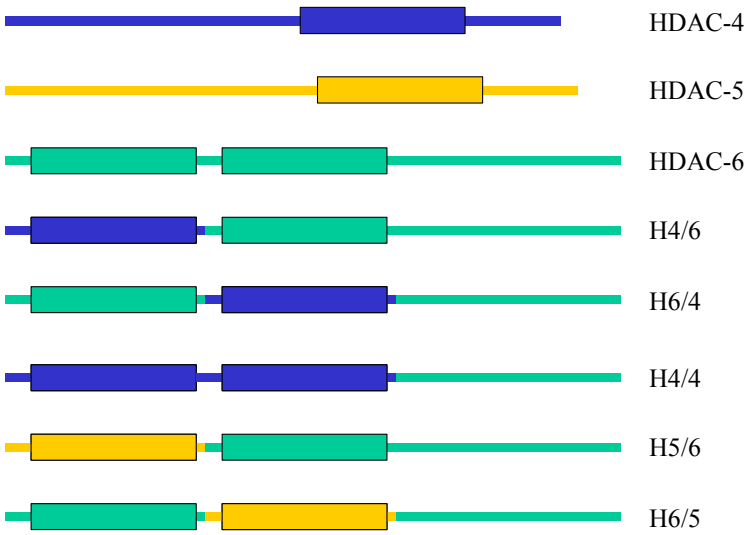
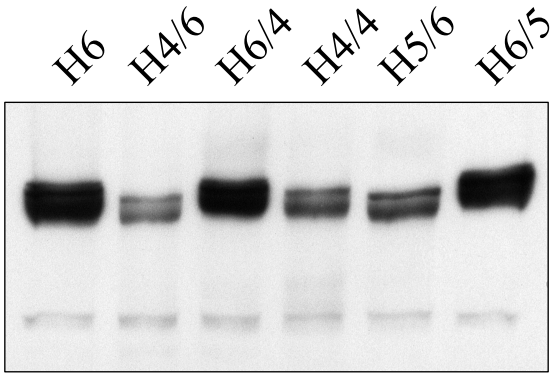
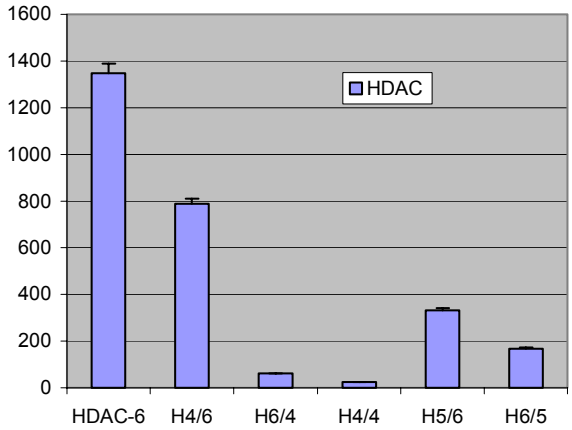


Fig2.2.4 Generation of artificial chimeric HDACs







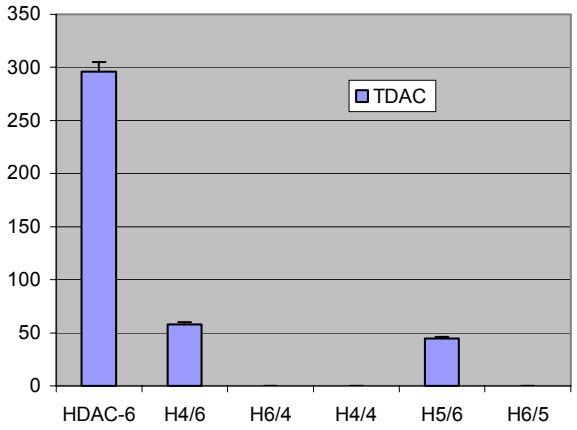
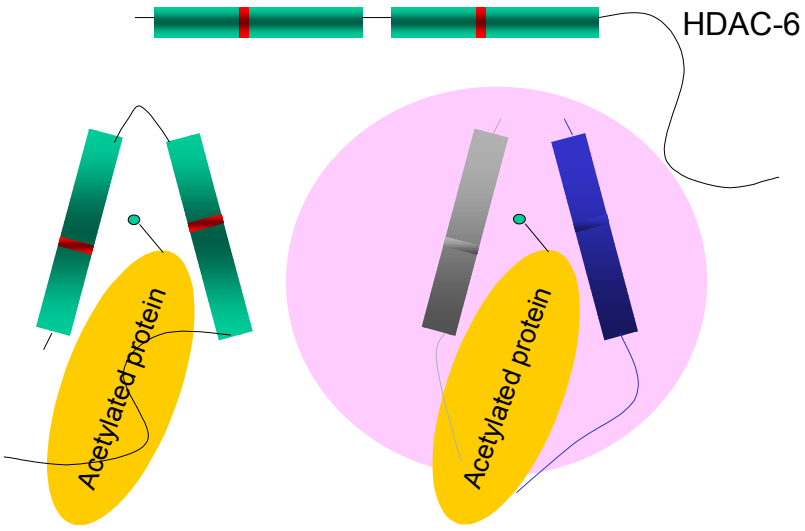


Fig2.2.5 Hypothetic models for protein deacetylation and design of novel HDAC inhibitors

A



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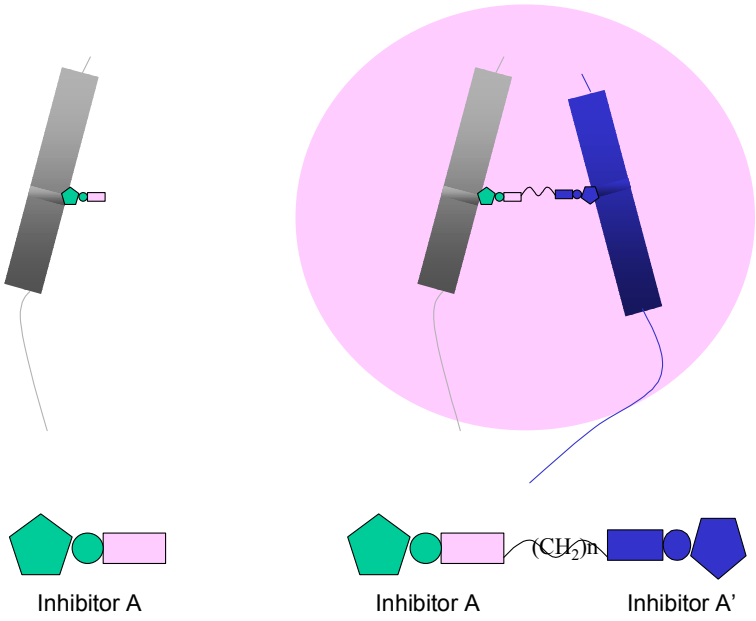


Purification of baculo-mHDAC-6 protein

To investigate the deacetylation catalyzed by HDAC-6, it is necessary and important to make the recombinant proteins including wild type and different mutants. Bacterial expression of HDAC-6 has been tried (data not shown; Khochbin, personal communication), but there are two major problems: first, the bacterial-made HDAC-6 is inactive (for both tubulin and histones); second, most of the recombinant proteins are insoluble. All of these may due to the improper folding of eukaryotic proteins in E.coli. Specially, since the catalytic center of hdac need Zn^{2+} ion, bacterial recombinant HDAC-6 may not fold hdac domain correctly. This has been observed with several other mammalian HDACs. Therefore, baculo-expression might serve as the better choice.

To make the baculo-expression vector, first of all, 6XHis amino acids have been inserted just before the stop-code of the mouse HDAC-6 cDNA, which made a short 6XHis tag on the C-terminal of mHDAC-6 protein. Since it is small and at the end, the disturbing of whole structure by extra 6XHis tag is minimized. Then, the 6XHis tagged mouse HDAC-6 was cloned into pDEST^{TM}8MCS1A baculo transfer Plasmids (kind gift from Haidi Zhang) with polyhedron promoter. The whole coding region was sequenced and several mutations were found comparing with the published sequence (PubMed). But later it is confirmed that the published sequence is wrong because of the sequencing problem. Bacmids for insect cell transformation were generated using the Bac-To-Bac Baculovirus Expression System (Life Technologies) in DH10Bac bacterial and were subsequently transfected into sf-9 cells. The expression of active baculo-mHDAC-6 was tested in sf-9 cells by western blotting and immunoprecipitation/HDAC assay (data not shown). For protein overexpression, insect sf-9 cells were infected with virus (1:10 dilution and 10ml for one 75cm^2 bottle) and collected 3 days later.

Strategy I: Inactive preparation (adapted from Zhang et al., 2002)

Cells (ca. 4 x 10^7 from 4X75cm^2 bottles) were resuspended in 4 ml of buffer W100 (30mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM ß-mercaptoethanol, 10% glycerol and 0.5% Triton X-100) containing 1x protease inhibitor cocktail without EDTA (Roche) and broken by homogenizing 40 times. Lysates were centrifuged at 17,000 g for 30 min and the supernatant was mixed with 400 µl Talon affinity resin (Clontech) pre-washed with buffer W100. After rotating for 3 h at 4°C, the resin was washed with buffer W100 (3 x 10 ml and each for 10 min). Protein was eluted with 4 ml of buffer W100 containing 40 mM imidazol and 1 mM DTT instead of ß-mercaptoethanol. The eluate was directly added to 400 µl of Ni/NTA beads (Qiagen) pre-washed with buffer W100, containing 40 mM imidazol and 1 mM DTT. Following 3 h at 4°C, the beads were washed similarly to Talon beads but with buffer W100 containing 40 mM imidazol and 1 mM DTT and the protein eluted with 2 ml of buffer W100 containing 100 mM imidazol and 1 mM DTT. The eluate was dialyzed against buffer D (as buffer W100 but containing 50% glycerol, 0.1% Triton X-100 and 1 mM DTT) (2 x 2 h, 1 L each time) and stored at –20°C.

As shown in Fig A, the combination of Ni and Talon beads gave much better purity (more than 90% pure) than either single affinity purification. Because of the intrinsic differences on binding specificity and strength, the nonspecific binding could be minimized using this protocol. But the most important problem with this protocol is that the HDAC-6 protein was inactive. As the crude enzyme prepared by Ni beads direct pull-down is as active as endogenous overexpressed enzyme, the enzyme must lost activity during the purification. Subsequently, $MgCl_2$, ß-mercaptoethanol, Triton X100,

and imidazol were tested for the effect on enzymatic activity of HDAC-6 either from baculo- or mammalian-overexpressed enzymes. As shown in Fig B , ß-mercaptoethanol and imidazol could inhibit HDAC-6 activity. Moreover, the inhibition by imidazol is irreversible since after remove the imidazol by extensive washing the activity cannot be recovered (data not shown). The inhibition by imidazol might be explained by its stronger ion affinity than His amino acids that are key amino acids in hdac catalytic core to bind Zn^{2+} and mediate catalytic reaction. Therefore, during the elution, large amount of imidazol could remove the Zn^{2+} in the hdac catalytic core and irreversibly inactive the enzyme.

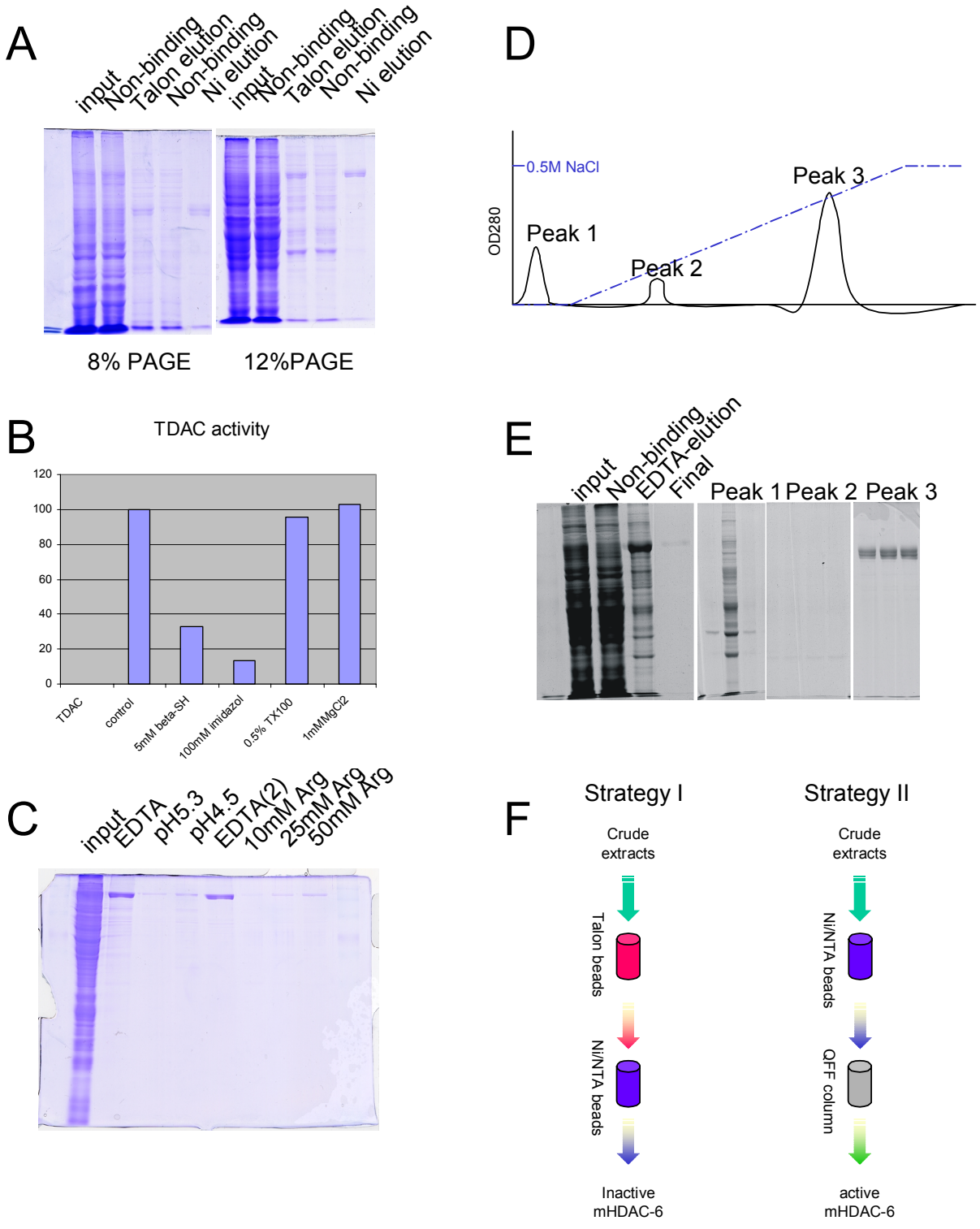
Strategy II: EDTA elution

To make the active recombinant mouse HDAC-6 protein, first of all, DTT and ß-mercaptoethanol were omitted from the W100 buffer. Second, other elution conditions were tested. A. 0.1 M EDTA pH8.0; B. different phospho-buffer (100mM NaH_2PO_4, 10mM Tris, 200mM NaCl, 10% Glycerol) with pH4.5 or 5.9, then 0.1 M EDTA pH8.0; C. Arginine 10, 25, or 50 mM. As shown in Fig C, the binding on Ni beads is too strong to be efficiently eluted by either pH or arginine. But EDTA can almost fully recover the bonded HDAC-6 protein and after dialysis the eluted HDAC-6 is still fully active (data not shown). The specific activity after pH and EDTA elution is lower than only EDTA, which suggested that pH changes might be bad for activity. Moreover, comparing the Talon beads, elution by EDTA from Ni beads has better purifity (data not shown).

To further make pure recombinant mHDAC6 protein, a QFF column (Q Sepharose Fast Flow, 1ml, Amersham) was used. (Several other kinds of columns were also tested and compared.) Concentrated samples (either by dialysis or centri-prep 50) were loaded on the Bio-Rad dual flow system. Then a gradient from 0 to 500 mM NaCl gradient (in 30mM Tris.HCl pH7.5) was used for elution. Flow rate is 1ml/min. As shown in Fig D and E, mHDAC-6 protein was eluted at ca. 400mM NaCl. And most of the contaminated proteins were eluted in peak 1 and there is an additional contamination peak 2 at ca. 100mM NaCl. (Notice: if overload, the separation will be bad.). To further concentrate elution, centricon-100 was used and this might lead to the loss of protein (see below).

Midi-scale purification had been made from ca. 20X 75cm2 bottles of infected sf-9 cells. The first step purification by Ni beads and EDTA elution is very efficient in that about 100% of the activity was recovered. But the final activity is only ca.3% left, which is due to the last step using centricon-100. To overcome this, centri-prep 50 should have been used instead.

Fig2.2.6 Purification of baculo-mHDAC6 protein



2.3 Analysis of HDAC-6 deficient mice and cells

2.3.1 Abstract

Posttranslational modifications play important roles in regulating protein structure and function. After the identification of histone acetyltransferases (HATs) and histone deacetylases (HDACs), acetylation/deacetylation of histone tails has been shown to be directly involved in the modulation of chromatin structure, thereby in gene transcription, DNA replication, genome stability, etc. In collaborating with other chromatin modifications and remodeling activities, dynamic and highly regulated acetylation/deacetylation on histone tails forms "histone code". HDAC-6 was identified as a class II HDACs, which has unique structure in that it contains two hadc domains. Recently HDAC-6 has been shown to be able to use both histone and tubulin substrates. *In vitro* and *in vivo*, HDAC-6 deacetylases acetylated lysine 40 in α-tubulin, therefore directly modulate the acetylation of microtubules. To investigate the *in vivo* functions of HDAC-6 we targeted HDAC-6 gene by homologous recombination in ES cells and generated a knockout and a floxed allele. HDAC-6 deficient mice were obtained and these mice are viable and fertile and do not do not show an obvious phenotype. Yet, tubulin acetylation is dramatically increased in most of tissues from those mice. This suggested that tubulin hyperacetylation is not essential for normal development. We found HDAC-6 is not important for the germ cell development in mouse testis but might be involved in the regulation of acrosome reaction activity and sperm activity. In addition we found HDAC-6 is involved in the formation of aggresomes in both a mouse Mallory body model and in 3T3 cells, which might be due to the retarded protein ubiquitination and/or transport along the microtubules. Moreover, HDAC-6 expression is upregulated during denervation-induced muscle atrophy and HDAC-6 deficient mice are more resistant to muscle atrophy. To dissect the different functional domains of HDAC-6, we reintroduced rescue mutants into HDAC-6 deficient cells and various transgenic mice, which contain different HDAC-6 mutants, are being made.

2.3.2 Introduction

Chromatin structure plays central roles in all eukaryotic processes involving genomic DNA, such as DNA replication, repairing, recombination, gene transcription. The modifications of the basic chromatin units, nucleosomes, are directly involved in the modulation of chromatin structure and therefore gene transcription. Specially, the acetylation on N-terminal tails of histones has been shown to affect nucleosome stability and is generally considered to create a chromatin environment conducive to gene activation (Cheung, 2000). Although the correlation between histone acetylation and gene transcription regulation has been suggested in the mid-1960s, it was not until recently that the enzymes responsible for adding or removing acetyl groups on histones were identified. Acetylation of histones was found to be catalyzed by proteins that had been studied in other contexts, for example as transcriptional coactivators, and which are now called histone acetyltransferases (HATs). Several of these HATs are however also able to acetylate non-histone proteins and the list of their new substrates continues to grow. Conversely, histone deacetylases (HDACs) are proteins that catalyze the removal of acetyl groups from histones and thereby lead to gene repression. To date, 18 HDACs have been identified in humans, and their activities have been implicated in transcription, cell cycle progression, gene silencing, differentiation, DNA replication, and the DNA damage response (reviewed in Thiagalingam et al., 2003).

Major groups of HDACs include the RPD3/HDA1 superfamily, the Silent Information Regulator 2 (SIR2) family and the HD2 family. RPD3/HDA1-like HDACs are found in all eukaryotic genomes and are further divided into two classes: Class I HDACs (HDAC-1, -2, -3, and -8) are similar to yeast RPD3 protein; Class II HDACs (HDAC-4, -5, -6, -7, -9 and -10) are homologous to yeast HDAC1 protein. The SIR2 family of HDACs is distinctive in that it has no structural similarity to other HDACs and requires NAD as a cofactor. The fourth family, the HD2-type HDACs, were first identified in maize and appear to be present only in plants (Lusser et al., 1997) but not in animals or fungi, and are distantly related to cis-trans isomerases found in insects, *S. cerevisiae* and parasitic apicomplexans. HDACs exist in cells as part of large molecular weight complexes that are recruited to specific promoters via their interactions with sequence-specific DNA binding proteins. These include the nuclear hormone receptors, the E box binding factors, and the methylcytosine binding protein MeCP2. Class I HDAC-1 and HDAC2- are found in the SIN3 and NURD/Mi2 complexes. HDAC-3 is associated with corepressor N-CoR and SMRT mediating transcriptional repression by the thyroid hormone receptor and oncoprotein v-ErbA. Class II HDACs -4, -5, -7 can interact with N-CoR, SMRT, and BcoR, an additional corepressor that mediates repression by BCL-6. Although the role of each HDAC in gene transcription repression has been extensively studied *in vitro* and in culture cells, the *in vivo* functions of HDACs are largely still unclear. Recently, HDAC-1 has been shown to be essential for the mouse early embryo development in that targeted disruption of both HDAC-1 alleles results in embryonic lethality before E10.5 due to severe proliferation defects and retardation in development (Lagger et al., 2002). On the other hand, Olson and colleges showed that mutant mice lacking the class II HDAC, HDAC-9, are sensitized to hypertrophic signals and exhibit stress-dependent cardiomegaly (Zhang et al., 2002).

HDAC-6 was identified as the homologue of yeast HDA1 histone deacetylase. Interestingly, it contains two hdac catalytic domains in the N-terminal part whereas a Zinc-UBP domain, which interacts with ubiquitin and ubiquitined proteins, is in the C-terminal part. Like other class II HDACs, HDAC-6 mainly localizes in the cytoplasm, but also can shuttle between nucleus and cytoplasm. This is regulated by a N-terminally located nuclear export signal (NES) and maybe other uncharacterized mechanisms. Suprisingly, HDAC-6 was not found in any class I and -II containing repressor complexes, which suggested it may have different regulation and maybe substrates other that other HDACs. Recently, we and also two other groups independently found that HDAC-6 interacts with tubulin and microtubule in the cytoplasm. Futhermore, HDAC-6 is able to deacetylate acetylated lysine 40 in α-tubulin *in vitro* and *in vivo*, and therefore modulate the acetylation of microtubules. Moreover, HDAC-6 also can deacetylate histone N-terminal tail. We showed that artifical recruiment of HDAC-6 to promoter could repress the trancription activity and this repression activity is dependent on the deacetylase activity. Recently, HDAC-6 was shown to interact with CBFA1/Runx2 and this interaction could recruit HDAC-6 into the nucleus and repress the transcription of promoters such p21 promoter in osteblasts. The C-terminal Zinc-UBP domain on HDAC-6 has been shown to interact with mono-ubiquitin, polyubiquitin, and ubiquitinated proteins. In addition HDAC-6 interacts with various ubiquitin E3 ligases. All of these suggests HDAC-6 might be involved in the regulation of protein ubiquitination. Recently, the group of T. Yao found HDAC-6 colocalized with ubiquitin-positive aggresomes in cells after proteosome inhibitor treatment and with Lewis body in the brain of Parkison's patients. Loss of HDAC-6 blocks the formation of aggresome by slowing down the transport of damaged ubiquitinated proteins along microtubules to centromere; this requires both hadc domains and Zinc-UBP domain. HDAC-6 also interacts with dyneins and may serve as a bridge to recruit ubiquitinated proteins, which bind to C-

terminal Zinc-UBP domain on HDAC-6, to dynein motors on microtubules. In summary, HDAC-6 contains at least three distinct, possibly interrelated, functions: deacetylation of tubulin and microtubules and therefore regulation of microtubule dynamics; deacetylation of histone tails and therefore repression of gene transcription; interaction with ubiquitin and ubiquitinated proteins and therefore involvement in protein ubiquitination/degradation. To further dissect these functions *in vivo*, we generated HDAC-6 deficient mice and cells. We found that HDAC-6 is the major tubulin deacetylase *in vivo* but is not essential for normal embryo and adult development.

2.3.3 Results

Part I. Gene targeting for HDAC-6

The HDAC-6 gene localizes on the X-chromosome of both human and mouse. Analysis of Celera database and direct sequencing of BAC clone showed that HDAC-6 contains 28 exons and spans ~17 kb (UniGene and data not shown). To construct the targeting vector, a 6.7kb genomic DNA, which encodes part of the first catalytic hdac domain, was subclone from the BAC. Then exons 7 to 10 within the first hdac domain are flanked by a single loxP site and a floxed Neo^R TK cassette (Fig2.3.1). The cre-mediated recombination will destroy the first catalytic domain of HDAC-6 and lead to a frameshift, which will produces a truncated protein.

The targeting vector was eletroporated into E14 ES cells and clones were selected by neomycin. Screening PCR involves primer A, which localizes outside of targeting vector, and primer B in the TK. From 238 clones, 28 clones were identified as positives by PCR. Subsequently, southern blotting was used to confirm the correct targeting. Two different probes were used and the targeted allele was confirmed (Fig2.3.2). Notice: the w.t. band comes from the contaminating genomic DNA from feeder cells and targeted cells only contain one targeted alle but no w.t. alle since these cells are derived from line E14, which has a male genotype. Moreover, all three loxP sites were PCR amplified, sequenced and found to be correct (data not shown).

To get k.o. and floxed alle, we transiently transfect pCMV-Cre plasmid to achieve cre-mediated DNA recombination. Negative selection with gamcyclovir was used to select against TH and thus to enrich for clones having deleted the Neo.TK cassette. Theoretically, after cre mediated recombination between 3 loxP sites there will be 5 different genotypes together with w.t. alle from feeder cells. Three PCR primers were designed to screen positives (Fig2.3.3). Several clones were identified to contain k.o. allele in which both exons 7~9 and the floxed Neo^R TK cassette were removed. In a few clones, only the floxed Neo^R TK cassette was removed whereas in the rest two loxP sites remained flanking exons 7~9. In all these clones, no Cre was detected by PCR (data not shown). To get rid of contaminated w.t. genomic from feeder cells, positives were cultured on poly-lysine coated plates without feeder for more than 5 passages. Then genomic DNA were purified and used for southern blot. As shown in Fig.2.3.4, clone 126 was identified as a floxed clone having deleted the tk-Neo cassette but retaining all HDAC-6 exons, while clone 124 was found to be knockout. By western blot analysis, HDAC-6 protein expression level is similar in w.t. and floxed cells, but completely absent in k.o. cells (fig.2.3.4 and data not shown). Absence of HDAC-6 resulted in elevated alpha tubulin acetylation without change in the protein level of alpha or beta-tubulin. Strikingly, the acetylation of histone H3 or H4 did not appear to be affected by the complete absence of this HDAC. Wild type or HDAC-6 deficient ES cells did not

show any obvious morphological difference and both could differentiate *in vitro* equally well into embryoid bodies, indicating that in ES cells at least HDAC-6 is not essential (result part I).

Both floxed and k.o. ES cell lines were injected into C57BL/6 mouse blastocysts and chimeras derived from both cell lines were used to generate heterozygous and homozygous mice. The genotype of offspring derived from HDAC-6 heterozygous intercrosses was determined by Southern blot analysis or PCR. Both HDAC-6 floxed and knockout animals were obtained at the expected Mendelian ratio, were viable and fertile and showed no obvious phenotype (data not shown).

Part II. general characterization of HDAC-6 k.o. mice and establishment of HDAC-6 deficient 3T3 cells

HDAC-6 k.o. mice were born normally and the ratio between k.o. and w.t. appears normal (data not shown), which suggested HDAC-6 is not essential for embryonic development. Adult k.o. mice also do not show obvious phenotypes. To check whether targeted mice really functionally lost HDAC-6, western blot was done for HDAC-6 and acetylated tubulin. As shown in fig2.3.5.A, HDAC-6 protein is completely lost in all tissues from k.o. mice. HDAC-6 protein is highly expressed in testis and also significantly in brain, liver, and skeletal muscle. Interestingly, in most tissues, except brain, the tubulin acetylation increased dramatically in the absence of HDAC-6. Specially, in heart and spleen, in which only very little HDAC-6 protein is expressed comparing with testis, the hyperacetylation of tubulin is similar as in testis. This result showed successful functional ablation of HDAC-6 in k.o. mice and evidences the important role of HDAC-6 in the control of tubulin acetylation level. This also demonstrated for the first time that HDAC-6 is the key deacetylase controlling the level of tubulin acetylation *in vivo*. Interestingly, in brain, the loss of HDAC-6 did not lead to significant increase of tubulin acetylation. This may be explained by the dominance of acetylase activities in the brain since the tubulin acetylation level is already very high there. It is also possible that the activity of HDAC-6 is tightly controlled in the neuron cells and it is temporally and spatially restricted to specific loci. For this the localization of HDAC-6' protein and activity in brain will be done. These data also suggested that hyperacetylation of tubulin in various mouse tissues does not significant affect the development and differentiation.

To better characterize the *in vivo* function of HDAC-6, we derived mouse fibroblast cells (MEFs) and 3T3 cell lines from k.o. mice. MEFs were derived from individual embryos and characterized by PCR. Only males were selected, based on sex typing of the Sry gene (data not shown). Three k.o. and three w.t. MEFs were used to prepare RNA and proteins at early passages (less than 6 passages). In the k.o. cells, the expression of exons 7~10 is completely abolished (fig2.2.6.A) and alternative splicing from exon 6 to exon 11 continues and lead to readingframe shift (data not shown). Importantly, the expression of other HDACs is not changed in the absence of HDAC-6. We also used those RNAs to check the global transcripts by microarray analysis. In agreement with that, HDAC-6 is mainly localized in the cytoplasm of MEF cells and there is no significant changes with histone acetylation, we could not find any significant difference from mRNA microarray analysis (data not shown). As shown fig2.3.6.B, in the absence of HDAC-6, tubulin acetylation increased dramatically. Moreover, after TSA treatment, the tubulin hyperacetylation in k.o. cells did not increase further (data not shown). All of these confirmed that in the normal culture condition, the role of HDAC-6 is mainly in the cytoplasm to deacetylate tubulin and microtubule but not in the nucleus to control gene expression.

Finally, following standard 3T3 protocol, spontaneously immortalized 3T3 cell lines were established, which are used for *in vitro* studies.

By western blot, we also checked the effect of tubulin hyperacetylation on other tubulin modifications. Surprisingly, we found that Tyr-tubulin and Glu-tubulin did not change (Fig2.3.7) although Glu-tubulin is also a marker for stabilized tubulin as well as acetylated tubulin. We are checking other tubulin modifications as well as the effect of tubulin acetylation on the association of MAPs.

To dissect the individual function domains of HDAC-6 *in vivo*, we setup a rescue system to reintroduce wild type and mutant mouse HDAC-6 back into HDAC-6 k.o. cells. Since the cells deficient of HDAC-6 can hardly be transfected for unknown reason(s) (data not shown), we used a retrovirus plasmid----pMSCV-EGFP, which contains EGFP as selection marker. Both wild type and mutant mouse HDAC-6 cDNAs were cloned into pMSCV-EGFP and retrovirus were packaged in Pheonix cells. After infection, GFP positive cells were sorted either as a pool or as single cells in 96-well plates. As shown in Fig2.3.7, the expression of reintroduced HDAC-6 varied in different clones and can be hardly detected in pools (data not shown). We got 2 to 3 fold overexpression of HDAC-6 in w.t. cells. And in clone 15F5, we got similar expression level of reintroduced HDAC-6 as in w.t. cells. In clone 15F5 and 15 F1, only 1 to 5 % of w.t. expression level was achieved. Then we check whether tubulin hyperacetylation can be reversed after rescue. Indeed, in clone 15F5, tubulin acetylation is reduced to similar level as in w.t. Finally, genotyping was used to confirm that the rescued clones have same genotypes as k.o. cells. The expression of different mutants and deletions was checked in transiently transfected 293T cells (data not shown) and the positive rescued clones are being established now.

2.3.4. Discussion

HDAC-6 was identified originally as a homologous of yeast histone deacetylase HDA1. Although *in vitro* it has been shown that HDAC-6 possesses histone deacetylase activity, its various distinct characteristics suggested that HDAC-6 might have different substrates/functions other than other HDACs. Recently, HDAC-6 has been identified as the first tubulin deacetylase, which could deacetylate acetylated Lysine40 in alpha tubulin both *in vitro* and *in vivo*. Most importantly, specific inactivation of HDAC-6 both *in vitro* and *in vivo* in several cell types/organs led to tubulin hyperacetylation but not histone hyperacetylation. In this report, we generated knockout of HDAC-6 in mice. In these HDAC-6 deficient mice, tubulin hyperacetylation was detected in most of tissues; yet, no obvious phenotypes were observed. This suggested that tubulin hyperacetylation might be not essential for the mouse embryogenesis and adult development & differentiation. MEF and 3T3 cells were derived from HDAC-6 deficient embryos. Also in those cells tubulin acetylation, but not histone acetylation, was increased dramatically in the absence of HDAC-6. Reintroducing wild type HDAC-6 into the knockout cells could completely rescue the tubulin acetylation. HDAC-6 deficient 3T3 cells also showed reduced formation of aggresome upon inhibition of proteosome activity (data not shown). To further dissect if this phenotype is due to the tubulin hyperacetylation and/or the role of HDAC-6 in ubiquitination regulation, the rescue mutants are being generated. In more carefully investigation, we found that HDAC-6 mice showed slowed formation of aggresome in DDC-induced formation of Mallory body model (data not shown). Moreover, we also found HDAC-6 expression is induced during muscle atrophy and HDAC-6 mice are more resistant to muscle atrophy , which may be due to the slowed protein degradation mediated by ubiquitination (see below). We also checked the possible

roles of HDAC-6 in spermatogenesis, mammary development, B & T cell development, and bone development. In summary, we generated first *in vivo* mammalian tubulin hyperacetylation model and the preliminary results suggested that HDAC-6 is not essential for the embryo and adult development and might be involved in regulation of microtubule dynamics and protein degradation.

***In vivo* tubulin hyperacetylation model for tubulin**

Microtubules are cylindrical cytoskeletal structures that are found in all eukaryotic cells types and participate in a great variety of cellular processes, including mitosis, ciliary and flagellar motility, intracellular transport of vesicles and organelles, and possibly in determining morphology of certain cells. The ability of microtubules to quickly polymerize and depolymerize, a process known as dynamic instability, places regulation of microtubule dynamics at the center of active research. Tubulin proteins, the building blocks of microtubules, are subject to several types of evolutionarily conserved post-translational modifications, including detyrosination, acetylation, generation of Δ2-tubulin, phosphorylation, polyglutamylation, and polyglycylation. Most of these modifications are reversible and all, except acetylation, occur at the highly variable carboxyl termini of tubulin α and β subunits. Acetylation is mostly associated with stable microtubular structures such as axonemes, and it occurs after microtubule assembly. On the basis of the electron-crystallographic structure, it has been predicted that the modified residue points towards the lumen of the microtubule (Nogales, 1999). Generally, acetylation can happen quickly - almost immediately - and acetylated tubulin therefore does not necessarily demarcate old microtubules. Some correlation has been found between α-tubulin acetylation and microtubule stability. Acetylated microtubules commonly resist drug-induced disassembly but not cold-induced disassembly, although in some cells a subset of acetylated microtubules is cold-resistant. It is still unclear, however, how the intracellular spatial organization of acetylated microtubules is determined. The *in vivo* role of acetylated microtubules in cells remains an important unanswered question. *In vivo*, overexpression of a non-acetylatable α-tubulin variant in *Chlamydomonas* (Kozminski et al., 1993), or complete elimination of tubulin acetylation by site-directed mutagenesis of the usually acetylated lysine residue to arginine in *Tetrahymena* (Gaertig et al., 1995), had no observable phenotype. Also, disruption of the HDAC-6 gene in embryonic stem cells, which led to highly increased tubulin acetylation levels, did not significantly affect cell proliferation or differentiation (Zhang et al., 2003). Although these results indicate that tubulin acetylation/deacetylation is not generally essential for cell survival, it has been reported to affect specialized functions. For example, a role for tubulin acetylation in cell motility has been proposed on the basis that HDAC-6 overexpression increased the chemotactic movement of NIH-3T3 cells (Hubbert et al., 2002), whereas inhibition of HDAC-6 inhibited cell migration (Haggarty et al., 2003). Clearly, a knockout and overexpression of HDAC-6 mouse models will be very useful for the identification of the role of tubulin acetylation *in vivo*. In this study, we generated HDAC-6 deficient mice, which have dramatic tubulin hyperacetylation in most of studied tissues. Therefore, it provides the first mammalian model for the study of tubulin acetylation.

The most interesting argument concerning the tubulin acetylation is that if tubulin acetylation is just downstream of the microtubule stabilization. Various experiments have showed that tubulin gets acetylated after microtubules are stabilized. For example, recently Gudenson's group showed that integrin regulates the stability of microtubules via FAK and Rho signals (Palazzo, et al., 2004). The integrin-FAK signaling pathway may facilitate Rho-mDia signaling through GM1, or through a specialized membrane domain containing GM1, to stabilize MTs in the leading edge of

migrating cells, therefore is involved in the regulation of cell migration and motility. In the contrast, we have found that HDAC-6 deficient 3T3 cells showed enhanced migration and adhesion (data not shown) and this upregulation is abolished after the rescued expression of HDAC-6 in k.o. cells. This suggested that *in vivo* tubulin acetylation is not only the marker for microtubule stabilization but also could be one of the determinants of microtubule stability (data not shown and Matsuyama, et al., 2002). Our HDAC-6 deficient mouse and cells provide important models to investigate how acetylation might be involved in regulation of microtubule stability.

Another open question is that how acetylation may affect other posttranslational modifications on tubulins and the binding of MAPs. In the "histone code" hypothesis, different modifications on histone tails may affect each other and finally form a relatively stable modification pattern, which provides a specific interaction platform for other binding factor; therefore this "code" can be subsequently translated into a physiological output. It could be speculated that different modifications on tubulin might also influence each other and finally form a "tubulin code", which reflects the dynamic regulation of microtubule structure and functions. We are starting to test this possible hypothesis by checking the other modifications on hyperacetylated microtubules and if the acetylation may alter the binding of specific factors, such as MAPs, on microtubules.

HDAC-6 and ubiquitination regulation

Proteolysis is the important negative half of the live which keeps intracelluar homeostatis, regulates the signal transduction, serves as emegence sourse of nutrients, generates peptides for antigen presentation, etc. In eukaryotic organisms, these functions are carried out predominantly by the ubiquitin/proteasome system. Ubiquitin is a short peptide (76 a.a.) that can be covalently conjugated to specific protein substrates. For this, a chain reaction which employs three distinct ubiquitin ligases is required. There have been several hundreds E3 ligases indentified and it is believed that these E3 ligases confer the substrate specificity. After the rounds of ligation of ubiquitin, this ubiquitin chain targets the substrate proteins to a structure called proteasome, where the substrates are proteolyzed into small peptides.

Skeletal muscle hypertrophy/atrophy is an excellent model for protein synthesis/degradation balance, which determines muscular protein content, and thus its size and functional capacity. If protein degradation exceeds synthesis, muscle atrophy will occur, as seen after nerve injury or in various systemic diseases, including cancer, cachexia, sepsis, renal failure, and AIDS. During the skeletal muscle atrophy, the size of pre-existing muscle fibers decreases, which predominatly due to the increase in the rate of ATP-dependent ubiquitin-mediated proteolysis (reviewed in Watchko et al., 2002). For example, the amount of polyubiquitin conjugation per total protein measured increases during the atrophy; the rate of protein breakdown increases during the atrophy; the expression of distinct components of the ubiquitin pathway increases during the atrophy and inhibition of the proteasome blocks atrophy. Recent studies have shown that the expression of two muscle-specific ubiquitin E3 ligases, MuRF1 and MAFbx (also known as Atrogin-1), is upregulated in the different atrophy models. Mice deficient for either MuRF1 or MAFbx maintain the skeletal muscle mass as well as fiber size under the atrophy conditions. This finding demonstrated for the first time that inhibition of particular ubiquitin E3 ligases could moderate the amount of muscle loss after atrophy-inducing

stimulus. Therefore, MuRF1 or MAFbx might be attractive targets for pharmacological intervention. They also might be seen as early markers of skeletal muscle atrophy, aiding in the diagnosis of muscle disease.

The interaction between HDAC-6 and ubiquitinated proteins and association with deubiquitining enzymes (Seigneurin-Berny, et al., 2001, and Hook, et al., 2002) and several E3 ligases, including atrogin-1 (data not shown and Saadi Kochbin, personal communication) suggested that HDAC-6 might play important roles in regulation of ubiquitination-mediated protein degradation. We checked the expression of HDAC-6 during the denervation-induced muscle atrophy. Both protein and RNA expression level are increased after denervation in denervated muscles (data not shown, in collaboration with the lab of Laurent Schaeffer, ENS, Lyon). More interestingly, HDAC-6 deficient mice showed resistance to atrophy after denervation. The decrease in muscle mass was significantly slowed down in HDAC-6 deficient mice comparing with control mice. The average size of muscle fibers were also bigger in HDAC-6 deficient mice than control mice after denervation (data not shown). Together with the fact HDAC-6 interacts with atrogin-1 (Saadi Kochbin, personal communication), one of the key E3 ligases during muscle atrophy, we propose that HDAC-6 might serve as a key controller for the fate of the ubiquitinated proteins. In this model, HDAC-6 binds to E3 ligases together with ubiquitinated proteins and may be involved in regulation of the ubiquitination process. The nonspecific binding to the ubiquitinated proteins and the role in general ubiquitination-mediated protein degradation suggested that HDAC-6 act an important enzyme but not only a recruiting factor. It can be speculated that any changes which may affect the rurnover of the HDAC-6 in this regulation may be used as clinical targets for muscle atrophy. The deacetylase activity of HDAC-6 may be important for this regulation (data not shown), which suggested there might be other substrates of HDAC-6 in this regulation. First, HDAC6 might deacetylate critical lysines on the ubiquitinated substrate protein and therefore expose those lysines for ubiquitination. Indeed, the deacetylation of specific lysines may allow their subsequent ubiquitination. There are also emerging examples to show that acetylation of proteins, e.g. E2F and p53, may enhance their stability. Second, HDAC6 could control the activity and assemble of the ubiquitination machinery by deacetylating them.

HDAC-6 and aggresome formation

As the mechanisms which control the synthesis of biological micro- and macromolecules have been extensively studied as main topics of biology, the importance of the reverse side, turnover and degradation of biological molecules, are becoming more and more noticed. Especially, the highly regulated degradation of both functional and misfunctional proteins has been involved in various events in normal development, differentiation, and many diseases. Even under normal physiological conditions, there is continued production of misfolded proteins in unstressed cells. Mainly due to the existence of a cellular 'quality control' machinery, such as chaperoning nascent or unfolded proteins and selectively degrading improperly folded polypeptide before they can aggregate, the suppression of the formation of these aggregates, aggresomes, ensures the fidelity of transcription and translation, keeps the stability of intracellular and extracellular microenvironment. Protein aggregates are oligomeric complexes of non-native conformers that arise from non-native interactions among structured, kinetically trapped intermediates in protein folding or assembly. Protein aggregation is facilitated by partial unfolding during thermal or oxidative stress and by alterations in primary structure caused by mutation, RNA modification or translational misincorporation . Protein aggregates can be either structured (e.g. amyloid) or amorphous. In either case, they tend to be insoluble and metabolically stable under physiological

conditions. Their accumulation is tightly linked to neuronal degeneration or organ failure in many 'protein deposition' diseases. (e.g. amyloid diseases, Alzheimer's disease, Parkinson's disease, Huntington's disease and alcoholic liver disease). However, although the occurrence of these aggregates is not in doubt, their physiological significance is unclear but potentially of great importance.

Aggresomes form by a dynein-driven, microtubule-dependent centripetal transport of peripheral aggregates to an area surrounding the centrosome. The formation of aggresome requires the microtubule network and the microtubule-associated motor. The combination of tubulin deacetylase and ubiquitin-binding activity on HDAC-6 protein suggested that HDAC-6 might provide a direct link between microtubule dynamics and regulation of protein degradation. Recently it has been shown (Kawaguchi, et al., 2003) that HDAC-6 functions as an adaptor that links cargos of aggregated protein to the minus end-directed motor, cytoplasmic dynein. It was suggested that ubiquitinated protein aggregates bind to HDAC-6 via recognition of multi-ubiquitin by the ZnF domain, and to dynein/dynactin. Dynein is a minus end-directed motor, which moves ubiquitinated protein aggregates along microtubule tracks toward the microtubule-organizing center. Inactivation of HDAC-6 by siRNA abrogated the association between High molecular weight ubiquitin conjugates and dynein, and subsequently the formation of aggresome. Surprisingly, the catalytic activity of HDAC-6 has been shown to be important for aggresome formation. This again suggested that the role of HDAC-6 here is not only an adapter but also as an active enzyme.

As accumulation of protein aggregates in intracellular inclusion bodies is a prominent pathological feature common to nearly all neurodegenerative diseases, the inactivation of HDAC-6 on the aggresome formation in various neurodegenerative diseases might be interesting for pharmacological investigation. To do this, we are currently using several *in vivo* aggresome formation mouse models, such as amyloid diseases, Alzheimer's disease, Parkinson's disease, and Huntington's disease, to cross with HDAC-6 knockout mice. In addition, preliminary results from the alcoholic liver disease model suggested that the formation of mallory bodies in HDAC-6 deficient mice is delayed but not completely blocked (data not shown).

HDAC-6 and epigenetics

Although so far, the functions of HDAC-6 were mainly focused in tubulin acetylation and regulation of protein degradation, HDAC-6 might be still tightly linked to epigenetic research. HDAC-6 could deacetylate histone substrates *in vitro*. Artificially recruitment of HDAC-6 to promoter could repress the reporter activity and this repression is dependent on the catalytic activity of HDAC-6 (data not shown). Moreover, in some tissues, HDAC-6 is able to localize in the nucleus. For example, it has been shown Runx2/CBFA1 interacts with HDAC-6 and could recruit HDAC-6 into nucleus in osteoblast cells and thereby represses the transcription of p21 (Westendorf, et al., 2002). This suggests that HDAC-6 may bind to Runx2 in differentiating osteoblasts to regulate tissue-specific gene expression. Therefore, it is interesting to check the bone development and differentiation in the absence of HDAC-6. But preliminary results could not show any significant defects in bones of HDAC-6 knockout mice (data not shown). Detailed analysis is still going on. Moreover, we are mating HDAC-6 knockout mice with CBFA1 transgenic mice, in which high bone resorption had been found in cells of the osteoblastic lineage (Geoffroy, et al., 2002), to see if the phenotypes of overexpressing CBFA1 could be affected by loss of HDAC-6.

Moreover, HDAC-6 might be involved in the pharmacological effects of HDAC inhibitors. So far, most of HDAC inhibitors do not have selectivity for certain HDACs, therefore they not only increase histone acetylation, thereby affect gene transcription, but also affect acetylation of tubulin and other substrates. Although it is still not clear what are exact roles of inhibition of HDAC-6 in the effect of HDAC inhibitors, clinical application of HDAC inhibitors cannot avoid the effect of tubulin hyperacetylation. HDAC-6 deficient mice and cells provide nice models to understand the effect of HDAC inhibitors via inhibition of HDAC-6. Preliminary results suggested that the inhibition of HDAC-6 is in part of the effect of HDAC inhibitors in that in the absence of HDAC-6 the sensitivity of cells to TSA is alternated (data not shown). On the other hand, as HDAC-6 might be involved in the formation of aggresomes such as lews body, mallory body, etc., inhibition of HDAC-6 might be used as novo clinical targets for neuron degeneration diseases. This possibility is being investigating.

Dissect the different function domains in HDAC-6

As HDAC-6 has different functional domains, rescue experiments were designed to dissect if they are involved in distinct *in vivo* functions. Several mutants were used for either *in vitro*, in HDAC-6 deficient 3T3 cell, or *in vivo*, in HDAC-6 deficient mice. First, catalytic inactive mutants, which still have the ability to bind ubiquitin and ubiquitinated proteins, were used to check the function of tubulin acetylation. The M2 mutant, which has mutations in Zinc-UBP domain but still can deacetylate tubulins was also used. Several deletion mutants are also used. Most interestingly, a NES1 mutant which has mutation in nuclei export signal and could not be transported from nuclei to cytoplasm (Verdel et al., 2000) was used to force HDAC-6 stay in the nucleus, therefore deacetylate histone substrates. As shown in Fig 2.3.7, reexpression of HDAC-6 in k.o. 3T3 cells can efficiently reduce the tubulin hyperacetylation to similar level as in wild type cells. Interestingly, rescued cells also showed similar phenotypes as wild type cells in both migration and adhesion assays (data not shown). This suggested that those cells were functionally rescued. The rescued cells by different mutants are under establishing and they will provide first evidence to dissect the *in vivo* functions of HDAC-6. In addition, overexpression model and rescue experiments are also being done *in vivo*. Using BAC transgenic technique, both wild type HDAC-6 BAC and modified mutant BACs will be used for transgenic mice. Overexpression of wild type HDAC-6 from BAC transgene will mimic the endogenous expression pattern and probably will lead to tubulin hypoacetylation in some of the tissues. This will provide first "tubulin hypoacetylation" mammalian model. The BAC rescue mutants will be crossed with HDAC-6 knockout mice to check the functional rescue *in vivo*.

2.2.5 Materials and Methods

Targeting of the mHDAC-6 gene in ES cells

The backbone of the targeting vector was subcloned from a BAC clone containing the mHDAC-6 gene (Means, 2000). The exons encoding the core of the first hdac domain were floxed by a single loxP site and a cassette expressing the neomycin resistance gene and thymidine kinase gene flanked by two loxP sites. E14 ES cells were eletroporated with the targeting vector and correctly targeted ES clones were identified by PCR and Southern blot analysis. The targeted ES cell clone No 223 was used for transient transfection (electroporation) with the Cre-expressing plasmid pCMV-Cre in order to mediate recombination between the loxP sites. Gancyclovir was used to negatively select against clones having retained the tk-neo cassette and clones containing a deleted or a floxed mutant allele were identified by PCR and Southern blotting.

Establish the MEF cells and 3T3 cell lines

Mouse embryo fibroblasts were isolated from E13.5 mouse embryos. Sex genotyping was used to select only male embryos. HDAC-6 knockout lines were further identified by PCR genotyping and western blot. For microarray and western blot experiments, MEFs between 4~8 passages were used. 3T3 cell lines were established followed standard 3T3 protocol. The cells were plated at density of 3X105 cells per 5cm plate and spited every 3 days for about 20 passages.

Infection of 3T3 cells by retrovirus

Wild type and mutant mouse HDAC-6 cDNA were cloned into pMSCV.EGFP. The retrovirus was packaged in Pheonix cells followed standard protocol. Both wild type and knockout 3T3 cells were infected. The infected cells were kept in culture for 2 weeks and single GFP-positive cells were sorted into 96-well plates. The positives were identified by western blot and PCR.

Western blot

Protein extracts were made from either MEF/3T3 cells or mouse tissues. Protein lysates were resolved by electrophoresis on 8% or 12% SDS-PAGE gels and subsequently to nitrocellulose membranes (Bio-Rad). Antibodies used are: mHDAC-6 (Verdel, et al., 2000), TU2.1 for alpha-tubulin or TU6-11 for acetylated tubulin (Sigma), Glu-α-Tubulin and Tyr-α-Tubulin (Synaptic system).

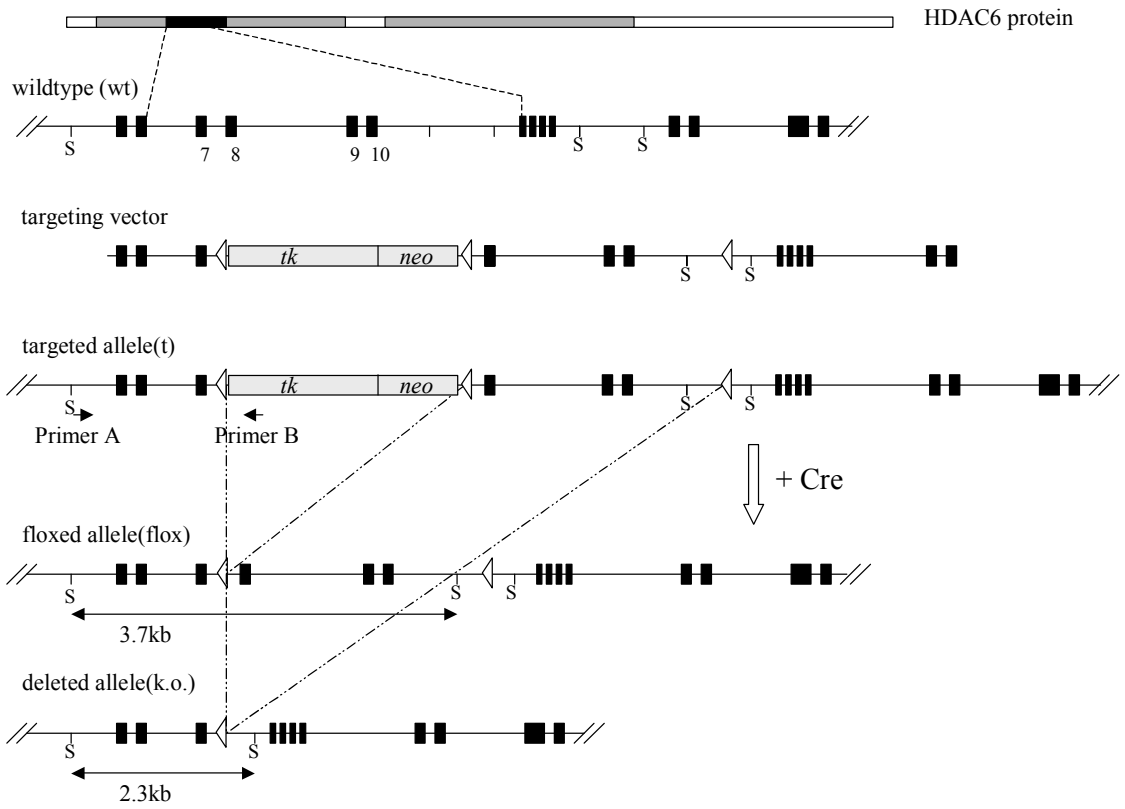


Fig2.3.1 Strategy for conditional and conventional knockout of HDAC-6

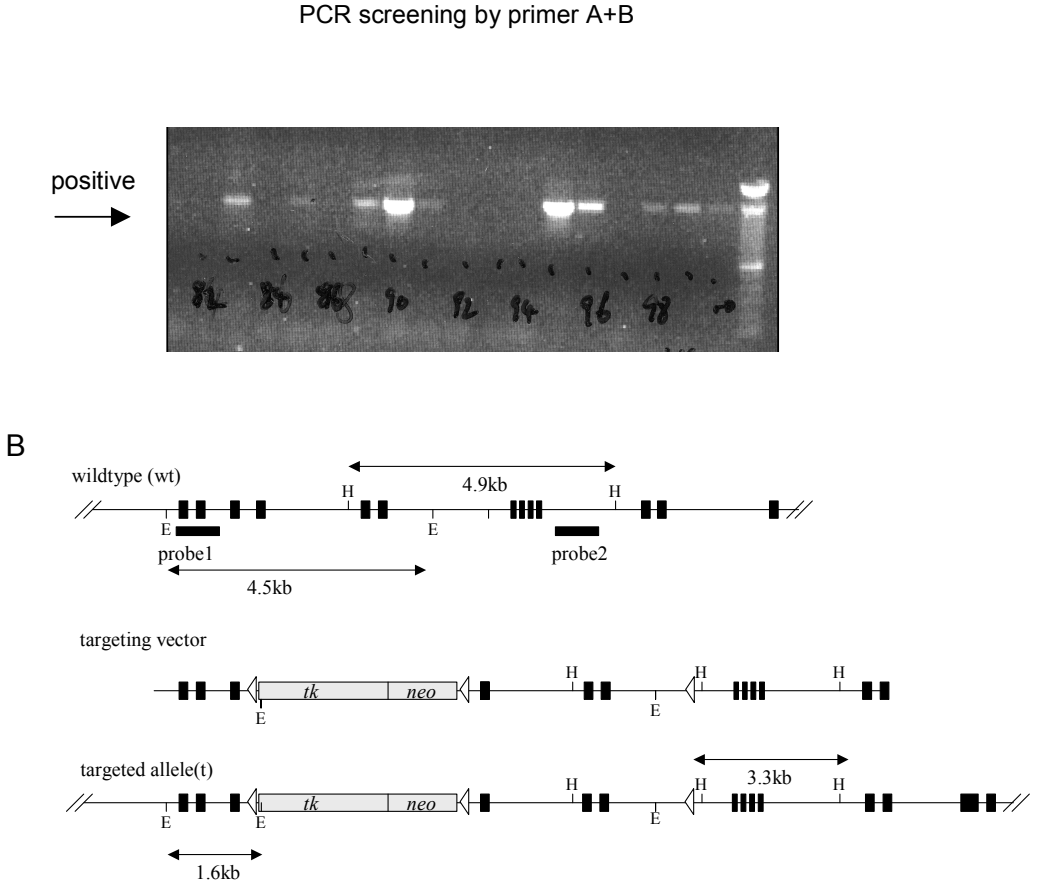


Fig2.3.2 Identification of the positive targeted ES clones by PCR and Southern blot

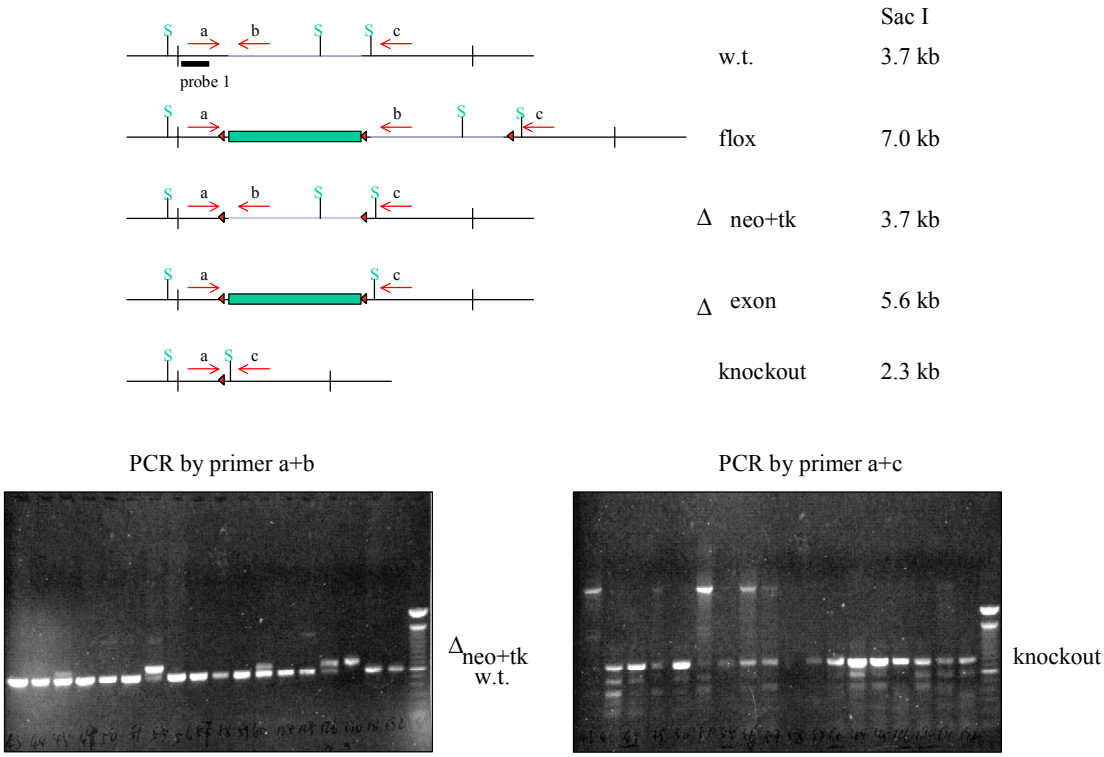


Fig2.3.3 *In vitro* cre-mediated recombination to get both null and floxed HDAC-6 alle

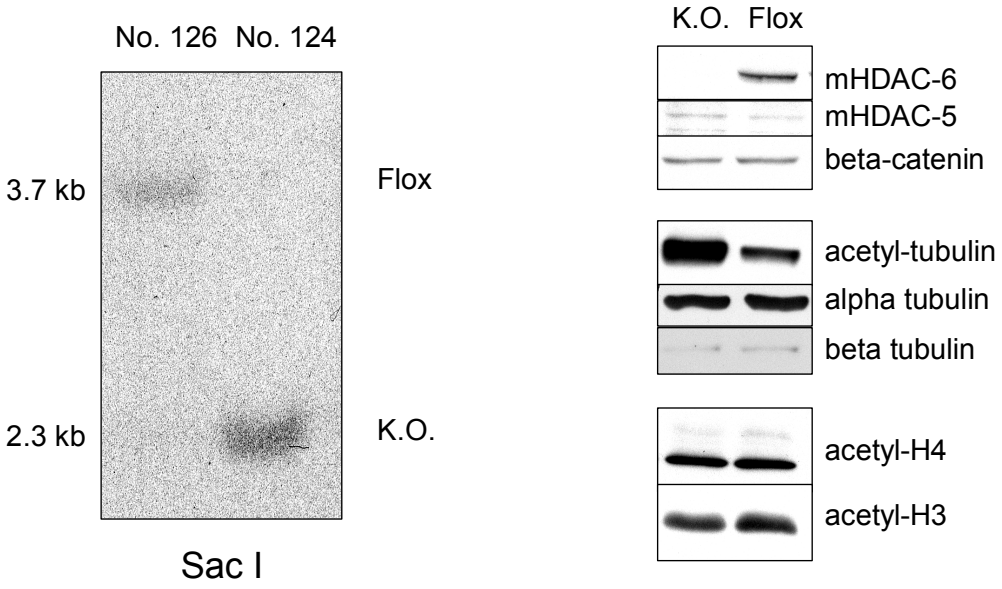


Fig2.3.4 HDAC-6 is the main tubulin deacetylase in ES cells but not for general histone deacetylation.

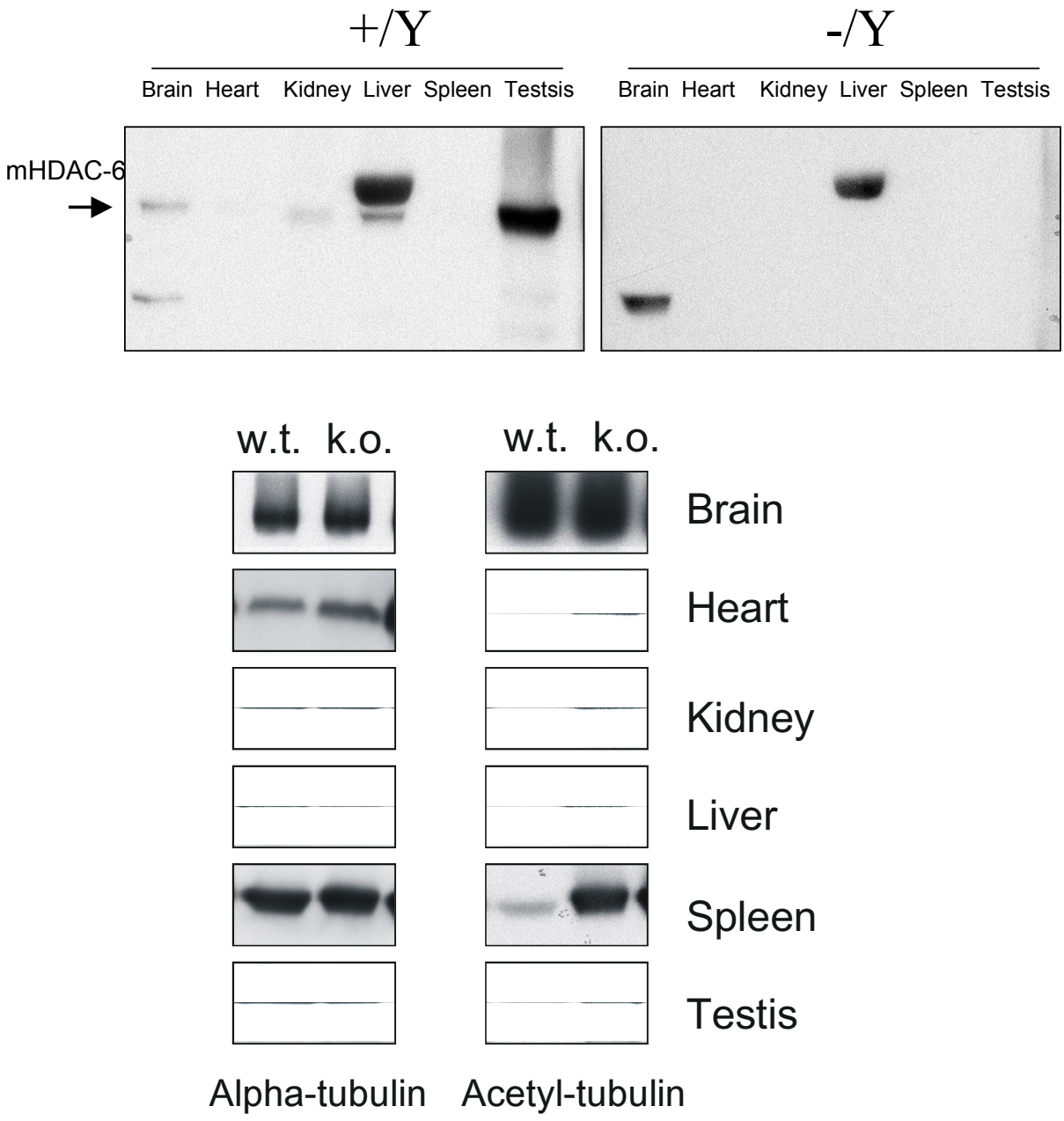


Fig2.3.5 loss of HDAC-6 protein leads to hyperacetylation in several mouse tissues

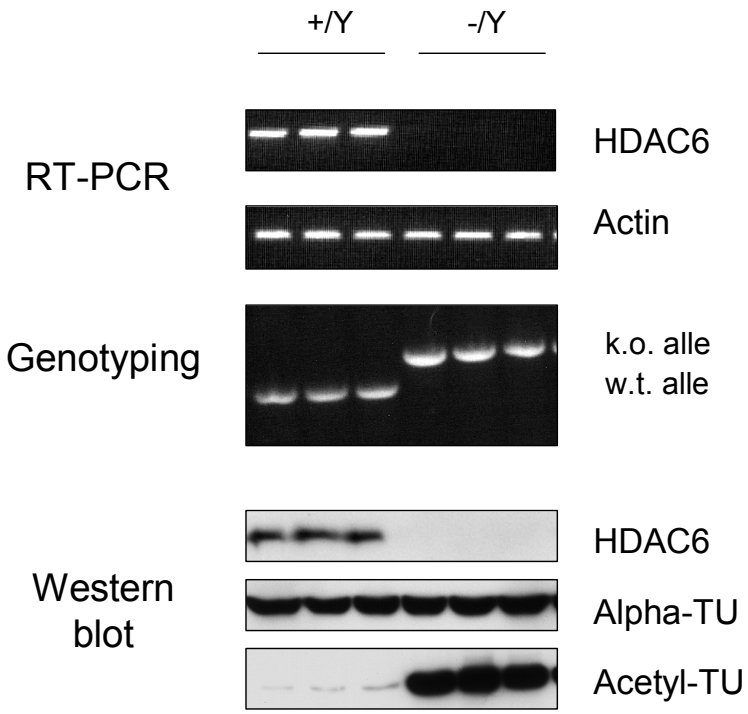


Fig2.3.6 Characterization of MEF cells derived from HDAC-6 knockout male embryos

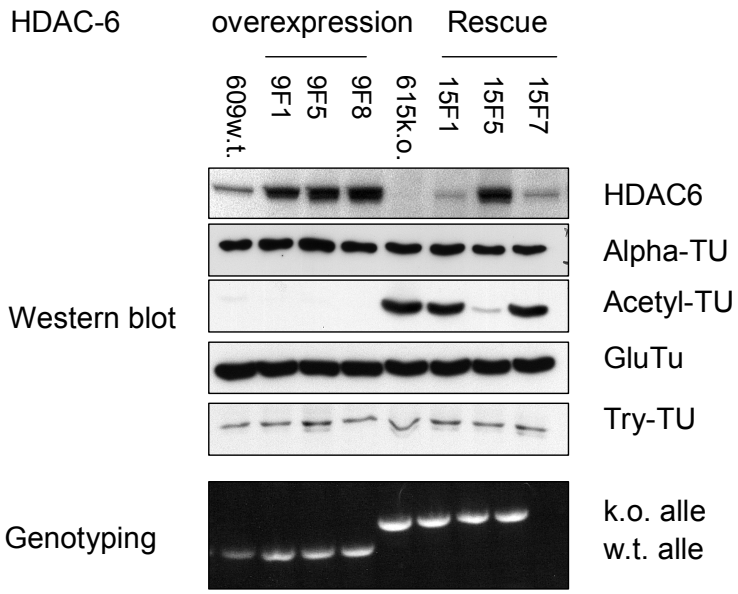


Fig2.3.7 Rescue and overexpression 3T3 cell lines derived from HDAC-6 knockout cells

2.4 Conditional knockout of HDAC-1

2.4.1 Abstract

Acetylation/deacetylation on histone N-terminal tails, together with other modifications such as methylation, phosphorylation, ubiquitination, forms an epigenetic "histone code", which has been proposed to be one of the key determinators of chromatin structure and genome regulation. The identification of enzymes which directly acetylate and deacetylate protein substrates has opened the possibility to understand the *in vivo* functions of protein acetylation. Histone deacetylase 1 (HDAC-1) is one of the major regulators of chromatin structure and gene expression. Tight control of HDAC-1 expression is essential for development and normal cell cycle progression. Since so far most of the functions of HDAC-1 have only been investigated *in vitro*, we generated conditional and conventional knockout mouse models to dissect the *in vivo* functions of HDAC-1. The HDAC-1 genomic locus was successfully targeted, and by *in vivo* cre-mediated DNA recombination, both knockout and floxed allele were obtained. Double floxed MEFs and 3T3 cells were established *in vitro*. By cre-mediated recombination, HDAC-1 deficient 3T3 cells were obtained. Surprisingly, the global histone modifications did not change significantly; in addtion, examination of the acetylation status of specific lysine residues showed that only few acetylation sites on H2B, H3, and H4 changed. Moreover, microarray analysis showed the expression of only a few genes was altered and most of the previously identified HDAC-1-target genes did not change. Tissue-specific ablation of HDAC-1 in several different tissues is currently being done. In addition, HDAC-1 +/- mice are being crossed with APC^{min} mice to investigate the possible role of HDAC-1 in carcinogenesis.

2.4.2 Introduction

Histone acetylation is a dynamic process directed by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Transcription factors recruit coactivators with HAT activity to regulatory DNA elements, whereas transcriptional repressors recruit corepressors with HDAC activity. Nucleosomal histones at or near regulatory elements that are associated with coactivators with HAT activity, become highly acetylated, resulting in the remodeling of chromatin structure. These highly acetylated histones also could be deacetylated rapidly by HDACs accessing these sites. Dynamically acetylated histones are not confined to regulatory regions but are located along the coding region of genes and, in some cases, with a chromatin domain. Histone deacetylation plays a central role in the regulation of genes linked to virtually all biological processes.

Major groups of HDACs include the RPD3/HDA1 superfamily, the Silent Information Regulator 2 (SIR2) family and the HD2 family. RPD3/HDA1-like HDACs are found in all eukaryotic genomes and are further divided into two classes: Class I HDACs (HDAC-1, -2, -3, and -8) are similar to yeast RPD3 protein; Class II HDACs (HDAC-4, -5, -6, -7, -9 and -10) are homologous to yeast HDAC-1 protein. The SIR2 family of HDACs is distinctive in that it has no structural similarity to other HDACs and requires NAD as a cofactor. The fourth family, the HD2-type HDACs, were first identified in maize and appear to be present only in plants (Lusser et al., 1997) but not in animals or fungi, and is distantly related to cis-trans isomerases found in insects, S. cerevisiae and parasitic apicomplexans. HDACs exist in cells as part of large molecular weight complexes that are recruited to specific promoters via their interactions with sequence-specific

DNA binding proteins. These include the nuclear hormone receptors, the E box binding factors, and the methylcytosine binding protein MeCP2, etc. Class I HDAC-1 and HDAC2- are found in the SIN3 and NURD/Mi2 complexes. HDAC-3 is associated with corepressor N-CoR and SMRT mediating transcriptional repression by the thyroid hormone receptor and oncoprotein v-ErbA. Class II HDACs -4, -5, -7 can interact with N-CoR, SMRT, and BcoR, an additional corepressor that mediates repression by BCL-6. Althrough the role of each HDACs in gene transcription repression has been extensively studied *in vitro* and in culture cells, the *in vivo f*unctions of HDACs are largely still unclear.

HDAC-1, a class I family member, was the first mammalian deacetylase to be identified (Taunton et al., 1996) and is the most extensively characterized HDAC to date. There is considerable evidence linking HDAC-1 to cell growth and tumorigenic transformation. HDAC-1 is known to be induced by growth stimuli in mouse T cells and fibroblasts (Bartl et al., 1997). In addition, HDAC-1 levels were found to be elevated in highly proliferative tissues, embryonic stem cells, and many transformed cell lines, suggesting a link between HDAC-1 and proliferation. HDAC-1 expression is also increased in human gastric and prostate cancers (Choi et al., 2001; Patra et al., 2001). More recently, HDAC-1 has been shown to be induced by hypoxia (Kim et al., 2001). On the contrary, HDAC-1 levels were found to drop during the differentiation process in a range of different cell systems.

A variety of transcription factors including Mad, p53, and unliganded hormone receptors can recruit HDAC-1 to specific genomic regions, thereby mediating the re-pression of corresponding target genes. The identification of p53 as an HDAC-1 target, directly implicates this co-regulator in the process of malignant transformation. Luo et al. (2000) showed that HDAC-1 deacetylates p53 and abrogates p53-mediated apoptosis, and has, therefore, an important role to play in tumor progression. Similarly, HDAC-1 has been shown to deacetylate other transcription factors and thus exert its repressive effect, including Sp1, E2F1, and MyoD. In the study by Mal et al. (2003), HDAC-1 was shown to deacetylate the muscle specific transcription factor MyoD and prevent the cells from differentiating, while maintaining an undifferentiated phenotype and en-hancing cell proliferation. However, as soon as the myogenic differentiation program became active, HDAC-1 disassociated from MyoD and allowed the expression of differentiation-inducing genes. Moreover, HDAC-1 elimination through protein degradation resulted in cell cycle arrest and induction of a differentiated phenotype in a wide panel of breast cancer cell lines (Zhou et al., 2000).

The *in vivo* function of HDAC-1 was completely unknown until recently (Lagger et al., 2002). In this report, HDAC-1 was shown to be a major deacetylase in mouse embryonic stem (ES) cells and its loss results in a substantial reduction of cellular HDAC activity and specific changes in histone modifications. The related enzymes HDAC-2 and HDAC-3 are up-regulated in HDAC-1-null cells, but cannot compensate for the loss of HDAC-1 function. Targeting of both HDAC-1 alleles leads to embryonic lethality before E10.5. HDAC-1-deficient embryos and HDAC-1-null ES cells have proliferation defects and display increased levels of a subset of cyclin-dependent kinase (CDK) inhibitors.

To investigate the role of HDAC-1 in post-embryonic development and carcinogenesis, we generated floxed HDAC-1 alle and knockout alle by gene targeting and *in vivo* cre-mediated recombination. Double floxed 3T3 cells were established from embryos and subsequently used to generate HDAC-1 deficient cells. Surprisingly, HDAC-1 deficient cells did not shown obvious changes in global histone modification although the total HDAC activity was significantly

reduced. Moreover, HDAC-1 deficient cells only have subtle changes in gene expression and did not show expression changes in proliferation related genes such as p21cip1, p27kip1 etc.

2.4.3 Results

To construct the targeting vector, exon 6, which encodes part of the core hdac domain of HDAC-1, was flanked by a single loxP site and a floxed Neo^R cassette (Fig.2.4.1). The cre-mediated recombination will destory the catalytic domain of HDAC-1 and lead to reading frameshift, which will lead to premature of truncated protein from this locus. Targeting vector was eletroporated into line E14 ES cells and clones were selected by neomycin. Two screening PCRs involve 5' primer, which localizes outside of targeting vector, and 3' primers in first loxP site and the Neo^R cassette, respectively. From 168 clones, only 1 clone was identified as postive by both PCRs (Fig.2.4.2). Subsequently, southern blotting was used to confirm the correct targeting. Moreover, all three loxP sites were PCR amplified and sequenced to be correct (data not shown).

Correctly targeted ES cell line was injected into C57BL/6 mouse blastocysts and derived chimeras were used to generate heterozygous and homozygous mice. The floxed animals were obtained with a frequency as the expected Mendelian ratio, and were viable and fertile and appeared to have a normal phenotype (data not shown).

To remove the Neo^R cassette and generate both knockout and floxed HDAC-1 allele, the EIIaCre transgenic mouse, which has cre expression restricted to oocytes and preimplantation stages of the embryo (Lakso et al., 1996; Holzenberger et al., 2002), was used to produce *in vivo* partial and/or total excision of loxP flanked sequences in HDAC-1 loci. In the absence of its natural E1A coactivator, the expression of genes under the control of the adenoviral EIIa promoter is known to be restricted to oocytes and preimplantation stages of the embryo. As shown in Fig.2.4.3.A, targeted HDAC-1 mice were crossed with EIIaCre mice and double positive F1s were selected by PCR genotyping. As expected, mating to EIIa-Cre resulted in first generation progeny harboring mosaicism due to Cre action past the zygote stage. The genetic mosaicism of all double transgenics was evaluated by PCR genotyping using DNA from tail biopsy samples (Fig.2.4.3.B). To eliminate the neomycin resistance cassette and to produce total k.o. mice in the subsequent segregation experiments, we selected mosaic mice with high prevalence of neo-excision (Δneo), and others with almost complete recombination (knockout alleles). Finally both floxed and knockout alle were successfully obtained from the segregation experiments. Both genotypes were confirmed by southern blot and PCR genotyping (data not shown). Western blot experiments showed that the expression of HDAC-1 was reduced about 50% in the heterozygous mice (data not shown).

To make the HDAC-1 deficient cells, first of all, double floxed MEF cells were established from HDAC-1 fl/fl embryos. Subsequently, following standard 3T3 protocol, spontaneous immortilized 3T3 lines were established. The genotypes of both floxed cells and wild type cells were confirmed by PCR and southern blot (Fig.2.4.4.A and data not shown). The deletion of HDAC-1 was tested by transient transfection of cre expression plasmids into double floxed cells. As shown in Fig.2.4.4.B, 48 hours after transfection, cre-positive cells specificially lost HDAC-1 protein in the nucleus.

Furthermore, double floxed 3T3 cells were transiently transfected with pMSCV-Cre-EGFP, which contains EGFP as selection marker. EGFP postive cells were sorted into 96 well plates as single cell and individual clones were established. Western blot and PCR genotyping were used to identify knockout cell lines. As shown in Fig.2.4.5., three independent cell lines were found shown to be deficient of HDAC-1 protein. PCR also confirmed that those cell lines indeed have a completely knockout alle. Both western blot and PCR were used to confirm that Cre is absent in those cell lines since it has been shown that continuous expression of the Cre recombinase in cultured cells lacking exogenous lox sites can cause decreased growth, cytopathic effects, and chromosomal aberrations. (Loonstra et al., 2001; Silver and Livingston, 2001).

By RT-PCR analysis, we confirmed the ablетion of HDAC-1 mRNA but did not find any significant expression changes with other HDACs (Fig.2.4.5.A). This was also confirmed by microarray analysis of those cell lines (data not shown). In HDAC assay, we found the total HDAC activity was reduced by about 30% (Fig.2.4.5.B). Further detailed HDAC activity assays on specific HDAC-containing complexes are being done.

Finally, we checked the global histone modifications in the absence of HDAC-1. Suprisingly, we could not detect significant difference of globle histone acetylation on H2A, H2B, H3 and H4 (Fig.2.2.5) as well as methylation and phosphorylation on H3. HPLC-MS analysis also could not detect significant changes on the global acetylation pattern (data not shown). By modification specific antibodies, we found the acetylation of most lysine sites were not changed except Lys9, Lys14 on H3, Lys5, Lys8 on H4, and H2B lys20. Further chromatin-immunoprecipitation experiments are being done to chech the acetylation pattern on specific genomic loci.

2.4.4 Discussion

Histone deacetylase 1 (HDAC-1) is one of the major regulatosr of chromatin structure and gene expression. Numbers of genes have been shown to be directly and indirectly regulated by HDAC-1. Therefore, tight control of HDAC1 expression is essential for development and normal cell cycle progression. Since so far most of the functions of HDAC-1 have only been investigated *in vitro*, we generated conditional and conventional knockout mouse model to dissect the *in vivo* functions of HDAC-1. HDAC-1 genomic locus was successfully targeted and by *in vivo* cre-mediated DNA recombination, both knockout and floxed alle were obtained. Double floxed MEFs and 3T3 cells were established in vitro. By cre-mediated recombination, HDAC-1 deficient 3T3 cells were obtained. Supringly, the global histone modifications did not change significantly whereas only few acetylation sites on H2B, H3, and H4 changed. Moreover, microarray analysis showed the expression of only a few genes were alterated and most of known HDAC-1-target genes did not change (data not shown). Tissue-specific ablетion of HDAC-1 in several different tissues are being done. In addition, HDAC-1 +/- mice are being crossed with APC^{min} mice to investigate the possible role of HDAC-1 in carcinogenesis.

The genetic dissection of the functions of HDAC-1 in various mouse tissues is only possible after the establishment of the conditional knockout system. The compleletly loss of HDAC-1 in mice results early embryonic lethal before E10.5 and this suggested HDAC-1 plays an important role, which could not be compensated by other HDACs, during early

embryo development. The early embryonic lethality have limited the understanding of the *in vivo* functions of HDAC-1 in post-embryonic development and differentiation. With our conditional knockout system, we are crossing the floxed mice with numbers cre-expressing mouse lines. For example, HDAC-1 has been shown to be involved in the transcription regulation of ribosomal gene (Zhou et al., 2002) and cell cycle regulators (e.g. p21). To investigate the possible function of HDAC-1 in liver function, such as regeneration, we are crossing our mice with Mx-Cre mice which get completely ablation of floxed gene in liver after induction. We are also crossing the mice with CD19-Cre and Nestin-Cre to dissect the possible roles of HDAC-1 in B-cell and nervous system development, respectively.

Since HDAC-1 deficient embryos die very early before E10.5, the establishment of MEFs from HDAC-1 knockout embryos has not been sucussful (C. Seiser, personal communication). Our HDAC-1 deficient 3T3 cells are the first HDAC-1 knockout somatic cell lines. Comparing the phenotype of the HDAC-1 -/- 3T3 cells with that of HDAC-1 deficient ES cells, there are numbers differences. First of all, we did not observe significant growth inhibition in the HDAC-1 deficient 3T3 cell lines. In agreement with this, we also did not find elevated levels of the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. These observations suggested that HDAC-1 might have different roles in undifferentiated cells (e.g. embryonic stem cells) versus differentiated cells (e.g. fibroblasts). HDAC-1 levels were found to be elevated in highly proliferative tissues, embryonic stem cells, and several transformed cell lines. Moreover, disruption of the HDAC-1 gene resulted in reduced proliferation of mouse embryos and embryonic stem cells (Lagger et al., 2002), whereas overexpression of HDAC-1 led to impaired proliferation of murine fibroblasts (Bartl et al., 1997). On the other hand, inhibition of transformed cell lines by HDAC inhibitors could inhibit the proliferatioin and re-differentiate tumor cells, whereas HDAC inhibitors have acceptable toxicity to normal differentiated cells. All of these suggest HDAC-1 might be important for the maintenance of the undifferentiation status but might be not essential for the differentiated cells. The second conflict is that in the absence of HDAC-1, the global acetylation did not change in the 3T3 cells although the total HDAC activity in cells was reduced significantly. As shown in Lagger et al. (2002), the acetylation of bulk histones as detected on acidic Triton–urea gels was not significantly affected by the loss of HDAC-1 in ES cells, but western blot analysis with modification-specific antibodies revealed increased acetylation levels of a subset of histones H3 and H4, as well as concomitant changes in histone H3 phosphorylation and methylation, in HDAC-1 deficient ES cells. In contrast, we did not observe any significantly global changes on histone modifications by either western blot or HPLC-MS analysis (data not shown). This evidence also supports that HDAC-1 might have different roles in undifferentiated cells versus differentitated cells. We plan to investigate the specific histone modifications at different genomic loci by chromatin immunoprecipitation.

HDACs are becoming one of the most interesting clinical targets for tumor therapy. Histone deacetylase inhibitors induce differentiation, cell cycle arrest, or apoptosis in transformed cell lines, processes most likely involving transcription activation. Moreover, normal cells were found to be less sensitive to effects caused by inhibition of HDACs (Kim et al., 1999). Importantly, several experiments employing rodent models for cancer have shown that HDAC inhibitors significantly reduce the growth of tumors and metastases *in vivo* . Notably, several of the compounds tested lacked considerable side effects at doses where tumor growth was inhibited markedly, which may suggest that they preferentially affect tumor cells, rather than causing general toxicity to individual organs or to the whole organism.

Colon cancer is one of the most studied tumors in human and mouse. The role of HDACs in colon cancer has been suggested based on the evidences from epidemiology to molecular mechanism. Epidemiologically, since Burkitt's initial

observation in 1971 that a diet high in fiber is associated with a low incidence of colon cancer (Burkitt, 1971), there have been many studies (Fuchs et al., 1999; Wargovich and Levin, 1996) that have sought to confirm this correlation. Butyrate, a four-carbon short-chain-fattic-acid (SCFA), has been shown to mediate the most profound protective effects of a high-fiber diet (Folino, 1995; D'Argenio et al., 1996). Interestingly butyrate is known to induce general histone hyperacetylation but, more specifically, on H3 and H4 species through a noncompetitive and nonspecific inhibition of HDACs. *In vivo*, in rats fed a high-fiber diet, high butyrate levels were correlated with histone hyperacetylation in colonic epithelial cells (Boffa et al., 1992). Subsequently, p21, G1 cell cycle inhibitor, has been identified as the key molecule required for butyrate-mediated cellular growth arrest (Archer and Hodin, 1999). Clinically, the level of acetylated histone H4 expression was shown to be reduced in 70% of gastric carcinomas in comparison with nonneoplastic mucosa, while the total amount of histone did not differ significantly between tumor and normal tissues, indicating global hypoacetylation in gastric cancer (Yasui et al., 2003). As HDAC-1 is thought be one of the most important HDACs, we are interested to check if loss of HDAC-1 could partially mimic the effect of butyrate in a mouse colorectal cancer model, APC^{min} mice. We have backcrossed HDAC-1 heterozygous mice to C57/BL6 by more than five generations. Now they are mated with APC^{min} mice to get double modified mice, which will be used to check the development of intestinal neoplasia. Moverover, colon-specific cre and HDAC-1 floxed mice will also be used for this research.

2.4.5 Materials and Methods

Targeting of the mHDAC-1 gene in ES cells

The exon 6 encoding the core of the hdac domain were floxed by a single loxP site and a cassette expressing the neomycin resistance gene flanked by two loxP sites. E14 ES cells were electroporated with the targeting vector and correctly targeted ES clones were identified by PCR and Southern blot analysis. The targeted ES cell clone No 149 was injected into C57BL/6 mouse blastocysts and chimeras derived from this cell line were used to generate heterozygous and homozygous mice. To delete the neomycin casstte and generate knockout alle, targeted mice were mated with EIIa-Cre mice. The chimeras which contain both Cre and targeted alle were genotyped by PCR and southern blot. Postives were backcrossed with BL/6 mice to get germline transmission. Resulted knockout alle and floxed alle were identified by PCR and Southern blotting.

Establish the MEF cells and 3T3 cell lines

Mouse embryo fibroblasts were isolated from E13.5 mouse embryos. Sex genotyping was used to select only male embryos. HDAC-1 fl/fl lines were further identified by PCR genotyping. 3T3 cell lines were established followed standard 3T3 protocol. The cells were plated at density of $3X10^5$ cells per 5cm plate and splited every 3 days for about 20 passages. Fl/Fl 3T3 cells were transiently transfected with pMSCV-Cre.EGFP. After 48 hours, GFP postives were sorted into single cells in 96 well plates. Individual clones were amplified and checked for HDAC-1 and Cre expression by western blot.

Immunofluoresence and immunoblotting

For cre and HDAC-1 staining, cells were fixed by PFA and stained with HDAC-1 monoclonal antibody (gift from Dr. Ceiser) and Cre (gift from Na Li).The DNA was counterstained by PI or DAPI.

Protein lysates were resolved by electrophoresis on 8% or 12% SDS-PAGE gels and subsequently to nitrocellulose membranes (Bio-Rad). Core histones were extracted from cells by standard acid extaction. 1 ug core histones were seperated by 14% SDS-PAGE. Antibodies used are: HDAC-1 and DM1A (Sigma), acetylated H3 and acetylated H4 (Upstate). The whole pannel of acetylated histone antibodies came from Upstate and part of them are gifts from Ni and Liu.

Fig.2.4.1 knockout strategy for conditional and conventional knockout of HDAC-1

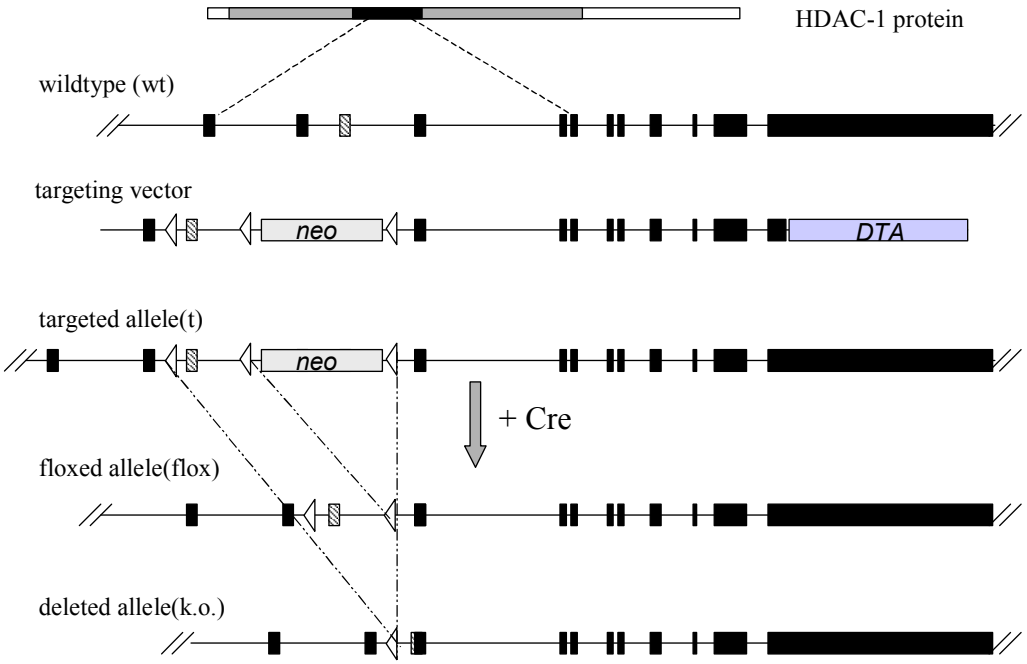


Fig.2.4.2 Isolate the positive targeted ES clones by Southern blot and PCR

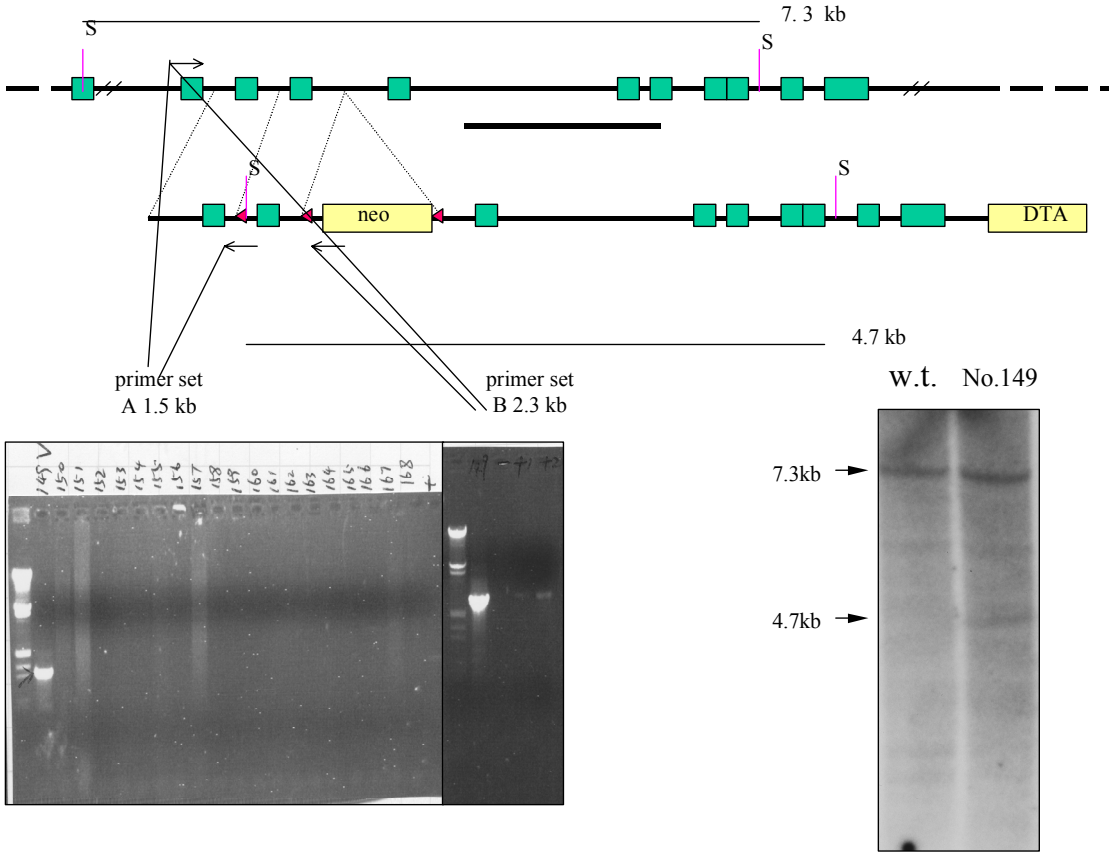


Fig.2.4.3 In vivo cre-mediated recombination to get both null and floxed HDAC-1 alle

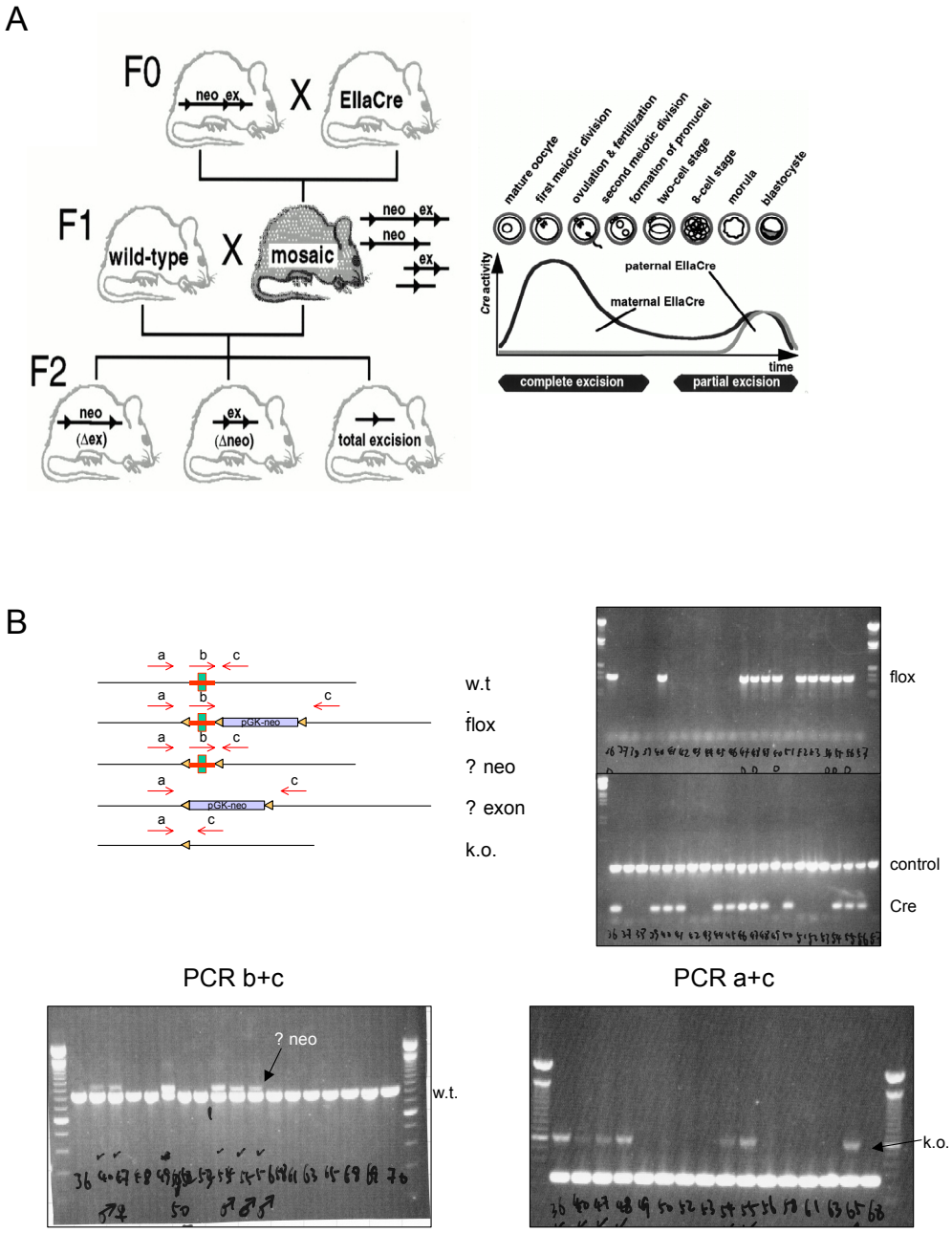
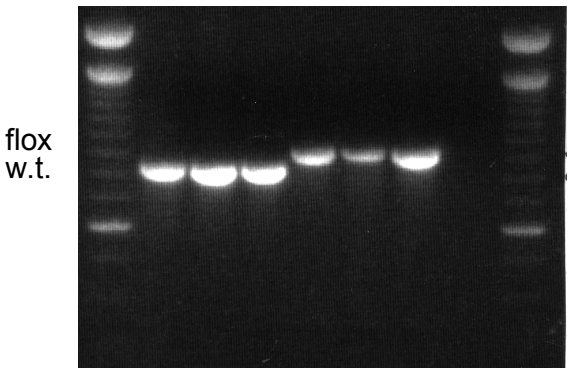


Fig.2.4.4 establish the flox/flox 3T3 cell lines from MEFs and test the cre-mediated loss of HDAC-1 in flox/flox cells

A



B

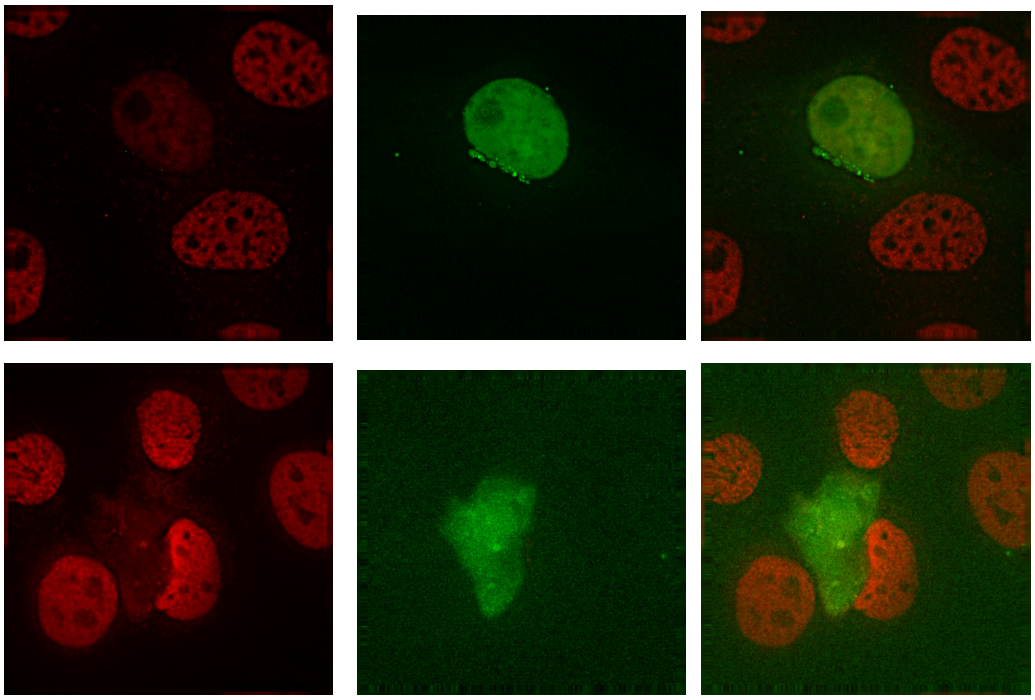
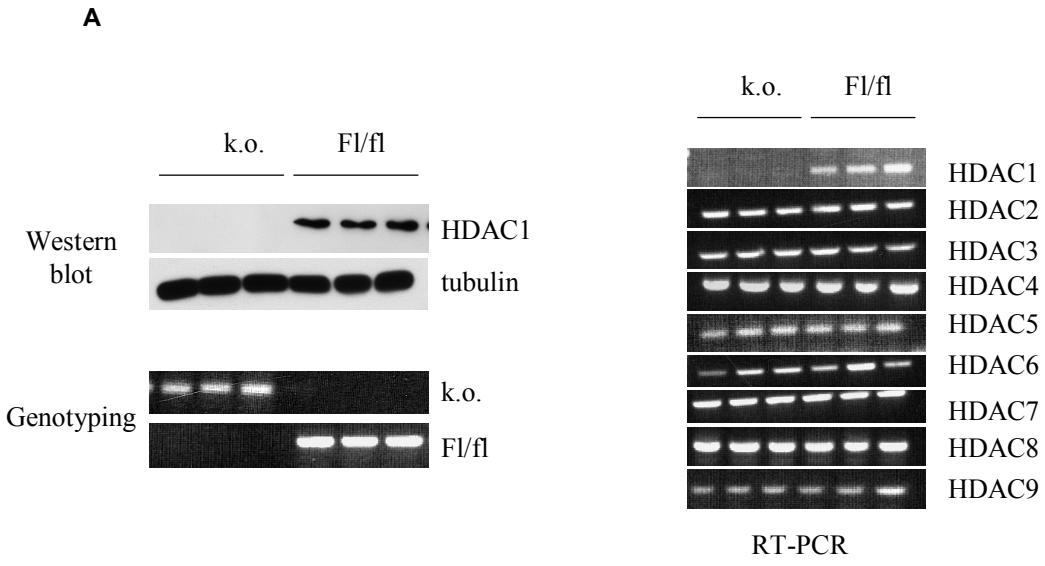


Fig.2.4.5 establish the HDAC-1 deficient 3T3 cells



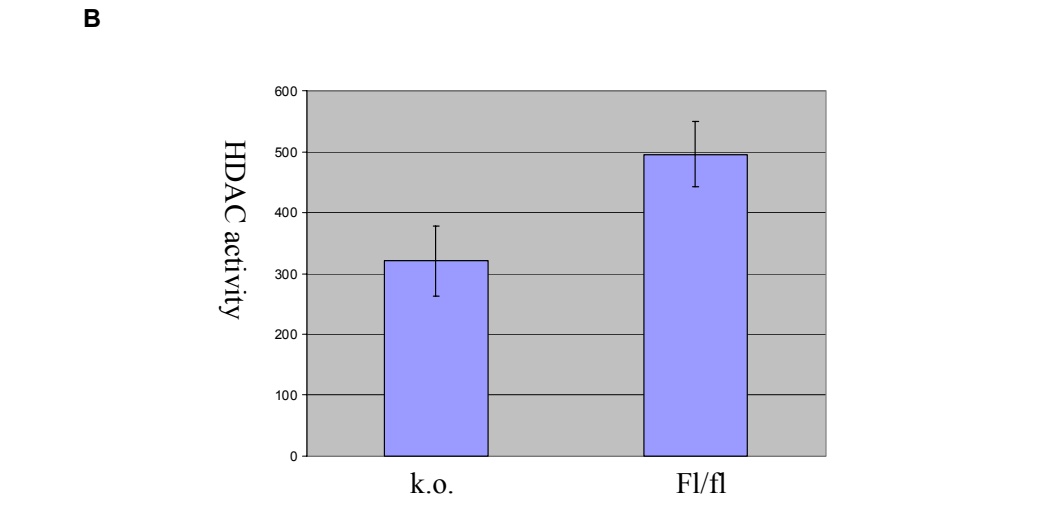
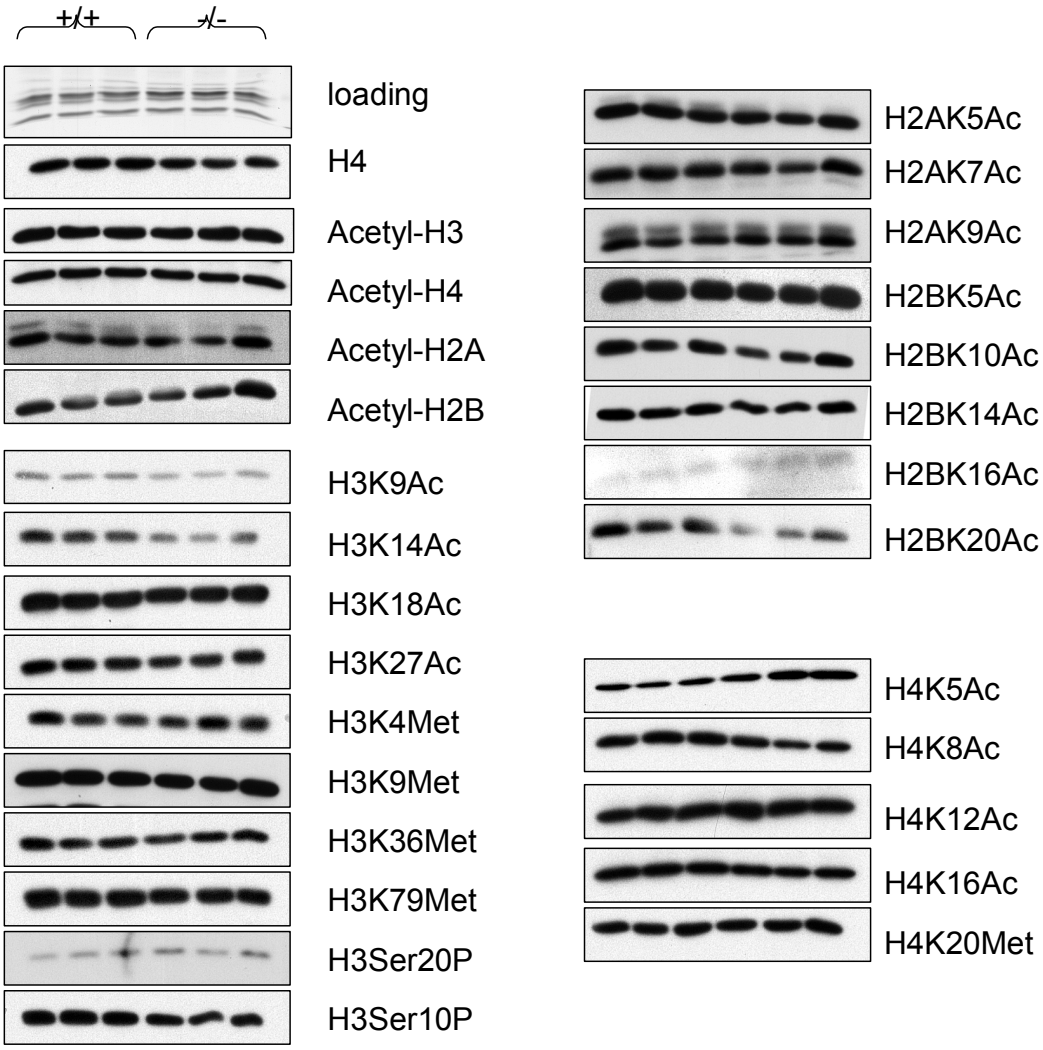


Fig.2.4.6 Histone modifications in HDAC-1 deficient 3T3 cells



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