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Nonhistone protein acetylation as cancer therapy targets

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Abstract

Acetylation and deacetylation are counteracting, post-translational modifications that affect a large number of histone and nonhistone proteins. The significance of histone acetylation in the modification of chromatin structure and dynamics, and thereby gene transcription regulation, has been well recognized. A steadily growing number of nonhistone proteins have been identified as acetylation targets and reversible lysine acetylation in these proteins plays an important role(s) in the regulation of mRNA stability, protein localization and degradation, and protein–protein and protein–DNA interactions. The recruitment of histone acetyltransferases (HATs) and histone deacetylases (HDACs) to the transcriptional machinery is a key element in the dynamic regulation of genes controlling cellular proliferation, differentiation and apoptosis. Many nonhistone proteins targeted by acetylation are the products of oncogenes or tumor-suppressor genes and are directly involved in tumorigenesis, tumor progression and metastasis. Aberrant activity of HDACs has been documented in several types of cancers and HDAC inhibitors (HDACi) have been employed for therapeutic purposes. Here we review the published literature in this field and provide updated information on the regulation and function of nonhistone protein acetylation. While concentrating on the molecular mechanism and pathways involved in the addition and removal of the acetyl moiety, therapeutic modalities of HDACi are also discussed.

Keywords

acetylation; cancer therapy; deacetylation; epigenetics; HDAC; HDAC inhibitor; histone deacetylase; nonhistone acetylation

Eukaryotic DNA, histones and histone-like proteins are assembled into chromatin, a highly organized, dynamic nucleoprotein complex that plays a significant role(s) in the regulation

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of cellular homeostasis [1,2]. The tails and the globular domains of nucleosomal histones can be modified by acetylation, phosphorylation, methylation, ubiquitination, sumoylation and, less commonly, by citrullination and ADP-ribosylation. These post-translational modifications can alter DNA–histone interactions or the binding of proteins, such as transcription factors, to chromatin [2,3]. Histone acetylations represent one of the best characterized post-translational modifications with profound functional implications for a wide range of cellular processes [4].

Acetylation levels of histone tails are maintained by the opposing, yet well balanced, activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone hyperacetylation leads to a more open chromatin structure associated with active gene transcription [2,5]. This is due to reduced ionic interactions of the positively charged histone tails with the negatively charged DNA backbone and reduced internucleosomal interactions [2,6]. Remarkably, HDAC enzymes are not exclusively targeted towards histones. A steadily growing number of nonhistone proteins have been described to be subject to reversible acetylation by HATs and HDACs. Among these non-histone targets are transcription factors, hormone receptors, signal transducers, chaperones and proteins of the cytoskeleton [4]. Dynamic acetylation of nonhistone proteins modulates a wide variety of cellular events that are involved in many biological processes such as cell proliferation, cell survival and apoptosis [3,5–8]. Moreover, HATs and HDACs were found to be deregulated in cancer [4] and aberrant expression of HDACs has been observed in various tumor types [9,10]. It should be pointed out that a target protein may be acetylated by different HATs at different sites, which often exert divergent or opposite effects. For example, high-mobility group (HMG)-A proteins have been found to be integral components of enhanceo somes. Acetylation by p300/cAMP response element binding protein (CREB)-binding protein (CBP) leads to enhance osome disruption and hence, transcriptional repression [11], whereas acetylation of HMG-A by p300/CBP-associated factor (PCAF) results in enhanceosome assembly and transcription activation [12]. Therefore, an altered balance of protein acetylation appears to contribute to cell transformation and cancer development [13,14].

Acetylation of nonhistone proteins has been demonstrated to modulate protein functions by altering their stability, cellular localization and protein–nucleotide/protein–protein inter actions. Well-characterized targets of nonhistone acetylation include important cellular factors such as p53, nuclear factor-κB (NF-κB), p65, CBP, p300, STAT3, tubulin, PC4, GATA factors, nuclear receptors, c-Myc, hypoxia-inducible factor (HIF)-1α, FoxO1, heat-shock protein (Hsp)-90, HMG, E2F, MyoD, Bcr–Abl, the FLT3 kinase, c-Raf kinase and so on [4,14,15]. Interestingly, many nonhistone proteins targeted by acetylation are relevant for tumorigenesis, cancer cell proliferation and immune functions [1,2,14,16]. Therefore, re-expression of downregulated genes essential for growth arrest and cell death, as well as the alteration of aberrant acetylation patterns of nonhistone proteins, is considered a viable approach for cancer therapy [3,4].

While histone acetylation and its roles in transcription regulation have been covered by many excellent review articles, non-histone acetylation has not been thoroughly discussed. Here we provide an updated summary on the following topics concerning the regulation and function of nonhistone acetylation: the classification, expression and activity of HDAC enzymes; the well-characterized nonhistone acetylation targets and their impact on cell signaling, transcription and protein stability; HDAC involvement in cancer, apoptosis and cell cycle control; and HDAC inhibitors (HDACi) and their potential application in cancer treatment. Although this review focuses on nonhistone acetylation and individual acetylation targets are described separately, we should bear in mind that acetylation and deacetylation of histones as well as nonhistone proteins are regulated through an interconnected network and different epigenetic modifications actually function in a highly coordinated manner.

Acetylation & deacetylation

Nonhistone protein acetylation is a widespread phenomenon among eukaryotes. Co-translational N^{α}-terminal acetylation is one of the most frequent protein modifications, occurring on approximately 85% of eukaryotic proteins [3,10]. A less common, but perhaps more important, form of protein acetylation takes place post-translationally. Acetylation of several proteins is known to occur on the ε-amino group of the lysine residues. This type of acetylation of histones weakens the histone–DNA contact and lysine acetylation is highly reversible and tightly controlled by the two counteracting enzymatic activities: those of HAT and HDAC [3]. Factor acetyltransferases have been reported to acetylate transcriptional activators, coactivators, basal factors and nonhistone chromosomal proteins with remarkable substrate specificity similar to histone acetylation [7]. Early studies suggested that many lysine residues in histones are abundantly acetylated and that this alters the histone–histone interactions as well as histone–regulatory protein interactions [1,2,13,14]. These events lead to an open, more conducible chromatin structure, which facilitates the process of transcription [15]. In humans, there are 18 potential deacetylase enzymes that are capable of removing acetyl groups and maintaining the equilibrium of lysine acetylation in different proteins [4,16].

Classes & families of HDAC

Histone acetyltransferases exist in nearly all organisms and can be classified into four main classes and two families – the classical and silent information regulator 2 (Sir2)-related protein (sirtuin) families. This classification is based on sequence similarity to yeast deacetylases and their cofactor dependency. In humans, members of the classical family include HDAC-1, -2, -3 and -8 (class I), and HDAC-4, -5, -6, -7, -9 and -10 (class II) (Table 1) [2,17]. Class II HDACs share homology to yeast HDAC-1 and Hos3 and can be further subdivided into class IIa (HDAC-4, -5, -7 and -9) and class IIb HDACs (HDAC-6 and -10), which contain two catalytic sites. HDAC-11 shares sequence conservation with Rpd3 and Hda1 and is placed in class IV [18]. Expression of class II and IV HDACs is restricted to certain tissues, where they shuttle between the nucleus and cytoplasm. Class I, II and IV HDACs are Zn^{+}-dependent, while the class III HDACs (SirT1–7), the homologs of the yeast SirT2 protein, require NAD^{+} for their activity [2].

While classical HDACs can be found in the cytosol and in the nucleus, no localization to mitochondria has been described so far [4,19]. Within the class I HDACs, HDAC-1, -2 and -8 are primarily found in the nucleus, whereas HDAC-3 is found in the nucleus, cytoplasm and cell membrane. Class II HDACs are able to shuttle in and out of the nucleus depending on different signals. HDAC-6 is a cytoplasmic, microtubule-associated enzyme.

Cellular functions & regulation of HDACs

Histone deacetylases often exist as components of multiprotein complexes such as the transcriptional co-repressors mSin3, nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) [20–22]. Using a library of fluorigenic tetrapeptide substrates, HDACs were ranked according to their substrate specificity: HDAC-8 > HDAC-1 > DAC-3 > HDAC-6 [21]. HDAC-1 and -2 are frequently found in complexes with Sir3, NuRD, N-CoR, mSirN3A, Ni-2/NRD and/or CoREST. These complexes are targeted to specific genomic regions by interaction with DNA-binding factors (Table 1). The following observations indicate diverse functions of the various HDACs [22]: different embryonic development stages have shown divergent HDAC-expression patterns, supporting a role for HDAC in embryogenesis and tissue differentiation; HDAC-3 modulates the functions of transcription factors such as TFII-1 and is critical for repression of multiple nuclear receptors (NRs); HDAC-1 interacts with MyoD and serves as a repressor

for proliferating myoblasts; aberrant expression of HDAC-1 confers resistance to sodium butyrate-mediated apoptosis in melanoma cells through a p53-mediated pathway [23]. Johnson *et al.* observed that HDAC-1, -2 and -3 coimmunoprecipitated with the ATP-dependent chaperone protein Hsp-70 [24]. HDAC-6 deacetylates tubulin and modulates cell migration [25].

A series of HDAC-knockout models have been generated with various defects. Targeted disruption of HDAC-1 results in embryonic lethality and reduced proliferation despite increased expression of HDAC-2 and -3 [26]; HDAC-4-knockout mice display premature ossification due to the excessive proliferation of chondrocytes [27]. HDAC-4 acts as a repressor of chondrocyte hypertrophy by interacting with the myocyte-specific enhancer factor 2C transcription factor [28]. HDAC-5 has been demonstrated to interact with myocyte enhancer factor 2 (MEF2). MEF2 participates in diverse gene-regulatory programs during muscle- and neural-cell differentiation, cardiac morphogenesis, blood vessel formation and growth responsiveness. HDAC-5 knockout leads to inhibition of the target genes' expression. These mice also develop cardiac hypertrophy. HDAC-6 plays a critical role in the cell response to misfolded protein stress. Knockdown of HDAC-6 induces Hsp-90 acetylation and reduction of the Hsp-90 chaperone activity [29]. HDAC-7 knockouts have defects in the maintenance of vascular integrity [30]. HDAC-7 inhibits the expression of the orphan nuclear receptor Nur77 and affects the apoptosis of T cells [31]. HDAC-9-knockout mice are sensitive to hypertrophic signals and develop cardiac hypertrophy with advanced age [32].

Cleavage factor Im (CFIm)-25, a component of mammalian CFIm, and poly(A) polymerase (PAP), a polyadenylating enzyme for the pre-mRNA, are acetylation targets. The residues acetyl ated in these proteins were mapped onto the regions required for interaction with each other. Whereas CBP acetylated these proteins, HDAC-1, HDAC-3, HDAC-10, SirT1 and SirT2 were involved in *in vivo* deacetylation. Shimazu and co-workers reported that HDACs regulate the 3′-end processing machinery and modulate the localization of PAP through the acetylation and deacetylation cycle [33]. These findings provide new insights into the role of protein acetylation in not only transcriptional initiation and elongation but also termination. There are still considerable gaps in our knowledge on the biological roles of the different HDACs.

Catalytic activities of HDACs are governed on multiple stages such as post-translational modifications, protein–protein interactions, availability of metabolic cofactors and subcellular localization (Table 2) [34]. Schuettengruber *et al.* reported that the murine *HDAC-1* promoter is autoregulated by the HDAC inhibitor (HDACi) trichostatin A (TSA), which is involved in the SP1 binding sites and CCAAT box [35]. In the T cells, IL-2 is responsible for murine HDAC-1 induction. It has been reported that the *HDAC-4* promoter is regulated by SP1/SP3 transcription factors [36]. Subcellular localization of HDAC-4, -5 and-7 is modulated by phosphorylation [36]. HDAC-4 phosphorylation and its nucleocytoplasmic shuttling depend upon CaMKIV [37]. Protein kinase D1 is important for phosphorylation of HDAC-7 and its nuclear export [37]. Myosin phosphatase dephosphorylates HDAC-7 and thus promotes its nuclear localization [38].

Effects of nonhistone protein acetylation

Acetylation can affect a single factor in multiple ways. For example, acetylation of transcription factors can change their protein–protein interactions, protein turnover, protein localization and DNA-binding ability (Table 3) [39]. Also, depending on the protein and acetylation site, the same acetyl group may exert different, or even opposite, effects. For example, acetylation enhances sequence-specific DNA binding for nonhistone proteins such

as p53, NF-κB, E2F, erythroid Kruppel-like factor (EKLF), p50 and PC4 [40–44], whereas it reduces DNA binding of other factors such as FoxO1, HMGI (Y), p65 and so on [3,14,45]. The ability to activate or repress the DNA-binding ability often depends on the site of acetylation. If the acetylation sites lie in a DNA-binding domain, acetylation may repress the DNA-binding and if they are adjacent to a DNA-binding domain, it may activate DNA binding [3,14]. The following sections summarize the acetylation effects on several cell functions.

Signaling & transcription

p53—The p53 protein is a sequence-specific DNA-binding transcription factor known to maintain cellular homeostasis [46]. p53 can be acetylated by distinct acetyltransferases at multiple lysines: K120, K^{164}, K^{320}, K^{370}, K^{372}, K^{373}, K^{381}, K^{382} and K^{386} (Table 3) [47]. The resulting effects on p53 activity are still controversial [46]. Acetylation of K^{120} promoted by hMOF and Tip60 is able to mediate the expression of genes involved in DNA damage-induced apoptosis. Acetylation of K^{320} and poly ubiquitination of p53 apparently activate transcription. Additionally, p53 acetylated at K^{382} recruits CBP via its bromodomain to further stimulate transcription machinery, presumably binding through the increased DNA affinity to the target genes by acetylated p53 (Figure 1) [3]. Following the discovery of p53–HAT complexes, it was reported that p53 also interacts with HDAC-1, possibly through Sin3 or MTA2 proteins [48]. It was subsequently shown that HDAC-1 deacetylates p53 *in vitro* and *in vivo* [49]. Moreover, a pre acetylated p53 peptide was deacetylated by wild-type HDAC-1 but not by a deacetylase mutant. Furthermore, overexpression of HDAC-1 greatly reduced the *in vivo* acetylation level of p53. Finally, the activation potential of p53 on the *BAX* promoter, a natural p53-responsive system, is reduced in the presence of HDACs.

Interestingly, Mdm2 can promote p53 deacetylation by recruiting a separate complex containing HDAC-1 [50]. In addition to HDAC-1, a class III HDAC, SirT1, binds to and deacetylates p53 [51]. Studies indicate that SirT1 is involved in chromatin remodeling, gene silencing and the DNA-damage response. SirT1 can deacetylate nonhistone proteins, including various transcription factors involved in growth regulation, stress responses and endocrine signaling. SirT1-mediated deacetylation also suppresses DNA damage-induced, p53-dependent apoptosis [52]. In addition, Jin *et al.* reported that SirT2 interacts with 14–3-3 b/c proteins [53]; this interaction represents a novel negative regulatory mechanism for p53 besides the well-characterized Mdm2-mediated repression. Because lysine residues acetylated in p53 overlap with those that are ubiquitinated, acetylation serves to promote p53 stability. Unacetylated lysines are targets for ubiquitination catalyzed by Mdm2, which ultimately leads to the destruction of p53. Thus, p53 acetylation is critical for both the efficient recruitment of transcriptional complexes to promoter regions and the activation of p53 target genes *in vivo* [54]. On the other side, the deacetylation of p53 may provide a quick-acting mechanism to stop p53 function when the transcriptional activation of target genes is no longer needed.

The global transcriptional coactivator PC4 is known to be acetylated specifically by p300 *in vitro* and *in vivo* in humans [45]. Acetylation of PC4 enhances its DNA-binding ability. Phosphorylation of PC4 was found to negatively regulate the acetylation, presenting an intriguing case for the phosphorylation-mediated inhibition of acetylation [45]. In most cases, phosphorylation tends to exert positive effects on acetylation of the same proteins [55]. PC4 directly interacts with p53 *in vitro* and *in vivo* and the interaction promotes the sequence-specific DNA binding of p53 [56]. PC4 also induces the expression of p53-responsive genes and thereby enhances p53-dependent apoptosis. Since both p53 and PC4 are acetylated, it would be interesting to find out the role of reversible acetylation of these

two proteins in the regulation of cellular homeostasis [57]. The deacetylation pathway of PC4 has not been understood, but initial data suggest that PC4 may be a target of HDAC-1 [57].

Han *et al.* reported that p300 interacts with SirT2 and acetylation of SirT2 by p300 relieves the inhibitory effect of SirT2 on the transcriptional activity of p53 [58]. These observations demon strate that p300 can inactivate SirT2 by acetylation and that p300 may regulate the activity of p53 indirectly through SirT2 in addition to its direct modification of p53. Deacetylation of p53 by SirT2 decreases the ability of p53 to transcriptionally activate the cell cycle inhibitor p21, which causes cells to re-enter the cell cycle following successful DNA repair.

Tubulin—Although acetylation of α-tubulin was found in mammalian cells two decades ago (Table 3), the acetyltransferase responsible remained unidentified for many years [59]. Stable microtubules contain high levels of acetyl ated α-tubulin. By contrast, dynamic microtubules, in which tubulin subunits are actively added or subtracted, such as at the leading edge of a migrating cell, are largely hypo-acetylated. HDAC-6 [60,61] and SirT2 [62] are able to deacetylate lysine 40 of α-tubulin and control the level of tubulin acetylation and assembly of the microtubule network. Deacetylation of α-tubulin by HDAC-6 and SirT2 enhances the stability of microtubules and consequently cell motility and other biological processes.

An interesting role of tubulin and tubulin-modifying deacetylases is their influence on the aggregation of misfolded proteins – the so-called aggresome formation, which serves to protect cells from stress and cell death [63]. HDAC-6 also deacetylates Hsp-90, pointing to a broader role of this enzyme in protein folding [64]. HDAC-6 contributes to the degradation of aggresomal proteins because it is able to bind to motor proteins (polyubiquitinated and dynein) as an adaptor protein to transport misfolded proteins along microtubules into aggresomes for lysosome-mediated degradation. Misfolded proteins can also be eliminated by the ubiquitin–proteasome pathway and the role of acetylation in this pathway is not clear. Small-molecule inhibitors of HDAC-6 inhibit the aggresome machinery and hence synergize with the proteasome inhibitor, which makes HDAC-6 an interesting target for new selective inhibitors. Inhibition of HDAC-6 results in a lower stability of the microtubules and consequently, increased cell stress and cell death. The inactivation of HDAC-6 reduces tumor formation in mice and HDAC-6-deficient cells are more resistant to oncogenic transformation [65,66]. Therefore, inhibition of HDAC-6 and SirT2 represents an interesting approach for the treatment of cancerous diseases.

Acetylated α-tubulin is present at the immune synapse, the junction between a T cell and antigen-presenting cell. The immune synapse also contains HDAC-6. By deacetylating tubulin, HDAC-6 aids in the remodeling of the synapse, which regulates the organization of adhesion and signaling molecules. The HDAC-6-mediated reorganization may attenuate the lymphocyte activation signaling cascade [67].

Nuclear receptor—Nuclear receptors (NRs) regulate gene expression by their association with HDAC complexes (Table 3). Hormones induce dramatic hyperacetylation at endogenous target genes by p300/CBP. This hyperacetylation is transient and coincides with the hormone-induced gene activation. The acetyltransferase, ACTR, can be acetylated by p300/CBP and PCAF [68]. The acetylation neutralizes the positive charges of the two lysine residues (629, 630) adjacent to the core LXLL motif of ACTR and disrupts the association of HAT–co-activator complexes with promoter-bound estrogen receptors. Thus, cofactor acetylation is a novel regulatory mechanism in hormonal signaling for transcription modulation.

The estrogen receptor (ER), peroxisome proliferator-activated receptor-γ (PPARγ) and androgen receptor (AR) are members of the NR superfamily. AR and ER are acetylated by HAT at a motif that is conserved across species and the NR family [3,8]. Acetylation of the NR appears to govern the ligand sensitivity and hormone antagonist responses. Mutagenesis of the six distinct AR-phosphorylation sites led to the identification of a single site capable of regulating HDAC responsiveness [69]. The gluco-corticoid receptor (GR) is a ligand-dependent transcriptional regulator that mediates a panoply of physiological, developmental and behavioral processes [70]. GR integrates, coordinates and responds to numerous cellular signals to accomplish its diverse functions. The GR is acetylated after ligand binding. Mutagenesis of the acetylated residues (K494/495) reduced GR acetylation and GR-mediated repression of NF-κB activity [71].

The short heterodimer partner (SHP) represses the actions of AR and ER. Unlike other NRs, SHP does not have a DNA-binding domain. Biological activities of SHP repression include competition with co-activators for the AF2-binding domains of NR, a dimerization partner [14]. HDAC-1, -3 and -6 each binds directly to SHP. Moreover, HDAC-1 and SHP can form complexes with both AR and ERα. However, it is not clear whether the HDAC recruited by SHP acts directly on the NR or on chromatin [72].

STAT proteins—Mammalian STAT proteins are a family of transcription factors consisting of seven members including STAT1–4, STAT5a, STAT5b and STAT6. It has been reported that the STAT proteins activate genes containing a γ-activating sequence (GAS) and interferon-stimulated response element (ISRE) in their promoters, thereby modulating cellular processes such as survival, apoptoapoptosis, cell proliferation and differentiation (Table 3) [3]. Outcomes of STAT-mediated reactions are tightly regulated by negative-feedback loops and tyrosine dephosphorylation [68]. STAT1 can be acetylated by CBP within its DNA-binding domain [5]. Overexpression of HDAC-1, -2 or -3 enhances STAT1-dependent gene expression upon cytokine stimulation. On the other hand, treatment with HDACi or specific siRNAs blocks the expression of interferon-responsive genes [73]. Acetylated STAT1 binds to the p65 subunit of NF-κB, which decreases p65 DNA binding and leads to downregulation of NF-κB target genes [5]. Tang reported that acetylated STAT2 acts as an adaptor for STAT1 and the STAT1/Ac-STAT2 heterodimer can interact with IFN-α receptor 2 or interferon-regulatory factor (IRF)-9 [46]. The expression of STAT1/STAT2-dependent genes is strongly suppressed upon HDAC inhibition [30,74]. In another study, Shankaranarayanan *et al.* correlated acetylation of STAT6 with the transcription of reticulocyte 15-lipoxygenase-1 [75]. p300/CBP-dependent acetylation of STAT3 may facilitate STAT3 dimerization, resulting in DNA binding and the transcriptional activation of STAT3 target genes cyclin D1, *Bcl-xL* and *c-myc* [75].

GATA factors—The transcriptional activator GATA1 plays an important role in hematopoietic cell differentiation. p300-mediated acetylation of GATA1 enhances its DNA-binding ability (Table 3). Since GATA1–DNA and Ac-GATA1–DNA complexes have differential mobility, presumably acetylation induces a conformational change of the protein [76]. The two highly conserved lysine-rich motifs within the central zinc finger domain of GATA1 are acetylated by p300/CBP. Mutation of these lysine-rich motifs in an erythroid cell lineage abrogated the cell differentiation, indicating that CBP-mediated acetylation of GATA1 is a key modification for its *in vivo* function.

GATA-2, another member of the zinc finger transcription factor family, expressed in hematopoietic stem cells and progenitors, is essential for cell survival. GATA-2 also exists as an acetylated protein *in vivo*. GATA-2 acetylation by p300 and GCN5 enhances its DNA-binding ability and transcriptional synergism with p300 [77]. GATA-2 directly associates with HDAC-3, but not HDAC-1 [78], and HDAC-3 can suppress the transcription potential

of GATA-2. Deletion analyses showed that amino acids 270–393, encompassing two entire zinc fingers of GATA2, are required for binding to HDAC-3. Reciprocally, amino acids 132–180 of HDAC-3 are required for binding to GATA2. Both factors co-localized in the nucleus. GATA-2 also interacts with HDAC-5, but not with the other class II HDACs (HDAC-4 and -6). This indicates that a tissue-specific transcription factor can selectively interact with HDAC family members and such selectivity may define some tissue-specific function.

High-mobility group proteins—High-mobility group proteins bind preferentially to distorted DNA and provoke bending in linear DNA [79]. These proteins are divided into three subfamilies including HMG-A1/A2, HMG-B1/B2 and HMG-N1/N2 [80]. Among the HMG proteins, HMG-B1 and -B2 are the most extensively studied. Both the proteins are acetylated by CBP at lysine 2 [81], resulting in increased DNA-bending ability (Table 3). HMG-B1 is a chromatin component that, when leaked out by necrotic cells, triggers inflammation. In addition, HMG-B1 is also acetylated at lysine 11 *in vivo* [82]. HMG-B1 can be secreted by activated monocytes and macrophages and functions as a late mediator of inflammation. Bonaldi *et al.* showed that HMG-B1 shuttles actively between the nucleus and cytoplasm [83]. Monocytes and macrophages acetylate HMG-B1 extensively upon activation with lipopolysaccharide.

The main function of HMG-A1/A2 is the regulation of IFN-β production in response to viral infection upon acetylation by CBP and PCAF at lysines 65 and 71, respectively [84]. Acetylation by PCAF is connected with positive transcriptional regulation of the *IFN*-β gene promoter. The termination of the interferon response occurred following acetylation of lysine 65 by CBP owing to the decreased affinity of HMG-A1 for DNA [85]. An epigenetic alteration of HMG-N2 is related to tumorigenesis. In human adenocarcinoma HT29 cells, HDAC inhibitory butyrate induces HMG-N2 acetylation and downregulates HMG-N2 binding to chromatin, leading to modified gene expression [86]. Sex-determining region Y (SRY) is another HMG protein acetylated by p300. Acetylation increases SRY nuclear localization and DNA binding [86]. Conversely, deacetylation of SRY by HDAC-3 through the HMG box leads to a loss of nuclear localization [87].

E2F—Cell cycle regulator E2F (E2F-1, -2 and -3) forms a heterodimer with DP1 protein and is known to regulate S-phase-specific genes. PCAF acetylates one of the E2F family members, E2F1 at the DNA-binding domain, which increases the DNA-binding activity of the protein and thus E2F-mediated transcriptional activation (Table 3) [42]. Acetylation also stabilizes E2F1 protein. When complexed with retinoblastoma protein (Rb), E2F acts as a transcriptional repressor [88]. This interaction prevents E2F from getting acetylated. The Rb–HDAC complex deacetylates E2F1, indicating that HDACs and HATs act antagonistically on their non-histone substrates, as they do on histones. Interestingly, Rb itself is phosphorylated by G1-CDKs and acetylated by p300/CBP. While Rb acetylation does not affect its interaction with E2F, the modification increases its binding to MDM2. Thus, HAT- and HDAC-mediated Rb acetylation may define a new cell cycle control mechanism through protein–protein interactions [89].

MyoD—Myogenesis relies critically on the MyoD transcriptional factor. MyoD activity is regulated by acetylation at three evolutionarily conserved lysine residues (lysine 99, 102 and 104) [90]. Acetylation activates the transactivating ability of MyoD by inducing a conformational change that increases its affinity for DNA targets (Table 3). Although MyoD appears to interact with both the co-activators p300/CBP and PCAF, forming a multimeric protein complex associated with the promoter region, it can only be specifically acetylated by PCAF [91]. In myogenesis, MyoD binds the gene promoters, stimulating the expression

of cyclin inhibitor p21 and certain muscle-specific genes. The acetylation of MyoD was found to enhance its DNA-binding activity and hence, transcription.

FoxO transcription factors—The mammalian family of FoxO proteins (FoxO1, 3, 4 and 6) belongs to the forkhead family of transcription factors. In the absence of insulin or growth factors, the FoxO proteins are located in the nucleus, triggering gene expression to regulate stress resistance, metabolism, cell cycle arrest and apoptosis. The p300/CBP- and PCAF-mediated acetylation diminishes the DNA-binding ability of FoxO proteins, in turn, reducing their activity [92]. In response to oxidative stress, SirT1 mediates deacetylation of FoxO1. SirT1-mediated FoxO1 deacetylation can increase lifespan and promote cellular survival [93]. Under conditions of caloric restriction, higher NAD^+ levels could increase SirT1 activity towards FoxO1 and the resulting modulation of FoxO1 functions may contribute to SirT2-mediated lifespan extension.

NF-κB—To ensure a transient transcriptional response, cells have evolved mechanisms to regulate the proper termination of inducible transcription factor function. NF-κB, a heterodimer of p65 (RelA) and p50 proteins, is controlled by regulating its subcellular localization through its interaction with IκBα. Chen *et al*. showed that p300/CBP acetylates the RelA subunit at lysines 218, 221 and 310 [89]. Acetylation of K^{221} and K^{310} is required for the full activity of p65 [94]. SirT1-driven deacetylation of p65 K^{310} inhibits transcription of NF-κB target genes [95]. Likewise, HDAC-1 and HDAC-3 deacetylate p65 at either K^{221} or K^{310}, resulting in the inhibition of NF-κB. Additionally, K^{122} and K^{123} acetylation reduces p65 DNA-binding affinity by increasing its IκBα interaction and nuclear export [96]. Recently, Buerki *et al.* identified a specific set of genes that are differentially regulated by TNF-α treatment when comparing wild-type and K^{314} and K^{315} mutant p65 using microarray ana lysis [97]. Acetylation of p65 thus regulates the NF-κB-dependent gene expression. Acetylation of p50 increases the NF-κB DNA binding towards target sequences, an action accompanied by increased p300 enrollment and activation of target-gene transcription [98].

Other nonhistone substrates—Many viral proteins are constitutive acetylation targets. Tat is a transactivator protein of HIV that plays an important role in HIV replication by binding to the trans-activator responsive region sequence of leader RNA [99]. Acetylation of Tat by p300 at K^{50} and K^{51} in its RNA-binding region decreases its affinity for the trans-activator responsive region sequences and leads to induction of transcription from the long terminal repeat by strengthening the elongation (Table 3) [100,101]. Adenoviral transforming protein E1A is acetylated by p300/CBP and PCAF at K239. Acetylated E1A can bind to the carboxyl-terminal binding protein (CtBP) and modulate global acetyltransferase activities, resulting in abnormal cell signaling and gene expression [102,103]. Moreover, acetylation impairs the capacity of E1A to bind importin-α3, resulting in cytosolic localization and thus, affects multiple cytoplasmic processes [104].

PTEN is an important phosphatase involved in cell signaling via phosphoinositols and the AKT/PI3 kinase pathway. Acetylation of PTEN by the HAT PCAF can stimulate its phosphatase activity; conversely, deacetylation of PTEN by SirT1 deacetylase and HDAC-1 can repress its activity [105,106].

Carboxyl-terminal binding protein participates in the regulation of cell differentiation, apoptosis, oncogenesis and development [107]. CtBP interacts with the p300 bromodomain and inhibits its transcriptional activity. It also interacts with nuclear hormone receptor co-repressor RIP140 and the acetylation of RIP140 inhibthe acetylation of RIP140 inhib the acetylation of RIP140 inhibits its interaction with CtBP [1]. Acetylation of adenoviral protein E1A inhibits its interaction with CtBP leading to alleviation of transcriptional

repression mediated by CtBP [21]. The orphan NR SF-1 regulates the development and differentiation of steroidogenic tissues. Acetylation of SF1 by GCN5 regulates its transcriptional activity and stabilizes the protein (Table 3). Inhibition of deacetylation by TSA increases SF1-mediated transcriptional activation and nuclear export of SF1 protein [108].

Yin Yang 1 (YY1) is a nuclear protein with sequence-specific DNA-binding and transactivation/transrepression activities [109]. YY1 is able to interact with several HDACs and forms a multiprotein complex with co-activators and co-repressors [110]. The interaction between HDAC-1 and YY1 is phosphorylation dependent [111]. A discrete domain located within residues 261–333 of YY1 is necessary and sufficient for the recruitment of HDAC. Acetylation of 261–333 of YY1 suppresses its DNA-binding activity and hence, the transcriptional activity [112].

Erythroid Kruppel-like factor is an erythrocyte-specific transcription factor regulating normal hematopoiesis (Table 3). p300/CBP acetylates EKLF at residues 288 and 302, which reside in the transactivation and zinc finger domains, respectively [113], and activates adult β-globin gene expression. While acetylation does not affect the EKLF DNA-binding affinity, it enhances EKLF association with the SWI–SNF complex, resulting in an open chromatin structure at the β-globin promoter. Lysine 302 acetylation enables the interaction of EKLF with Sin3A and this complex represses transcription in a gene-specific manner [65,114].

mRNA stability

A HDACi-mediated decrease in endothelial nitric oxide synthase (eNOS) levels interferes with endothelial cell function [115]. The eNOS generates nitric oxide, a key second messenger in inflammatory diseases. Side effects of this free radical are cytotoxic through lipid, DNA and protein damage. The HDACi-mediated reduction of the half-life of eNOS mRNA is nevertheless sufficient to decrease eNOS protein levels. Furthermore, the HDACi TSA has been demonstrated to decrease the RNA stability of DNA methyltransferase-1 and -3B [116,117], which results in a significant reduction of *de novo* DNA methylation. HDACi can additionally decrease the expression of ERα [118] and tyrosine hydroxyl ase [119] by modulation of ERα mRNA stability. MicroRNAs (miRNAs) are noncoding RNAs that, through RNA interference, are involved in multiple cellular processes such as differentiation and apoptosis. Recent studies have demonstrated that deregulated miRNA expression contributes to the malignant phenotype. The mechanisms behind these effects on mRNA stability remain to be identified. Perhaps, dynamic protein acetylation affects mRNA turnover via RNase and/or mRNA stabilizing factors, which usually bind to the 3′-UTR of mRNA [119]. The HDACi LAQ-824 rapidly alters the levels of many miRNA species assessed. Interestingly, the study also observed that HDACi can modulate post-transcriptional processes [120]. The functional consequence of altered miRNA expression upon HDACi treatment is not yet understood in detail.

Protein stability

Acetylation regulates protein stability in a refined mode and by surprisingly diverse mechanisms [39]. Acetylation of lysines can block ubiquitination at the same residue, thereby preventing proteasomal degradation. This was first suggested for tumor-suppressor protein p53, which is tightly controlled by the Mdm2 E3 ligase-driven proteasomal degradation pathway. Acetylation abrogates complex formation between p53 and Mdm2, whereas an unacetyl atable p53 mutant strongly interacts with Mdm2, resulting in p53 degradation [46]. In an overexpression system, Mdm2 formed a HDAC-1-containing complex binding to p53. Recruitment of HDAC-1 might thereby link two enzymatic

activities promoting p53 degradation [50,52,121]. It is a little surprising that positive regulation of p53 levels by HDACi has not been reported. This could be owing to the fact that the HDACi-insensitive SirT1 probably represents the major p53 deacetylase [3,50]. Accordingly, SirT inhibitor treatment leads to p53 hyperacetylation. The combined effect of HDACs and SirTs on p53 stability remains to be investigated.

Nonproteasomal and proteasomal degradation may also be facilit ated by acetylation. For example, acetylation of non-proteasomal protein HNF-6 increases its half-life, whereas a mutant incapable of being acetylated is degraded non-proteasomally [122]. Similarly, SV40 large T-Ag stability is controlled by CBP, HDAC-1, HDAC-3 and SirT1. In this case, HDACi-initiated signaling enhances a proteasome-independent degradation of T-Ag [33].

Acetylation of HIF-1α, an important angiogenesis regulator, at K532 by ARD1 was reported to induce its degradation [123]. HIF-1α is steadily ubiquitinated by the E3 ligase pVHL and degraded by the proteasome under normoxic conditions [1]. HIF-1α acetylation facilitates interaction with pVHL and its degradation [124]. It has been reported that the metastasis-associated protein MTA1 forms a complex with HDAC-1 and is able to bind, deacetylate and stabilize HIF-1α [13]. However, other studies link an HDACi-mediated HIF-1α decrease to pVHL- and proteasome-independent degradation [124] or to the function of class II HDACs [125], probably suggesting multiple pathways regulating HIF-1α stability under different conditions [8].

TGF-β signaling is mediated through the Smad family of transcription factors. He *et al.* provided evidence that Smad7 is a potent *in vivo* inhibitor for signal transduction of the TGFβ super-family during development and maintenance of homeostasis of multiple epithelial tissues [126]. Acetylation of Smad7 by p300 on lysines 64 and 70 leads to increased protein stability by preventing the ubiquitination of overlapping lysines by the ubiquitin ligase Smurf1 [127]. Overexpression of HDAC-1 significantly increases the amount of ubiquitinated Smad7, leading to an overall decrease in the protein half-life [128]. HDAC-1 and -3 bind strongly to Smad7, HDAC-2, -5 and -6 bind weakly and HDAC-4 does not bind at all. Overexpression of HDAC-1 significantly increases the amount of ubiquitinated Smad7, leading to an overall decrease in the protein half-life [128].

HDAC & cancer

HDAC expression in cancer cells

Histone deacetylases are associated with a number of well-character ized cellular oncogenes and tumor-suppressor genes, leading to aberrant recruitment of HDAC activity, altered gene expression and the development of specific forms of leukemia and lymphoma [129]. The oncoprotein that is encoded by one of the translocation-generated fusion genes in acute promyelocytic leukemia, *PML*–retinoic-acid receptor (*RAR*)-α, represses transcription by associating with a co-repressor complex that contains HDAC activity. In non-Hodgkin's lymphoma, the transcriptional repressor lymphoma-associated zinc finger-3/B cell lymphoma (LAZ3/BCL6) is overexpressed and associated with aberrant transcriptional repression through recruitment of HDAC, leading to lymphoid oncogenic transform ation. Acute myeloid leukemia (AML)-m2 subtype is associated with the t(8;21) chromosomal translation, which produces an AML1–ETO fusion protein, a potent dominant transcription repressor acting through its recruitment of HDAC activity.

The most extensively studied model of differentiation in which HDACs play an important role is that of myoblast differentiation, which has also led to a greater understanding of how class II HDACs (e.g., HDAC-4 and HDAC-5) are regulated. Class II HDACs are localized in either the cytoplasm or nucleus. Phosphorylation of HDAC-4 at the N-terminus by CaMK

leads to sequestration of HDAC-4 by 14–3-3 proteins and its active transport out of the nucleus. Inactive HDAC-4 is a target of the Ras signal-transduction cascade, whereby phosphorylation (presumably at different residues) leads to either translocation into the nucleus or release from sequestration [130]. The hallmarks of the malignant phenotype include the loss of differentiated status and a decreased reliance on exogenous growth factors. Mutations resulting in constitutive activation of signal-transduction pathways, such as the Ras pathway, are among the most frequent genetic changes in cancer cells [130,131]. Therefore, a constitutively active form of Ras could lead to the nuclear localization of HDACs and consequently, alteration of gene transcription. The link between altered HDAC activity and tumorigenesis is probably best demonstrated in acute promyelotic leukemia. The retinoic-acid receptor transcription factors RARα and its heterodimerization partner RXR bind to retinoic acid response elements (RAREs) and, in the absence of retinoids, repress transcription through a complex involving SIN3/HDAC, N-CoR and SMRT.

Increased HDAC-1 levels have been detected in gastric cancers, esophageal squamous-cell carcinoma and hormone-refractory prostate cancer [132,133]. Increased HDAC-2 expression was found in colon cancer. Increased levels of class II HDAC enzymes (HDAC-6) have been linked to better survival in breast cancer, but reduced expression of class II HDAC enzymes HDAC-5 and HDAC-10 has been associated with poor prognosis in lung cancer patients [67,134]. The potential role of the sirtuins in regulating AR function has been investigated. SirT1 inhibited cellular proliferation in AR-expressing prostate cancer cell lines but not in cells that do not express AR [70], demonstrating that their interaction is physiologically relevant. Inhibition of SirT1 with antagonists increased androgen-regulated gene transcription. Importantly, inhibitors of endogenous SirT1 (Sirtinol, splitomycin and nicotinamide) induced endogenous *AR* gene expression. The repression of AR activity by SirT1 required the catalytic function of SirT1. The AR lysine residues that are acetylated by p300 serve as substrates for SirT1-mediated deacetylation [135]. The ability of SirT1 to deacetylate the AR and repress its activity might provide a novel and effective modality for cancer therapy.

HDAC involvement in apoptosis

Acetylation of p53 at distinct sites regulates different cellular activities performed by p53. Acetylation at position 373 in p53 by p300/CBP leads to cell apoptosis whereas acetylation at residue 320 by PCAF leads to cell cycle arrest [136]. Under stressed conditions, acetylation levels of p53 increase, leading to its activation. Acetylation of p53 is also controlled at the deacetylation level by HDAC-1, HDCA-3 and SirT1. Inhibitors of SirT1 and HDAC-2 enhance p53 acetylation and thereby induce p53-mediated apoptosis [137,138]. The p53 homolog p73 is also acetylated and activated in response to DNA damage and potentiates apoptosis [139].

The DNA end-joining protein Ku70 prevents apoptosis by sequestering a pro-apoptotic protein, Bax, from mitochondria [140]. However, the acetylation of Ku70 disrupts its interaction with Bax and elevates Bax-mediated apoptosis. STAT1 has been known to repress NF-κB-mediated cell signaling. Acetylated STAT1 interacts with NF-κB, thereby preventing its DNA-binding ability, nuclear localization and expression of antiapoptotic genes [5]. These examples clearly indicate that acetylation of nonhistone proteins may induce or inhibit apoptosis depending upon the protein and physiological status. Detailed information from this area of research would facilitate the design of novel therapeutic strategies capable of activating apoptosis in malignant cells.

HDAC & cell cycle regulation

Crucial stages of the cell cycle are generally controlled through transcriptional regulation of a subset of genes, which are in turn regulated by the acetylation/deacetylation of histone and non-histone proteins. One notable example is the regulation of *C-myc* gene expression and cell cycle progression. C-myc regulates the expression of several genes involved in growth promotion by associating with its DNA-binding partner Max. p300 associates with C-myc and promotes C-myc stabilization, independent of p300-mediated acetylation, whereas C-myc acetylation increases its turnover [141]. Another cell cycle regulatory protein, cyclin D1, plays key regulatory roles during G1 phase and is overexpressed in many cancers. Cyclin D1 interacts with PCAF and facilitates the association of ER and PCAF. Overexpression of PCAF results in cyclin D1-dependent regulation of ER activity [142]. Interestingly, cyclin D1 expression is downregulated by the HDAC-1 complex recruited to its promoter by SMAR1, a matrix attachment region-binding protein [143]. Indeed, one well-recognized effect of HDACi is its potent action on cell cycle regulation.

Acetylation by small inhibitors of HDAC can affect the cell cycle by direct or indirect alteration of p21 and pRb. p21 is a potent cyclin-dependent kinase (CDK) inhibitor and its expression is controlled by p53. Under DNA damage conditions, p53 induces p21 expression, which results in growth arrest and cell apoptosis [144]. It has been well observed that HDACi induced p21 expression and that the induction was mediated by Sp1/Sp3 and ATM [145]. On the other hand, HuR proteins bind to the 3′-UTR and stabilize p21 mRNA, resulting in increased p21 expression [146]. HDACi butyrate increased p21 mRNA stability depending on *de novo* protein synthesis in HepG2 cells [147]. Moreover, pRb is able to interact with mSin3 and HDACs and regulate cyclin A and E expression, which evokes apoptosis [148]. The underlying mechanisms for the interplay among HDACs, HuR, p21 and pRB are not clearly understood.

HDAC inhibitors

Interestingly, the discovery of small-molecule HDACi preceded the discovery of HDACs. Sodium butyrate was the first compound identified to induce acetylation of histone [149]. Later, TSA, a fungal antibiotic [150], valproic acid (VA), already used in the treatment of epileptic diseases [151] and several other compounds were identified as HDACi. To date, several classes of HDACi have been proven to have potent and specific anticancer activities in preclinical studies [152–154]. Natural or synthetic small molecular compounds have been used as biological switches for probing into the functional mechanism of HDACs [155]. These compounds can be divided into six groups based on their structure, including hydroxamic acids (e.g., TSA) and suberoylanilide hydroxamic acids (SAHAs); cyclic tetrapeptides (e.g., trapoxin, apicidin and HC-toxin); depsipeptides (e.g., FK228); short-chain fatty acids (e.g., butyrate and VA); synthetic pyridyl carbamate (e.g., MS-275); synthetic benzamide derivatives (e.g., tacedinaline) and ketones (e.g., trifluoromethyl ketone and α-ketomides) (Box 1). Most of these upregulate the expression of tumor suppressors (such as p53 and p21) and block cell cycle progression [156].

Since epigenetic changes critically contribute to cancer onset and progression, HDACi were soon considered promising anti-cancer drugs [157]. Indeed, at the cellular level, HDACi can induce differentiation, programmed cell death, cell cycle arrest, senescence, apoptosis, reactive oxygen species (ROS) production and cell death [2,158]. For example, in preclinical studies, SAHA has been found to induce cell death by producing ROS [159]. Furthermore, Rosato *et al*. observed that HDACi MS-275 exerts dose-dependent growth arrest and differentiation at low drug concentrations and a marked induction of ROS, mitochondrial damage, caspase activation and apoptosis at higher concentrations [160]. HDACi are thought to interact with the catalytic domain of HDACs to block substrate recognition,

resulting in restoration of the expression of relevant genes that are silenced in malignancies [161]. HDACi were found to reduce tumor invasiveness, angiogenesis and metastasis (Figure 2). An additional promising effect of HDACi for cancer therapy is their selective toxicity against tumor cells compared with normal cells [6,162]. However, HDACi appear to be equally competent at promoting the acetylation of nonhistone proteins [14]. While there have been attempts to reveal aberrant gene-expression patterns in tumors, less information is available for differences in the acetylation patterns between normal and cancer cells and the effects of HDACi.

The fact that HATs and HDACs are deregulated in various cancers [69] suggests that anomalous acetylation occurs and may be corrected with HDACi treatment. Choudhary *et al.* used high-resolution mass spectrometry to identify 3600 lysine acetylation sites on 1750 proteins and quantified acetylation changes in response to the HDACi SAHA and MS-275 [163]. Acetylation of these sites preferentially targets large macro molecular complexes involved in diverse cellular processes, such as gene expression, RNA signaling, DNA-damage repair, cell cycle progression, cytoskeleton function, splicing, nuclear transport and actin nucleation, protein chaperones and ribosome formation. These findings suggest that the regulatory scope of lysine acetylation is broad and comparable with that of other major post-translational modifications.

Romidepsin and SAHA target many proteins whose structure and function are altered by acetylation, including chromatin-associated histones, nonhistone gene-transcription factors and proteins involved in regulation of cell proliferation, migration and death [164]. In clinical trials, both HDACi have shown significant anticancer activity against both hematologic and solid tumors at doses well tolerated by patients. SAHA and romidepsin have been approved by the US FDA for the treatment of CTCL. Other analogs of both drugs have shown unacceptable toxicity [164,165].

Histone deacetylase inhibitors appear to be highly effective in targeting endothelial cells. HDACi upregulate gene expression of p21WAF1/CIP1, which induces cell cycle arrest and down-regulates gene expression of survivin, an inhibitor of apoptosis, in proliferating endothelial cells [166]. TSA inhibits the VEGF-induced expression of VEGF receptors VEGFR1, VEGFR2 and neuropilin in a dose-dependent and reversible fashion. TSA and SAHA upregulate the expression of semaphorin III, a VEGF protein competitor, at both mRNA and protein levels [167]. The HDACi NVP-LAQ824 blocks mRNA and protein expression of the pro-angiogenic tyrosine kinase receptor Tie 2, as well as the Tie 2 ligand Ang-2, and results in reduced endothelial cell proliferation, tube formation and invasion into Matrigel plugs. In the mouse subcutaneous prostate and breast cancer models, a combination of NVP-LAQ824 with the VEGF receptor tyrosine kinase inhibitor PTK787 induced 80–85% inhibition of tumor growth without overt toxicity [166]. Butyrates upregulate cell adhesion molecules, including intercellular adhesion molecule-1 and E-selectin, on the surface of endothelial cells. Downregulation of eNOS in endothelial cells has also been shown to be critical for the antiangiogenic activity of the HDACi VA [168].

Isoform-specific inhibition of HDACs remains a challenging task [16]. TSA-induced acetylation of STAT3 was accompanied by enhanced nuclear localization of STAT3 [169]. Moreover, several genes under the control of STAT3 are indeed downregulated by HDACi and inhibition of this pro-oncogenic factor may contribute to the actions of HDACi [2]. Other pan-HDACi are SAHA, LAQ-824, MGCD0103 and LBH-589 [170]. One study indicated that tubacin can work as an HDAC-6-specific inhibitor [171]. VA has been demonstrated to be as a class I selective inhibitor [151], while MS-275 and depsipeptide are selective towards only a subset of class I HDACs [16]. In particular, HDAC-8 has been shown to play an important role in neuroblastoma pathogenesis [16]. Treatment with an

undisclosed HDAC-8-selective small-molecule inhibitor induced differentiation of neuroblastoma cells *in vitro* [172]. Additional isoform-selective HDACi are under development. Such selective compounds will not only provide new investigative tools for molecular biology, but might also represent new candidate drugs for cancer treatment. Whether or not strictly isoform-specific HDACi would have additional therapeutic benefits is controversial [173].

Recently, Suzuki designed isoform-selective HDACi using the homology model of HDAC-6, one of the HDAC isoforms [174]. HDAC-6-insensitive inhibitor NCH-51, and HDAC-6-selective inhibitor NCT-10, were successfully identified as isoform-selective HDACi with potential therapeutic benefits. A novel butyrate-derived HDACi (OSU-HDAC-42) has recently been tested in the transgenic adenocarcinoma of the mouse prostate model. Interestingly, OSU-HDAC-42 achieved a remarkable suppression of prostate tumorigenesis, preventing the occurrence of macro scopic prostate tumors and poorly differentiated carcinomas [52,175]. An inhibition of HDAC-6 by such inhibiinhibitors results in a lower microtubule stability and consequently in increased cell stress and cell death.

Endometrial cancer is the most common malignancy of the female reproductive tract and treatment of advanced disease is a difficult challenge for clinicians. Treatment with single-agent paclitaxel results in median progression-free intervals of 7.3 months [66,176]. Dowdy *et al.* found that TSA and paclitaxel synergistically inhibited the proliferation of serous endometrial cancer cells [66]. Accompanying this effect was the dramatic activation of the apoptosis cascade. One of the most important mechanisms by which paclitaxel inhibits tumor growth is by microtubule stabilization. Importantly, they showed that, when added to paclitaxel, TSA causes a marked increase in micro tubule stabilization, indicating that these two agents work in a cooperative fashion in endometrial cancer cells. The effects of the TSA/paclitaxel combination on apoptosis and tubulin acetylation have subsequently been confirmed in a mouse xenograft model. There was a significant reduction in tumor weight in the mice treated with the drug combination compared with those treated with either agent alone. The ability of TSA to potentiate the anticancer effects achieved by paclitaxel may have important implications for the treatment of women with endometrial cancer and possibly for patients harboring other malignancies with limited sensitivity to paclitaxel. Chen *et al.* reported that the pretreatment of prostate cancer cells with HDACi (TSA, SAHA, MS-275 and OSU-HDAC-42) led to increased Ku70 acetylation and reduced Ku70 DNA-binding affinity without disrupting the Ku70–Ku80 heterodimer formation [177]. The impaired Ku70 function diminished the cellular capability to repair DNA double-strand breaks (DSBs) induced by bleomycin, doxorubicin and etoposide, thereby enhancing their cell-killing effect [177]. This sensitizing effect was most prominent when cells were treated with HDACi and DNA-damaging agents sequentially.

A recent review discussed the cancer chemopreventive properties of three reported dietary HDACi [178], namely butyrate, diallyl disulfide and sulforaphane (SFN). In general, these dietary agents are weak ligands and must be present in much higher concentrations than TSA or SAHA in order to inhibit HDAC activity. The diallyl disulfide compound, derived from garlic [179], was reported to inhibit HDAC activity following metabolic conversion to *S*-allylmercaptocysteine (SFN-Cys) [180]. SFN is found in a wide variety of cruciferous vegetables including broccoli, cabbages, watercress and Brussels sprouts. SFN and phenylhexyl isothiocyanate are among the synthetic isothiocyanates that have been shown to be HDACi and have antitumor activities *in vitro* and *in vivo* [169,181], whereas the SFN parent compound had no effect on HDAC unless it was incubated with cells to allow for metabolic conversion [182]. Interestingly, isothiocyanate was recently found to have dual epigenetic effects as both a HDACi and a demethylating agent [181].

Expert commentary

The growing number of nonhistone acetylation targets has opened new avenues for research. The regulation and function of acetylation in nonhistone compared with histone proteins are still understudied and many aspects remain to be investigated. To date the number of identified acetylated nonhistone proteins may be far below the actual number that exist in the *in vivo* acetylome. Switches between acetylation and alternative post-translational modifications at the same lysine residue appear to play a critical role. The functional consequences of acetylation can be as variable as their targets. It is difficult to predict the effect of acetylation of proteins at multiple sites without experimental testing. Animal as well as human data must be interpreted in the context of the counter balancing actions of HDAC and ACT. Acetylation appears to often occur in protein complexes containing multiple subunits and cofactors. The resulting substrate specificity and enzymatic activity are often difficult to reconstitute *in vitro*. Despite these technical challenges, research on acetylation of nonhistone substrates such as transcription factors and cellular and viral proteins has drawn broad attention. The dynamic nature and the diversified function of acetylation/deacetylation of these proteins have been increasingly appreciated. Specifically, more research efforts should be directed to elucidate the mechanisms by which acetylation is regulated and to determine how nonhistone acetylation affects mRNA stability and the localization, interaction, degradation and function of proteins.

Protein acetylation and gene expression regulate protein stability, which can influence cellular signaling, cell proliferation and apoptosis. Consequently, HDACs have been recognized as potential targets for developing effective anticancer therapeutics. HDACi have shown the potential for amelioration of disease states by promoting cell cycle arrest and apoptosis through acetylation. The first generation of HDACi is FDA approved and has been demonstrated to have a low toxicity profile in comparison with traditional cytotoxic agents. New HDACi, now in Phase I and II clinical trials, appear to be even better tolerated. Acetylation of histone and nonhistone proteins can interfere with other post-transcriptional modifications, such as ubiquitination, and prevent or induce the proteasomal degradation of (proto-) oncoproteins, such as HIF-1α and c-Myc. Moreover, HDACi-induced abrogation of chaperone function correlates with enhanced degradation of client proteins, such as FLT3, Bcr–Abl, mutated p53 or ERα. Analyzing how HDACi influence the fine-tuned abundance of enzymes of the proteasomal pathway and induce the turnover of cancer-relevant proteins, such as AML1–ETO, PML–RARα, HDAC-2, or cyclin D1, provides further insights into the actions of these compounds. Given the pleiotropic effects HDACi have on multiple pathways, such data will aid in understanding how HDACi specifically affect different cell types. Moreover, data from *in vitro* experiments, animal models and clinical studies on HDACi are often explained solely based on their effects on histones and transcription control. In the future, studies will fill this gap of knowledge and the effects of nonhistone acetylation will be taken into account in the development of new HDACi.

Five-year view

Acetylation of nonhistone proteins regulates a wide variety of cellular events. In addition, it is highly likely that many of these reactions require formation of a complex by multiple proteins. Despite the wide array of possible outcomes, the fast technical advancement has made it possible to dissect the individual effects of the acetylation status of different sites through empirical testing. More information will become available through the establishment of mRNA/protein expression and signaling databases. Given the deregulation of HDAC and extensive involvement of aberrant protein acetylation in tumorigenesis, epigenetic research will become an ever increasing presence in the field of cancer research

and therapeutics. This will facilitate comparison between normal and diseased subjects, not just in cancer research, but in the study of medical disorders as well.

Technical innovation will lead to further streamlining and/or automation of studies on protein acetylation. Recombinant proteins, new antibodies and molecular labeling techniques, such as mass spectrometry, can be applied for *in vitro* experiments as well as *in vivo* using engineered animal models. Although acetylation lysine-specific antibodies have been used for the collection of evidence of acetylation, such antibodies may not be as specific for acetylation as advertised. Undesirable false-positive or -negative results may result from the nonspecificity of these antibodies. This will make it necessary to use other means, such as *in vivo* labeling or mass spectrometry, to confirm the antibody-based findings. These developments will significantly speed up the process of determining the impact of nonhistone proteins on cell signaling, transcription, mRNA/protein stability and the cell cycle and apoptosis.

It is particularly exciting that, owing to its dynamic nature, protein acetylation is a reversible process. Epigenetic inhibitors could possibly correct aberrant acetylation patterns and ameliorate the diseased state. The growing number of identified acetylation target proteins beyond chromatin will provide a large pool of potential targets for therapeutic interference. Development of drugs with specificity to subclasses of tumor-selective transferases will also occur in the near future. More research will uncover important differences in splice variants, which will also be exploited by drug development. Preliminary data support synergy between these inhibitors and traditional cytotoxic agents. Future drug regimens will include combinations of these agents with improved efficacy and lower toxicity.

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Box 1. Histone deacetylase inhibitors

Hydroxamic acid-derived compounds

- Trichostatin A
- Suberoylanilide hydroxamic acid
- M-carboxycinnamic acid bishydroxamide
- Azelaic bis-hydroxamic acid
- NVP-LAQ824
- LBH589
- Oxamflatin
- PXD101
- Scriptaid
- Pyroxamide Cyclic tetrapeptides
- Depsipeptide (FK228, FR901228)
- Apicidine
- Trapoxin
- HC-toxin
- Chlamydocin
- Depudesin
- CHAPS Short-chain fatty acids
- Valproic acid
- Phenyl butyrate
- Phenyl acetate
- Sodium butyrate
- AN-9 (Pivanexs)
- Synthetic pyridyl carbamate derivative
- MS-275 Synthetic benzamide derivatives
- CI-994 (*N*-acetyldinaline)
- Tacedinaline Ketones
- Trifluoromethyl ketone
- α-ketomides

Key issues

- Nonhistone proteins undergo aberrant acetylation that may modulate a wide variety of cellular events that are involved in critical biological processes such as gene expression, cell signaling, mRNA stability, protein folding, cytoskeleton assembly, enzymatic activity, protein localization, protein stability and protein–protein interactions.
- Hundreds of nonhistone proteins have been identified as acetylation targets. Effects of acetylation/deacetylation on their capacity for DNA binding, protein–protein interaction and cellular signaling have been characterized. 2D gel electrophoresis and LC/MS-MS ana lysis have been used to identify acetylated proteins.
- p53 acetylation favors DNA binding and transcriptional activation of tumor-suppressor genes. Similar mechanisms have been suggested for other transcription factors, GATA factors, MyoD, E2F1 and many other proteins.
- The recruitment of histone acetyltransferases (HATs) and histone deacetylases (HDACs) to the transcriptional machinery is a key element in the dynamic regulation of genes controlling cellular proliferation and differentiation. Histone acetyltransferase and HDAC often modify protein acetylation status in multiprotein complexes.
- Gene silencing can be reversed using small-molecule inhibitors of HDAC, some of which are either US FDA approved or currently in Phase I and II clinical trials. Several tested HDAC inhibitors (HDACi) have shown tolerable side effect profiles.
- HDACi exhibit potent inhibitory and stimulatory effects on the cell cycle and cell apoptosis, respectively. Preclinical studies suggest that HDACi may synergize with other cytogenic agents to alter chromatin structure, gene-expression patterns and tubulin acetylation and inhibit tumor growth in animal models.

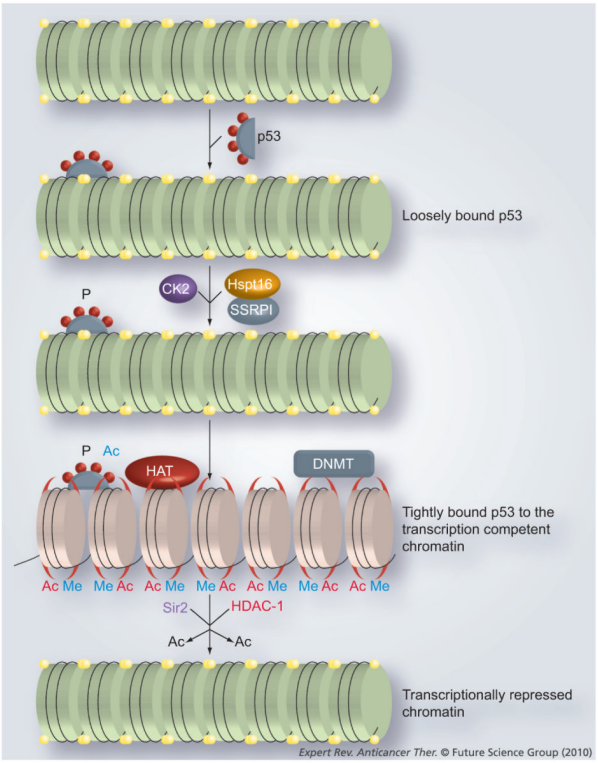

Figure 1. Acetylation regulates p53 function

p53 loosely binds to the repressed, tightly packed chromatin. A cascade of events initiated with the phosphorylation of p53, followed by recruitment of acetyltransferases and methyltransferases takes place, leading to p53 acetylation. Acetylated p53 binds tightly to the acetylated and methylated chromatins. The overall effects are relaxation of chromatin and transcription activation. Deacetylation of p53 and nucleosomal histones by HDAC-1 or SirT2 reverses the process and leads to chromatin tightening and transcription repression. Ac: Acetyl group; HAT: Histone acetyltransferase; HDAC: Histone deacetylase.

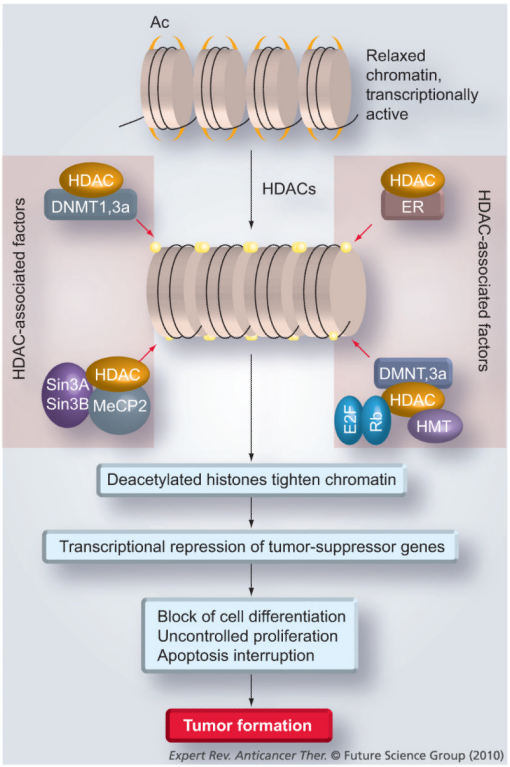

Figure 2. Histone deacetylase recruitment and transcription repression

Histone octamers are represented by circles and the DNA is shown in black lines. HDAC is recruited to gene promoters by nonhistone proteins including methylated DNA-binding protein MeCP2, ER, methyl transferases (DNMT) and transcription factors (E2F, Rb). HDAC removes the acetyl group from nonhistone as well as histone proteins, which leads to chromatin conformational change and transcription repression of tumor-suppression genes. The loss of tumor-suppressor gene products contributes to the block of cell differentiation, uncontrolled cell proliferation, interrupted apoptosis and, ultimately, tumor formation.
ER: Estrogen receptor; HDAC: Histone deacetylase;
Rb: Retinoblastoma protein.

Table 1

Classification and characteristics of histone deacetylases.

Parameter	Class I	Class IIa	Class IIb	Class III and IV
Yeast HDAC	RPD3	HDA1	HDA1	SirT2
Human HDAC	HDAC-1–3, -8	HDAC-4, -5, -7, -9	HDAC-6, -10	SirT1–7, HDAC-11
Molecular weight (kDa)	–	120–135	120–135	40–50 kDa
Size (no. of amino acid residues)	HDAC-1 (483), HDAC-2 (488), HDAC-3 (428), HDAC-8 (377)	HDAC-4 (1084), HDAC-5 (1122), HDAC-7 (855), HDAC-9 (1011)	HDAC-6 (1215), HDAC-10 (669)	HDAC-11 (347)
Distribution	Ubiquitous	Brain, heart	Testis, liver, kidney	Unknown
Localization	Nuclear	Nuclear/ cytoplasmic	Mostly cytoplasmic	Nuclear
Chromosomal localization	HDAC-1 (1p34.1), HDAC-2 (6q21), HDAC-3 (5q31), HDAC-8 (Xq13)	HDAC-4 (2q37.2), HDAC-5 (17q21), HDAC-7 (12q13), HDAC-9 (7p21-p15)	HDAC-6 (Xp11.22–23), HDAC-10 (22q13.31-q13.33)	HDAC-11 (3p25.2)
Target substrates	Histones, p53, NF-κB	Histones	Histones, tubulin, Hsp	Histones, tubulin p53, TAF
Catalytic sites	One	One	HDAC-6, two and HDAC-10, two	Two
Protein complexes	NuRD, SIN3	–	–	–
Co-repressor	N-CoR, SMRT	N-CoR, SMRT	–	–
Interacting proteins	Rb, p53, MyoD, NF-κB, SP11, BRCA1, DNMT1, DNMT3A-B, MBD2–3, MECP2, ATM	MEF2	Tubulin, Hsp	p53
Co-factor	Zn	Zn	Zn	NAD^+
Knockout phenotype	HDAC-1, embryonic lethal, increased histone acetylation, increase in p21 and p27, HDAC-2, cardiac defect	HDAC-4, defects in chondrocyte differentiation HDAC-5 and -9, cardiac defect HDAC-7, maintenance of vascular integrity, increase in MMP10	–	–
Cellular function	Participate in Sin3, NuRD, Co-REST complex (HDAC-1, -2); participate in SMRT/N-CoR (HDAC-3)	Interaction with SMRT/N-CoR and the co-repressors BcoR and CtBP (HDAC-4)	Tubulin deacetylase (HDAC-6) Recruitment other HDACs (HDAC-10)	Repression of p53 activity Regulate the 3′-end processing machinery of mRNA (SirT1 and SirT2)

CtBP: Carboxyl-terminal binding protein; HDAC: Histone deacetylase; Hsp: Heat-shock protein; MEF: Myocyte enhancer factor; N-CoR: Nuclear receptor co-repressor; NF-κB: Nuclear factor-κB; Rb: Retinoblastoma protein; SMRT: Silencing mediator for retinoid and thyroid hormone receptor.

Table 2

Nonhistone proteins known to be direct substrates for HDAC.

Substrate	Category	HDAC	Function of acetylation	Ref.
α-tubulin	Cytoskeleton	HDAC-6, SirT2	Modulates microtubule depolymerization	[183] [14]
β-catenin	Cell adhesion, transcription regulator	HDAC-6	Regulates β-catenin function in a promoter-dependent manner	[184]
TCF	DNA-binding factor	HDAC-3	Increases nuclear localization of high-mobility group (HMG) protein	[185]
p53	Tumor suppressor	HDAC-1, SirT2	Decreases DNA binding and transcriptional activity	[51]
MyoD	Muscle cell differentiation	HDCA-1	Acetylation regulates transactivation and the conversion of naive fibroblasts to muscle cells	[90]
E2F	Transcription factor/cell cycle	HDAC-1	Acetylation increases DNA-binding affinity	[186]
Hsp90	Chaperone protein	HDAC-6	Affects the maturation of glucocorticoid receptors	[187]
HIF1α	Transcription factor	HDAC-7	Regulates degradation in the proteasome	[123]
GATA	Transcription factor	HDAC-3, -4, -5	Affects erythroid differentiation	[14]
YY1	Transcription factor	HDAC-1, -2, -3	Acetylation promotes stable HDAC–YY1 interaction Acetylation regulates YY1–DNA binding	[112]
NF-κB	Transcription factor	HDAC-3, SirT1	Promotes the p65–IκB interaction and inhibits NF-κB-mediated transcription	[95]
Estrogen receptor	Steroid hormone receptor	HDAC-1	Increases cell proliferation	[188]
pRb	Tumor suppressor	HDAC-1	Regulates the specific interaction with E2F-1	[189]

HDAC: Histone deacetylase; HIF1α: Hypoxia-inducible factor 1α; Hsp: Heat-shock protein; NF-κB: Nuclear factor-κB; TCF: T-cell factor; pRb: Retinoblastoma protein; YY1: Yin Yang 1.

Table 3

Biochemical and cellular activities regulated by acetylation.

Biological implication	Nonhistone proteins affected by acetylation	
	Upregulation	*Downregulation*
Protein stability	p53, p73, Smad7, c-Myc, Runx3, AR, H2A.z, E2F1, NF-E4, ER81, SREBP1a, HNF6, BACE1	GATA1, HIF-1α, pRb, SV40 T-Ag
DNA binding	p53, SRY, STAT3, GATA transcription factors, E2F1, p50 (NF-κB), Erα, p65 (NF-κB), c-Myb, MyoD, HNF-4, AML1, BETA2, NF-E2, KLF13, TAL1/SCL, TAF(I)68, AP endonuclease	YY1, HMG-A1, HMG-N2, p65 (NF-αB), DEK, KLF13, Fen-1
Transcriptional regulation	Transcriptional activation: p53, HMG-A1, STAT3, AR, ERα (basal), GATA transcription factors, EKLF, MyoD, E2F1, p65 (NF-αB), GR, p73, PGC1α, MEF2D, GCMa, PLAG1, PLAGL2, Bcl-6, α-catenin, KLF5, Sp1, BETA2, Cart1, RIP140, TAF(I)68	Transcriptional inactivation ERα (ligand-bound), HIF-1α, STAT1, FoxO1, FoxO4, RIP140
Protein–protein interactions	STAT3, AR, EKLF, importin A, STAT1, TFIIB, α-tubulin, actin, cortactin	p65(RelA), Ku70, heat-shock protein 90
DNA repair	p53, E2F, TDF	NAIL2, FEN1, Pol b
Protein localization	PCAF, SRY, CtBP2, POP-1, HNF-4, PCNA subnuclear WRN, PCNA	c-Abl, p300, PAP
mRNA stability	p21, Brm	Tyrosine hydrolase (Th), eNOS
Enzymatic activity	p300, ATM	PTEN, HDAC1, Mdm2, ACS, Neil2, Polα
Mitochondrial proteins	ACS, Sod1/2	Profilin I, Thioredoxin
Viral proteins	SV40 T-Ag	E1A, HIV Tat, S-HDAg, L-HDAg

AR: Androgen receptor; EKLF: Erythroid Kruppel-like factor; ER: Estrogen receptor; GR: Glucocorticoid receptor; HMG: High-mobility group; NF-κB: Nuclear factor κB; SRY: Sex-determining region Y.