Modulation of posttranscriptional and posttranslational regulatory processes by histone deacetylase inhibitors

Dissertation

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“Success is going from failure to failure

without a loss of enthusiasm.”

Winston Churchill

1. Summary

Acetylation is a very critical posttranslational modification *in vivo*. Targeting lysine residues of various proteins crucially modulates protein functions and interactions. The inhibitors of histone deacetylases are promising anticancer drugs – currently in clinical testing. We could show that the treatment of cells with these histone deacetylase inhibitors is able to modulate cell fate by enhancing conditions that trigger apoptosis by two distinct mechanisms.

Firstly, VPA and co-treatment with the chemotherapeutic agent HU induce expression of the pro-apoptotic BH3-only protein BIM. The enhanced BIM expression arises from an increase in transcription of the *BIM* gene in an AP1-dependent manner. This effect occurs in cultured cells and in primary head and neck cancer cells from patients. We observed that the effect of apoptosis induction following treatment is mainly dependent on the level of BIM protein. This suggests that this therapy might be useful in the clinic.

Secondly, incubation of cancer cells with various histone deacetylase inhibitors reduces expression of the class III deacetylase SIRT1. SIRT1 carries out various cellular functions including stress responses and metabolic regulation. During stress conditions, SIRT1 expression is enhanced favouring cell survival by inhibiting apoptosis. We could show for the first time that inhibitors of the classical protein deacetylases (class I, II and IV), that do not target class III enzyme activity, unexpectedly target the class III deacetylase SIRT1 by decreasing its protein levels. The reduced SIRT1 protein amount upon inhibitor treatment is due to changes in *SIRT1* mRNA stability. The mRNA binding protein HuR is responsible for this effect. Histone deacetylase inhibitor treatment reduces the cytosolic amount of HuR. Additionally, its binding affinity for SIRT1 mRNA decreases significantly leading to SIRT1 mRNA decay. Furthermore, we identified three novel phosphorylation sites within HuR upon inhibitor treatment. Conceivably, these trigger the changed characteristics of HuR towards *SIRT1* mRNA. The loss of SIRT1 upon histone deacetylase inhibitor treatment leads to enhanced sensitivity of cell towards apoptotic stimuli.

Enhancing conditions of hyperacetylation in cancer cells triggers an adaptation of the cellular proteome favouring cell death and sensitivity of cancer cells towards further therapeutics. In sum this work provides valuable information for the treatment of cancer cells with histone deacetylase inhibitors in combination with other chemotherapeutics.

2. Zusammenfassung

Als posttranslationale Modifikation spielt die Acetylierung *in vivo* eine entscheidende Rolle. Die Neutralisierung positiver geladener Lysinreste verschiedenster Proteine kann deren Funktion sowie die Interaktion mit anderen Proteinen entscheidend beeinflussen. Die Inhibitoren der Proteindeacetylasen sind vielversprechende Chemotherapeutika die sich momentan in klinischen Studien befinden. Wir konnten zeigen, dass die Behandlung von Krebszellen mit diesen Hemmstoffen im Stande ist, den apoptotischen Zelltod durch zwei verschiedene Mechanismen zu verstärken.

Zum einen induziert der Deacetylaseinhibitor VPA zusammen mit dem Chemotherapeutikum HU die Transkription und letztendlich die Expression des pro-apoptotischen Proteins BIM. Dieser Effekt ist sowohl in kultivierten Zellen als auch in primären Kopf und Hals-Tumorzellen von Patienten nachweisbar. Die apoptoseinduzierende Wirkung der Inhibitorgabe ist vorwiegend von der Menge des zellulären BIM Proteins abhängig. Dies gibt Hinweise auf die klinische Bedeutung dieser Therapie.

Ebenso reduziert die Inkubation mit verschiedenen Histondeacetylaseinhibitoren die Expression der Klasse III Deacetylase SIRT1 in verschiedenen Krebszelllinien. SIRT1 moduliert viele verschiedene Zellfunktionen einschließlich Apoptose und Stoffwechsel. Unter Stress wird die SIRT1 Expression erhöht und damit das Zellüberleben durch eine Hemmung der Apoptose gesichert. Wir konnten zum ersten Mal zeigen, dass Inhibitoren der klassischen Proteindeacetylasen (Klasse I, II und IV), welche nicht die Aktivität der Klasse III beeinflussen, dennoch die Klasse III Deacetylase SIRT1 ins Visier nehmen durch eine Verminderung der SIRT1 Proteinmenge. Die inhibitorinduzierte Reduktion der SIRT1 Proteinlevel ist auf eine Änderung der SIRT1 mRNA-Stabilität zurückzuführen. Hierfür scheint das mRNA-bindende Protein HuR verantwortlich zu sein. Eine Histondeacetylaseinhibitor-Behandlung reduziert den cytosolischen Anteil von HuR. Der SIRT1 mRNA Abbau wird höchstwahrscheinlich durch den Verlust der Bindungsaffinität von HuR gegenüber der SIRT1 mRNA ausgelöst. Zusätzlich identifizierten wir drei bisher unbeschriebene hemmstoffinduzierte Phosphorylierungsstellen innerhalb von HuR. Es ist denkbar, dass diese Veränderungen die HuR-Charakteristika in Bezug auf die *SIRT1* mRNA bedingen. Der Verlust von SIRT1 nach Inhibitorbehandlung erhöht die Zellsensitivität gegenüber apoptoseauslösenden Bedingungen.

Die Induktion der Hyperacetylierung in Krebszellen moduliert das zelluläre Proteom dahingehend, dass es Apoptose fördert und Krebszellen gegenüber weiteren Einflüssen sensibilisiert. Zusammenfassend bietet diese Arbeit wertvolle Grundlagen für die erfolgreiche Behandlung von Krebszellen mit Histondeacetylase-Inhibitoren in Kombination mit anderen Chemotherapeutika.

3. INTRODUCTION

3.1. Acetylation as an important posttranslational modification

The classical 20 amino acids are the building blocks of every eukaryotic protein. They cover a wide variety of chemical characteristics providing the basis for different structures. Protein structures depend on hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions. The resulting secondary, tertiary and quaternary structures define the functionality of all cellular proteins.

Protein functions are modulated by protein amounts and the abundance of co-factors as well as by posttranslational modifications. For example, serine or threonine residues are phosphorylated by kinases. Furthermore, lysine residues can be modified by acetylation, (mono-, di- and tri-) methylation, ubiquitination, sumoylation and neddylation (see Figure 1). These sometimes competing modifications alter the structure of the lysine moiety. At physiological pH, unmodified lysine residues are protonated and positively charged. The addition of acetyl groups neutralises the charge and also creates a novel surface for protein interactions. In the past, critics stated that only a limited or specialized number of proteins become acetylated and that biologically important acetylation sites of low abundance remain undetected because of a large background of non-acetylated proteins (Choudhary *et al.*, 2009). Now, more robust techniques open new possibilities to identify protein targets for acetylation. One approach is to purify acetylated proteins by an acetylation-specific antibody and to further identify acetylated peptides by mass spectrometry (Kim *et al.*, 2006). Recently, Choudhary et al. identified more than 3600 acetylation sites in 1750 proteins. This approach raised the number of known acetylated proteins extremely. In comparison this the size of the phosphoproteome and suggests that acetylation can similarly

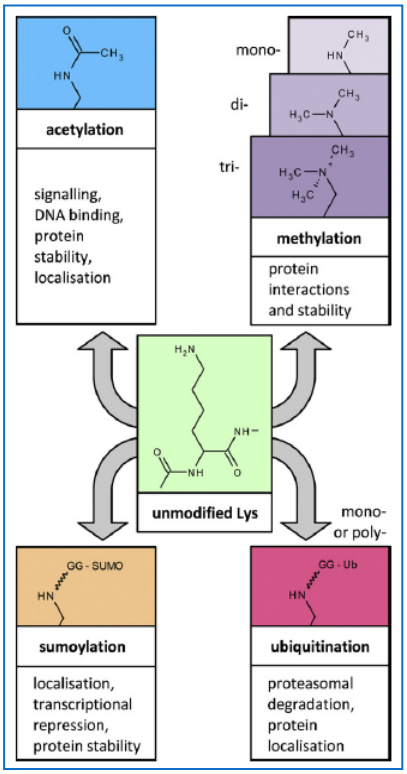

Figure 1 Lysine modifications.
Lysine residues are subject to posttranslational modifications with varying functional consequences. Switching between modifications allows to alter protein function. (Spange *et al.*, 2009)

affect protein characteristics (Choudhary *et al.*, 2009; Cohen and Yao, 2004; Olsen *et al.*, 2006 Yang, 2008 #78).

Histones condense DNA in the nucleus. Since these proteins were the first identified acetylated proteins, acetylation was only considered in the context of histone modification and transcriptional control. Indeed, acetylating und deacetylating enzymes are important transcriptional co-activators and co-repressors (Xu *et al.*, 1999). Nonetheless, the discovery of an increasing number of acetylated non-histone proteins and the finding that acetylation-modifying enzymes exert crucial functions outside of the nucleus, revealed much broader regulatory potentials for reversible acetylation. Depending on the cellular compartment (nucleus, cytosol and mitochondria), motifs for acetylation differ extremely (Choudhary *et al.*, 2009). The classic motif for acetylation is the conserved LxxLL motif whereby adjacent lysine residues are targeted. The reason for this could be a different subset of acetylases and deacetylases recognising different sets of substrates.

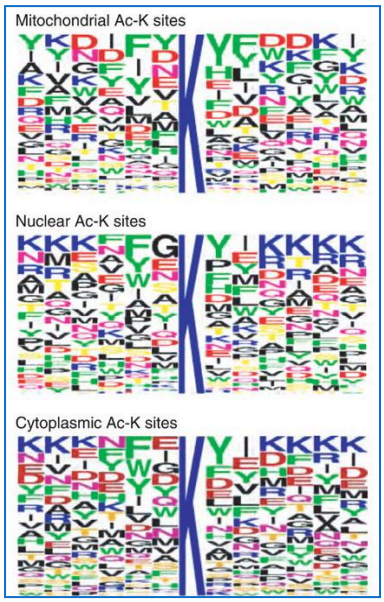

Figure 2 Acetylation motifs in different subcellular compartments Sequence logo plots represent normalized amino acid frequencies for ±6 amino acids from the lysine acetylation site (Olsen *et al.*, 2006).

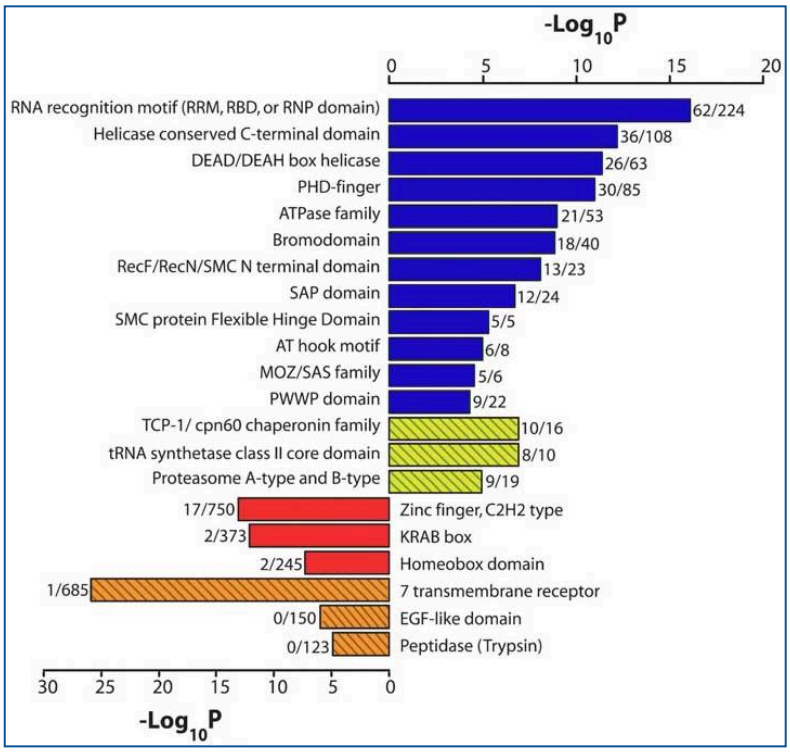

Figure 3: Domain architecture of acetylated proteins.
blue bars = protein families and domains that are significantly overrepresented; red bars = underrepresented domains in the acetylome as compared with those in the entire proteome; yellow + orange striped bars = cytoplasmic domains (Choudhary *et al.*, 2009).

Additionally, the architecture of acetylated proteins shows that certain domains are overrepresented in acetylated proteins (Choudhary *et al.*, 2009) (see Figure 3). These acetylation events might be important for the assembly of multiprotein machineries and for specific cellular functions. Figure 3 indicates that proteins containing RNA recognition motifs show a prevalence for acetylation.

The regulatory power of the acetylome is further amplified through its crosstalk with other modifications, including phosphorylation of serine, threonine or tyrosine residues (S/T-P) and other lysine-based modifications (K-X), where X can be ubiquitination, methylation, neddylation, or sumoylation (Norris *et al.*, 2009). Several networks, relying on acetylation, phosphorylation and others, interact with each other (see figure 2). Taken together, all this demonstrates that complex networks of posttranslational modifications can modulate protein functions. This observation raises the question how these modifications interact with each other and form dynamic programmes regulating cellular fate. However, there is no unified consequence of protein acetylation. The outcome is always context-dependent and needs to be elucidated for every single protein. Therefore, the impact of acetylation can only be deciphered experimentally.

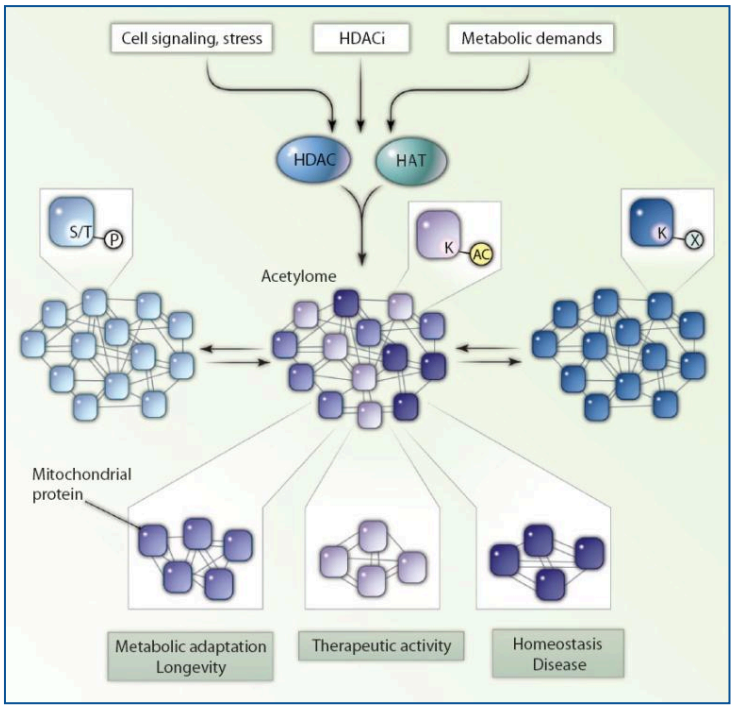

Figure 4 The acetylome undergoes dynamic changes in response to cell signalling, stress, metabolic demands, and HDAC inhibitor (HDACi) treatment (Norris *et al.*, 2009).

3.1.1 Protein acetylases and –deacetylases

The dynamics of protein acetylation are regulated by the opposing enzymatic activities of protein acetylases (HATs) and protein deacetylases (HDACs). Due to their first identified substrates, the protein deacetylases are also named histone deacetylases. The more correct term would be lysine or protein deacetylase. Since the discovery of the first HAT enzyme, the yeast Hat1 (Kleff *et al.*, 1995), a lot of attention has been drawn to these enzymes. HATs are evolutionarily conserved from yeast to man and form multiple subunit complexes (Kimura *et al.*, 2005). In mammals, over 30 HATs display distinct substrate specificities for histones and non-histone proteins. HATs mainly exerting transcription-related acetylation are grouped into five families: GNAT, MYST, p300/CBP, basal/general transcription factors and nuclear receptor cofactors (Roth *et al.*, 2001). CBP and p300 are the most intensively studied HATs and are often found within the same complexes. Apparently, p300 seems to have the broadest spectrum of substrates which includes histones as well as non-histone proteins (Kimura *et al.*, 2005).

The deacetylase super family is much more homogenous then the HAT family. HDACs are grouped into two distinct families. The “classical family” of zinc-dependent HDACs is structurally related to the yeast Hda1/Rpd3 proteins (de Ruijter *et al.*, 2003). The second family, the sirtuins, consists of the NAD^+-dependent yeast Sir2 homologues (Haigis and Guarente, 2006). Histone deacetylases are further grouped into four classes according to their phylogenetic conservation (Gregoretti *et al.*, 2004). Class I, II and IV HDACs are members of the classical HDAC family. Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are orthologues of the yeast Rpd3 enzyme. The yeast Hda1 homologues represent the mammalian class II HDACs that can be subdivided into class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10). Class IIa HDACs does not seem to exert HDAC activity (Fischle *et al.*, 2002). It is suggested that any HDAC activity associated with these proteins reflects co-purification of class I HDACs. The main difference between those two subgroups relies on their catalytic centre. Whereas class I possesses a Tyr moiety, class IIa harbours a His residue at the corresponding site. The important role for this moiety is demonstrated by the fact that an amino acid substitution in the catalytic centre of class IIa HDACs unleashed a ~1000 fold increase in class IIa catalytic activity compared to their wild type counterparts (Lahm *et al.*, 2007). HDAC11 shares equal sequence conservation with

Rpd3 and Hda1, and is therefore grouped as the currently solitary member into class IV (Gao *et al.*, 2002). Homologues of yeast Sir2, SIRTs, represent the class III deacetylases, which are termed sirtuins in mammals (SIRT1 to 7) (Yang and Seto, 2008). An overview of the domain structures of all mammalian protein deacetylases is shown in Figure 5.

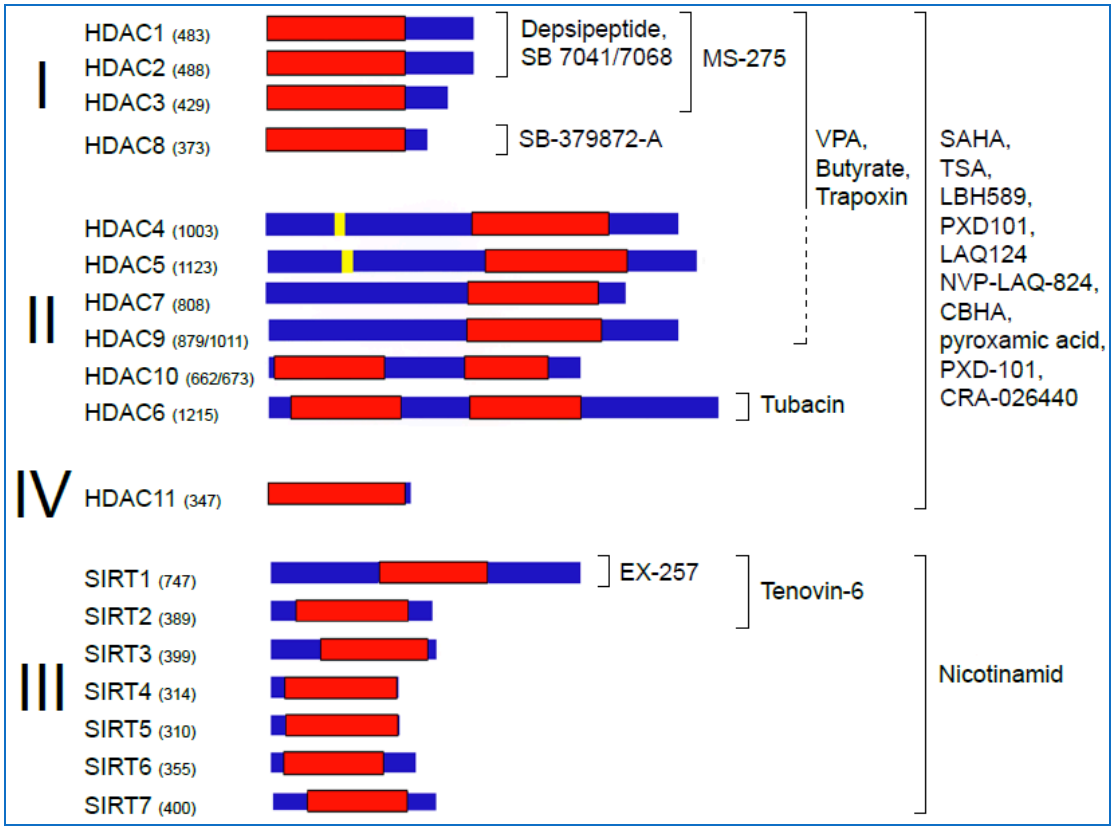

Figure 5: Members of the histone deacetylase superfamily and inhibitors

The classical HDACs in class I, II and IV and the sirtuins (class III) all contain a deacetylase domain (red). The size of the protein, denoted in amino acids, is stated next to the protein name. Known inhibitors for single HDAC and HDAC subgroups are depicted at the right side (Lichtman M., 2005).

3.1.2 Protein deacetylases as chemotherapeutic targets

The impact of acetylation on critical regulators of cell signalling and cell fate is enormous. Many diseases show abnormal gene expression. These changes may be corrected by targeting the enzymes that catalyse acetylation/deacetylation processes

Hence, this predestines HDACs and HATs, which dynamically target the acetylome, as drug targets in cancer and neurodegenerative diseases, such as Parkinson's and Alzheimer's disease. Currently, more than 100 clinical trials assess the efficacy of HDACi in a clinical setting (Bolden *et al.*, 2006; Garber, 2007; Kazantsev and Thompson, 2008; Müller and

Krämer, 2010; Xu *et al.*, 2007). The HDACi suberoylanilide hydroxamic acid (SAHA) is in clinical use against the cutaneous T cell lymphoma (CTCL). Of note, drugs used successfully in therapeutic settings for different diseases proved to have HDACi activity. For example, valproic acid (VPA), used in the treatment of epilepsy, bipolar disorder, and less commonly major depression is able to inhibit class I and IIa HDACs. Moreover, Methotrexate, which is used for treating human cancers, inhibits HDAC1 and 2 as well as overall HDAC activity, increasing histone acetylation (Yang *et al.*, 2010).

How HDACi achieve therapeutic effects is an area of ongoing research. The ability to identify "acetylation targets" of HDACi could potentially provide a window into the understanding of acetylation events relevant to therapeutic activity. In many cases the relevance of these drugs in the fight against cancer still awaits the results of phase III trials. Moreover, HDAC inhibitors are potent reprogramming agents for the generation of pluripotent stem cells (Huangfu *et al.*, 2008).

3.2 SIRT1

Our metabolism eventually degrades fatty acids, carbohydrates and proteins to a single and versatile intermediate, acetyl-CoA. This same intermediate is also used during the modification of proteins at their lysine residues known to regulate gene expression. Therefore, acetylation and NAD^+-dependent deacetylation emerged as directly connecting the intracellular energy state and cellular fate (Finkel *et al.*, 2009). The NAD^+-dependent sirtuin deacetylases are homologs of the *Sir2* gene in *S. cerevisiae*. In sum, the activity of the sirtuin family is linked to the metabolic state of the cell.

Among the homologs of the yeast Sir2 protein, SIRT1 is the best-characterised member in humans. The seven human sirtuins are distributed in the whole cell with SIRT1, 6 and 7 being in the nucleus, SIRT2 in the cytosol and SIRT3, 4 and 5 in the mitochondria (Michishita *et al.*, 2005). While the classical HDACs only perform deacetylating reactions, some members of the sirtuin family (SIRT1, 2, 4 and 6) can use NAD^+ to ADP-ribosylate target proteins. The ADP-ribosylation activity of several sirtuins is >1000-fold lower than their deacetylation activity, therefore the physiological relevance of the ADP-ribosylation activity is not clear (Du *et al.*, 2009). An overview of localisation, substrates and functions of different sirtuins can be seen in Table 1

Table 1: Overview: localisation, substrates and proposed function of the human sirtuins (based on Frojdo *et al.*, 2008; Haigis and Sinclair, 2010; Kong *et al.*, 2009; Lavu *et al.*, 2008)

	Localisation	Substrates	Function
SIRT1	Nucleus/ Cytoplasm	histones H1/H3/H4, p53, FOXO proteins, NFκB, KU70, MyoD, CBP, COUP-TF, CTIP2, NCoR, p300, BCL6, BCL11A, PGC1α, MEF2D, eNOS, ACS1, E2F1, AR, p73, SMAD7, NBS1, RB, TLE1, IRS2, LXR, AROS, HIC1, SUV39H1, WRN, DBC1, TORC2, AceCS1, HIV-Tat, HSF-1, NBS1, PCAF, PPARγ, TAF $_{I}$68	stress regulation, genome integrity, DNA repair, longevity, senescence, apoptosis, metabolism, inflammation, neuroprotection
SIRT2	Cytoplasm, Nucleus	histone H4, α-Tubulin	cell cycle, genome integrity
SIRT3	Mitochondria	histone H4, AceCS2	Acetyl-Co-synthesis, thermogenesis, longevity
SIRT4	Mitochondria	GDH	insulin secretion
SIRT5	Mitochondria	N/A	N/A
SIRT6	Nucleus	DNA-Pol β	DNA repair
SIRT7	Nucleoli	RNA-Pol I	Ribosomal RNA-transcription

3.2.1 SIRT1 functions

SIRT1 can deacetylate proteins in the presence of its cofactor nicotinamide adenine dinucleotide (NAD^+). Hereby, it catalyzes the conversion of NAD^+ and acetylated lysine to nicotinamide, 2'-O-acetyl-ADP-ribose (OAADPr) and the deacetylated lysine (see Figure 6). Of note, it is the only sirtuin in the nucleus with a reasonable deacetylase activity (Michishita *et al.*, 2005).

Next to its histone targets (mainly histone H3(K9 & K14), histone H4(K16) (Imai *et al.*, 2000) and the linker histone H1 (Vaquero *et al.*, 2004)) SIRT1 deacetylates many non-histone proteins (see Table1 for details) (Lavu *et al.*, 2008). Through its multitude of intracellular targets, SIRT1 regulates epigenetic gene silencing, DNA repair and recombination, apoptosis, cell cycle and senescence, microtubule organisation as well as fat and glucose metabolism.

In sum, SIRT1 activities provide stress resistance and cell survival, which in turn could play crucial roles in processes like neuroprotection, inflammation, and longevity as well as in cancer development (Anekonda and Reddy, 2006; Baur *et al.*, 2006; Labinskyy *et al.*, 2006; Michan and Sinclair, 2007; Saunders and Verdin, 2007).

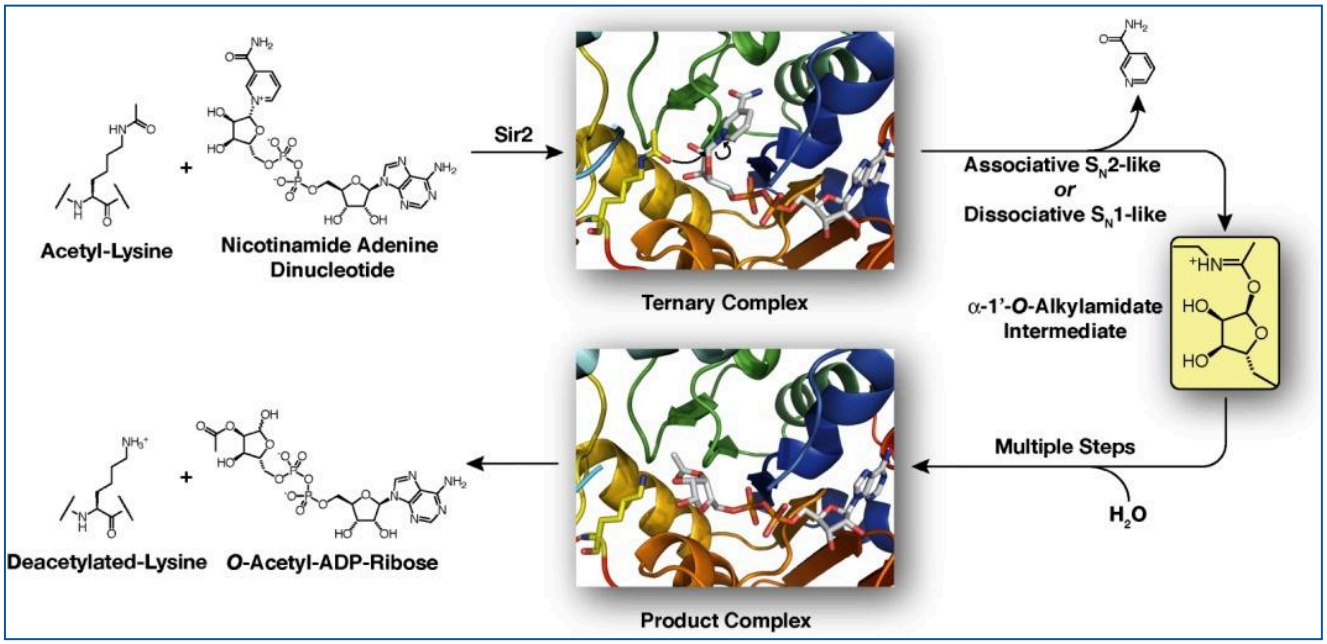

Figure 6: Proposed enzymatic mechanism of SIRT1 deacetylase activity

Acetyl-lysine substrate and NAD+ bind to SIRT1 - forming a ternary complex. After binding, the reaction proceeds and releases nicotinamide. The O-alkylamidate intermediate is then hydrolyzed through multiple steps to form a product complex from which deacetylated-lysine product and O-acetyl-ADP-ribose are released. (Smith and Denu, 2006)

In yeast, the Sir2 protein links the energy status of the cell directly to longevity. One hypothesis of ageing mechanisms in yeast suggests that extrachromosomal ribosomal DNA circles accumulating in the mother cell by inadvertent recombination cause senescence. Increased SIRT1 activity in turn suppresses recombination of rDNA by compacting its chromatin structure (Guarente, 2000). Therefore, caloric restriction (CR) can interfere with senescence via increasing Sir2 activity by an altered NAD^+/NADH ratio.

In mammals, SIRT1 is also thought to play a role in CR-associated longevity. Activated SIRT1 modulates many aspects of glucose and lipid homeostasis by deacetylating key metabolic molecules like PPARγ, PGC-1α, IRS-2, CRTC2, UCP-2, AceCS1, STAT3 and TORC2, showing its pivotal role in the regulation of metabolism (see Figure 7). The deacetylation of PGC1α by SIRT1 activates its transcriptional activity and thus induces the expression of target genes involved in gluconeogenesis and fatty acid oxidation. Deacetylation of FOXO1 also increases its transcriptional activity and promotes the expression of gluconeogenic genes in the liver, of insulin in the pancreas, and of adiponectin in adipose tissues preventing illnesses like diabetes mellitus. Therefore, SIRT1 is often linked to the positive effects of restricted calorie intake not only due to its NAD^+-dependency. Hence, its action is associated

with extending lifespan and improving health and survival (Baur *et al.*, 2006; Cohen *et al.*, 2004b; Lavu *et al.*, 2008; Yu and Auwerx, 2009).

Besides its metabolic functions, SIRT1 triggers cell survival under stress conditions by multiple targets. SIRT1 is the main deacetylase of p53. In its deacetylated state the ability of p53 to trans-activate target genes (e.g. p21, Bax) is attenuated (Luo *et al.*, 2001; Vaziri *et al.*, 2001).

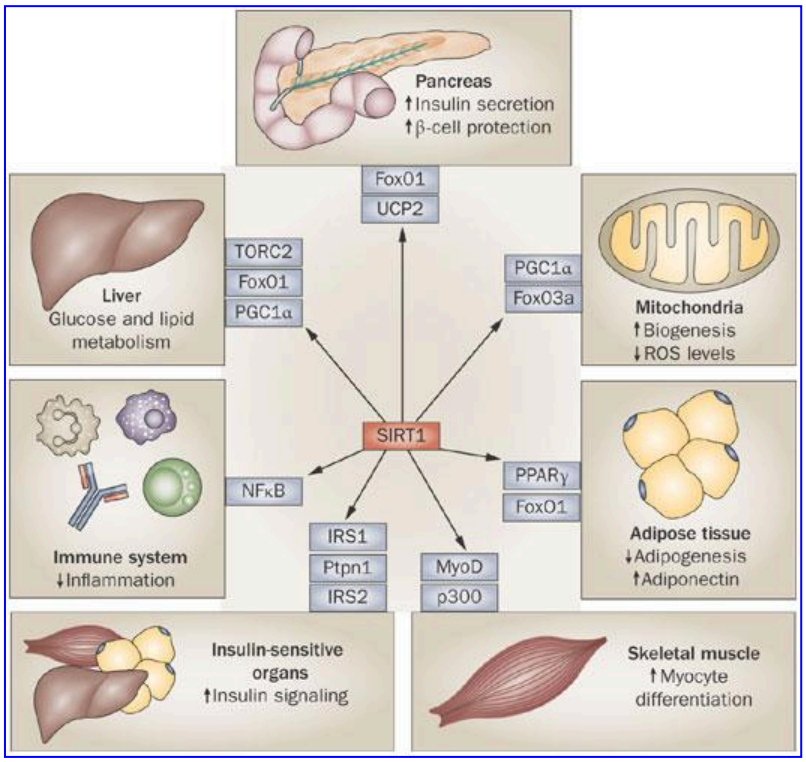

Figure 7 SIRT1 affects inflammatory responses, insulin secretion, hepatic metabolism of glucose and lipids, adipogenesis and adiponectin secretion, mitochondrial homeostasis and ROS levels, the insulin signalling pathway, and myogenesis (Liang *et al.*, 2009).

In addition to p53, p73 is a SIRT1 target, both proteins mediate the cellular response to genotoxic stress and thus SIRT1 is a critical regulator of cells exposed to environmental and chemotherapeutic stimuli (Yang *et al.*, 2007). SIRT1-mediated deacetylation of the DNA repair-associated Ku70 activates DNA repair processes and inhibits Bax-induced apoptosis (Cohen *et al.*, 2004b). Moreover, SIRT1 deacetylates several proteins of the FOXO (FOXO1, 3a, 4 and 6) transcription factor family. Non-acetylated FOXO proteins are no longer active. Consequently, a downregulation of FOXO transcriptional targets like the pro-apoptotic BIM and an upregulation of the stress response gene GADD45 occurs in cells (Brunet *et al.*, 2004). Promoting cell survival by these various mechanisms favours a central role of SIRT1 as stress regulator.

3.2.2 SIRT1 and cancer

The role of SIRT1 in cancer is discussed controversially. It enables cells to survive stressful conditions by the modulation of signalling pathways promoting apoptosis or autophagy. Some kinds of cancer seem to depend on high SIRT1 levels favouring cell growth and survival. Conceivably, a wide variety of solid tumours and leukaemias exhibit increased SIRT1 expression compared to healthy tissue (Bradbury *et al.*, 2005; Chang *et al.*, 2009; Chen *et al.*, 2005; Hida *et al.*, 2007; Jung-Hynes *et al.*, 2009; Kuzmichev *et al.*, 2005; Lim, 2006; Mariadason, 2008). Overexpression of this protein can be relevant for oncogenesis and chemotherapeutic responses. For example, a glioblastoma-derived cell line overexpresses SIRT1 and a knockdown of SIRT1 in these cells effectively enhanced radiosensitivity and apoptosis *in vivo* and *in vitro*. Additionally, other hallmarks of cancer cells decreased, including cell migration ability, tumour volume and colony formation resulting in enhanced survival of mice bearing the tumour (Chang *et al.*, 2009). Another common hallmark of human cancers notably is the loss of histone H4 Lys16 acetylation, whereby SIRT1 is the major deacetylase of H4 Lys16 (Fraga *et al.*, 2005). Therefore, enhanced SIRT1 activity can be linked to the loss of site-specific deacetylation (Hajji *et al.*, 2010).

On the other hand, enhanced SIRT1 expression showed a promoting effect on tumour development and maintenance in a β-catenin-driven mouse model (Firestein *et al.*, 2008). In another tumour cell model a strong expression of SIRT1 is associated with a deregulation of pRb1 signalling that promotes cell growth and inhibits apoptosis (Hida *et al.*, 2007; Wang *et al.*, 2006). Moreover, SIRT1 overexpression epigenetically silences tumour suppressor genes, like *E-cadherin, SFRP1/2, CRB1* and *MLH1*. As a consequence SIRT1 inhibition induces re-expression of the stated genes without changing their promoter DNA methylation status (Pruitt *et al.*, 2006). SIRT1 even affects the multidrug resistance of tumours. SIRT1 also regulates the expression of the multidrug resistance 1 (MDR1) gene. Thereby, it can promote chemo-resistance linked to poor prognosis (Chu *et al.*, 2005). Overexpressed SIRT1 additionally seems to increase the expression of telomerase, which extends chromosome ends and thereby blocks cellular senescence (Lin and Elledge, 2003).

SIRT1 is suitable as a therapeutic target because it can affect multiple critical pathways causally linked to human diseases. Both inhibitors and activators of SIRT1 could be of benefit for patients depending on the individual disease and perhaps also on its specific stage.

Targeting SIRT1 activity can be seen as a double-edged sword – to lose or overexpress SIRT1 under normal conditions is unfavourable. Under pathological conditions, SIRT1 activators are favourable in the therapy of metabolic diseases. Small molecule inhibitors of SIRT1 are rated as novel anti-cancer agents, especially by inducing senescence-like growth arrest and a strong pro-apoptotic effect in cancer cells (Heltweg *et al.*, 2006; Lara *et al.*, 2009; Mai *et al.*, 2005; Olaharski *et al.*, 2005).

3.3 SIRT1 regulation

As a key regulator of cellular signalling, SIRT1 is tightly controlled. In recent years, a complex regulatory network controlling SIRT1 actions emerged. Various stress-signalling pathways regulate SIRT1 at multiple steps including transcription, mRNA stability, translation, posttranslational modifications and recruitment of binding partners as well as cofactors.

3.3.1 Transcriptional regulation of SIRT1

The expression of the *SIRT1* gene is controlled by various external stimuli, including upregulation via starvation, caloric restriction, oxidative stress and DNA damage or downregulation by hypoxia. The SIRT1 promoter comprises a small CpG island (350 bp) and numerous transcription factor binding sites. Additionally, SIRT1 regulates its own transcription. Known transcriptional regulators of the *SIRT1* promoter include p53, E2F1, FOXO3A and the HIC1–CtBP repressor complex. SIRT1 associates with all of these factors and regulates their activity via deacetylation resulting in a feedback mechanism.

The tumour suppressor p53 functions as a repressor for the SIRT1 promoter. In the absence of nutrients, *SIRT1* transcription is induced through nuclear translocation of FOXO3a, which interacts with p53 and thereby inhibits the suppressive activity of p53 (Nemoto *et al.*, 2004; Zschoernig and Mahlknecht, 2008). The transcription factor HIC1 (hypermethylated in cancer 1) represses SIRT1 expression via SIRT1-HIC1 complexes. In cancer cells as well as during ageing the HIC1 promoter is hypermethylated and thereby epigenetically silenced. Loss of HIC1 might promote tumourigenesis by upregulation of SIRT1 (Bzduch and Behulova, 1992; Chen *et al.*, 2005; Lim, 2007; Milner, 2009) and might be causal or the aberrantly high promoter activity of SIRT1 in tumour cells (Okazaki *et al.*, 2010; Zschoernig and Mahlknecht, 2008) (see Figure 8).

3.3.2 Posttranslational regulation of SIRT1

The posttranslational regulation of SIRT1 involves modifications of SIRT1 as well as SIRT1 binding factors that modulate its activity.

Regulation of SIRT1 enzymatic activity can intrinsically be achieved by phosphorylation as well as sumoylation. The three JNK1-targeted phosphorylation sites S27, S47 and T530 increase nuclear localization of SIRT1 and its enzymatic activity towards histones (Nasrin *et al.*, 2009). Elevated SIRT1 protein levels in cancer cells have recently been attributed to SIRT1 protein stability. This is correlated with stress-induced JNK2-dependent SIRT1 phosphorylation at S27 (Ford *et al.*, 2008). SIRT1 protein stability may also play a role in the progressive loss of SIRT1 associated with aging. Comparing mouse embryonic fibroblasts (MEFs) of different passages indicates that SIRT1 protein levels, but not *SIRT1 mRNA*, decrease rapidly with increasing cell passages (Sasaki *et al.*, 2006). Immortalisation of these cells restored the level of SIRT1 protein to that of early passage MEFs. It could be speculated that the phosphorylation status of SIRT1 differs in these cells resulting in a more stable SIRT1. Additionally, CK2-dependent phosphorylation of S154, S649, S651 and S683 increases SIRT1 binding affinity towards its targets (Kang *et al.*, 2009). So far, no functional role for other CK2-targeted sites (S659 & S661) could be detected (Zschoernig and Mahlknecht, 2009). Alongside SIRT1 phosphorylation, the C-terminal K734 is subject to modification with small ubiquitin-related modifier (SUMO). Sumoylation increases SIRT1 activity. When this lysine is replaced with an arginine, SIRT1 deacetylase activity towards acetylated p53 and p73 is almost completely abolished. DNA damage and oxidative stress promote desumoylation of SIRT1 by SENP1 and thereby decrease SIRT1 activity (Yang *et al.*, 2007).

SIRT1 binding proteins can modulate its enzymatic activity. Recently, DBC1 (deleted in breast cancer 1) has been identified as a specific negative SIRT1 regulator. DNA damage and oxidative stress-induced binding of DBC1 to SIRT1 suppresses its action and favours p53 acetylation (Zschoernig and Mahlknecht, 2008). Complementary, an active regulator of SIRT1 protein (AROS) has been found to directly bind the SIRT1 N-terminus and increase its enzymatic activity towards p53 (Kim *et al.*, 2007; Verdin, 2007). So far, it is not known how the combination or differential regulation of DBC1 and AROS affects SIRT1 activity. Taken together, SIRT1 regulation is complex and protein levels do not necessarily represent SIRT1 protein activity.

3.3.3 Small molecule modulators of SIRT1

As mentioned above, SIRT1 activity is highly dependent on NAD^+-cofactor availability. During conditions of caloric excess NAD^+-levels can be depleted resulting in inactive SIRT1. The same situation can be generated by a strong induction of NAD^+ competing enzymes like the DNA repair-associated PARP. A massive induction of DNA damage strongly activates PARP and depletes NAD^+-levels and can therefore inhibit SIRT1 functions and promote cell death (Pillai *et al.*, 2005).

Small molecule activators as well as inhibitors of SIRT1 are considered as therapeutics in different disease states. The most prominent SIRT1 activator is the polyphenol resveratrol (Howitz *et al.*, 2003). The favourable effects of resveratrol in mammals are attributed to increased SIRT1 activity including increased lifespan, protection from obesity in mice placed on a high-calorie diet with increased insulin sensitivity and protection from metabolic diseases (Baur *et al.*, 2006; Lagouge *et al.*, 2006). Recent results indicate that resveratrol indirectly activates SIRT1 (Pacholec *et al.*, 2010; Tang, 2010). Other small molecule activators like quercetin, piceatannol are interesting drugs for metabolic diseases showing positive effects on glucose homeostasis and insulin sensitivity in obesity mouse models (Guarente, 2006; Liang *et al.*, 2009).

On the other hand, SIRT1 inhibitors are more likely to be beneficial in malignant disorders. They could mainly be useful for the treatment of cancers by increasing p53 activity which induces apoptosis and stops the formation of tumours. Tenovin-6, a SIRT1 and SIRT2 specific inhibitor, increases p53 acetylation and has been shown to decrease tumour growth *in vivo* as a single agent – potentially providing new therapeutic options (Lain *et al.*, 2008).

3.3.4 Posttranscriptional regulation of SIRT1

3.3.4.1 mRNA binding proteins

The first hint that posttranscriptional regulation is crucial for the control of SIRT1 came from the finding that the AU-rich element-binding protein HuR stabilises *SIRT1 mRNA*. So far, no further mRNA binding proteins are known to regulate the *SIRT1 mRNA* level. The mainly nuclear HuR binds to and protects the 3´UTR of *SIRT1 mRNA* from degradation. Multiple HuR binding sites can often be found within one mRNA. Targeting *c-fos, c-myc, cox-2, TNF-α, GM-CSF, β-catenin, eotaxin, p27, cyclin A, cyclin B1, cyclin D1, p21, p27, p53* and *SIRT1 mRNA*,

HuR has been suggested to critically affect cell proliferation, tumourigenesis, senescence and stress responses (Abdelmohsen *et al.*, 2007a; Abdelmohsen *et al.*, 2007b). While under certain conditions HuR binds closely to one mRNA, it dissociates from another resulting in complete destabilization of the transcript (Abdelmohsen *et al.*, 2007b; Wilusz and Wilusz, 2007).

An siRNA mediated knockdown of HuR resulted in a complete loss of SIRT1 protein (Abdelmohsen *et al.*, 2007b). Therefore, regulation and modulation of HuR function are closely related to SIRT1 expression levels. Remarkably, the conformation of HuR's three RNA recognition motifs (RRMs), HuR localisation as well as its phosphorylation state critically affect *SIRT1 mRNA* stability (Abdelmohsen *et al.*, 2007b; Kim *et al.*, 2008a; Wilusz and Wilusz, 2007) (see Figure 8).

Oxidative stress activates checkpoint kinase Chk2 through phosphorylation and promotes HuR phosphorylation at S88, S100 and T118. In turn, this results in dissociation of the HuR–SIRT1-mRNA complex. Decreased levels of HuR and increased levels of Chk2 synergize to decrease *SIRT1* mRNA and protein levels during senescence (Abdelmohsen *et al.*, 2007b). Furthermore, the cell cycle-dependent Cdk1 phosphorylates nuclear HuR at S202, leading to its nuclear retention by binding to 14-3-3 proteins. Hence, *SIRT1* mRNA stability decreases in the G_2/M-phase (Kim *et al.*, 2008a).

3.3.4.2 micro RNAs

Next to proteins, the role of miRNAs in the regulatory network of mRNA fate attracts an increasing number of researchers. These small regulatory RNAs primarily bind within the 3´UTR of mRNAs. Their grade of complementarity is responsible for the suppressing effect. High similarity causes siRNA-like mRNA degradation whereas low sequence similarity inhibits translation.

Several miRNAs targeting the 3´-UTR of *SIRT1 mRNA* are identified so far: the strongly p53-dependent miR-34a (Yamakuchi *et al.*, 2008); the hypoxia related regulator miR-199a (Rane *et al.*, 2009); the ageing associated miR-217 (19786632); the nutrition related miR-132 (Strum *et al.*, 2009); miR-200b/c (Tryndyak *et al.*, 2010) and miR-449a (Lize *et al.*, 2010) (see Figure 8).

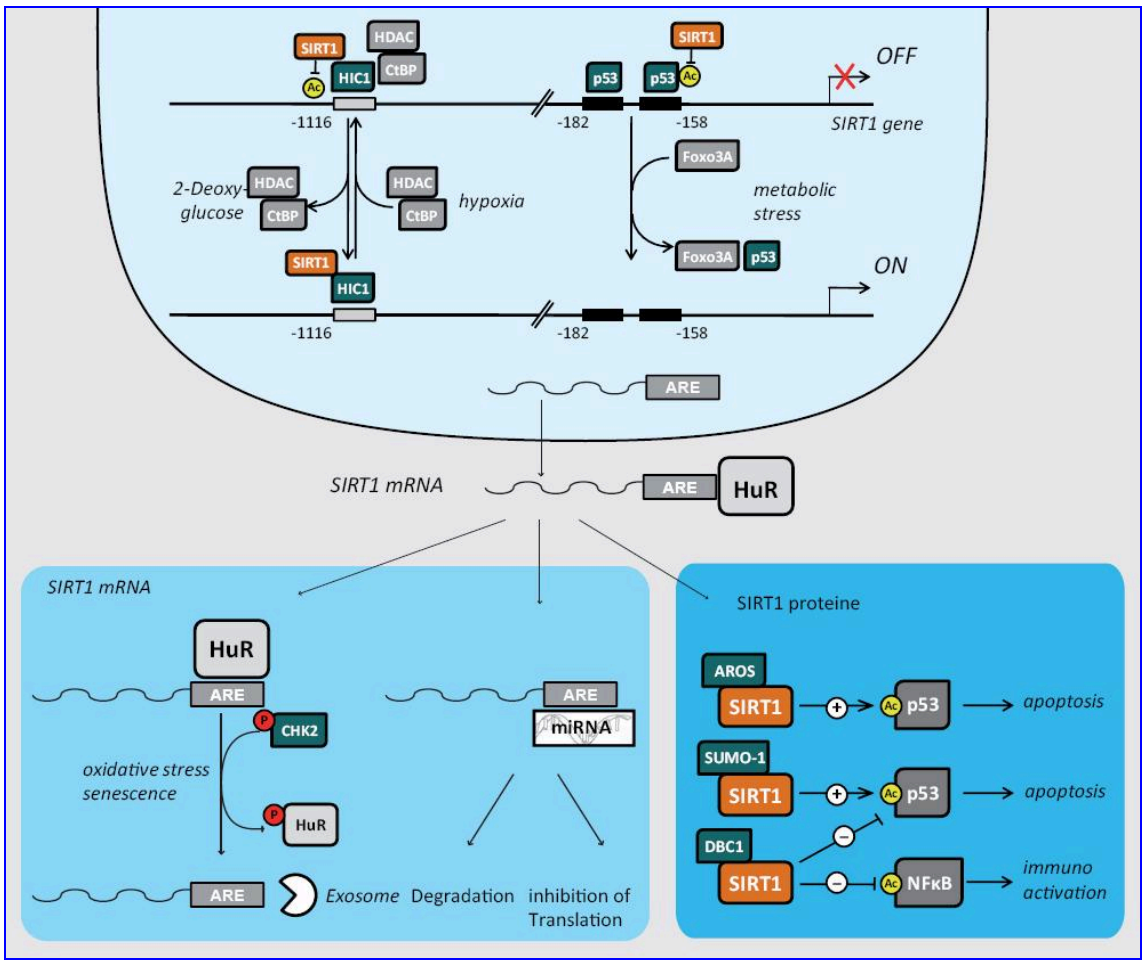

Figure 8: complex regulation of SIRT1 activity

The promoter of SIRT1 is regulated by various transcription factors including SIRT1 itsself, HIC1, p53 and FOXO3A. Additionally, SIRT1 mRNA is regulated by the RNA binding protein HuR and diverse microRNAs. Finally, SIRT protein activity is regulated positively and negatively by interacting proteins such as AROS and DBC1 or posttranslational modifications like SUMO.

3.4 BIM

Another crucial factor in the regulation of apoptosis and consequently in the survival of tumour cells is the Bcl-2 protein family member BIM (B cell lymphoma 2 interacting mediator of cell death). Defective apoptosis not only promotes tumourigenesis, but also can confound chemotherapeutic response. The tumour suppressor BIM has been shown to determine cancer cell sensitivity in vivo.

BIM contains a Bcl-2 homology domain 3 (BH3) characteristic for the BH3 only proteins. Like the other members of the Bcl-2 family it forms hetero- and homodimers to act as an apoptotic regulator. BIM appears to act as a 'death ligand' which can only neutralize certain members of the pro-survival Bcl-2 sub-family (O'Connor *et al.*, 1998).

3.4.1 BIM function

BIM acts as an apoptotic activator dimerised with other Bcl-2 members (e.g. Bcl2, Bcl2L1/Bcl-$_{XL}$, and Mcl1). In healthy unstressed cells BIM binds to microtubules via the LC8 dynein light chain. Upon stress signalling BIM participates in the permeabilisation of the outer mitochondrial membrane.

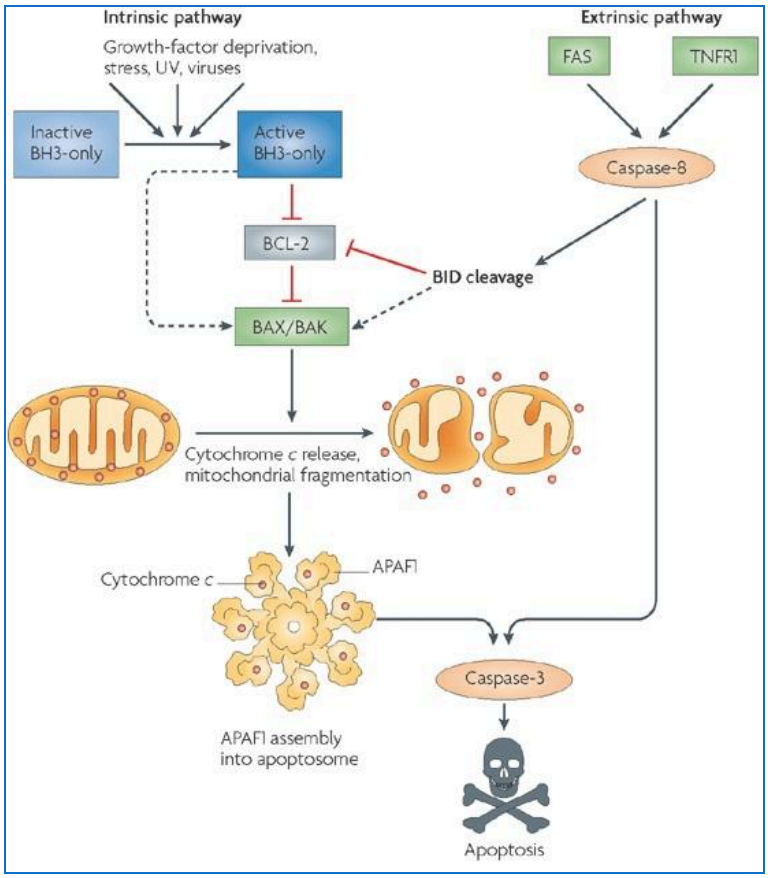

Figure 9 apoptosis induction
Apoptosis can be induced by cell surface receptors, (Fas, or TNFR1) (extrinsic pathway), or by various stress/genotoxic agents (intrinsic pathway) resulting in the activation of caspases (Youle and Strasser, 2008).

Hereby, pro-apoptotic content is released into the cytoplasm where it activates the downstream apoptotic machinery (see Figure 9) (Hendrickson *et al.*, 2008; Youle and Strasser, 2008). The intrinsic apoptotic pathway starts with BH3-only protein induction or their posttranslational activation resulting in an inhibition of Bcl-2 family members. In turn, this activates Bax and Bak activation which promotes apoptosis. Interestingly, some BH3-only proteins (e.g. BIM, Puma) directly activate Bax and/or Bak. Once activated Bax and Bak promote cytochrome c release, which leads to the assembly of Apaf1 into the apoptosome. Hereby, caspase 9 becomes activated to cleave and thereby activate downstream executioner caspases like caspase 3. Caspases cleave multiple substrates, activate DNases and orchestrate the programmed cell death. The extrinsic apoptotic pathway can bypass the

mitochondrial step and activate caspase 8 directly, which also leads to caspase 3 activation (Youle and Strasser, 2008).

Several drugs, especially chemotherapeutics, target apoptotic pathways or trigger apoptosis indirectly to kill cancer cells efficiently. In these cells, growth factor signalling like the epidermal growth factor (EGF) pathway is often deregulated. Oncogenic kinase mutations affecting the MAPK pathway and the PI3K/Akt pathway can result in a cancerous behaviour of cells. Upon induction of this pathway the downstream activation of ERK1/2 leads to a phosphorylation of numerous cytoplasmic and nuclear substrates involved in cell survival and proliferation. ERK-mediated phosphorylation of c-myc, ELK1, and other transcription factors favours the expression of growth promoting genes (Wang *et al.*, 2007). ERK-mediated phosphorylation of Bcl-2 proteins can inhibit apoptosis.

EGFR inhibitors such as the kinase inhibitor Gefitinib trigger apoptosis by inhibition of the downstream Ras-ERK pathway. Consequently, BIM is up-regulated, targeting all its pro-survival relatives. The apoptotic affect can be further enhanced by the addition of the BH3 mimetic ABT-737, which also targets Bcl-2 and Bcl-$_{XL}$, enabling Bax to permeabilise the mitochondrial membrane. The potential benefit of simultaneously inhibiting oncogenic kinases and inhibiting Bcl-2 action is most promising in the therapy of solid tumours (Hendrickson *et al.*, 2008). The inhibition of BCR-ABL by Imatinib has similar effects (see Figure 10). The activity of BIM is predominantly regulated by its protein level. As mentioned above, Ras signalling inhibits BIM expression (Cragg *et al.*, 2007).

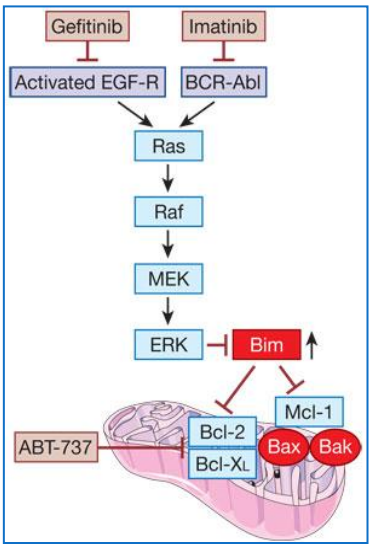

Figure 10 Targeting of the BH3 only protein BIM by anticancer drugs (Cragg *et al.*, 2007).

Manuscript Overview 1

Acetylation of non-histone proteins modulates cellular signalling at multiple levels.

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This review focuses on the posttranslational acetylation of non-histone proteins, which determines vital regulatory processes. A steadily growing number of identified acetylated non-histone proteins demonstrate that reversible lysine acetylation affects mRNA stability, and the localisation, interaction, degradation and function of proteins. Interestingly, most non-histone proteins targeted by acetylation are relevant for tumourigenesis, cancer cell proliferation and immune functions. Here, we summarise the complex effects of dynamic alterations in the cellular acetylome on physiologically relevant pathways.

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Review

Acetylation of non-histone proteins modulates cellular signalling at multiple levels

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ABSTRACT

This review focuses on the posttranslational acetylation of non-histone proteins, which determines vital regulatory processes. The recruitment of histone acetyltransferases and histone deacetylases to the transcriptional machinery is a key element in the dynamic regulation of genes controlling cellular proliferation and differentiation. A steadily growing number of identified acetylated non-histone proteins demonstrate that reversible lysine acetylation affects mRNA stability, and the localisation, interaction, degradation and function of proteins. Interestingly, most non-histone proteins targeted by acetylation are relevant for tumourigenesis, cancer cell proliferation and immune functions. Therefore inhibitors of histone deacetylases are considered as candidate drugs for cancer therapy. Histone deacetylase inhibitors alter histone acetylation and chromatin structure, which modulates gene expression, as well as promoting the acetylation of non-histone proteins. Here, we summarise the complex effects of dynamic alterations in the cellular acetylome on physiologically relevant pathways.

Contents

Abbreviations: BMAL1, brain and muscle ARNT-like 1; CBP, CREB-binding protein; CLOCK, circadian locomoter output cycles protein kaput; ErbB2, erythroblastic leukaemia viral oncogene homolog 2; FOXO, forkhead box; HIC1, hypermethylated in cancer 1; HIF-1α, hypoxia-inducible factor 1α; HNF, hepatocyte nuclear factor; MEF2A, myocyte enhancer factor 2A; NF-κB, nuclear factor kappa B; PARP, poly(ADP-ribose) polymerase; PCAF, p300/CBP associated factor; pVHL, von Hippel–Lindau protein; Rpd3, reduced potassium dependency 3; SIRT, silent mating type information regulation 2 homolog; SRC1α, v-src sarcoma viral oncogene homolog; SRY, sex determining region Y; STAC, sirtuin activating compounds; STAT, signal transducers and activators of transcription; SUMO, small ubiquitin related modifier; Tip60, HIV Tat interacting protein 60 kDa; Ubc8, ubiquitin conjugating enzyme 8; YY1, Yin and Yang protein 1.

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1. Histone acetylation

Eukaryotic DNA, histones and histone-like proteins are assembled into nucleosomes. Histones, the main protein component of chromatin, not merely play a role in packaging DNA. The tails and the globular domains of 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. Histone hyperacetylation provides a more open chromatin structure correlating with gene transcription (Durrin et al., 1991). This is due to reduced ionic interactions of the positively charged histone tails with the negatively charged DNA backbone and reduced internucleosomal interactions. Additionally, modified histones generate specific binding sites for protein interactions, for example with transcription factors and histone acetyltransferases (HATs). Binding of HATs to acetylated lysine moieties via bromodomains can provide a feed-forward mechanism for acetylation. Still, the recruitment of additional factors is required to initiate transcription (Kuo et al., 1998). Via such mechanisms, various proteins with gene activating and gene silencing activity can be recruited to individual promoters.

The term “histone code” has been coined for the combinatorial diversity of posttranslational histone modifications (Fischle et al., 2003; Strahl and Allis, 2000). However, in recent discussions the influence of histone acetylation on transcriptional activation – including the “histone code”-theory – loses weight in explaining transcriptional activation. From this point of view, a single acetylation event may either directly govern processes like transcription, replication and DNA repair mechanisms, or might alternatively be integrated within the overall chromatin context. Because histone acetylation itself directly modulates the chromatin structure, it is hard to discriminate between acetylation as a “code” signal for transcriptional initiation and direct effects towards chromatin structure (Turner, 2007). HDACi treatment induces global protein acetylation, but changes the expression of only 2–10% of human genes significantly, with almost equal numbers of genes upregulated and downregulated (Daly and Shirazi-Beechey, 2006; Gray et al., 2004). Promoters can be both, induced (e.g., p21) and suppressed (e.g., SRC1α) following HDACi treatment, although ChIP analysis revealed a similar histone acetylation pattern (Ellis et al., 2008). Hence, histone hyperacetylation is not a reliable predictor of gene activity. Moreover, histone deacetylase inhibitor (HDACi)-mediated histone hyperacetylation has been reported to affect non-transcribed peripheral chromatin stronger than central euchromatin, which in some cases even responded with histone hypoacetylation (Rada-Iglesias et al., 2007).

2. Histone deacetylases (HDACs) and histone deacetylase inhibitors

2.1. HDACs and SIRTs

HATs catalyse the transfer of an acetyl group from acetyl-CoA to the ε-NH_2 group of the amino acid side chain of lysine residues. Acetylation of lysine residues at the ε-NH_2 is highly dynamic. The first deacetylase activity was identified back in the 1960s (Inoue and Fujimoto, 1969), soon after the discovery of histone acetylation and its potential role in the regulation of gene expression (Allfrey et al., 1964; Phillips, 1963). Since histones were the first identified targets of deacetylases, these enzymes were termed histone deacetylases. The large and continuously growing number of non-histone targets undoubtedly demonstrates that histones are only some of the many substrates of HATs and HDACs. Therefore, referring to HDACs and HATs as lysine deacetylases/acetylases (KDACs/KATs) or protein deacetylases/acetylases (PDACs/PATs) appears to be more precise. However, throughout this review we will continue to use the traditional term HDAC.

HDACs can be grouped into two distinct families. The “classical family” of zinc-dependent HDACs are structurally related to the yeast Hda1/Rpd3 proteins (de Ruijter et al., 2003), and the second one consists of the NAD^+-dependent yeast Sir2 homologues (Haigis and Guarente, 2006). Histone deacetylases are further grouped into four classes according to their phylogenetic conservation (Gregoretti et al., 2004). Class I, II and IV HDACs are the classical HDAC family. Homologues of yeast Sir2, SIRTs, represent the class III deacetylases, also commonly termed sirtuins in mammals. Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are proteins orthologous to the yeast Rpd3 enzyme. The yeast Hda1 homologues of mammalian class II HDACs can be subdivided into class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10). HDAC11 shares equal sequence conservation with

Rpd3 and Hda1, and is therefore grouped as a currently solitary member into class IV. Class III HDACs represent a phylogenetically conserved group with seven members in man (SIRT1 to 7) (Gao et al., 2002; Yang and Seto, 2008).

Class I HDACs consist of 350–500 amino acid residues (≈50 kDa) and are ubiquitously expressed. Besides their deacetylase (DAC) domain, spanning approximately 300 residues, they carry a small C-terminal part that is often subject to posttranslational modifications, like phosphorylation, ubiquitination and sumoylation (de Ruijter et al., 2003; Krämer et al., 2001; Yang and Seto, 2008). Class II HDACs are considerably larger, consisting of about 1000 residues. The class IIa deacetylases possess an Hda1-like catalytic domain in the C-terminus and additional N-terminal extensions, which harbour multiple domains and regulatory sites. The class IIb deacetylases HDAC6 and HDAC10 have an N-terminal catalytic domain (Yang and Seto, 2008). In contrast to other deacetylases, HDAC6 further contains a C-terminally located second functional DAC domain. Most classical histone deacetylases form large high molecular weight complexes of up to 1–2 MDa (Yang and Seto, 2008). These complexes contain multiple corepressors harbouring chromatin remodelling activities and the ability to bind sequence specific transcription factors (Grozinger and Schreiber, 2002). Whilst classical HDACs can be found in the cytosol and in the nucleus, no localisation to mitochondria has been described so far (de Ruijter et al., 2003; Yang and Gregoire, 2005; Yang and Seto, 2008).

Sirtuins make use of a different mechanism of catalysis. Instead of using an electrophilic Zn^{2+} ion to directly hydrolyse the amide bond with water, they transfer the acetyl group to the cosubstrate NAD^+ yielding two products, nicotinamide and 2′-*O*-acetyl-ADP-ribose (Denu, 2005). This reaction depends on the NAD^+/NADH ratio. Metabolism thus may provide a mechanism to regulate SIRTs. Moreover, some SIRTs catalyse ADP-ribosylation (Frye, 1999; Michan and Sinclair, 2007). The seven human SIRT paralogs are ubiquitously expressed (Michishita et al., 2005). SIRT1, 6 and 7 are nuclear proteins with differential subnuclear distribution. SIRT6 is linked with heterochromatic regions and SIRT7 found in nucleoli. Although SIRT1 is mainly found in the nucleoplasm, it neither occurs in heterochromatin nor in nucleoli. SIRT1 also carries out some cytoplasmic functions (Jin et al., 2007), SIRT2 localises to the cytoplasm, and SIRT3, 4 and 5 are mitochondrial proteins (Michishita et al., 2005).

Due to its interactions with several transcription factors, SIRT1 is involved in multiple regulatory processes. These typically are apoptotic and stress responses, linked to a variety of diseases including cancer (Michan and Sinclair, 2007). SIRT1 furthermore affects neuroprotection, tumour suppression, inflammation, and longevity (Anekonda, 2006; Baur et al., 2006; Labinskyy et al., 2006; Saunders and Verdin, 2007). SIRT3, 4, and 5 are regulators of mitochondrial processes, metabolism, and longevity (Guarente, 2008).

2.2. HDACi

Of note, the discovery of HDAC inhibitors (HDACi) preceded the discovery of HDACs. Sodium butyrate was the first compound identified to induce histone acetylation (Riggs et al., 1977). Later, Trichostatin A (TSA), a fungal antibiotic (Yoshida et al., 1990), valproic acid (VPA), already used in treatment of epileptic diseases (Göttlicher et al., 2001), and several other compounds were identified as HDACi. These agents fall into diverse structural classes: Hydroxamic acid derivatives, carboxylates, benzamides, electrophilic ketones, cyclic peptides and a few substances not assignable to these groups. A pharmacophoric model for the actions of HDACi has been suggested (Miller et al., 2003).

Since epigenetic changes critically contribute to cancer onset and progression, HDACi were soon considered as promising anticancer drugs (Bolden et al., 2006; Krämer et al., 2001; Warrell et al., 1998; Yoo and Jones, 2006). Indeed, at the cellular level HDACi can induce differentiation, cell cycle arrest, senescence, apoptosis, reactive oxygen species (ROS)-production and mitotic cell death. In vivo, HDACi were found to reduce tumour invasiveness, angiogenesis and metastasis. An additional very promising effect of HDACi for cancer therapy is their selective toxicity against tumour cells compared to normal cells (Bolden et al., 2006; Minucci and Pelicci, 2006; Xu et al., 2007). However, inhibiting deacetylation not only affects chromatin structure. HDACi equally promote the acetylation of non-histone proteins (Caron et al., 2005; Glozak et al., 2005), which can determine the interactions, localisation, and stability of these proteins. Whilst there are attempts to reveal aberrant gene expression patterns in tumours, less information is available for differences in the acetylation patterns between normal and cancer cells and the effect of HDACi. The fact that HATs and HDACs are deregulated in various cancers (Bolden et al., 2006; Das and Kundu, 2005) gives a clear hint that anomalous acetylation takes place, which might be corrected by therapeutic HDACi treatment.

Isoform-specific inhibition of HDACs remains a challenging task (Khan et al., 2008). TSA inhibits all HDACs roughly to the same extent. Other pan-HDACi are suberoylanilide hydroxamic acid (SAHA), LAQ-824 and LBH-589. A class I-selective inhibitor is VPA (Göttlicher et al., 2001), whilst MS-275 and depsipeptide are selective towards only a subset of class I HDACs (Khan et al., 2008). HDAC6-specific HDACi also exist, e.g., tubacin (Haggarty et al., 2003). Additional isoform-selective HDACi are available or under development. Such compounds will not only provide interesting new tools for molecular biology, but might also represent new candidate drugs for cancer treatment. It is, however, controversially discussed whether strictly isoform-specific HDACi would have therapeutic benefits (Karagiannis and El-Osta, 2007).

2.3. SIRT inhibitors (SIRTi) and sirtuin activating compounds (STACs)

Because of the need for NAD^+ as a cosubstrate carba-NAD^+ and nicotinamide are non-competitive inhibitors of SIRT proteins (Denu, 2005; Grubisha et al., 2005; Schmidt et al., 2004). However, the large number of other NAD^+-dependent enzymes could evoke side effects of such agents (Belenky et al., 2007; Grubisha et al., 2005). By chemical genetic screening, compound libraries of small organic molecules were analysed for inhibition of Sir2 in yeast (Bedalov et al., 2001; Grozinger et al., 2001). Sirtinol and splitomicin were found to be efficient SIRTi in eukaryotic cells (Araki et al., 2004; Bedalov et al., 2001; Fulco et al., 2003; Yeung et al., 2004). Apart from inhibitors, activators of SIRTs (STACs) have also been identified. The best characterised and most potent one is resveratrol. This plant polyphenol increases lifespan in several animal models, an effect also observed upon overexpression of Sir2 or its orthologs (Howitz et al., 2003; Wood et al., 2004). In vivo, artificial activation of sirtuins equally mimics caloric restriction, which is linked to positive effects on physiological condition and longevity (Baur et al., 2006).

3. Histone acetyltransferases (HATs) and histone acetyltransferase inhibitors (HATi)

3.1. HATs

Since the discovery of the first HAT enzyme, the yeast Hat1 (Kleff et al., 1995), a lot of attention has been drawn to these enzymes.

HATs are evolutionarily conserved from yeast to man and form multiple subunit complexes (Kimura et al., 2005). Unlike HDACs, HATs are more diverse in structure and function (Yang, 2004). In mammals, over 30 HATs display distinct substrate specificities for histones and non-histone proteins. HATs do not acetylate lysine moieties randomly. Crystal structure analysis identified a potential recognition motif, in which glycine is followed by an acetylatable lysine (GKxxP) (Bannister et al., 2000; Rojas et al., 1999). However, this motif has serious limitations in predicting non-histone protein acetylation. For example, a proteomic survey identified different sets of preferentially acetylated amino acid stretches in mammalian proteins. Nuclear non-histone proteins frequently possess an asparagine in the −1 position and a histidine in the +1 position, whilst histones show a tendency for a lysine or acetylated lysine in ±4 positions. The subset of acetylated mitochondrial proteins additionally has histidine or tyrosine at the +1 position. These variations could be due to the localisation of target-specific HATs (Kim et al., 2006), and cytoplasmic proteins do not demonstrate a strictly conserved acetylation consensus motif at all.

In contrast to HDACs, the HAT classification is less clear. They are grouped into two general classes: A- and B-type HATs, of which A-type HATs mainly carry out transcription-related acetylation. These are further grouped into five families: GNAT, MYST, p300/CBP, basal/general transcription factors and nuclear receptor cofactors (Roth et al., 2001). Nuclear A-type HATs are chiefly found in conserved, cooperatively acting high-molecular-weight complexes (Grant and Berger, 1999). The cytoplasmic B-type HATs acetylate de novo synthesised free histones, promoting their nuclear localisation and deposition onto newly synthesised DNA (Allis et al., 1985; Ruiz-Carrillo et al., 1975). Many HATs show a distinct pattern of substrate specificity, even towards histones, depending on the subunit composition of HAT complexes and the specific recruitment to the target sites of acetylation (Waterborg, 2002). HAT complexes also affect chromosome decondensation, DNA-damage repair and the acetylation of non-histone targets (Lee and Workman, 2007). Many HATs possess an evolutionarily conserved protein module specifically recognising acetyl-lysines—the bromodomain which directs chromatin associated proteins to acetylated histones (Dhalluin et al., 1999; Lee and Workman, 2007).

The most intensively studied HATs are CBP and p300. Both contain a bromodomain and are often found within the same complexes. Apparently, p300 seems to have the broadest substrate acceptance for histones and non-histone proteins (Kimura et al., 2005). The GNAT family (Gcn5 related *N*-acetyltransferases) includes Gcn5 and PCAF (p300/CBP associated factor), which are important for transcriptional initiation. Elp3 is involved in transcriptional elongation and Hat1 in histone deposition and telomeric silencing (Grant, 2001). Members of the MYST family serve as catalytic subunits in Tip60, HBO1 and MOZ/MORF complexes (Lee and Workman, 2007). There are additional enzymes that carry an acetyl transferase activity. Because of their lower sequence similarity they cannot be grouped into any of these families. Numerous HATs furthermore undergo functionally relevant auto-acetylation (Thompson et al., 2004).

3.2. HATi

Comparatively little attention has been drawn to inhibitors of acetyltransferases (HATi), as HATs are rarely considered as drug targets. A reason for this could be the promising use of HDACi in various diseases like leukaemia and other haematological disorders. Currently, only a small number of HATi is known. Synthetic peptide-CoA conjugates showed HAT inhibitor potential (Lys-CoA for p300 and H3-CoA-20 for PCAF) (Lau et al., 2000), but they lack cell permeability. The cell permeable polyphenol curcumin was shown to specifically inhibit p300 activity in the micromolar range. Other naturally occurring compounds like anacardic acid and garcinol are non-specific inhibitors of p300/CBP and PCAF (Balasubramanyam et al., 2003). Furthermore, the quinoline derivative MC1626 (Smith et al., 2007) and isothiazolones (Stimson et al., 2005) act as inhibitors of Gcn5, PCAF and p300, respectively.

4. Non-histone targets of HDACs and HATs—the acetylome

Lysine side chains can be acetylated, methylated (mono-, di- or trimethylation), ubiquitinated (mono- or polyubiquitination), sumoylated and ADP-ribosylated (Merrick and Duraisingh, 2007). These rivalling and reversible posttranslational modifications are regulated by a complex interplay of different enzymes. Reversible acetylation of lysine ε-amino groups crucially modulates protein function und cellular networks (Fig. 1). In eukaryotic cells, acetylation is among the most common covalent modifications and ranks similar to the important master switch phosphorylation (Kouzarides, 2000). Acetylation apparently shows a broader substrate spectrum than phosphorylation, and far fewer acetylases than kinases have been described. Intriguingly, no acetylation cascades have been identified to date.

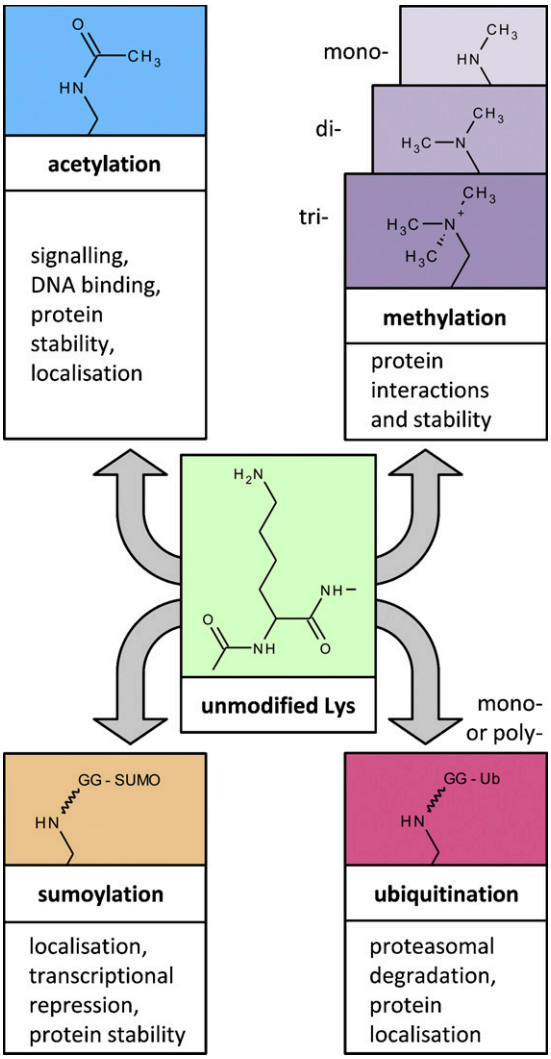

Fig. 1. Lysine modifications. Lysine residues are subject to posttranslational modifications with varying functional consequences. Switching between modifications allows to alter protein function.

Hundreds of proteins are known to be modified by acetylation. Surely, there exist many more acetylated proteins than those identified until now, and acetylation can change protein characteristics and functions enormously. In general, acetylation changes the electrostatic state of lysine from positive to neutral and increases the size of the amino acid side chain. Acetylation can equally affect enzymatic activities, as acetylated lysines exhibit slightly different preferences for secondary structures than unacetylated lysines. Different covalent modifications can furthermore compete for the same lysines important for signalling or the subcellular localisation of a protein (Kim et al., 2006). Additionally, the acetylation of lysines can create new docking sites for protein–protein interactions, for example via recognition by the bromodomain. Hence, acetylation can determine protein function at multiple levels.

Protein acetylation patterns appear to be very organ specific (Iwabata et al., 2005). To identify acetylated proteins, in several studies lysine-acetylated peptides and proteins were immunopurified, and investigated by 2D gel electrophoresis and HPLC/MS analysis (Iwabata et al., 2005; Kim et al., 2006; Xie et al., 2007). More than 60 transcription factors and many other proteins involved in DNA repair and replication, metabolism, cytoskeletal dynamics, apoptosis, nuclear import, protein folding and cellular signalling were found to be acetylated (Cohen and Yao, 2004; Kouzarides, 2000; Sterner and Berger, 2000; Yang and Gregoire, 2007). Since 277 lysine acetylation sites were identified in 133 mitochondrial proteins, acetylation is also a very abundant posttranslational modification in mitochondria (Kim et al., 2006). In sum, these modified proteins are called the acetylome (see Table 1).

5. Acetylation regulates multiple processes from gene expression to protein activity

Acetylation can affect signalling pathways and thereby alter cell fate and function. mRNA splicing, mRNA transport, mRNA integrity, translation, protein activity, protein localisation, protein stability and interactions are regulated by acetylation. Hence, acetylation can interfere with every step of regulatory processes from signalling to transcription to protein degradation.

5.1. Signalling and transcription

The process of gene expression has been closely linked to acetylation. In addition to histone acetylation, the acetylation of non-histone proteins is important. For example, acetylation of transcription factors within their DNA-binding domain has been described. In the cases of E2F1, YY1 and many more, this results in altered DNA binding affinity (Lamonica et al., 2006; Martinez-Balbas et al., 2000). However, acetylation can equally inhibit the DNA binding of transcription factors, providing an explanation why HDACi do not generally increase gene expression. Not all proteins are regulated by acetylation of the protein itself. For example, HDAC6-mediated Hsp90 deacetylation enables activation of the glucocorticoid receptor by ligands (Kovacs et al., 2005). The following examples demonstrate the complex consequences of acetylation on cellular signalling and their effects on transcriptional activity.

5.1.1. STAT proteins

Mammalian STATs are a family of transcription factors consisting of seven members. These are STAT1, STAT2, STAT3, STAT4,

Table 1
Selected acetylated proteins

Biological implication	Proteins affected by acetylation	
Protein stability	Acetylation increases stability p53, p73, Smad7, c-Myc, Runx3, AR, H2A.z, E2F1, NF-E4, ER81, SREBP1a, HNF6, BACE1	Acetylation decreases stability GATA1, HIF-1α, pRb, SV40 T-Ag
DNA binding	Increased DNA binding p53, SRY, STAT3, GATA transcription factors, E2F1, p50 (NFκB), Erα, p65 (NFκB), c-Myb, MyoD, HNF-4, AML1, BETA2, NF-E2, KLF13, TAL1/SCL, TAF(I)68, AP endonuclease	Decreased DNA binding YY1, HMG-A1, HMG-N2, p65 (NFκB), DEK, KLF13, Fen-1
Gene expression	Transcriptional activation p53, HMG-A1, STAT3, AR, ERα (basal), GATA transcription factors, EKLF, MyoD, E2F1, p65(NFκB), GR, p73, PGC1α, MEF2D, GCMa, PLAG1, PLAGL2, Bcl-6, β-Catenin, KLF5, Sp1, BETA2, Cart1, RIP140, TAF(I)68	Transcriptional inactivation Erα (ligand-bound), HIF-1α, STAT1, FOXO1, FOXO4, RIP140
Protein interactions	Enhanced STAT3, AR, EKLF, Importin A, STAT1, TFIIB, α-Tubulin, actin, cortactin	Decreased p65(RelA), Ku70, Hsp90
Localisation	Ac → nucleus PCAF, SRY, CtBP2, POP-1, HNF-4, PCNA Sub-nuclear WRN, PCNA	Ac → cytosol c-Abl, p300, PAP
mRNA stability	Increased p21, Brm	Decreased Tyrosinhydrolase (Th), eNOS
Enzymatic activity	Enhanced p300, ATM	Decreased PTEN, HDAC1, Mdm2, ACS, Neil2, Polβ
Mitochondrial proteins	ACS (Ac-CoA-Synthetase), Sod1/2, Profilin I, Thioredoxin; multiple components of metabolic and oxidative phosphorylation machinery	
Viral proteins	E1A, S-HDAg, L-HDAg, HIV Tat, SV40 T-Ag	

Selected non-histone proteins and functional consequences of their acetylation (Arányi et al., 2007; Cohen and Yao, 2004; Das and Kundu, 2005; Dokmanovic et al., 2007; Glozak et al., 2005; Ito et al., 2007; Rössig et al., 2002; Sadoul et al., 2008; Xu et al., 2007; Yamamichi et al., 2005; Yang and Gregoire, 2007; Zhang and Dent, 2005).

STAT5A, STAT5B and STAT6. Specific cytokines and growth factors activate JAK kinases. These phosphorylate STATs, which induces their translocation into the nucleus. STATs are known to activate genes containing GAS or ISRE response elements in their promoters, thereby modulating biological processes like cell proliferation, survival, apoptosis and differentiation. STAT-mediated effects are tightly regulated by negative feedback loops. Only tyrosine dephosphorylation inactivates STATs directly (Mertens and Darnell, 2007).

STAT6 was the first STAT protein shown to undergo acetylation and its acetylation correlates with the transcription of reticulocyte-type 15-lipoxygenase-1 (Shankaranarayanan et al., 2001). STAT1 can be acetylated by CBP within its DNA binding domain (Krämer et al., 2006). Acetylation of STAT1 might negatively regulate its functions, as overexpression of HDAC1, HDAC2, or HDAC3 enhances STAT1-dependent gene expression upon cytokine stimulation, and inhibition of HDACs with HDACi or specific siRNAs blocks the expression of IFN-responsive genes (Chang et al., 2004; Klampfer et al., 2004; Nusinzon and Horvath, 2003). Acetylated STAT1 furthermore binds to the NF-κB subunit p65 which decreases p65 DNA binding, nuclear localisation and expression of anti-apoptotic NF-κB target genes in transformed cells. Via this mechanism, acetylation of STAT1 K^{410} and K^{413} regulates NF-κB activity and thus ultimately apoptosis (Krämer et al., 2006) (Fig. 2B). Acetylated STAT2 was reported to act as an adaptor for STAT1 and the conformation of the STAT1/Ac-STAT2 heterodimer might be critical for binding to the IFNα receptor 2 or the interferon regulatory factor IRF9 (Tang et al., 2007). This finding is, however, hard to reconcile with several studies clearly showing that the STAT1/STAT2 dependent activation of genes is strongly suppressed upon HDAC inhibition (Chang et al., 2004; Klampfer et al., 2004; Nusinzon and Horvath, 2003). The cytokine-dependent acetylation of STAT3 by p300/CBP may facilitate STAT3 dimerisation, resulting in DNA binding and transcriptional activation of STAT3 target genes (Yuan et al., 2005) involved in cell growth and cell survival, like cyclin D1, bcl-x_L, and c-myc (Wang et al., 2005; Yuan et al., 2005) (Fig. 2C). However, such a finding contrasts the well-established anti-proliferative functions of HDACi, and the mechanistic details and physiological relevance of STAT3 acetylation are discussed controversially (O'Shea et al., 2005).

5.1.2. NF-κB

The inducible transcription factor NF-κB plays a central role in immune responses, inflammation, cell survival, differentiation and proliferation. The NF-κB/Rel family consists of p50, p52, p65 (RelA), c-Rel and RelB, which form homo- or heterodimers (Xiao, 2004). The p50/p65 heterodimer is the most frequently found combination in mammals. Inactive NF-κB complexes are retained in the cytoplasm by the IκB inhibitor. The activation of the IκB kinase (IKK) results in IκB phosphorylation triggering its ubiquitination and proteasomal degradation. Free NF-κB translocates to the nucleus where it binds to target sequences. This promotes or inhibits transcription through coactivator or corepressor recruitment (Hayden and Ghosh, 2008).

Posttranslational modifications of NF-κB dimers have been shown to alter their interactions with co-activators. Phosphorylated p65 preferentially interacts with p300/CBP, resulting in p65 acetylation at multiple sites. Acetylation of K^{221} and K^{310} is associated with an increased transcription of NF-κB target genes (Chen and Greene, 2004), and is required for the full activity of p65 (Chen et al., 2002). This is also supported by the observation that SIRT1 driven deacetylation of p65 K^{310} inhibits transcription of NF-κB target genes (Yeung et al., 2004). Likewise, HDAC1 and HDAC3 deacetylate p65 at either K^{221} or K^{310}, resulting in the inhibition of NF-κB. The p300-mediated acetylation of K^{314} and K^{315} in p65 had no obvious effect on NF-κB DNA binding or localisation. However, microarray analysis identified a specific set of genes differently regulated by TNFα treatment when comparing wild type and K^{314} and K^{315} mutant p65 (Buerki et al., 2008). Additionally, K^{122} and K^{123} acetylation reduces p65 DNA binding affinity accompanied with increased IκB interaction and nuclear export (Kiernan et al., 2003). Site-specific p300-mediated acetylation of p65 thus regulates the specificity of NF-κB dependent gene expression (Greene and Chen, 2004; Kiernan et al., 2003) (Fig. 2A). The situation appears less complicated for p50. Acetylation of p50 (K^{431}, K^{440} and K^{441}) promotes higher DNA binding affinity towards NF-κB target sequences correlating with increased p300 recruitment and transcriptional activation (Deng and Wu, 2003; Deng et al., 2003).

NF-κB is deregulated in a large number of diseases. HDACi and SIRTi application should result in NF-κB hyperacetylation and modulation of NF-κB target gene expression. Indeed, HDACi have been shown to repress NF-κB signalling and expression of several NF-κB target genes (Huang et al., 1997; Inan et al., 2000; Krämer et al., 2001). Others though showed that HDACi enhanced NF-κB-dependent gene expression in the presence of TNFα (Adam et al., 2003; Ashburner et al., 2001; Quivy et al., 2002; Vanden Berghe et al., 1999), or even that a HATi induced the repression of NF-κB target genes (Sung et al., 2008). A possible caveat to assess the in vivo relevance of p65 acetylation is that this modification could only be detected upon overexpression of a HAT protein in certain studies (Chen and Greene, 2003). Hence, there is debate on which lysine in NF-κB p65 is actually acetylated under physiological conditions.

5.1.3. p53

The tumour suppressor p53 is a key player in cellular signalling and stress responses. This transcription factor can both, positively or negatively regulate the expression of genes contributing to cell cycle arrest, senescence and apoptosis (Vousden and Lane, 2007). The phosphorylation and acetylation status of p53 has a major impact on its functions, with phosphorylation of p53 stimulating its acetylation (Sakaguchi et al., 1998). The p53 protein can be acetylated by distinct acetyltransferases at different lysines: K^{120}, K^{164}, K^{320}, K^{370}, K^{372}, K^{373}, K^{381}, K^{382} and K^{386} (Li et al., 2007; Tang et al., 2008). The resulting effects on p53 activity are still discussed controversially (Zhao et al., 2006). Especially for C-terminal acetylation of p53 there is dispute whether or not acetylation increases DNA binding (Espinosa and Emerson, 2001; Gu and Roeder, 1997; Sakaguchi et al., 1998). Lately, mutation at major acetylation sites (K^{120}, K^{164} and in the C terminus) was shown to result in a complete loss of p53 activity towards p21 transcription, whereas single site mutations were compensated by acetylation at the remaining functional residues (Tang et al., 2008). Acetylation of K^{120} mediated by Tip60 and hMOF seems to be important for the expression of genes favouring apoptosis after DNA damage. Here, the acetylated lysine contributes to coactivator recruitment (Sykes et al., 2006; Tang et al., 2006). Acetylation of K^{320} and polyubiquitination of p53 apparently stimulates transcription (Knights et al., 2006; Le Cam et al., 2006). Additionally, p53 acetylated at K^{382} recruits CBP via its bromodomain to further activate transcription, suggesting that p53 acetylation increases its DNA binding affinity followed by transcriptional activation of its target genes (Mujtaba et al., 2004).

As p53 acetylation might favour DNA binding and transcriptional activation (Fig. 2C), this mechanism has likewise been suggested for the transcription factors SRY (Thevenet et al., 2004), GATA factors (Boyes et al., 1998; Hayakawa et al., 2004; Yamagata et al., 2000), E2F1 (Martinez-Balbas et al., 2000; Marzio et al., 2000), MyoD (Sartorelli et al., 1999) and many others.

5.1.4. FOXO transcription factors

Acetylation has equally been found to inhibit gene expression. For example, acetylation can disrupt DNA binding and transcrip-

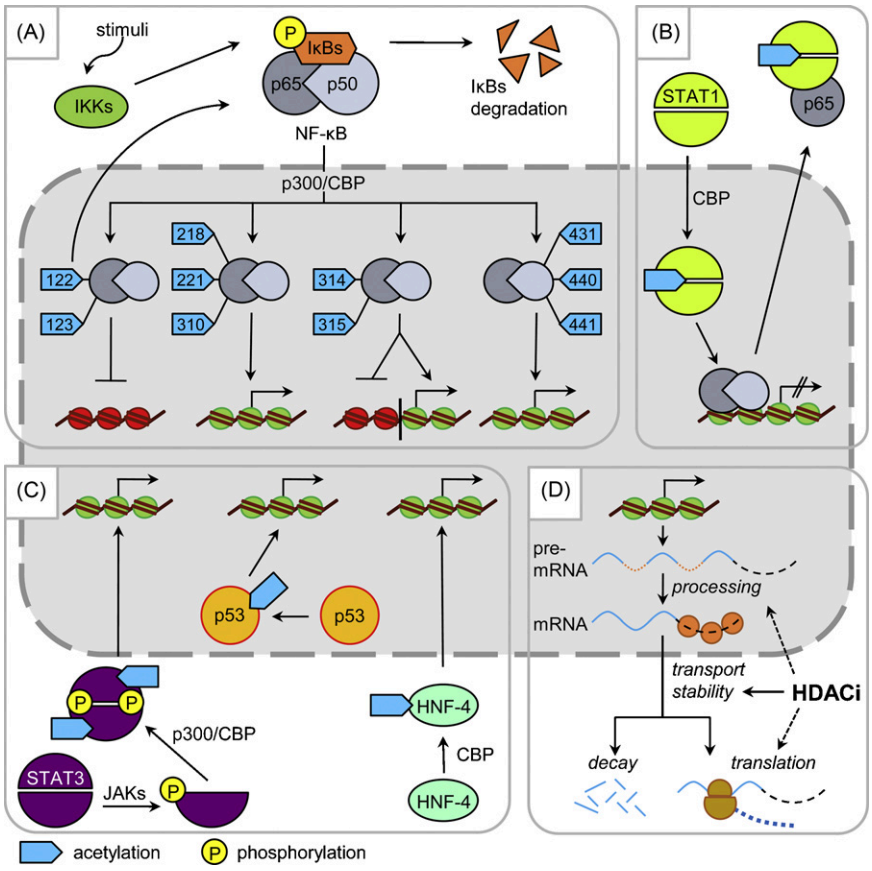

Fig. 2. Effects of acetylation on signalling, transcription and posttranscriptional events. (A) p300/CBP-mediated site-specific acetylation of p65 or p50 has a major impact on the transcriptional activity of NF-κB in vitro. Acetylation of p65 (K^{218}, K^{221} and K^{310}) or p50 (K^{431}, K^{440} and K^{441}) activates transcription. The acetylation of p65 on K^{122} and K^{123} inhibits its DNA binding, favours IκB binding and results in the cytoplasmic accumulation of NF-κB. Acetylation of p65 on K^{314} and K^{315} regulates a specific set of NF-κB target genes. (B) Acetylation of STAT1 induces its interaction with p65. The resulting complex dissociates from DNA, translocates into the cytoplasm and inhibits the transcriptional activity of NF-κB. (C) Acetylation of transcription factors can affect dimerisation (e.g., STAT3), DNA binding affinity (e.g., p53) or subcellular localisation (e.g., HNF-4) and thereby transcriptional activity. (D) HDACi alters the cellular acetylation state of various proteins including factors needed for pre-mRNA processing or translation. Additionally HDACi have been shown to alter mRNA stability.

tional activation by the FOXO transcription factors, as well as for YY1 (Yao et al., 2001), and the ligand-bound, activated ERα (Wang et al., 2001) (for additional proteins see Table 1).

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. Upon insulin/growth factor stimulation, the Akt kinase phosphorylates the FOXO proteins, resulting in nuclear export and inhibition of FOXO action (Carter and Brunet, 2007). Besides phosphorylation, acetylation of FOXO proteins functions as a second pathway of negative control (Calnan and Brunet, 2008). The p300/CBP- and PCAF-mediated acetylation diminishes the DNA binding ability of FOXO proteins, in turn reducing their activity (Calnan and Brunet, 2008). In response to oxidative stress SIRT1 mediates deacetylation of FOXO1. However, it is not that clear whether this deacetylation automatically leads to FOXO activation, as FOXO acetylation can have diverse effects towards different promoters (Daitoku et al., 2004; Fukuoka et al., 2003; van der Heide and Smidt, 2005). Nonetheless SIRT1-mediated FOXO deacetylation can promote cellular survival and increase lifespan (van der Horst et al., 2004). Under conditions of caloric restriction, higher NAD^+-levels could increase SIRT1 activity towards FOXO, and the resulting modulation of FOXO functions may contribute to Sir2 family mediated lifespan extension.

5.2. Posttranscriptional regulation

Posttranscriptional control is mainly mediated by cis-acting RNA elements located in the 5′- and 3′-untranslated regions of mRNAs (5′-UTRs and 3′-UTRs) that can be targeted by *trans*-acting RNA binding proteins (Grzybowska et al., 2001; Pesole et al., 2001). The control of mRNA stability is often under-rated as a mechanism in regulation of gene expression. In fact, the initiation of transcription is just one side of the medal. The other side includes pre-mRNA processing, mRNA transport, mRNA stability and finally translation. In the end, transcriptional activation not necessarily results in increased protein levels. It is estimated that about 50% of the changes in gene expression actually are alterations in mRNA stability rather than “direct” transcriptional control (Cheadle et al., 2005). Actions of HDACi towards such posttranscriptional control mechanisms have not been the focus of research so far (Fig. 2D).

5.2.1. Pre-mRNA processing

Acetylation has been suggested to have an impact on pre-mRNA processing, including RNA splicing as well as 5′- and 3′-processing. Interestingly, the heterogeneous ribonucleoprotein A1, which has a potential role in splicing by binding to unprocessed pre-mRNA, can be acetylated (Kim et al., 2006). Moreover, an impact of acetylation on proteins involved in RNA cleavage and polyadenylation has been described for CFIm25, a component required for 3′ RNA cleavage and polyadenylation, as well as poly-(A)-polymerase (PAP), a

polyadenylating enzyme involved in pre-mRNA 3′-end processing. Actually, acetylation decreased interactions between PAP and the CFIm complex, and PAP acetylation disrupts its binding to the importin-α/β complex, resulting in cytosolic accumulation (Shimazu et al., 2007).

5.2.2. Acetylation-mediated regulation of mRNA stability and translation

The endothelial nitric oxide synthase (eNOS) generates nitric oxide, a key second messenger in inflammatory diseases. Side effects of this free radical are cytotoxic effects through lipid, DNA and protein damage. The majority of the literature favours a protumourigenic role of eNOS, which is in contrast to its potential anti-tumourigenic functions (Ying and Hofseth, 2007). TSA evokes eNOS promoter activity and causes a constant transcript rate in nuclear run-on experiments. The HDACi-mediated reduction of the half-life of eNOS mRNA is nevertheless sufficient to decrease eNOS protein levels. Consequently, an HDACi-mediated decrease in eNOS levels interferes with endothelial cell function (Rössig et al., 2002). Furthermore, the HDACi TSA has been shown to decrease the mRNA stability of DNA methyltransferase-1 and -3B (Januchowski et al., 2007; Xiong et al., 2005), which results in a significant reduction of de novo DNA methylation. HDACi can additionally decrease the expression of estrogen receptor α (De los Santos et al., 2007) and tyrosine hydroxylase (Arányi et al., 2007) by modulation of mRNA stability. The mechanisms behind these effects on mRNA stability remain to be identified. Perhaps, dynamic protein acetylation affects mRNA turn-over via an RNase and/or mRNA stabilising factors, which usually bind to the 3′-UTR of mRNA (Arányi et al., 2007).

MicroRNAs are non-coding RNAs that regulate mRNA stability by RNA interference. The HDACi LAQ-824 rapidly alters the levels of 40% of >60 different assessed miRNA species. This finding also supports the idea that HDACi can modulate posttranscriptional processes (Scott et al., 2006). The functional consequence of altered miRNA expression upon HDACi treatment remains to be understood in detail.

Until now, no major impact of protein acetylation on translational control has been described. Remarkably though, two translation factors (EF1α and eIF-5A) can be targeted by acetylation and this may regulate translation (Kim et al., 2006).

5.3. Protein modification and stability

5.3.1. Acetylation switches

Various posttranslational modifications target lysine residues, raising the possibility of modification-based switches. The targeted replacement of one modification by another allows the functional adaptation of proteins. Alternative acetylation/ubiquitination determines protein stability, whereas rivalling acetylation/sumoylation likely affects protein activity, with sumoylation of transcription factors being often linked to transcriptional repression (Geiss-Friedlander and Melchior, 2007). Accordingly, acetylation switches at the ΨKxE SUMO consensus sites have been reported for the transcription factor Sp3 (Sapetschnig et al., 2002), the HIC1 tumour suppressor (Stankovic-Valentin et al., 2007) and the HAT p300 (Bouras et al., 2005). The phosphorylation-regulated sumoylation–acetylation switch (SAS) occurring on the transcription factor MEF2A has been well characterised (Fig. 3A). In MEF2A, ΨK^{403}xExxSP is a phosphorylation-dependent sumoylation motif (PDSM) where phosphorylation of the serine residue triggers sumoylation of the lysine residue. Ca^{2+}-dependent dephosphorylation at S^{408} by calcineurin causes loss of sumoylation, allowing acetylation, which inhibits postsynaptic differentiation (Shalizi et al., 2006).

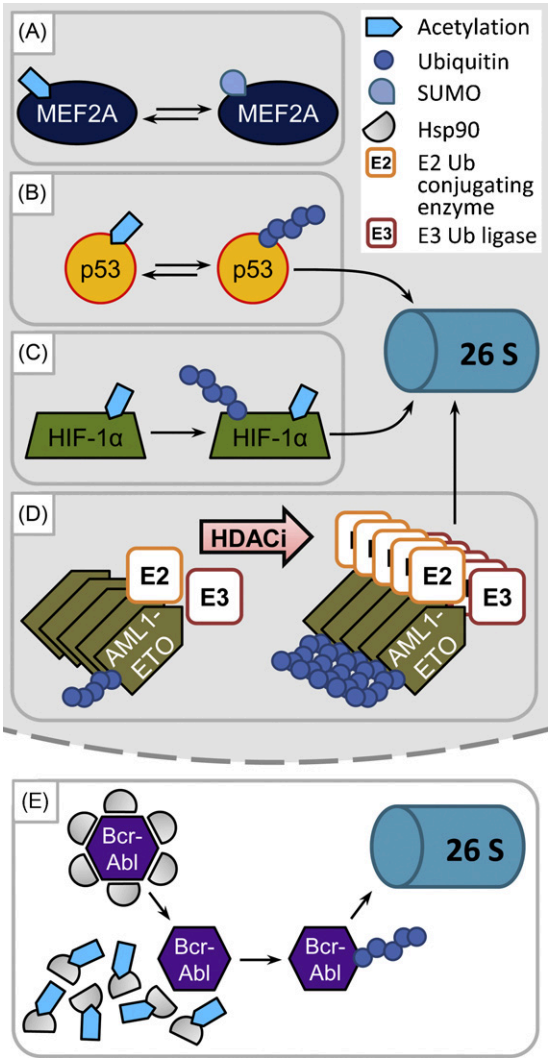

Fig. 3. Interplay of acetylation and other protein modifications. (A) Posttranslational modifications can target the same lysine residue, as exemplified for the SUMO-Acetylation switch (SAS) occurring on MEF2A. (B) Ubiquitination and acetylation differentially regulate proteasomal degradation, as ubiquitin conjugation at p53 lysines is blocked by acetylation. Other examples are Smad7, p73 and c-Myc. (C) Protein acetylation leads to enhanced degradation, for example of HIF-1α. (D) The HDACi-mediated transcriptional upregulation of E2 and/or E3 enzymes of the ubiquitin-proteasome pathway acts in concert to specifically induce protein degradation, e.g., of HDAC2, and of the leukaemia fusion proteins AML1-ETO, and PML-RARα. (E) HDACi can lead to Hsp90 hyperacetylation, which correlates with Hsp90 target protein degradation in the cytosol.

5.3.2. Acetylation can prevent ubiquitination and proteasomal degradation

Acetylation regulates protein stability in a sophisticated manner and by surprisingly diverse mechanisms (Fig. 3) (Sadoul et al., 2008). Acetylation of lysines can block ubiquitination at the same residue thereby preventing proteasomal degradation. This was first suggested for p53, which is tightly controlled by the Mdm2 E3 ligase driving proteasomal degradation of p53. PCAF and p300/CBP catalyse acetylation of C-terminal p53 lysine residues (K^{320}, K^{370}, K^{372}, K^{373}, K^{381} and K^{382}), which overlap with ubiquitination sites (Fig. 3B) (Ito et al., 2002). Acetylation abrogates complex formation between p53 and Mdm2, whereas an unacetylatable p53 mutant strongly interacts with Mdm2 resulting in p53 degradation (Tang et al., 2008). In an overexpression system, Mdm2 formed an HDAC1 containing complex binding to p53. Recruitment of HDAC1 (Juan et al., 2000; Luo et al., 2000) might thereby link two enzymatic activ-

ities promoting p53 degradation (Ito et al., 2002). It is nonetheless very surprising that positive regulation of p53 levels by HDACi has not been reported, yet. This could be due to the fact that the HDACi-insensitive SIRT1 likely represents the major p53 deacetylase (Luo et al., 2001). Accordingly, SIRTi lead to p53 hyperacetylation. The combined effect of HDACs and SIRTs on p53 stability remains to be analysed.

Similar acetylation–ubiquitination mechanisms are described for the p53 homolog p73 (Bernassola et al., 2004), Smad7 (Grönroos et al., 2002; Kume et al., 2007; Simonsson et al., 2005), Runx3 (Jin et al., 2004) and c-Myc (Vervoorts et al., 2003). Numerous HATs (CBP, Tip60, Gcn5 and PCAF) were reported to acetylate c-Myc at multiple lysines, preventing its ubiquitination and proteasomal degradation (Patel et al., 2004; Vervoorts et al., 2003). In contrast, p300 dependent acetylation of c-Myc resulted in enhanced degradation (Faiola et al., 2005). These results indicate that acetylation of different lysines affects targets in divergent ways (Table 1).

5.3.3. Acetylation can increase proteasomal degradation

Acetylation of proteins can equally promote enhanced degradation (Fig. 3C). Acetylation of the important angiogenesis regulator HIF-1α at K^{532} by ARD1 was reported to induce its degradation (Jeong et al., 2002). HIF-1α is steadily ubiquitinated by the E3 ligase pVHL and degraded by the proteasome under normoxic conditions (Lee et al., 2004). HIF-1α acetylation facilitates interaction with pVHL and its degradation (Jeong et al., 2002). Although hypoxia usually induces stabilisation of HIF-1α, HDACi lead to the destabilisation of HIF-1α protein levels even under hypoxic conditions (Jeong et al., 2002). In addition, the metastasis-associated protein MTA1 forms a complex with HDAC1 that is able to bind, deacetylate and stabilise HIF-1α (Yoo et al., 2006). However, other studies link an HDACi-mediated HIF-1α decrease to pVHL- and proteasome-independent degradation (Kong et al., 2006) or to class II HDACs (Qian et al., 2006), likely suggesting multiple pathways regulating HIF-1α stability (Bilton et al., 2006). The transcription factor GATA1 (Fig. 3E) (Hernandez-Hernandez et al., 2006) and pRb (Leduc et al., 2006) are other proteins preferentially degraded after acetylation (Table 1).

5.3.4. Acetylation can lead to non-proteasomal degradation

Non-proteasomal degradation is also affected by acetylation. For example, the acetylation status of the SV40 large T-Ag is controlled by CBP, HDAC1, HDAC3 and SIRT1, and HDACi enhance a proteasome-independent degradation of T-Ag (Shimazu et al., 2006). Also, acetylation of HNF-6 increases its half-life, whereas an unacetylatable mutant is degraded non-proteasomally (Rausa et al., 2004).

5.3.5. HDACi can influence protein stability by regulation of the ubiquitination machinery

The expression of various enzymes required for proteasomal degradation pathways is affected by acetylation. This mechanism was first identified for an HDAC itself. HDAC2 is selectively degraded upon treatment with VPA (Hrzenjak et al., 2006; Krämer et al., 2003; Tou et al., 2004). Ubc8 and RLIM are the E2 and E3 enzymes for ubiquitination of HDAC2, and VPA induces expression of Ubc8. TSA, a general class I and II HDACi, also enhances Ubc8 expression, but additionally triggers degradation of RLIM. Therefore, the overall levels of HDAC2 remain constant in the presence of such an HDACi (Krämer et al., 2003). Notably, HDAC2 is suggested both as a pharmacodynamic marker and as a target of VPA as a cancer therapeutic (Atmaca et al., 2007; Bug et al., 2005; Heinzel and Krämer, in press).

The oncogenic fusion proteins AML1-ETO and PML-RARα critically contribute to leukaemia. Notably, HDACi treatment also favours their proteasomal degradation (Fig. 3D) (Krämer et al., 2008b; Yang et al., 2007), which is equally linked to increased Ubc8 levels (Krämer et al., 2008b). In addition to relieving misdirected transcriptional repression by direct inhibition of HDACs, targeting leukaemia fusion proteins for proteasomal degradation (Fig. 3D) can hence be considered as a promising therapeutic strategy against acute myeloid and promyelocytic leukaemias.

Correct folding of proteins by chaperones, especially Hsp90, also has a major impact on protein stability. HDACi that inhibit HDAC6 induce Hsp90 hyperacetylation (Aoyagi and Archer, 2005; Bali et al., 2005; Scroggins et al., 2007), and degradation of several Hsp90 client proteins. Among the proteins described to be downregulated by HDACi via the HDAC6-Hsp90 axis (Fig. 3F) are several (proto-) oncogenes like the leukaemia fusion protein Bcr-Abl (George et al., 2005; Nimmanapalli et al., 2003), the Flt3 kinase (Bali et al., 2004), the c-Raf kinase (Yu et al., 2002), and the receptor tyrosine kinase ErbB2 (Fuino et al., 2003). However, combinatory treatment with HDACi and an Hsp90 inhibitor results in synergistically enhanced protein degradation, suggesting that both agents act on at least partially distinct pathways (George et al., 2005). In addition, Hsp90 acetylation and reduced complex formation between Hsp90 and its client proteins after depletion of HDAC6 hardly evokes destabilisation of these client proteins (Bali et al., 2005). Even more surprisingly, expression of an acetylation-mimicking Hsp90 mutant does not show notable effects on the stability of its target proteins (Scroggins et al., 2007).

5.3.6. Acetylation as a folding control mechanism in the endoplasmatic reticulum

Acetylation at seven lysine residues of the beta-site amyloid precursor protein (APP) cleavage enzyme (BACE1) seems to provide folding control through its ER-Golgi transition. Non-acetylated BACE1 is cleared out of the ER by proteasome-independent degradation, exclusively allowing acetylated BACE1 to translocate to the Golgi apparatus (Costantini et al., 2007). This finding not only demonstrates a new function for acetylation, but could also give insights to the role of APP and BACE1 in Alzheimer disease.

5.4. Acetylation and regulation of the cell cycle and circadian rhythms

5.4.1. Acetylation and cell cycle control

HDACi-mediated acetylation events can affect the cell cycle by indirect or direct modulation of p21, retinoblastoma protein (pRb) and E2F transcription factors.

p21 (WAF1, CDKN1) is a potent cyclin-dependent kinase inhibitor that belongs to the Cip/Kip family of Cdk inhibitors. Its expression is tightly controlled at the transcriptional level by p53. Under stress conditions, like DNA damage, p53 induces p21, which results in growth arrest. One of the major benefits of HDACi in cancer therapy was suggested to be p21 upregulation resulting in cell cycle arrest or apoptosis (Ocker and Schneider-Stock, 2007). HDACi-induced p21 expression is thought to be independent of p53, but dependent on Sp1/Sp3 and other factors, e.g., ATM (Ju and Muller, 2003) or c-Myc (Li and Wu, 2004). Nevertheless, p21 induction may also prevent apoptosis by inhibition of caspase activation (Heinzel and Krämer, in press; Krämer et al., 2008a; Suzuki et al., 1999). Transcriptional activation does not seem to be the only mechanism responsible for the HDACi-mediated increase in p21 protein levels. The influence of mRNA stability on p21 expression is an established concept, traced back to the binding of Hu proteins or other factors targeting the 3′-UTR. This stabilises p21 mRNA, resulting in increased p21 expression (Kim et al., 2005; Yang et al., 2004; Yano et al., 2005). Indeed, posttranscriptional regulation of p21 by butyrate has been described and different HDACi increased p21 mRNA stability depending on de novo protein synthesis in HepG2

cells. The underlying mechanism, however, is still unclear (Hirsch and Bonham, 2004).

E2F proteins and pRb represent decisive regulators of cell cycle control and progression. E2F1 binds to pRb in a cell-cycle dependent manner, which prevents interactions of E2F1 with the transcriptional machinery. Growth factor-induced phosphorylation of pRb by cyclin-dependent kinases releases E2F1. Free E2F1 facilitates the G1/S transition and S-phase by expression of its target genes (Sun et al., 2007). HDACi have been shown to interfere with E2F1 activity by downregulation of different cyclins, c-Myc and Cdc25A and upregulation of p21 (Abramova et al., 2006). On the other hand, E2F1 is acetylated at three lysines by p300, which stabilises the E2F protein and increases its specific DNA binding (Ianari et al., 2004; Martinez-Balbas et al., 2000). Acetylation of these lysines evokes the accumulation of ubiquitinated, though stable E2F1 (Galbiati et al., 2005).

pRb itself recruits mSin3 and HDACs to counteract the expression of cyclins A and E, which evokes cycle arrest (Zhang et al., 2000). Equally, acetylation of pRb on K^{873} and K^{874} hampers its cyclin E-Cdk2 dependent phosphorylation and the following insufficient pRb phosphorylation leads to cell cycle arrest. This is proposed to regulate differentiation-specific functions of pRb (Nguyen et al., 2004), which could provide a different explanation for the HDACi-induced growth arrest of cells: Hyperacetylated pRb would continue to block E2F1 and thereby prevent cell cycle progression.

5.4.2. Acetylation and circadian function

Mammalian CLOCK and BMAL1 are transcription factors connected to the circadian system (Kondratov et al., 2003). The circadian rhythm dictates a daily periodicity of approximately 24 h in the biochemical, physiological or behavioural processes of mammals. Polymorphisms in the CLOCK gene are associated with sleep disorders (Pirovano et al., 2005). CLOCK and BMAL1 form a heterodimer that binds to E-box enhancer elements, for example in the promoter of the PER1 gene (Motzkus et al., 2007). Recently, CLOCK has been shown to have intrinsic HAT activity. Beside effects on chromatin structure, CLOCK acetylates BMAL1 at K^{537}. Further, BMAL1 undergoes rhythmic acetylation in the mouse liver, correlating with the downregulation of CLOCK-controlled genes. This seems to be mediated by acetylated BMAL1, which recruits CRY1 that in turn accomplishes transcriptional repression (Hirayama et al., 2007). Therefore, CLOCK-induced acetylation of its binding partner and the resulting repression of CLOCK target genes could contribute to a circadian auto-regulatory feedback loop, which may be affected by HDACi.

5.5. Acetylation affects metabolism and mitochondria

Unexpectedly, about 20% of mitochondrial proteins were shown to be acetylated (277 acetylation sites in 133 proteins). The lysine acetylation recognition motif differs between mitochondrial and nuclear or cytosolic proteins (Kim et al., 2006) and the large number of acetylated mitochondrial proteins could regulate metabolism. SIRT3, 4 and 5 are the only known deacetylases in mitochondria and their activity might be controlled by the cellular NAD^+/NADH ratio (Grubisha et al., 2005). The cosubstrates for such acetylation and deacetylation reactions, acetyl-CoA and NAD^+, are the key indicators of the cellular energy state. This suggests a possible regulatory mechanism in which the energy state of the cell can control the cellular acetylome or acetylation states of mitochondrial proteins. In addition, the acetylation of the metabolically central cytoplasmic acetyl-coenzyme A synthetase (ACS) within its catalytic centre at K^{609} decreases its enzymatic activity (Starai et al., 2002). Therefore, sirtuins could regulate the cellular metabolism in response to nutrient availability and exhibit a function as a metabolic master switch (Schwer and Verdin, 2008). Hence acetylation can provide a tight control and response to metabolic changes.

5.6. Viral proteins

Many viral proteins can be targeted by acetylation (Table 1). Because the viral genome encodes only a very limited number of proteins, their acetylation could have a major impact on virus–host interactions.

5.6.1. HIV Tat

The HIV transactivator protein Tat plays a critical role in HIV replication by binding the leader RNA (TAR sequence) of the viral genome. Tat is acetylated by p300 at K^{50} and K^{51} in its RNA binding region (Ott et al., 1999), and by PCAF at K^{28} in its activator domain (Kiernan et al., 1999). Acetylation of Tat by p300 decreases the binding affinity between Tat and TAR sequences. This releases Tat and thereby enhances transcription from the LTR by promoting elongation (Kiernan et al., 1999). Deacetylation of Tat by SIRT1 allows its rapid recycling to TAR, which is critical for the repeated replication of the viral genome. Cycles of Tat acetylation by p300 and deacetylation by SIRT1 appear to regulate viral transcription. SIRT1 therefore acts as a coactivator during Tat transactivation (Pagans et al., 2005). HATi could inhibit Tat acetylation in the first place (Balasubramanyam et al., 2004), next to specific inhibitors of SIRT1 that could abolish Tat acetylation and deacetylation cycles required for HIV replication. HDACi are furthermore discussed as agents targeting latent viral reservoirs (Mai, 2007).

5.6.2. E1A

The adenoviral transforming protein E1A is acetylated by p300/CBP and PCAF at K^{239}. Acetylation of E1A inhibits p300/CBP-dependent transcription by interacting with and specifically inhibiting p300 activity (Deng et al., 2005). In its acetylated state, E1A can also bind to the carboxyl-terminal binding protein (CtBP) (Molloy et al., 2006). As a consequence, viral diseases can modulate global HAT activity, resulting in abnormal cellular signalling and gene expression. Furthermore, acetylation impairs the ability of E1A to bind importin-α3, resulting in cytosolic localisation. The cytoplasmic fraction of acetylated E1A may in turn affect multiple cytoplasmic processes (Madison et al., 2002).

6. Conclusion

Aberrant lysine acetylation has been reported in malignant cells (Yang, 2004), and HATs and HDACs are closely linked to severe diseases such as cancer, neurodegeneration, cardiovascular disorders, inflammatory lung diseases, as well as to ageing (Blander and Guarente, 2004; Carrozza et al., 2003; Heinzel and Krämer, in press; Ito et al., 2007; McKinsey and Olson, 2004; Saha and Pahan, 2006). The previous view that HDACi modulate gene expression mainly by histone acetylation appears to be too narrow. The growing number of identified acetylatable targets beyond chromatin provides a whole new world of regulatory mechanisms. Furthermore, switches between acetylation and other posttranslational modifications at the same lysine residue play a critical role. The functional consequences of acetylation can be almost as variable as their targets. It is not possible to predict the effect of acetylation of proteins at multiple sites without experimental testing, and the number of identified acetylated proteins up to date surely is below the actual number representing the in vivo acetylome. In vitro approaches have to be interpreted keeping in mind that acetyltransferases and deacetylases act primarily in protein complexes. These contain multiple subunits and cofactors that crucially

control substrate specificity and enzymatic activity that can hardly be reconstituted in vitro. However, despite technical challenges, acetylation research draws attention to the highly interesting field of posttranslational modifications and their critical impact on cellular functions. HDACi could possibly correct aberrant acetylation patterns and ameliorate disease states. In the future, acetylation of non-histone targets should be considered as a crucial molecular mechanism of tumour-selective HDACi therapy.

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Manuscript Overview 2

HDAC target the posttranscriptional regulation of SIRT1 by a modulation of HuR

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Submitted

Here, we show that histone deacetylase inhibitors (HDACi) decrease expression of SIRT1 at the mRNA and protein levels. We demonstrate acetylation-dependent posttranscriptional regulation of SIRT1 via the RNA binding protein HuR, which controls the stability of SIRT1 mRNA. This finding reveals novel mechanistic insights into the control of protein expression by HDACi.

Histone deacetylase inhibitors target tumour cells by posttranscriptional regulation of SIRT1

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Abstract

The histone deacetylase (HDAC) enzyme family controls eukaryotic transcription, cell proliferation and apoptosis. The evolutionary conserved class III HDAC SIRT1 critically controls various cellular functions promoting resistance to apoptotic stimuli. The dysregulation of HDAC activity is linked to oncogenesis and increased survival rates of tumour cells. Identifying mechanisms regulating HDACs is therefore of utmost importance for cancer research. Here, we show that histone deacetylase inhibitors (HDACi) decrease expression of SIRT1 at the mRNA and protein levels. We demonstrate acetylation-dependent posttranscriptional regulation of SIRT1 via the RNA binding protein HuR, which controls the stability of *SIRT1* mRNA. HDACi evoke nuclear translocation and posttranslational modification of HuR. Subsequently, the affinity of HuR for *SIRT1* mRNA is altered favouring *SIRT1* mRNA degradation. We identify an unexpected link between class I/II HDAC inhibitors, which do not block the activity of the class III enzyme SIRT1, and reduced SIRT1 expression. Thus, HDACi indirectly target this epigenetic regulator via posttranscriptional regulation. This finding reveals novel mechanistic insights into the control of protein expression by HDACi.

Keywords: acetylation, histone deacetylase inhibitor, protein deacetylase SIRT1, HUR, mRNA stability, phosphorylation

Introduction

HDACs are important regulators of gene expression and cellular signalling. According to their size and cofactor usage, the HDAC family is grouped into four classes (Bolden *et al.*, 2006). Transcriptionally active chromatin is hyperacetylated. Silent chromatin is hypoacetylated, which results in a more condensed form. Consequently, access of the transcriptional machinery to DNA target sequences is impaired (Kouzarides, 2007). Nevertheless, inhibiting HDACs does not simply result in a net increase in gene expression (Spange *et al.*, 2009). Moreover, acetylation of lysine residues can modulate the interaction, localisation, stability and enzymatic activity of histones and non-histone proteins. Remarkably, acetylation is in scope and consequences comparable to the phosphorylation of proteins (Norris *et al.*, 2009; Spange *et al.*, 2009).

Class III HDACs, also termed sirtuins, are NAD^+-dependent-enzymes linked to metabolism, ageing, apoptosis, differentiation and stress responses in several species (Finkel *et al.*, 2009). SIRT1 is often linked to positive effects of calorie restriction. It targets a variety of cellular substrates like p53, FOXO proteins, NFκB-p65, NCOR, histones H1 and H4, KU70, p300, BCL11A, PGC1α, eNOS, E2F1, AR, p73, SMAD7, RB, SUV39H1, COUP-TF (Lavu *et al.*, 2008; Liu *et al.*, 2009). Its multitude of intracellular targets mediate stress resistance and cell survival which in turn are connected to metabolic control, inflammation and longevity as well as cancer development (Donmez and Guarente). Thus, the activity of SIRT1 has been implicated in improved organismic health and survival (Baur *et al.*, 2006). Enhanced SIRT1 expression also showed beneficial effects on tumour development and maintenance in a β-catenin-driven mouse model of colon cancer (Firestein *et al.*, 2008).

Although SIRT1 activators are considered for therapy of metabolic diseases, SIRT1 overexpression or unbalanced activation is linked to cancer by increasing cell survival and stress resistance of cancer cells (Jiang, 2008; Lavu *et al.*, 2008). For example, SIRT1 can inhibit p53 function by deacetylation (Tang *et al.*, 2008). Furthermore, high SIRT1 levels can

inhibit apoptosis by targeting FoxO1 (Jung-Hynes and Ahmad, 2009), E2F1 (Wang *et al.*, 2006) and p73 (Dai *et al.*, 2007). Moreover, loss of acetylation at Lys16 of histone H4 is a hallmark of human cancer pointing to an enhanced SIRT1 activity or a decreased activity of the acetyltransferase hMOF (Hajji *et al.*, 2010). A wide variety of cancer tissues, from solid tumours to leukaemia, exhibit increased SIRT1 expression compared to healthy tissue (Hida *et al.*, 2007). According to the multi-hit model of cancer development, SIRT1 seems to be a tumour growth promoter once other critical molecular changes have taken place. Of note, sirtuin inhibitors like salermide show a strong cancer-specific proapoptotic effect (Lara *et al.*, 2009).

Dysregulated protein acetylation is a frequent feature of human cancers and can result in unbalanced cell cycle control and aberrant gene expression. Therefore, enzymes regulating the acetylation status represent attractive targets for cancer therapy. Pan-HDAC inhibitors like Trichostatin A (TSA), LBH589 or LAQ824, inhibit all classical HDACs (class I, II and IV), whereas other HDACi like valproic acid (VPA) or sodium butyrate inhibit a subgroup of these deacetylases. Therapeutic effects of HDACi include induction of differentiation, cell cycle arrest, senescence, apoptosis and mitotic cell death (Minucci and Pelicci, 2006).

Due to their evolutionary origin, the class III HDACs – the sirtuins – have a different catalytic mechanism that cannot be inhibited by the classical HDACi. Nevertheless, we were interested in potential indirect effects of classical, chemotherapeutically active HDACi on SIRT1. Here we show that HDACi decrease SIRT1 protein level dramatically. Our data indicate that, this reduction is due to altered posttranscriptional regulation of *SIRT1* mRNA and posttranslational modification of its binding partner HuR. HDACi modulate the cellular distribution and binding affinity of HuR favouring cytosolic mRNA decay of *SIRT1* transcripts. We propose a novel mechanism by which HDACi affect sirtuins and achieve a more vulnerable cancer cell condition via reduced expression of SIRT1 in a therapeutic setting.

Results

HDACi decrease SIRT1 protein levels

HDACi block the catalytic activity of class I/II HDACs and promote the proteasomal degradation of the class I enzyme HDAC2 (Krämer *et al.*, 2003). We observed that incubation of cells with HDACi like TSA, VPA or butyrate also reduced the protein levels of the class III deacetylase SIRT1 drastically (Fig. 1A). Fig. 1B shows that this effect was time-dependent and reproducible in several different tumour cells. The SIRT1 loss was already noticeable after 24 hours, which is consistent with previously published findings (Hajji *et al.*, 2010; Kyrylenko *et al.*, 2003). Under these conditions, HDACi did not lead to degradation of the housekeeping protein tubulin (Fig. 1A, B).

Given that HDACi are able to increase proteasomal degradation of proteins (Buchwald *et al.*, 2009; Krämer *et al.*, 2003), we tested whether inhibitors of the 26S proteasome (Lactacystin and ALLN) can prevent the decrease of SIRT1 levels upon HDACi treatment. Since co-treatment with proteasome inhibitors could not counteract the HDACi-induced SIRT1 reduction (Fig. 1C), we could rule out an HDACi-dependent proteasomal degradation of SIRT1.

Additionally, HDACi can induce apoptosis of cancer cells (Bolden *et al.*, 2006) and SIRT1 is a target of caspase-1, -3, -8 and -9 (Ohsawa and Miura, 2006). Therefore, we blocked caspase activity with the pan-caspase inhibitor Z-VAD-FMK and analysed SIRT1 protein levels. Since this compound permitted detection of a weak signal for SIRT1 in cells treated with HDACi, apoptosis appeared as a minor cause of SIRT1 degradation (Fig. 1D). Thus, proteasomal degradation and caspases do not contribute significantly to attenuation of SIRT1 in cells exposed to HDACi.

HDACi target SIRT1 at the posttranscriptional level

These findings argue for a regulation of SIRT1 at the mRNA level. Indeed, we noticed that HDACi reduced *SIRT1* mRNA levels (Fig. 2A). This result is consistent with published data (Kyrylenko *et al.*, 2003) and suggests that HDACi reduce SIRT1 protein expression by affecting *SIRT1* mRNA levels.

Based on these results, we analysed the putative regulation of the *SIRT1* promoter by HDACi. We tested different *SIRT1* promoter fragments towards their activity after HDACi treatment in the pGL3-luciferase reporter system. For this purpose, we subcloned fragments of the SIRT1 core promoter (1000 bp (A) to 100 bp (F)) containing the transcription start site (Fig. 2 B upper panel). These constructs showed a higher basal transcriptional activity compared to the pGL3vector containing only the SV40 promoter. Inclusion of the 5´UTR resulted in increased overall promoter activity. Unexpectedly, all *SIRT1* promoter fragments independent of the presence of the 5´UTR showed increased luciferase activity after incubation with HDACi (Fig. 2 B). RNA controls from the same cells, run in parallel, however showed a decrease in endogenous *SIRT1* mRNA levels (data not shown).

A nuclear run-on assay (NRO) revealed an almost unchanged frequency of transcriptional initiation of the endogenous *SIRT1* promoter *in vivo*, while *SIRT1* mRNA levels declined (Fig. 2C). The lack of induction of *SIRT1* promoter activity in the NRO as compared to the luciferase assay is likely due to the use of the whole promoter context in the NRO versus small, non-chromatinised promoter fragments in the reporter assay. The fact that *SIRT1* mRNA expression does not decrease in either case indicates that the loss of *SIRT1* may depend on the regulation of mRNA stability.

Having excluded impaired transcriptional induction of the *SIRT1* gene, we tested whether HDACi reduce the half-life of *SIRT1* mRNA. After blocking RNA synthesis with the RNA polymerase II inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), we analysed the stability of *SIRT1* mRNA by quantitative real time-PCR of DRB and HDACi/DRB co-

treated cells. The *SIRT1* mRNA stability was unaltered with DRB alone or in combination with butyrate at two and four hours after treatment (Fig. 2D). By blocking protein synthesis with cycloheximide (CHX) we determined that de novo protein synthesis is not necessary for the effects of HDACi on SIRT1 (Fig. 2E). We therefore conclude that the effect of HDACi on SIRT1 expression is direct and does not require de novo synthesis of proteins or RNAs.

HDACi alter the subcellular localisation of HuR

The AU-rich element binding protein HuR (ELAV) crucially regulates the stability of the *SIRT1* mRNA. It binds at several sites within the 1.8 kb 3´-UTR of the SIRT1 mRNA and favours SIRT1 expression by enhancing *SIRT1* mRNA stability (Abdelmohsen *et al.*, 2007b). The conformation of its three RNA recognition motifs (RRMs), its localisation as well as its phosphorylation state critically determine HuR's effects on mRNA stability (Abdelmohsen *et al.*, 2007b; Kim *et al.*, 2008). HuR binds to newly synthesised mRNA in the nucleus and is co-exported into the cytosol. Cytosolic HuR protects mRNA from degradation and maintains translation. We hence speculated that HDACi might reduce *SIRT1* mRNA levels by affecting this protein. Fluorescence microscopy and Western Blot experiments revealed that HDACi treatment reduced the cytosolic amount of HuR, while overall HuR levels remained stable (Fig. 3 A & B).

Although an impact of HDACi treatment on HuR localisation has also been reported by others (Pryzbylkowski *et al.*, 2008; Wang *et al.*, 2004), the overall change in HuR localisation appears insufficient to fully account for the *SIRT1* mRNA loss in cells incubated with HDACi. Moreover, the typical $NaAsO_2$-induced cytosolic accumulation of RNA degrading P-bodies (Fig. 3 A lower panel) is not seen in HDACi-treated cells, which disfavours a general induction of the cytosolic mRNA decay machinery by such agents.

HDACi alter the mRNA binding ability and phosphorylation state of HuR

Modulation of the HuR affinity for *SIRT1* mRNA would be a more specific mechanism, than a change in HuR localisation. By an RNP-IP approach we tested whether HDACi affect the *SIRT1* mRNA binding capacity of HuR. As a positive control for reduced HuR binding affinity towards *SIRT1* mRNA we treated cells with H_2O_2, which induced Chk2-dependent phosphorylation of HuR resulting in dissociation of the HuR-*SIRT1* mRNA-complex (Abdelmohsen *et al.*, 2007b). After a 24 hour incubation period with HDACi, specific binding of HuR to *SIRT1* mRNA was no longer detectable (Fig. 4 A). Thus, this effect is linked to the HDACi-induced SIRT1 loss upon HDACi treatment.

Mechanisms affecting the binding affinity of HuR for its target mRNAs can be modulated by HuR binding factors or by posttranslational modifications. These observations, together with the stable levels of HuR in HDACi-treated cells, suggest an HDACi-induced posttranslational modification of HuR. Lysine acetylation is an obvious candidate for an HDACi-induced posttranslational modification of HuR. However, immunoprecipitation and Western blotting revealed no acetylation of HuR (data not shown).

Since phosphorylation of HuR influences its *SIRT1* mRNA binding affinity strongly, we analysed immunoprecipitated HuR by mass spectrometry. This analysis revealed that HuR(S202) was phosphorylated in control cells, whereas treated cells showed no phosphorylation at this site. Additional sites critical for the HuR-RNA binding affinity are the Chk2 (checkpoint kinase-2)-dependent phosphorylation sites HuR(S88, S100, T118). HuR phosphorylated at these sites displayed reduced *SIRT1* mRNA affinity following oxidative stress (Abdelmohsen *et al.*, 2007b). No phosphorylation was detectable at these sites, even though the corresponding peptides were traceable. This suggests a Chk2-independent mechanism that lowers HuR-SIRT1 mRNA binding affinity. Additionally, our mass spectrometry approach identified three novel HDAC-responsive HuR phosphorylation sites, at HuR(S41, S142, S197) (Fig. 4B).

Effects of HDACi on cancer cells partially depend on attenuation of SIRT1

Given that SIRT1 favours cancer cell survival, loss of SIRT1 upon HDACi treatment could provide therapeutic benefits. To test whether SIRT1 is relevant for anti-proliferative effects of HDACi we overexpressed SIRT1 in Hela cells and treated them with TSA to block all classical HDACs. The ectopic SIRT1 expression vector did not include the 3´UTR of SIRT1. Consequently, it could not be targeted posttranscriptionally. Ectopic SIRT1 expression promoted cell growth in comparison to the control (Fig. 5A). In addition to higher proliferation rates, SIRT1 overexpression favoured cell viability upon TSA treatment. The effect of SIRT1 overexpression was most prominent after a 72 hour incubation period. Hence, SIRT1 loss upon HDACi treatment could in part account for the therapeutic impact of HDACi therapy.

Discussion

Alteration of mRNA stability by HDACi affects gene expression

The proper regulation of deacetylases appears to be critical for the maintenance of homeostasis. Accordingly, dysregulated HDAC activity has been found in certain severe human malignancies (Bolden *et al.*, 2006; Krämer, 2009). HDACs are known to crucially regulate eukaryotic gene expression via deacetylation of histones and other proteins targeting transcription. Their inhibition by HDACi alters the expression of 2–10% of human genes significantly, with almost equal numbers of genes up- and down-regulated (Müller and Krämer, 2010). On the other hand, approximately 50% of inducible changes in gene expression are caused by alterations in mRNA stability rather than “direct” transcriptional control (Cheadle *et al.*, 2005). It is unknown to which extent posttranscriptional mechanisms change gene expression in response to HDACi.

As a key regulator of cellular signalling, SIRT1 is tightly controlled. Recently, a complex network regulating SIRT1 transcription, mRNA stability, posttranslational modifications, co-factors and binding proteins has been identified. When analysing the effect of HDACi on

mRNA stability we found that while SIRT1 mRNA half-life is drastically reduced by DRB, it cannot be further decreased by HDACi co-treatment (Fig. 2D). This finding suggests that SIRT1 mRNA destabilisation by HDACi is established at later time points. Alternatively, the DRB-blocked RNA synthesis may override HDACi-induced effects on SIRT1. HDACi as well as DRB may act on the same targets, which excludes additive effects. It is equally possible that further RNA-dependent destabilising pathways are needed to enhance *SIRT1* mRNA degradation (Rössig *et al.*, 2002). Of note, effects of HDACi on posttranscriptional control mechanisms just recently became a topic of interest. Our report adds SIRT1 to the few proteins like eNOS, p21, ERα, BRM, GATA3, Claudin-1, DNMT-1, -3B that are posttranscriptionally regulated by HDACi (Spange *et al.*, 2009).

HDACi can alter SIRT1 mRNA stability via modulation of HuR

3´-UTR binding proteins modulate mRNA stability. HuR has been suggested to critically affect cell proliferation, tumourigenesis, senescence and stress responses by targeting *c-FOS, c-MYC, COX-2, Tnf-α, GM-CSF, β-catenin, eotaxin, p27, cyclin A, cyclin B1, cyclin D1, p21, p27, p53* and *SIRT1* mRNA (Abdelmohsen *et al.*, 2007a; Abdelmohsen *et al.*, 2007b). While under certain conditions HuR binds tightly to some mRNAs, it dissociates from others resulting in complete destabilisation of the transcript (Abdelmohsen *et al.*, 2007b). HuR posttranscriptionally regulates SIRT1 expression levels critically, which is evidenced by the fact that an siRNA-mediated knock-down of HuR results in a complete loss of SIRT1 protein expression. This observation furthermore shows that *SIRT1* mRNA is subject to high turnover rates (Abdelmohsen *et al.*, 2007a).

Here, we reveal the novel finding that HDACi evoke dissociation of HuR from *SIRT1* mRNA and consequently its decay. Thus, we not only provide a new member to the handful of proteins controlled posttranscriptionally by HDACi (Spange *et al.*, 2009), our findings even suggest a mechanism responsible for this process.

We also considered that different transport mechanisms as well as binding partners were found to affect the cellular distribution of the mainly nuclear HuR protein. Our findings are consistent with reports showing a decrease of cytoplasmic HuR levels in cells exposed to

HDACi alone or in combination with aza-cytidine (Pryzbylkowski *et al.*, 2008; Wang *et al.*, 2004). AMP-activated protein kinase (AMPK) might be involved in the HDACi-dependent regulation of SIRT1 by HuR. Upon activation of AMPK, p300 becomes phosphorylated which in turn triggers acetylation of importin-α1. In this case, acetylation favours binding to its nuclear import factor importin-β. Additionally, AMPK phosphorylates importin-α1 creating a binding site for HuR. The formed complex containing importin-β/importin-α1/HuR translocates into the nucleus. HDACi enhance acetylation of importin-α1, promoting importin-β binding (Bannister *et al.*, 2000). Basal activity of AMPK without further activation may be sufficient to phosphorylate a certain cytosolic HuR fraction, which in turn translocates to the nucleus. Ultimately, these points argue for an HDACi-induced shift in the cellular HuR distribution independent of AMPK activation.

In addition to localisation, phosphorylation of HuR interferes with its binding capacity for mRNAs (Abdelmohsen *et al.*, 2007b). Phosphorylation of HuR(S202) enhances nuclear retention and promotes association of target transcripts with HuR (Abdelmohsen *et al.*, 2007a). This site is targeted by the cell-cycle dependent kinase 1 (CDK1) favouring nuclear localisation and retention of HuR during the G2/M-Phase (Kim *et al.*, 2008). Phosphorylation at HuR(S202) was hence observed only in asynchronously growing control cells in different cell cycle stages, e.g. with activated CDK1 in the G2/M-phase (Fig. 4B). HDACi like butyrate or VPA induce G1-cell cycle arrest via induction of p21 preventing CDK1 activity (Krämer *et al.*, 2008). This could explain why there is no detectable HuR(S202) phosphorylation in HDACi-treated cells. Therefore, the HDACi effect is independent of the G_2/M-induced phosphorylation at HuR(S202).

Regarding SIRT1 mRNA the additional HDACi-induced phosphorylation of HuR(S41, S142) (Fig. 4B), located within the RNA binding domains (RRM), may be responsible for the reduced binding affinity. Moreover, phosphorylation of HuR(S197), which is positioned close to HuR(S202), could interfere with mRNA binding as well as the subcellular localisation of HuR. Our results add a new layer to the understanding how HuR regulates *SIRT1* mRNA

stability in response to protein acetylation. HDACi critically influence HuR phosphorylation and mRNA stability of SIRT1. Similar regulatory processes might apply to other enzymes and modulators.

HDACi-mediated SIRT1 loss and cancer sensitivity

Accumulating evidence supports a tumour-promoting role for SIRT1 (Ford *et al.*, 2005). Since SIRT1 knockout mice show no elevated tumour rates after induction of skin papillomas, SIRT1 does not act like a classical oncogene. Simple overexpression of SIRT1 does equally not per se lead to tumourigenesis (Lavu *et al.*, 2008). Nevertheless, tumour tissues very often show elevated SIRT1 levels (Stunkel *et al.*, 2007), which correlates with poor survival prognosis of cancer patients (Jang *et al.*, 2008). Deacetylation of the tumour suppressors p53, p73 and Ku70 likely contributes to such tumour-promoting functions of SIRT1 (Cohen *et al.*, 2004; Luo *et al.*, 2001).

Congruent with such findings, tumour suppressor proteins including p53, HIC1 and DBC1 repress SIRT1. During ageing and cancer development, the HIC1 promoter can undergo hypermethylation and epigenetic silencing. Consequently, SIRT1 expression is expected to rise in ageing tissues, where it might increase the survival of damaged cells and cancer risk (Campisi and Yaswen, 2009). Indeed, inhibition of SIRT1 as well as its siRNA-mediated knock-down impairs the growth of multiple cancer cell lines (Kamel *et al.*, 2006; Stunkel *et al.*, 2007) as well as tumours (Kojima *et al.*, 2008).

Classical HDACi do not block the catalytic activity of class III HDACs (Müller and Krämer, 2010). We could nonetheless demonstrate that these inhibitors have a strong effect on SIRT1 expression levels in different cancer cell lines. Hence, decreased expression of SIRT1 may contribute to the anti-tumourigenic effects of HDACi (Hajji *et al.*, 2010). Transient or persistent attenuation of SIRT1 decreases the stress resistance of transformed cells, which may explain why HDACi are more effective in killing cancer cells when combined with chemotherapeutic drugs inducing cellular stress (Hajji *et al.*, 2010; Müller and Krämer, 2010). Hence, the view on whether HDACi target class III HDACs requires a novel definition.

Materials and methods

Cell Culture, Transfections, Luciferase assay and Proliferation

All cells (293T, Hela, NB4, U3A, P19, 2F and Cos7) were maintained in RPMI medium supplemented with 10% FCS (PAA), 1% penicillin/streptomycin. All cell lines were cultured at 37°C in a 5% CO_2 atmosphere. 293T cells were seeded at $2.5*10^4$/24-Well and transfected with 0.025 µg pGL3 derived promoter vectors using PEI (Sigma). Luciferase assays were carried out as described with *SIRT1* promoter constructs (Göttlicher *et al.*, 2001). Hela cells were transfected using SIRT1 plasmid and Turbofect (Fermentas). The SIRT1 expression vector has been described before (Narala *et al.*, 2008). Cell viability was determined by using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT, Sigma) and was represented as percentage of the cell viability of untreated cultures.

Drugs and chemicals:

The HDACi and other inhibitors were purchased as indicated: sodium butyrate (Merck), valproic acid (Sigma) and TSA (Sigma), cycloheximide (CHX, Sigma), Z-VAD-FMK (Z-VAD, Bachem), lactacystein (Lac, Santa Cruz), ALLN (Sigma), sodium orthovanadate (Na_3VO_4), sodium fluoride (NaF), sodium pyrophosphate (NaPP, Sigma), 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB, Sigma), H_2O_2 (Merck), 4,5 dimethyl-2-yl 2,5-diphenyl tetrazolium bromide (MTT) (Sigma), Luciferin (Promega).

Preparation of cell lysates, immunoprecipitation and immunoblotting

For Western blot analysis, all NETN lysates were assessed by BCA assay (Thermo) for protein concentrations. They were size-fractionated by SDS-PAGE and transferred onto PVDF membranes. Antibodies were obtained from Santa Cruz Biotechnology: SIRT1 sc15404, HuR sc5261; Sigma: Tubulin #T5168; Upstate Biotechnology: HDAC1 05-614. After secondary antibody incubation, signals were detected by enhanced chemiluminescence. All Western blots were probed for Tubulin to ensure equal sample loading. Densitometric analysis of protein bands was performed with

Abobe Photoshop software. To define the relative density in each sample, the average grey value of the specific protein band was normalised to the signal for Tubulin.

Real Time PCR

RNA was isolated using Trizol (PeqLab). 2 µg of RNA were reverse transcribed using using oligo-dT primer and RevertAid M-MuLV Reverse Transcriptase (Fermentas). Semi-quantitative RealTime-PCR (sq-RT-PCR) was performed using the ABsolute QPCR SYBR Green Fluorescein Mix (Thermo) with SIRT1 primers ATTGTTATTGGGTCTTCCCTCA (fw) and CATCACAGTCTCCAAGAAGCTC (rev). RT-PCR linearity was controlled with GAPDH primers TGCACCACCAACTGCTTAGC (fw) and GGCATGGACTGTGGTCATGAG (rev).

Nuclear run-on assay

Nuclear run-on assay was performed as described (Hartmann *et al.*, 2009). Radiolabeled RNA was hybridized with a Hybond-N nylon membrane (Amersham) containing immobilized fragments of *GAPDH* (1 µg of a 558 bp fragment generated with primers ACCACAGTCCATGCCATCAC (fw) and TCCACCACCCTGTTGCTGTA (rev)) and *SIRT1* (2 µg of a 402 bp PCR-fragment generated with primers CTGGGGAAGGAGACAATGG (fw) and GCGAGAGTCTCCCGACCT (rev)). Hybridisation was performed overnight at 65°C with 1 x 10^6 c.p.m. labeled RNA per sample using 3 ml of the Rapid-hyb buffer (Amersham) according to the manufacturer's recommendations. Signals were quantified by phosphoimager (Fujifilm).

RNP-IP

Immunoprecipitation (IP) of endogenous RNA-protein complexes was performed as described (Abdelmohsen *et al.*, 2007b). The RNA isolated from IP material was reverse-transcribed using oligo-dT primer and RevertAid M-MuLV Reverse Transcriptase (Fermentas). RT-PCR was done as described above.

Immunofluorescence staining and fluorescence microscopy

Hela cells were fixed using 4% PFA and permeabilised with PBS + 0,25% Triton X-100. After blockading, samples were incubated with primary antibody anti-HuR followed by incubation with secondary antibody conjugated with Alexa488 (Invitrogen, Karlsruhe Germany).

Subsequently, cells were covered with ProLong® Gold antifade reagent (Invitrogen, Karlsruhe Germany) containing DAPI. Microscopic analysis was performed with the ApoTome deconvolution system® (Carl Zeiss, Göttingen). Images were processed using Abobe Photoshop software.

Mass spectrometry

HuR was immunoprecipitated from Hela cell lysates with phosphatase inhibitors NaF, Na_3VO_4 and NaPP, and samples were separated by SDS-PAGE. Protein gels were stained with Coomassie stain kit (Invitrogen) and appropriate gel sections were subjected to in-gel digestion with trypsin as described (Shevchenko *et al.*, 1996) with one modification: reduction and oxidation of thiol groups was performed with a mixture of tributylphosphine and 4-vinylpyridine (Sigma-Aldrich). The trypsin in-gel protein digests were analysed with LC-ESI-MS equipment consisting of a MDLCÔ chromatography system (GE Healthcare, Munich, Germany) online coupled to a Finnigan LTQ mass spectrometer (Thermo Electron Corporation, Dreieich, Germany). The HPLC was equipped with a Zorbax 300SB 5 µM, 5×0.3 mm trapping column and a Zorbax 300SB 5 µM, 150×0.075 mm separation column (Agilent, Böblingen, Germany). The separation of peptides on the HPLC occurred by applying a linear gradient running from 0% to 47% acetonitrile, followed by a stepwise elution with 84% acetonitrile in 0.1% formic acid, each under control of Unicorn software (GE Healthcare, Munich, Germany). The LTQ was operated under control of Xcalibur 1.4 software (Thermo ElectronCorporation, Dreieich, Germany). For processing of the mass spectra and the final protein identification the BioWorks 3.2 software (Thermo Electron Corporation, Dreieich, Germany) and the National Center for Biotechnology Information human protein database were used. Site-specific phosphorylation analysis was performed by scanning pSer and pThr for loss of phosphoric acid (neutral loss of 98) in the respective MS/MS spectra.

Acknowledgements

We would like to thank H. Vaziri for the SIRT1 expression plasmid.

Figures:

Figure 1

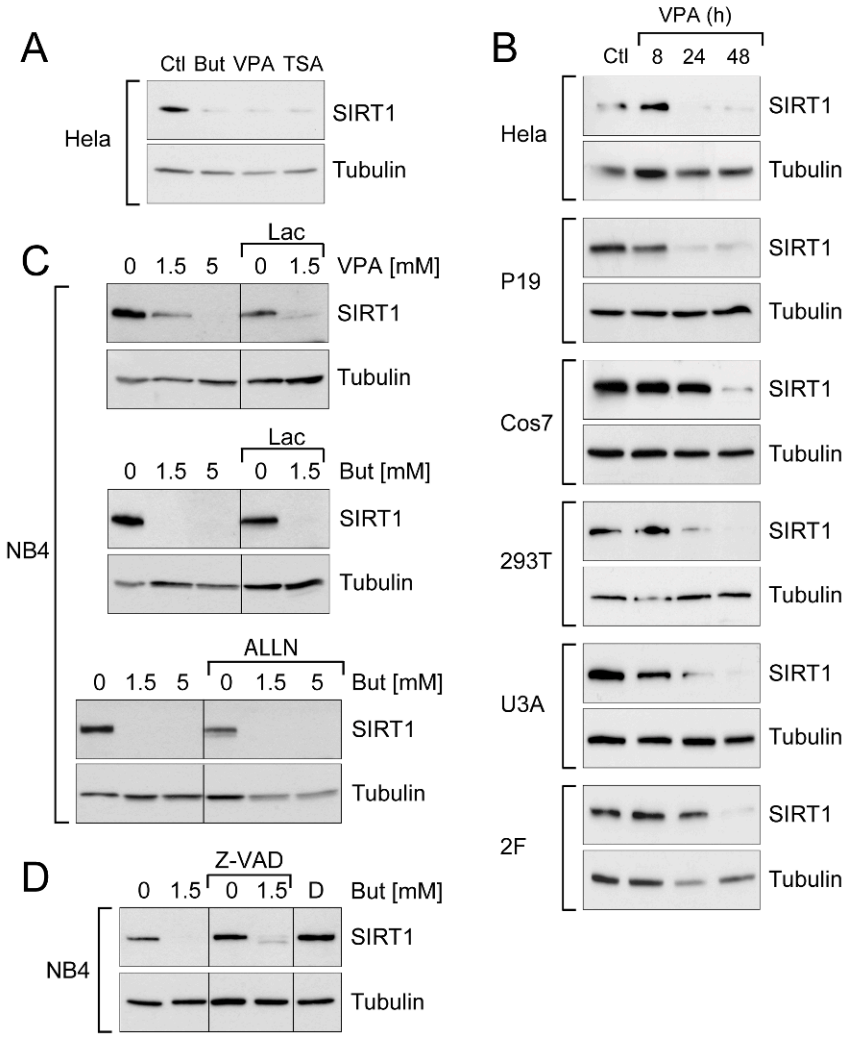


Figure 1 HDACi reduce SIRT1 protein levels

(a) Western Blot analysis of SIRT1 protein level in lysates from Hela cells treated with 3 mM butyrate, 3 mM VPA and 100 nM TSA for 24 h. Tubulin was used as loading control.

(b) Western Blot analysis of SIRT1 protein levels in lysates from various cell lines treated with VPA (1.5 mM) for the indicated time periods (8, 24 and 48 h).

(c) NB4 cells were either co-treated with butyrate (1.5 mM)/ VPA (1.5 mM) and the proteasome inhibitors lactacystein (10 µM)/ALLN (1 µg/ml) or with these agents alone for 24 h. Protein levels in lysates were analysed by Western blot.

(d) NB4 cells were co-incubated with butyrate (1.5 mM) or Z-VAD (20 µM) alone or in combination for 24 h. Protein levels were analysed by Western blot. (D = DMSO, solvent control for Z-VAD)

Figure 2

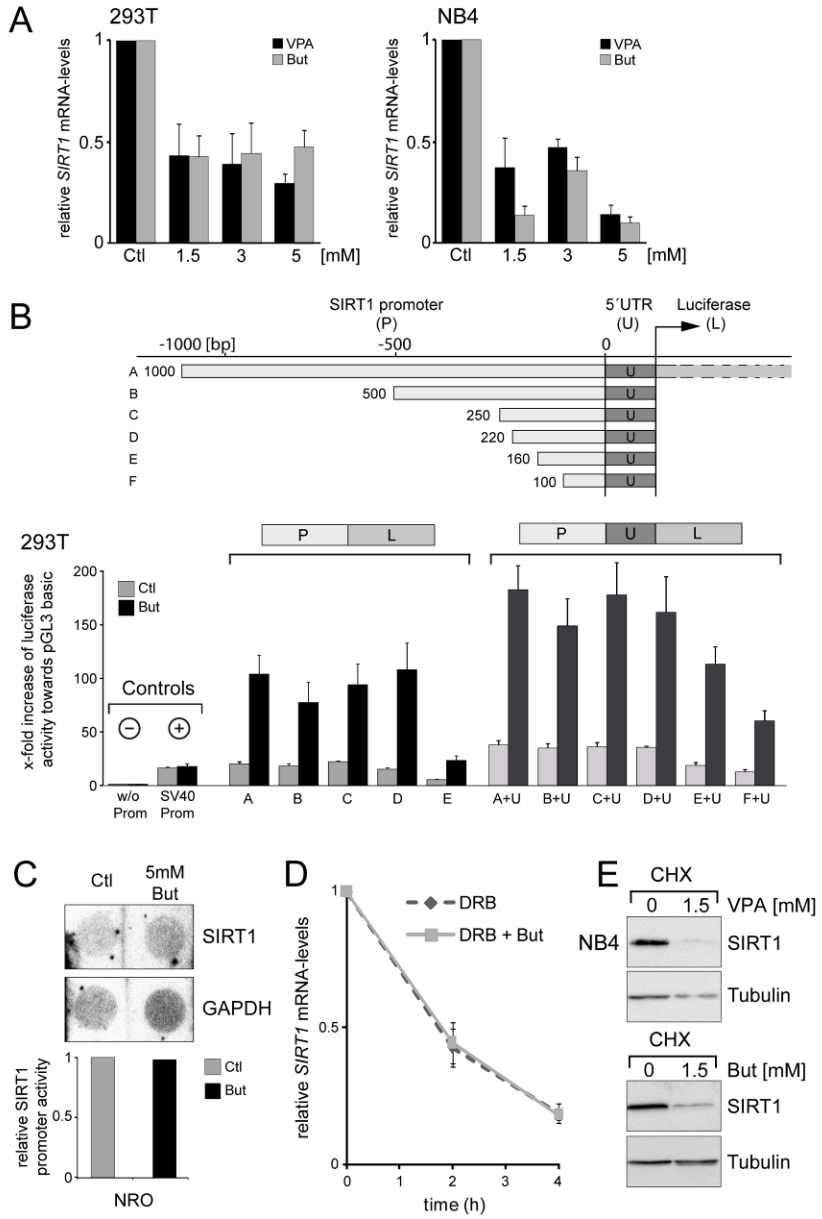


Figure 2 HDACi reduce SIRT1 protein levels by decreasing *SIRT1* mRNA stability

(a) After a 24 h incubation of 293T and NB4 cells, *SIRT1* mRNA levels were analysed by sq-RT-PCR at different concentrations (1.5; 3 and 5 mM) of butyrate or VPA. mRNA levels measured in untreated cells were set as 1. Data were normalised to GAPDH. (means ± SEM., n = 3)

(b) Analysis of the *SIRT1* promoter was carried out with the pGL3 basic luciferase reporter system. A series of fragments (from 1000 bp (A) to 100 bp (F); without 5´-UTR or with 5´-UTR (U) were subcloned into a promoterless luciferase vector (pGL3 basic) (panel B, upper part). Data were normalised to the activity of a co-transfected SV40 β-galactosidase plasmid to ensure comparable transfection conditions; negative control: promoterless vector (pGL3 basic), positive control: SV40 promoter containing vector (pGL3 Promoter). (means ± SEM., n = 3)

(c) Transcription rates of *SIRT1* and *GAPDH* in 293T cells treated for 24 h with 5 mM butyrate were analysed by NRO. Upper panel, Representative autoradiographies; Lower panel, densitometric analysis.

(d) Hela cells were treated with the mRNA synthesis inhibitor DRB (75 µM) alone or in combination with butyrate (3 mM) for 2 and 4 h. *SIRT1* mRNA half-life was evaluated by sq-RT-PCR . Data were normalised to GAPDH. (means ± SEM., n = 7)

(e) NB4 cells were incubated for 24 h with the protein synthesis inhibitor cycloheximide (CHX, 1 µg/ml) in co-treatment with the HDACi butyrate or VPA.

Figure 3

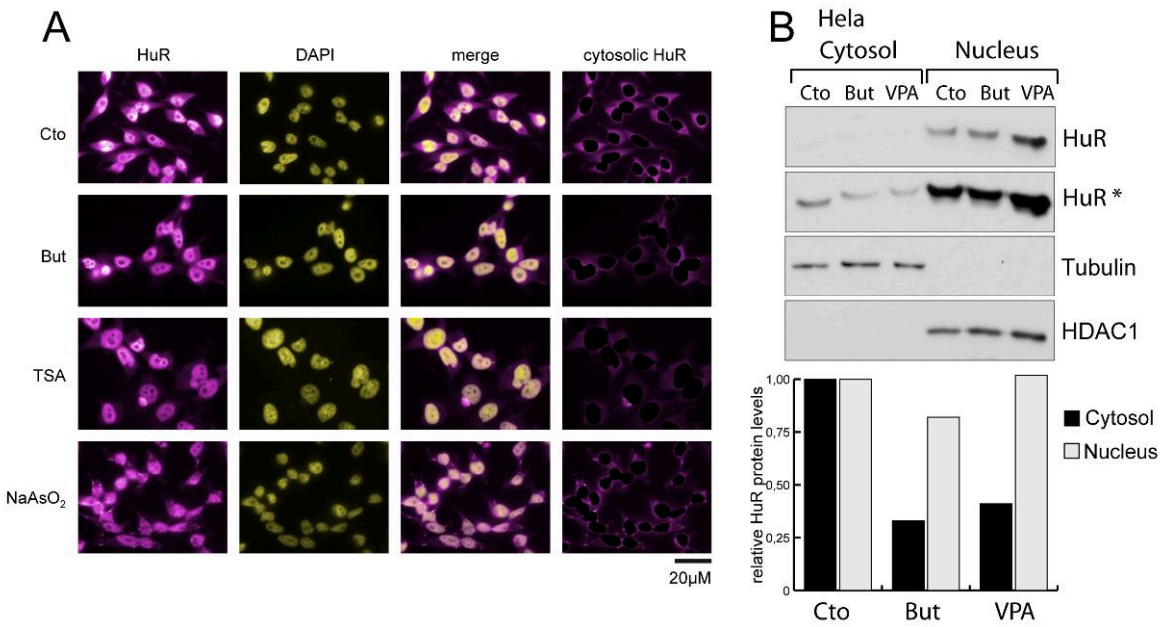


Figure 3 HDACi treatment affects HuR subcellular localisation and mRNA binding affinity

(a) The subcellular localisation of HuR upon HDACi treatment (3 mM butyrate and 100 nM TSA) was analysed in Hela cells after 24h by microscopy. P-bodies were induced by incubation with 0.5 mM $NaAsO_2$ for 45 minutes. The DNA dye DAPI was used as a control to visualise the nucleus. The mainly nuclear HuR complicated the detection and quantification of the cytosolic HuR levels. Therefore, the nuclear areas were removed in the right panel to facilitate the detection of cytosolic HuR levels.

(b) After a 24 h incubation of Hela cells with 3 mM butyrate or 3 mM VPA, cytoplasmic and nuclear extracts were analysed for the subcellular localisation of HuR (short and long (*) exposure). Loading controls for the cytosolic compartment (Tubulin) and for the nuclear compartment (HDAC1) were used to ensure proper fractionation. Densitometric analysis of the HuR protein bands in the different subcellular compartments is included (lower part).

Figure 4

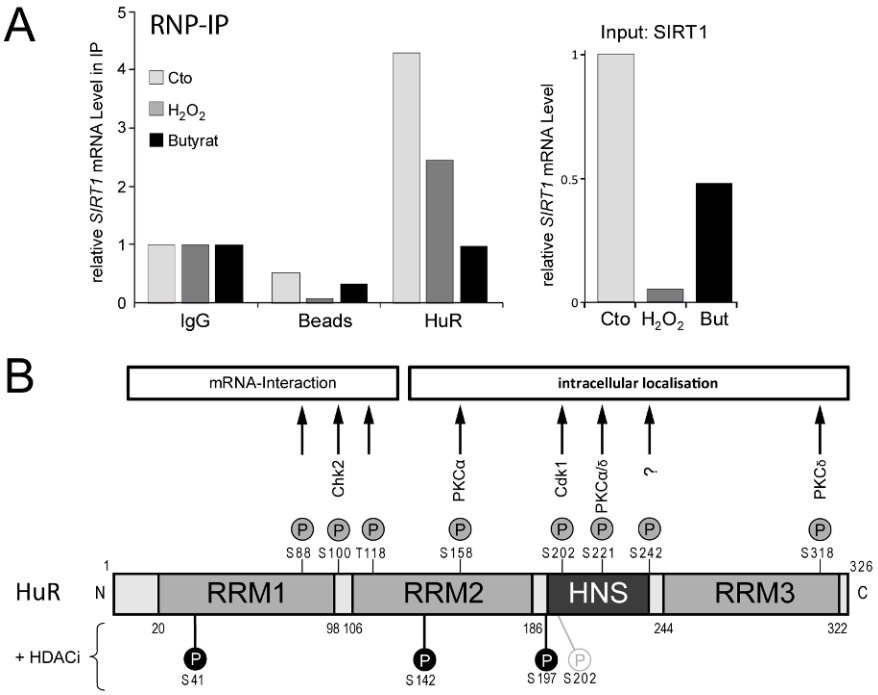


Figure 4 The phosphorylation pattern of HuR changes in response to HDACi treatment

(a) After incubating 293T cells for 24 h with 3mM butyrate, lysates were subjected to RNP-IP with an antibody specific for HuR. The depicted levels of HuR-bound *SIRT1* mRNA after HDACi treatment represent data from one representative experiment (n=3). H_2O_2 treatment was used as a positive control for the decrease of *SIRT1* mRNA binding to HuR.

(b) Schematic diagram of the HuR domain structure is depicted including published phosphorylation sites, the associated kinases and functional consequences. HuR was immunoprecipitated from Hela cell lysates after 24 h incubation with 3 mM But, separated by SDS-PAGE and further analysed by mass spectrometry. Phosphorylation sites identified by this approach are shown underneath the HuR domain structure.

Figure 5

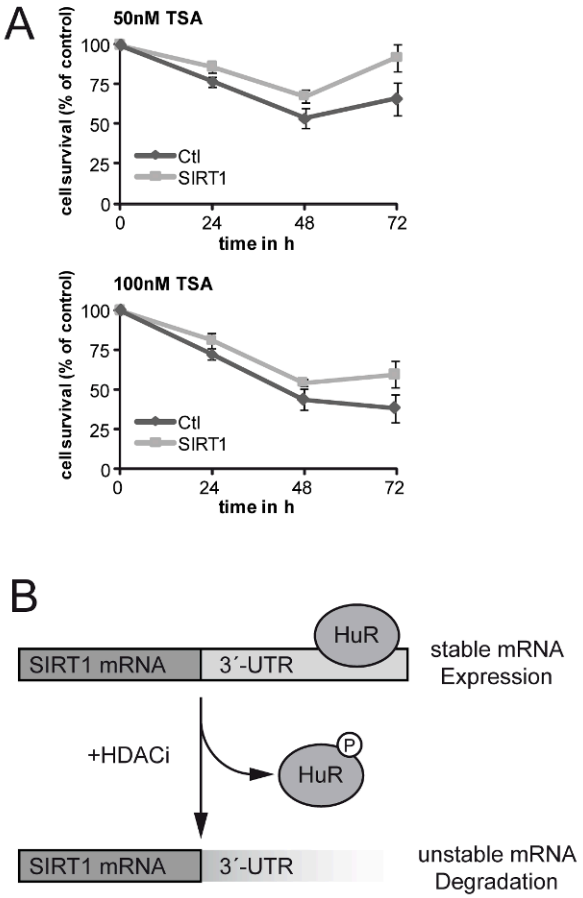
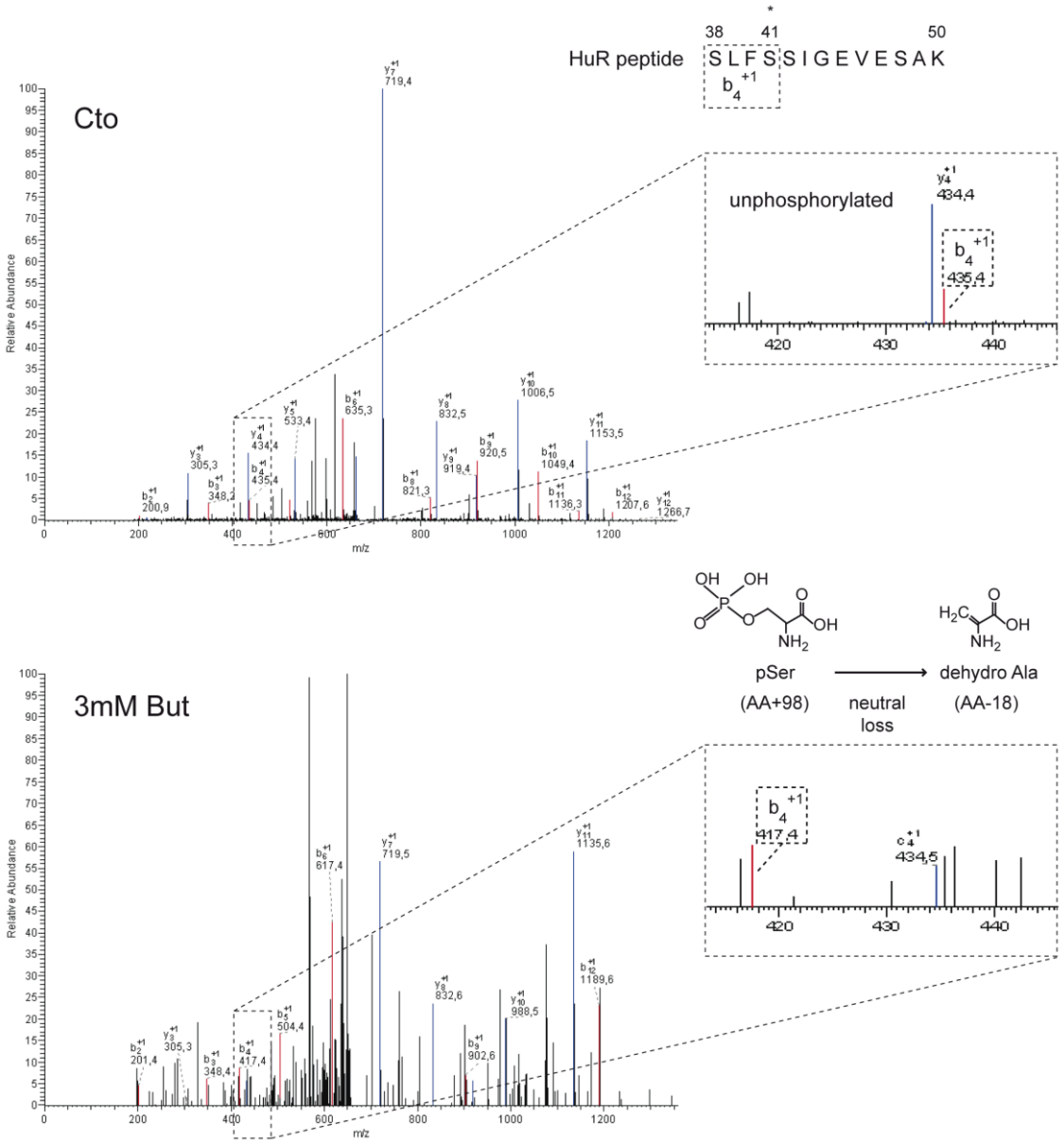


Figure 5 SIRT1 increases survival of HDACi-treated cells

(a) Hela cells were transfected either with control plasmid or pBabe-Ires-Neo-SIRT1-R. Cell survival was assessed in response to TSA (50 nM and 100 nM) after 24 h, 48 h and 72 h treatment by MTT assay.

(b) HuR protects and assures expression of *SIRT1* mRNA by binding to its 3´-UTR. Under HDACi treatment HuR becomes phosphorylated and dissociates from the mRNA. *SIRT1* mRNA devoid of HuR is prone to degradation by the mRNA decay machinery.

supplemental figure 1



Supplementary Figure 1 Mass spectrometric analysis of HuR
The HuR peptide SLFSSIGEVESAK (AA 38-50) was identified in control and treated lysates of Hela cells by MS. The ESI-MS blots (left panels) and an enlarged image detail (right panels) of this particular peptide show the fragment ions of the b- and y-series. The further MS/MS analysis was used for localisation of a particular phosphorylation event within the peptide. Treated cells show a mass loss of 18 at the SLFS peptide ion (b_4^{+1}) in treated cells. This characteristic neutral loss of the peptide confers to a phosphorylation event at Ser41 under HDACi-treatment. The identification of phosphorylated peptides was shown exemplarily at this peptide and applied to the other peptides accordingly.

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Manuscript Overview 3

Hydroxyurea and valproic acid synergize in the potent killing of head and neck cancer cells by downregulating EGFR and triggering BIM-induced apoptosis.

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Submitted

We show that the histone deacetylase inhibitor valproic acid (VPA) combined with the ribonucleotide reductase inhibitor hydroxyurea (HU) efficiently elicit HNSCC cancer cell deathVPA/HU enhances expression of the pro-apoptotic BCL-2 family protein BIM through transcriptional activation. We provide a molecular rationale for the potent anti-cancer activities of this drug combination, which efficiently eliminates HNSCC in murine tumour xenografts.

Drug-induced head and neck cancer cell killing by downregulating EGFR and triggering BIM-induced apoptosis

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Key words: apoptosis, BH3-only protein, chemotherapy, hydroxyurea, oral cancer, therapy resistance, tumor xenograft, valproic acid.

Abbreviations: α, anti; BCL, B cell lymphoma protein; BIM, B cell lymphoma 2 interacting mediator of cell death; ERK, extracellular signal regulated protein kinase; FFPE, formalin fixed paraffin embedded; HDACi, histone deacetylase inhibitor; HNSCC, head and neck squamous cell carcinoma; HU, hydroxyurea; IF, immunofluorescence; IHC, immunohistochemistry, i.p., intraperitoneally; PCD, programmed cell death; RNAi, RNA interference; T, trichostatin A; B, sodium butyrate; VPA, sodium valproat; STAT, Signal transducer and activator of transcription; vs, versus.

Hereby, we confirm that all authors agree with the submission of the report. The authors declare that there are no conflicts of interest.

ABSTRACT

Background

Head and neck squamous cell carcinomas (HNSCC) are characterized by resistance to therapy due to the overexpression of anti-apoptotic proteins and the epidermal growth factor receptor (EGFR), counteracting improvement of long-term survival.

Methods

Employing HNSCC cell lines, freshly isolated tumor cells and murine tumor transplantation models, we show that the histone deacetylase inhibitor valproic acid (VPA) combined with the ribonucleotide reductase inhibitor hydroxyurea (HU) efficiently trigger HNSCC cancer cell death. *In vitro*, VPA/HU treatment is superior in cancer cell killing when compared to cisplatin or the EGFR inhibitors cetuximab and gefitinib. VPA/HU enhances expression of the pro-apoptotic BCL-2 family protein BIM through AP-1 mediated transcriptional activation, whereas PUMA and BAX levels are not increased. The pro-apoptic activity of BIM in HNSCC was confirmed by ectopic overexpression and RNAi-mediated depletion studies. Also, significantly elevated BIM levels ($p<0.01$) were detectable in the apoptotic tumor centers *versus* proliferating tumor margins in HNSCC patients (n=31), underlining BIM's clinical relevance. Importantly, VPA/HU treatment additionally reduces expression and cell surface localization of EGFR.

Conclusion

We provide a molecular rationale for the potent anti-cancer activies of this drug combination, which efficiently eliminates HNSCC in murine tumor xenografts. A major advantage promoting clinical studies with VPA/HU is the fact that both agents have already been tested in the clinics, whereas other anti-tumor strategies targeting BCL-2 family members have unknown toxicity profiles and uncertain clinical efficacy. The VPA/HU combination will be of therapeutic interest most likely also for other tumor entities characterized by therapy resistance and EGFR activation.

INTRODUCTION

With a worldwide annual incidence of more than 640,000 cases, head and neck cancer is the sixth most common malignant neoplasm in humans (1, 2). The majority of head and neck squamous cell carcinoma (HNSCC) is induced by chronic exposure to a surplus of carcinogens enclosed in all forms of tobacco, synergized by heavy alcohol consumptions and/or is associated with oncogenic human papillomaviruses (3, 4). HNSCC is characterized by local tumor aggressiveness, high rate of early recurrences and development of second primary carcinomas (3). Loco-regional relapse after therapy is the major cause of death despite modern disease management strategies (5, 6). Hence, long-term survival rates, especially for advanced HNSCC (30-40%), have not improved significantly over the last decades (3, 6). Currently, EGFR-targeting agents, such as antibodies or tyrosine kinase inhibitors gained major clinical attention (3, 7). Despite encouraging developments, EGFR-directed therapies are effective only in a relatively small percentage of cancer patients underlining the need for additional treatment options (7).

Therapy resistance favoring recurring or advanced-stage HNSCC mainly results from failure of the tumor cells to undergo chemoradiation-induced apoptosis (1, 3). Particularly, the intrinsic or mitochondrial pathway of programmed cell death (PCD) plays an important role for killing cancer cells in response to various therapies, and is controlled by interactions among pro- and anti-apoptotic BCL-2 protein family members (8, 9). Pro-survival proteins like BCL-XL and BCL-2 inhibit apoptosis by binding and neutralizing the activities of the pro-apoptotic multidomain proteins BAX and BAK as well as the BH3 domain-only proteins BIM, BIK, NOXA, and PUMA (8-10).

Overexpression of anti-apoptotic BCL-2 proteins and apoptosis inhibitors like Survivin is known for HNSCC and plays a critical role for therapy resistance and overall survival (8, 9, 11). Consequently, strategies for neutralizing these cytoprotective factors involve shifting the cellular balance of anti- versus pro-apoptotic proteins in favor of the latter. As a consequence, proteins such as BIM (*B cell lymphoma 2 interacting mediator of cell death*) participate in the formation of a pore that permeabilizes the mitochondrial outer membrane.

Pro-apoptotic contents are exposed to the cytoplasm where they engage various aspects of the downstream apoptotic machinery (8, 9). Hence, permeabilization of the mitochondrial outer membrane is considered the point of commitment to cancer cell death.

In this respect, histone deacetylase inhibitors (HDACi), such as VPA, have emerged as promising chemotherapeutic agents by inducing a wide range of anti-tumoral activities, including induction of cell cycle arrest and apoptosis (12). HDACi can correct aberrant genomic and non-genomic signaling by chromatin remodeling as well as histone/protein modifications (13). The (pre)clinical efficacy of HDACi has been examined in various studies (12, 14, 15). Likewise, the ribonucleotide reductase inhibitor hydroxyurea (HU) sensitizes tumors to cancer therapy-induced apoptosis and has been used to treat HNSCC (16). However, it has not been investigated whether the combination of VPA and HU may be applicable for the treatment of HNSCC nor have molecular mechanisms underlying its potential anti-tumoral activity been resolved in detail.

Our study demonstrates for the first time that this drug combination efficiently eliminates HNSCC cancer cells by evoking expression of the pro-apoptotic protein BIM and by downregulation of EGFR. This potent dual anti-tumoral activity strongly suggests the clinical exploitation of this novel drug combination as a strategy to counteract therapy resistance in HNSCC.

MATERIAL AND METHODS

Cells, transfections and luciferase assay

Cultivation of the indicated head and neck cancer and other tumor cell lines has been described in detail *(17-21)* (Supplementary Table SI). Cell lines constitutively expressing shRNA directed against BIM or a scrambled control were generated by transfection of pHR-THT-BIMshRNA-SFFV-eGFP or pHR-THT-scr_shRNA-SFFV-eGFP *(22)*, respectively. Cells were selected by addition of puromycin (1 µg/ml; Sigma Aldrich, Munich, Germany). Cells were transfected using PEI (Sigma Aldrich, Munich, Germany) or Lipofectamine (Invitrogen, Karlsruhe, Germany). Luciferase assays were carried out as stated *(19, 23)*. All reporter experiments were performed out in triplicate and repeated thrice.

Microscopy and image analysis

Observation, image analysis and quantification of protein localization were performed as described *(24)*. DNA/cell nuclei were visualized by Hoechst 33258 staining (Sigma Aldrich, Munich, Germany) according to *(24)*. At least 100 fluorescent cells were analyzed in three independent experiments.

Patients, tissue sampling and primary tumor cell isolation

Biopsies of patients diagnosed with HNSCC and treated at the Departments of Oral and Maxillofacial Surgery and ENT of the University Hospitals in Frankfurt and Mainz were analyzed. Tumor specimens were collected from primary tumors of patients who underwent surgery. All cases were clinically and histologically diagnosed according to established criteria including grading and TNM-classification (Supplementary Table SII). Studies of human tissue biopsies were performed according to the requirements of the local ethics committee, and informed consent was obtained in accordance with the Declaration of Helsinki. For the isolation of primary cancer cells, tumor specimens were cut into small pieces and enzymatically digested with collagenase typeI/hyaluronidase (Sigma Aldrich, Munich, Germany) in RPMI-1640 (Invitrogen, Karlsruhe, Germany) at 37°C overnight. Following digestion, dissociated cells were passed through a cell strainer, and epithelial cancer cells separated from stromal cells by MACS® separation using CD326 (EpCAM)

MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. Cells were propagated for one week as described *(25)* and subjected to analysis.

Drug treatment and clonogenic survival assay

Cells were treated with VPA, trichostatin A (T), sodium butyrate (B), HU, or cisplatin (Sigma Aldrich, Munich, Germany) as described *(15, 25)*. The EGFR antagonists gefitinib (Tocris Bioscience, Ellisville, USA) and cetuximab (ImClone, New York, NY, USA) were applied for 48 h. For folony formation assays, $1x10^3$ cells/T25 flask were seeded in triplicate. 24 h later, cells were treated with the indicated compounds or PBS control and further cultivated for 10 days. Drug-containing medium was replaced every day. Cells were fixed and stained with Giemsa. Colonies containing >50 cells were counted automatically using a colony counter (Oxford Optronics, Oxford, United Kingdom). Data shown are calculated from the mean values of three independent experiments.

Antibodies (Ab)

Ab were: α-PUMA (4976) (NEB Cell Signaling, Frankfurt, Germany); α-Survivin (Novus NB 500-201; Novus Biologicals, Littleton, CO); anti-ß-Actin (A2066), α-BIM (B7929), anti-alpha-Tubulin (T5168) (Sigma Aldrich, Munich, Germany); α-BCL-XL (66461A), α-Caspase-8 (9745), -9 (9501) (Pharmingen); cleaved Caspase-3 (9664) (Cell Signaling); α-BAX (sc-20067), α-Caspase-3 (sc-7272/-7148), α-EGFR (sc-81449), α-ERK1/2 (sc-135900), α-STAT3 (sc-482) (Santa Cruz Biotechnology, Heidelberg, Germany). Appropriate HRP-, Cy3- or FITC-conjugated secondary antibodies (Sigma Aldrich, Munich, Germany; Santa Cruz Biotechnology, Heidelberg, Germany) were used.

Protein extraction, immunoblot analysis and immunofluorescence

Preparation of whole lysates from cells or tissue, co-immunoprecipitations and immunoblotting were carried out as described *(18, 19)*. Equal loading of lysates was controlled by reprobing blots for Actin or Tubulin as described *(19)*. Immunofluorescence was performed as described in detail *(19, 24)*.

Immunohistochemistry (IHC)

Tissue samples or transfected cell pellets were formalin fixed, paraffin embedded (FFPE) and processed for IHC as described *(17, 18)*. For antigen retrieval, sections were treated in a pressure cooker with Tris buffer (10 mM, pH9.0) for BIM or were treated with proteinase K (S3020, DakoCytomation, Glostrup, Denmark) for 8 min at room temperature for EGFR detection. Sections were incubated with primary Ab (α-BIM, 1:800; α-EGFR 1:50) overnight at 4°C. For visualization, the EnVision® detection system (Dako GmbH, Hamburg, Germany) was applied as described *(17)*. Sections were counterstained with hematoxylin. Negative control slides without primary Ab were included for each staining. For quantification, sections were scanned at low power to identify areas of positivity and three random fields were selected. Expression levels for BIM were scored semi-quantitatively based on staining intensity and distribution using the immunoreactive score (IRS) *(17)*. IRS=SI (staining intensity) x PP (percentage of positive cells). SI is assigned as 0, negative; 1, weak; 2, moderate; 3, strong. PP is defined as 0, negative; 1, <5%; 2, 6–30%; 3, 31–60%; and 4, >60% positive cells.

Measurement of apoptosis, cell cycle and viability

Assessment of apoptosis was performed by quantifying Caspase-3-dependent hydrolysis of a fluorogenic substrate and by immunoblot-based detection of cleaved caspases *(19)*. Apoptotic cells were visualized by analyzing mitochondrial integrity using the *PromoKine Mitochondrial Apoptosis Staining Kit* (PromoCell; Heidelberg, Germany), staining of fragmented nuclei with Hoechst dye or TUNEL-staining according to *(26)*. Briefly, 200 cells from three separate images were inspected and the percentage of apoptotic cells determined. Cell viability was calculated employing MTT-assays and the electric sensing zone method according to *(18, 19)*. Cell cycle profiles were obtained by FACS-mediated analysis of prodidium iodide (PI) stained cells according to (15).

Xenograft tumors

FaDu cells ($2x10^6$) cells were implanted into both flanks of four-week-old female NMRI *nu/nu* mice (Harlan Winkelmann, Hamburg, Germany) *(26)* and were allowed to establish for

seven days followed by treatment for 14 d. VPA/HU (350 mg/kg, 750 mg/kg body weight) or PBS control was administered intraperitoneally (i.p.) everly second day as described *(23)*. Mice were randomized into groups (4 mice/group) such that the average tumor volumes across the groups were equal. Tumor growth was monitored using calipers to calculate tumor volumes according to the formula: length x π width^2 x 0.52. Animals were euthanized at the end of the study, and the tumors processed for IHC analysis as described *(26)*. All animal experiments were approved by the Institutional Animal Care and Use Committees at the Universities of Erlangen and Mainz.

Statistical analysis

For all experiments stating *p*-values, a paired Student's t-test was performed. Unless stated otherwise, *p*-values represent data obtained from three independent experiments done in triplicate. *p*-values <0.05 were considered as significant.

Plasmids and RNAi

The expression construct for human BIM_{EL}, pCDNA4/TO-BIM_{EL}, was described *(27)*. For expression of a BIM_{EL}-GFP fusion, BIM_{EL} cDNA was PCR amplified and cloned into pc3-GFP (pc3BIM_{EL}-GFP) as described *(24)*. pGL3-luciferase reporter constructs containing the *BIM* promoter, MYB, E2F or AP1 binding sites have been described *(28)*. Lentiviral vectors constitutively expressing shRNA directed against BIM or a scrambled control, pHR-THT-BIMshRNA-SFFV-eGFP or pHR-THT-scr_shRNA-SFFV-eGFP, respectively were reported *(22)*.

RESULTS

VPA and HU synergize in the killing of HNSCC tumor cells and loss of clonogenicity

Cell lines representing HNSCC from different anatomical sites (Supplementary Table SI) were treated with VPA and HU alone and in combination. MTT assays revealed that although VPA and HU individually inhibited proliferation in a dose-dependent manner, co-administration of VPA/HU was most effective (Figure 1A and B; Supplementary Table SI). Similar results were obtained using a clonogenic cell survival assay (Figure 1C). FACS analysis showed that the VPA/HU combination potently induced apoptosis and confirmed that HU induced S-phase arrest (Figure 2A; Supplementary Figure S1A). Induction of cell death was already evident using a single dose of 0.3 mM VPA/HU and was not dependent on repetitive drug administration (Figure 2A). VPA/HU-induced apoptosis was further confirmed by independent experimental approaches. Immunoblot analysis showed enhanced cleavage of Caspases-3, -8 and -9 (Figure 2B; Supplementary Figure S1B). Also, increased Caspase-3 activity was detectable in lysates from treated cells, which could be counteracted by the pan-Caspase inhibitor Z-VAD-FMK (Figure 2B). The observed cleavage of Caspase-9, the loss of mitochondrial integrity, and DNA-fragmentation upon treatment strongly imply that the intrinsic apoptosis pathway is responsible for VPA/HU induced cell death (Figure 2C; Supplementary Figure S1C). Data for representative cell lines are shown. Similar results were obtained for other cell lines tested (Supplementary Table SI; data not shown).

Induction of the pro-apoptotic protein BIM by VPA/HU treatment correlates with cell death

When analyzing the effects of VPA/HU treatment on the levels of pro- and anti-apoptotic BCL-2 proteins, we observed increased BIM levels (Figure 3A). Although both drugs slightly induced expression of BIM, the effect was most prominent using the VPA/HU combination, correlating with enhanced apoptosis. The doses required to induce appreciable Caspase-3 activation and apoptosis were comparable to those necessary to induce BIM expression (Figure 2 and 3A). Notably, this effect was not restricted to VPA as treatment with other HU/HDACi combinations, such as with TSA or butyrate, also resulted in BIM induction and

cell death (Supplementary Figure S1D; data not shown). Another BH-3-only protein, p53-upregulated modulator of apoptosis (PUMA), was recently reported to mediate apoptosis induced by EGFR inhibitors in HNSCC cells (10). In contrast to the strong induction of BIM by VPA/HU, immunoblot analysis revealed no enhanced expression of PUMA, BAX, and BCL-2/BCL-xL (Figure 3G and data not shown). Moreover, VPA/HU could induce BIM in p53-negative PC3 cells, and BIM induction could be verified by independent methods in p53-mutant FaDu cells (Supplementary Figure S1E, S2A).

BIM is critical for VPA/HU-induced apoptosis in HNSCC cells

To demonstrate that enhancing BIM levels triggers apoptosis in HNSCC cell lines, we first performed ectopic overexpression studies. Transfection of plasmids encoding a BIM_{EL}-GFP fusion or untagged BIM_{EL}, the longest BIM isoform (196 amino acids), resulted in efficient cell death (Figure 3B and not shown).

In order to confirm direct relevance of BIM for VPA/HU-induced apoptosis, we used RNAi to deplete endogenous BIM. HNSCC cells with attenuated BIM expression displayed enhanced proliferation linked to reduced basal apoptosis rates (Figure 3C and data not shown). Furthermore, compared to the scrambled-control, cell lines with BIM levels reduced by BIM-specific shRNAs showed significantly enhanced resistance to VPA/HU-induced cell death, as verified by analyzing Caspase-3 activation, TUNEL-staining and loss of mitochondrial integrity (Figure 3D; Supplementary Figure S2B). Collectively, these results provide strong evidence that BIM is critical for the VPA/HU-induced killing of HNSCC cells.

VPA/HU enhances BIM expression via AP1-dependent transcriptional activation

Increased BIM levels (Figure 3E) could be the result of transcriptional activation *(28, 29)*. Transfection of a BIM promoter-containing luciferase reporter revealed that VPA/HU indeed stimulated BIM transcription (Figure 3F). To date, several transcription factors have been reported to regulate BIM transcription *(9, 28)*. VPA/HU-induced BIM expression was observed also in cells bearing inactive p53 (FaDu) or p53-negative cells (PC3) (Supplementary Figure S1D and E), and a BIM reporter containing an inactivated MYB-binding site was still responsive to VPA/HU treatment (Supplementary Figure S2C).

Transfection of HNSCC cells with E2F- or c-JUN/FOS (AP1)-dependent reporter constructs finally demonstrated the crucial role of AP1 for the VPA/HU-mediated transcriptional activation of BIM (Supplementary Figure S2D). Although pharmacological inhibition of ERK signaling was critical for BIM expression in B-RAF/K-RAS mutant lung tumor cells (9), VPA/HU did not affect ERK levels but still evoked enhanced BIM expression in our cell models (Supplementary Figure S2E).

BIM expression in tumor biopsies from head and neck carcinoma patients

To show that BIM is expressed also in HNSCC patients, we first visualized BIM expression by IHC in tumor biopsies (n=31). Using the immunoreactive score (IRS) *(17)*, significantly elevated BIM levels ($p<0.001$) were observed in cancer cells in the apoptotic tumor centers *versus* proliferating tumor margins (Figure 4A and 4B). Hence, regulated BIM expression appears to be relevant for disease progression and outcome. Second, to definitely demonstrate that BIM is induced by VPA/HU also in primary tumor cells triggering apoptosis, we tested cancer cells freshly isolated from HNSCC patients. Treatment of such tumor cells with VPA/HU resulted in enhanced BIM levels and cancer cell death (Figure 4C).

VPA/HU attenuates expression and cell surface localization of EGFR. The EGF receptor is overexpressed in epithelial malignancies and represses BIM expression *(9, 30)*. As EGFR-targeting strategies are currently tested in the clinics for HNSCC, we investigated the effects of VPA/HU treatment on this receptor. Interestingly, immunoblot analysis revealed that the combination of VPA/HU efficiently reduced the levels of total and phosphorylated EGFR (Figure 5A). To further examine the intracellular localization of EGFR, cells were treated with VPA/HU or PBS, FFPE and examined by IHC analysis. This analysis not only confirmed the reduction of EGFR levels, but also showed that such treatment attenuated the cell surface localization of the receptor and enhanced BIM expression (Figure 5B). As a control, VPA/HU treatment appears not to cause a general attenuation of pro-survival proteins, as STAT3 levels, an important factor for head and neck carcinogenesis *(31)*, were not significantly affected (Supplementary Figure S2E). Collectively, these data provide evidence for a hitherto

unknown molecular mechanism explaining the potent anti-cancer activity of the VPA/HU combination.

VPA/HU efficiently suppresses HNSCC tumor growth in murine xenotransplantation models

Prior to testing the anti-tumoral activity of VPA/HU in murine models, we first evaluated the cell killing activity of VPA/HU and of chemotherapeutic drugs currently used in the clinics. In our HNSCC cell culture models, VPA/HU treatment was more effective in triggering cell death, when compared to the EGFR inhibitors cetuximab and gefitinib or the DNA-damaging agent cisplatin (Supplementary Figure S2F).

These *in vitro*-results encouraged us to examine whether VPA/HU treatment also inhibits tumor growth *in vivo*. Using a xenograft model, established FaDu tumors were treated with VPA/HU (350 mg/kg, 750 mg/kg body weight) or PBS control i.p. for 14 days. Administration of VPA/HU to FaDu tumor-bearing mice significantly inhibited tumor growth ($p<0.001$) (Figure 5C). To visualize whether drug treatment also enhanced BIM levels and caused EGFR attenuation *in vivo*, tumors from treated and control animals were analyzed by IHC. Enhanced BIM levels and reduced EGFR expression were observed in tumors from VPA/HU treated animals compared to those from control mice (Figure 5D). The above data not only confirmed the potent anti-cancer activity of the VPA/HU combination *in vivo*, but also demonstrated the *in vivo*-relevance of the molecular mechanisms identified in our cell culture models.

DISCUSSION

Employing comprehensive *in vitro* and *in vivo* models we here demonstrate for the first time that combining HDACi with a ribonucleotide reductase inhibitor potently kills HNSCC. Although such agents have been shown to individually affect tumor cells *(16, 32)*, the (pre)clinical anti-tumor activities of the VPA/HU combination as well as the underlying molecular mechanisms have not been investigated so far for HNSCC.

Treatment of malignant cells with HDACi can induce a wide range of anticancer effects including apoptosis, cell cycle arrest, differentiation as well as immunomodulatory effects *(12, 33-35)*. Hence, numerous HDACi have been tested in the clinics or are currently the subject of ongoing early-phase clinical trials, including HNSCC *(14, 36-38)*. Since HDACi monotherapies seem not to be effective against solid tumors, their full therapeutic potential will be best realized through combination with other anticancer agents *(15, 34)*. However, most reports do not provide a well-defined molecular rationale for combining an HDACi with a given agent. Moreover, the molecular and biological events that underpin any observed additive or synergistic combination effects are largely lacking *(15, 34)*.

In contrast, we here provide convincing evidence that activation of the proapoptotic BH3-only protein BIM is a key regulator for VPA/HU-induced tumor cell death. This conclusion is based on several lines of evidence: First, freshly isolated tumor cells from HNSCC patients responded to VPA/HU administered at therapeutically achievable levels with BIM induction and apoptosis. These results strongly support an expectable clinical efficacy of VPA/HU independent from the fact that permanent tumor cell lines may differ dramatically from primary tumor cells at their molecular level *(39)*. Furthermore, VPA/HU induced BIM upregulation, induction of apoptosis and loss of the clonogenic potential of HNSCC cell lines derived from different anatomical sites. This finding will be clinically relevant as SCC from different anatomical regions, like the hypopharynx or the oral cavity, differ drastically in their clinical prognosis and response (6). Second, ectopic expression and RNAi experiments convincingly demonstrated that BIM is essential for VPA/HU-induced cancer cell death. Third, VPA/HU efficiently prevented progression of HNSCC tumors in nude mice correlating

with enhanced BIM levels. The tumor growth delay achieved with the combination treatment was highly significant compared with the untreated control, without major toxicity.

BIM-evoked apoptosis can be crucial for epithelial tumor cell death triggered by anti-cancer therapeutics *(9, 40, 41)*. Consistent with this notion, we found strong induction of the intrinsic cell death pathway via mitochondrial Cytochrome C and Caspase-9. Although the BH-3-only protein PUMA was recently reported to mediate apoptosis of HNSCC cell lines induced by EGFR tyrosine kinase inhibitors (10), VPA/HU-mediated PCD did not require induction of the p53 target genes PUMA and BAX. Notably, HNSCC cells with attenuated BIM expression even displayed enhanced proliferation. Our finding suggests that lowering endogenous pro-apoptotic factors not only increases tumor cell survival but also proliferation is potentially relevant for HNSCC therapy response and progression. Although we have shown that BIM plays a major role in death signaling, this does not rule out the additional participation of other BCL-2 family members and/or other apoptosis inhibitor proteins (1, 10). Remaining apoptosis rates of HU/VPA-treated cells with attenuated BIM expression are probably mediated via alternative pro-apoptotic proteins and/or the activation of the extrinsic death pathway *(10, 42)*.

As we did not observe increased BCL-2/BCL-xL levels upon VPA/HU treatment potentially neutralizing increased BIM expression, it is conceivable to speculate that the addition of BH3 mimetics, such as ABT-737, may not further boost tumor cell death. In contrast, killing of *B-RAF* mutant lung tumor cells required BIM induction by inhibition of ERK signaling combined with ABT-737, whereas *B-RAF* wild type cancer cells were even largely resistant to this treatment *(29)*. As the frequency of *RAF/RAS* mutations in HNSCC is rather low *(43, 44)*, VPA/HU is thus likely to be clinically more effective when compared to certain other attempts to alter BCL-2 family members (9).

Increased BIM levels could be the result of reduced proteasomal degradation or enhanced transcriptional activation *(8, 9, 45)*. We found that VPA and HU activate BIM at the transcriptional level. To date, several transcription factors, including p53, E2F, c-JUN/FOS (AP1), MYB, RUNX3, and FOXO3A have been reported to regulate *BIM* transcription *(9, 28,*

46). Our data collectively support a crucial role of AP1 for VPA/HU-mediated transcriptional activation of *BIM* in HNSCC. This finding seems to be of clinical relevance as in contrast to p53, which is mutated in the majority of HNSCC *(47)*, the AP1 system is intact in cancer patients *(48)*. S-phase-dependent induction of c-JUN could be a mechanism enhancing BIM expression *(49)*. Of note, the activity of c-JUN is repressed by the HDAC3-NCoR complex *(50)*, which is particularly sensitive to inhibition by VPA *(12, 23)*. Phosphorylation of c-JUN by JUN kinase (JNK) permits dissociation of HDAC3 and c-JUN-dependent transcription *(50)*. HU has been shown to activate JNK *in vivo (51)*, which thereby could contribute to c-JUN-dependent *BIM* induction. Our results do not contradict the reported activation of BIM via E2F and MYB, as these data were collected in different cell systems with different stimuli *(28)*.

HNSCC tumors are often characterized by deregulated EGFR signaling due to receptor overexpression, activating receptor mutations and aberrant downstream signaling cascades. Survival is secured by the sequential phosphorylation and activation of MEK and ERK kinases, leading to stabilization of MCL-1, activation of BCL-2, and degradation of BIM. Pharmacogenetic approaches interfering with EGFR signaling trigger apoptosis by enhancing BIM expression (9). Importantly, we demonstrate for the first time that VPA/HU treatment efficiently reduced not only EGFR levels, but also attenuated its cell surface localization *in vitro* and in tumor xenografts. The underlying mechanisms remain to be resolved in detail. These may involve HU-induced replication arrest, known to attenuate oncogenic tyrosine kinase signaling *(52)*, and/or the E3 ubiquitin ligase c-CBL, which controls EGFR ubiquitination and lysosomal degradation *(53)*.

A major advantage allowing now to expedite clinical studies using VPA/HU for the treatment of HNSCC is the fact that both agents have already been used in the clinics *(16, 37)*. Thus, one can rely on an extensive knowledge on the therapeutically most effective dose and pharmacodynamics of these drugs *(32, 38)*. The safety profiles of HDACi and HU have been favorable, especially in comparison to traditional cytotoxic chemotherapy. Toxicities common to most HDACi tested as well as to HU, are fatigue, nausea, and diarrhea *(16, 32, 37, 38, 54)*. Compared with other anti-tumor strategies targeting BCL-2 family

members *(29)*, for which toxicity profiles and clinical efficacy are not yet known, this advantage will allow to swiftly translating our findings into the clinical arena. Although recent targeting strategies such as antibodies directed against EGFR (cetuximab) or the Vascular Endothelial Growth Factor (bevacizumab), have gained major attention, the clinical response rates to such therapies are rather low (7, 16). In addition, whereas these agents are expensive and often show a suboptimal pharmacodynamic profile, VPA and HU are cheap and stable drugs, which can be administered orally (14, 16). Moreover, as the VPA/HU combination also attenuates EGFR, it may represent a contingency treatment option for patients acquiring resistance to EGFR-targeting approaches *(55)*. Although not examined in this study, suffice it to say that the VPA/HU combination may be of therapeutic interest also for other tumor entities, characterized by therapy resistance and EGFR overexpression, such as colon cancer.

Despite our pre-existing knowledge and the potent dual anti-tumor activity of VPA/HU with tolerable toxicity in mice shown here, it remains now to be demonstrated that this drug combination shows therapeutic efficacy without dose-limiting toxicities in the clinic.

NOTES

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FIGURES

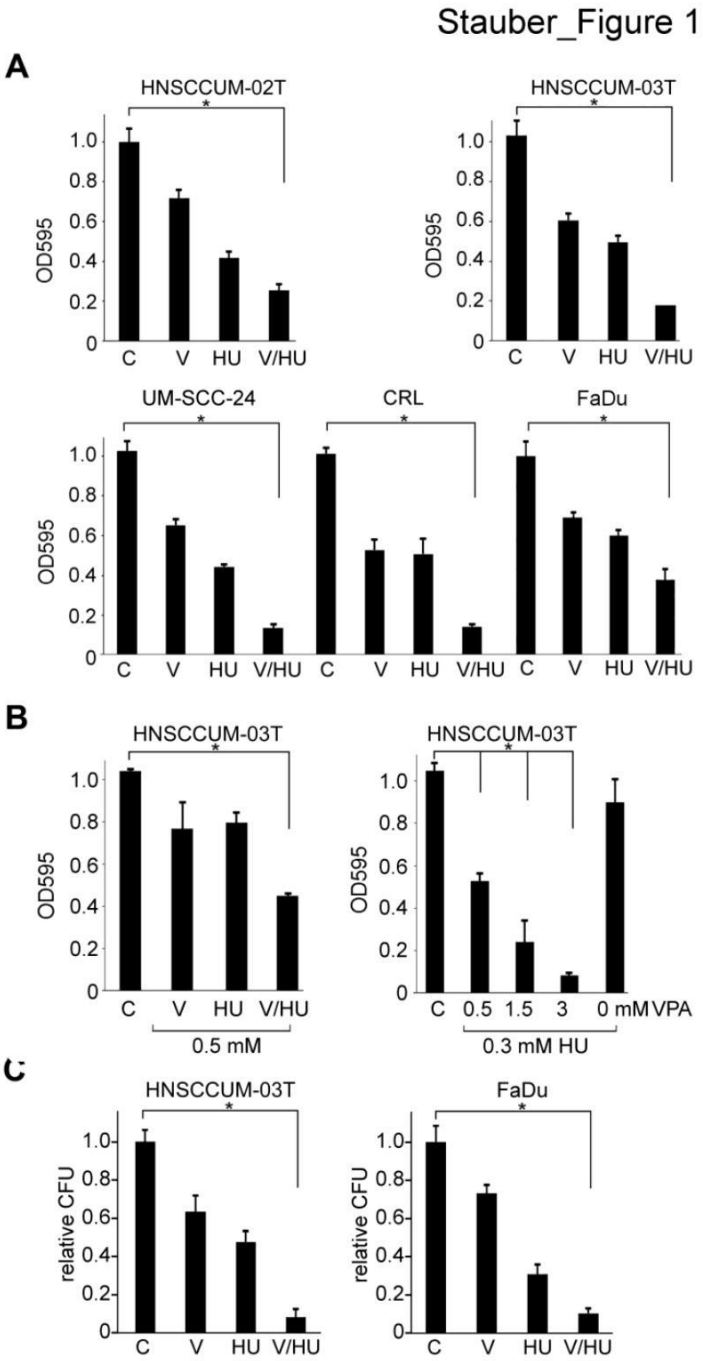

Fig. 1. VPA and HU synergize in HNSCC cells growth inhibition and loss of clonogenicity. Columns, mean; bars, ±SD from three independent experiments. **A**) Indicated cell lines were treated with VPA (V), hydroxyurea (HU), VPA/HU (1.5 mM each) or PBS (C; set as 1) for 48 h, and proliferation was analyzed by the MTT assay. **B**) Treatment was performed with the indicated drug combinations or PBS (C; set as 1). Cell proliferation was assessed with MTT. **C**) VPA/HU affects clonogenic cell survival. Cells were seeded and 24 h later treated with the indicated compounds or PBS. Surviving colonies were counted 10 d later and displayed as colony forming units (CFU) relative to the PBS control (C; set as 1).

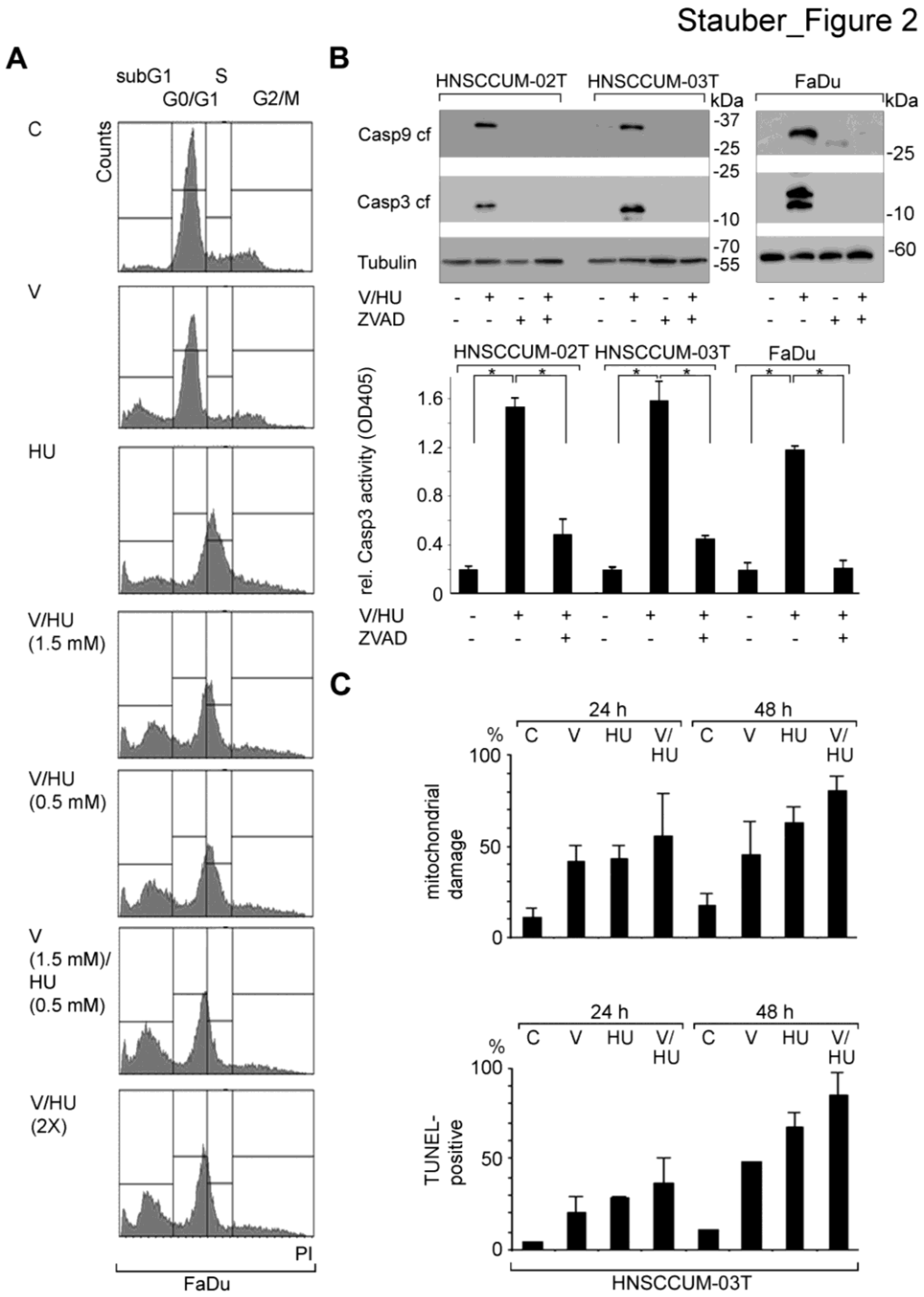

Fig. 2. VPA and HU efficiently trigger apoptosis in HNSCC tumor cells. **A**) Drug-induced apoptosis was determined by measuring the sub-G1 population by flow cytometry (PI staining) 48 h post treatment. Induction of cell death was already evident using a single dose of VPA/HU (1.5 mM/0.5 mM) and was not further enhanced by additional drug administration after 24 h (VPA/HU 2X). **B**) VPA/HU treatment (1.5 mM each; 48 h) induced caspases activation sensitive to the pan-Caspase inhibitor Z-VAD. Immunoblot analysis demonstrated cleavage of Caspase-3 and -9 (upper panel; tubulin, loading control. Apoptosis was quantified by measuring Caspase-3 activity in cell lysates (lower panel). **C**) VPA/HU-induced cell damage shown by analyzing mitochondrial integrity and by TUNEL-staining. The VPA/HU combination (1.5 mM each) caused significant mitochondrial damage already 24 h post treatment, resulting in loss of dimeric *MitoCapture* dye staining (upper panel). TUNEL-staining revealed VPA/HU-induced DNA-damage indicative of apoptotic cells (lower panel).

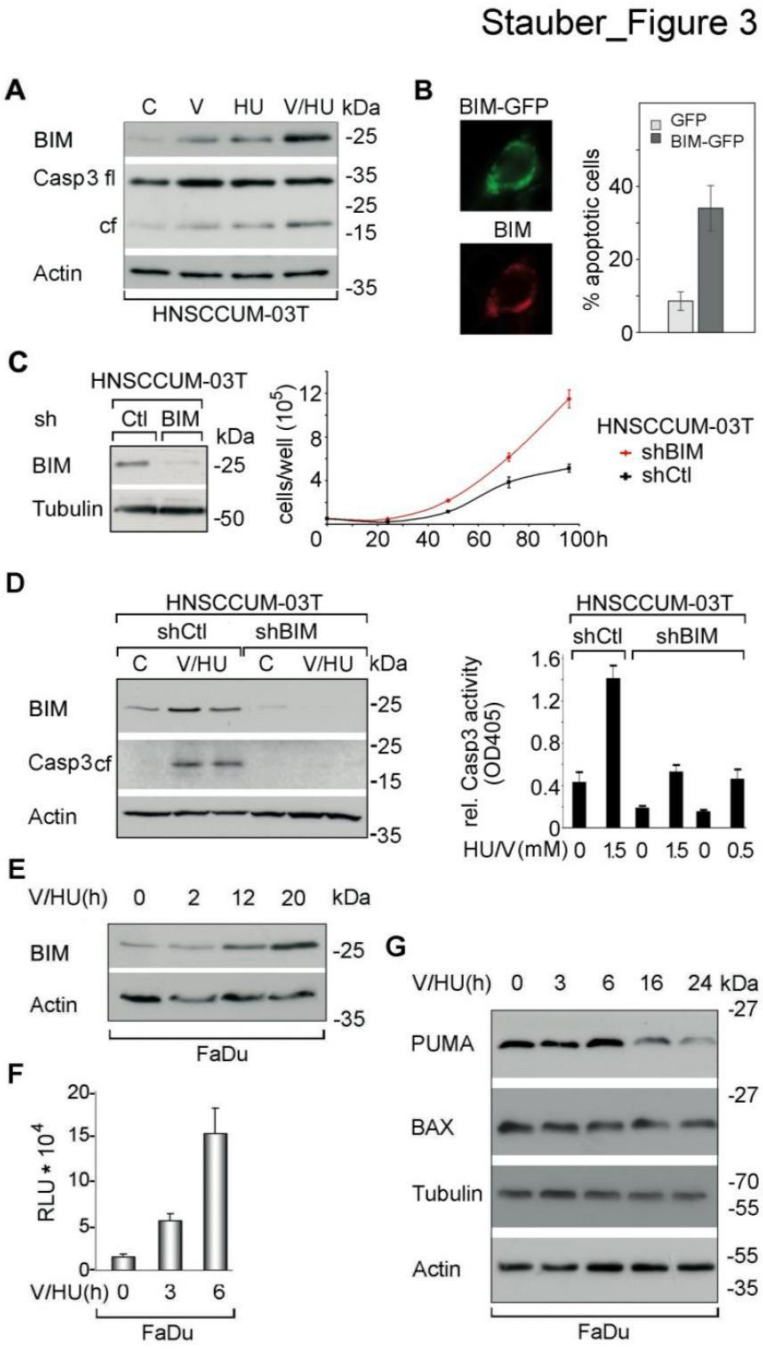

Fig. 3. VPA/HU-treatment specifically induces the BCL-2 family protein BIM modulating cell proliferation and apoptosis. **A**) Cells were drug treated (1.5 mM; 20 h) and expression levels of the indicated proteins were visualized by immunoblot. Actin served to control equal loading of cell lysates. **B**) Cell death induction by ectopic expression of BIM_{EL}-GFP. BIM_{EL}-GFP was visualized 24 h post transfection in FaDu cells by direct and indirect immunofluorescence using α-BIM Ab. **C**) Downregulation of BIM in HNSCCUM-03T cells stably transfected with BIM- (shBIM) *vs* scrambled-shRNA (shCtl) verified by immunoblot. Counting revealed that cells with attenuated endogenous BIM levels displayed enhanced proliferation. **D**) Decreased VPA/HU-induced apoptosis (1.5 mM each, 24 h) in BIM-depleted cells shown by immunoblot analyses for BIM and cleaved Caspase-3 (left), as well as by quantification of enzymatic Caspase-3 activity in cell lysates (right). **E**) Immunoblot revealed that VPA/HU (1.5 mM each) induced BIM in a time-dependent manner. **F**) VPA/HU-mediated transcriptional activation was monitored by analyzing luciferase activity. FaDu cells transfected with a BIM reporter were treated with VPA/HU (1.5 mM each). **G**) In contrast to the strong induction of BIM levels by VPA/HU, correlating with Caspase-3 cleavage, no enhanced expression of PUMA and BAX was induced by VPA/HU. Actin and Tubulin served as loading controls. Columns, mean; bars, ±SD from three independent experiments.

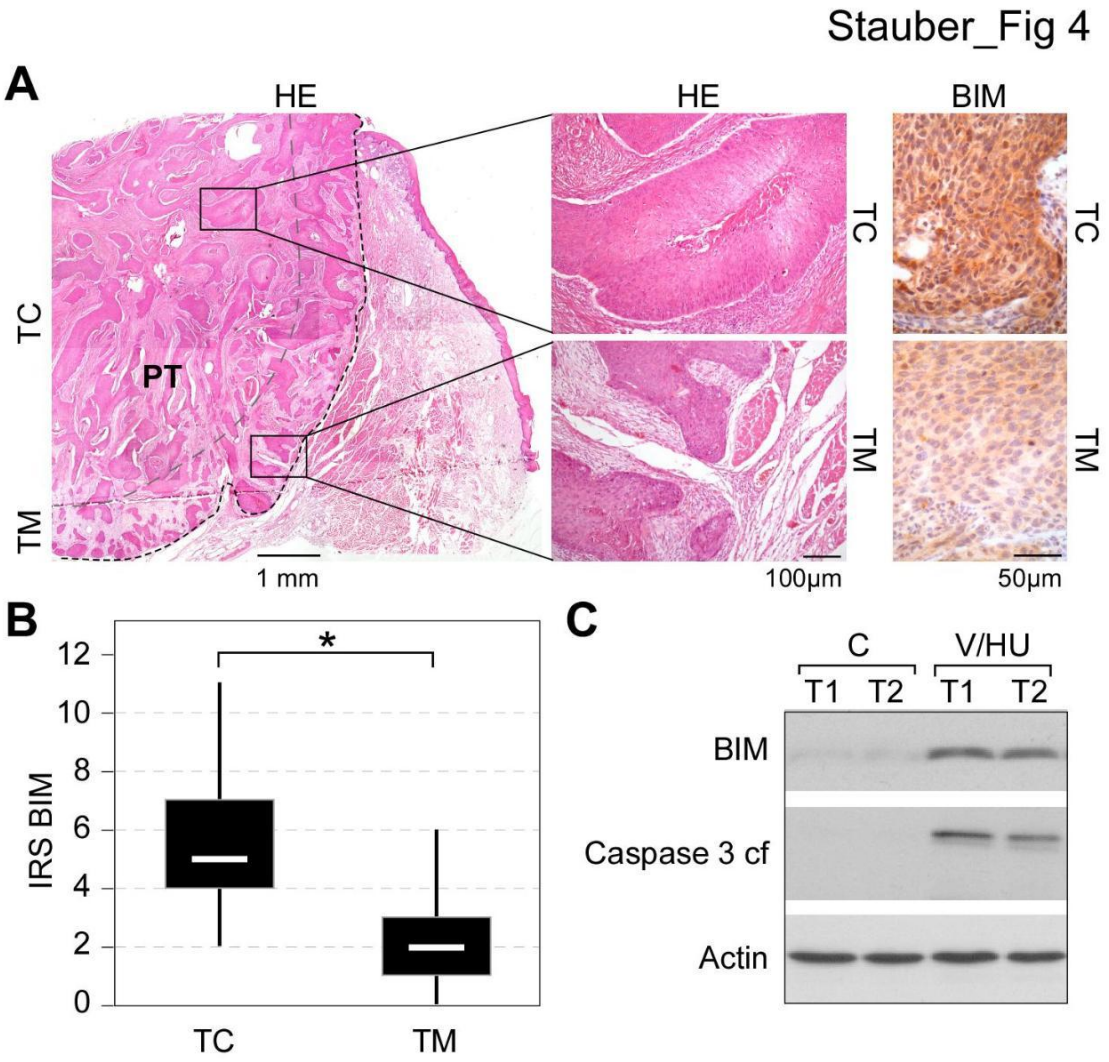

Fig. 4. BIM expression in tumor biopsies from head and neck carcinoma patients. **A**) Detection of BIM in HNSCC tumor centers (TC) *vs* proliferating tumor margins (TM). Representative example of an oral SCC (G2, pT3, pN0) stained with hematoxylin/eosin (HE) (left panel) and immunohistochemical visualization of BIM using α-BIM Ab (right panel). **B**) Box plot (with range) for BIM IRS reveals enhanced BIM expression in the TC in HNSCC patient biopsies (*p<0.001; n=31). **C**) Treatment of freshly isolated tumor cells from two patients (T1: Hypopharynx, G2, pT3, pN0; T2: oral cavity, G3, pT3, pN0) with VPA/HU (1.5/0.5 mM) for 48 h resulted in BIM induction and Caspase-3 activation. Indicated proteins were detected by immunoblot analysis. Actin served as loading control.

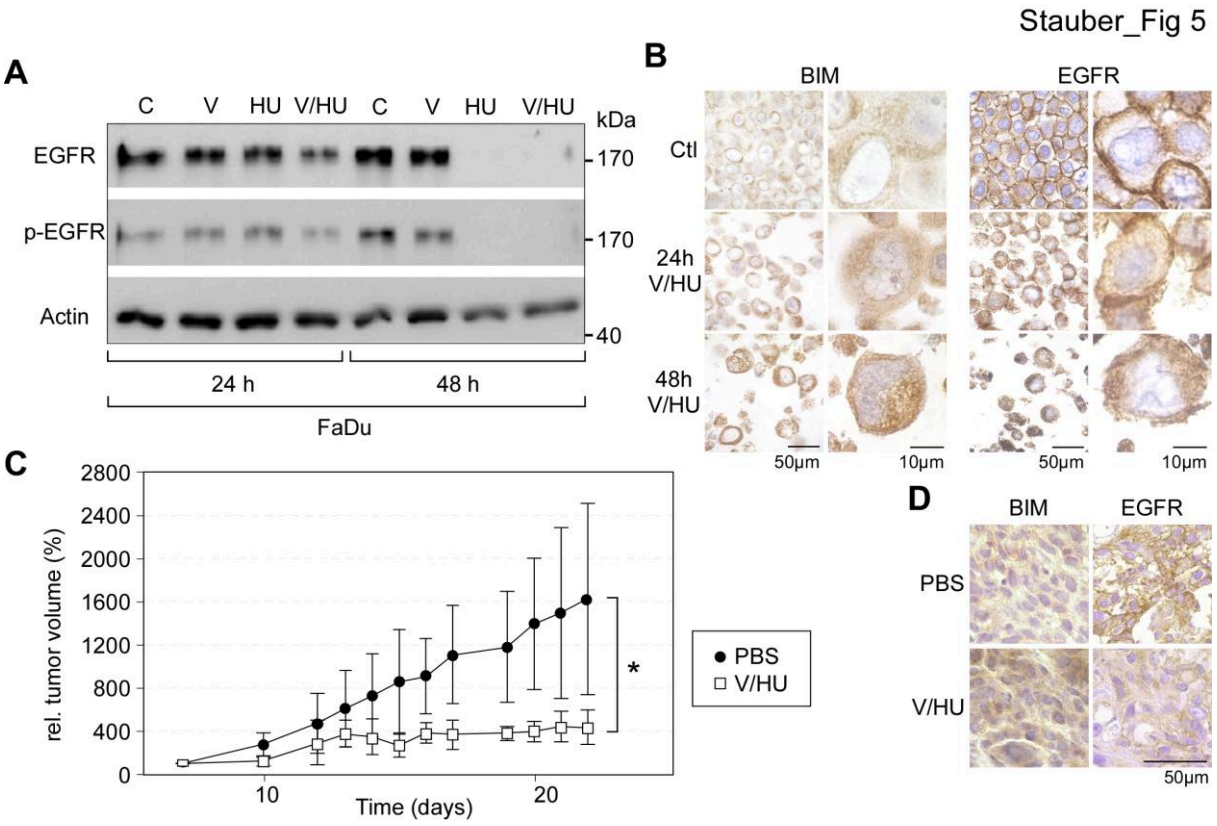

Fig. 5. Effects of VPA/HU on EGFR and suppression of HNSCC tumor growth. **A**) FaDu cells were treated with V, HU, VPA/HU (1.5 mM each) or PBS (C). Expression of proteins indicated was analyzed by immunoblotting. Actin served to control equal loading. VPA/HU treatment effectively reduced the levels of total and phosphorylated EGFR. **B**) FaDu cells treated with VPA/HU (1.5 mM each) were FFPE and used for IHC analysis employing EGFR- or BIM-specific Ab. Treatment resulted in reduced expression and cell surface localization of the EGFR as well as increased BIM levels. **C**) VPA/HU suppressed the growth of FaDu HNSCC xenograft tumors. Growth curve of tumors subjected to VPA/HU (i.p., 350 mg/kg and 750 mg/kg body weight) or PBS control. Nude mice were inoculated with FaDu tumor cells. When tumors had reached the target size of 0.1 cm^3, mice were treated once every second day for 14 days. *p<0.001, *n*=4 animals per treatment group, data are mean±SD. **D**) Enhanced BIM and reduced EGFR levels in xenograft tumors at the end of VPA/HU treatment. BIM and EGFR expression was visualized by IHC.

SUPPLEMENTARY MATERIAL

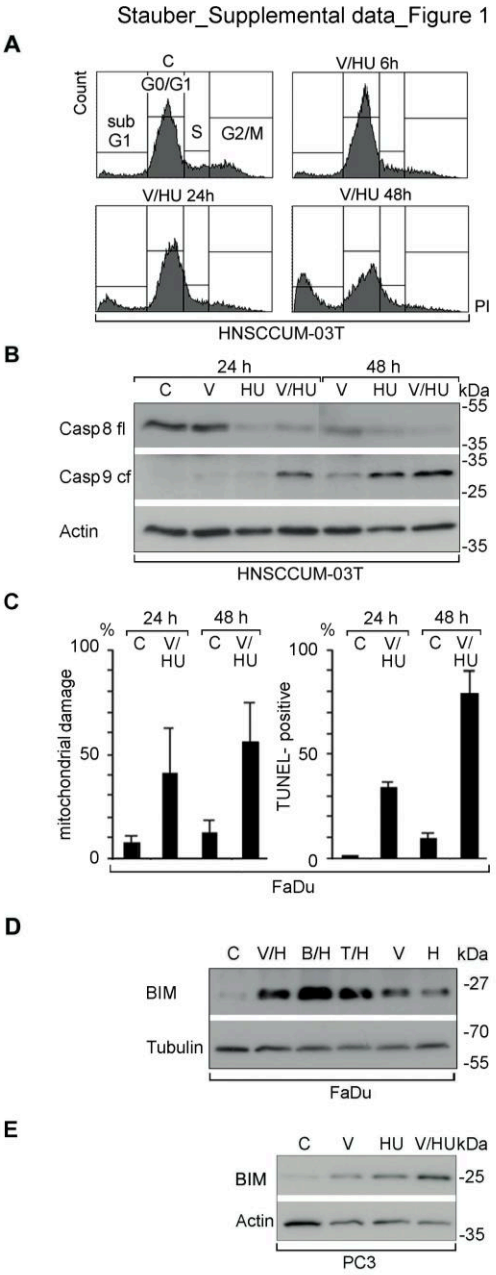

Fig. S1. A) Effect of VPA/HU treatment (1.5 mM each) on HNSCCUM-03T cells analyzed by flow cytometry (PI staining). The increase in the sub-G1 population, indicative of apoptosis, was most prominent 48 h post treatment. **B**) Indicated drug treatment (VPA, HU; 1.5 mM each) of HNSCCUM-03T cells resulted in cleavage of Caspase-8 and -9 demonstrating activation of PCD demonstrated by immunoblot. **C**) VPA/HU-induced cell damage was shown by analyzing mitochondrial integrity and by TUNEL-staining of FaDu cells. VPA/HU treatment (1.5 mM each) caused significant mitochondrial damage already 24 h post treatment, resulting in loss of dimeric *MitoCapture* dye staining. TUNEL-staining revealed VPA/HU-induced DNA-damage characteristic for apoptotic cells, which was most prominent 48 h post treatment. **D**) The combination of HU with HDACi synergize in BIM-induction. FaDu cells were treated with V (1.5 mM), HU (1.5 mM), V/HU (1.5 mM each), trichostatin A (T, 100nM) and HU (1.5 mM), sodium butyrate (B, 1.5 mM) and HU (1.5 mM) for 24 h. BIM expression was analyzed by immunoblot. Tubulin served as the loading control. **E**) VPA/HU-treatment results in enhanced BIM expression in p53-negative PC3 cells. Cells were treated with V (1.5 mM), HU (1.5 mM) or V/HU (1.5 mM each) for 20 h. BIM expression was visualized by immunoblot analysis. Actin served as the loading control.

Stauber_Supplemental data_Figure 2

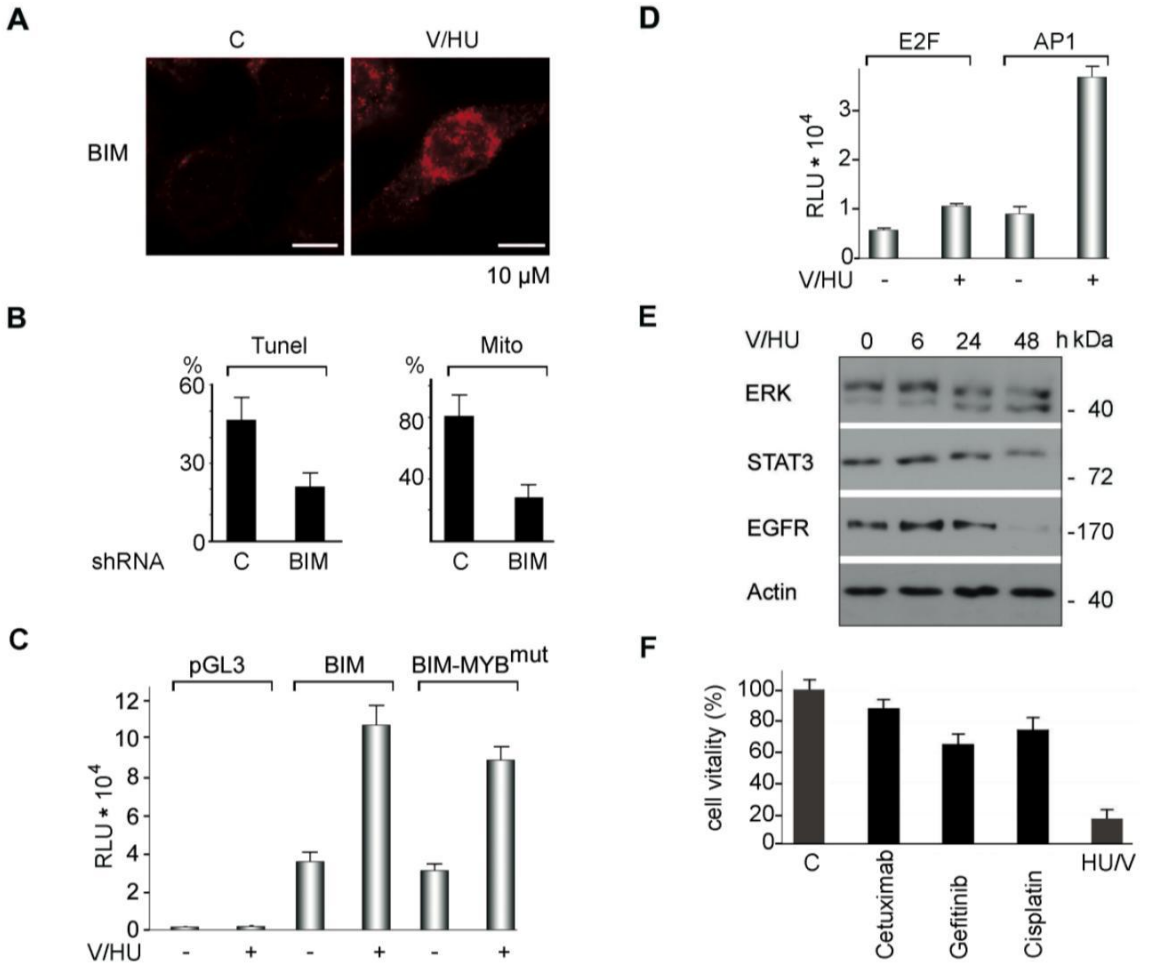

Fig. S2. A) VPA/HU-induced (1.5 mM each, 24 h) BIM expression *versus* PBS control (C) in HNSCCUM-03T cells was visualized by indirect immunofluorescence using BIM-specific Ab followed by staining with a Cy3-conjugated secondary Ab. **B**) BIM depletion attenuates VPA/HU-induced apoptosis. Decreased VPA/HU-induced apoptosis (1.5 mM each, 24 h) in cells stably expressing BIM-(shBIM) *vs* scrambled-shRNA (shCtl) is shown by TUNEL-staining or by analyzing mitochondrial integrity (Mito). **C**) In contrast to the empty control vector (pGL3), VPA/HU treatment resulted in transcriptional activation of the *BIM*-reporter as well as of a reporter containing a *BIM* promoter with an inactivated MYB-binding site (BIM-MYB^{mut}). **D**) VPA/HU promotes AP1-dependent transcriptional activation of the *BIM* promoter. FaDu cells transfected with the indicated reporter constructs were treated with VPA/HU (+; 1.5 mM each, 6 h) or PBS control (-). Transcriptional activation was monitored by quantifying luciferase activity (RLU). Columns, mean; bars, ±SD from three independent experiments. **E**) Time-dependent effects of VPA/HU-treatment on survival pathways in FaDu cells. Expression of the indicated proteins was visualized by immunoblot. Actin was used to monitor equal loading of cell lysates. In contrast to the strong reduction of EGFR levels, no significant effect on ERK or STAT3 levels were detectable. **F**) Evaluation of the cytotoxic activity of VPA/HU and chemotherapeutic drugs. FaDu cells were treated with PBS (C, set at 100%), cetuximab (10 mg/ml), gefitinib (20 µM), cisplatin (10 µM) or VPA/HU (1.5 mM each) for 48 h and the numbers of viable cells counted. Columns, mean; bars, ±SD from three independent experiments.

Supplementary Table SI

Anatomical origin, EGFR status, VPA/HU-induced BIM expression and apoptosis of cell lines used in the study.

A

HNSCC cell line / anatomical origin	**EGFR expression**	**VPA/HU-induced**	
		BIM expression	**Apoptosis***
Naso-/Oropharynx			
Nasopharynx			
Detroit 562	high	+	++
Tonsils			
UMB-SCC-864	high	+	+
UMB-SCC-969	high	+	+
Tongue			
HNSCCUM-02T	high	+	++
SCC-4 (CRL-1624)	high	+	++
Hypo-/Laryngopharynx			
Hypoharynx			
FaDu	high	+	+
Piriform sinus			
HNSCCUM-03T	high	+	++
Larynx			
UM-SCC-5	high	+	++
UM-SCC-10B	high	+	++
UM-SCC-24	high	+	++
UM-SCC-33	high	+	++

B

Cancer cell line / anatomical origin	**EGFR expression**	**VPA/HU-induced**	
		BIM expression	**Apoptosis***
Prostate			
*PC3 (*B-Raf WT)	high	+	++
Lung			
A549 (B-Raf/K-RAS mut)	high	+	++

*****Cell viability was calculated employing MTT-assays.

Supplementary Table SII

Tumor stages and clinical characteristics of 31 HNSCC patients examined by IHC analysis. *n.k.: not known; M: male; F: female.*

A

Case	Staging		Grading	Age	Sex
	pT	pN			
1	T2	N2	G2	47	M
2	T4	N2	G2	68	M
3	T4	N1	G2	46	M
4	T1	N2	G3	57	M
5	T2	N3	G2	56	M
6	T2	N1	G3	50	M
7	n.k.	n.k.	n.k.	n.k.	n.k.
8	T3	N1	G2	48	M
9	T3	N2a	G2	58	M
10	T3	N2	G2	56	M
11	T2	N3	G2	57	M
12	T2	N2	G3	56	M
13	T4	N3	G3	56	M
14	T2	N2	G2	47	F
15	T2	N1	G2	58	F
16	T3	N2	G2	58	F
17	T3	N2	G2	72	M
18	T3	N1	G2	49	M
19	T4	N2c	G3	66	M
20	T2	N2b	G2	53	M
21	T4	N2	G2	51	M
22	T3	N1	G2	47	M
23	T3	N2	G2	56	M
24	T4	N1	G2	42	F
25	T4	N3	G3	56	M
26	T3	N2	G2	49	M
27	T3	N1	G2	58	M
28	T1	N2	G3	57	M
29	T2	N3	G2	57	M
30	T2	N2	G2	47	M
31	T4	N2	G2	80	M

B

Parameter	Anatomical site	
	Naso-/Oro-pharynx (*N*)	Hypo-/Laryngo-pharynx (*N*)
pT1/2	10	2
pT3/4	12	6
N0/1	5	3
N2/3	17	5
G1	0	0
G2	17	6
G3	4	2
Σ	22	8

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4. Discussion

4.1 Acetylation as a regulator

Posttranslational modifications of lysine residues can strongly interfere with protein function (Merrick and Duraisingh, 2007). In general, acetylation changes the electrostatic state of lysine from positive to neutral, increasing the hydrophobicity and the size of the amino acid side chain. Via such alterations, acetylation can affect enzymatic activity. Secondly, acetylated lysines exhibit slightly different preferences for secondary structures than unacetylated lysines. Thirdly, the different modifications like acetylation, sumoylation, ubiquitination and methylation can compete for the same lysines important for signalling pathways. Fourthly, a modification within localisation signals can change the subcellular distribution of a protein (Kim *et al.*, 2006). Additionally, the acetylation of lysines can create a new docking site for protein-protein interactions, for example recognition by bromodomains. In sum, acetylation can determine protein function, including protein stability, protein structure, DNA binding capacity and protein-protein interactions.

In eukaryotic cells, acetylation is among the most common covalent modifications and ranks almost equivalently to the important master switch phosphorylation (Kouzarides, 2000). Up to now, thousands of acetylated proteins have been identified even though far fewer acetylases than kinases have been described. Acetylation apparently shows a broader substrate spectrum than phosphorylation. Therefore, its targets cover transcription factors and many other proteins involved in DNA repair and replication, metabolism, cytoskeletal dynamics, apoptosis, nuclear import, protein folding and cellular signalling (Choudhary *et al.*, 2009; Cohen and Yao, 2004; Kouzarides, 2000; Sterner and Berger, 2000; Yang and Gregoire, 2007).

In sum, these modified proteins are called the acetylome. Acetylation can affect signalling pathways and thereby alter cell fate and function. Acetylation can activate transcription independent from histone acetylation by orchestration of transcription factors and the transcriptional machinery. Moreover, mRNA splicing, mRNA transport, mRNA integrity, translation, protein activity, protein localisation, protein stability and interactions are regulated by acetylation.

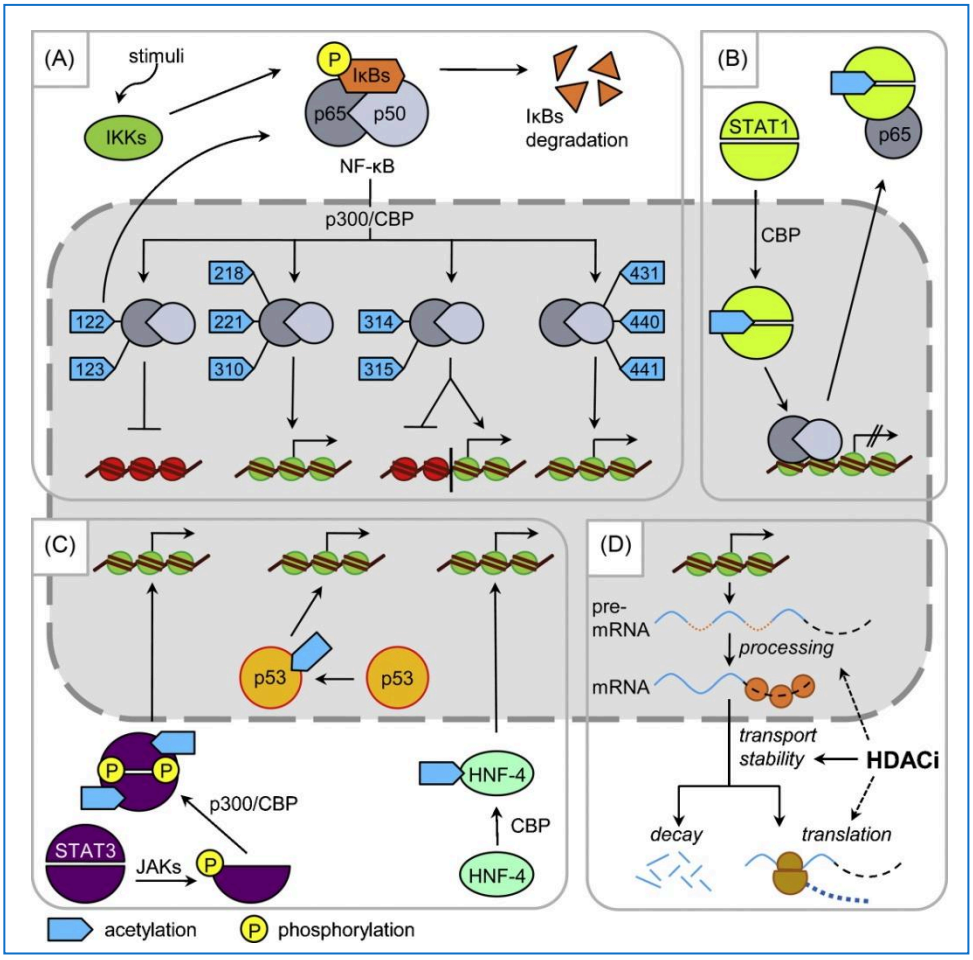

Figure 11 Effects of acetylation on signalling, transcription and posttranscriptional events.

(A) p300/CBP-mediated site-specific acetylation of p65 or p50 has a major impact on the transcriptional activity of NF-κB *in vitro*. Hereby, sitespecific acetylation can activate or inhibit transcription.

(B) Acetylation of STAT1 induces its interaction with p65. The resulting complex dissociates from DNA, translocates into the cytoplasm and inhibits the activity of NF-κB. (C) Acetylation of transcription factors can affect dimerisation (e.g., STAT3), DNA binding affinity (e.g., p53) or subcellular localisation (e.g., HNF-4) and thereby transcriptional activity. (D) HDACi alters the cellular acetylation state of various proteins including factors needed for pre-mRNA processing or translation. Additionally HDACi have been shown to alter mRNA stability (Spange *et al.*, 2009).

In short: Acetylation can interfere with every step of regulatory processes from signalling to transcription to protein degradation. The proper regulation of deacetylases appears to be critical for the maintenance of homeostasis, which is exemplified by deregulated HDAC activity in certain severe human malignancies (Bolden *et al.*, 2006; Glozak and Seto, 2007; Marks and Xu, 2009).

4.2 HDACi affect SIRT1 by targeting HuR

The long way from newly synthesised RNA to its translation into a protein is a multi-step process. Posttranscriptional control is mainly mediated by cis-acting RNA elements located in the 5'- and 3'-untranslated regions of mRNAs (5'-UTRs and 3'-UTRs) that can be targeted by trans-acting RNA binding proteins (Grzybowska *et al.*, 2001; Pesole *et al.*, 2001). The control of mRNA stability is often under-rated as a mechanism for the regulation of gene

expression. In fact, the initiation of transcription is just one side of the medal. The other side includes pre-mRNA processing, mRNA transport, mRNA stability and finally translation into protein. In the end, transcriptional activation does not necessarily result in increased protein levels.

HDACs are known to crucially regulate eukaryotic gene expression via deacetylation of histones and other proteins targeting transcription. The addition of HDACi is thought to enhance transcriptionally active chromatin, which is hyperacetylated. Consequently, access of the transcriptional machinery to DNA target sequences is simplified (Kouzarides, 2007; Rosenfeld *et al.*, 2006). Nevertheless, inhibiting HDACs does not simply result in a net increase in gene expression {Glozak, 2007 #27; Spange *et al.*, 2009). Their inhibition by HDACi alters the expression of 2–10 % of human genes significantly, with almost equal numbers of genes up- and down regulated (Daly and Shirazi-Beechey, 2006; Gray *et al.*, 2004). To which extent posttranscriptional events contribute to the changes in gene expression is unknown. It is estimated that about 50% of all changes in gene expression actually are alterations in mRNA stability rather than “direct” transcriptional control (Cheadle *et al.*, 2005). Actions of HDACi towards such posttranscriptional control mechanisms have not been the focus of research so far, whereas other proteins like eNOS, p21, ERα, Brm, DNMT-1, -3B, GATA3 and now also SIRT1 have been shown to be posttranscriptionally regulated by HDACi (Licata *et al.*, 2009; Spange *et al.*, 2009) (Figure 11 D).

As a key regulator of cellular signalling, SIRT1 is tightly controlled. In recent years, a complex network regulating SIRT1 action emerged including its transcription, co-factors, binding proteins and posttranslational modifications modulating SIRT1 function. Additionally, HuR posttranscriptionally regulates its expression levels. An siRNA-mediated knockdown of HuR resulted in a complete loss of SIRT1 protein expression. This and the fact that the RNA-Polymerase II inhibitor DRB drastically reduces *SIRT1 mRNA* half life suggests that *SIRT1 mRNA* is subject to high turnover rates. The stabilising action of HuR depends on its subcellular distribution as well as on its ability to bind mRNA tightly. Many different transport mechanisms as well as HuR binding partners affect the cellular distribution of the mainly nuclear protein. The cytoplasmic fraction of HuR is thought to protect mRNA from the cytosolic mRNA degradation machinery by binding to the 3´-UTR. Exposing cells to HDACi

decreases cytoplasmic HuR levels. The change in HuR localisation mediated by HDACi alone or in combination with aza-cytidine has already been described (Pryzbylkowski *et al.*, 2008; Wang *et al.*, 2004). In this context, the AMP-activated protein kinase (AMPK) is critically involved in the subcellular distribution of HuR. Upon AMPK activation p300 becomes phosphorylated. This enhances p300 activity, triggering acetylation of importin-α1(K22). This in turn favours the binding to its nuclear import factor importin-β. Additionally, AMPK phosphorylates importin-α1(S105) creating a binding site for HuR. The posttranslational modification-dependent complex of importin-β/ importin-α1/HuR translocates into the nucleus (see Figure 12). Regarding the effect of HDACi, they are thought to enhance CBP/p300-dependent acetylation of importin-α1(K22). Hence, this promotes the binding to its cofactor importin-β by neutralising an unusual basic residue within the IBB interaction domain (Bannister *et al.*, 2000). Additionally, phosphorylation on importin-α1(S105) is equally important as acetylation of importin-α in the nuclear import process of HuR. It is unclear whether acetylation alone or only in combination with phosphorylation of importin-α can trigger its nuclear localisation. The binding affinity of importin-α1 towards HuR is enhanced upon phosphorylation of serine 105 (Wang *et al.*, 2004). An importin-α mutant of S105 was not able to locate HuR to the nucleus.

Moreover, phosphorylation at either HuR(maybe S202) or importin-α(S105), which increases their binding affinity towards each other, can be discussed. Nevertheless, HDACi alone triggered HuR nuclear localisation without increasing AMPK enzymatic activity after 6h treatment (Wang *et al.*, 2004). The basal activity of AMPK without further activation may be sufficient to phosphorylate a certain cytosolic HuR fraction, which in turn translocates to the nucleus. Eventually, this points to an HDACi-induced shift in the cellular HuR distribution independent from AMPK activation.

Another mechanism triggering nuclear localisation of HuR is the CDK1 dependent phosphorylation of HuR(S202). In a cell cycle-dependent manner – the phosphorylation at site 202 of HuR enhances the binding of 14-3-3 protein which leads to a nuclear retention of HuR during the G2/M-Phase (Kim *et al.*, 2008a) (see Figure 12). Our mass spectrometric analysis revealed that in control cells HuR(S202) is phosphorylated, whereas Butyrate-treated cells showed no phosphorylation at this site. The phosphorylation at HuR(S202) was observed only in untreated cells, which consist of a mix of asynchronously growing cells in

different cell cycle stages with activated CDK1 in the G2/M-phase. HDACi like Butyrate or VPA have been shown to induce G1-cell cycle arrest via p21 induction preventing CDK1 activity (Krämer *et al.*, 2008). This could explain why there is no detectable HuR(S202) phosphorylation in HDACi treated cells. Therefore, the HDACi effect is independent of the G2/M-induced phosphorylation at HuR(S202). Additional transport mechanisms depicted in Figure 12 comprise the PKCα/δ-dependent phosphorylation at HuR(S221) triggering a cytoplasmic localisation.

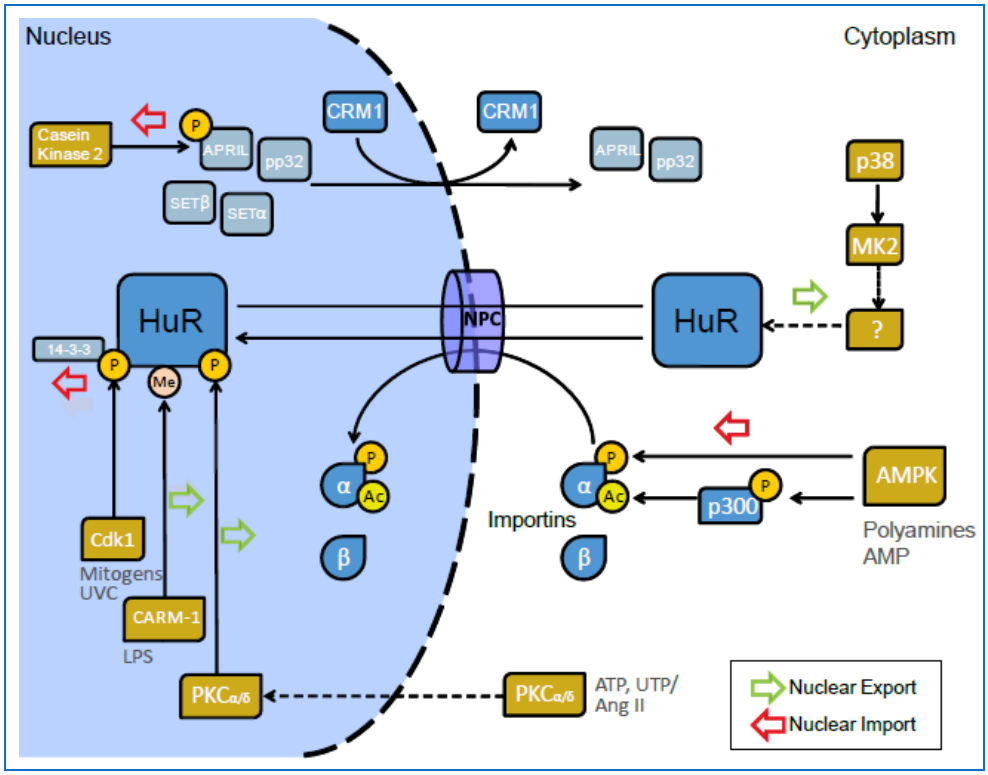

Figure 12 HuR – regulation of the subcellular HuR Transport Depicted are several transport mechanisms targeting the subcellular localization of HuR. The main export factor is CRM1 which exports HuR in combination with different cargo proteins (APRIL, pp32, SETα and SETβ). Importin α1 and β serve as import factors. These transport mechanisms can be further stimulated or abrogated by distinct signalling pathways that target the posttranslational modification of HuR or its transport factors. The green and red arrows depict whether certain pathways trigger a cytosolic or nuclear localisation of HuR.

Moreover, phosphorylation at HuR(S197) which we identified, is located close to HuR(S202) and could interfere with mRNA binding or localisation. Kim et al. mutated different phosphorylatable amino acids in the hinge region of HuR. There was no significant shift in the basal subcellular localisation of the HuR(S197A) mutant (Kim *et al.*, 2008b). It is

possible that under conditions of cellular stress this phosphorylation site can interfere with HuR localisation.

In addition to localisation, posttranslational modifications interfere with HuR RNA binding capacity (Abdelmohsen *et al.*, 2007b). As mentioned, phosphorylation of HuR(S202) not only enhances nuclear retention, it also shows an increased association of target transcripts with HuR (Kim *et al.*, 2008a). Moreover, H_2O_2-induced DNA damage activates the kinase Chk2, which in turn phosphorylates HuR at distinct sites (S88, S100 & T118) resulting in a loss of HuR-*SIRT1 mRNA* binding affinity. This SIRT1-specific mechanism leads to a strong decline in SIRT1 levels (Abdelmohsen *et al.*, 2007b). HDACi do not affect these sites in our approach. Hence, it is likely that other posttranslational modifications of HuR are responsible for the decline of mRNA binding. Presumably, the additional phosphorylation at HuR(S41 & S142) within the RNA binding domains could be responsible for the loss of HuR affinity towards *SIRT1* mRNA upon HDACi treatment. The posttranslational modifications could also indirectly affect mRNA binding by creating novel surfaces for protein interactions modulating protein or RNA binding.

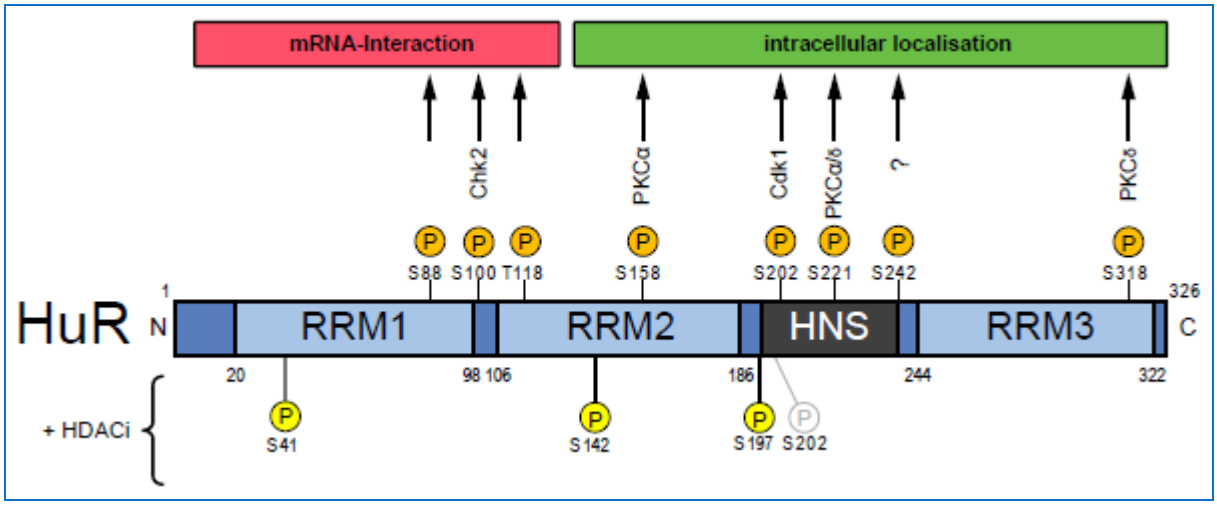

Figure 13 HuR - phosphorylated sites HuR phosphorylation sites above the domain structure indicate published phosphorylation sites, the associated kinases and functional consequences. The Sites underneath the domain structure represent phosphorylation sites identified in our mass spectrometrical approach of 24 h incubated (3 mM But) Hela cells.

How HDACi induce the phosphorylation of different sites in HuR (see Figure 13 – sites under domain structure) is still unknown. HDACi do not directly target kinases. Therefore, indirect pathways activated by HDACi are probably causing a kinase activation or

phosphatase inactivation triggering HuR phosphorylation. All 3 sites have so far not been described as phosphorylated or targeted by specific kinases.

HuR has so far not been described to be targeted by HDACs. Immunoprecipitated cytosolic HuR shows HDAC activity in a RNA dependent way. This suggests that HuR indirectly binds an RNA-associated deacetylase. They suggested HDAC6 because it is the major HDAC in the cytosol (Scott *et al.*, 2008). Our effect is detectable under conditions not inhibiting HDAC6. Therefore, we can rule out that the co-immunoprecipitated, putative HDAC6 plays a role in our system. Nevertheless, there are correlations between an increase in *claudin-1* and *HDAC-2 mRNA* expression throughout all stages in colon cancer patients. Inhibition of claudin-1 expression by HDAC-2-specific small interfering RNA further supported the role of HDAC-2 in this regulation. In this context, HDAC inhibitors decrease *claudin-1 mRNA* stability mediated by its 3′-UTR (Krishnan *et al.*, 2010).

HuR protects and assures expression of *SIRT1 mRNA* by binding to its 3´-UTR. Under HDACi treatment HuR dissociates from the mRNA and becomes phosphorylated.

4.3 Further possible HDACi-mediated posttranscriptional regulation scenarios

Together with proteins, small noncoding RNAs can control the fate of mRNA. The role of miRNAs (micro RNAs) in this regulatory network is under intensive investigation. These endogenous highly conserved RNAs (21 – 23nt) primarily bind within the 3´-UTR of mRNAs. Their extent of complementarity causes siRNA-like mRNA degradation or inhibition of translation of the targeted mRNA.

HDACi can modulate the expression of proteins as well as regulatory RNAs. Nearly a third of all human genes are estimated to be regulated by miRNAs (Bartel, 2004). By modulating the expression of miRNAs, HDACi may also effect protein expression in a more indirect way. Only a very limited number of studies focused on miRNA expression under HDACi treatment. HDACi can change the miRNA expression pattern similar to other protein coding genes (Bandres *et al.*, 2009; Lee *et al.*, 2009; Scott *et al.*, 2006). A profile of miRNA expression in LAQ824-treated SKBr3 cells showed that the expression of the SIRT1 targeting miR-34a slightly decreases, whereas expression of miR-200c increases (Scott *et al.*, 2006). SAHA incubation induces miR-132 expression drastically in A549 cells (Lee *et al.*, 2009).

Therefore, this specific miRNA should be analysed in further investigations in the SIRT1 HDACi regulation process.

Additionally, SIRT1 expression can be changed upon HDACi-induced downstream effects like cell cycle arrest. This is the case for the SIRT1-targeting miR-34a, which is cyclically expressed in Hela cells, with the lowest levels in the G1/S-phase (Zhou *et al.*, 2009). Therefore, it does not seem to play a major role in our system. Hela cells showed no increase in miR-34a while NB4 cells upregulated miR-34a. Moreover, this shows that HDACi can target miRNAs in a cell type-specific context (see Supplementary Figure 1).

Further investigations need focus on the interdependent regulation of genes by 3´UTR binding proteins and miRNAs. HuR has been shown to repress c-Myc through an interdependent mechanism with let7 miRNA. Both binding sites are located next to each other. This suggests a regulatory model wherein HuR inhibits c-Myc expression by recruiting let-7-loaded RISC (RNA miRNA-induced silencing complex) to the c-Myc 3'UTR (Kim *et al.*, 2009).

4.4 HDACi affect BIM

As mentioned, BIM is a crucial regulator of apoptosis. Therefore, its expression and activity is tightly controlled in normal and cancer cells preventing cell death. BIM activity is repressed by growth factor signalling pathways, especially the extracellular signal-regulated kinase 1/2 (ERK1/2) and protein kinase B (PKB) pathways. In tumour cells these oncogene-regulated pathways inhibit BIM action, thereby promoting tumour cell survival.

Transcription of the BIM gene is normally repressed by serum, growth factors and cytokines, and increases upon the withdrawal of these factors (Bouillet *et al.*, 1999; Ewings *et al.*, 2007; Whitfield *et al.*, 2001). In this context, FOXO3A, inhibited by ERK1/2 and PKB, induces BIM transcription (Gilley *et al.*, 2003). Hence, the inhibition of either the ERK1/2 or PKB pathway is sufficient to increase BIM mRNA in many cell types. The expression and activation of BIM seems to be a common response to some chemotherapeutics. Indeed, BIM emerged as an important mediator of tumour cell death in response to chemotherapeutics (Gillings *et al.*, 2009).

Increased BIM levels could be the consequence of reduced proteasomal degradation or enhanced transcriptional activation, mRNA stability or translation (Hendrickson *et al.*,

2008; Youle and Strasser, 2008)). We found that VPA and HU activate BIM at the transcriptional level. To date, several transcription factors, including p53, E2F, c-JUN/FOS (AP1), MYB, FOXO1/3A, and RUNX3 have been reported to regulate BIM transcription (Biswas *et al.*, 2007; Hendrickson *et al.*, 2008). Our data collectively support a crucial role of AP1 for VPA/HU-mediated transcriptional activation of BIM in HNSCC. This finding seems to be of clinical relevance as in contrast to p53, which is mutated in the majority of HNSCC (Poeta *et al.*, 2007), the AP1 system appears to be intact also in cancer patients (Mishra *et al.*, 2010; Weber *et al.*, 2007). S-phase-dependent induction of c-JUN could be a mechanism enhancing BIM expression (Yogev *et al.*, 2006). Of note, the HDAC3-NCoR complex (12853483), which is particularly sensitive to inhibition by VPA, represses the activity of c-JUN (see Figure 5). Phosphorylation of c-JUN by JUN kinase (JNK) permits dissociation of HDAC3 and c-JUN-dependent transcription (Weiss *et al.*, 2003). HU has been shown to activate JNK *in vivo* (Yan and Hales, 2008), which thereby could contribute to c-JUN-dependent BIM induction. Also the VPA/HU combination treatment of HNSCC cells enhances BIM expression via AP1-dependent transcriptional activation (Biswas *et al.*, 2007; Cragg *et al.*, 2008). Although ERK signalling was reported to be critical for BIM expression VPA/HU did not affect ERK levels in our cell models.

4.5 HDACi target cancer cells in parallel ways by SIRT1 and BIM

4.5.1 HDACi therapy

Treatment of malignant cells with HDACi can induce a wide range of anticancer effects including apoptosis, cell cycle arrest, and differentiation targeting tumour cells at different stages of cancer development (Bolden *et al.*, 2006; Frew *et al.*, 2009).

Hence, numerous HDACi have been tested in the clinic or are currently the subject of ongoing clinical trials, including HNSCC (Blumenschein *et al.*, 2008; Bots and Johnstone, 2009; Prince *et al.*, 2009; Schrump, 2009). Since HDACi monotherapies do not seem to be effective against solid tumours, their full therapeutic potential would be best realised through combination with other anticancer agents (Bolden *et al.*, 2006; Krämer *et al.*, 2008). However, most reports do not provide a well-defined molecular rationale for combining an HDACi with a given agent. Moreover, the molecular and biological events that underpin any observed additive or synergistic combination effects are largely lacking (Bolden *et al.*, 2006;

Krämer *et al.*, 2008). HDACi modulate various signal transduction pathways. Probably, the crosstalk of all these pathways in sum contributes to cancer cell sensitisation and apoptosis induction. The co-treatment of HDACi with other chemotherapeutics seems to be favoured above monotherapies. HDACi make cancer cells more vulnerable towards apoptosis and cell death (Hajji *et al.*, 2010; Iacomino *et al.*, 2008). This work provides two possible contributors to the effect of sensitisation established by the concurrent regulation of SIRT1 and BIM.

4.5.2 SIRT1

Although SIRT1 has been reported as a tumour suppressor in colon cancer by deacetylating the tumour promoter β-catenin (Firestein *et al.*, 2008), most evidence supports a tumour-promoting role for SIRT1 (Ford *et al.*, 2005). SIRT1 knockout mice showed no elevated tumour rates after induction of skin papillomas by the classical two-stage carcinogenesis protocol. Therefore, SIRT1 does not act like a like a classical tumour-suppressor gene. Strikingly, tumour tissue very often shows elevated SIRT1 levels (Stunkel *et al.*, 2007), which correlate with poor survival prognosis (Jang *et al.*, 2008). Deacetylation of the tumour suppressors p53, p73 and Ku70 likely contributes to the tumour-promoting functions of SIRT1 (Cohen *et al.*, 2004a; Dai *et al.*, 2007; Luo *et al.*, 2001). Following the multi-hit model of cancer development, SIRT1 seems to be a tumour growth promoter coming into play after aberrant changes have taken place.

SIRT1 is repressed in non-malignant cells by tumour suppressor proteins including p53, HIC1, DBC1, and Chk2. During ageing and cancer development, the HIC1 promoter can undergo hypermethylation and epigenetic silencing (Chen *et al.*, 2005). In this cells SIRT1 expression is expected to rise, where it might enhance the survival of damaged cells and cancer risk (Campisi and Yaswen, 2009). The simple increase of SIRT1 expression does not per se lead to tumorigenesis (Lavu *et al.*, 2008; Zeng *et al.*, 2009). Nevertheless, inhibition of SIRT1 as well as its siRNA-mediated knockdown have been shown to have growth inhibitory effects on multiple cancer cell lines (Kamel *et al.*, 2006; Solomon *et al.*, 2006; Stunkel *et al.*, 2007) and tumour growth (Chang *et al.*, 2009; Kojima *et al.*, 2008).

Beside knockdown, the subcellular localisation of SIRT1 is important. The mainly nuclear protein can have a cytosolic localisation in normal cells as well as cancer cells (Moynihan *et al.*, 2005; Stunkel *et al.*, 2007; Tanno *et al.*, 2007). While only a small

percentage stays in the nucleus targeting well-established factors, cytosolic SIRT1 may affect a completely different set of yet undiscovered functional proteins. In consequence, "carcinogenic" SIRT1 functions should be considered in the discussion of tumour suppressing or promoting actions of SIRT1 in tumour development. Indeed, deacetylation of cytosolic cortactin by SIRT1 has been shown to promote cell migration (Zhang *et al.*, 2009).

So far, classical HDACi have not been described to impair the catalytic activity of class III sirtuins (Müller and Krämer, 2010). We could clearly show that these inhibitors have a strong effect on SIRT1 expression levels in different cancer cell lines. Hence, decreased expression of SIRT1 may contribute to the anti-tumourigenic effects of HDACi (Hajji *et al.*, 2010). Transient or persistent attenuation of SIRT1 decreases the stress resistance of transformed cells. Such an observation may explain why HDACi are more effective in killing cancer cells when combined with chemotherapeutic drugs inducing cellular stress (Hajji *et al.*, 2010; Müller and Krämer, 2010).

4.5.3 BIM

BIM has attracted increasing attention as a plausible target for tumour therapy. It promotes anoikis of many tumour cells, such as lung cancer, breast cancer, osteosarcoma, melanoma and HNSCC. Various chemotherapeutic agents mediate cell death via BIM activation. Hence, BIM suppression supports metastasis and chemoresistance. BIM-targeted therapies offer benefits including the selective of treatment for tumour cells (Akiyama *et al.*, 2009).

The work in manuscript 3 provides convincing evidence that activation of the pro-apoptotic protein BIM is essential for efficient VPA/HU-induced tumour cell death. This result is applicable to therapy conditions because freshly isolated tumour cells from HNSCC patients responded to VPA/HU administered at therapeutically achievable levels with BIM induction and apoptosis. Additionally, ectopic expression and RNAi experiments convincingly demonstrated that BIM is essential for VPA/HU-induced cancer cell death. Moreover, VPA/HU efficiently prevented progression of HNSCC tumours in nude mice correlating with enhanced BIM levels. BIM-evoked apoptosis is crucial for epithelial tumour cell death triggered by several anticancer therapeutics (Hendrickson *et al.*, 2008; Tan *et al.*, 2005).

HNSCC tumours are often characterised by deregulated EGFR signalling due to receptor overexpression, activating mutations in the receptor itself and/or downstream signalling cascades. In these cells survival is secured by the sequential phosphorylation and activation of MEK and ERK kinases, leading to stabilisation of MCL-1, activation of BCL-2, and degradation of BIM. Approaches interfering with EGFR signalling trigger apoptosis also by enhancing BIM expression (Hendrickson *et al.*, 2008). Importantly, we demonstrate for the first time that VPA/HU treatment efficiently reduced not only EGFR levels, but also attenuated its cell surface localisation. The underlying mechanisms remain to be resolved in detail. These may include HU-induced replication arrest, known to attenuate oncogenic tyrosine kinase signalling (Shields *et al.*, 2008), and/or the E3 ubiquitin ligase c-CBL, which controls EGFR ubiquitination and lysosomal degradation (Pennock and Wang, 2008).

4.5.4 Perspectives

BIM as well as SIRT1 are important regulators of cell fate and survival. Our findings and the literature suggest that inducing anti-apoptotic SIRT1 and lowering endogenous pro-apoptotic factors like BIM is a common mechanism enhancing tumour cell survival and proliferation. Targeting both of these factors is of potential clinically relevance not only for HNSCC therapy.

We described a so far completely new regulation mechanism for SIRT1. Our results add a new layer to the understanding how HuR regulates SIRT1 mRNA stability in response to protein acetylation. HDACi critically influence HuR phosphorylation and mRNA stability of SIRT1. Similar regulatory processes might apply to other enzymes and modulators. Hence, the view on whether HDACi target class III HDACs requires a re-evaluation. Additionally we found that the pro-apoptotic BIM is enhanced upon HDACi treatment especially by transcriptional induction in an AP1-dependent manner.

Although we have shown that BIM plays a major role in apoptotic signalling, this does not rule out the additional participation of other BCL-2 family members and/or other apoptosis-regulating proteins like SIRT1 (Li *et al.*, 2009; Lippert *et al.*, 2007). The induction of BIM alone may not be sufficient for significant tumour cell death, as BIM is more likely to act in concert with other BH3-only proteins, or other death pathways, when new targeted therapeutics are used in combination with traditional chemotherapy agents (Gillings *et al.*,

2009). Residual apoptosis rates of HU/VPA-treated cells with attenuated BIM expression are probably mediated via alternative pro-apoptotic proteins and/or the activation of the extrinsic pathway (Gillenwater *et al.*, 2007; Inoue *et al.*, 2008). However, the mechanisms of HDAC inhibitor-induced apoptosis are incompletely understood. Several pathways are accounted for this effect. Whose of their contributing factors or the interplay of all/subgroup factors concur to the apoptotic effects needs to be investigated further.

Additionally, we show for the first time the potent tumour cell killing activity of combining the HDACi VPA with the ribonucleotide reductase inhibitor HU against HNSCC. Although VPA and HU individually have been shown to target cancer cells in various malignancies (Lane and Chabner, 2009; Seiwert *et al.*, 2008), the (pre)clinical anti-tumour activity of the VPA/HU combination for HNSCC und the underlying molecular mechanisms have not been investigated so far.

SIRT1 has been reported to be involved in the acetylation of FOXOs and the expression of BIM (Brunet *et al.*, 2004; Motta *et al.*, 2004; Yang *et al.*, 2005). HDACi treatment, which decreases SIRT1, favours the acetylation of FOXO1 which in turn activates BIM expression. This pathway is able to induce apoptosis in response to treatment with the HDACi depsipeptide (Yang *et al.*, 2009). BIM, a BH3-only pro-apoptotic protein, was significantly upregulated by depsipeptide in cancer cells, and BIM's function in depsipeptide-induced apoptosis was confirmed by knockdown of BIM with RNAi. Thus, it is important to clarify whether acetylation of FOXO1 is involved in HDAC inhibitor-induced apoptosis via SIRT1 and BIM.

Both, SIRT1 and BIM, are critical regulators of apoptosis and cell fate. We showed that both of them can be targeted by HDACi. The underlying mechanisms that alter gene expression differ extremely. We could show that in the case of SIRT1, HDACi critically reduce mRNA stability by modulating the binding affinity and localisation of HuR. On the other hand, BIM expression is up-regulated at the transcriptional level in an AP1-dependent manner. The parallel change in expression favours conditions that enhance apoptosis especially of tumour cells (see Figure 14). This amplifies the cancer specific effects of HDACi treatment and is able to sensitise cancer cells towards the additional treatment with other chemotherapeutics providing a basis therapy with less side effects.

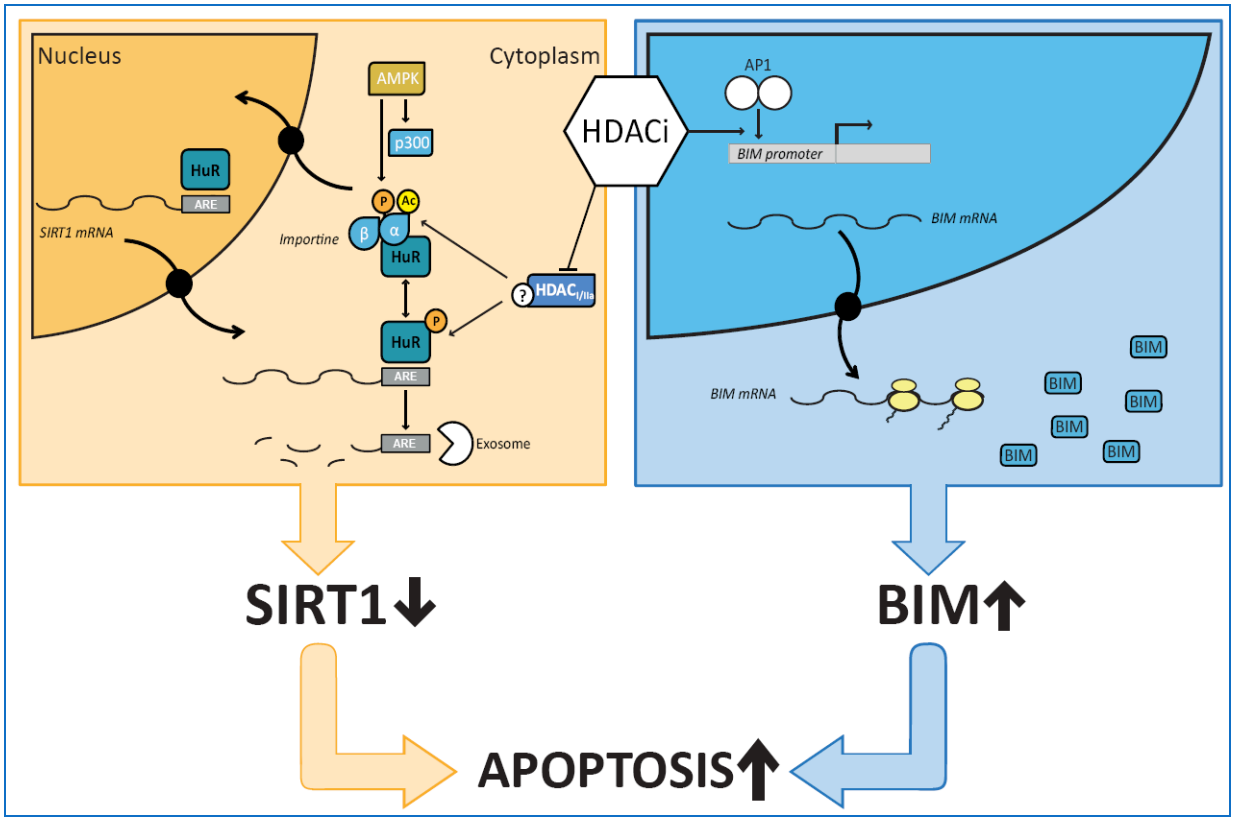

Figure 14 HDACi affect cancer cell death by a parallel regulation of SIRT1 and BIM

HDACi down-regulate SIRT1 expression posttranscriptionally. In this case (left side), the shift in acetylation status of the cell alters the subcellular HuR localisation as well as its binding affinity towards SIRT1 mRNA. On the other hand (right side), HDACi increase BIM expression by enhancing BIM transcription in an AP1-dependent manner. Both proteins regulate apoptosis. Both changes in gene expression (SIRT1 = downregulated / BIM = upregulated) favour the induction of apoptosis – resulting in an enhanced apoptosis rate of cancer cells.

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Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University Jena or to any other University.

Jena, June 6 th 2010 .....

Stephanie Spange

Contribution to manuscripts

Manuscript 1 Acetylation of non-histone proteins modulates cellular signalling at multiple levels

The manuscript was designed and written by Tobias Wagner and myself as first author. The following points and the associated pictures were written by myself. Tobias Wagner wrote the chapters 2 about HDACs and HDACi as well as point 5.3 about protein modification and stability. The stated parts stated underneath were written by myself.

1. Histone acetylation
3. Histone acetyltransferases (HATs) and histone acetyltransferase inhibitors (HATi)
4. Non-histone targets of HDACs and HATs—the acetylome
5. Acetylation regulates multiple processes from gene expression to protein activity
5.1. Signalling and transcription
5.2. Posttranscriptional regulation
5.4. Acetylation and regulation of the cell cycle and circadian rhythms
5.5. Acetylation affects metabolism and mitochondria
5.6. Viral proteins
6. Conclusion

Manuscript 2 Histone deacetylase inhibitors target tumour cells by posttranscriptional regulation of SIRT1

The manuscript was designed and written by myself as first author. The work was supervised by O.H. Krämer and T. Heinzel. Martin Wieczorek performed the experiments for panel 3 and in part for the RNP-IP experiments of HuR of panel 4a. The mass spectrometrical analysis was carried out by Bernhard Schlott (Supplemental Figure S1). With exception of the above listed, all experiments were performed by myself.

Manuscript 3 Hydroxyurea and valproic acid synergize in the potent killing of head and neck cancer cells by downregulating EGFR and triggering BIM-induced apoptosis.

I contributed with the generation of several stable cell lines Cell lines (SK37, FaDu, HNSCCUM-03T cells) constitutively expressing shRNA directed against BIM or a scrambled control. They were generated by transfection of pHR-THT-BIMshRNA-SFFV-eGFP or pHR-THT-scr_shRNA-SFFV-eGFP. Cells were selected by addition of puromycin (1 µg/ml) over several weeks. These cells were then further used for experiments depicted in Figure 3 analysing the effectof HDACi and HU towards cells caspase3 activity

Prof. Thorsten Heinzel

Curriculum Vitae

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Submitted: Spange S, Wieczorek M, Schlott B, Krämer OH, Heinzel T, Histone deacetylase inhibitors target tumour cells by posttranscriptional regulation of SIRT1, submitted to Oncogene

Stauber R, Knauer S, Habtemichael N, Bier C, Spange S, Weisheit S, Nonnenmacher F, Schweiter A, Engels K, Reichardt S, Krämer OH Hydroxyurea and valproic acid synergize in the potent killing of head

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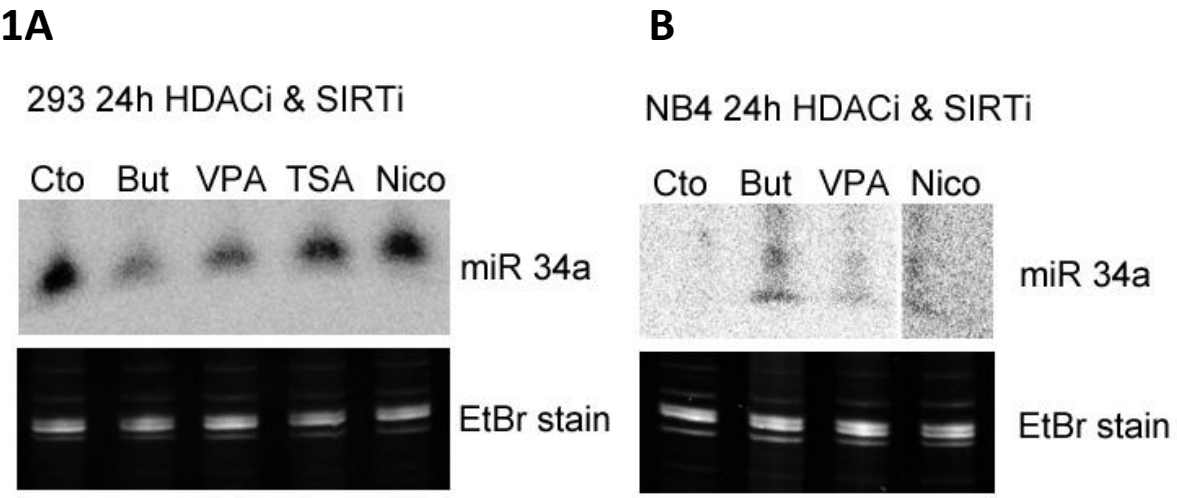
certificate „Bedingungen erfolgreicher Teamarbeit"
certificate „Moderation effektiver Gruppenarbeit"
certificate „Scientific artwork"
certificate „Scientific Posters"

Supplementary Material



Supplementary Figure 1 Detection of miR34a after 24h HDACi and SIRTi inhibitor treatment of 293 and NB4 cells.

Whole RNA was isolated from 24h treated cells (3mM Butyrate, 3mM VPA, 100nM TSA and 5mM Nicotinamide). 30µg were separated on a denaturating Northern Blot gel for small RNAs. After semidryblotting onto a nitrocellulose membrane – the membrane was hybridised for 16h at 42°C with radiolabeled (P^{32}) RNA probes to detect miR-34a. The same gel, stained with Ethidium bromide, served as loading control to ensure equal sample loading (lower panel).