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*Ph.D. thesis*

# HDAC4: studying the pro-oncogenic role in human immortalized fibroblasts

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*"Everyone is a genius.*
*But if you judge a fish by its ability to climb a tree,*
*it will live its whole life believing that it is stupid"*

*Albert Einstein*

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# ABSTRACT

In vitro transformation of primary human fibroblasts has been commonly used to understand the specific steps required to generate neoplastic cells following the ordered introduction of cellular and viral oncogenes and/or the down-modulation of tumor suppressor genes. In this thesis I have applied this strategy to explore the pro-oncogenic function of class IIa HDACs. Class IIa histone deacetylases deregulation can contribute to cancer development and progression in different ways. However their real involvement in tumor biology is still debated. To clarify this issue I have investigated the role of HDAC4, a representative member of this class, in human immortalized foreskin fibroblast (BJ/hTERT). I have demonstrated that HDAC4 negatively influences the isolation of clones after retroviral infection. This effect is MEF2-independent and is in part due to the activation of an apoptotic response. Through the generation of BJ/hTERT cells expressing BCL-xL, a Bcl-2 family member characterized by a pro-survival function, it was possible to isolate clones expressing HDAC4 mutated in the 14-3-3 binding sites, suggesting that HDAC4 deregulation can elicit apoptosis. Isolated clones were characterized, and alterations in the cell cycle profile were not observed. However strong repressive forms of HDAC4 were also subject to intense proteolytic degradation. The apoptotic response and the proteasome-mediated degradation were also described using a doxycycline-inducible system. In this case the nuclear resilient mutants of HDAC4 render BJ/hTERT cells more susceptible to apoptosis only when triggered by DNA damage and protein synthesis inhibition, but not by proteasome inhibitors or oxidative stress. In addition all the nuclear resident mutants evidenced a higher rate of proteasomal degradation.

Finally, ectopically expressing in BJ/hTERT cells, a form of HDAC4 mutated only in NES sequence (HDAC4-L/A), allowed the isolation of clones characterized by a MEF2-repressed phenotype. This mutation causes the accumulation of the deacetylase in the nuclear compartment, without interfering with 14-3-3 binding. This result suggests a possible implication of these adaptor proteins in the HDAC4 anti-proliferative activity. In parallel murine fibroblast expressing the HDAC4-L/A mutant acquire the ability to growth in an anchorage-independent manner.

Overall this thesis sheds some light on the HDAC4 potential of eliciting oncogenic conversion also in human cells managed also by the 14-3-3 binding.

# Introduction

## 1. Epigenetics

The term "epigenetics" was originally coined by Conrad Waddington to describe the heritable changes in a cellular phenotype that were independent of alteration in the DNA sequence (Waddington, 1959) Chromatin structure and organization define the state of the genomic information influencing the ability of genes to be expressed or silenced. The basic functional unit of chromatin is the nucleosome, which comprise 147 base-pair (bp) of DNA wrapped around a core of positively charged proteins called histones. Basically, chromatin can be organized in two different ways: i) heterochromatin, which is an highly condensed, late to replicate and contains silenced genes; and ii) euchromatin, which adopts an open conformation and contains most of the active genes (Du Toit, 2012) (Dawson and Kouzarides, 2012).

These conformations are characterized by different epigenetic modifications that occur in the DNA and/or in the histone tails, protruding from the nucleosomes (Kouzarides, 2007). Changes in the chromatin status of specific genes can lead to their repression or activation. Several histone post-translational modifications (PTMs) have been described, including acetylation, methylation, phosphorylation, sumoylation, ubiquitylation, ADP ribosylation, deimination, proline isomerization and many others (Bannister and Kouzarides, 2011; Berger, 2007). All together the histone PTMs constitute the so-called "histone code" that is read and recognized by additional proteins in order to regulate gene expression (Strahl and Allis, 2000). These proteins are called epigenetics regulators and can be divided into distinct groups based on broad functions: epigenetic writers place epigenetic marks on DNA or histones; these marks are removed by epigenetic erasers and recognized by epigenetic readers (Falkenberg and Johnstone, 2015) (Figure 1).

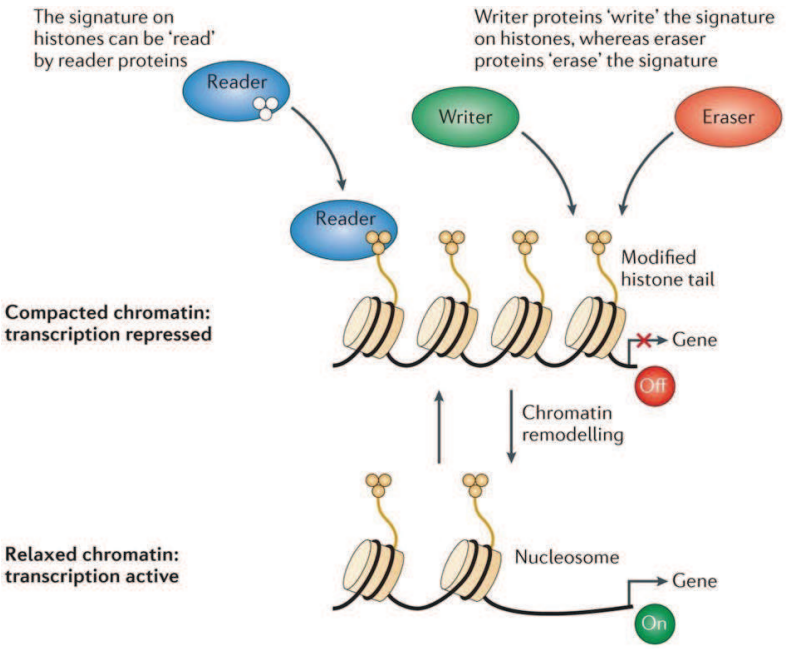


**Figure 1**. *Epigenetic regulators.* Chromatin architecture and status are defined by different chemical groups attached to DNA or to the histone tails. Epigenetic regulators are proteins involved in the regulation and interpretation of chromatin status. The function of each group of proteins is described in the figure. Adopted from (Hojfeldt et al., 2013)

Around fifty years ago, Vincent Allfrey and colleagues discovered the lysine acetylation of histones, demonstrating that acetylation of the ε-amino group of lysine residues on histones could play a role in gene expression (Allfrey et al., 1964; Gershey et al., 1968). The acetylation neutralizes the positive charge of the histone lysine residues, relaxing the chromatin conformation and augmenting chromatin accessibility.

On histones, acetylation is generally associated with gene activation. On the opposite, the removal of acetyl groups induces chromatin condensation and gene transcriptional repression (Haberland et al., 2009). It is well established that lysine acetylation also occurs in a considerable number of non-histone proteins, such as transcription factors and cytoplasmic proteins, governing protein-protein interaction and also affecting gene transcription and other cellular processes (Glozak and Seto, 2007). Lysine acetylation is a reversible modification controlled by the antagonistic action of two families of enzymes, histone acetylases (HATs) and histone deacetylases (HDACs). HATs promote the transfer of acetyl groups from acetyl CoA to the ε-amino group of the lysine residue. On the contrary, HDACs catalyze the removal of the acetyl group from the acetylated residue, releasing an acetate molecule (Yang and Seto, 2007). The fine regulation of histones' PTMs and epigenetic changes on DNA are important mechanisms for cells to have an appropriate patterns of gene expression during

different cellular programs, and an altered expression of epigenetic regulators have been described in cancer and in several human pathologies (Dawson and Kouzarides, 2012; Falkenberg and Johnstone, 2015).

## 2. Histone deacetylase family

$N^{\varepsilon}$-acetylation was extensively studied in the past decade and it is known that this modification not only occurs on the histone tails. (Choudhary et al., 2009; Choudhary et al., 2014). In mammals 18 HDACs have been identified and characterized. They are grouped into four different classes, based on the sequence homologies to yeast orthologues (Figure 2). The four classes differ in structure and also for the sub-cellular localization and the enzymatic activity (Martin et al., 2007; Yang and Seto, 2008).

Class I HDACs family consists of HDAC 1, 2, 3 and 8, which share homology with Rpd3 (Yang and Seto, 2008). These HDACs are expressed ubiquitously in all tissues and predominantly localized in into the nucleus of the cells. They also display a high $Zn^{2+}$-dependent enzymatic activity toward histone substrate. They posses relatively simple structure (Figure 2) with short conserved C-terminus deacetylase domain and a brief amino-terminal region (Martin et al., 2007).

Class II is constituted by 6 members: HDAC4, 5, 6, 7, 9, and 10, which show homology to the Hda1 yeast orthologus (Yang and Seto, 2008). They are also $Zn^{2+}$-dependent proteins and have a tissue-specific expression in particular in muscle, brain, neurons, bone, thymocytes and endothelium (Martin et al., 2007). On the basis of their structural and functional features this class has been further subdivided in two subclasses: class IIa and class IIb. The first comprises HDAC4, 5, 7 and 9. These deacetylases display a very large N-terminal domain, which is involved in the interaction with other cofactors and differently from the others HDAC classes they are subjected to a nuclear-cytoplasmic shuttling, as a mechanism of functional regulation. Moreover they also have a conserved acetylates domain structurally located in the C-terminus of the proteins, but strangely they do not show its own enzymatic activity (Haberland et al., 2009; Martin et al., 2007). Class IIb comprises instead the other two members: HDAC6 and 10. Conversely, they are enzymatically active on some substrate such as for example tubulin (Martin et al., 2007).

Class III groups Sirt 1, 2, 3, 4, 5, 6 and 7. Also known as Sirtuins (Sir2 like-protein), they are homologous with Sir2 in yeast (Yang and Seto, 2008). They are widely expressed and localize in different subcellular compartments. Sirtuins differentially from other deacetylases require $NAD^+$ as cofactor. They show a broad range of biological function such as regulation of oxidative stress, DNA repair, regulation of metabolism and aging (Bosch-Presegue and Vaquero, 2014).

Finally, only HDAC11 belongs to class IV. HDAC11 contains conserved residues in the catalytic core region that are shared by both class I and class II HDACs with small amino- and carbossi-terminal regions. Its expression is enriched in kidney, brain, testis, heart and skeletal muscle, but its function has been less studied (Haberland et al., 2009).

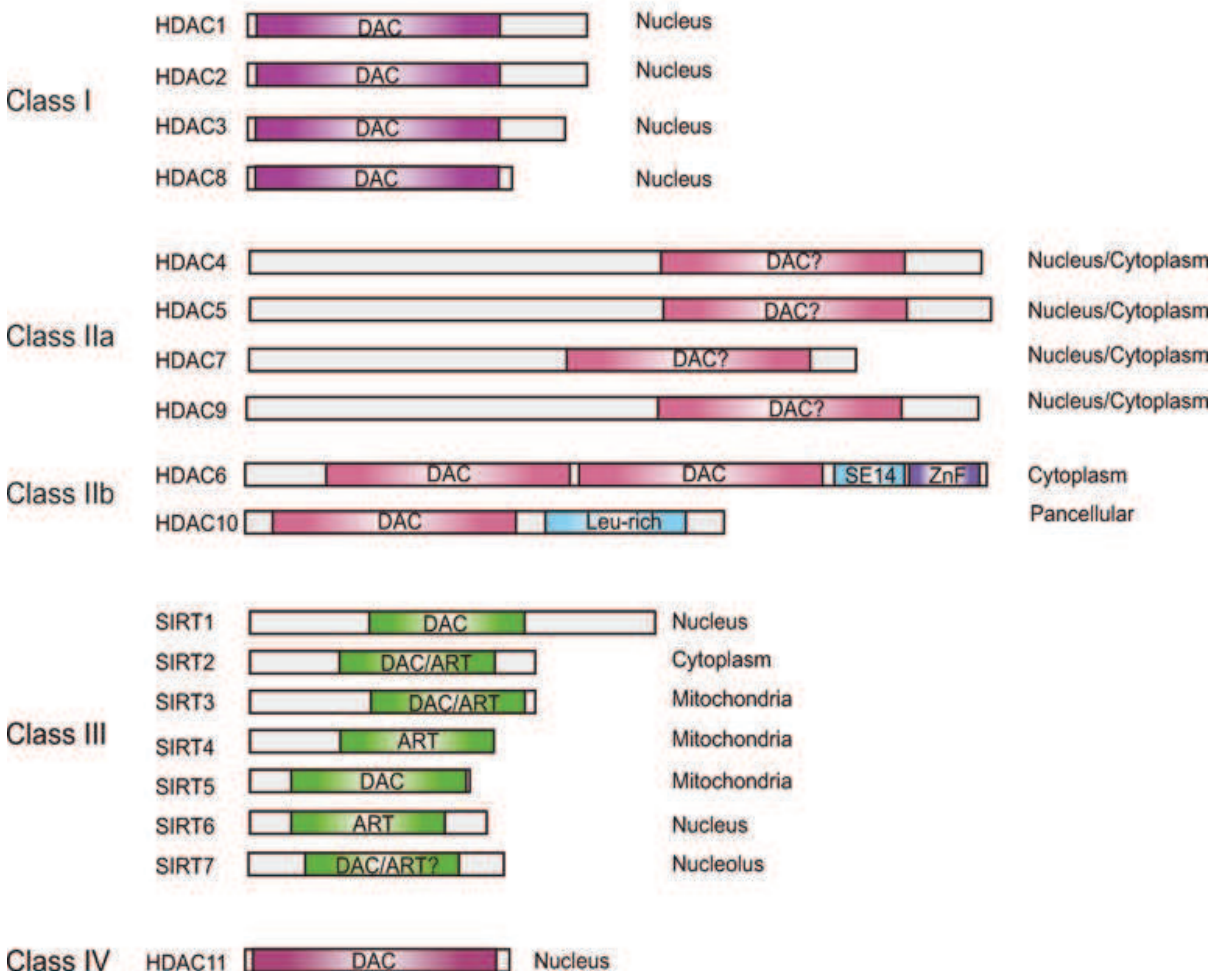


**Figure 2.** *HDACs in humans*. The 18 different HDACs proteins are subdivided in various families according to the homologies to the yeast orthologous. A schematic representation of the different classes and their structure are showed. Class IIa HDACs are characterized by an extended N-terminal domain, which on the opposite is very small in the other sub-families, and by an inactive deacetylase domain localized in the C-terminal end. Each HDACs show a typical intracellular localization. The expression levels differ also from various tissues and organs. Adopted from Clocchiatti et al 2011.

# 3. Class IIa histone deacetylases

## 3.1 Structure

Structurally, class IIa subfamily is characterized by a bipartite model (Figures 2 and 3) with a conserved C-terminal region and an extended N-terminal domain (Zhang et al., 2008), both absent in the other classes, justifying their classification into a different sub-group. The N-terminal region has an extension of around 600 amino acid (aa) in HDAC4, taking this as the major representative member of this class (Figure 3), however in the other members this region is between 500 and 600 aa long (Martin et al., 2007). Homology of the N-terminus, among the four members of this class scores between 30-45% of identity, except for some amino acids involved in specific functions (Di Giorgio et al., 2015; Martin et al., 2007).

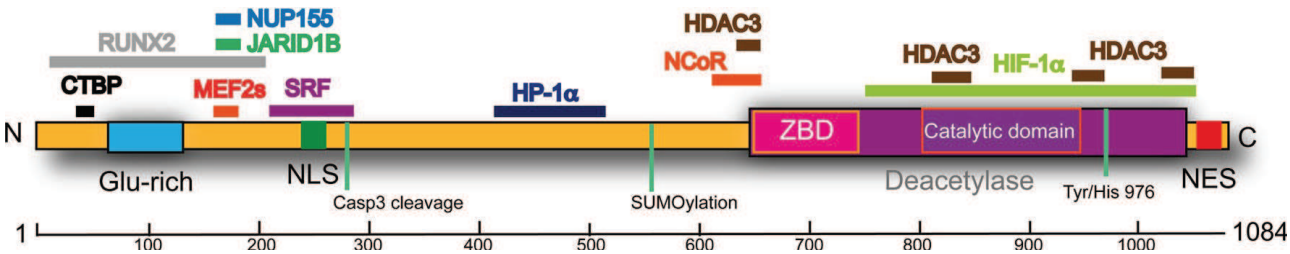


**Figure 3**. *Class IIa structure representation.* Schematic representation of class IIa HDACs highlighting the principal domains. As prototype of class IIa HDAC4 was selected. Certain interaction partners, as well as the relative HDAC4 sequences involved, are illustrated. The NLS represent the NLS1 that is the principal domain involved in the nuclear import (adopted from Di Giorgio 2015).

The amino-terminal domain regulates the nuclear import, because there are two nuclear localization signals (NLSs). NLS1 (residue 244–279) (Figure 2) is the major NLS and is rich in basic amino acids, while NLS2 (residue 1–117) is a minor NLS (Nishino et al., 2008). These two regions are able to interact with importin-α and to mediate nuclear accumulation of the protein (Grozinger and Schreiber, 2000; Nishino et al., 2008).

Within this large region is also sited a coiled–coil glutamine-rich domain that is peculiar of the family (Figure 2). This domain in HDAC4 have 26 glutamine residues, is organized in a single α-helix and is involved in the formation of homo- and hetero-dimers (Martin et al., 2007). Curiously, this region is conserved in HDAC4, 5 and 9 but is absent in HDAC7. This peculiar domain of class IIa HDACs is important because is an adaptor domain devoted to

protein-protein interaction. Most of the interactors are with DNA-binding proteins, indeed HDAC4 lacks a DNA binding domain and any association with nucleic acids needs to be mediated by a partner (Wang et al., 1999). In addition, the N-terminal region contains specific residues that are subjected of various PTMs, such as proteolytic cleavage (Bakin and Jung, 2004; Liu et al., 2004; Paroni et al., 2004), ubiquitination (Li et al., 2004), sumoylation (Kirsh et al., 2002) and most importantly phosphorylation. The latter is important because there are certain residues that act as docking site for 14-3-3 proteins, influencing the nuclear-cytoplasmic-shuttling of the class IIa, which is one of the best characterized strategy for the regulation of these deacetylases (Grozinger and Schreiber, 2000; Martin et al., 2007; Wang et al., 2000) (see below).

The C-terminal domain contains the catalytic deacetylase domain that is ineffective on acetylated lysine residues. The HDAC domain is made up of approximately 400 residues arranged into 21 α-helix and 10 β-strands organized in a single domain, structured around a central catalytic $Zn^{2+}$ ion (Schuetz et al., 2008). It comprises 2 aspartates and a histidine that coordinate this $Zn^{2+}$ while 2 other aspartates (Figure 3a), another histidine, a serine and a leucine coordinate two potassium ions (Bottomley et al., 2008; Vannini et al., 2004). This conformation is also conserved in class I HDACs, but despite this similarity, class IIa possess a bigger active site compared to class I HDACs (Figure 3b) (Di Giorgio et al., 2015; Lobera et al., 2013). The structural peculiarity responsible for this difference is the mutation of the tyrosine into a histidine, position 976 in HDAC4 (Lahm et al., 2007) (Figure 3). Histidine is sterically less cumbersome and induces the relaxation of the structure. As a consequence, this histidine is far from the central $Zn^{2+}$ and is not able to form hydrogen bonds with the intermediate of the enzymatic reaction (Figure 3a). The intermediate is, therefore, very unstable, thus resulting in an ineffective reaction. Nevertheless, class IIa can efficiently process alternative substrates such as trifluoroacetyl-lysine, this probably due to the presence of the trifluoro group that should destabilize the amide bond, hence favoring the reaction even in the absence of transition-state stabilization (Lahm et al., 2007). Importantly, replacing back the Tyr generate class IIa HDACs with a catalytic efficiency 1,000-fold higher compared to the wild-type form (Bottomley et al., 2008; Lahm et al., 2007). Nonetheless, this mutant does not show enhanced repression respect to the wild-type, at least in the instance of MEF2-dependent transcription, a well-known class IIa partner (Fischle et al., 2002).

Class IIa HDAC possess another distinctive feature that is the existence of an additional Zinc Binding Domain (ZBD). The ZBD consists in a β-hairpin surrounded by two antiparallel

β-strands, forming a pocket-like structure that accommodates a second ‘‘structural’’ zinc ion (Schuetz et al., 2008). In the case of HDAC4 three cysteines (667, 669, 751) and one histidine (675), conserved only among class IIa HDACs, coordinate this $Zn^{2+}$ and made the so-called ‘‘core’’ of the domain (Bottomley et al., 2008) (Figure 3c). Importantly, respect to the Apo-structure where Cys 669 and His 675 coordinates the zinc ion, in the inhibited status, these residues were replaced by His 665 and His 678 in the coordination of the $Zn^{2+}$ (Di Giorgio et al., 2015). This domain is extremely flexible and the oxidation of the cysteines involved in $Zn^{2+}$ coordination (667 and 669 in HDAC4) is sufficient to free the metal, with the consequent opening and deconstruction of the ZBD (Bottomley et al., 2008). Because this domain is head-to-head to the active site (Figure 3c), it contributes to make the class IIa HDACs’ catalytic site more accessible than that of class I HDACs (Figure 3b) and does not allow the formation of an efficient hydrophilic tunnel necessary for the release of the acetate reaction product (Bottomley et al., 2008; Vannini et al., 2004).

In addition the C-terminal region is characterized by the presence of a nuclear exporting signal (NES) important for the export of HDACs from the nucleus and hence for the intracellular trafficking and regulation of the enzymes. This sequence is able to interact with CRM-1, a nuclear protein also known as exportin-1, capable of mediating the transit from the nucleus to the cytoplasm of a target protein through the interaction with nucleoporin. The C-terminal region is also important for the interaction with the protein complex HDAC3-SMRT/NCoR (Fischle et al., 2002) responsible for the enzymatic activity reveled after class IIa purification form human cells.

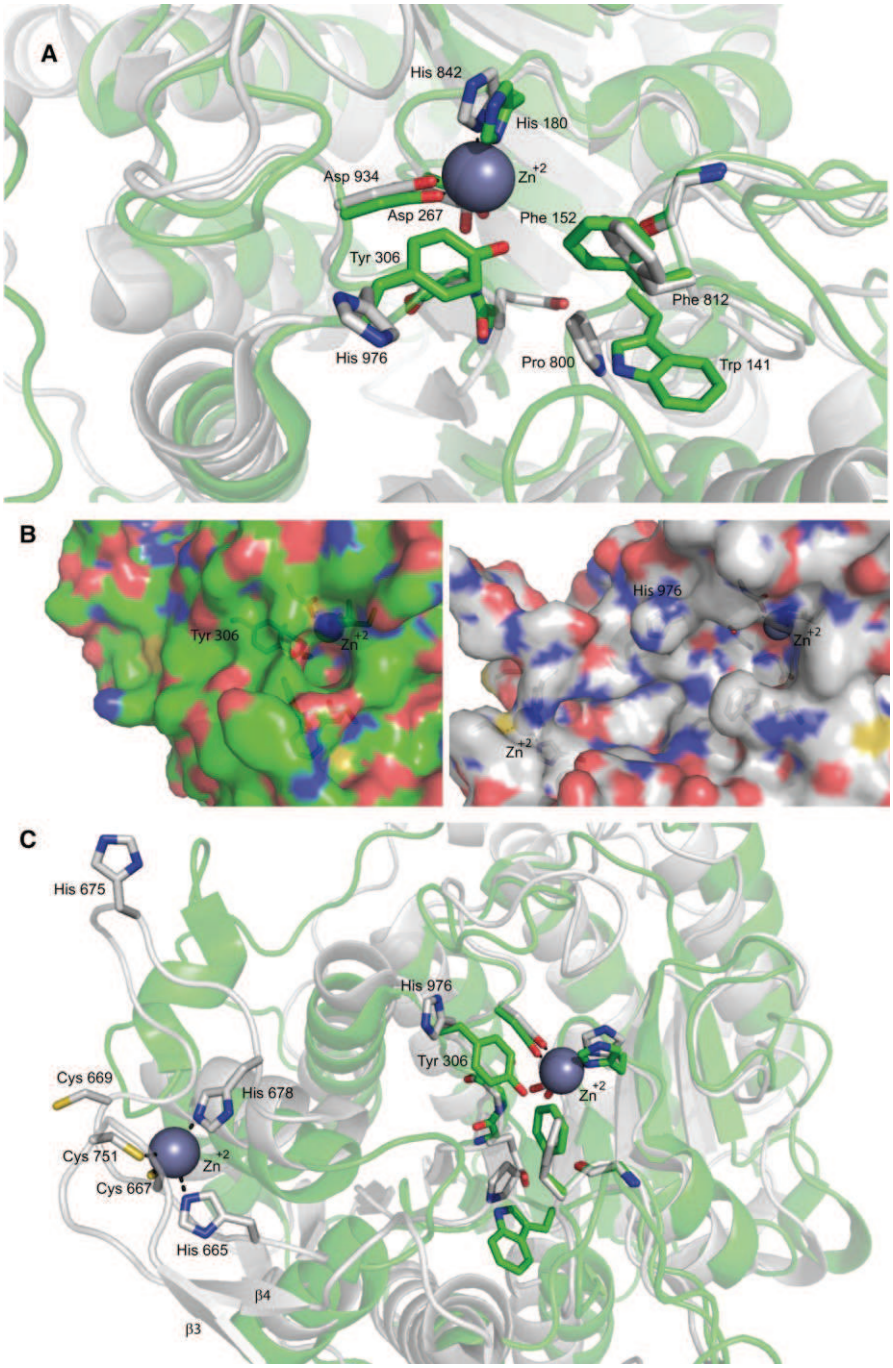


**Figure 3**. *Representation of class I and class IIa catalytic sites (A,B) and the zinc binding domain (C)*. (*A*). Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (green) and of class IIa HDAC4 (white) catalytic sites. As mentioned in the text the His 976 is rotated away from the active site differently from Tyr 308 in HDAC8. (*B*) Surface representation of class I HDAC8 (green) and class IIa HDAC4 (white) catalytic sites. The figure shows the hydrophilic tunnel necessary for the release of the reaction product in HDAC8 (green), while in HDAC4 (white) the His/Tyr substitution prevents tunnel formation. (*C*). Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (green) and of class IIa HDAC4 (white) catalytic site (right) and zinc binding domain of HDAC4 (left). β3 and β4 are the two antiparallel β -strands involved in the formation of the pocket-like structure in the zinc binding domain. Importantly, His 665 and His 678 in this inhibitor bound structure are replaced by Cys 669 and His 675 in the coordination of the zinc ion in the Apo-structure. Unfortunately the crystallization of Apo-HDAC4 was unsuccessful and these differences are deduced from crystallographic studies of the mutant GOF (H976Y) of HDAC4 (Bottomley et al., 2008).

## 3.2 Regulation of class IIa HDACs activity

Cells use different strategies to influence class IIa HDACs functions. Transcriptional mechanisms have been described to control class IIa HDACs activities under different conditions, but also modulations at translation level, via specific microRNAs contribute to coordinate HDACs levels.

### *3.2.1 Transcriptional and post-transcriptional regulation*

The transcriptional control of HDACs genes was not deeply investigated up today. A limited number of studies are reported in literature concerning this argument. Nevertheless, the transcription seems to be under the control of different mechanisms (Di Giorgio and Brancolini 2016 in press). For each class IIa HDACs member a set of splicing variants are present but for only few of them a regulation or function has been described (Di Giorgio and Brancolini 2016 in press).

HDAC4 expression is repressed by mithramycin (Liu et al., 2006). This chemical compound is produced by *Streptomyces plicatus* and is able to bind GC-rich DNA sequences displacing Sp transcription factors (Sleiman et al., 2012). HDAC4 proximal promoter is characterized by the presence of high GC content and in fact binding of mithramycin interferes with Sp1-dependent transcription causing HDAC4 down-regulation (Liu et al., 2006; Sleiman et al., 2012). Furthermore, also HDAC7 promoter seems to be under the control of Sp1 during differentiation (Zhang et al., 2010).

This mechanism works in parallel with other additional circuits operating in a tissue-specific manner. This is the case of atrophic muscles, where denervation raises HDAC4 levels, thus influencing a metabolic shift (Tang et al., 2009). Due to the increased levels, HDAC4 re-localizes in the nucleus where induces myogenin activation, which in turn induces the deacetylase expression, thus alimenting a forward-feedback mechanism (Tang et al., 2009). A similar, but negative, feedback mechanism operates during muscle differentiation (Haberland et al., 2007). In this case MEF2A, C and D, the foremost studies TFs partners of class IIa HDACs, are able to bind the promoter of HDAC9, induce its expression and thus fine-tuning their own expression (Haberland et al., 2007). Other evidences of transcriptional

regulation strategy were investigate in the case of stemness-maintenance (Addis et al., 2010), and others remain to be define (Barneda-Zahonero et al., 2013; Vega et al., 2004).

The regulation of class IIa transcription impacts both on the synthesis of mRNA, but also on the control of the mRNA stability. MicroRNAs act at post-transcriptional level influencing both mRNA half-life and translation efficiency. Several miRNAs have been recognized to affect the expression of class IIa HDACs (Di Giorgio and Brancolini 2016 in press). Among them, miR-1 was the first reported to targeting HDAC4 (Chen et al., 2006). The study was achieved using a muscle differentiation model where miR-1 by targeting the 3'-UTR of HDAC4 mRNA promoted myogenesis (Chen et al., 2006). The effect was explained as a block in translation, since HDAC4 mRNA levels were not affected. After this first observation additional reports described the repressive role of miR-1 on HDAC4 also in other cellular contexts (Datta et al., 2008b; Nasser et al., 2008). In muscle also miR-206 and miR-29 regulates HDAC4 activity (Winbanks et al., 2011). The levels of these two miRNAs can be down-regulated by TGF-β activation. Over-expression of both microRNAs resulted in the translational repression of HDAC4 in muscle cells promoting differentiation (Winbanks et al., 2011). miR-206 expression is also enhanced during denervation where it is up-regulated by MyoD in order to buffer HDAC4 increase and thus favoring a possible re-innervation (Williams et al., 2009). About miR-29, its expression could be promoted by HDAC4 itself creating a negative-feedback loop (Mannaerts et al., 2013). Another miRNA sharing this feedback circuit is miR-220a. This miRNA target HADC4 3'-UTR region like miR-1, but with negative impact on mRNA levels; the deacetylase in turn, decreases miRNA-220a transcription disturbing the binding of Sp-1 to its promoter (Yuan et al., 2011b). In hepatocellular carcinoma (HCC) down-regulation of miR-200a enhances cells proliferation and migration, whereas its up-regulation inhibits these processes (Yuan et al., 2011b). Also miR-140 targets HDAC4 and it is involved in the onset of the endochondrial ossification in mice (Tuddenham et al., 2006). Moreover, in osteosarcoma and colon cancer cells, it is able to inhibit proliferation and increase chemoresistance, by inducing p53 and p21 and this response is less evident in cells characterized by mutations in p53 (Song et al., 2009).

Several other miRNAs can influence HDAC4 expression in different contexts (for recent review see Di Giorgio and Brancolini 2016 in press): miR-22 (Huang et al., 2013; Lu et al., 2015), miR-365 (Guan et al., 2011), miR-9 (Davila et al., 2014), miR-2861 (Li et al., 2009), miR-483-5p (Han et al., 2013) and miR-125a-5p (Nishida et al., 2011). Other members of class IIa are subjected to miRNA regulation as well: HDAC5 by miR-2861 (Fischer et al.,

2015) and miR-9* (Roccaro et al., 2010). HDAC7 expression is under the regulation of miR-140-5p and miR-34 involved, respectively, in the metastasis of tongue carcinoma and in resistance to therapy in breast cancer cells (Kai et al., 2014; Wu et al., 2014). Up to now only one miRNA against HDAC9 was identified: miR-188, which controls the osteogenetic program (Li et al., 2015).

Taking together, these evidences suggest that different miRNAs specifically target class IIa HDACs to modulate specific cellular responses and biological functions in different cell types.

### *3.2.2 Post-translational regulation*

The function, activity, and stability of proteins can be controlled by PTMs. Class IIa HDACs family is subjected to a wide spectrum of modifications after the translational process (Wang et al., 2014)Di Giorgio and Brancolini, 2016 in press). It's known that the integration of all PTMs is an important code to understand the regulatory network operating on these enzymes involved in various physiological responses. Dissection of the modulation patterns operating on class IIa HDACs is mandatory to unveil all the cellular responses monitored by these enzymes.

#### *Phosphorylation and the sub-cellular localization*

An important and peculiar strategy adopted by cells to modulate class IIa HDACs activity is to shuttle these enzymes between the nucleus and the cytoplasm (Fischle et al., 2002). Since their repressive function is mainly exerted inside the nucleus, the cytoplasmic accumulation is in general considered as a negative regulation favored when transcription needs to be switched on (Clocchiatti et al., 2013a; Martin et al., 2007). This strategy could be considered as an immediate and quickly adaptive response to external stimuli. The nucleo/cytoplasmic distribution of class IIa HDACs is controlled by two separate domains: i) the NLS presents in the N-terminal region and ii) NES located in the C-terminal part (Grozinger and Schreiber, 2000; Nishino et al., 2008). As above mentioned, NLS 1/2 and NES sequences, when are properly exposed, are able to interact with importin-α and CRM-1 respectively, important determinants to control the distribution in the two sub-cellular compartments of class IIa HDACs (McKinsey et al., 2001). In fact, inhibition of the exportin-1 receptor nuclear

transporter by leptomycin B treatment determines the nuclear accumulation of class IIa HDACs, suggesting that these proteins are continuously transported out from the nucleus.

In 2000's it was demonstrated for the first time that HDAC4 and HDAC5 are able to interact with 14-3-3 proteins and this interaction mediates their sub-cellular localization and hence influences the repressive power (Grozinger and Schreiber, 2000). This interaction is mediated by different serine residues, located in the amino-terminal region, that after the attachment of a phosphate group act as docking sites for 14-3-3 dimer binding. Nowadays, its well know that this control requires the phosphorylation of at least three (four for HDAC7) serine residues well-conserved among the family members (HDAC4: Ser246, 467, 632; HDAC5: Ser259, 497, 661; HDAC7: Ser155, 181, 321, 446; HDAC9: Ser220, 451, 611). 14-3-3 dimers can influence class IIa HDACs localization through different mechanisms: i) by masking the N-terminal NLS and thus preventing recognition of importin-α; ii) unmasking the C-terminal NES, thus facilitating the nuclear export or iii) a combined action of these two mechanisms. Regarding the latter, in cardiomyocites, it was demonstrated that HDAC4 is phosphorilated by two different splicing isoforms of the Calcium/calmodulin-dependent protein kinase II delta (CaMKII δ), one localized in the nucleus and the other one in the cytoplasm (Nishino et al., 2008). This report provides a clear view of the 14-3-3 mechanism because Nishino and colleagues demonstrated that class IIa/14-3-3 interaction can occurs both in the nucleus and in the cytoplasm. Moreover they have also highlighted that 14-3-3 binding in the nucleus facilitates HDAC's export and, on the contrary in the cytoplasm, this binding prevents the nuclear import.

Different kinases are able to affect class IIa HDACs sub-cellular localization. The first family, historically described to modulate HDACs nuclear export, was the previously cited CaM kinase family. In particular, in an in vitro model of muscle cell differentiation the activation of CaMKI promotes cytoplasmic accumulation of class IIa HDACs favoring nuclear export (McKinsey et al., 2000). Not only CaMKI but also CaMKIV can monitor the subcellular localization of the deacetylases (Karamboulas et al., 2006). Although CaMKI and CaMKIV are promiscuous class IIa HDACs kinases, CaMKII manifest a specific activity against HDAC4 (Backs et al., 2006; McKinsey et al., 2001). In fact only HDAC4 has a CaMKII peculiar docking site centered on Arg601 (Backs et al., 2006). CaMKI and IV phosphorylate all class IIa HDACs and show preference for residues 246 and 467 in HDAC4 (and the corresponding aa in other deacetylases), while CaMKII preferentially phosphorylates serines 467 and 632 of HDAC4 (Backs and Olson, 2006; Backs et al., 2006). HDAC4 is also

able, thanks to its capability to form heterodimers with HDAC5 and HDAC9, to make them susceptible to CaMKII activity. On the contrary HDAC7, which lack the glutamine rich-region, does not oligomerize with HDAC4 and hence is not influenced by this kinase. The calcium-mediated export of class IIa HDACs is involved in the regulation of many physiological processes, such as myogenesis, hypertrophy and neuronal survival (Bolger and Yao, 2005; Metrich et al., 2010; Shalizi et al., 2006). In 2005 Bolger and Yao discovered that the CaMKII-mediated HDAC4 export in the cytoplasm exerts a pro-survival role (Bolger and Yao, 2005). Accordingly, the pharmacological inhibition of CaMK with KN-93 causes the nuclear accumulation of HDAC4 and the induction of apoptosis (Bolger and Yao, 2005). In general, the pro-survival effect associated with HDAC4 nuclear export, depends on the activation of a MEF2-transcriptional response (Shalizi et al., 2006).

Another serine/threonine kinase, also belonging to the same CaMK superfamily, is PKD. This kinase is a downstream effector of PKC pathway (Matthews et al., 2006). It is involved as an important regulator of class IIa HDACs during muscle remodeling (Kim et al., 2008) and cardiac hypertrophy (Vega et al., 2004). PKD takes also part in the B-lymphocyte maturation (Matthews et al., 2006) and in the T-lymphocytes thymus selection and apoptosis (Parra et al., 2005). In all these circumstances phosphorylation of class IIa HDACs induces their cytoplasmic accumulation and hence the de-repression of the target genes.

In addition to CaMK and PKD, another important group of kinases govern the phosphorylation status of class IIa HDACs. The master regulator of this group is the well known tumor suppressor LKB-1, which operates on a spectrum of downstream kinases including AMP activated protein kinase (AMPK), microtubule affinity regulating kinases (MARK), SNF-related kinases (SNRK), NUAK family, BR-serine/threonine kinases (BRSK) and salt inducible kinases (SIK) (Hardie, 2011). Recent studies reported that MARK kinases phosphorylate class IIa HDAC members on a specific and conserved residue (i.e. Ser246 in HDAC4, Ser259 in HDAC5 and Ser159 in HDAC7). This base-line phosphorylation facilitates the subsequent signal-dependent phosphorylation by other kinases of the remaining residues required for 14-3-3 binding (Dequiedt et al., 2006).

The metabolic state of the cells can also influence class IIa HDACs localization and functions, in particular under conditions of metabolic stress and upon energy depletion, with concomitant increase of AMP levels, AMPK kinase is activate inside the cells (Liang and Mills, 2013). After activation, AMPK is able to phosphorylate class IIa HDACs, thus inducing their nuclear export (van der Linden et al., 2007). Accordingly, from a physiological

point of view, the stress induced after physical exercise determines the export of HDAC4 and HDAC5 and this correlates with the activation of AMPK and CaMKII (McGee et al., 2008). Recently, McGee and colleagues have reported that during exercise the regulation of HDAC5 is under the supervision of an additional kinases, that is PKD (McGee et al., 2014). Inhibition of LKB1/AMPK pathway causes a nuclear re-localization of class IIa HDACs, a key event in order to promote the activation of gluconeogenesis in the liver (Mihaylova et al., 2011). In this context, after glucagon release, HDAC5 is accumulated in the nucleus and here it associates with HDAC3 deacetylase and activates FOXO1/3 that in turn stimulate the transcription of key enzymes of gluconeogenesis (Mihaylova et al., 2011).

The other class of kinases which activity is modulated during metabolic changes is the SIK family. This group consists of 3 proteins named: SIK1, SIK2 and SIK3 (Katoh et al., 2004). Several papers reported the involvement of SIKs in HDACs re-localization also independently from a metabolic regulation (Berdeaux et al., 2007; Walkinshaw et al., 2013). SIK1 is able to phosphorylate and to re-localize HDAC5 out of the nucleus to switch on MEF2 transcriptional program, thus influencing the myogenic program (Berdeaux et al., 2007). In the adipose tissue modulation of SIK2 influences HDAC4 phosphorylation in a multiprotein complex, which comprises also CREB-regulated transcription co-activator 2 and 3, as well as PP2A. This complex is under the supervision of PKA and is involved in the regulation of GULT4 transcription and glucose uptake (Henriksson et al., 2015). About SIK3, Yang group described that in HEK293 cells this kinase together with SIK2 causes a cytoplasmic re-localization of HDAC5 and HDAC9, but not for HDAC4 and HDAC7 (largely cytoplasmic). Moreover, while SIK2 promotes the nuclear export through the phosphorylation of 14-3-3 consensus sites, SIK3 is effective also on the 14-3-3 Ser/Ala mutants, thus demonstrating that SIK3-mediated export is both kinase activity and classical 14-3-3 binding sites independent (Walkinshaw et al., 2013). Finally, while SIK2 causes the de-repression of MEF2 and stimulates myogenesis in C2C12 cells, SIK3 is incompetent towards MEF2 activation (Walkinshaw et al., 2013). Whether these differences reflect cell lineage specific features or others conditions is currently unknown.

Phosphorylation is a reversible PTM, which can be rapidly reverted by a phosphatase activity. This modulation can impact also on class IIa regulation/localization. In fact in 2000's it was demonstrated that calyculin A, an inhibitor of PP1 and PP2a phosphatases, promoted the nuclear export of HDAC4 reducing the interaction with importin-α (Grozinger and Schreiber, 2000). Some years later, in 2008, the effective contribution of PP2A to class IIa

HDACs cytoplasmic accumulation was proved (Kozhemyakina et al., 2009; Martin et al., 2008; Paroni et al., 2008). PP2A is able to bind the N-termial region of HDAC4. Here, PP2A recognizes and dephosphorilates the NLS1 domain, specifically on Ser 298, causing a nuclear accumulation of HDAC4. Ser 298 is also a target residue for GSK3β kinase, which regulates, under starved conditions, the phosphorylation-dependent poly-ubiquitylation of HDAC4 and with its consequent degradation (Cernotta et al., 2011). As consequence, PP2A-mediated dephosphorilation may therefore also protect HDAC4 from the nuclear degradation. Another serine residue controlled by PP2A is the 246 (in HDAC4). It has been shown that in chondrocytes, the parathyroid hormone-related peptite (PTHrP) suppresses MEF2 and RUNX2 transcriptional activities via PP2A, which dephosphorylates HDAC4 on Ser246. This modification results in chondrocytes hypertrophy. (Kozhemyakina et al., 2009). PP2A activity does not influence nuclear accumulation of HDAC4 during apoptosis. In fact, the amino-terminal fragment (aa 1-289) generated by caspase cleavage enters in the nucleus without requiring the PP2A-mediated dephosphorylation and retains its effectiveness of repression on MEF2 (Paroni et al., 2007). ATM, a kinase mutated in the Ataxia telangiectasia syndrome, modulates PP2A activity. Here, HDAC4 exhibits nuclear localization and represses MEF2 and CREB transcription factors, thus inducing both heterochromatinization and neurodegeneration (Li et al., 2012).

PP1α e PP1β are two others kinases that mediate class IIa HDACs phosphorylation. The first one is able to interact also with MEF2 and to retain HDAC4 into the nucleus causing a repression of the MEF2-dependent gene expression (Perry et al., 2009). PP1β, instead acts in a multiprotein complex together with MYPT1, that are constituent of the myosin phosphatase complex. In smooth muscle, this complex is able to desphosphorylate HDAC7, thus stimulating its nuclear retention (Parra et al., 2007; Walkinshaw et al., 2013). Beyond this classic model of regulation, class IIa HDACs localization and functions can also be affected by other independent mechanisms.

*14-3-3 independent regulation of the subcellualr localization*

Phospho-mediated regulation of class IIa HDACs is a complex issue and sometimes phosphorylation does not correlate with cytoplasmic localization. 14-3-3 docking sites are not the only residues involved in shuttling regulation, as demonstrated few years ago using a combinatorial proteomics approach and phospho-mutant screening by Greco and colleagues (Greco et al., 2011). They have figured out that for HDAC5 there are at least 17

phosphorylation sites, 13 of which do not encompass the consensus for 14-3-3 proteins (Greco et al., 2011). In particular, the phosphorylation of serine 279 is essential to induce the nuclear import of the protein. This residue is conserved among all class IIa HDACs, with the exception of HDAC7 (Greco et al., 2011). The same residue is also target by the kinase Dirk1B, but in this case the phosphorylation reduces nuclear accumulation (Deng et al., 2005). PKA and CDK5 are other two kinases capable of phosphorylate Ser279 on HDAC5. The effect on localization is puzzling and deserves further confirmation, since PKA retains HDAC5 into the nucleus (Ha et al., 2010) but, on the contrary, CDK5 promotes nuclear export in neurons (Taniguchi et al., 2012). This opposite influence on HDAC5 localization could be explained by the existence of other kinases targeting additional residues. Another evidence that support the role of Ser279 in mediating the 14-3-3-independent shuttling arises from a manuscript demonstrating that the Mirk/Dirk1B complex is able to phosphorylate HDAC5 on this residue (Deng et al., 2005). This serine leaves in the NLS region and is also conserved in HDAC4 and HDAC9 but not in HDAC7. During the cell cycle, HDAC4, HDAC5 and HDAC9, but not HDAC7 can be phosphorylated by Aurora B kinase, respectively on Ser 265, Ser 278, and Ser 242. These phosphorylations allow the re-localization of deacetylases at the mitotic midzone during late anaphase, and in the midbody during cytokinesis (Guise et al., 2012). This phosphorylation-dependent re-localization abolishes the interaction with the NCoR complex, thus limiting part of class IIa deacetylase activity (Guise et al., 2012). Moreover, RAS oncogene was found to promote the nuclear localization of HDAC4 by stimulating its phosphorylation by extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Zhou et al., 2000). However, experiments demonstrating the direct phosphorylation of HDAC4 by ERK1/2 are still lacking and the specific PTMs involved are still mysterious.

Another mechanism participating in the regulation of class IIa HDACs depends on the redox condition. Under oxidizing environment, a disulfide bridge is formed between cysteines 667 and 669 (Ago et al., 2008). Cysteine 667 lies in the binding site for the structural zinc ion (Di Giorgio et al., 2015; Hudson et al., 2015). In a reducing environment, these two residues and the coordinated zinc ion fold the protein, bringing the ZBD in contact with the NES. In this way the CRM1 binding site is masked and the nuclear export is blocked. In the presence of oxidants, these cysteines are oxidized, the zinc is no longer coordinated, the NES is exposed to CRM1 and the protein is exported into the cytoplasm (Ago et al., 2008). This

mechanism acts during cardiac hypertrophy, a pathological condition characterize by an increase in the intracellular ROS (Haworth et al., 2012; Oka et al., 2009).

*Other strategies of regulation*

The control of class IIa HDACs activities is not limited to their sub-cellular localization. Additional PTMs can act on these HDACs. Ubiquitin-dependent degradation and proteolytic cleavage can influence class IIa HDACs behavior. Initially it was demonstrated that HDAC7 degradation occurs mainly in the cytoplasm after its phosphorylation-mediated export from the nucleus (Li et al., 2004). The UPS-mediated HDAC7 cytoplasmic degradation was recently confirmed during endochondral ossification (Bradley et al., 2015). HDAC7, during chondrocytes maturation represses MEFs and RUNX2 in order to avoid endochondral ossification. Indeed HDAC7 is exported into the cytoplasm where it is degraded by the UPS and liberates β-catenin increasing the proliferation rate (Bradley et al., 2015). In muscle, degradation of class IIa HDACs plays a pivotal role during fiber type switching from fast and glycolytic to slow and oxidative (Potthoff et al., 2007b). In this case the degradation of HDAC4 and HDAC5, on the contrary takes place within the nucleus (Potthoff et al., 2007b). Nuclear degradation was also described in untransformed cells exposed to serum starvation (Cernotta et al., 2011). In this case GSK3β phosphorylates HDAC4 on serine 298 and this PTMs acts as a priming event required for its poly-ubiquitylation and nuclear degradation (Cernotta et al., 2011). In another study using a knock-out mouse model for HDAC4 and HDAC5, it has been pointed out that during osteoclast differentiation poly-ubiquitylation and degradation of HDAC4 is mediated by SMURF3 E3-ligase (Obri et al., 2014).

Degradation of class IIa HDACs can also pass through lysosomes. In a rat osteoblastic cell line, stimulation with parathyroid hormone causes HDAC4 PKA-dependent phosphorylation on Ser740, its nuclear export and finally the degradation through the lysosomal pathway (Shimizu et al., 2014).

Class IIa HDACs are also subject to non-reversible processing, this refers to proteolytic cleavage. HDAC4 is cleaved by caspase 2 and 3 (Paroni et al., 2004) while HDAC7 is processed by caspase8 (Scott et al., 2008), on residue 289 and 375 respectively. In both cases the cleavage products increase the apoptotic rate (Paroni et al., 2004; Scott et al., 2008), but only the HDAC4 amino-terminal fragment retains a repressive influence against MEF2-dependent transcription (Paroni et al., 2004). Another proteolytic cleavage was observed on HDAC4 during the hypertrophic response in the heart (Backs et al., 2011). In cardiomyocites,

PKA activation causes the cleavage of HADC4 between the residues 201 and 202, by an unknown protease. The amino-terminal fragment generated, accumulated in the nucleus and it was able to repress MEF2 transcription being incompetent for SRF repression (Backs et al., 2011). Among class IIa HDACs, only HDAC4 posses the binding site for PKA, located in the C-terminal region of the protein (residues 638-651). The anti-hypertrophic effect of PKA was sufficient to antagonize the pro-hypertrophic action of CaMKII, without affecting cardiomyocite survival (Backs et al., 2011).

SUMOylation is an additional PTMs that can occur on class IIa HDACs. HDAC4, HDAC5 and HADC9 can be SUMOylated on lysine 559, 605 and 549 respectively (Kirsh 2002 EDG). Conversely HDAC7, probably because this deacetylase does not possess the glutamine-rich domain, is not subjected to such modification (Guo et al., 2007). HDAC4, in particular, becomes SUMOylated by the SUMO E3 ligase RanBP2 on the nucleopore complex during the nuclear/cytoplasmic shuttling (Kirsh et al., 2002). SUMOylation increases the interaction with HDAC3 and therefore the class IIa repressive capability (Kirsh et al., 2002). Class IIa HDACs are not merely target of SUMO E3-ligases, but several evidences indicate that they could promote SUMOylation of some partners. Gregorie and coworkers have been demonstrated that class IIa HDACs are involved in the activation of Ubc9 SUMO E2-ligase (Gregoire et al., 2006). Through this mechanism, class IIa HDACs can promote SUMOylation of different proteins like as MEF2s (Gregoire et al., 2006), promyelocytic leukemia protein (PML) (Gao et al., 2008) and two nuclear receptors: LXRα/NR1H3 and LXRβ/NR1H2 (Lee et al., 2009).

## 3.3 Biological functions

### *3.3.1 Binding partners*

Class IIa HDACs have emerged as a transcriptional co-repressors (Wang et al., 1999), even if an ineffective deacetylase domain characterizes them. To exert their function on DNA, class IIa need to reside on chromatin. Since they lack a DNA binding domain, their recruitment on specific regulatory DNA regions is dependent on the ability to interact with different partners. As in part discussed above, class IIa HDACs are able to cooperate with a wide array of transcription factors (Clocchiatti et al., 2013a; Martin et al., 2007) and could thus potentially

control a plethora of genetic programs. Among the large list of class IIa interactors, the most characterized are members of the MEF2 family transcription factors, which fine tune differentiation, cell growth and survival (Potthoff et al., 2007b). Comparative in vitro binding studies have testified that the affinity of interaction, between HDAC4 and MEF2C or HDAC4 and other putative partners such as SRF and RUNX2, differs enormously. MEF2 can be proposed as the preferred class IIa transcriptional partner (Paroni et al., 2007). The interaction with MEF2 involves 18 amino acid (residues 166-188 in HDAC4) located in the amino-terminal portion, specifically in the glutamine-rich region of HDACs, and are highly conserved among the four members of this sub-group (Guo et al., 2007). As aforementioned, HDAC7 does not possess the glutamine-rich domain, but it is still able to bind MEF2 because it retains the key residues necessary for interaction, that are leucines 128 and 133 (Han et al., 2005). The binding between MEF2s and class IIa HDACs takes place both in the nucleus and in the cytoplasm. The interaction with cytoplasmic MEF2s stimulates the nuclear import of class IIa HDACs (Borghi et al., 2001). The fact that MEF2 are the favorite class IIa partners is highlighted also from in vivo studies. The phenotypes of each knockout mice for class IIa HDAC family members can be explained as the effect of MEF2 hyper-activation in bone (HDAC4), heart (HDAC5/9) and cardiovascular system (HDAC7), in relation to the district in which the single HDACs are more expressed (Chang et al., 2004; Chang et al., 2006; Vega et al., 2004).

RUNX2 transcription factor is another protein capable to interact with class IIa HDACs (Kang et al., 2005; Martin et al., 2007). This transcription factor is involved in the regulation of chondrocytes hypertrophy (Vega et al., 2004; Zheng et al., 2003) and also in endochondral bone ossification (Yoshida et al., 2004). Among class IIa HDACs, HDAC4 and HDAC5 are the main repressors of RUNX2-mediated transcription. Differently from MEF2 regulation, class IIa HDACs in addition to negatively regulate RUNX2 transcriptional program hindering their DNA-binding ability (Martin et al., 2007), they also stimulates RUNX2 UPS-mediated degradation (Jeon et al., 2006).

To the same family of MEF2s, belongs also another class IIa HDACs partner that is SRF (Chang et al., 2006).

Members of the nuclear factor activated T cells (NFAT) family of transcription factors are able to interact with class IIa. In particular, NFAT3c transcriptional activity is repressed by HDAC4, HDAC5 and HDAC9 (Dai et al., 2005). In contrast to the interaction with MEF2,

the recruitment of class IIa HDACs by NFAT is indirect and relies on a bridging cofactor, the chaperone mammalian relative of DnaJ (MRJ) (Dai et al., 2005).

An important co-repressor, which cooperates with class IIa HDACs is CtBP. This protein interacts not only with class IIa members, but also with HDAC1 and HDAC3. The binding to CtBP is required, at least in part, for the repression of MEF2-mediated transcription. In fact, mutations of the CtBP-binding domain in MITR, a C-terminal deleted HDAC9 splice variant, abolishes its interaction with CtBP and impairs, but does not eliminate, the ability of MITR to inhibit MEF2-dependent transcription (Zhang et al., 2001).

Other important partners of class IIa HDACs are the members of the FOXO family of transcription factors. The regulation of FOXO TFs seems to be both negative and positive (Clocchiatti et al., 2013a). In T lymphocytes it has been reported that class IIa HDACs inhibit FOXOP3 functions (Zhou et al., 2008), while on the contrary Mihaylova described that class IIa HDACs when in complex with HDAC3 stimulate FOXO1/3 deacetylation and thus their activation (Mihaylova et al., 2011).

The list of class IIa HDACs partners is very long and inside that there are further proteins such as HP-1α and SUV39H1, two structural component of heterochromatin that are essential for DNA packaging (Zhang et al., 2002b), JARID1B which is co-regulated with HDAC4 during mammary gland morphogenesis (Barrett et al., 2007) and TRPS1 a regulator of chondrocyte proliferation and differentiation, which silencing effect was almost completely rescued by HDAC4 over-expression (Wuelling et al., 2013). Another interactor of HDAC4 defined by immunoprecipitation is the activating transcription factor 4 (ATF4) (Kikuchi et al., 2015). In this study, conducted in multiple myeloma cells, the authors showed that HDAC4 interacts with ATF4 and inhibits activation of endoplasmic reticulum (ER) stress-associated pro-apoptotic transcription factor CHOP (C/EBP homologous protein). HDAC4 knockdown or inhibition can enhance apoptosis under ER stress condition by up-regulating both ATF4 and CHOP. Accordingly, HDAC4 knockdown showed modest cell growth inhibition (Kikuchi et al., 2015).

All these class IIa binding partners, described till now, are associated among each other by the fact that are nuclear localized (for review see Martin et al 2007). But, as aforementioned, class IIa HDACs can also lie in the cytoplasm where they are also able to interact with other partners carrying out additional functions. Hypoxia-inducible factor (HIF) is one of them. In particular HIF-1α is a transcription factor that are highly degraded under normal oxygen condition while is activated during hypoxia. In the latter case, class IIa HDACs bind HIF-1α

in the cytoplasm deacetylating and thus stabilizing the transcription factor (Kato et al., 2004). Similarly to HIF-1α, also STAT1 was reported being deacetylated by HDAC4 in the cytoplasm (Stronach et al., 2011a). It seems that HDAC4 could mediate resistance to chemotherapy mainly through the modulation of the JAK-STAT pathway (Kaewpiboon et al., 2015). Instead, in the cytoplasm of muscle cells HDAC4 acts on MEKK2, a member of the MAPK family. HDAC4-mediated deacetylation provokes MEKK2 activation that culminates in muscle remodeling during denervation (Choi et al., 2012).

To assess their repressive functions class IIa HDACs needs to be associated with class I HDACs and to reside in the nucleus. As aforementioned this interaction involves the deacetylase domain of class IIa proteins. It has been demonstrated that class IIa HDACs enzymatic activity is mainly due because of the recruitment of class I enzyme in a multiprotein complex together with SMRT and NCoR (Fischle et al., 2002). Recently has been shown, by in vitro studies using recombinant proteins, that the amino acid involved in the coordination of ZBD (i.e. 667, 675 and 751) are essential for the interaction with SMRT-NCoR complex (Hudson et al., 2015). In the cytoplasm the interaction with HDAC3 is weak and thus it seems that class IIa HDACs don't have a cytoplasmic activity (Fischle et al., 2001). Not only the C-terminal region is important for class IIa HDACs repressive function. The first evidence come from MITR, a N-terminal splice variant of HDAC9, able to repress MEF2-dependet transcription (Zhang et al., 2001). Furthermore, the amino-terminal fragment of HDAC4, generated after caspase cleavage (aa1-289) is a stronger repressor of RUNX2 and SRF than the full-length protein (Paroni et al., 2007). Another recent example described the major repressive function of the N-terminal fragment, in particular on some apoptotic genes showing a pro-survival role of HDAC4 (Guo et al., 2015), Guo and colleagues reported that in mice affected by retinisis pigmentosa, a blindness caused by rod cells death, the overexpression of the N-terminal fragment (1-251) is able to reduce rod cells death. They reported that HDAC4 1-251 is able to suppress apoptosis because of a major stability of the protein fragment.

There are many class IIa HDACs binding partners. It implies that the presence of the peculiar extended N-terminal domain is important because it mainly mediates interaction with other proteins. Similarly also the deacetylase domain is important for the modulation of the dynamic protein-protein interactions network. This complexity could justify both the long list of interactors as well as the various genetics programs regulated by class IIa HDACs.

### *3.3.2 Regulation of skeletogenesis*

Most of the bones in vertebrate skeleton are formed from cartilaginous template in which chondrocytes undergo hypertrophy, followed by apoptosis. Thereafter, osteoblast, blood vessel and other cell types invade and produce the bone matrix in the space lefts by chondrocytes. HDAC4 plays a central role during skeleton formation and in vivo it is expressed in pre-hypertrophic chondrocytes (Vega et al., 2004). It has been reported that mice deficient in HDAC4 die within two weeks from birth displaying a deregulated osteogenesis, resulting from premature and ectopic bone calcification. This defect results from excessive hypertrophic chondrocyte differentiation and inadequate endochondral ossification (Vega et al., 2004). Initially the HDAC4 null phenotype was explained by an altered regulation of RUNX2 (Vega 2004 EDG), but some years later this pathway has been redefined, thanks to other observations discovering the essential role of MEF2 activation in this developmental process (Arnold et al., 2007). The excessive endochondral ossification exhibited by *Hdac4* null mice can be partially rescued after deletion of one *Mef2c* allele (Arnold et al., 2007). However, HDAC4-mediated regulation of RUNX2 intervenes in this differentiating program. VEGF is a target gene of RUNX2, and it is implied in the vascularization step. HDAC4, through the inhibition of RUNX2, is able to inhibit VEGF thus blocking the endochondral bone ossification (Zelzer et al., 2001). Therefore, HDAC4 participates in the TGF-β mediated inhibition of osteoblast differentiation (Kang et al., 2005). The complete deletion of TRPS1, a multi zing finger nuclear regulator of chondrocytes proliferation and differentiation able to bind HDAC4, causes defect in cell cycle progression that could almost rescued by HDAC4 over-expression (Wuelling et al., 2013).

### *3.3.3 Regulation of cardiomyogenesis and of the vascular system*

A variety of intrinsic and extrinsic stimuli, such as stress, exercise or cardiovascular disorders, provoke an adaptation response of cardiac cells, becoming enlarged because of hypertrophic growth (Backs and Olson, 2006). MEF2 integrates many stress signals in the adult myocardium and regulates the expression of numerous fetal cardiac genes (McKinsey et al., 2002). All class IIa members are detectable in mouse heart suggesting a functional role for the MEF2 class IIa HDACs axis in cardiac hypertrophy (Zhang et al., 2002a). Indeed, expression of constitutively repressive mutants of HDAC4, 5 and 9 prevents hypertrophic gene expression in primary rat cardiomyocytes (Backs and Olson, 2006; Vega et al., 2004; Zhang et al., 2002a). In contrast, disruption of HDAC9 leads to hyperactivation of MEF2-

dependent transcriptional activity in response to pathologic cardiac hypertrophic signals (Zhang et al., 2002a). Curiously, mice lacking *Hdac4* or *Hdac9* are vital, but exhibit exacerbated cardiac hypertrophy triggered by hormonal stress-related signals (Chang et al., 2004) showing spontaneous onset of cardiac hypertrophy with advancing age (Zhang et al., 2002a). Furthermore, the contemporary loss of *Hdac5* and *Hdac9* causes perinatal death due to heart developmental abnormalities (Chang et al., 2004). Interestingly, cardiac hypertrophy is characterized by altered levels of intracellular calcium, condition that activates $Ca^{2+}$-dependent kinases such as the CaMK family and the PKD. These kinases, as aforementioned, are all involved in controlling class IIa HDACs intracellular shuttling (Backs et al., 2006; Vega et al., 2004). In cardiomyocites, CaMKII activation induces nuclear export of HDAC4, with consequent de-repression of MEF2 and NFAT, thus promoting a hypertrophic growth (Backs et al., 2006). In miR-22 null mice, cardiac miR22 was found to be essential for hypertrophic growth in response to stress, through directly targeting of Sirt1 and HDAC4 (Huang et al., 2013).

Class IIa HDACs, play also a key role during the differentiation of vascular structures (Chang et al., 2006). Mice deficient in *Hdac7* show embryonic lethality at day 11, resulting from a failure to form tight junctions in the developing circulatory system, which affect its integrity leading to dilatation and hemorrhages (Chang et al., 2006). This phenotype could be explained by the over activation of MEF2 that causes the up-regulation of the matrix metalloprotease 10 (MMP10) (Chang et al., 2006). Moreover, the silencing of *HDAC7* in HUVEC cells dramatically affects the generation of capillary structure in vitro, thus confirming the essential role of this HDAC in the regulation of angiogenesis (Mottet et al., 2007).

It has also been demonstrated that HDAC7 could be regulated by vascular endothelial grow factor (VEGF) (Wang et al., 2008). In this case, the regulation is mediated by PKD, that through the phosphorylation of 14-3-3 docking sites induces the nuclear export of HDAC7 and thus the activation of VEGF-mediated gene expression. These target genes can be dependent, as well as independent, from MEF2 transcription factors. Overall they influence the proliferation and migration capability of endothelial cells (Wang et al., 2008).

### *3.3.4 Muscle differentiation*

Class IIa HDACs and MEF2 transcription factors act as main regulators in skeletal muscle development (McKinsey et al., 2000). In particular HDACs function as the fine tuning regulator of the differentiation program. MEF2s alone do not posses intrinsic myogenic activity. They need to cooperate with other transcription factors, like as the basic helix-loop-helix (eg. MyoD), which instead own this ability (Olson et al., 1990). In fact MEF2 proteins act to amplify and potentiate the myogenic differentiation program (Potthoff and Olson, 2007). This dependence from other myogenic factors does not mean that MEF2s are not necessary during muscle differentiation. In fact muscle specific knock-out mice of *Mef2c* is lethal because of rapidly deterioration of myofibers after birth (Potthoff et al., 2007a). Also HDACs are important in this differentiation program. Class IIa HDACs localize into the nucleus repressing the MEF2-dependent transcriptional program in undifferentiated cells. When cells, following the exposure to differentiative stimuli stop to proliferate, class IIa HDACs are translocated into the cytoplasm and thus MEF2s target genes are activated and myoblast undergo to myotubes differentiation (Lu et al., 2000). The calcium-dependent kinases CaMK II and IV act during muscle differentiation to trigger HDAC4 and HDAC5 phosphorylation and their cytoplasmic accumulation (McKinsey et al., 2000). This effect elicits the dissociation of MEF2-HDAC repressive complex provoking the de-repression of MEF2 target genes.

Class IIa HDACs intervene also in the regulation of skeletal muscle remodeling. In fact it is reported that after denervation HDAC4, which is present at the neuro-muscolar junction., is up-regulated and translocated into the nucleus, where it promotes muscular atrophy by repressing DUSH2, a negative regulator of myogenin (Cohen et al., 2007). Consequently, also myogenin target genes, like the E3-ligase Atrogin-1 and MURF1, are activated (Moresi et al., 2010). All these mechanisms activate the atrophy-program and, in parallel, also miR-206 expression, which targets the 3'UTR of HDAC4 and HDAC5. This response is part of a negative feed-back loop that try to re-induce muscle innervations (Moresi et al., 2010). Moreover, also HDAC9 participates in atrophy progression, but on the contrary of HDAC4, it is down-modulated. Importantly the *Hdac9* knock-out mice are exaggeratedly sensitive to the denervation responses in skeletal muscle (Mejat et al., 2005). In this case, it is important to consider that *Hdac9* expression, as part of a negative feed-back loop, is also regulated by MEF2 (Haberland et al., 2007), which acts on producing more myogenin and hence inducing atrophy.

In atrophy condition, class IIa deacetylases were described to be involved also in metabolism regulation, through their influence on the fiber type shift (Tang et al., 2009). In denervated muscles, Tang and colleagues reported that HDAC4 acts as strong repressor of glycolysis, probably through MEF2 repression, and as strong activator of oxidative gene program, favoring the switch from fast-glycolytic type II to the slow-oxidative ones (type I) (Tang et al., 2009).

### *3.3.5 The immune system*

Class IIa HDACs affect also the development and the function of the immune system (Parra, 2015). They take part in the process of negative selection in the thymus, to eliminate the self-reacting T-cells (Dequiedt et al., 2003). Among the various class IIa HDACs, HDAC7 is the most expressed in developing thymocytes at the $CD4^+$ $CD8^+$ double-positive stage (Dequiedt et al., 2003). Under basal condition, HDAC7 localizes in the nucleus, where it exerts transcriptional repressive functions (Dequiedt et al., 2003; Kasler and Verdin, 2007). In this context, HDAC7 was found to repress the transcription of the orphan nuclear receptor Nurr77, leading to the inhibition of apoptosis that cause the negative selection of T cells (Dequiedt et al., 2003). After T-cell receptor activation, PKD1 is activated and it phosphorylates HDAC7, which is exported into the cytoplasm, thus resulting in the activation of Nur77. This gene is a MEF2 target involved in the induction of apoptosis of T cells (Parra et al., 2005). HDAC7 was subsequently reported to be involved not only in repression of Nur77, but also in the transcriptional regulation of a large number of genes involved in both positive and negative selection of thymocytes (Kasler and Verdin, 2007). More recently Kasler and colleagues presented evidences for the role of HDAC7 in T-cell development. Using a conditional knock-out mouse model, they demonstrated that specific deletion of *Hdac7* in double positive thymocytes results in a significant positive selection of single-positive $CD4^+$ cells (Kasler et al., 2011). HDAC7 is also crucial for the proper function of cytotoxic T cells (Navarro et al., 2011). HDAC7 is not the only class IIa HDAC involved in the regulation of T cells. HDAC9 plays a central role in the control of T regulatory cells (Treg). *Hdac9* KO mice present higher number of T-reg and increased immune suppressive functions. This effect is due to the unrestricted activity of Foxp3, an important transcription factor for development and function of these regulatory cells (Tao et al., 2007). Also in B cells the engagement of the B cell receptor promotes the activation of PKD, which drives class IIa HDACs out of the nucleus (Matthews et al., 2006). Recently, it has been reported

that *HDAC7* is highly expressed in pre-B lymphocytes but is not present in myelod cells such as macrophages (Tao et al., 2007). In this lineage HDAC7 interacts with MEF2C and is recruited to the promoter of macrophage genes in B-cell precursor (Tao et al., 2007). Knocking-down *HDAC7* in B lymphocytes leads to the de-repression of myeloid genes, indicating that HDAC7 may be an essential lineage specific-transcriptional repressor (Tao et al., 2007).

### *3.3.6 Neurons*

An increasing body of evidences indicate a crucial role for class IIa HDACs in physiological and pathological neuronal functions (Parra et al., 2015). HDAC4 is expressed at high level in neurons, where it's mainly located in the cytoplasm (Majdzadeh et al., 2008). Mice deficient of *Hdac4*, in addition of having severe skeletal abnormalities they also exhibit brains that are 40% smaller than the control (Majdzadeh et al., 2008). Recently an important role of HDAC4 in neuronal synaptic plasticity and memory formation was proposed (Kim et al., 2012; Sando et al., 2012). Kim and co-workers showed that a specific deletion of *Hdac4* in mice forebrain resulted in the impairment of memory, behavioral learning and long-term synaptic plasticity defects (Kim et al., 2012). Sando et al., found that, when present in the nucleus, HDAC4 governs the gene transcriptional program characteristic of the central synapses, affecting information processing in the brain (Sando et al., 2012). Interestingly, alteration of HDAC4 physiological activities through the expression a truncated catalytic domain form of the protein, specifically in the forebrain of transgenic mice resulted in defects in spatial learning and memory (Sando et al., 2012). The potential neurotoxicity of HDAC4 was confirmed by its functional abnormalities coupled to different neurodegenerative disease (for review see Falkenberg et al., 2015). A very interesting report was on ataxia telangiectasia (ATM) disorder (Li et al., 2012), in which it was demonstrated the double face of HDAC4 medal. It means that Li and colleagues described two opposite functions of HDAC4 in neurons. In particular nuclear-resident HDAC4 exhibits neurotoxic effect with induction of the apoptotic response (i.e. activation of caspase-3). The other face of the medal was the protective function of the HDAC4 cytoplasmic fraction, that hinder the activation of caspase-3 and improved the motor behavior of ATM mice (Li et al., 2012). The protective function was also acquired by blocking the nuclear accumulation of HDAC4 though the inhibition of PP2A a well known phosphatase able to induce HDAC4 nuclear import (Li et al., 2012).

Also HDAC5 participates in neuronal development programs. Two studies of the same group showed that HDAC5 is involved in axonal regeneration (Cho et al., 2015; Cho et al., 2013). It has been reported that axon injury induces the nuclear export of HDAC5 in a calcium- and PKC-dependent manner (Cho et al., 2013). The cytoplasmic accumulation is necessary for axon regeneration as testified by the expression of a nuclear resident phosphor-mutant HDAC5, which interferes with axon regeneration (Cho et al., 2013). The CNS-dependent regulation of HDAC9 was observed too (Sugo et al., 2010). In fact, *Hdac9* is expressed in the mouse cerebellar cortex during post natal cortical development (Sugo et al., 2010). After spontaneous neuronal activity, HDAC9 is exported to the cytoplasm leading activation of *c-Fos* gene and thus promoting dendritic growth (Sugo et al., 2010).

### *3.3.7 Metabolism*

Several kinases like as LKB1, AMPK or the SIK family, which hold in check class II HDACs are also well known metabolic regulators (Mihaylova et al., 2011; van der Linden et al., 2007). Hence, it is not surprising that class IIa HDACs govern central aspect of metabolism. Mihaylova and colleagues reported that HDAC4, HDAC5 and HDAC7, are expressed in the liver (Mihaylova et al., 2011). During fasting, the release of glucagon in the liver activates the gluconeogenesis, at least in part through the inhibition of the AMP-kinase (Mihaylova et al., 2011). The inhibition of AMPK causes a massive accumulation of class IIa HDACs in hepatocytes' nucleus. Here, they associate with HDAC3 that deacetylates and activates FOXO transcription factors (FOXO1 and FOXO3). FOXO proteins, then stimulate the transcription of two key enzymes of the gluconeogenesis, the glucose-6-phosphatase and the phosphoenolpyruvate carboxy kinase (Mihaylova et al., 2011). Knock-down of class IIa HDACs, in murine liver cells, results in inhibition of Foxo target genes, lower blood glucose levels and augmented glycogen storage (Mihaylova et al., 2011). In parallel, in a study performed in *Drosophila*, it was observed that during feeding conditions, HDAC4 is phosphorylated and localized into the cytoplasm (Wang et al., 2011). The kinase responsible for such re-localization is SIK3. During fasting SIK3 becomes inactivated, resulting in the dephosphorylation and nuclear translocation of HDAC4, thus allowing FOXO deacetylation (Wang et al., 2011). Recently, it has been shown that also SIK2, another member of the SIK family kinases, is able to modulate HDAC4 localization in the adipose tissue. Here HDAC4 is involved in the regulation of the GLUT4 transcription and glucose uptake (Henriksson et al., 2015). Furthermore, HDAC4 has been characterized as an immune-metabolic sensor. In

particular under over-nutrition, leptin reduces the expression inflammatory genes under the control of NF-kB through HDAC4 nuclear accumulation (Luan et al., 2014). Accordingly, HDAC4 variants have been associated with both body mass index and waist circumference (Luan et al., 2014) and its expression is down-modulated in the fat from obese subjects (Abu-Farha et al., 2013).

HDAC9 is another class member, which can contribute to adipogenesis and obesity (Chatterjee et al., 2014; Chatterjee et al., 2011). HDAC9-deficient pre-adipocytes show accelerated adipogenic differentiation, indicating that HDAC9 may act as a negative regulator of adipogenesis (Chatterjee et al., 2011). The same laboratory demonstrated a role for HDAC9 in obesity (Chatterjee et al., 2014). In response to chronic caloric excess, the differentiation of pre-adipocytes into functional adipocytes is compromised. After administration of a chronic high-fat diet in mice, *HDAC9* deficiency was found to result in improvement of adipogenic differentiation and establishment of a better metabolic state with a diminished weight gain, and have improved glucose tolerance, insulin sensitivity and reduced hepatosteatosis (Chatterjee et al., 2014).

## 3.4 Class IIa HDACs and cancer

A Class IIa histone deacetylases role in tumor pathology is not clear depicted. From literature emerges a dual role of the members of this family of HDACs. Various reports described them as positive regulators of cell growth and cancer progression, whereas other studies define class IIa as onco-suppressive factors (Barneda-Zahonero and Parra, 2012; Clocchiatti et al., 2011) (Figure 5).

Initial observation pointed-out significant mutations of HDAC4 in human breast cancer samples (Sjoblom et al., 2006). In addition, a genome-wide approach evidenced HDAC4 homozygous deletion in melanoma cell lines (Stark and Hayward, 2007). HDAC4 expression was also up-regulated within a sub-group soft tissue sarcomas, specifically in leiomyosarcoma (Di Giorgio et al., 2013). Class IIa HDACs were described to be associated with poor prognosis of estrogen receptor positive ($ER^+$) breast tumors (Clocchiatti et al., 2013b). Mutation of HDAC7 has been reported in non-Hodgking lymphoma (Morin et al., 2011). High level of cytoplasmic HDAC7 have been observed in pancreatic cancer patient (Weichert,

2009). Similarly, in children with acute lymphoblastic leukemia (ALL), high level of HDAC7 and HDAC9 expression were associated with poor prognosis (Moreno et al., 2010). The up-regulated expression of HDAC9 was associated also with poor survival in medulloblastoma patients (Milde et al., 2010). HDAC9 levels were on the contrary highly repressed in glioblastoma (Clocchiatti et al., 2011). Conversely HDAC7 and HDAC5 were activated and over-expressed in glioblastoma (Clocchiatti et al., 2011). Together with HDAC9, also HDAC5 was found to be over-represented in high-risk medulloblastoma patients, demonstrating a relation between its expression and poor survival (Milde et al., 2010). Finally, HDAC4 was recently described as a prognostic marker in glioma tumors (Cheng et al., 2015). In this report low levels of HDAC4 were correlated to the low-grade of gliomas. Hence, HDAC4 was proposed as a new prognostics marker which can refine the prognosis of glioma (Cheng et al., 2015).

A link between class IIa HDACs expression and cancer was also established by multiple studies on cell lines. These studies suggest that, depending on the cellular context, class IIa HDACs can act either as pro-proliferative factors or as a tumor suppressors (Barneda-Zahonero and Parra, 2012; Clocchiatti et al., 2011) and again a clear picture could not be depicted. Class IIa HDACs pro-oncogenic activity is supported by a series of evidences underling their participation in cell proliferation. First of all the involvement of class IIa HDACs in regulation of cell growth could be easily supposed from the phenotype of the knock-out mice (Vega et al., 2004). In fact, HDAC4 -/- mice are characterized by premature and ectopic endochondral ossification (Vega et al., 2004). Because of an inner balance of HDAC4 regulation inside the cells, its dysfunction could be deleterious. Stimulation with mitogens, like EGF in osteoblasts, can modify this equilibrium, inducing an increase in HDAC4, thus enhancing RUNX2 repression and hence osteoblasts proliferation (Zhu et al., 2011b). Both HDAC4 and HDAC7 oncogenic potential was recently well demonstrated in mouse fibroblasts (Di Giorgio et al., 2013). The transforming capability depends mainly, from the modulation of a limited set of genes, most of which are MEF2 target (Di Giorgio et al., 2013). Over-expression of HDAC4 or HDAC7 nuclear resident forms induced acquisition of tumorigenic phenotype with high proliferation rate, cytoskeleton modification, high mobility, ability to growth in an anchorage-independent manner and tumor formation in mice (Di Giorgio et al., 2013). The involvement of HDAC4 in cancer cells growth was also demonstrated in p53 deficient HeLa cells (Cadot et al., 2009). In this report HDAC4 silencing showed a negative effect specifically on cancer cell line proliferation, whereas no influence

was observed in normal fibroblasts. In particular, it was shown that HDAC4 down-modulation causes G2/M arrest because of defects in chromosomes segregation (Cadot et al., 2009). This effect was also demonstrated in colorectal carcinoma cells where HDAC4 is necessary to promote efficient mitotic segregation of chromosomes. Similarly its down-modulation induces an accumulation in the G2 phase (Wilson et al., 2008). These effects mostly depend on the capability of HDAC4 to repress p21/CDKN1A transcription in a p53-independent manner (Wilson et al, 2008). Both the nuclear localization and the deacetylase activity are required for HDAC4-mediated repression of p21 (Wilson et al., 2008). HDAC4 was identified as a repressor of p21 expression also in breast cancer cells, through the binding to FOXP3 transcription factor (Liu et al., 2009). HDAC4 p21-mediated oncogenic behavior was highlighted in gastric cancer (Kang et al., 2014). In particular HDAC4 was able to repress p21 and its silencing decrease proliferation of cancer cells line and arrest cells in G1 phase, because of induction of p21. HDAC4 silencing induces also autophagy and apoptosis.

Deregulation of HDAC4 expression was also observed in HCC where its levels are increased because of a lack of post-transcriptional controls mediated by the miR-1, miR-22 and miR200a (Datta et al., 2008a; Yuan et al., 2011b; Zhang et al., 2010). In all these cases HDAC4 down-regulation reduced HCC cells proliferation. Interestingly, HDAC4 negatively regulates the transcription of miR200a, by repressing Sp1 TF. As a consequence of HDAC4 destabilization, miR200a can influence the acetylation of p21/CDKN1A promoter and its transcription (Yuan et al., 2011a). The down-regulation of miR-1 and the coupled increase in HDAC4 levels were also observed in lung cancer (Nasser et al., 2008).

HDAC4 appears to be linked with the resistance to chemotherapy. In breast cancer HDAC4 determines the resistance to 5-fluorouracil, a common drug utilized for anti-tumor therapy, through the deacetylation of the SMAD4 promoter and the repression of its transcription (Yu et al., 2013). In ovarian tumor cells resistant to cisplatin, HDAC4 is over-expressed together with STAT1 and depletion of both proteins is sufficient to re-sensitize cancer cells. Mechanistically, HDAC4-STAT1 interaction takes place only in the cytoplasm of resistant cells. Under this condition HDAC4 deacetylases STAT1, thus promoting its phosphorylation and nuclear import. Furthermore STAT1 deacetylation seems to be independent from HDAC3 (Stronach et al., 2011b). As aforementioned, HDAC4 in the cytoplasm interacts also with HIF-1α inducing its transcriptional activation, through the prevention of its degradation, and thus promoting the expression of VEGF and angiogenesis

in cancer (Qian et al., 2006). Binding of HDAC4 to HIF-1 generates a complex that regulated glycolysis and cytotoxic stress of cell adaptation to hypoxic conditions (Geng et al., 2011).

In contrast to these pro-oncogenic activities, HDAC4 shows an opposite behavior in lymphomas, being its down-modulation linked to leukemogenesis. In this tumor miR-155 is up-regulated and one of its direct target is HDAC4. In agreement, over-expression of HDAC4 in diffuse large B-cell lymphoma results in a reduction of cell proliferation, lower clonogenic potential and induction of apoptosis (Sandhu et al., 2012).

Among the class IIa members, HDAC5 was similarly reported to have a contradictory role in cancer. In U2OS osteosarcoma cells, SY5Y neuroblastoma cells and MCF7 breast carcinoma cells it inhibits cell proliferation when ectopically expressed (Huang et al., 2002). These tumor-suppressive roles were largely dependent on the activation of the tumor necrosis factor (TNF) pathway, followed by the induction of apoptosis (Huang et al., 2002). Secretion of TNFα and of other pro-inflammatory cytokines was influenced by HDAC5 in macrophage after inflammatory stimulus (Poralla et al., 2015).

On the contrary, HDAC5 was recently identified as a promoter of osteosarcoma progression, through the up-regulation of TWIST1 expression, with a still unknown mechanism (Chen et al., 2014). The oncogenic role was described also in HCC (Feng et al., 2014). In this situation low levels of HDAC5 inhibit cells cancer proliferation by the induction of cell cycle arrest and apoptosis (Fan et al., 2014). Peixoto and colleagues reported another mechanism by which HDAC5 participates in cancer cell growth. They showed that HDAC5 is necessary for replication fork advancement. In particular they demonstrated that the silencing of HDAC5 induced a slowing down of the cell cycle progression, because of the replication fork stalling. This condition induced DNA double-strand breaks and the activation of apoptosis (Peixoto et al., 2012). In neuroblastoma cells HDAC5 can block differentiation and can induce proliferation (Sun et al., 2014). In particular HDAC5 is induced by N-MYC, and in a feedback loop HDAC5 stabilizes N-MYC protein. Both proteins, when assembled in a complex are able to repress a set of tumor suppressor genes (Sun et al., 2014). Previously, in 2007 the same group reported that TLX, an orphan nuclear receptor, recruits HDAC5 and HDAC3 on its target promoter genes, including the tumor suppressor p21 and PTEN, turning off their transcription (Sun et al., 2007).

As aforementioned for HDAC5, HDAC9 is positively associated with high-risk medulloblastoma (Milde et al., 2010). Its silencing in medulloblastoma cell lines decreases cell growth and induces apoptosis with caspase activation (Milde et al., 2010). By contrast,

qRT-PCR studies in brain tumors have reveled a down-regulation of HDAC9 in glioblastoma compared to low-grade astrocytoma and normal brain (Clocchiatti et al., 2011). Altered expression of HDAC9 together with HDAC7 was observed also in bone marrows of children with acute lymphoblastic leukemia, associated with bad tumor prognosis (Moreno et al., 2010). Nowadays, the role of HDAC9 in tumor progression and how it impact on cell growth and survival is not clear, further investigations are necessary for a more clear picture.

Using a high-throughput approach, HDAC7 was identified as a potential oncogene for hematopoietic cells (Rad et al., 2010). Its contribution to cancer cells proliferation was proposed to be partially due to the regulation on c-Myc (Zhu et al., 2011a). Silencing of HDAC7 resulted in a significant G1/S cell cycle arrest in different cancer cell lines. This cell cycle block occurred through the suppression of c-Myc expression and the augmentation of p21 and p27 protein levels. Interestingly, it was noted that HDAC7 silencing induces cellular senescence, revised by c-Myc re-expression (Zhu et al., 2011a).

Differently from HDAC4, HDAC7 is able to interact with HIF1-α but is not involved in its activation. It seems to be necessary for the HIF-1α mediated repression of cyclin D1 expression (Wen et al., 2010). It has been suggested that this mechanism nourishes chemoresistance. Another important strategy that has been proposed being important for the effect on tumorigenesis of class IIa HDACs involves the β-catenin pathway. In particular, Margariti and co-workers figured out that either over-expression or down-regulation of HDAC7 prevent G1/S phase transition and decrease the proliferation rate of HUVEC cells (Margariti et al., 2010). This intrinsic contradiction was explained by the fact that HDAC7 over-expression suppresses endothelial cells proliferation through the retention of β-catenin in the cytoplasm and the down-regulation of cyclin D1. On the opposite HDAC7 deficiency enhances nuclear translocation of β-catenin, increasing Rb levels, which results in hypertrophic cells (Margariti et al., 2010). An explanation for this paradox arises from a study on smooth muscle cells (Zhou et al., 2011). Here it has been demonstrated that HDAC7 exists in 2 isoforms: spliced and unspliced. Usually, HDAC7 lies in the cytoplasm as unspliced isoform, where binds and hold in check β-catenin, thus preventing its nuclear translocation. After growth stimuli HDAC7 is spliced, the new isoform releases β-catenin, thus permitting its translocation in the nucleus, where it is able to activate a set of genes that induce cells proliferation (Zhou et al., 2011). This pathway was confirmed in chondrocytes. Here the down-regulation of HDAC7 increases proliferation because of β-catenin activation with the consequent induction of cyclin D3 and repression of p21/CDKN1A (Bradley et al., 2015).

Clearly class IIa seem to influence cell growth in lineage-dependent manner. For example in breast cancer cells the triple silencing of HDAC4, HDAC5 and HDAC9 determines a decrease in the proliferation of MCF7-ER$^+$ cells but not of MDA-MB-231 ER$^-$ cells (Clocchiatti et al., 2013b). The anti-proliferative effect of the triple silencing was the consequence of an apoptotic response elicited by Nur77 up-regulation (Clocchiatti et al., 2013b). Recently, it has been reported that HDAC7, influences the proliferation of breast epithelial cells (Clocchiatti et al., 2015). HDCA7 seems to have a pro-oncogenic effect, sustaining the proliferation of acini in a 3D culture model. In this model HDAC7 levels are under the regulation of HER2 signaling. This effect is mediated by the down-regulation of MEF2-mediated transcription (Clocchiatti et al., 2015). On the opposite, in particular type of ALL, the pre-B-ALL, and in B-cell lymphoma HDAC7 is down-modulated and exhibits an onco-suppressive role (Barneda-Zahonero et al., 2015). In this case the effect of HDAC7 over-expression was the activation of the apoptotic response and the down-regulation of an important oncogene such as c-Myc. Both the HDAC7 amino-terminus, containing the MEF2 binding site, as well as the catalytic domain are important to exert this pro-apoptotic function. The importance of c-Myc down-modulation was described by the rescue of cells viability after the re-introduction of c-Myc in cells overexpressing HDAC7 (Barneda-Zahonero et al., 2015).

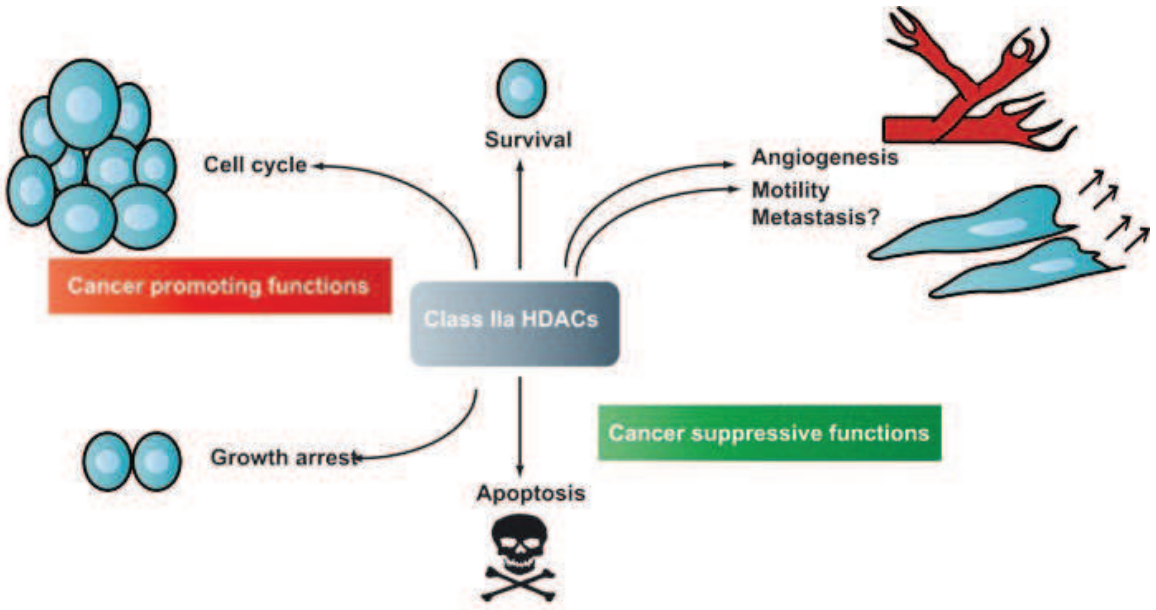


**Figure 5** *Class IIa HDACs influences on cell fates in relation to cancer.* Schematic representation of the different influences exerted by Class IIa on cancer-related cellular functions. Class IIa HDACs can participate in different cancer-related processes. According to the context they could behave as tumor promoter or tumor repressive players. (from Clocchiatti et al, 2011)

Overall these studies indicate that, depending from the cellular context, class IIa HDACs can display different influences on the transformation process. It is possible that the number of genes and the signaling pathways governed by class IIa HDACs can vary depending from the cell lineages. Epigenetic stages, the gene expression landscape and mutational status could in principle swift toward pro-oncogenic or tumor suppressive outcomes. Certainly, further investigations are important to better clarify the roles of each class IIa HDACs member.

## 3.5 Transformation of human cell lines

Definition of the genetic alterations driving the conversion of normal cells into malignant cells is a process commonly defined as "transformation". It is an important *in vitro* approach to clarify the basic mechanisms of cancer development. The vast majority of these experiments were assessed with mouse cell lines and only a limited set of studies were performed with human cells. It is empirically known that human cells are more resistant to oncogenic transformation (Akagi et al., 2003). More than 30 years ago, through extensive studies in rodent models, it was established that primary rodent cells can be transformed by two oncogenic "hits", such as the combination of ectopic expression of *c-myc* and the activated H-RAS or either the activated H-RAS and the adenovirus protein E1A or also the activated H-RAS and simian virus 40 (SV40) (Land et al., 1983; Ruley, 1983); whereas the normal human cells cannot be fully transformed by such oncogenes combination or even by additional ones (Akagi et al., 2003; Rangarajan et al., 2004). These observations suggest that mouse and human cells possess distinct requirements to fully alter the proliferative features and, from an evolutionary point of view, human cells have acquired strong mechanisms counteracting the malignant transformation process. It has been speculated that the different susceptibility to transformation between human and mouse cells may be due to differences in DNA repair capacity, response to oxidative stress, maintenance of genome stability or epigenetic controls of gene expression (Akagi et al., 2003).

Empirically, keeping in culture primary mouse and human fibroblasts some differences can be appreciated. Primary mouse cells after few passages tend to acquire a particular phenotype characterized by enlarged and flattened morphology with simultaneous block of the cell cycle, typical of the onset of senescence, a defensive mechanism against uncontrolled proliferation. But after 8 passages, some clones of spontaneously immortalized fibroblast began to emerge.

On the contrary, primary human fibroblasts have a longer lifespan because the senescent-like morphology appears only after 50 cell divisions. Once entered in this growth-arrested status, human fibroblasts remain in most of the cases forever because it is an irreversible mechanism (Rangarajan et al., 2004).

Senescence can also be induced by oncogenes both in primary rodent and human cells. In particular the first and among the foremost studied oncogene able to induce senescence in primary mouse and human fibroblast was H-RAS (Serrano et al., 1997). The phenotype observed after RAS activation is peculiar, since, initially it is characterized by a boost of growth, then cells acquire the senescent-like phenotype with a specific morphology and markers. The positivity of associated acidic-β-galactosidase enzyme activity assay, the positivity for DNA-damage foci (i.e. γ-H2AX) and the presence of peculiar senescence-associated heterochromatin foci (SAHF) are features of OIS (oncogene induced senescence) (Di Micco et al., 2006). More recently also Akt was discovered to induce senescence in fibroblast, but the phenotype is completely different, because is not characterized by the initial growth phase, it showed absence of DNA damage hallmarks and SAHF foci, instead the morphology is always flattened and spread and cells have the cell cycle blocked (Astle et al., 2012; Kennedy et al., 2011).

A debated characteristic distinguishing mouse from human cells behavior about the transformation process is the regulation of the telomerase catalytic subunit (TERT) that control telomeres length. Commonly, TERT expression in tightly suppressed in the somatic cells of humans (Kim et al., 1994), whereas it is constitutively expressed in mouse somatic cells (Prowse and Greider, 1995). The constitutive expression of TERT and hence the long telomere have been assumed to be the biological basis for the relatively frequent spontaneous immortalization observed in mouse cells, which is supposed to be a prerequisite for malignant transformation (Hahn et al., 2002). In fact, the ectopic expression of the human TERT (hTERT) in various human cell lineages was capable of immortalization, avoiding the replicative senescence (Bodnar et al., 1998). However, in the same year Seger and colleagues reported that hTERT is dispensable for human transformation, because combing the ectopic expression of adenovirus E1A, activated RAS and MDM2 without the additional expression of TERT was sufficient to elicit a transformed phenotype (Seger et al., 2002). They also add, that proceeding with cell cultivation the induction of hTERT arise spontaneously, thus they conclude that in humans TERT is important for maintenance of tumor and not for its own

onset (Seger et al., 2002). So, it seems that TERT in human malignancies has a role, but the mechanisms by which it acts still remains to be clarified.

In summary it is widely accepted that human and mouse cells need a set of genetic changes to get transformation, which are substantially very different (Hahn et al., 1999). In fact as explained above, the two hints model valid for rodent primary cells is not sufficient to transform human fibroblasts. The minimal model used to study human transformation require at least three hits: hTERT, SV40 expression (including LT and ST) and H-RAS activation (Akagi et al., 2003; Hahn et al., 1999). Another model used, is the aforementioned overexpression of E1A, RAS oncogenic mutation plus the p53 loss (or MDM2 overexpression) (Seger et al., 2002). In both cases there are oncoviral proteins, SV40 and E1A, which are not physiologically present inside the cells, touching various cellular pathways. Hence, these models are not perfect to study the transformation process and human cancer.

RAS mutations are frequently observed in different human cancers and its activation is a fundamental prerequisite for the transformation process. In fact also some of its downstream partners (Raf, Ral and PI3K) are sufficient to transform human cells in various combination with SV40 and hTERT (Rangarajan et al., 2004). To gain oncogenic transformation, it is not only important to engage proliferative signaling pathways, but it is also relevant to overcome mechanisms, which protect cells from uncontrolled replication. These safeguard mechanisms are represented by the above mentioned OIS, all the death stimuli such as apoptosis and necrosis, the cell-cycle checkpoints and the immune system surveillance (de Visser et al., 2006; Kastan and Bartek, 2004; Kerr et al., 1994). All these mechanisms are controlled by a group of genes with suppressive functions against tumor onset and growth.

Cellular senescence depends critically on two powerful tumor suppressor pathways: the p53 and pRb/p16INK4a pathways (Campisi and d'Adda di Fagagna, 2007; Serrano et al., 1997). In particular, RAS-arrested cells are characterized by augmented levels of p53 and p16INK4a proteins (Serrano et al., 1997). p16INK4a is able to directly associate with CDK4 and CDK6 and to inhibit their activities (Serrano et al., 1993). The primary function of the CDKs is to phosphorylate the retinoblastoma tumor suppressor protein (Rb) allowing the progression of the cell cycle towards the S phase (Weinberg, 1995). The over-expression of p16INK4a inhibits the cyclin-dependent kinases and leads to G1 cell cycle arrest. Primary murine fibroblasts lacking either p53 or p16INK4a are transformed by the oncogenic *ras* alone, on the contrary in human fibroblasts the inactivation of p53 or p16INK4a alone with

concomitant RAS activation is not sufficient to bypass OIS (Serrano et al., 1997). Accordingly, as discussed above, human fibroblast exhibit a stronger resistance to the uncontrolled proliferation and in fact to get the fully transformed phenotype is necessary to inactivate both p53 and pRb/p16INK4a pathways (permitted by the overexpression of SV40 LT), together with the ectopic expression of hTERT and mutated RAS (Serrano et al., 1997). In many instance p53 and Rb are activated to promote senescence by products of the *INK4a/ARF* locus (Lowe and Sherr, 2003). This locus encodes two tumor suppressor: the already cited p16INK4a and p14ARF (p19ARF in mice), expressed from partially overlapping nucleotides sequences read in alternative reading frame. p16INK4a inactivate the Rb pathway by inhibiting CDKs, as described above. On the contrary, p14ARF increases the growth suppressive function of p53 by interfering with its negative regulator Mdm2. Both p16INK4a and p14ARF accumulates in senescent cells, in fact mutations that affect the *INK4a/ARF* locus are frequently found in cancer and immortalized cells (Lundberg et al., 2000).The most studied onco-suppressive gene, called also the guardian of the genome, is p53. In the absence of cellular stress, the p53 protein is maintained at low steady-state levels and exerts very little effect on cell fate. However, in response to various types of stress, p53 becomes activated. p53 activation is reflected in elevated protein levels, as well as augmented biochemical capabilities. As a consequence of p53 activation, cells can undergo marked phenotypic changes, ranging from increased DNA repair to senescence and apoptosis (Oren, 2003). p53 was found to be mutated in numerous cancer types, this implies that in the transformation process it plays a fundamental part (Muller and Vousden, 2013).

Although p53 has a key role in human cancer, combining the activation of RAS, with the down-regulation of p53 in human foreskin fibroblast (BJ) is able to bypass OIS but is insufficient to fully transform cells (Boehm et al., 2005). Adding to this setting (RAS activation + p53 loss) the over-expresson of c-Myc, which as mentioned above in mouse induce transformation already in combination with RAS mutation, is still again not sufficient to transforms human fibroblast, also with the hTERT expression (Boehm et al., 2005). To observe at least the anchorage-independent growth capability of human immortalized fibroblast it is necessary to add at the p53 loss and activation of RAS background one of the protein encoded by the SV40 genome, that is small T antigene (ST) which modulates PP2A phosphatase activity (Ahuja et al., 2005). On the contrary to obtain the complete transformation and to permit also the formation of tumors in xenograft mice, it has been demonstrated that is necessary also the ectopic expression of another protein of the SV40

virus, in that case is the large T antingen (LT) (Rangarajan et al., 2004). The LT protein, regulates completely different cellular pathways (Ahuja et al., 2005). In particular LT inhibit two very important pathways: the p53 and the pRB through different protein domains (Ahuja et al., 2005). This suggests that the further hint necessary to induce malignant transformation in human cells is the loss of the pRB cellular control. Ragarajan and coworkers suggest that in BJ cells expressing hTERT, the minimal combination to form tumor in nude mice is: ectopic expression of activated RAS, over-expression of ST protein (it means a deregulation of PP2A activity) and the loss of both p53 and pRB pathways (Rangarajan et al., 2004). This result is in conflict with Sager and colleagues in with they say that p53 (over-expression of MDM2) and pRB loss are sufficient to transform human cells in RAS activated background, even though without the hTERT over-expression (Seger et al., 2002). Importantly, differences in transformation can be observed among human fibroblasts isolated from different sources (Boehm et al., 2005). Both reports where assessed using functional deletion mutants of the viral E1A and SV40 proteins. These oncoproteins operate by interfering with different growth suppressive cellular pathways and the effect is indirect.

From all these reports emerge clearly how human cells are much resistant, compared to murine cells, to transformation and how many pathways need be de-regulated in order to get the fully malignant phenotype.

# Materials and methods

## Plasmid construction and retroviral infection

pWZL-Hygro constructs expressing HDAC4 and its mutants were previously described (Cernotta et al., 2011), Bcl-xL was subcloned into pBabe-Puro restricted by using EcoRI restriction site (NEB). Retroviral vectors were transfected in the amphotropic packaging cell line HEK293T with the calcium phosphate method (15µg of each construct were transfected). After 48h post-transfection, viral supernatants were collected, filtered, supplemented with 8 µg/ml polybrene, and combined with fresh medium in order to infect BJ cells. Following 24h of infection with virons, cells were selected with hygromycin (150µg/mL) for 5 days.

pCW (Tet-One System "All-in-one" purchuased from Addgene) plasmids expressing HDAC4 doxycycline-inducible transgenes were generate by PCR from the pWZL-Hygro constructs and subsequently cloned using NehI/BamHI-BglII restriction sites (NEB).

## Cell cultures and reagents

BJ/Tert cells were cultured in Earl's Salt Minimal Essential Medium (EMEM) (Hyclone) completed with non-essential amino-acids (NEAA, Hyclone). All other cell lines were grown in Dulbecco modified Eagle medium (DMEM; Lonza). All medium were supplemented with 10% fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100U/ml), and streptomycin (100µg/ml) (Lonza). For analyses of cell growth $2x10^4$ cells were seeded and the medium was changed every 2 days.

In all trypan blue exclusion assays, at least 300 cells from three independent samples were counted for each data point. Data were represented as arithmetic mean±SD for at least three independent experiments.

## Immunofluorescence

Cells were fixed with 3% paraformaldehyde and nuclei were stained with To-Pro®3 stain (Thermo-Fisher). The cells were imaged with a Leica confocal scanner SP equipped with a 488 λ Ar laser and a 543 to 633 λ HeNe laser.

## Western Blotting

Proteins obtained after an SDS denaturating lysis and sonication were transferred to a 0.2-μm pore- sized nitrocellulose membrane and incubated with the specific primary antibodies. After several washes, blots were incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Sigma) for 1h at room temperature. Finally, blots were developed with Super Signal West Dura, as recommended by the vendor (Pierce). For primary antibody stripping, blots were incubated for 30 min at 60°C in stripping solution (62.5mM Tris-HCl pH 6.8, 2% SDS) containing 100 mM β-mercaptoethanol.

## Reagents and Antibodies

The following chemicals were used (the final concentrations are indicated), 2c (2μM) (Cersosimo et al., 2015); Bortezomib (250nM), Leptomycin and Camptothecin (10μM) (all from LC laboratories); Doxycycline, 2,3- Dimethoxy-1,4-naphthoquinone (DMNQ) (10μM), dimethyl sulfoxide (DMSO) (all from Sigma-Aldrich), SMAC mimetic (Lecis et al., 2012) (100nM), TRAIL (100ng/mL). Primary antibodies: GFP (Paroni et al., 2004), Actin (Sigma-Aldrich), RAS and E1A(M73) (Abcam), RACK1 (Santa Cruz Biotechnology)

## RNA extraction and quantitative qRT-PCR

Cells were lysed using Tri-Reagent (Molecular Research Center). A total of 1 μg of total RNA was retro-transcribed by using 100U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) analyses were performed using Bio-Rad CFX96 and SYBR Green technology. The data were analyzed by use of a comparative threshold cycle using GAPDH and HPRT (hypoxanthine phosphoribosyltransferase) as normalizer genes. All reactions were done in triplicate.

## Soft agar assays.

Equal volumes of 1.2% agar and DMEM were mixed to generate 0.6% base agar. A total of 40.000 NIH-3T3 cells expressing the different transgenes were seeded in 0.3% top agar, followed by incubation at 37°C in humidified conditions. The cells were grown for 15 days, and the culture medium was changed twice per week. Foci were visualized by using MTT staining.

## Statistics

For experimental data, a Student *t* test was used. A *P* value of 0.05 was chosen as the statistical limit of significance. Unless otherwise indicated, all of the data in the figures are arithmetic means ± the standard deviations from at least three independent experiments. *=p<0.05; **=p<0.01; ***p=<0.005

# Results

## 1. HDAC4 prevents isolation of clones following retroviral infection of immortalized human fibroblasts

The role of HDAC4 in tumor development is still not clear. Hence to further prove the involvement of these epigenetic regulators in the process of tumorigenesis, we investigated the capability of HDAC4 to elicit in vitro transformation of human fibroblasts. We decided to use the sequential insertional approach, in order to identify the essential oncogenic combination requested to acquire a fully transformed phenotype in vitro. Human foreskin fibroblasts (BJ) were selected as a model of study. Since, primary human fibroblasts are characterized by a long-lifespan, after a certain number of passages in culture they undergo to senescence (Rangarajan et al., 2004). To overcome this physiological replicative block, BJ cells have been engineered to constitutively express the catalytic subunit of the human telomerase (hTERT), which contributes to bypass the senescent status and permits the establishment of an immortalized cell population.

Initially BJ/TERT cells stably expressing HDAC4 were generated. Cells were engineered to express different forms of HDAC4. In particular, in addition to the wild-type allele, 3 mutant proteins were ectopically expressed: i) a super-repressive mutant, herein referred as TM, in which the three serine residues, which upon phosphorylation are required for the binding to 14-3-3 proteins are mutated into alanine (S246A, S467A, S632A) (Grozinger and Schreiber, 2000); ii) a nuclear mutant with highest repressive capability, where in addition to the three serines mutated into alanines (TM), it brings a point mutation in the NES sequence (herein referred quadruple mutant/QM). This additional mutation consists in a substitution of the lysine 1062 in an alanine (L1062A), which favours the nuclear retention (McKinsey et al., 2001) and finally iii) an HDAC4 mutated in the 14-3-3 docking site but carries also a small deletion in the MEF2-binding site, between the aa 166 and 184 (Di Giorgio et al., 2013). This mutation generates a nuclear resident HDAC4, unable to complex MEF2 transcription factor and was named HDAC4-TMΔMEF2 mutant. GFP and E1A were used as negative and

positive control respectively. All the over-expressed proteins, with the exception of E1A, were GFP-tagged, in order to easily discriminate them from the endogenous ones.

To get a stable expression of the ectopic HDAC4 proteins we decided to transduce BJ/hTERT cells with retroviral vectors. After virus particles formation, virions were used to infect cells for 24h. The transduced cells were selected with hygromycin for 5 days and the positive clones in each plate were counted at the end of the selection. As showed in figure 7A the forced expression of TM, TMΔMEF2 and QM mutants have a strong negative effect on the number of clones/plate emerged after 5 day of selection (Figure 7A). The latter mutants led to a reduction of over 50% of the number of clones counted respect to WT form, which is characterized by a slight reduction when compared to GFP-infected cells. Moreover, keeping the isolated clones in fresh cultivation medium for additional 5 days, it was observed further dramatic drop in the number of resistant clones when the mutated forms of HDAC4 (TM, TMΔMEF2 and QM) were transduced (Figure 7A–10 Days).

This result suggests that in human fibroblasts expressing hTERT, introduction of different forms of HDAC4 incompetent for 14-3-3 binding could affect cell survival. Also the number of clones expressing the WT allele was further reduced after 10 days of culture, thus indicating a less pronounced but still present spontaneous cell death (Figure 7A-10 Days). These results suggest a possible implication of HDAC4 in the activation of a cell death program.

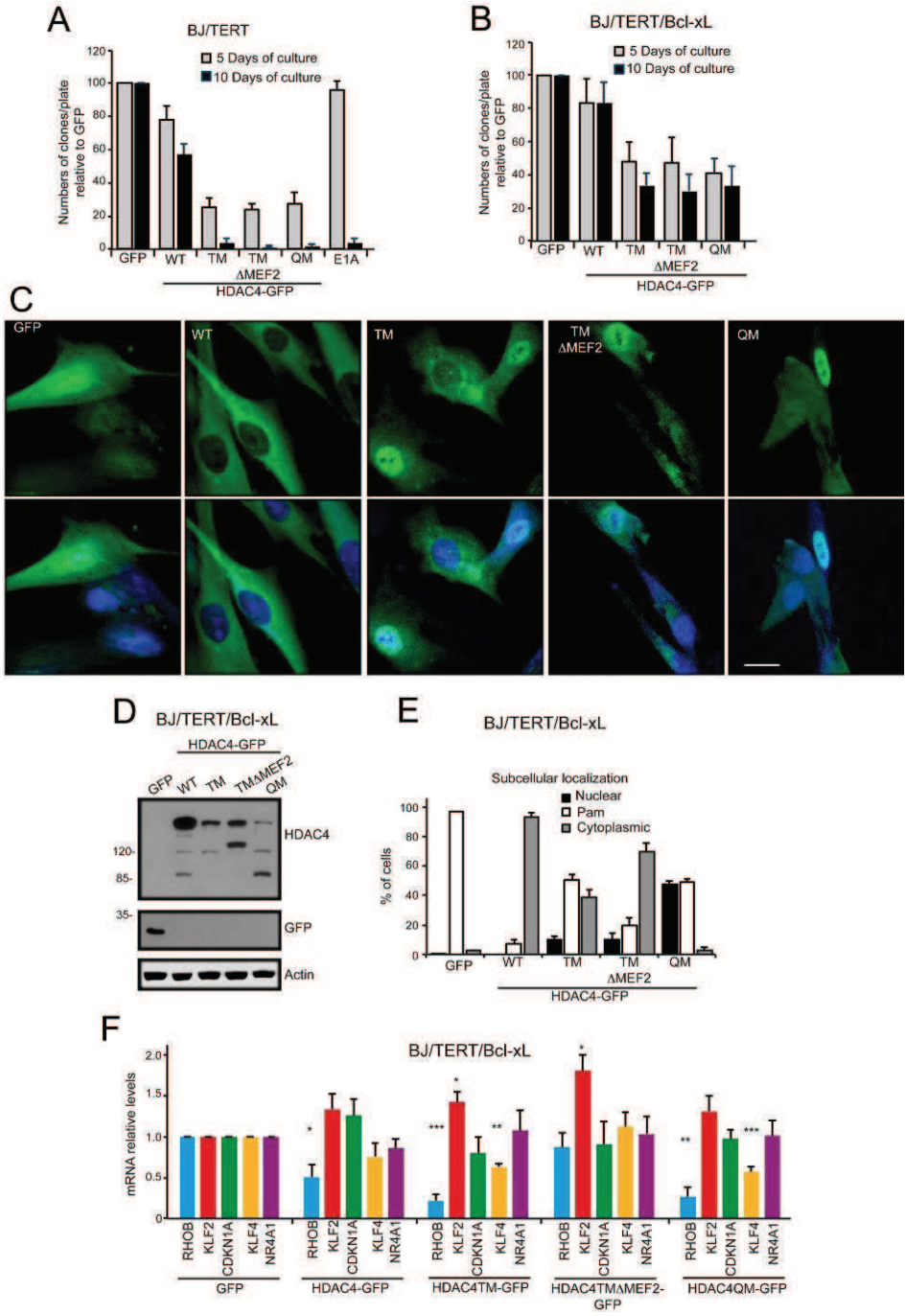


**Figure 7.** *Negative influence of HDAC4 on cell survival and proliferation in BJ/hTERT cells.* A/B) Percentage of clones, obtained after retroviral infection of BJ/hTERT (A) and BJ/hTERT/BCL-xL (B) cells, counted after 5 day of hygromycin selection, and after 5 additional days of culture in normal medium. C) Confocal pictures of BJ/TERT/BCL-xL cells expressing the different HDAC4-GFP proteins. Nuclei were stained with TO-PRO-3 iodide staining. D) Immunoblot assay were performed to visualize the levels of expression of the different transgenes. The antibody used was anti-GFP in order to detect the GFP-tagged HDAC4 E) Quantification of exogenous HDAC4-GFP subcellular localization in BJ/TERT/BCL-xL cells. For each experiment, at least 300 cells were counted (n=3). F) The mRNA levels of selected HDAC4 target genes were measured using qRT-PCR in BJ/TERT/BCL-xL fibroblasts. The mRNA levels were relative to the GFP-expressing cells.

The involvement of HDAC4 in apoptotic cell death was previously demonstrated in 2004 (Paroni et al., 2004). It was reported that the amino-terminal nuclear fragment generated after caspase cleavage is able to repress MEF2-transcrptional program and to trigger the apoptotic cell death.

To prove that the ectopic expression of nuclear HDAC4 alleles is able to render BJ/hTERT more susceptible to apoptotic cell death, we generated BJ/hTERT cells stably expressing BCL-xL, a member of the Bcl-2 family of proteins, which acts as a pro-survival protein by preventing the release of mitochondrial factors that trigger the intrinsic apoptotic response. To assess this hypothesis the retroviral infections with HDAC4-WT and its derivative mutants TM, TMΔMEF2 and QM was performed in cells overexpressing BCL-xL. The over-expression of BCL-xL allowed the isolation of a significant higher number of clones, both at 5 and 10 days from selection for each transgene, inducing a partial rescue of cell survival as evidenced by the percentage of clones/plate relative to the GFP scored (Figure 7B compare with Figure 7A). Accordingly, after isolation, almost all the resistant clones are able to growth in normal medium and were propagated (Figure 7B-10 Days).

The expression and localization of the relative transgenes in BJ/TERT/BCL-xL was verified by immunofluorescence (Figure 7C) and immunoblotting (Figure 7D). The immunoblot assay highlighted that the TM and the QM mutants were expressed at lower levels. Furthermore bands with low molecular weight, possibly corresponding to proteolytic cleavage products were evident, particularly in the case of the QM mutant (Figure 7D). The intracellular localization of each HDAC4-GFP protein was also quantified (Figure 7E). It is interestingly to note that with the exclusion of WT all the others HDAC4 alleles should be characterized by a nuclear accumulation, but inexpertly they also showed a cytoplasmic localization, stronger in the case of TM and of TMΔMEF2 mutants. This effect could be explained by the fact that some 14-3-3 independent mechanisms of HDAC4 regulation exist in this cell line. Furthermore it is also possible that in the case of QM the binding with 14-3-3 protein causes a steric cumbersome that block the interaction with the importin-α of HDAC4, hindering the nuclear import of the protein (Grozinger and Schreiber, 2000). In literature it is also reported that the interaction with cytoplasmic MEF2 TFs contributes to nuclear localize HDAC4 (Borghi et al., 2001) thus explain also in part the TMΔMEF2 cytoplasmic fraction. Furthermore it should also take into account the observed proteolytic processing (Figure 7D) of the HDAC4 that could generate fragments incompetent for nuclear localization.

Finally, through qRT-PCR, were assessed the repressive ability of HDAC4 mutants (Figure 7F). The selected target genes were also well known MEF2 target (Di Giorgio et al., 2013). The nuclear resilient forms of HDAC4 represented by TM and QM mutants were able to repress strongly the selected target genes (i.e. RHOB and KLF4) respect to the WT form. Accordingly, the mutant lacking the MEF2 binding site did not influence the mRNA levels of the same targets (Figure 7F).

On the other hand, none of the HDAC4 mutants showed an effect on cell cycle profile (data not shown) as recently observed in other cell lineages (Clocchiatti et al., 2015; Di Giorgio et al., 2015; Wilson et al., 2008), which is mirrored by the absence of p21/CDKN1A mRNA down-regulation (Figure 7F).

## 2. HDAC4 biological effect on BJ/Tert is not linked neither to aggresome formation nor to viral infection or LTR repression

Class IIa HDACs structure is characterized by the presence, in the N-terminal domain, of the glutamine-rich region (Figure 3). As discussed in the introduction, this domain is basically involved in protein-protein interaction and in the formation of dimers. It has been demonstrated that over-expression of HDAC4 induced the formation of aggresome and subsequent ubiquitin-mediated degradation (Cernotta et al., 2011). In order to exclude the possibility that aggregates formation and accumulation elicits an unfolded protein response (UPR) which in turns activates apoptosis thus hindering clones isolation in BJ/hTERT fibroblasts, we decided to stably express HDAC7. This class IIa HDAC is the sole that do not possess the glutamine-rich region and hence it is not susceptible to aggresome formation. BJ/hTERT cells were infected with the wild-type form of HDAC7 and also with the nuclear super-repressive mutant named HDAC7 S/A, that carried the substitution of the 4 serine residues, necessary for the 14-3-3 binding, into alanine, mimicking the HDAC4-TM mutant. Also HDAC7 constructs were GFP-tagged. GFP alone was considered as negative control. Cells were selected with hygromycin for 5 days and clones were counted on each plate (Figure 8A). Ectopic expression of HDAC7-WT did not influence clones number formation, while on the contrary HDAC7 mutated in the 14-3-3 binding sites induced a significant reduction of the number of clones during the selection process, similarly to HDAC4-TM

expression. After additional 5 days in culture, cells expressing HDAC7-S/A undergo to spontaneous cell death. This experiment indicates that the anti-proliferative effects of 14-3-3 mutant versions of class IIa HDACs is independent from the glutamine-rich region and aggresome formation (Figure 8A-10 Days).

A connection between HDAC4 and virus seems to exist (Lomonte et al., 2004; Palmisano et al., 2012; Shirakawa et al., 2013). HDAC4 acts as a repressor against viral gene, because HDAC4 could elicit some epigenetic modifications inducing the formation of heterochromatin through the interaction with viral protein. Hence, we wondered whether HDAC4 could influence viral particles formation and/or transduction or could repress viral promoters regulating the genes encoded by the retroviral constructs, thus hindering its own expression. To assess this hypothesis we performed an infection with HDAC4 constructs on HEK-293 cells. Cells were infected with all the HDAC4 proteins (WT, TM, TMΔMEF2 and QM) tagged with GFP and virus particles were kept in contact with cells for 24h, then immediately collected and analyzed through FACS analysis in order to score the GFP positive cells. Figure 8B showed the percentage of GFP positive cells. Although the rate of infection is not high, the results suggest that all the different HDAC4 constructs were able to infect BJ/hTERT cells with the same percentage/efficiency, even if the HDAC4 mutant lacking the MEF2 binding site showed a slight, but statistically significant, capability to infect cells (Figure 8B).

On the other hand considering the ability of HDAC4 to repress viral genes (Shirakawa et al., 2013) we hypothesized that HDAC4 could exert its repressive ability on the LTR viral promoter governing the hygromicyn resistance gene thus hindering its expression and hence the isolation of positive clones. To verify this point, we transiently transfect HEK-293 cells with different plasmids. Three different conditions were performed. The empty pWZL retroviral vector, used in the previous infection, carrying the resistance gene under the control of an LTR viral promoter was transfected in all the conditions. The pEGFPc1vector expressing the GFP protein was also used in the three conditions as control of transfection. Finally, HDAC4-TM tagged with FLAG peptite (pFLAG-HDAC4-TM) was co-trasnfected with the others transgenes. pFLAG-HDAC4/WT was co-transfected in parallel in order to compare the repressive ability of the HDAC4 when subjected to nuclear/cytoplasmic shuttling. pFLAG empty plasmid was used as a negative control condition. 24h hours after transfection cells were collected and the relative mRNA levels of HDAC4 and hygromycin

gene were quantified by qRT-PCR. The results showed that relative to the GFP control, HDAC4 is higher express both for WT and TM mutants (Figure 8C). The levels of the hygromycin mRNA in the case of WT are not be subjected to substantial variation while the HDAC4 TM nuclear proteins unexpectedly activated hygromycin transcription (Figure 8D) excluding any repressive effect of HDAC4 on LTR viral promoter regulating the expression of hygromycin gene resistance.

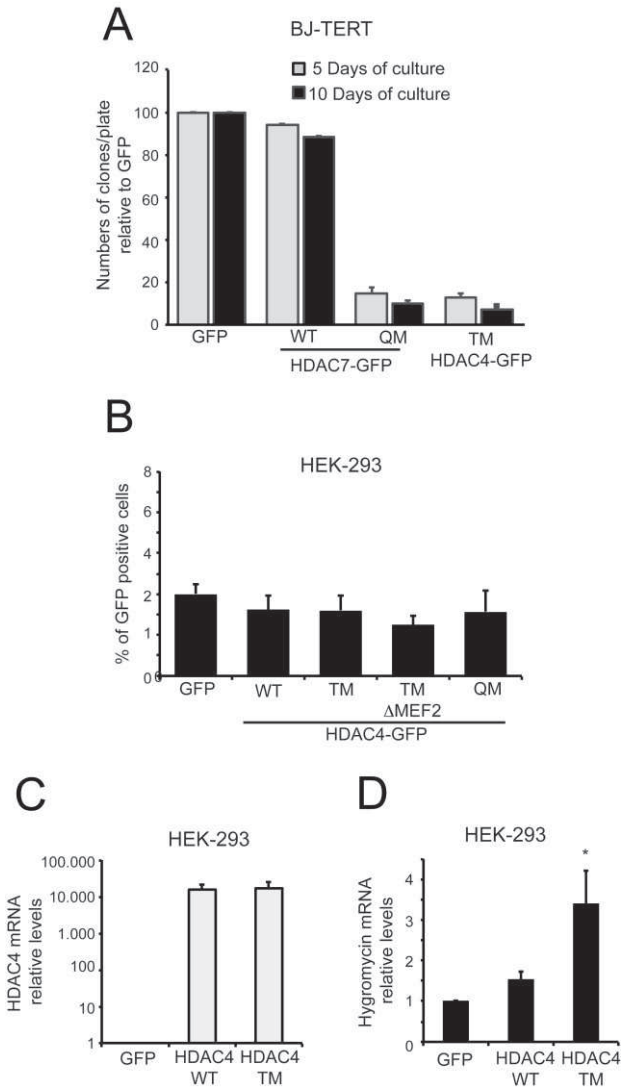


**Figure 8.** *Characterization of the anti-proliferative effect of HDAC4 mutants in 14-3-3 binding sites.* A) Percentage of clones, obtained after retroviral infection of BJ/hTERT cells with HDAC7-WT and S/A mutant, counted after 5 days of hygromycin selection, and after 5 additional days of culture in normal medium. B) HEK293 GFP positive cells scored with FACS analysis after retroviral infection with the indicated constructs. C/D) mRNA levels of HDAC4-WT and TM (C) and of hygromycin (D) were measured with qRT-PCR in HEK-293 cells.

Together these data suggest that the adverse effect of HDAC4 on clones isolation is not linked neither to aggresome formation and UPR response, nor to the viral particles transduction or to the repression of LTR promoter regulating the hygromycin gene. A high-throughput approach based on shRNA library or CRISPR technology could be more helpfully in order to identify genes and the possible pathways involved in this phenomenon.

## 3. DNA-damage induction and proteins synthesis inhibition trigger apoptosis in BJ/hTERT expressing nuclear HDAC4.

To further investigate the hypothesis that HDAC4 mutated for 14-3-3 binding render human immortalized fibroblasts more susceptible to apoptosis, we adopted a different experimental approach. We generated a doxycycline-inducible system to exogenously express WT, TM, TMΔMEF2 and QM forms of HDAC4 in BJ/hTERT cell line and to get a conditional expression among the various HDAC4 mutants thus avoiding the negative effect of the constitutive expression during clones isolation. In this case the empty vector was used as a negative control and the oncoviral gene E1A was used as the positive one.

After retroviral infection all the different constructs did not affect cell survival (data not shown). Next, the protein levels of the different transgenes were assessed by immunoblot analysis after 6h of doxycycline induction (Figure 9A). With the exception of HDAC4/-TM, which showed a reduced level of expression, the WT and the other mutant proteins were expressed at comparable levels. To assess the repressive activity of each HDAC4 form, a qRT-PCR analysis was carried out (Figure 9B). Three genes KLF2, RHOB and p21/CDKN1A, previously recognized as targets of MEF2-HDAC axis were selected. The qRT-PCR analysis confirmed that the super-repressive mutants (TM and QM) show a stronger repressive effect respect to the WT. Curiously in BJ/hTERT cells up-regulation of HDAC4 was insufficient to repress KLF2 expression (Figure 9B). As expected the HDAC4-TMΔMEF2 was unable of suppressing these genes. CDKN1A mRNA levels were not regulated by induction of the different HDAC4 proteins, by contrast E1A suppressed its expression (Figure 9B).

Additionally, an immunofluorescence assay was performed in order to assess both the expression levels and the intracellular localization of the different isoforms. Representative

pictures are shown in figure 10A. As evidenced from the quantitative analysis(Figure 10B) the localization of the HDAC4-TM mutant is not completely localized into the nucleus, but is more nuclear respect to the same isoform constitutively expressed in BJ/hTERT/BCL-xL (compare Figure 7C) Since the HDAC4-QM is completely accumulated into the nucleus this suggests that a secondary mechanism, in addition to the classical 14-3-3 binding, can affect the intracellular trafficking of HDAC4. Furthermore to evaluate the nuclear/cytoplasmic trafficking we treated cells with leptomycin B, an inhibitor of CRM-1-dependent nuclear export. The block of the nuclear export induced a rapid nuclear accumulation of all WT and of TM mutants (Figure 10B), thus indicating that HDAC4 proteins are subjected to nuclear/cytoplasmic shuttling and that CRM-dependent export operates also in the case of HDAC4 mutants defective in the 14-3-3 binding.

The ectopic expression of HDAC4-TM in mouse-immortalized fibroblast induces the acquisition of a transformed phenotype, including a greater proliferative potential (Di Giorgio et al., 2013). To assess whether the HDAC4 mutants influence the BJ/hTERT proliferation, we analyzed the growth profile of each HDAC4 protein. As emerge from the figure 9C, the expression of the HDAC4 mutants for 10 days did not significant influence cell proliferation. By contrast the E1A oncogene confers a significant proliferative growth advantage as expected.

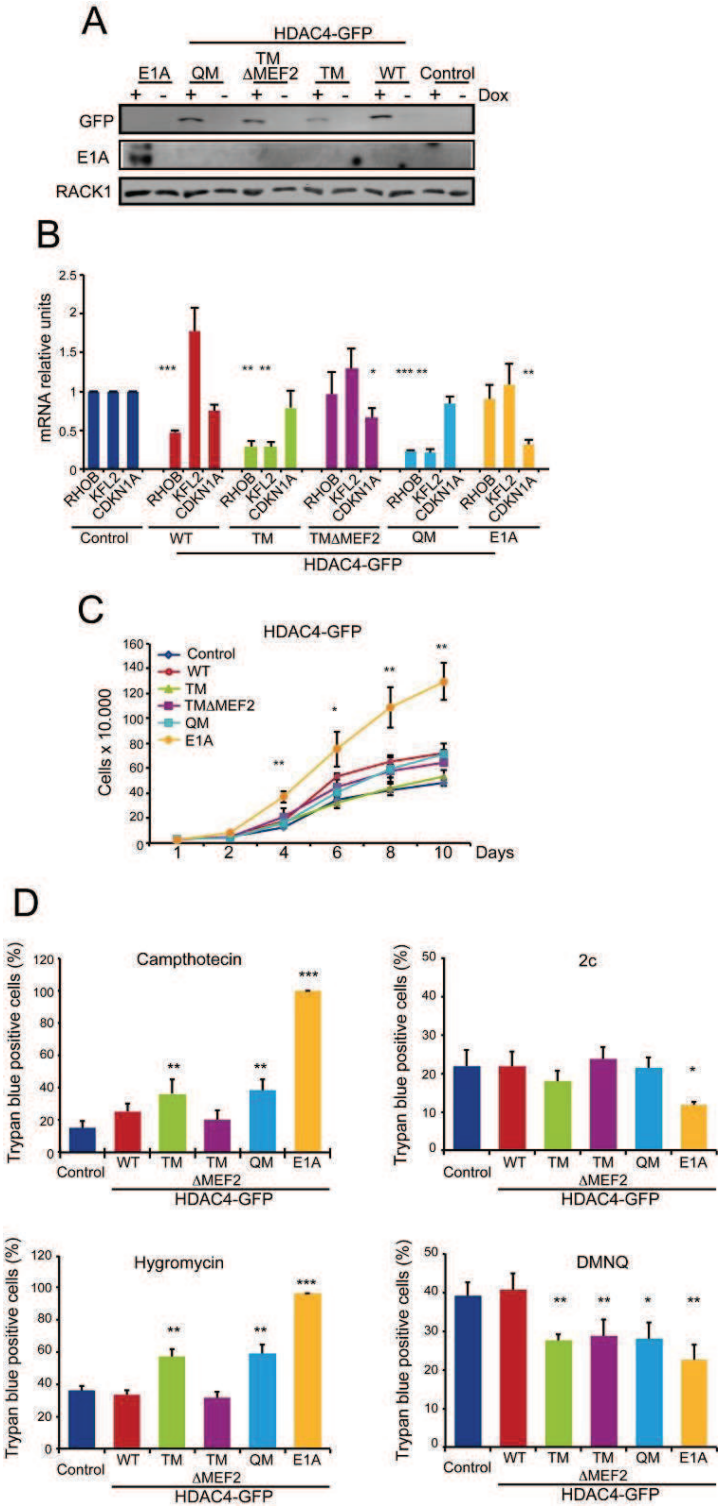


**Figure 9.** *Doxycycline-inducible HDAC4 characterization.* A) BJ/hTERT cells stably integrating the doxycycline-inducible vectors encoding the relative transgene, were treated with doxycycline (600ng) and protein levels were assessed by immunoblot after 24h of induction. B) mRNA levels of the selected MEF2-HDAC target genes were measured following 24h with doxycycline treatment. C) Cell growth assay in BJ/hTERT with continuous doxycycline induction. D) BJ/hTERT cells engineered with the doxycycline-inducible system were pre-treated with doxycycline for 24h and then with the indicated molecules. The appearance of cell death was scored with trypan blue staining after 48 hours.

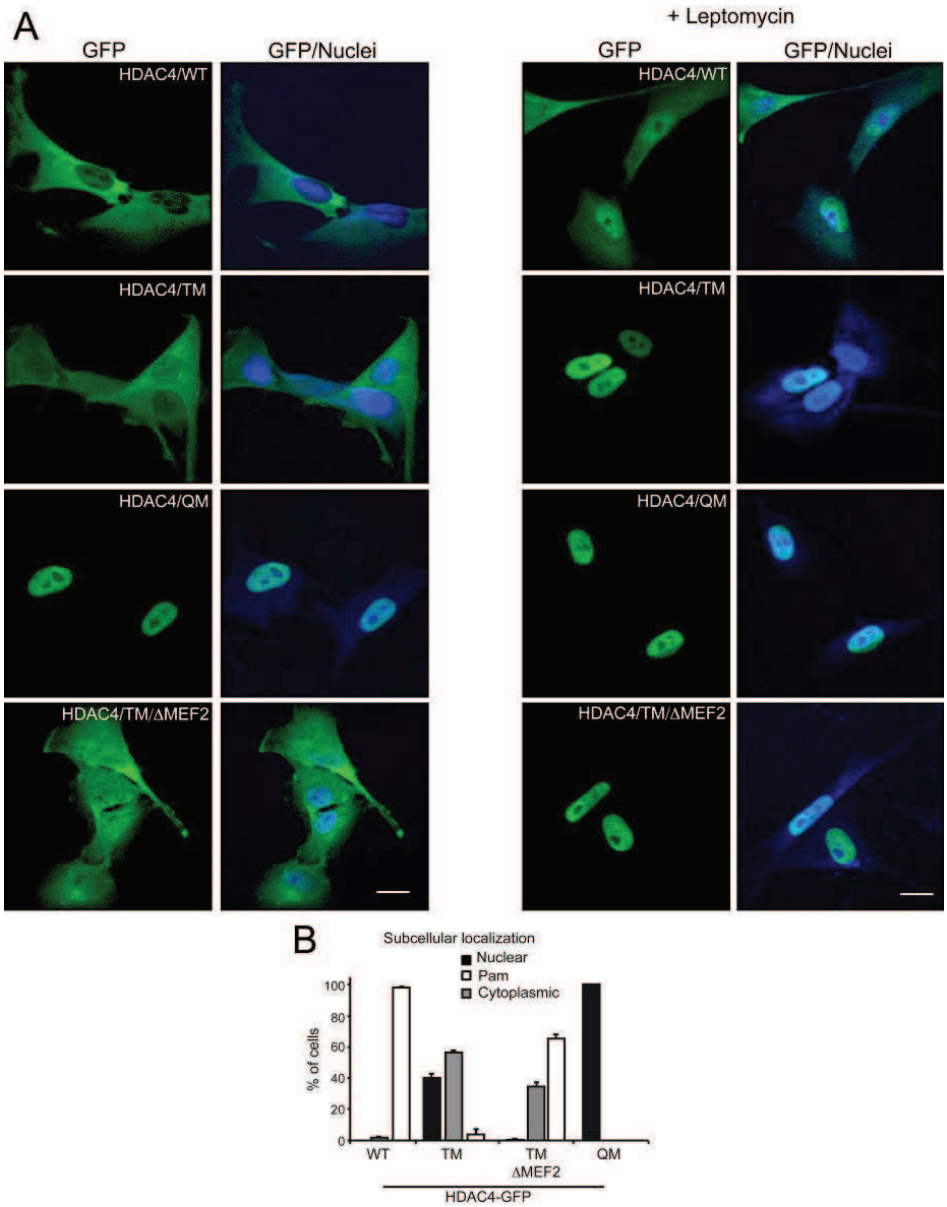


**Figure 10.** *Subcellular localization of inducible HDAC4*. A) Confocal pictures of BJ/hTERT cells 24h after doxycycline induction (left panels) or with additional leptomycin B treatment (5 ng/ml) for 3h. Immunofluorescence analysis was performed to visualize HDAC4 subcellular localization. B) Quantification of the subcellular localization of the different HDAC4 isoforms. Nuclei were stained with TO-PRO-3 iodide staining. For each experiment at least 300 cells were counted (n=3).

Unexpectedly, induction of HDAC4 mutated in 14-3-3 binding sites was insufficient to trigger cell death (data not shown). Hence to clarify whether the different HDAC4 isoforms are able to influence the susceptibility to apoptosis, cells were treated with different stimuli in order to engage different death pathways (Figure 9D). Expression of the different HDAC4

proteins was induced with doxycicline 24h before the treatment with the specific death stimuli.

2c is a synthetic isopeptidases inhibitor that triggers apoptosis in several cell lines (Cersosimo et al., 2015). Unexpectedly, 2c did not induce a stronger apoptotic response (Figure 9F/2c). This effect could be justified by the fact that these cells are not fully tumorigenic.. By contrast, in the presence of DMNQ, a molecule that stimulate a necrotic cell death, the induction of HDAC4 isoforms mutated in the 14-3-3 binding sites generates a slightly protective effect on cells (Figure 9F/DMNQ).

HDAC4 was reported to be involved in DNA damage response (Basile et al., 2006). For this reason we decided to treat cells with camptothecin, a molecule able to induce DNA damage. After treatment with camptothecin cells showed a different response to trypan blue assay, in particular TM and QM mutants were characterized by a higher percentage of cell death relative to WT protein and controls. This increased death response possibly depends on MEF2 repression since the mutant lacking the MEF2 binding site behaves like the negative control (Figure 9F/Camptothecin). As expected the E1A oncogene strongly increased cell death in response to DNA damage. Similar results were obtained when cells were treated with hygromycin, an inhibitor of the protein synthesis, the same compound used for the selection of cells after retroviral transduction. Also in this case the TM and QM mutants showed an increase in the percentage of positive trypan blue cells, and the TMΔMEF2 allele did not shown any increase (Figure 9F/Hygromycin).

To gain further insight into the apoptotic response elicited by HDAC4 exogenous expression, we decided to treat cells with a stimulus engaging the extrinsic apoptotic pathway (Figure 11). A combination of SMAC mimetic and TRAIL, the ligand of death receptors, or either agent alone were used. In this case, the single treatment with SMAC or TRAIL alone, following the doxycycline induction of HDAC4-WT and TM mutant, did not produce any effect on cell death. The full engagement of the extrinsic pathway by the combination of the two stimuli elicited apoptosis but this response was not influenced by the different HDAC4 isoforms (Figure 11).

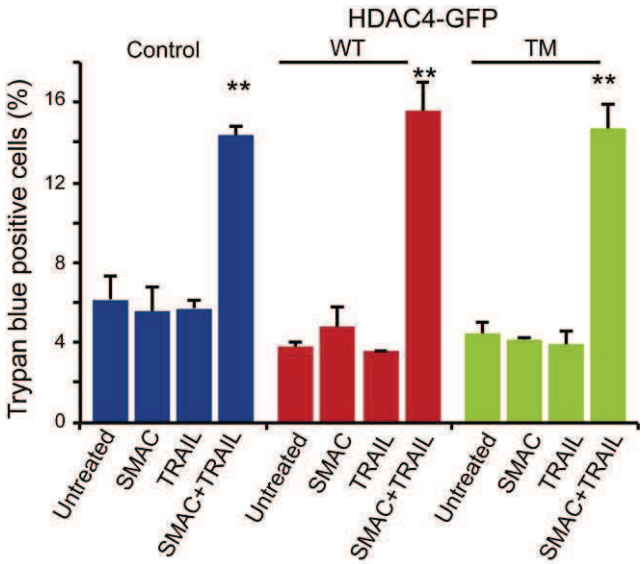


**Figure 11.** *BJ/hTERT cells apoptosis in response to an extrinsic apoptotic stimulus.* BJ/hTERT cells, after 24h pre-treatment with doxycycline, were incubated with SMAC mimetic and recombinant TRAIL for additional 24h. Appearance of cell death was scored by trypan blue staining. At least 300 cells were counted for each replicate (n=3).

## 4. The intrinsic instability of the HDAC4 14-3-3 mutants could explain their limited impact on cell survival and proliferation

This study points to a profound difference in terms of cell proliferation when the HDAC4 mutated in the 14-3-3 sites were expressed constitutively or transiently upon induction with doxocycline. To clarify this incongruence we decided to verify the protein levels of the different HDAC4 isoforms during a prolonged period of induction, we monitored the protein levels of each HDAC4 isoform by immunoblot (Figure 12A). We decide to check the level of expression at the same time point of the growth curve. It was evident how the expression levels in a longer period were different among the different proteins. In particular it is interestingly that both the stronger MEF2-repressive mutants (TM and QM) were much less expressed, subjected to proteolitic cleavage and their levels decline through the time although the cells were cultured in the presence of doxycycline (Figure 12A).

The ubquibitin-mediated degradation of nuclear HDAC4 was previously demonstrated by Cernotta and colleague (Cernotta et al., 2011). To confirm this hypothesis cells were treated with bortezomib, an inhibitor of the proteasome at different time points (Figure 12B). Already after 4 hours of bortezomib treatment, the levels of the HDAC4-TM mutant were overtly

augmented. This stabilization was less evident in the case of the HDAC4-WT form (Figure 12B). This result indicates that a nuclear HDAC4 mutated in the 14-3-3 binding sites is prone to be targeted for proteasomal degradation. This rapid turn-over could in part justify the absence of an effect on cell proliferation and survival when the expression of the different HDAC4 isoforms was performed with an inducible system.

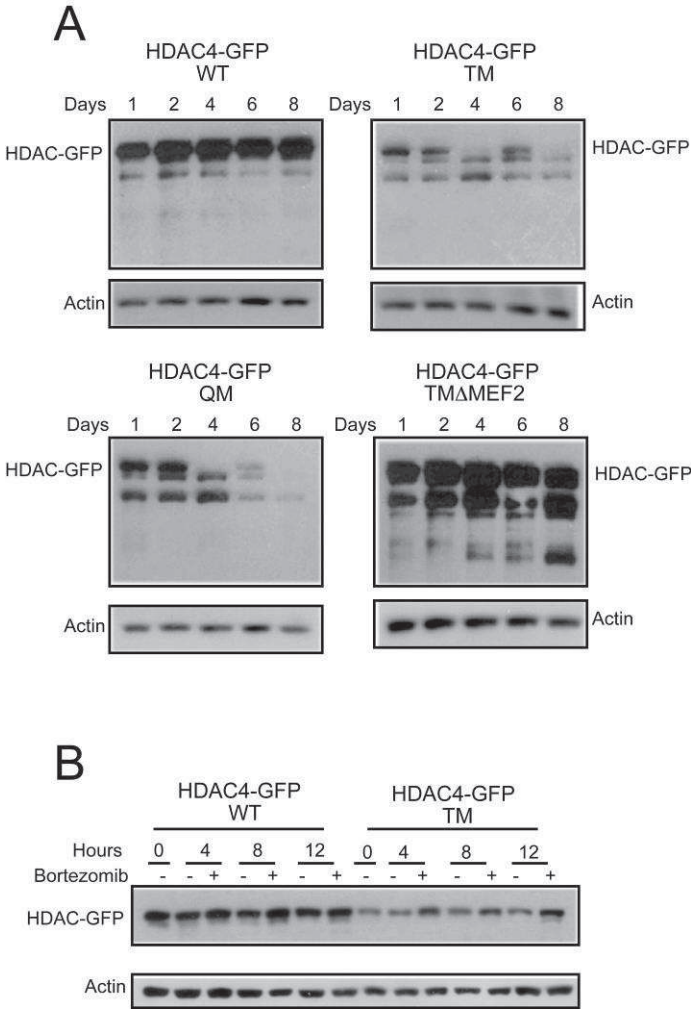


**Figure 12.** *HDAC4 is subjected to proteasomal degradation.* A) BJ/hTERT cells were inducted with doxycycline for 8 days and the expression levels were detected at the indicated time points. B) Cells were pre-treated with doxycycline for 24h and then bortezomib was added to culture medium. Protein levels were assessed by immunoblot at the indicated time points.

## 5. HDAC4 mutated in the NES sequence rescues clones formation in BJ/hTERT cells and allow the anchorage-independent growth of NIH-3T3

HDAC4 functions are modulated by various mechanisms as discussed in the introduction with the foremost characterized being the nuclear/cytoplasmic shuttling mediated by 14-3-3 proteins. The binding with 14-3-3 can occur both in cytoplasm as well in the nucleus preventing HDAC nuclear import in the first case and facilitating the export in the other one (Nishino et al., 2008). 14-3-3 binding on HDAC4 could also influence HDAC4 functions because of steric hindrance which could prevent the recruitment of other co-factors and/or the recruitment of HDAC4 on other protein complexes. To investigate whether this aspect could play a role in the HDAC4 pro-death effect, well evident in the case of the constitutive expression as scored by number of clones isolated or following the co-treatment with certain apoptotic insults (in the case of the inducible expression), we generated a GFP-tagged construct which encode for HDAC4 with an alanine instead of a lysine residue in position 1062 (HDAC4-L/A). This mutation lies in the NES sequence inducing HDAC4 to be localized inside the nuclear compartment, without interfering with 14-3-3 binding.

After retroviral infection, we generated BJ/hTERT cells stably expressing this transgene. Cells were selected with hygromycin for 5 day in order to isolate only the positive clones expressing the ectopic construct. Following the selection the number of clone/plates generated were counted. HDAC4-TM was used as positive control and the GFP alone was considered as the negative one. Figure 13A shows how the expression of the nuclear resident HDAC4-L/A does not affect the number of clones isolated after retroviral infection (5 days). The number of clones/plates was also counted after further 5 days of culture in normal medium (10 days). All clones generated were able to grow and they did not shown overt markers of cell death (Figure 13A). Subsequently, protein levels were assessed by immunoblot analysis and as evidenced in figure 13B all the constructs evidence a comparable expression levels. Considering the repressive function of HDAC4, we also assessed the ability of HDAC4-L/A mutant to repress at transcriptional levels the selected MEF2-target genes (Figure 13C). Compared to HDAC4-WT the L/A mutant shows a stronger repressive potential, with the exception for CDKN1A, as already described for TM and QM proteins.

As aforementioned, HDAC4-TM isoform was previously characterized for the ability to confer a transformed phenotype in mouse NIH-3T3 fibroblasts both in vitro and in vivo (Di Giorgio et al., 2013). Moreover, taking into account that human fibroblast are more resistance to oncogenic transformation (Akagi et al., 2003), we decided to generate NIH-3T3 mouse fibroblast stably expressing HDAC4-L/A mutant in order to clarify its ability to induce the oncogenic transformation in murine cells. Following the generation of stable cell lines integrating the TM and L/A alleles, protein expression levels were evaluated with immunoblot (Figure 13D). Here, GFP and RAS expressing cells were considered as negative and positive controls respectively. At transcriptional level the repressive ability was evaluated through a quantitative RT-PCR (Figure 13E). Relative to the TM allele, the L/A mutant shows a stronger repressive ability versus RhoB and NR4A1 target genes and less pronounced against KLF4. Here again, CDKN1A mRNA levels were not modulated by the NES mutated protein (Figure 13E). One of the hallmark conferred by HDAC4-TM to murine fibroblasts is the ability to growth in an anchorage-independent manner. We performed a soft agar experiment to assess this feature in the case of HDAC4-L/A mutant. As shown in figure 13F, HDAC4-L/A expressing cells developed a significant number of colonies in soft agar, similarly to H-RAS/G12V and HDAC4-TM expressing cells. All these data suggest that, the HDAC4 mutated only in the exporting sequence is able to influence somehow the previously described negative effect on human fibroblast clones isolation in vitro. In addition, HDAC4-L/A permitted the acquisition of an important oncogenic feature, which is the ability of immortalized mouse fibroblasts to growth in an anchorage–independent manner. Further investigations are necessary to better characterize the mechanism below this phenotype.

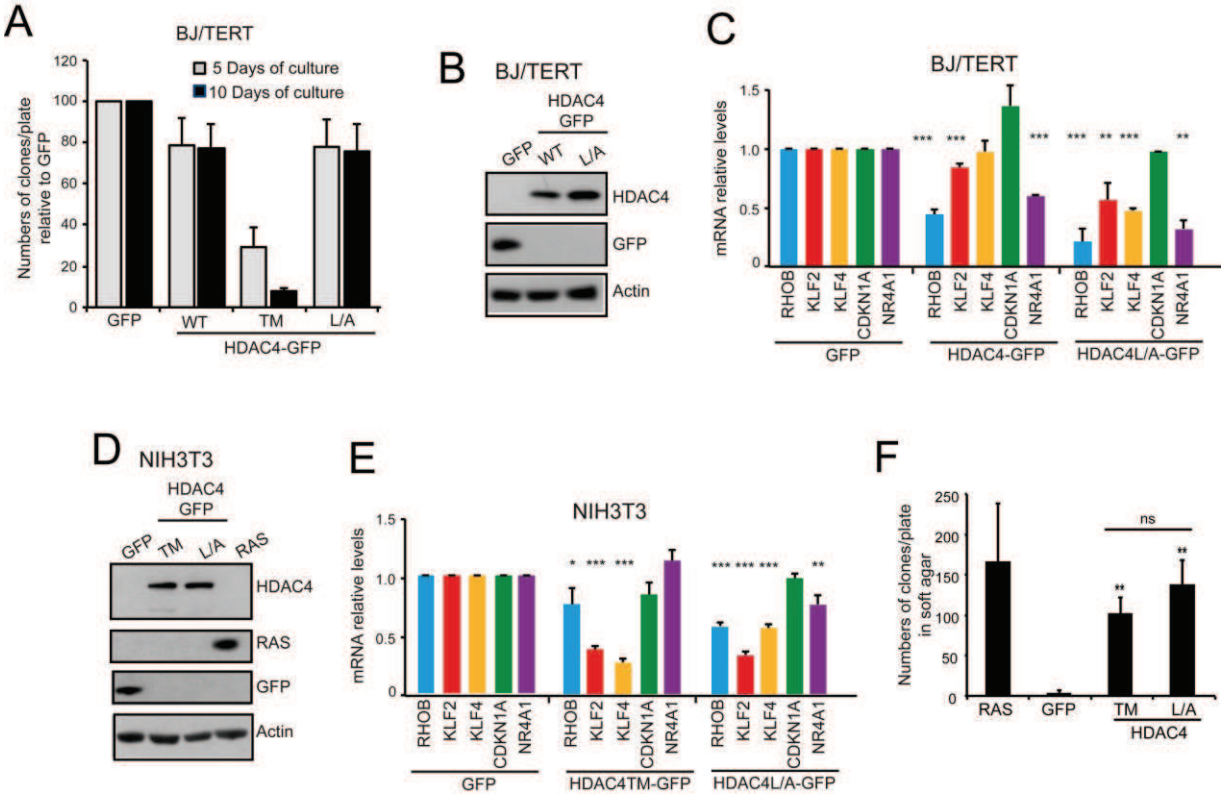


**Figure 13.** *HDAC4-L/A characterization in human and mouse fibroblasts.* A) Percentage of clones, obtained after retroviral infection of BJ/hTERT cells, counted after 5 day of hygromycin selection (gray bar), and after 5 additional days of culture in normal medium (black bar). B/D) Immunoblot assay were performed to visualize the levels of expression of the different transgenes in BJ/hTERT (B) and in NIH-3T3 (D) fibroblasts. The antibody used was anti-GFP in order to detect the GFP-tagged HDAC4. C/E) The mRNA expression levels of five HDAC4 target genes were measured using qRT-PCR in BJ/hTERT (C) and mouse NIH-3T3 (E) fibroblasts. The mRNA levels were relative to the GFP-expressing cells. F) Quantitative results of colony formation in soft agar of NIH-3T3 cells expressing the indicated transgenes. **, $P < 0.01$; ***, $P < 0.001$

# Discussion

Despite several years of studies, the role of class IIa HDACs during the neoplastic transformation is still not completely understood. Reports on class IIa HDACs are copious and sometimes controversial. In summary a dual role emerges from the literature. Various studies described these deacetylases as positive regulators of cell growth and cancer progression, whereas other studies propose to class IIa onco-suppressive activities (Barneda-Zahonero and Parra, 2012; Clocchiatti et al., 2011). The large body of these studies was conducted on immortalized human cancer cell lines (Barneda-Zahonero et al., 2015; Huang et al., 2002), and the involvement of class IIa was associated with their ability to influence cell proliferation. Despite these evidences, a direct in vitro demonstration that class IIa are involved in the acquisition of cancer hallmarks was performed only in 2013 by Di Giorgio and colleagues (Di Giorgio et al., 2013). In this paper the authors demonstrated in NIH-3T3 mouse immortalized fibroblasts, that the over-expression of a nuclear resilient HDAC4, mutated in 14-3-3 sites elicits the induction of a tumorigenic phenotype characterized by elongated morphology, loss of contact inhibition, anchorage-independent growth and tumorigenicity in xenograft assay.

With the attempt to implement the state of the art about the relationship between class IIa and cancer, we decided to investigate whether in vitro tumorigenic potential of HDAC4 could be translated also in human immortalized foreskin fibroblasts. We decided to take advantage from a sequential insertional approach in order to identify the essential oncogenic combination requested to gain a fully transformed phenotype, in cooperation with HDAC4.

Starting with the ectopic expression of the 14-3-3 mutant of HDAC4 in BJ/hTERT, the first evidence described was a negative effect on cell survival scored as clones isolation after retroviral infection. Subsequently, it was postulated an activation of a cell death program, because of the drastic drop in the number of isolated clones during the first 10 days of propagation. The different mutants studied are characterized by mutations of three serine residues, involved in 14-3-3 binding, into alanine. We selected this kind of mutations in order to have a hyper-active form of the protein derived from its forced localization into the nucleus (i.e. TM and QM). We also utilized the TMΔMEF2 construct which is unable to bind MEF2

and thus helps to discriminate between MEF2-dependent and independent effects. The negative effect on cell survival was observed with all three mutants, thus suggesting that this effect is linked to the absence of 14-3-3 binding and is not MEF2-dependent.

An involvement of HDAC4 in the apoptotic response was previously described. In particular, it was demonstrated that HDAC4 is a substrate of caspase-2 and -3 (Paroni et al., 2004). Cleavage of HDAC4 occurs at Asp 289 and disjoins the carboxy-terminal fragment, localized into the cytoplasm, from the amino-terminal fragment, which accumulates into the nucleus. In the nucleus, the caspase-generated fragment of HDAC4 is able to trigger cytochrome *c* release from mitochondria and cell death in a caspase-9–dependent manner. This pro-death effect was proposed being dependent on the repressive activity towards MEF2 target genes. We demonstrated that the co-expression of BCL-xL, a member of Bcl-2 family with pro-survival functions, rescues in part the negative effect on clones survival, described for HDAC4 mutated forms and allows also to propagate the isolated clones expressing the mutated deacetylases. Although the assessed percentage of infect cells was not high (Figure 8), among the different constructs no differences were observed in terms of infected cells, but the levels of expressed proteins detected were lower with nuclear localized mutants respect to the WT allele. In addition the 14-3-3 mutated isoforms were characterized by an intense proteolytic fragmentation (Figure 7D). Nevertheless, nuclear localized isoforms showed a greater repressive ability on MEF2-target genes compared to wt, thus indicating that the lower amount of proteins expressed maintains its stronger repressive ability. Even though all these three forms are characterized by mutations in 14-3-3 binding sites, which should result in nuclear localization because of the inability to bind 14-3-3 proteins and are not able to be translocated in the cytoplasm, they showed a conspicuous cytoplasmic fraction when stably expressed in BJ/hTERT/BCL-xL cells. This fact could be explained by the existence of additional mechanism of sub-cellular regulation mediated by other aminoacid residues. For example serine 279 is a residue conserved among all class IIa HDACs, with the exception of HDAC7, which leaves in the NLS region (Greco et al., 2011). This residue is targeted by various kinases such as Dirk1B, PKA and CKD5 and controversial roles were described. Dirk1B and CDK5 reduce nuclear accumulation (Deng et al., 2005; Taniguchi et al., 2012), while PKA retains class IIa HDACs into the nucleus (Ha et al., 2010). This opposite influence could be explained by the existence of other kinases targeting additional residues.

Another evidence that supports the role of Ser279 in mediating the 14-3-3-independent shuttling arises from a manuscript demonstrating that the Mirk/Dirk1B complex is able to phosphorylate HDAC5 on this residue (Deng et al., 2005). In addition, the proteolytic activity hitting these HDAC4 mutants could result in some protein fragments not competent for nuclear localization. Furthermore, in the case of the TMΔMEF2 mutant the inability to bind MEF2 could also reduce the nuclear fraction. Also the environmental influence could play a role in HDACs sub-cellular distribution. The redox condition could influence so much HDAC4 localization in fact a disulfide bridge is formed between cysteines 667 and 669 under oxidizing condition, on the contrary in a reducing environment, these two residues and the coordinated zinc ion fold the protein, bringing the ZBD in contact with the NES. In this way the CRM1 binding site is masked and the nuclear export is blocked (Ago et al., 2008).

HDAC4 is involved in various cellular functions, including the maintenance of silencing against exogenous genomes (Palmisano et al., 2012). We have demonstrated that HDAC4 in BJ/hTERT human fibroblasts is not able to engage a repressive activity against the viral genome and virus particles formation.

In order to dissect in more detail the anti-proliferative effect of the 14-3-3- mutant versions of HDAC4, we decided to adopt a different investigation strategy, by generating a conditional expression model, using the doxycicline-dependent system. This strategy allowed the investigation of the cell cycle profile, which did not present any differences among the different HDAC4 mutants. This absence of phenotype could depend on the high rate of proteasomal degradation showed by the HDAC4 nuclear forms (Figure 12). Concerning the apoptotic response, only when treated with specific apoptotic insults induction of HDAC4-TM and QM mutants rendered cells more susceptible to apoptosis. Interestingly apoptosis was triggered only by the induction of DNA damage and the inhibition of the protein synthesis of HDAC4 mutants augmenting the cell death rate.

Further investigations are necessary to clarify why the increased responsiveness to apoptosis is linked only to certain stimuli and in particular is important to discover the partners playing with HDAC4, because our results seem to exclude MEF2 involvement.

Surprisingly a HDAC4 mutated in the NES sequence when introduced in BJ/TERT did not explicated any overt anti-proliferative effect although it maintained the ability to repress MEF2 transcription targets (Figure 13). This result in conjunction with the pro-apoptotic effect of the TMΔMEF2 mutant indicates that MEF2-repression is unrelated to the anti-

proliferative activity of the ectopically expressed 14-3-3 mutants. It is important also to consider that the NES mutation allows the formation of a complex between the 14-3-3 proteins and HDAC4 and this complex could hinder the formation of another complex that could activate the anti-proliferative response. Vice versa it is possible that interaction with 14-3-3 proteins is necessary to activate some survival pathway.

Because of a higher resistance to in vitro oncogenic transformation, mainly due to the need of human fibroblast to have a combination of different oncogenic hits (Akagi et al., 2003), and also to complete the state of art about the role of HADC4 in mouse oncogenic transformation we decide to engineered NIH 3T3 immortalized mouse fibroblasts with HDAC4-L/A allele in order to define a plausible oncogenic potential of this HDAC4 mutant. In parallel with the characterization, which showed a comparable repressive ability of MEF2 selected targets respect to the already characterized TM and QM isoforms, the acquisition of a transformed phenotype was observed. NES mutated HDAC4 allows NIH 3T3 to growth in an anchorage-independent manner forming a slight higher number of colonies in soft agar assay than TM allele (Figure 13). This phenotype should be better characterized in the future.

The L/A mutant can represent an interesting tool to investigate the pro-oncogenic potential of class IIa HDACs in human cells. In this context this study can be considered a preliminary investigation aimed to tackle the question in the complexity of the human refractoriness to transformation. A very interesting evidence emerged from this thesis is that HDAC4 is not well tolerated by human fibroblast perhaps because of its involvement in cell cycle progression. Human cells, which have evolved multiple options to counteract the oncogenic transformation, reject the hyper-active forms of the protein both by translocating the deacetylases into the cytoplasm and by inducing their degradation. Another important aspect is that in order to get a phenotype, HDAC4 seems need to reach a threshold expression level. Hence, it is important to develop new expression systems which are able to produce a significative amount of protein and better maintained together with the inhibition of the UPS. Although future studies will be directed to find the minimal essential combinations of oncogenes needed to get a fully transformed phenotype together with the L/A mutant in human fibroblasts, a reverse approach consisting in the silencing of HDAC4 using the new CRISPR/Cas9 technology could be more useful to understand the oncogenic potential of this class of proteins in human cells.

# Additional work

During the three years of the PhD program I have been focused also in other experimental works. In particular I have been involved in the investigation of MEF2s TFs in the regulation of the cell cycle.

We have discovered a complex regulation of MEF2s during cell cycle progression. MEF2s are activated at the G0/G1 transition by phosphorylation to orchestrate the expression of the immediate early genes in response to growth factor stimulation. We have observed that, MEF2 activities are subsequently down-regulated during G1 progression. MEF2s down-regulation is mainly mediated by the interaction with the E3 ligase F-box protein SKP2, which induces degradation of these TFs through the ubiquitin proteasome system. The SKP2-MEF2 interaction is mediated by a cyclin-dependent kinase 4 (CDK4)/cyclin D1 complex which is able to phosphorylate specific MEF2 residues inducing the binding to SKP2. MEF2 anti-proliferative effects were manly operated via the CDK inhibitor p21/CDKN1A gene. This cell cycle regulator is a MEF2 target gene. In particular my work has been focused on the investigation of MEF2 binding on CDKN1A promoter. Initially, through a bioinformatics approach, different MEF2 binding sites on CDKN1A promoter were predicted. Next, to confirm which of the putative binding sites were effectively recognized by MEF2s, chromatin immunoprecipitation assays (ChIP) were performed. Two different MEF2s members were evaluated: MEF2C and MEF2D. These TFs were able to bind within the first intron of the CDKN1A gene at +2,1 kb from the TSS. Importantly, this genomic region was previously characterized by ENCODE project as enriched in histone H3 lysine 27 acetylation (H3K27ac) and histone H3 lysine 4 monomethylation (H3K4me1), two markers of open chromatin. Finally, I demonstrated that the presence of MEF2D was important to regulate the H3K27ac in the genomic region bound by the transcription factor this suggest a possible implication of MEF2 in maintaining the open chromatin status.

These results will be not discussed in this thesis, since the work has been already published (see the enclosed published manuscript).

# References


Abu-Farha, M., Tiss, A., Abubaker, J., Khadir, A., Al-Ghimlas, F., Al-Khairi, I., Baturcam, E., Cherian, P., Elkum, N., Hammad, M.*, et al.* (2013). Proteomics analysis of human obesity reveals the epigenetic factor HDAC4 as a potential target for obesity. PLoS One *8*, e75342.

Addis, R.C., Prasad, M.K., Yochem, R.L., Zhan, X.C., Sheets, T.P., Axelman, J., Patterson, E.S., and Shamblott, M.J. (2010). OCT3/4 Regulates Transcription of Histone Deacetylase 4 (Hdac4) in Mouse Embryonic Stem Cells. J Cell Biochem *111*, 391-401.

Ago, T., Liu, T., Zhai, P., Chen, W., Li, H., Molkentin, J.D., Vatner, S.F., and Sadoshima, J. (2008). A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. Cell *133*, 978-993.

Ahuja, D., Saenz-Robles, M.T., and Pipas, J.M. (2005). SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene *24*, 7729-7745.

Akagi, T., Sasai, K., and Hanafusa, H. (2003). Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. Proc Natl Acad Sci U S A *100*, 13567-13572.

Allfrey, V.G., Faulkner, R., and Mirsky, A.E. (1964). Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A *51*, 786-794.

Arnold, M.A., Kim, Y., Czubryt, M.P., Phan, D., McAnally, J., Qi, X., Shelton, J.M., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007). MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell *12*, 377-389.

Astle, M.V., Hannan, K.M., Ng, P.Y., Lee, R.S., George, A.J., Hsu, A.K., Haupt, Y., Hannan, R.D., and Pearson, R.B. (2012). AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancy. Oncogene *31*, 1949-1962.

Backs, J., and Olson, E.N. (2006). Control of cardiac growth by histone acetylation/deacetylation. Circ Res *98*, 15-24.

Backs, J., Song, K., Bezprozvannaya, S., Chang, S., and Olson, E.N. (2006). CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest *116*, 1853-1864.

Backs, J., Worst, B.C., Lehmann, L.H., Patrick, D.M., Jebessa, Z., Kreusser, M.M., Sun, Q., Chen, L., Heft, C., Katus, H.A.*, et al.* (2011). Selective repression of MEF2 activity by PKA-dependent proteolysis of HDAC4. J Cell Biol *195*, 403-415.

Bakin, R.E., and Jung, M.O. (2004). Cytoplasmic sequestration of HDAC7 from mitochondrial and nuclear compartments upon initiation of apoptosis. J Biol Chem *279*, 51218-51225.

Bannister, A.J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res *21*, 381-395.

Barneda-Zahonero, B., Collazo, O., Azagra, A., Fernandez-Duran, I., Serra-Musach, J., Islam, A.B., Vega-Garcia, N., Malatesta, R., Camos, M., Gomez, A.*, et al.* (2015). The transcriptional repressor HDAC7 promotes apoptosis and c-Myc downregulation in particular types of leukemia and lymphoma. Cell Death Dis *6*, e1635.

Barneda-Zahonero, B., and Parra, M. (2012). Histone deacetylases and cancer. Mol Oncol *6*, 579-589.

Barneda-Zahonero, B., Roman-Gonzalez, L., Collazo, O., Rafati, H., Islam, A.M.M.K., Bussmann, L.H., di Tullio, A., De Andres, L., Graf, T., Lopez-Bigas, N.*, et al.* (2013). HDAC7 Is a Repressor of Myeloid Genes Whose Downregulation Is Required for Transdifferentiation of Pre-B Cells into Macrophages. Plos Genetics *9*.

Barrett, A., Santangelo, S., Tan, K., Catchpole, S., Roberts, K., Spencer-Dene, B., Hall, D., Scibetta, A., Burchell, J., Verdin, E.*, et al.* (2007). Breast cancer associated transcriptional repressor PLU-1/JARID1B interacts directly with histone deacetylases. Int J Cancer *121*, 265-275.

Berdeaux, R., Goebel, N., Banaszynski, L., Takemori, H., Wandless, T., Shelton, G.D., and Montminy, M. (2007). SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. Nat Med *13*, 597-603.

Berger, S.L. (2007). The complex language of chromatin regulation during transcription. Nature *447*, 407-412.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349-352.

Boehm, J.S., Hession, M.T., Bulmer, S.E., and Hahn, W.C. (2005). Transformation of human and murine fibroblasts without viral oncoproteins. Mol Cell Biol *25*, 6464-6474.

Bolger, T.A., and Yao, T.P. (2005). Intracellular trafficking of histone deacetylase 4 regulates neuronal cell death. J Neurosci *25*, 9544-9553.

Borghi, S., Molinari, S., Razzini, G., Parise, F., Battini, R., and Ferrari, S. (2001). The nuclear localization domain of the MEF2 family of transcription factors shows member-specific features and mediates the nuclear import of histone deacetylase 4. Journal of Cell Science *114*, 4477-4483.

Bosch-Presegue, L., and Vaquero, A. (2014). Sirtuins in stress response: guardians of the genome. Oncogene *33*, 3764-3775.

Bottomley, M.J., Lo Surdo, P., Di Giovine, P., Cirillo, A., Scarpelli, R., Ferrigno, F., Jones, P., Neddermann, P., De Francesco, R., Steinkuhler, C.*, et al.* (2008). Structural and functional analysis of the human HDAC4 catalytic domain reveals a regulatory structural zinc-binding domain. J Biol Chem *283*, 26694-26704.

Bradley, E.W., Carpio, L.R., Olson, E.N., and Westendorf, J.J. (2015). Histone deacetylase 7 (Hdac7) suppresses chondrocyte proliferation and beta-catenin activity during endochondral ossification. J Biol Chem *290*, 118-126.

Cadot, B., Brunetti, M., Coppari, S., Fedeli, S., de Rinaldis, E., Dello Russo, C., Gallinari, P., De Francesco, R., Steinkuhler, C., and Filocamo, G. (2009). Loss of histone deacetylase 4 causes segregation defects during mitosis of p53-deficient human tumor cells. Cancer Res *69*, 6074-6082.

Campisi, J., and d'Adda di Fagagna, F. (2007). Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol *8*, 729-740.

Cernotta, N., Clocchiatti, A., Florean, C., and Brancolini, C. (2011). Ubiquitin-dependent degradation of HDAC4, a new regulator of random cell motility. Mol Biol Cell *22*, 278-289.

Cersosimo, U., Sgorbissa, A., Foti, C., Drioli, S., Angelica, R., Tomasella, A., Picco, R., Semrau, M.S., Storici, P., Benedetti, F.*, et al.* (2015). Synthesis, characterization, and optimization for in vivo delivery of a nonselective isopeptidase inhibitor as new antineoplastic agent. J Med Chem *58*, 1691-1704.

Chang, S.R., McKinsey, T.A., Zhang, C.L., Richardson, J.A., Hill, J.A., and Olson, E.N. (2004). Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Molecular and Cellular Biology *24*, 8467-8476.

Chang, S.R., Young, B.D., Li, S.J., Qi, X.X., Richardson, J.A., and Olson, E.N. (2006). Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. Cell *126*, 321-334.

Chatterjee, T.K., Basford, J.E., Knoll, E., Tong, W.S., Blanco, V., Blomkalns, A.L., Rudich, S., Lentsch, A.B., Hui, D.Y., and Weintraub, N.L. (2014). HDAC9 knockout mice are protected from adipose tissue dysfunction and systemic metabolic disease during high-fat feeding. Diabetes *63*, 176-187.

Chatterjee, T.K., Idelman, G., Blanco, V., Blomkalns, A.L., Piegore, M.G., Jr., Weintraub, D.S., Kumar, S., Rajsheker, S., Manka, D., Rudich, S.M.*, et al.* (2011). Histone deacetylase 9 is a negative regulator of adipogenic differentiation. J Biol Chem *286*, 27836-27847.

Chen, J., Xia, J., Yu, Y.L., Wang, S.Q., Wei, Y.B., Chen, F.Y., Huang, G.Y., and Shi, J.S. (2014). HDAC5 promotes osteosarcoma progression by upregulation of Twist 1 expression. Tumour Biol *35*, 1383-1387.

Chen, J.F., Mandel, E.M., Thomson, J.M., Wu, Q.L., Callis, T.E., Hammond, S.M., Conlon, F.L., and Wang, D.Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet *38*, 228-233.

Cheng, W., Li, M., Cai, J., Wang, K., Zhang, C., Bao, Z., Liu, Y., and Wu, A. (2015). HDAC4, a prognostic and chromosomal instability marker, refines the predictive value of MGMT promoter methylation. J Neurooncol *122*, 303-312.

Cho, Y., Park, D., and Cavalli, V. (2015). Filamin A is required in injured axons for HDAC5 activity and axon regeneration. J Biol Chem *290*, 22759-22770.

Cho, Y., Sloutsky, R., Naegle, K.M., and Cavalli, V. (2013). Injury-induced HDAC5 nuclear export is essential for axon regeneration. Cell *155*, 894-908.

Choi, M.C., Cohen, T.J., Barrientos, T., Wang, B., Li, M., Simmons, B.J., Yang, J.S., Cox, G.A., Zhao, Y., and Yao, T.P. (2012). A direct HDAC4-MAP kinase crosstalk activates muscle atrophy program. Mol Cell *47*, 122-132.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M.L., Rehman, M., Walther, T.C., Olsen, J.V., and Mann, M. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science *325*, 834-840.

Choudhary, C., Weinert, B.T., Nishida, Y., Verdin, E., and Mann, M. (2014). The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol *15*, 536-550.

Clocchiatti, A., Di Giorgio, E., Demarchi, F., and Brancolini, C. (2013a). Beside the MEF2 axis: Unconventional functions of HDAC4. Cell Signal *25*, 269-276.

Clocchiatti, A., Di Giorgio, E., Ingrao, S., Meyer-Almes, F.J., Tripodo, C., and Brancolini, C. (2013b). Class IIa HDACs repressive activities on MEF2-depedent transcription are associated with poor prognosis of ER(+) breast tumors. Faseb J *27*, 942-954.

Clocchiatti, A., Di Giorgio, E., Viviani, G., Streuli, C., Sgorbissa, A., Picco, R., Cutano, V., and Brancolini, C. (2015). The MEF2-HDAC axis controls proliferation of mammary epithelial cells and acini formation in vitro. J Cell Sci *128*, 3961-3976.

Clocchiatti, A., Florean, C., and Brancolini, C. (2011). Class IIa HDACs: from important roles in differentiation to possible implications in tumourigenesis. J Cell Mol Med *15*, 1833-1846.

Cohen, T.J., Waddell, D.S., Barrientos, T., Lu, Z., Feng, G., Cox, G.A., Bodine, S.C., and Yao, T.P. (2007). The histone deacetylase HDAC4 connects neural activity to muscle transcriptional reprogramming. Journal of Biological Chemistry *282*, 33752-33759.

Dai, Y.S., Xu, J., and Molkentin, J.D. (2005). The DnaJ-related factor Mrj interacts with nuclear factor of activated T cells c3 and mediates transcriptional repression through class II histone deacetylase recruitment. Molecular and Cellular Biology *25*, 9936-9948.

Datta, J., Kutay, H., Nasser, M.W., Nuovo, G.J., Wang, B., Majumder, S., Liu, C.G., Volinia, S., Croce, C.M., Schmittgen, T.D.*, et al.* (2008a). Methylation mediated silencing of MicroRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res *68*, 5049-5058.

Datta, J., Kutay, H., Nasser, M.W., Nuovo, G.J., Wang, B., Majumder, S., Liu, C.G., Volinia, S., Croce, C.M., Schmittgen, T.D.*, et al.* (2008b). Methylation mediated silencing of microRNA-1 gene and its role in hepatocellular carcinogenesis. Cancer Res *68*, 5049-5058.

Davila, J.L., Goff, L.A., Ricupero, C.L., Camarillo, C., and Oni, E.N. (2014). A Positive Feedback Mechanism That Regulates Expression of miR-9 during Neurogenesis (vol 9, e94348, 2014). PLoS One *9*.

Dawson, M.A., and Kouzarides, T. (2012). Cancer epigenetics: from mechanism to therapy. Cell *150*, 12-27.

de Visser, K.E., Eichten, A., and Coussens, L.M. (2006). Paradoxical roles of the immune system during cancer development. Nat Rev Cancer *6*, 24-37.

Deng, X., Ewton, D.Z., Mercer, S.E., and Friedman, E. (2005). Mirk/dyrk1B decreases the nuclear accumulation of class II histone deacetylases during skeletal muscle differentiation. J Biol Chem *280*, 4894-4905.

Dequiedt, F., Kasler, H., Fischle, W., Kiermer, V., Weinstein, M., Herndier, B.G., and Verdin, E. (2003). HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity *18*, 687-698.

Dequiedt, F., Martin, M., Von Blume, J., Vertommen, D., Lecomte, E., Mari, N., Heinen, M.F., Bachmann, M., Twizere, J.C., Huang, M.C., *et al.* (2006). New role for hPar-1 kinases EMK and C-TAK1 in regulating localization and activity of class IIa histone deacetylases. Molecular and Cellular Biology *26*, 7086-7102.

Di Giorgio, E., Clocchiatti, A., Piccinin, S., Sgorbissa, A., Viviani, G., Peruzzo, P., Romeo, S., Rossi, S., Dei Tos, A.P., Maestro, R., *et al.* (2013). MEF2 is a converging hub for histone deacetylase 4 and phosphatidylinositol 3-kinase/Akt-induced transformation. Mol Cell Biol *33*, 4473-4491.

Di Giorgio, E., Gagliostro, E., and Brancolini, C. (2015). Selective class IIa HDAC inhibitors: myth or reality. Cell Mol Life Sci *72*, 73-86.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A., *et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Du Toit, A. (2012). Chromatin: Defining heterochromatin. Nat Rev Mol Cell Biol *13*, 684-685.

Falkenberg, K.J., and Johnstone, R.W. (2015). Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders (vol 13, pg 673, 2014). Nat Rev Drug Discov *14*, 219-219.

Fan, J., Lou, B., Chen, W., Zhang, J., Lin, S., Lv, F.F., and Chen, Y. (2014). Down-regulation of HDAC5 inhibits growth of human hepatocellular carcinoma by induction of apoptosis and cell cycle arrest. Tumour Biol *35*, 11523-11532.

Feng, G.W., Dong, L.D., Shang, W.J., Pang, X.L., Li, J.F., Liu, L., and Wang, Y. (2014). HDAC5 promotes cell proliferation in human hepatocellular carcinoma by up-regulating Six1 expression. Eur Rev Med Pharmacol Sci *18*, 811-816.

Fischer, S., Paul, A.J., Wagner, A., Mathias, S., Geiss, M., Schandock, F., Domnowski, M., Zimmermann, J., Handrick, R., Hesse, F., *et al.* (2015). miR-2861 as novel HDAC5 inhibitor in CHO cells enhances productivity while maintaining product quality. Biotechnol Bioeng *112*, 2142-2153.

Fischle, W., Dequiedt, F., Fillion, M., Hendzel, M.J., Voelter, W., and Verdin, E. (2001). Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. Journal of Biological Chemistry *276*, 35826-35835.

Fischle, W., Dequiedt, F., Hendzel, M.J., Guenther, M.G., Lazar, M.A., Voelter, W., and Verdin, E. (2002). Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell *9*, 45-57.

Gao, C.Z., Ho, C.C., Reineke, E., Lam, M., Cheng, X.W., Stanya, K.J., Liu, Y., Chakraborty, S., Shih, H.M., and Kao, H.Y. (2008). Histone deacetylase 7 promotes PML sumoylation and is essential for PML nuclear body formation. Molecular and Cellular Biology *28*, 5658-5667.

Geng, H., Harvey, C.T., Pittsenbarger, J., Liu, Q., Beer, T.M., Xue, C., and Qian, D.Z. (2011). HDAC4 protein regulates HIF1alpha protein lysine acetylation and cancer cell response to hypoxia. J Biol Chem *286*, 38095-38102.

Gershey, E.L., Vidali, G., and Allfrey, V.G. (1968). Chemical studies of histone acetylation. The occurrence of epsilon-N-acetyllysine in the f2a1 histone. J Biol Chem *243*, 5018-5022.

Glozak, M.A., and Seto, E. (2007). Histone deacetylases and cancer. Oncogene *26*, 5420-5432.

Greco, T.M., Yu, F., Guise, A.J., and Cristea, I.M. (2011). Nuclear import of histone deacetylase 5 by requisite nuclear localization signal phosphorylation. Mol Cell Proteomics *10*, M110 004317.

Gregoire, S., Tremblay, A.M., Xiao, L., Yang, Q., Ma, K., Nie, J., Mao, Z., Wu, Z., Giguere, V., and Yang, X.J. (2006). Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. J Biol Chem *281*, 4423-4433.

Grozinger, C.M., and Schreiber, S.L. (2000). Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc Natl Acad Sci U S A *97*, 7835-7840.

Guan, Y.J., Yang, X., Wei, L., and Chen, Q. (2011). MiR-365: a mechanosensitive microRNA stimulates chondrocyte differentiation through targeting histone deacetylase 4. Faseb J *25*, 4457-4466.

Guise, A.J., Greco, T.M., Zhang, I.Y., Yu, F., and Cristea, I.M. (2012). Aurora B-dependent regulation of class IIa histone deacetylases by mitotic nuclear localization signal phosphorylation. Mol Cell Proteomics *11*, 1220-1229.

Guo, L., Han, A., Bates, D.L., Cao, J., and Chen, L. (2007). Crystal structure of a conserved N-terminal domain of histone deacetylase 4 reveals functional insights into glutamine-rich domains. Proc Natl Acad Sci U S A *104*, 4297-4302.

Guo, X.Z., Wang, S.B., Xu, H.P., Ribic, A., Mohns, E.J., Zhou, Y., Zhu, X.J., Biederer, T., Crair, M.C., and Chen, B. (2015). A short N-terminal domain of HDAC4 preserves photoreceptors and restores visual function in retinitis pigmentosa. Nature Communications *6*.

Ha, C.H., Kim, J.Y., Zhao, J., Wang, W., Jhun, B.S., Wong, C., and Jin, Z.G. (2010). PKA phosphorylates histone deacetylase 5 and prevents its nuclear export, leading to the inhibition of gene transcription and cardiomyocyte hypertrophy. Proc Natl Acad Sci U S A *107*, 15467-15472.

Haberland, M., Arnold, M.A., McAnally, J., Phan, D., Kim, Y., and Olson, E.N. (2007). Regulation of HDAC9 gene expression by MEF2 establishes a negative-feedback loop in the transcriptional circuitry of muscle differentiation. Molecular and Cellular Biology *27*, 518-525.

Haberland, M., Montgomery, R.L., and Olson, E.N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet *10*, 32-42.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hahn, W.C., Dessain, S.K., Brooks, M.W., King, J.E., Elenbaas, B., Sabatini, D.M., DeCaprio, J.A., and Weinberg, R.A. (2002). Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol Cell Biol *22*, 2111-2123.

Han, A.D., He, J., Wu, Y.Q., Liu, J.O., and Chen, L. (2005). Mechanism of recruitment of class II histone deacetylases by myocyte enhancer factor-2. Journal of Molecular Biology *345*, 91-102.

Han, K., Gennarino, V.A., Lee, Y., Pang, K.F., Hashimoto-Torii, K., Choufani, S., Raju, C.S., Oldham, M.C., Weksberg, R., Rakic, P.*, et al.* (2013). Human-specific regulation of MeCP2 levels in fetal brains by microRNA miR-483-5p. Gene Dev *27*, 485-490.

Hardie, D.G. (2011). AMP-activated protein kinase-an energy sensor that regulates all aspects of cell function. Gene Dev *25*, 1895-1908.

Haworth, R.S., Stathopoulou, K., Candasamy, A.J., and Avkiran, M. (2012). Neurohormonal regulation of cardiac histone deacetylase 5 nuclear localization by phosphorylation-dependent and phosphorylation-independent mechanisms. Circ Res *110*, 1585-1595.

Henriksson, E., Sall, J., Gormand, A., Wasserstrom, S., Morrice, N.A., Fritzen, A.M., Foretz, M., Campbell, D.G., Sakamoto, K., Ekelund, M.*, et al.* (2015). SIK2 regulates CRTCs, HDAC4 and glucose uptake in adipocytes. J Cell Sci *128*, 472-486.

Hojfeldt, J.W., Agger, K., and Helin, K. (2013). Histone lysine demethylases as targets for anticancer therapy. Nat Rev Drug Discov *12*, 917-930.

Huang, Y., Tan, M., Gosink, M., Wang, K.K., and Sun, Y. (2002). Histone deacetylase 5 is not a p53 target gene, but its overexpression inhibits tumor cell growth and induces apoptosis. Cancer Res *62*, 2913-2922.

Huang, Z.P., Chen, J.H., Seok, H.Y., Zhang, Z., Kataoka, M., Hu, X.Y., and Wang, D.Z. (2013). MicroRNA-22 Regulates Cardiac Hypertrophy and Remodeling in Response to Stress. Circ Res *112*, 1234-U1103.

Hudson, G.M., Watson, P.J., Fairall, L., Jamieson, A.G., and Schwabe, J.W. (2015). Insights into the Recruitment of Class IIa Histone Deacetylases (HDACs) to the SMRT/NCoR Transcriptional Repression Complex. J Biol Chem *290*, 18237-18244.

Jeon, E.J., Lee, K.Y., Choi, N.S., Lee, M.H., Kim, H.N., Jin, Y.H., Ryoo, H.M., Choi, J.Y., Yoshida, M., Nishino, N.*, et al.* (2006). Bone morphogenetic protein-2 stimulates Runx2 acetylation. Journal of Biological Chemistry *281*, 16502-16511.

Kaewpiboon, C., Srisuttee, R., Malilas, W., Moon, J., Oh, S., Jeong, H.G., Johnston, R.N., Assavalapsakul, W., and Chung, Y.H. (2015). Upregulation of Stat1-HDAC4 confers resistance to etoposide through enhanced multidrug resistance 1 expression in human A549 lung cancer cells. Mol Med Rep *11*, 2315-2321.

Kai, Y., Peng, W., Ling, W., Hao, J.B., and Zhuan, B. (2014). Reciprocal effects between microRNA-140-5p and ADAM10 suppress migration and invasion of human tongue cancer cells. Biochem Bioph Res Co *448*, 308-314.

Kang, J.S., Alliston, T., Delston, R., and Derynck, R. (2005). Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. Embo Journal *24*, 2543-2555.

Kang, Z.H., Wang, C.Y., Zhang, W.L., Zhang, J.T., Yuan, C.H., Zhao, P.W., Lin, Y.Y., Hong, S., Li, C.Y., and Wang, L. (2014). Histone deacetylase HDAC4 promotes gastric cancer SGC-7901 cells progression via p21 repression. PLoS One *9*, e98894.

Karamboulas, C., Dakubo, G.D., Liu, J., De Repentigny, Y., Yutzey, K., Wallace, V.A., Kothary, R., and Skerjanc, I.S. (2006). Disruption of MEF2 activity in cardiomyoblasts inhibits cardiomyogenesis (vol 119, pg 4315, 2006). Journal of Cell Science *119*.

Kasler, H.G., and Verdin, E. (2007). Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes. Mol Cell Biol *27*, 5184-5200.

Kasler, H.G., Young, B.D., Mottet, D., Lim, H.W., Collins, A.M., Olson, E.N., and Verdin, E. (2011). Histone deacetylase 7 regulates cell survival and TCR signaling in CD4/CD8 double-positive thymocytes. J Immunol *186*, 4782-4793.

Kastan, M.B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kato, H., Tamamizu-Kato, S., and Shibasaki, F. (2004). Histone deacetylase 7 associates with hypoxia-inducible factor 1 alpha and increases transcriptional activity. Journal of Biological Chemistry *279*, 41966-41974.

Katoh, Y., Takemori, H., Horike, N., Doi, J., Muraoka, M., Min, L., and Okamoto, M. (2004). Salt-inducible kinase (SIK) isoforms: their involvement in steroidogenesis and adipogenesis. Mol Cell Endocrinol *217*, 109-112.

Kennedy, A.L., Morton, J.P., Manoharan, I., Nelson, D.M., Jamieson, N.B., Pawlikowski, J.S., McBryan, T., Doyle, B., McKay, C., Oien, K.A.*, et al.* (2011). Activation of the PIK3CA/AKT pathway suppresses senescence induced by an activated RAS oncogene to promote tumorigenesis. Mol Cell *42*, 36-49.

Kerr, J.F., Winterford, C.M., and Harmon, B.V. (1994). Apoptosis. Its significance in cancer and cancer therapy. Cancer *73*, 2013-2026.

Kikuchi, S., Suzuki, R., Ohguchi, H., Yoshida, Y., Lu, D., Cottini, F., Jakubikova, J., Bianchi, G., Harada, T., Gorgun, G.*, et al.* (2015). Class IIa HDAC inhibition enhances ER stress-mediated cell death in multiple myeloma. Leukemia *29*, 1918-1927.

Kim, M.S., Akhtar, M.W., Adachi, M., Mahgoub, M., Bassel-Duby, R., Kavalali, E.T., Olson, E.N., and Monteggia, L.M. (2012). An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. J Neurosci *32*, 10879-10886.

Kim, M.S., Fielitz, J., McAnally, J., Shelton, J.M., Lemon, D.D., McKinsey, T.A., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2008). Protein kinase D1 stimulates MEF2 activity in skeletal muscle and enhances muscle performance. Mol Cell Biol *28*, 3600-3609.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

Kirsh, O., Seeler, J.S., Pichler, A., Gast, A., Muller, S., Miska, E., Mathieu, M., Harel-Bellan, A., Kouzarides, T., Melchior, F.*, et al.* (2002). The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. EMBO J *21*, 2682-2691.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.

Kozhemyakina, E., Cohen, T., Yao, T.P., and Lassar, A.B. (2009). Parathyroid Hormone-Related Peptide Represses Chondrocyte Hypertrophy through a Protein Phosphatase 2A/Histone Deacetylase 4/MEF2 Pathway. Molecular and Cellular Biology *29*, 5751-5762.

Lahm, A., Paolini, C., Pallaoro, M., Nardi, M.C., Jones, P., Neddermann, P., Sambucini, S., Bottomley, M.J., Lo Surdo, P., Carfi, A.*, et al.* (2007). Unraveling the hidden catalytic activity of vertebrate class Ila histone deacetylases. P Natl Acad Sci USA *104*, 17335-17340.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature *304*, 596-602.

Lecis, D., Mastrangelo, E., Belvisi, L., Bolognesi, M., Civera, M., Cossu, F., De Cesare, M., Delia, D., Drago, C., Manenti, G.*, et al.* (2012). Dimeric Smac mimetics/IAP inhibitors as in vivo-active pro-apoptotic agents. Part II: Structural and biological characterization. Bioorg Med Chem *20*, 6709-6723.

Lee, J.H., Park, S.M., Kim, O.S., Lee, C.S., Woo, J.H., Park, S.J., Joe, E.H., and Jou, I. (2009). Differential SUMOylation of LXR alpha and LXR beta Mediates Transrepression of STAT1 Inflammatory Signaling in IFN-gamma-Stimulated Brain Astrocytes. Molecular Cell *35*, 806-817.

Li, C.J., Cheng, P., Liang, M.K., Chen, Y.S., Lu, Q., Wang, J.Y., Xia, Z.Y., Zhou, H.D., Cao, X., Xie, H.*, et al.* (2015). MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. J Clin Invest *125*, 1509-1522.

Li, H., Xie, H., Liu, W., Hu, R., Huang, B., Tan, Y.F., Liao, E.Y., Xu, K., Sheng, Z.F., Zhou, H.D.*, et al.* (2009). A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. J Clin Invest *119*, 3666-3677.

Li, J.L., Chen, J.M., Ricupero, C.L., Hart, R.P., Schwartz, M.S., Kusnecov, A., and Herrup, K. (2012). Nuclear accumulation of HDAC4 in ATM deficiency promotes neurodegeneration in ataxia telangiectasia. Nature Medicine *18*, 783-U182.

Li, X., Song, S., Liu, Y., Ko, S.H., and Kao, H.Y. (2004). Phosphorylation of the histone deacetylase 7 modulates its stability and association with 14-3-3 proteins. J Biol Chem *279*, 34201-34208.

Liang, J.Y., and Mills, G.B. (2013). AMPK: A Contextual Oncogene or Tumor Suppressor? Cancer Res *73*, 2929-2935.

Liu, F., Dowling, M., Yang, X.J., and Kao, G.D. (2004). Caspase-mediated specific cleavage of human histone deacetylase 4. J Biol Chem *279*, 34537-34546.

Liu, F., Pore, N., Kim, M., Voong, K.R., Dowling, M., Maity, A., and Kao, G.D. (2006). Regulation of histone deacetylase 4 expression by the SP family of transcription factors. Mol Biol Cell *17*, 585-597.

Liu, R., Wang, L., Chen, G., Katoh, H., Chen, C., Liu, Y., and Zheng, P. (2009). FOXP3 up-regulates p21 expression by site-specific inhibition of histone deacetylase 2/histone deacetylase 4 association to the locus. Cancer Res *69*, 2252-2259.

Lobera, M., Madauss, K.P., Pohlhaus, D.T., Wright, Q.G., Trocha, M., Schmidt, D.R., Baloglu, E., Trump, R.P., Head, M.S., Hofmann, G.A.*, et al.* (2013). Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. Nat Chem Biol *9*, 319-325.

Lowe, S.W., and Sherr, C.J. (2003). Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev *13*, 77-83.

Lu, J.R., McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2000). Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. Molecular Cell *6*, 233-244.

Lu, W., You, R., Yuan, X., Yang, T., Samuel, E.L., Marcano, D.C., Sikkema, W.K., Tour, J.M., Rodriguez, A., Kheradmand, F.*, et al.* (2015). The microRNA miR-22 inhibits the histone deacetylase HDAC4 to promote TH17 cell-dependent emphysema. Nat Immunol *16*, 1185-1194.

Luan, B., Goodarzi, M.O., Phillips, N.G., Guo, X., Chen, Y.D., Yao, J., Allison, M., Rotter, J.I., Shaw, R., and Montminy, M. (2014). Leptin-mediated increases in catecholamine signaling reduce adipose tissue inflammation via activation of macrophage HDAC4. Cell Metab *19*, 1058-1065.

Lundberg, A.S., Hahn, W.C., Gupta, P., and Weinberg, R.A. (2000). Genes involved in senescence and immortalization. Curr Opin Cell Biol *12*, 705-709.

Majdzadeh, N., Wang, L., Morrison, B.E., Bassel-Duby, R., Olson, E.N., and D'Mello, S.R. (2008). HDAC4 inhibits cell-cycle progression and protects neurons from cell death. Dev Neurobiol *68*, 1076-1092.

Mannaerts, I., Eysackers, N., Onyema, O.O., Van Beneden, K., Valente, S., Mai, A., Odenthal, M., and van Grunsven, L.A. (2013). Class II HDAC Inhibition Hampers Hepatic Stellate Cell Activation by Induction of MicroRNA-29. PLoS One *8*.

Margariti, A., Zampetaki, A., Xiao, Q., Zhou, B., Karamariti, E., Martin, D., Yin, X., Mayr, M., Li, H., Zhang, Z.*, et al.* (2010). Histone deacetylase 7 controls endothelial cell growth through modulation of beta-catenin. Circ Res *106*, 1202-1211.

Martin, M., Kettmann, R., and Dequiedt, F. (2007). Class IIa histone deacetylases: regulating the regulators. Oncogene *26*, 5450-5467.

Martin, M., Potente, M., Janssens, V., Vertommen, D., Twizere, J.C., Rider, M.H., Goris, J., Dimmeler, S., Kettmann, R., and Dequiedt, F. (2008). Protein phosphatase 2A controls the activity of histone deacetylase 7 during T cell apoptosis and angiogenesis. Proc Natl Acad Sci U S A *105*, 4727-4732.

Matthews, S.A., Liu, P., Spitaler, M., Olson, E.N., McKinsey, T.A., Cantrell, D.A., and Scharenberg, A.M. (2006). Essential role for protein kinase D family kinases in the regulation of class II histone deacetylases in B lymphocytes. Mol Cell Biol *26*, 1569-1577.

McGee, S.L., Swinton, C., Morrison, S., Gaur, V., Campbell, D.E., Jorgensen, S.B., Kemp, B.E., Baar, K., Steinberg, G.R., and Hargreaves, M. (2014). Compensatory regulation of HDAC5 in muscle maintains metabolic adaptive responses and metabolism in response to energetic stress. Faseb J *28*, 3384-3395.

McGee, S.L., van Denderen, B.J., Howlett, K.F., Mollica, J., Schertzer, J.D., Kemp, B.E., and Hargreaves, M. (2008). AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes *57*, 860-867.

McKinsey, T.A., Zhang, C.L., Lu, J.R., and Olson, E.N. (2000). Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature *408*, 106-111.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2001). Identification of a signal-responsive nuclear export sequence in class II histone deacetylases. Molecular and Cellular Biology *21*, 6312-6321.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2002). MEF2: a calcium-dependent regulator of cell division, differentiation and death (vol 27, pg 40, 2002). Trends Biochem Sci *27*, 271-271.

Mejat, A., Ramond, F., Bassel-Duby, R., Khochbin, S., Olson, E.N., and Schaeffer, L. (2005). Histone deacetylase 9 couples neuronal activity to muscle chromatin acetylation and gene expression. Nature Neuroscience *8*, 313-321.

Metrich, M., Berthouze, M., Morel, E., Crozatier, B., Gomez, A.M., and Lezoualc'h, F. (2010). Role of the cAMP-binding protein Epac in cardiovascular physiology and pathophysiology. Pflugers Arch *459*, 535-546.

Mihaylova, M.M., Vasquez, D.S., Ravnskjaer, K., Denechaud, P.D., Yu, R.T., Alvarez, J.G., Downes, M., Evans, R.M., Montminy, M., and Shaw, R.J. (2011). Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. Cell *145*, 607-621.

Milde, T., Oehme, I., Korshunov, A., Kopp-Schneider, A., Remke, M., Northcott, P., Deubzer, H.E., Lodrini, M., Taylor, M.D., von Deimling, A.*, et al.* (2010). HDAC5 and HDAC9 in medulloblastoma: novel markers for risk stratification and role in tumor cell growth. Clin Cancer Res *16*, 3240-3252.

Moreno, D.A., Scrideli, C.A., Cortez, M.A., de Paula Queiroz, R., Valera, E.T., da Silva Silveira, V., Yunes, J.A., Brandalise, S.R., and Tone, L.G. (2010). Differential expression of HDAC3, HDAC7 and HDAC9 is associated with prognosis and survival in childhood acute lymphoblastic leukaemia. Br J Haematol *150*, 665-673.

Moresi, V., Williams, A.H., Meadows, E., Flynn, J.M., Potthoff, M.J., McAnally, J., Shelton, J.M., Backs, J., Klein, W.H., Richardson, J.A.*, et al.* (2010). Myogenin and Class II HDACs Control Neurogenic Muscle Atrophy by Inducing E3 Ubiquitin Ligases. Cell *143*, 35-45.

Morin, R.D., Mendez-Lago, M., Mungall, A.J., Goya, R., Mungall, K.L., Corbett, R.D., Johnson, N.A., Severson, T.M., Chiu, R., Field, M.*, et al.* (2011). Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature *476*, 298-303.

Mottet, D., Bellahcene, A., Pirotte, S., Waltregny, D., Deroanne, C., Lamour, V., Lidereau, R., and Castronovo, V. (2007). Histone deacetylase 7 silencing alters endothelial cell migration, a key step in angiogenesis. Circ Res *101*, 1237-1246.

Muller, P.A., and Vousden, K.H. (2013). p53 mutations in cancer. Nat Cell Biol *15*, 2-8.

Nasser, M.W., Datta, J., Nuovo, G., Kutay, H., Motiwala, T., Majumder, S., Wang, B., Suster, S., Jacob, S.T., and Ghoshal, K. (2008). Down-regulation of Micro-RNA-1 (miR-1) in Lung Cancer SUPPRESSION OF TUMORIGENIC PROPERTY OF LUNG CANCER CELLS AND THEIR SENSITIZATION TO DOXORUBICIN-INDUCED APOPTOSIS BY miR-1. Journal of Biological Chemistry *283*, 33394-33405.

Navarro, M.N., Goebel, J., Feijoo-Carnero, C., Morrice, N., and Cantrell, D.A. (2011). Phosphoproteomic analysis reveals an intrinsic pathway for the regulation of histone deacetylase 7 that controls the function of cytotoxic T lymphocytes. Nat Immunol *12*, 352-361.

Nishida, N., Mimori, K., Fabbri, M., Yokobori, T., Sudo, T., Tanaka, F., Shibata, K., Ishii, H., Doki, Y., and Mori, M. (2011). MicroRNA-125a-5p Is an Independent Prognostic Factor in Gastric Cancer and Inhibits the Proliferation of Human Gastric Cancer Cells in Combination with Trastuzumab. Clin Cancer Res *17*, 2725-2733.

Nishino, T.G., Miyazaki, M., Hoshino, H., Miwa, Y., Horinouchi, S., and Yoshida, M. (2008). 14-3-3 regulates the nuclear import of class IIa histone deacetylases. Biochem Biophys Res Commun *377*, 852-856.

Obri, A., Makinistoglu, M.P., Zhang, H., and Karsenty, G. (2014). HDAC4 integrates PTH and sympathetic signaling in osteoblasts. J Cell Biol *205*, 771-780.

Oka, S., Ago, T., Kitazono, T., Zablocki, D., and Sadoshima, J. (2009). The role of redox modulation of class II histone deacetylases in mediating pathological cardiac hypertrophy. J Mol Med (Berl) *87*, 785-791.

Olson, E., Edmondson, D., Wright, W.E., Lin, V.K., Guenet, J.L., Simonchazottes, D., Thompson, L.H., Stallings, R.L., Schroeder, W.T., Duvic, M.*, et al.* (1990). Myogenin Is in an Evolutionarily Conserved Linkage Group on Human Chromosome-1q31-Q41 and Unlinked to Other Mapped Muscle Regulatory Factor Genes. Genomics *8*, 427-434.

Oren, M. (2003). Decision making by p53: life, death and cancer. Cell Death Differ *10*, 431-442.

Paroni, G., Cernotta, N., Dello Russo, C., Gallinari, P., Pallaoro, M., Foti, C., Talamo, F., Orsatti, L., Steinkuhler, C., and Brancolini, C. (2008). PP2A regulates HDAC4 nuclear import. Mol Biol Cell *19*, 655-667.

Paroni, G., Fontanini, A., Cernotta, N., Foti, C., Gupta, M.P., Yang, X.J., Fasino, D., and Brancolini, C. (2007). Dephosphorylation and caspase processing generate distinct nuclear pools of histone deacetylase 4. Molecular and Cellular Biology *27*, 6718-6732.

Paroni, G., Mizzau, M., Henderson, C., Del Sal, G., Schneider, C., and Brancolini, C. (2004). Caspase-dependent regulation of histone deacetylase 4 nuclear-cytoplasmic shuttling promotes apoptosis. Mol Biol Cell *15*, 2804-2818.

Parra, M. (2015). Class IIa HDACs - new insights into their functions in physiology and pathology. Febs J *282*, 1736-1744.

Parra, M., Kasler, H., McKinsey, T.A., Olson, E.N., and Verdin, E. (2005). Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. Journal of Biological Chemistry *280*, 13762-13770.

Parra, M., Mahmoudi, T., and Verdin, E. (2007). Myosin phosphatase dephosphorylates HDAC7, controls its nucleocytoplasmic shuttling, and inhibits apoptosis in thymocytes. Gene Dev *21*, 638-643.

Peixoto, P., Castronovo, V., Matheus, N., Polese, C., Peulen, O., Gonzalez, A., Boxus, M., Verdin, E., Thiry, M., Dequiedt, F.*, et al.* (2012). HDAC5 is required for maintenance of pericentric heterochromatin, and controls cell-cycle progression and survival of human cancer cells. Cell Death Differ *19*, 1239-1252.

Perry, R.L.S., Yang, C., Soora, N., Salma, J., Marback, M., Naghibi, L., Ilyas, H., Chan, J., Gordon, J.W., and McDermott, J.C. (2009). Direct Interaction between Myocyte Enhancer Factor 2 (MEF2) and Protein Phosphatase 1 alpha Represses MEF2-Dependent Gene Expression. Molecular and Cellular Biology *29*, 3355-3366.

Poralla, L., Stroh, T., Erben, U., Sittig, M., Liebig, S., Siegmund, B., and Glauben, R. (2015). Histone deacetylase 5 regulates the inflammatory response of macrophages. J Cell Mol Med *19*, 2162-2171.

Potthoff, M.J., Arnold, M.A., McAnally, J., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007a). Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. Molecular and Cellular Biology *27*, 8143-8151.

Potthoff, M.J., and Olson, E.N. (2007). MEF2: a central regulator of diverse developmental programs. Development *134*, 4131-4140.

Potthoff, M.J., Wu, H., Arnold, M.A., Shelton, J.M., Backs, J., McAnally, J., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2007b). Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. J Clin Invest *117*, 2459-2467.

Prowse, K.R., and Greider, C.W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. Proc Natl Acad Sci U S A *92*, 4818-4822.

Qian, D.Z., Kachhap, S.K., Collis, S.J., Verheul, H.M., Carducci, M.A., Atadja, P., and Pili, R. (2006). Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1 alpha. Cancer Res *66*, 8814-8821.

Rad, R., Rad, L., Wang, W., Cadinanos, J., Vassiliou, G., Rice, S., Campos, L.S., Yusa, K., Banerjee, R., Li, M.A.*, et al.* (2010). PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. Science *330*, 1104-1107.

Rangarajan, A., Hong, S.J., Gifford, A., and Weinberg, R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171-183.

Roccaro, A.M., Sacco, A., Jia, X.Y., Azab, A.K., Maiso, P., Ngo, H.T., Azab, F., Runnels, J., Quang, P., and Ghobrial, I.M. (2010). microRNA-dependent modulation of histone acetylation in Waldenstrom macroglobulinemia. Blood *116*, 1506-1514.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature *304*, 602-606.

Sandhu, S.K., Volinia, S., Costinean, S., Galasso, M., Neinast, R., Santhanam, R., Parthun, M.R., Perrotti, D., Marcucci, G., Garzon, R.*, et al.* (2012). miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the E mu-miR-155 transgenic mouse model. P Natl Acad Sci USA *109*, 20047-20052.

Sando, R., 3rd, Gounko, N., Pieraut, S., Liao, L., Yates, J., 3rd, and Maximov, A. (2012). HDAC4 governs a transcriptional program essential for synaptic plasticity and memory. Cell *151*, 821-834.

Schuetz, A., Min, J., Allali-Hassani, A., Schapira, M., Shuen, M., Loppnau, P., Mazitschek, R., Kwiatkowski, N.P., Lewis, T.A., Maglathin, R.L.*, et al.* (2008). Human HDAC7 harbors a class IIa histone deacetylase-specific zinc binding motif and cryptic deacetylase activity. J Biol Chem *283*, 11355-11363.

Scott, F.L., Fuchs, G.J., Boyd, S.E., Denault, J.B., Hawkins, C.J., Dequiedt, F., and Salvesen, G.S. (2008). Caspase-8 cleaves histone deacetylase 7 and abolishes its transcription repressor function. J Biol Chem *283*, 19499-19510.

Seger, Y.R., Garcia-Cao, M., Piccinin, S., Cunsolo, C.L., Doglioni, C., Blasco, M.A., Hannon, G.J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. Cancer Cell *2*, 401-413.

Serrano, M., Hannon, G.J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature *366*, 704-707.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Shalizi, A., Gaudilliere, B., Yuan, Z., Stegmuller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J.W., and Bonni, A. (2006). A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. Science *311*, 1012-1017.

Shimizu, E., Nakatani, T., He, Z., and Partridge, N.C. (2014). Parathyroid hormone regulates histone deacetylase (HDAC) 4 through protein kinase A-mediated phosphorylation and dephosphorylation in osteoblastic cells. J Biol Chem *289*, 21340-21350.

Sjoblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N.*, et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. Science *314*, 268-274.

Sleiman, S.F., Langley, B.C., Basso, M., Berlin, J., Xia, L., Payappilly, J.B., Kharel, M.K., Guo, H.C., Marsh, J.L., Thompson, L.M.*, et al.* (2012). Mithramycin Is a Gene-Selective Sp1 Inhibitor That Identifies a Biological Intersection between Cancer and Neurodegeneration (May, pg 6858, 2011). J Neurosci *32*, 6743-6743.

Song, B., Wang, Y., Xi, Y., Kudo, K., Bruheim, S., Botchkina, G.I., Gavin, E., Wan, Y., Formentini, A., Kornmann, M.*, et al.* (2009). Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. Oncogene *28*, 4065-4074.

Stark, M., and Hayward, N. (2007). Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. Cancer Res *67*, 2632-2642.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

Stronach, E.A., Alfraidi, A., Rama, N., Datler, C., Studd, J.B., Agarwal, R., Guney, T.G., Gourley, C., Hennessy, B.T., Mills, G.B.*, et al.* (2011a). HDAC4-Regulated STAT1 Activation Mediates Platinum Resistance in Ovarian Cancer. Cancer Res *71*, 4412-4422.

Stronach, E.A., Alfraidi, A., Rama, N., Datler, C., Studd, J.B., Agarwal, R., Guney, T.G., Gourley, C., Hennessy, B.T., Mills, G.B.*, et al.* (2011b). HDAC4-regulated STAT1 activation mediates platinum resistance in ovarian cancer. Cancer Res *71*, 4412-4422.

Sugo, N., Oshiro, H., Takemura, M., Kobayashi, T., Kohno, Y., Uesaka, N., Song, W.J., and Yamamoto, N. (2010). Nucleocytoplasmic translocation of HDAC9 regulates gene expression and dendritic growth in developing cortical neurons. Eur J Neurosci *31*, 1521-1532.

Sun, G., Yu, R.T., Evans, R.M., and Shi, Y. (2007). Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. Proc Natl Acad Sci U S A *104*, 15282-15287.

Sun, Y., Liu, P.Y., Scarlett, C.J., Malyukova, A., Liu, B., Marshall, G.M., MacKenzie, K.L., Biankin, A.V., and Liu, T. (2014). Histone deacetylase 5 blocks neuroblastoma cell differentiation by interacting with N-Myc. Oncogene *33*, 2987-2994.

Tang, H.B., Macpherson, P., Marvin, M., Meadows, E., Klein, W.H., Yang, X.J., and Goldman, D. (2009). A Histone Deacetylase 4/Myogenin Positive Feedback Loop Coordinates Denervation-dependent Gene Induction and Suppression. Mol Biol Cell *20*, 1120-1131.

Taniguchi, M., Carreira, M.B., Smith, L.N., Zirlin, B.C., Neve, R.L., and Cowan, C.W. (2012). Histone deacetylase 5 limits cocaine reward through cAMP-induced nuclear import. Neuron *73*, 108-120.

Tao, R., de Zoeten, E.F., Ozkaynak, E., Chen, C., Wang, L., Porrett, P.M., Li, B., Turka, L.A., Olson, E.N., Greene, M.I., *et al.* (2007). Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med *13*, 1299-1307.

Tuddenham, L., Wheeler, G., Ntounia-Fousara, S., Waters, J., Hajihosseini, M.K., Clark, I., and Dalmay, T. (2006). The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. Febs Letters *580*, 4214-4217.

van der Linden, A.M., Nolan, K.M., and Sengupta, P. (2007). KIN-29 SIK regulates chemoreceptor gene expression via an MEF2 transcription factor and a class II HDAC. EMBO J *26*, 358-370.

Vannini, A., Volpari, C., Filocamo, G., Casavola, E.C., Brunetti, M., Renzoni, D., Chakravarty, P., Paolini, C., De Francesco, R., Gallinari, P., *et al.* (2004). Crystal structure of a eukaryotic zinc-dependent histone deacetylase, human HDAC8, complexed with a hydroxamic acid inhibitor. Proc Natl Acad Sci U S A *101*, 15064-15069.

Vega, R.B., Matsuda, K., Oh, J., Barbosa, A.C., Yang, X.L., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J.M., Richardson, J.A., *et al.* (2004). Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell *119*, 555-566.

Waddington, C.H. (1959). Canalization of development and genetic assimilation of acquired characters. Nature *183*, 1654-1655.

Walkinshaw, D.R., Weist, R., Kim, G.W., You, L., Xiao, L., Nie, J., Li, C.S., Zhao, S., Xu, M., and Yang, X.J. (2013). The tumor suppressor kinase LKB1 activates the downstream kinases SIK2 and SIK3 to stimulate nuclear export of class IIa histone deacetylases. J Biol Chem *288*, 9345-9362.

Wang, A.H., Bertos, N.R., Vezmar, M., Pelletier, N., Crosato, M., Heng, H.H., Th'ng, J., Han, J., and Yang, X.J. (1999). HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. Mol Cell Biol *19*, 7816-7827.

Wang, A.H., Kruhlak, M.J., Wu, J., Bertos, N.R., Vezmar, M., Posner, B.I., Bazett-Jones, D.P., and Yang, X.J. (2000). Regulation of histone deacetylase 4 by binding of 14-3-3 proteins. Mol Cell Biol *20*, 6904-6912.

Wang, B., Moya, N., Niessen, S., Hoover, H., Mihaylova, M.M., Shaw, R.J., Yates, J.R., 3rd, Fischer, W.H., Thomas, J.B., and Montminy, M. (2011). A hormone-dependent module regulating energy balance. Cell *145*, 596-606.

Wang, S.S., Li, X.M., Parra, M., Verdin, E., Bassel-Duby, R., and Olson, E.N. (2008). Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7. P Natl Acad Sci USA *105*, 7738-7743.

Wang, Z., Qin, G., and Zhao, T.C. (2014). HDAC4: mechanism of regulation and biological functions. Epigenomics *6*, 139-150.

Weichert, W. (2009). HDAC expression and clinical prognosis in human malignancies. Cancer Lett *280*, 168-176.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Wen, W., Ding, J., Sun, W., Wu, K., Ning, B., Gong, W., He, G., Huang, S., Ding, X., Yin, P.*, et al.* (2010). Suppression of cyclin D1 by hypoxia-inducible factor-1 via direct mechanism inhibits the proliferation and 5-fluorouracil-induced apoptosis of A549 cells. Cancer Res *70*, 2010-2019.

Williams, A.H., Valdez, G., Moresi, V., Qi, X.X., McAnally, J., Elliott, J.L., Bassel-Duby, R., Sanes, J.R., and Olson, E.N. (2009). MicroRNA-206 Delays ALS Progression and Promotes Regeneration of Neuromuscular Synapses in Mice. Science *326*, 1549-1554.

Wilson, A.J., Byun, D.S., Nasser, S., Murray, L.B., Ayyanar, K., Arango, D., Figueroa, M., Melnick, A., Kao, G.D., Augenlicht, L.H.*, et al.* (2008). HDAC4 promotes growth of colon cancer cells via repression of p21. Mol Biol Cell *19*, 4062-4075.

Winbanks, C.E., Wang, B., Beyer, C., Koh, P., White, L., Kantharidis, P., and Gregorevic, P. (2011). TGF-beta Regulates miR-206 and miR-29 to Control Myogenic Differentiation through Regulation of HDAC4. Journal of Biological Chemistry *286*, 13805-13814.

Wu, M.Y., Fu, J.J., Xiao, X.L., Wu, J.B., and Wu, R.C. (2014). MiR-34a regulates therapy resistance by targeting HDAC1 and HDAC7 in breast cancer. Cancer Lett *354*, 311-319.

Wuelling, M., Pasdziernik, M., Moll, C.N., Thiesen, A.M., Schneider, S., Johannes, C., and Vortkamp, A. (2013). The multi zinc-finger protein Trps1 acts as a regulator of histone deacetylation during mitosis. Cell Cycle *12*, 2219-2232.

Yang, X.J., and Seto, E. (2007). HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene *26*, 5310-5318.

Yang, X.J., and Seto, E. (2008). The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol *9*, 206-218.

Yoshida, C.A., Yamamoto, H., Fujita, T., Furuichi, T., Ito, K., Inoue, K.I., Yamana, K., Zanma, A., Takada, K., Ito, Y.*, et al.* (2004). Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Gene Dev *18*, 952-963.

Yu, S.L., Lee, D.C., Son, J.W., Park, C.G., Lee, H.Y., and Kang, J. (2013). Histone deacetylase 4 mediates SMAD family member 4 deacetylation and induces 5-fluorouracil resistance in breast cancer cells. Oncol Rep *30*, 1293-1300.

Yuan, J.H., Yang, F., Chen, B.F., Lu, Z., Huo, X.S., Zhou, W.P., Wang, F., and Sun, S.H. (2011a). The histone deacetylase 4/SP1/microrna-200a regulatory network contributes to aberrant histone acetylation in hepatocellular carcinoma. Hepatology *54*, 2025-2035.

Yuan, J.H., Yang, F., Chen, B.F., Lu, Z., Huo, X.S., Zhou, W.P., Wang, F., and Sun, S.H. (2011b). The Histone Deacetylase 4/Sp1/microRNA-200a Regulatory Network Contributes to Aberrant Histone Acetylation in Hepatocellular Carcinoma. Hepatology *54*, 2025-2035.

Zelzer, E., Glotzer, D.J., Hartmann, C., Thomas, D., Fukai, N., Soker, S., and Olsen, B.R. (2001). Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. Mech Develop *106*, 97-106.

Zhang, C.L., McKinsey, T.A., Chang, S.R., Antos, C.L., Hill, J.A., and Olson, E.N. (2002a). Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell *110*, 479-488.

Zhang, C.L., McKinsey, T.A., Lu, J.R., and Olson, E.N. (2001). Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. Journal of Biological Chemistry *276*, 35-39.

Zhang, C.L., McKinsey, T.A., and Olson, E.N. (2002b). Association of class II histone deacetylases with heterochromatin protein 1: Potential role for histone methylation in control of muscle differentiation. Molecular and Cellular Biology *22*, 7302-7312.

Zhang, L., Jin, M., Margariti, A., Wang, G., Luo, Z.L., Zampetaki, A., Zeng, L.F., Ye, S., Zhu, J.H., and Xiao, Q.H. (2010). Sp1-dependent Activation of HDAC7 Is Required for Platelet-derived Growth Factor-BB-induced Smooth Muscle Cell Differentiation from Stem Cells. Journal of Biological Chemistry *285*, 38463-38472.

Zhang, Y., Fang, H., Jiao, J., and Xu, W. (2008). The structure and function of histone deacetylases: the target for anti-cancer therapy. Curr Med Chem *15*, 2840-2849.

Zheng, Q.P., Zhou, G., Morello, R., Chen, Y.Q., Garcia-Rojas, X., and Lee, B. (2003). Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. Journal of Cell Biology *162*, 833-842.

Zhou, B., Margariti, A., Zeng, L., Habi, O., Xiao, Q., Martin, D., Wang, G., Hu, Y., Wang, X., and Xu, Q. (2011). Splicing of histone deacetylase 7 modulates smooth muscle cell proliferation and neointima formation through nuclear beta-catenin translocation. Arterioscler Thromb Vasc Biol *31*, 2676-2684.

Zhou, L., Lopes, J.E., Chong, M.M.W., Ivanov, I.I., Min, R., Victora, G.D., Shen, Y.L., Du, J.G., Rubtsov, Y.P., Rudensky, A.Y.*, et al.* (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing ROR gamma t function. Nature *453*, 236-U214.

Zhou, X., Richon, V.M., Wang, A.H., Yang, X.J., Rifkind, R.A., and Marks, P.A. (2000). Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras. Proc Natl Acad Sci U S A *97*, 14329-14333.

Zhu, C., Chen, Q., Xie, Z., Ai, J., Tong, L., Ding, J., and Geng, M. (2011a). The role of histone deacetylase 7 (HDAC7) in cancer cell proliferation: regulation on c-Myc. J Mol Med (Berl) *89*, 279-289.

Zhu, J., Shimizu, E., Zhang, X., Partridge, N.C., and Qin, L. (2011b). EGFR signaling suppresses osteoblast differentiation and inhibits expression of master osteoblastic transcription factors Runx2 and Osterix. J Cell Biochem *112*, 1749-176

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# The Control Operated by the Cell Cycle Machinery on MEF2 Stability Contributes to the Downregulation of CDKN1A and Entry into S Phase

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**MEF2s are pleiotropic transcription factors (TFs) which supervise multiple cellular activities. During the cell cycle, MEF2s are activated at the $G_0/G_1$ transition to orchestrate the expression of the immediate early genes in response to growth factor stimulation. Here we show that, in human and murine fibroblasts, MEF2 activities are downregulated during late $G_1$. MEF2C and MEF2D interact with the E3 ligase F-box protein SKP2, which mediates their subsequent degradation through the ubiquitin-proteasome system. The cyclin-dependent kinase 4 (CDK4)/cyclin D1 complex phosphorylates MEF2D on serine residues 98 and 110, and phosphorylation of these residues is an important determinant for SKP2 binding. Unscheduled MEF2 transcription during the cell cycle reduces cell proliferation, whereas its containment sustains DNA replication. The CDK inhibitor p21/CDKN1A gene is a MEF2 target gene required to exert this antiproliferative influence. MEF2C and MEF2D bind a region within the first intron of *CDKN1A*, presenting epigenetic markers of open chromatin. Importantly, H3K27 acetylation within this regulative region depends on the presence of MEF2D. We propose that following the initial engagement in the $G_0/G_1$ transition, MEF2C and MEF2D must be polyubiquitylated and degraded during $G_1$ progression to diminish the transcription of the *CDKN1A* gene, thus favoring entry into S phase.**

In vertebrates, the family of MEF2s comprises 4 members—MEF2A, -B, -C, and -D—as well as some splicing variants (1). Common features of all MEF2 members are the MADS box (MCM1, agamous, deficiens, serum response factor) and the adjacent MEF2 domain positioned within the highly conserved amino-terminal region (1). These domains are involved in recognizing the YTA(A/T)$_4$TAR DNA motif, in mediating the formation of homo- and heterodimers, and in the interaction with different cofactors (1). The carboxy-terminal half is much less conserved. It encompasses the transactivation domains and the nuclear localization signal (2). The different family members exhibit specific but also overlapping patterns of expression, during either embryogenesis or adult life (1, 3). MEF2s are subjected to intense supervision by environmental signals, in order to couple the gene expression signature to the organism requirements (1). MEF2s oversee the expression of several genes, depending on and in cooperation with other transcription factors (TFs) (3, 4). In addition, MEF2s can also operate as repressors of transcription when in complexes with class IIa histone deacetylases (HDACs) (5, 6, 7, 8).

The extent of genes under the influence of MEF2s justifies the pleiotropic activities and the assorted cellular responses attributed to these TFs. During development, in general, expression of MEF2 is linked to the activation of differentiation programs (1). In various scenarios, the onset of MEF2 expression coincides with the withdrawal from the cell cycle (9). Specific ablation of MEF2C in neural/progenitor cells impacts differentiation but not their survival or proliferation (10). Also, in muscle, simultaneous ablation of different MEF2s impacts differentiation of satellite cell-derived myoblasts but does not alter proliferation (11).

In oncogene-transformed fibroblasts, induction of MEF2 transcription can trigger antiproliferative responses, which are responsible for reverting the tumorigenic phenotype (7). In other contexts, MEF2s seem to be involved in sustaining rather than inhibiting cell proliferation (12). During the cell cycle, MEF2 transcriptional activities are upregulated when quiescent cells are stimulated to re-enter $G_1$ (13). Here, they contribute to the expression of the immediate early genes in response to serum (14, 15). Paradoxically, signaling pathways elicited by growth factors, and in particular, the phosphoinositol 3-kinase (PI3K)/Akt pathway can also repress MEF2-dependent transcription (7). This repression is exerted mainly through the ubiquitin-dependent degradation of the TFs (7).

Overall, these results suggest that, during different proliferative stages, MEF2 transcriptional activities could be subjected to multiple and complex adaptations. To better understand the contribution of MEF2s to the regulation of cell growth, in this study we investigated MEF2C and MEF2D expression, regulation, and activities during distinct phases of the cell cycle, using murine and human fibroblasts as cellular models.

## MATERIALS AND METHODS

**Cell cultures and reagents.** BJ/TERT cells were cultured in Earle's salts minimal essential medium (EMEM) (HyClone) completed with nones-

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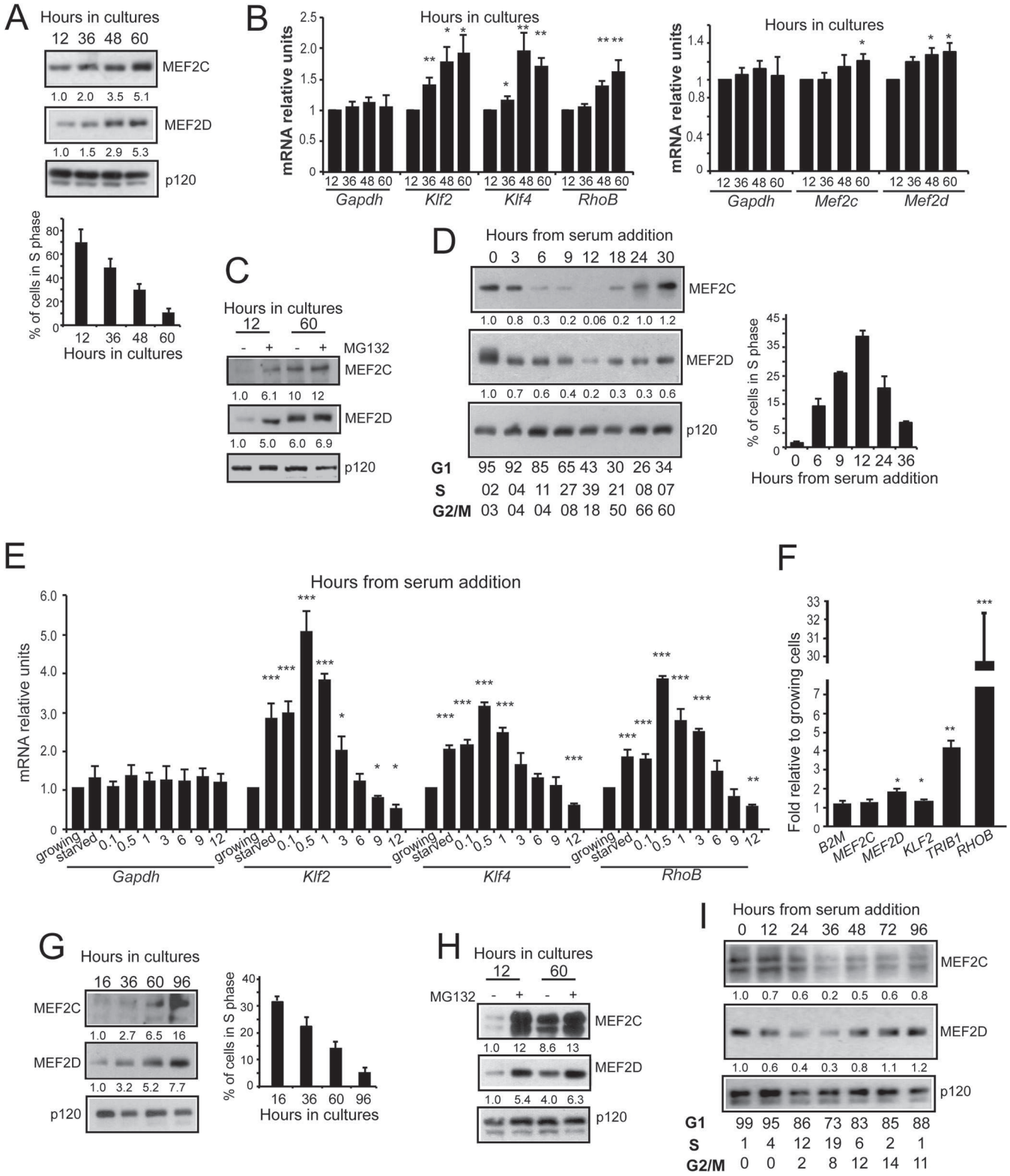



**FIG 1** MEF2C and MEF2D levels are regulated during the cell cycle. (A) Immunoblot and densitometric analysis of MEF2 family members in NIH 3T3 cells grown for the indicated times in 10% FCS. The fraction of cells synthetizing DNA was scored after BrdU staining. p120 was used as a loading control. (B) mRNA expression levels of three MEF2 target genes (*Klf2*, *Klf4*, and *RhoB*), *Mef2c*, and *Mef2d* in NIH 3T3 cells grown for the indicated times in 10% FCS. mRNA levels are relative to the first time point (12 h). *Gapdh* was used as a control gene. Data are means and SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$. (C) Immunoblot and densitometric analysis of MEF2C and MEF2D levels in NIH 3T3 cells grown for the indicated times in 10% FCS and treated for 10 h with MG132 or not treated, as indicated. p120 was used as a loading control. (D) Immunoblot and densitometric analysis of MEF2C and MEF2D in NIH 3T3 cells, reintroduced into the growth cycle with 10% FBS, after serum starvation for 48 h. Cellular lysates were collected at the indicated time points. Cytofluorimetric analysis of cell cycle

sential amino acids (NEAA; HyClone). All other cell lines were grown in Dulbecco modified Eagle medium (DMEM; Lonza). All media were supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Lonza). Cells expressing the inducible form of MEF2 were grown in DMEM (Sigma-Aldrich)/EMEM (Life Technologies) without phenol red. For analyses of cell growth, $10^4$ cells were seeded, and the medium was changed every 2 days.

The following chemicals were used: 20 μM LY294002 (LY), 10 μM PD9800591, 0.5 μM okadaic acid (LC Laboratories); 2.5 μM MG132, 1 μM 4-hydroxytamoxifen (4-OHT), 10 μg/ml cycloheximide (CHX), 5 μM roscovitine, 3 μM PD0332991, 1 μM p38i IV, 1 μM staurosporine, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 100 nM microcystin L1, 50 μM ATP, protease inhibitor cocktail (PIC), and dimethyl sulfoxide (DMSO) (all from Sigma-Aldrich); 100 nM Torin1 (Cayman); and 20 μM SKP2in [3-(1,3-benzothiazol-2-yl)-6-ethyl-7-hydroxy-8-(piperidin-1-ylmethyl)-4H-chromen-4-one] (UkrOrgSyntez Ltd.).

**Plasmid construction, transfections, retroviral/lentiviral infections, and silencing.** The pEGFPC2, pFLAG CMV5a, and pGEX-4T1 constructs expressing *MEF2C*, *MEF2D*, and *SKP2* were generated by PCR and subsequent cloning, using EcoRI/SalI restriction sites (NEB). Phosphodefective (Ser-Thr/Ala) and phosphomimicking (Ser/Asp) MEF2D mutants were generated using a Stratagene QuikChange Lightning kit (Agilent). The *MEF2D* and *MEF2D* S98A S110A deletion mutants were generated by PCR and cloned into pEGFPC2 and pGEX-4T1 plasmids. pWZL-Hygro-*MEF2-VP16-ER*, pWZL-Hygro-*MEF-ΔDBD-VP16-ER*, pWZL-Neo-*p53DN* (175H) were previously described (7). To generate pWZL-Hygro and pBABE-Puro plasmids expressing SKP2, SKP2DN, SKP2ΔDD (lacking the first 8 amino acids of the destruction domain), MEF2D-FLAG, and MEF2D-S98A/S110A, the relative cDNAs were subcloned into pWZL-Hygro and pBABE-Puro plasmids using the PCR method. The fidelity of all generated plasmids was verified by DNA sequencing.

pLKO plasmids (15897 and 274054, referred to here as 15 and 27) expressing short hairpin RNAs (shRNAs) directed against *MEF2D* were obtained from Sigma-Aldrich. For retroviral infection, HEK293 Ampho Phoenix cells were transfected with 12 μg of plasmid DNA. After 48 h at 32°C, virions were collected and diluted as appropriate to get the same multiplicity of infection (MOI) for all genes. For lentivirus-based knockdown, HEK-293 cells were transfected with 5 μg of VSV-G, 15 μg of Δ8.9, and 20 μg of pLKO plasmids. After 36 h at 37°C, virions were collected and opportunely diluted in fresh medium. Unless otherwise specified, all transfection experiments in 293 and IMR90-E1A cells were performed with a standard calcium phosphate method. Silencing of BJ/TERT and BJ/TERT/p53DN was performed with 73 nmol of *SKP2* small interfering RNAs (siRNAs) (GGUAUCGCCUAGCGUCUGA; Invitrogen) and 56 nmol of *CDKN1A* siRNAs (AGACCAGCAUGACAGAUUU; Qiagen).

**Production of recombinant proteins and immunoblots.** pGEX plasmids expressing wild-type MEF2D with amino acids 1 to 190 deleted, MEF2D S98A/S110A, MEF2D S98D/S110D, full-length SKP2, and Rb with amino acids 379 to 928 deleted (16) were transformed in BL-21 bacterial cells. Recombinant protein expression was induced with 1 mM IPTG at 30°C for 30 min, and proteins were purified using glutathione-Sepharose beads (GE Healthcare). Immunoblots were performed as previously described (17), and relative quantitative measurements were achieved by densitometric analysis of Western blot films, normalized to the corresponding p120 or p62/nucleoporin or CRADD (loading controls) values. Each immunoblot experiment was repeated at least twice with similar results, and each densitometric analysis refers to the figures.

**Immunoprecipitation and glutathione *S*-transferase (GST) pulldown.** Cells were lysed in a hypotonic buffer (20 mM Tris-HCl, pH 7.5; 2 mM EDTA; 10 mM $MgCl_2$; 10 mM KCl; and 1% Triton X-100) supplemented with protease inhibitors. For each immunoprecipitation 1 μg of antibody was used.

Portions (2 μg each) of recombinant proteins were used as baits in each pulldown experiment. MEF2D-green fluorescent protein (GFP) and SKP2-GFP were obtained from transfected HEK-293 cells, lysed with a hypertonic buffer containing 300 mM NaCl in order to destroy the complexes as much as possible. Pulldown was conducted at 4°C with rotation for 2 h.

**Antibodies.** Antibodies used were those against MEF2C C-17 (sc13268; Santa Cruz Biotechnology), VP16 (Santa Cruz Biotechnology), MEF2C CB (raised against a bacterially produced segment of MEF2C [amino acids 341 to 473]), MEF2D (BD Bioscience), extracellular signal-regulated kinase (ERK), phosphorylated ERK (pERK), AKT, pAKT (Ser473), RAN, nucleoporin p62, p120 (Cell Signaling), SKP2-8D9 (Life Technologies), p21 CP74 and FLAG M2 (Sigma-Aldrich), GFP (17), CRADD (18), ubiquitin (Covance), and H3K27ac (ab4729; Abcam).

**RNA extraction and quantitative qRT-PCR.** Cells were lysed using Tri-Reagent (Molecular Research Center). A total of 1 μg of total RNA was retrotranscribed by using 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) analyses were performed using Bio-Rad CFX96 and SYBR green technology (Resnova). The data were analyzed by use of a comparative threshold cycle using the $\beta_2$ microglobulin gene and *HPRT* (encoding hypoxanthine phosphoribosyltransferase) as normalizer genes. All reactions were done in triplicate.

**Cell cycle FACS analysis and BrdU assay.** For synchronization in $G_0/G_1$, NIH 3T3 cells and BJ/TERT cells were serum starved for 48 and 72 h, respectively, and then reactivated by addition of fetal calf serum (FCS). For fluorescence-activated cell sorting (FACS) analysis, cells were fixed with ethanol (overnight), treated with 10 μg RNase A (Applichem Lifescience), and stained with 10 mg propidium iodide (Sigma-Aldrich). Data were analyzed with Flowing software (http://www.flowingsoftware.com/). For S-phase analysis, cells were grown for 3 h with 50 μM bromodeoxyuridine (BrdU). After fixation, coverslips were treated with HCl. Mouse anti-BrdU (Sigma) was used as the primary antibody. Nuclei were stained with Hoechst 33258 (Sigma).

***In vitro* phosphorylation studies.** Cellular lysates from 2.5 million NIH 3T3 cells for each time point were obtained. Cells were lysed for 10 min in native buffer (10 mM HEPES [pH 7.4], 0.1% Triton, 20 mM $MgCl_2$, 1 mM MnCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], PIC, 10 mM NaFl, 5 mM $NaVO_4$, 0.5 μM okadaic acid, 100 nM microcystin L1). Two micrograms of GST fusion proteins bound to resin in GST-binding buffer (50 mM Tris [pH 7.4], 140 mM NaCl) was next added. The kinase reaction was carried out by incubating for 1 h at 30°C the glutathione-bound proteins with cellular lysates supplemented with 50 μM ATP and 1 μCi γ-ATP (Perkin-Elmer). After several washes, sample buffer was

parameters is provided in the lower panel. BrdU positivity is shown in the histogram. (E) mRNA expression levels of three MEF2 target genes (*Klf2*, *Klf4*, and *RhoB*) in NIH 3T3 cells collected 12 h after seeding (growing) or grown for an additional 48 h in 0.5% FBS (starved) and then reintroduced into the growth cycle for the indicated times. mRNA levels are relative to growing condition. *Gapdh* was used as a control. Data are means and SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. (F) mRNA expression levels of three MEF2 target genes (*KLF2*, *TRIB1*, and *RHOB*), *MEF2C*, and *MEF2D* in growing BJ/TERT cells (16 h) compared to density-arrested cells (96 h). mRNA levels are relative to the first time point (16 h). The $\beta_2$ microglobulin gene was used as a control gene. Data are means and SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. (G) Immunoblot and densitometric analysis of MEF2 family members in human BJ/TERT cells. Cellular lysates were collected at different times after seeding, as indicated. The fraction of cells synthetizing DNA was scored after BrdU staining. p120 was used as a loading control. (H) Immunoblot and densitometric analysis of MEF2 family members in BJ/TERT cells collected at different times after seeding and treated with the proteasome inhibitor MG132, as indicated. p120 was used as a loading control. (I) Immunoblot and densitometric analysis of MEF2 family members in BJ/TERT cells, starved for 72 h, reactivated with 10% FBS, and collected at different times after reactivation, as indicated. Cell cycle analysis results are provided in the lower panel.

added to the beads. When the recombinant cyclin-dependent kinases (CDKs) were used, 250 ng of cyclin D1/CDK4 or cyclin E1/CDK2 (Sigma-Aldrich) was incubated with GST-MEF2D proteins.

**Chromatin immunoprecipitation and promoter study.** The sequence of the *CDKN1A*-proximal promoter (10 kb upstream and 10 kb downstream from the transcription start site [TSS]) was recovered from ENCODE. The presence of a putative MEF2 binding site was predicted using CisterZlab (http://zlab.bu.edu/~mfrith/cister.shtml) and JASPAR (http://jaspar.binf.ku.dk/) algorithms.

For each chromatin immunoprecipitation (ChIP), $2.5 \times 10^6$ cells were used and ChIP was performed as previously described (7). Anti-MEF2C (CB), anti-MEF2C C-17, anti-MEF2D, anti-H3K27ac, and anti-FLAG M2 antibodies were used, and preimmune serum was used as an unrelated control.

**Statistics.** For experimental data, a Student *t* test was used. A *P* value of 0.05 was chosen as the statistical limit of significance. Unless otherwise indicated, data in the figures are arithmetic means and standard deviations from at least three independent experiments.

## RESULTS

**MEF2C and MEF2D protein stability is regulated during the cell cycle.** We recently showed that suppression of the PI3K/Akt pathway elicits the upregulation of MEF2C and MEF2D expression (7). This upregulation is mediated by the stabilization of MEF2 proteins, because of a reduced targeting to the ubiquitin-proteasome system (UPS). Regulation of MEF2 protein half-life could be a general phenomenon, linked not to PI3K-induced transformation alone but rather to distinct proliferative states of the cells. To explore this hypothesis, we decided to investigate the regulation of MEF2s during different proliferative conditions in untransformed cells. NIH 3T3 fibroblasts were selected for these studies, and we initially assessed MEF2 levels during growth arrest, as induced by density-dependent inhibition. Figure 1A illustrates that MEF2C and MEF2D levels increase when cells exit the cell cycle. Densitometric analysis further proved this upregulation. Analysis of BrdU incorporation confirmed entry into the quiescence state following contact inhibition. In parallel, levels of mRNAs of MEF2 target genes (*Klf2*, *Klf4*, and *RhoB*), including *Mef2c* and *Mef2d* themselves, rise during density-dependent growth inhibition (Fig. 1B). Experiments using MG132 proved that the UPS plays a key role in the control of MEF2s levels under the different growth conditions. Blocking the proteasome-mediated degradation efficiently augmented MEF2C and MEF2D levels only in growing cells (Fig. 1C). Finally, when $G_0$ serum-deprived cells were restimulated to grow, by addition of 10% FCS, MEF2C and MEF2D levels decreased as cells entered the S phase (Fig. 1D).

Previous studies demonstrated that MEF2s are engaged in the transcription of serum-induced immediate early genes (13, 14, 15). Hence, we decided to follow the expression levels of MEF2 target genes after serum stimulation of quiescent cells. The time course analysis (Fig. 1E) confirmed that at early times after serum addition, expression of these genes is augmented. Also, *Mef2c* and *Mef2d* mRNAs were upregulated, but at a very modest level (data not shown). However, these upregulations were transient, and 3 h after stimulation for *Klf2* and *Klf4*, or 6 h in the case of *RhoB*, mRNA levels were reduced compared to those in quiescent cells. These results are in agreement with the described downregulation of MEF2C and MEF2D proteins occurring during late $G_1$/S phase (Fig. 1D). Interestingly after 12 h of stimulation, when cells are mainly in S phase, expression of the MEF2 targets was significantly reduced compared to exponentially growing cells.

To confirm our observations, we also investigated the regulation of *MEF2C* and *MEF2D* genes during the cell cycle in human fibroblasts. Immortalized BJ cells expressing *TERT* gene were arrested in a density-dependent manner. Figure 1F shows that MEF2 target genes, in particular *TRIB1* and *RHOB*, were induced following density-dependent inhibition. Immunoblot analysis confirmed that MEF2C and MEF2D levels increase during growth arrest (Fig. 1G). The strong discrepancy between the changes in RNA and protein levels of MEF2C and MEF2D (compare Fig. 1F and G) further indicates the involvement of the UPS. In fact, as reported for murine fibroblasts, proteasomal inhibition increased MEF2C and MEF2D levels in growing cells, but it had a lower impact on density-arrested cells (Fig. 1H). Also in human fibroblasts, reintroduction of serum-deprived cells into the growth cycle was coupled to an S-phase-mediated MEF2C and MEF2D downregulation (Fig. 1I).

**SKP2 regulates MEF2C and MEF2D stability.** A key point of MEF2C and MEF2D regulation during the cell cycle is their targeting of the UPS. To identify the ubiquitin E3 ligase involved in such a task, we compared gene expression profiles of growing versus quiescent cells, as well as of cells transformed with RAS and PI3K oncogenes versus the normal counterpart. All conditions were marked by a decreased half-life of MEF2 proteins (7) (data not shown). Among the ubiquitin E3 ligases upregulated in both transformed and growing cells, we focused our attention on *SKP2* (S-phase kinase-associated protein 2), also known as *FBXL1* (19). qRT-PCR analysis revealed that expression levels of *Skp2* inversely correlate with the MEF2 target genes (*Klf2*, *Klf4*, and *RhoB*), during density-dependent inhibition and in RAS- or PI3K-transformed murine fibroblasts (Fig. 2A). Furthermore, analysis of publicly available gene expression profiles in different tumors revealed a significant inverse correlation between the expression of MEF2 target genes and *SKP2* in soft tissue sarcomas, gastric cancer, metastatic skin carcinoma, metastatic melanoma, and acute lymphoblastic leukemia (see Fig. S1 in the supplemental material).

To prove the relationships between SKP2 and MEF2s, we performed coexpression studies in human fibroblasts expressing the E1A oncogene. The amount of ectopically expressed MEF2C-GFP (Fig. 2B) was dramatically reduced in the presence of coexpressed SKP2, and proteasomal inhibition recovered its levels. Similarly, MEF2D-GFP levels were downregulated by the simultaneous coexpression of SKP2. Moreover, a deletion-containing version of the E3 ligase acting as dominant negative (ΔF box) (20) efficiently rescued MEF2D-GFP levels (Fig. 2C).

We next investigated whether MEF2D could interact with SKP2. MEF2D was selected for this analysis because of its higher expression, compared to MEF2C, in fibroblasts (21). After coimmunoprecipitation, a complex between endogenous MEF2D and SKP2 was purified from cells expressing SKP2-GFP, and the amount of MEF2D bound to SKP2 was dramatically increased following MG132 treatment (Fig. 2D). MEF2D-GFP expressed in 293 cells was polyubiquitylated, and coexpression with SKP2 increased this polyubiquitylation, whereas SKP2DN reduced it (Fig. 2E). When the dominant negative version of SKP2 was stably expressed in NIH 3T3 cells, levels of MEF2C and MEF2D proteins increased. In contrast, introduction of a hyperactive version of SKP2, SKP2ΔDD (22), caused a dramatic reduction of both MEF2C and MEF2D levels (Fig. 2F).

When NIH 3T3 cells are challenged with MG132 or with the

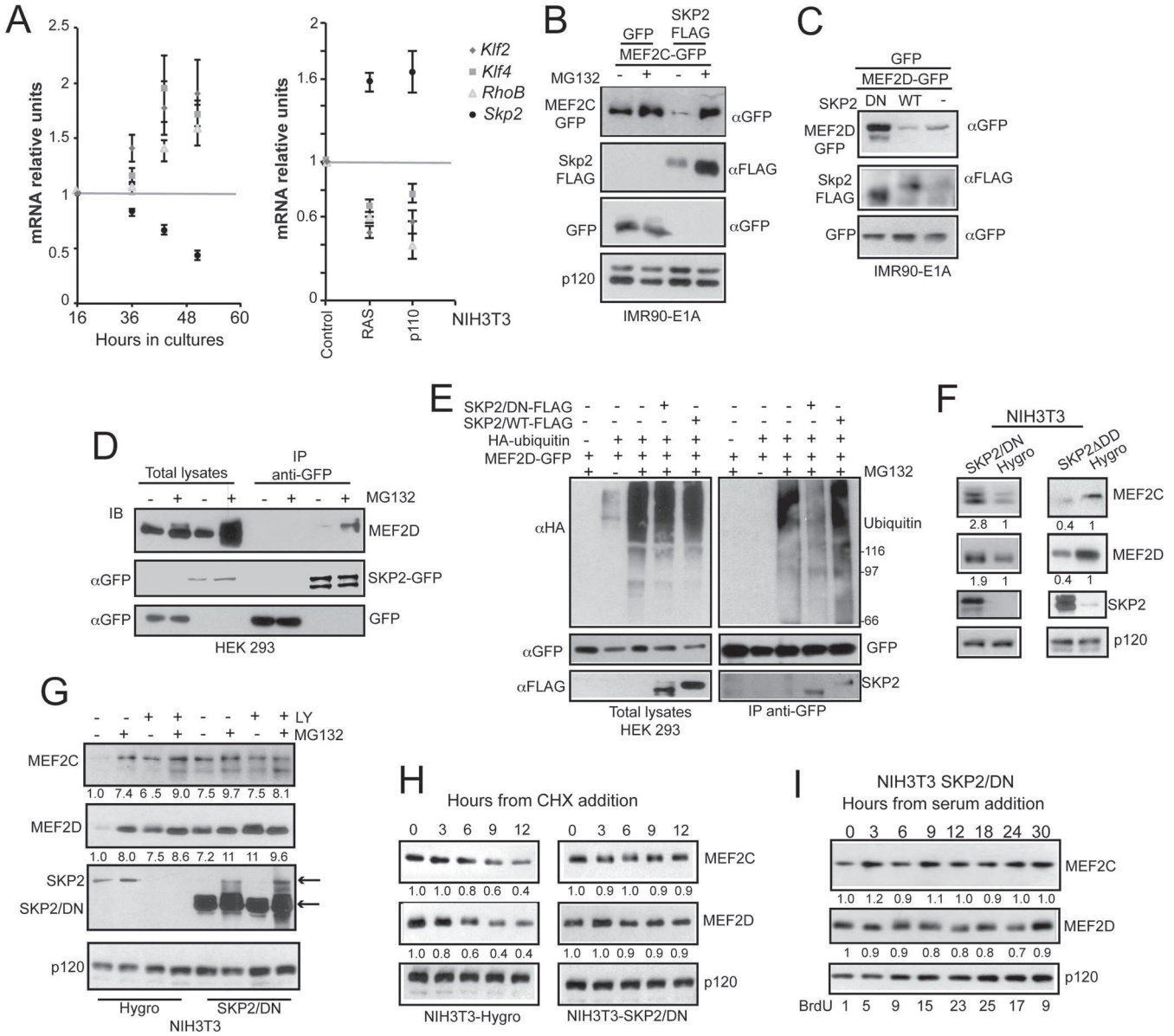



**FIG 2** SKP2 binds and mediates the ubiquitylation of MEF2C/D. (A) mRNA expression levels of MEF2 target genes (*Klf2*, *Klf4*, and *RhoB*) and of *Skp2* in NIH 3T3 cells grown for the indicated times in 10% FBS or expressing p110-CAAX and H-RAS. The scheme highlights the inverse correlation between MEF2 target expression levels and *Skp2*. (B) IMR90-E1A cells were transfected with pEGFP-N1-*MEF2C* (1 μg) and 2 μg of pFLAG-CMV5a *SKP2* or pFLAG-CMV5a *GFP* as a control. After 24 h, cells were treated with MG132 or left untreated, and after 12 h, cellular lysates were generated and subjected to immunoblot analysis using the indicated antibodies. p120 was used as a loading control. (C) IMR90-E1A cells were transfected with pEGFP-C2-*MEF2D* (1 μg), 2.5 μg of pFLAG-CMV5a-*SKP2*, pFLAG-CMV5a-*SKP2DN*, or empty pFLAG-CMV5a as a control and 200 ng of pEGFP-C2. After 36 h, cellular lysates were generated and subjected to immunoblot analysis using the indicated antibodies. (D) Cellular lysates from HEK-293 cells, transfected with 5 μg of pEGFP-N1-*SKP2* or with empty pEGFP-C2 plasmids and treated for 8 h with 2.5 μM MG132 or left untreated, were immunoprecipitated with an anti-GFP antibody. Immunoblots were performed using the indicated antibodies. (E) HEK-293 cells were cotransfected with the HA-ubiquitin gene (1 μg) and *MEF2D-GFP* (2 μg) or *GFP* and *SKP2-FLAG* or *SKP2DN-FLAG* or an empty plasmid (4 μg). Twenty-four hours later, cells were treated for 8 h with 2.5 μM MG132 or left untreated. GFP fusions were immunoprecipitated using an antibody against GFP and were subjected to immunoblotting using an antiubiquitin antibody. After being stripped, the filter was probed with anti-GFP and anti-FLAG antibodies. Inputs are included. (F) Immunoblot analysis of MEF2 family members and SKP2 in NIH 3T3 cells stably expressing the dominant negative form (DN) or the hyperactive form (ΔDD) of *SKP2* or the control gene (Hygro). Immunoblotting was performed using the indicated antibodies. p120 was used as the loading control. (G) Immunoblot and densitometric analysis of MEF2 family members and SKP2 in NIH 3T3 cells stably expressing the dominant negative form (DN) of SKP2 or the control gene (Hygro) and treated 12 h after the seeding with LY294002 for 24 h and for the last 12 h with MG132, or left untreated, as indicated. Untreated cells were harvested after 36 h from seeding. p120 was used as the loading control. The lower arrow points to ectopically expressed SKP2/DN. The higher arrow points to a band showing the same size of the endogenous SKP2. (H) Immunoblot and densitometric analysis of MEF2 family members in NIH 3T3 cells stably expressing the dominant negative form of *SKP2* (*SKP2DN*) or the control (*HYGRO*) and treated for the indicated times with 10 μg/ml of CHX. (I) Immunoblot and densitometric analysis of MEF2 family members in NIH 3T3 cells stably expressing *SKP2DN*, starved for 48 h, reactivated with 10% FBS, and collected at different times after stimulation, as indicated. BrdU positivity is shown at the bottom.

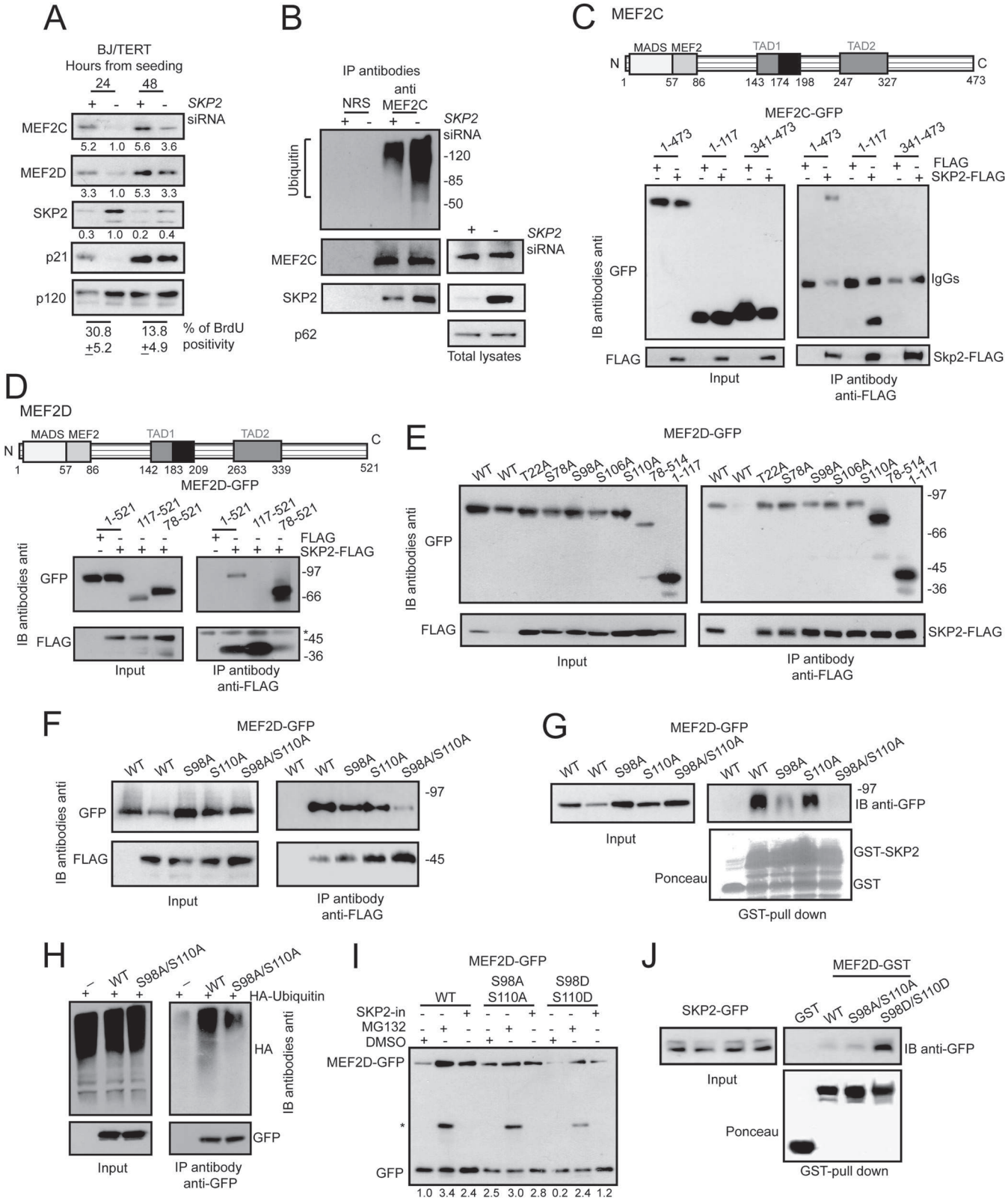



**FIG 3** Mapping of SKP2 binding to MEF2C/D and characterization of SKP2 interference. (A) Immunoblot and densitometric analysis of MEF2 family members SKP2 and p21 in BJ/TERT cells transfected for 36 h with *SKP2* siRNA. Transfections were performed 24 h or 48 h after seeding, as indicated. p120 was used as the loading control. BrdU positivity is shown at the bottom. (B) BJ/TERT cells were transfected with *SKP2* siRNA. After 24 h cells were treated for 8 h with 2.5 μM MG132 or left untreated. MEF2C complexes were immunoprecipitated using an antibody against MEF2C and were subjected to immunoblotting using an antiubiquitin antibody. After being stripped, the filter was probed with an anti-MEF2C antibody and an anti-SKP2 antibody. Inputs and p62 (nucleoporin), as

PI3K inhibitor LY294002 (LY), MEF2C and MEF2D abundance increases (7). In cells expressing SKP2/DN, the levels of the two MEF2s were higher than control levels. Treatment with LY, MG132, or both failed to further increase the quantities of these TFs (Fig. 2G). This result indicates that SKP2 is the crucial E3 ligase engaged by the PI3K pathway to switch off MEF2 activities. To further prove the contribution of SKP2, we also used cycloheximide (CHX). In proliferating cells, a block of protein synthesis elicited a reduction of MEF2C and MEF2D, already appreciable at 6 h from CHX addition. This reduction was abolished in cells expressing SKP2/DN (Fig. 2H). Finally, the downregulation of MEF2C and MEF2D observed in serum-deprived cells stimulated with 10% FCS was also abrogated in SKP2/DN-expressing cells (compare Fig. 2I and 1D). Not surprisingly, these cells exhibited a reduced ability to enter S phase after serum stimulation. In summary, these results indicate that SKP2 is a critical E3 ligase dictating MEF2C and MEF2D protein levels during the cell cycle.

**Molecular determinants of the MEF2-SKP2 interaction.** To further confirm the influence of SKP2 on MEF2 stability, we silenced its expression in human fibroblasts. Downregulation of SKP2 provoked the upregulation of both MEF2C and MEF2D proteins (Fig. 3A). The CDK inhibitor p21, a SKP2 substrate, was used as a positive control (23). Furthermore, we also proved that polyubiquitylation of MEF2C was reduced in SKP2-silenced cells (Fig. 3B).

SKP2 interacts with its substrates in a phosphorylation-dependent manner (19). In order to map the amino acid residues critical for this interaction, we initially performed a simple deletion analysis to circumscribe the region involved. In Fig. 3C and D, schematic representations of MEF2C and MEF2D TFs highlighting their principal domains are shown. Coimmunoprecipitation experiments proved that the region from positions 1 to 117 of MEF2C is sufficient for the interaction with SKP2 (Fig. 3C). In accordance, the carboxy terminus of MEF2D is dispensable for this interaction (Fig. 3D). Having identified in the MEF2 amino-terminal portion the region recognized by SKP2, we next applied *in silico* analysis to locate putative phosphorylation motifs. Again, as explained above, we focused the studies on MEF2D. T22, S78, S98, S106, and S110 of MEF2D (all conserved in MEF2C) resulted in the highest score as putative consensus phosphoacceptor sites.

Single phosphodead substitutions of MEF2D were generated, and the binding to SKP2 was tested after cotransfection of the relative cDNAs in 293 cells. Neither the Ala/Ser nor the Ala/Thr substitution in MEF2D abrogated the binding to SKP2. However, a slightly reduced interaction was observed when serine 98 and 110 were replaced with alanine (Fig. 3E). We next generated MEF2D with double dephosphomimetic substitutions. Simultaneous mutations of Ser 98 and 110 to Ala dramatically reduced the binding of MEF2D to SKP2 in 293 cells (Fig. 3F). Then, we used a GST pulldown assay to confirm the importance of Ser 98 and 110 for the interaction with SKP2. In the pulldown assay, the single substitution S98A diminished the binding to SKP2, whereas the double mutation S98A/S110A completely abrogated it (Fig. 3G). We also proved that polyubiquitylation of the S98A/S110A double mutant was largely compromised but not totally suppressed compared to that of the wild-type (Fig. 3H). Furthermore, MG132 and the SKP2 inhibitor increased the amounts of MEF2D-GFP and of the phosphomimetic double mutant (S98D/S110D) but not that of the S98A/S110A double mutant (Fig. 3I). Finally, the GST pulldown assay established that the interaction between SKP2 and the recombinant MEF2D was dramatically improved in the case of the phosphomimetic double mutant (Fig. 3J). These results indicate that phosphorylation of serines 98 and 110 plays an important role in the control of MEF2D stability, by mediating the interaction with SKP2.

**MEF2D is a substrate of CDK4.** To identify the kinases responsible for MEF2D phosphorylation on serines 98 and 110, we initially arranged an *in vitro* phosphorylation assay using crude cellular extracts from NIH 3T3 cells as a source of kinase activities. When a MEF2D-GST fusion protein comprising amino acids 1 to 190 was incubated with these cellular extracts in the presence of radiolabeled γ-ATP, it was phosphorylated (Fig. 4A). MEF2D-GST phosphorylation was augmented when extracts were obtained from cells in the $G_1$ phase compared to quiescent $G_0$ cells. Coomassie blue gel staining verified the amount of recombinant protein loaded. Under the same experimental conditions, GST alone was not phosphorylated. When the MEF2D-S98A/S110A double mutant was used, phosphorylation was reduced but not abrogated, thus indicating that the two serine residues are targets of some kinase and that additional amino acids can be phosphorylated *in vitro* (Fig. 4A). Since S98 and S110 share consensus phosphorylation sequences for several kinases (ERKs, mTOR, p38, CDK2, and cyclin-dependent kinase 4 [CDK4]), we tested whether the relative specific inhibitors could influence MEF2D phosphorylation in our *in vitro*



the loading control are included. (C) Scheme of MEF2C domains. The MADS and MEF2 domains and the two transcriptional activation domains (TADs) are indicated. HEK-293 cells were transfected with pEGFP-N1-*MEF2C* deletions (1.5 μg) and pFLAG-CMV5a-*SKP2* or pFLAG-CMV5a (4 μg) and treated for 8 h with 2.5 μM MG132. FLAG fusions were immunoprecipitated using an antibody against FLAG and were subjected to immunoblotting using an anti-GFP antibody. After being stripped, the filter was probed with an anti-FLAG antibody. Inputs are included. (D) Scheme of MEF2D domains. The MADS and MEF2 domains and the two TADs are indicated. HEK-293 cells were transfected with pEGFP-C2-*MEF2D* deletion mutants (1.5 μg) and pFLAG-CMV5a-*SKP2* or pFLAG-CMV5a (4 μg). Experimental treatments and immunoprecipitations were performed as for panel C. The asterisk marks the IgGs. (E) HEK-293 cells were transfected with pEGFP-C2-*MEF2D* deletion mutants and phosphodead mutants (1.5 μg) and pFLAG-CMV5a-*SKP2* or pFLAG-CMV5a (4 μg). Experimental treatments and immunoprecipitations were performed as for panel C. (F) HEK-293 cells were transfected with wild-type (WT) pEGFP-C2-*MEF2D* and single or double phosphodead mutants (1.5 μg) and pFLAG-CMV5a-*SKP2* or pFLAG-CMV5a (4 μg). Experimental treatments and immunoprecipitations were performed as for panel C. (G) GST pulldown assay. Cellular lysates from HEK-293 cells expressing WT or phosphodead mutant forms of MEF2D-GFP were incubated with 3 μg of recombinant GST-SKP2 or GST alone. (H) HEK-293 cells were cotransfected with the HA-ubiquitin gene (2 μg) and WT *MEF2D-GFP* or the double-phospho-mutant (4 μg) or *GFP* alone and treated for 8 h with 2.5 μM MG132 or left untreated. GFP fusions were immunoprecipitated using an antibody against GFP and were subjected to immunoblotting using an antiubiquitin antibody. After stripping, filter was probed with an anti-GFP antibody. Inputs are included. (I) Immunoblot analysis of MEF2D in IMR90-E1A cells transfected with the wild-type, the phosphomutant, and the phosphomimicking forms of MEF2D fused to GFP (2 μg) and with empty pEGFP-C2 (1 μg), used as the loading control. After 12 h cells were harvested, split in three, and treated for 12 h with DMSO, MG132, and the SKP2 inhibitor (SKP2-in), as indicated. (J) GST pulldown assay. Cellular lysates from HEK-293 cells expressing SKP2-GFP were incubated with 2 μg of GST, GST-MEF2D, or its phosphodead and phosphomimetic mutants, as indicated.

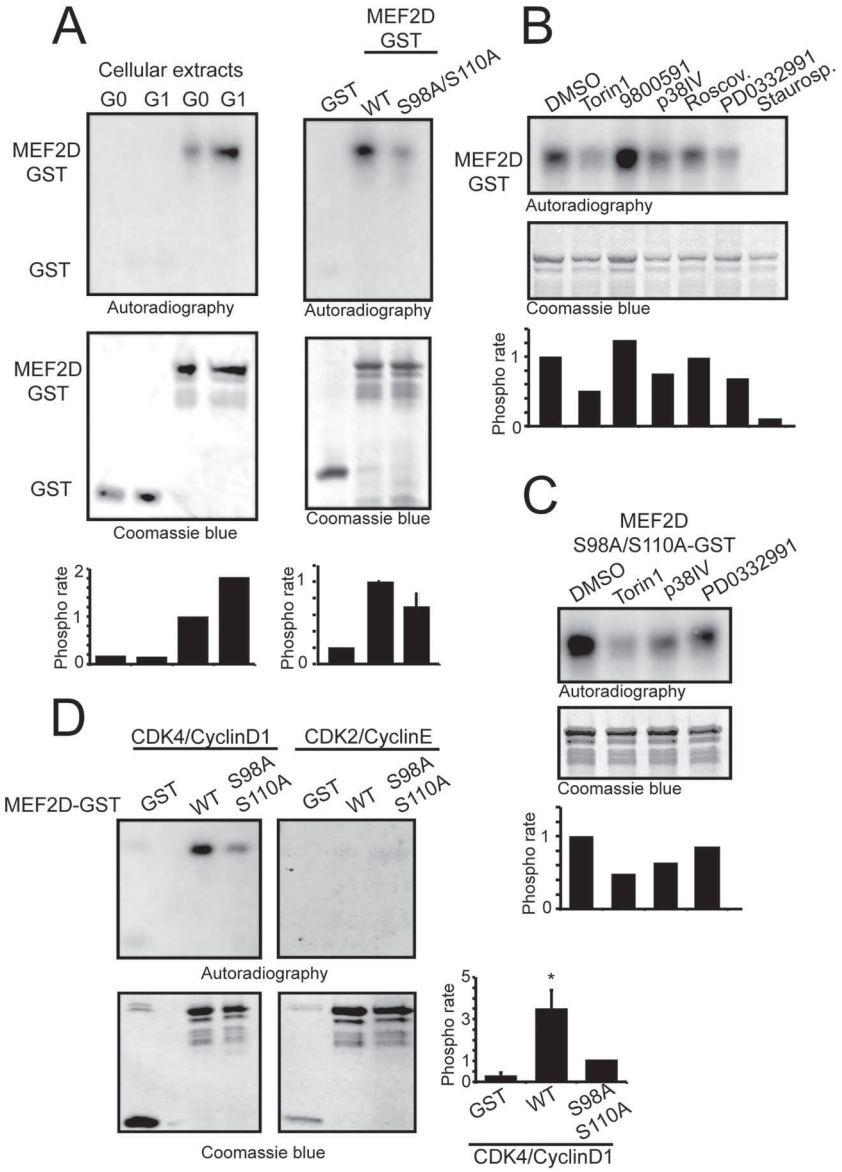



**FIG 4** MEF2D is phosphorylated by CDK4. (A) (Left) Autoradiography after *in vitro* phosphorylation of MEF2D-GST and GST as a control, using cellular extracts from serum-starved NIH 3T3 cells, incubated for 4 h with 10% FCS or left untreated. (Right) Autoradiography after *in vitro* phosphorylation of WT and phosphodead MEF2D-GST and GST alone using cellular extracts from NIH 3T3 cells that had been serum starved and treated for 4 h with 10% FCS. Coomassie staining was used as the loading control. Densitometric analysis is also shown. (B and C) Autoradiography after *in vitro* phosphorylation performed on GST-MEF2D (B) and the phosphodead mutant (C) in the presence of the indicated kinase inhibitors or DMSO. Crude cellular extracts were obtained from NIH 3T3 cells that had been serum starved and then treated for 4 h with 10% FCS. Coomassie staining was used as the loading control. Densitometric analysis is also provided. (D) Autoradiography after *in vitro* phosphorylation performed on GST-MEF2D and GST-MEF2D S98A/S110A, using recombinant cyclin D1/CDK4 or cyclin E1/CDK2. Coomassie staining was used as the loading control. Densitometric analysis is also provided. *, $P < 0.05$.

phosphorylation assay. Staurosporine was used as a positive control. Only mTOR, p38, and CDK4 inhibitors reduced MEF2D(1–190) phosphorylation, thus confirming the existence of multiple MEF2 kinases in the cellular extracts (Fig. 4B). Next, we used the MEF2D-S98A/S110A double mutant, in order to understand which kinase could be involved in the phosphorylation of these residues. The p38 and mTOR inhibitors effectively repressed phosphorylation of the double mutant, whereas the CDK4 inhibitor was the less efficient (Fig. 4C). This result suggests that CDK4 is the kinase that could be more specifically involved in the phosphorylation of S98 and S110, whereas mTOR and p38 are principally implicated in phosphorylating other residues. Finally, to verify the contribution of CDK4, the complex CDK4/cyclin D1 was tested for the ability to phosphorylate MEF2D-GST. To evaluate the specificity, the related kinase CDK2/cyclin E was used for comparison. Only CDK4/cyclin D1 was able to phosphorylate MEF2D *in vitro* and this phosphorylation was reduced but not abrogated when the S98A/S110A mutant was used (Fig. 4D), thus pointing to the existence of additional phosphorylation sites. The efficacy of both kinases was tested on GST-Rb (data not shown). In conclusion, the 1–190 fragment of MEF2D is the

target of multiple kinases in different residues, and CDK4/cyclin D1 can phosphorylate MEF2D on serines 98 and 110, as well as on additional residues.

**Roles of MEF2s in the regulation of cell cycle progression.** The discovery of the tight regulation of MEF2s stability during cell cycle progression prompted us to explore the effect of artificially altering MEF2 transcriptional activity on cell proliferation. We used the MEF2-VP16-ER chimera and, as a control, the MEF2-VP16-ER construct lacking the DNA-binding domain (ΔDBD, amino acids 58 to 86). NIH 3T3 cells expressing transcription-competent MEF2 are characterized by a reduced proliferative profile, as indicated by the reduction in cell numbers (Fig. 5A), the diminished percentage of cells incorporating BrdU (Fig. 5B), and the increased number of cells in the $G_1$ phase of the cell cycle (Fig. 5C). To strengthen this observation, we used a repressive version of MEF2 (24), generated by fusing the MADS/MEF2 domains to the transcriptional repressor Engrailed (MEF2-ENG). Cells expressing the repressive version of MEF2 increased their proliferation rate, as evidenced by (i) the increased number of cells (Fig. 5D), (ii) the highest percentage of BrdU incorporation (Fig. 5E), and (iii) the reduced number of cells in $G_1$ (Fig. 5F).

We next investigated the behavior of NIH 3T3 cells expressing the different MEF2 versions during the $G_0/G_1$ transition following readdition of serum to starved cells. In this study, we also compared the behavior of cells expressing SKP2ΔDD, a hyperactive version of the E3 ligase (Fig. 5G). As expected, the cell cycle profile of cells expressing the hyperactive SKP2 exhibited a high percentage of cells in S phase also under starvation, thus indicating their inability to enter $G_0$. In serum-starved cells expressing the repressive version of MEF2, cycling cells can still be detected (approximately 30% of the cells in S and $G_2$ phases). Readdition of serum elicited cell cycle reentry in GFP and ΔDBD cells, whereas in the presence of the transcriptionally active MEF2, the percentage of cells approaching S phase was diminished (Fig. 5G).

CDK inhibitors are key regulators of cell cycle progression (25). To evaluate a possible contribution of these inhibitors in transducing MEF2 antiproliferative signal, we took advantage of the profile of genes repressed in NIH 3T3 cells, after expression of a nuclear resident form of HDAC4, since the vast majority of these genes are MEF2 targets (7). Figure 5H shows that *Cdkn1a* was the sole CDK inhibitor significantly repressed by HDAC4. We next compared the expression patterns of some MEF2 targets and of *Cdkn1a* in the cell lines engineered to express the different MEF2 variants. *Cdkn1a* shows a pattern of expression similar to that of other MEF2 target genes, being increased in MEF2-VP16 and reduced in MEF2-ENG cell lines (Fig. 5I). The influence of the two MEF2 chimeras on p21 was confirmed also at protein levels (Fig. 5J). We also analyzed the pattern of *p21* expression following serum stimulation of starved cells. Similar to the other MEF2 targets, *Cdkn1a* mRNA levels were upregulated at starvation and slowly declined during the $G_1$/S transition (9/12 h from stimulation). Compared to the other MEF2 targets analyzed, *Cdkn1a* was induced to a much lesser extent at early times from serum stimulation (Fig. 5K).

Having defined a mechanism through which MEF2s could suppress cell proliferation, we wanted to confirm the growth inhibitory activity of MEF2 in human cells. We used BJ/TERT human fibroblasts expressing a mutated p53 allele, acting as dominant negative, and cells not expressing this allele. Since *CDKN1A* is an important p53 target gene, we wanted to exclude an involvement of this tumor suppressor in the antiproliferative activity elicited by MEF2s. Induction of MEF2-dependent transcription using the MEF2-VP16-ER chimera dramatically suppressed cell proliferation and DNA synthesis in a p53-independent manner (see Fig. S2A and B in the supplemental material). Upregulation of MEF2 target genes and of *CDKN1A* was confirmed following MEF2 transcriptional activation in both BJ/TERT and BJ/TERT/p53DN cells (see Fig. S2C and D in the supplemental material). Upregulation of CDKN1A was also verified by immunoblotting (see Fig. S2E in the supplemental material). Both analyses showed that MEF2-dependent upregulation of CDKN1A is p53 independent.

To unambiguously demonstrate the role of MEF2s in the control of cell cycle, we downregulated MEF2D expression following lentiviral infection. Two different shRNAs were evaluated (sh15 and sh27). In BJ/TERT/p53DN cells, MEF2D downregulation was coupled with the reduction of CDKN1A levels (Fig. 6A), an increase in DNA synthesis (Fig. 6B) and augmented cell proliferation (Fig. 6C). mRNA levels of MEF2 target genes and of *CDKN1A* were reduced in cells with impaired MEF2D expression (Fig. 6D). The specificity of the shRNAs against MEF2D with respect to other MEF2 members was also demonstrated (Fig. 6D).

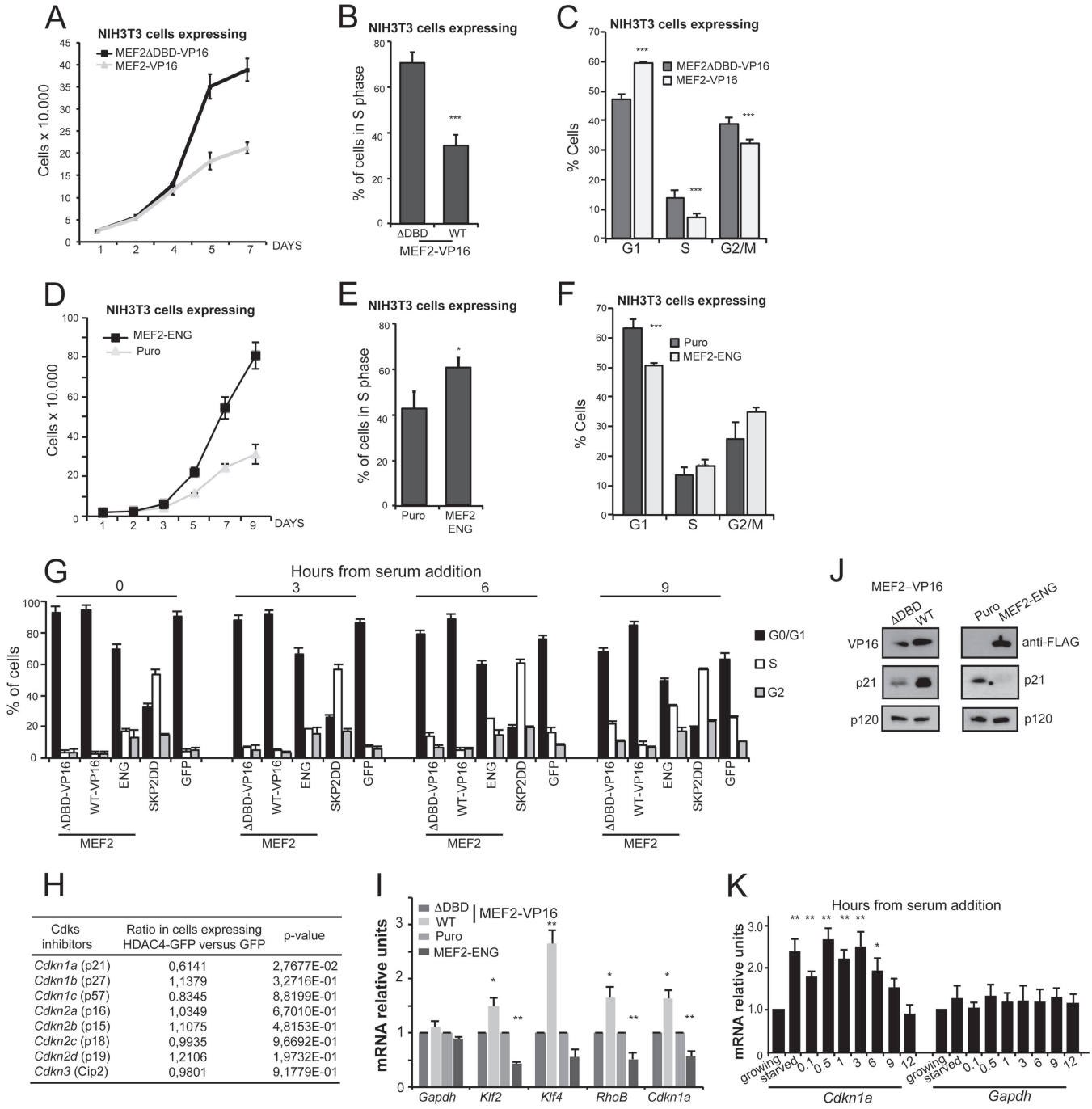
We again decided to verify the proproliferative effect of MEF2D downregulation in a different cell line. When BJ/TERT/E1A/RAS cells were used, the results were confirmed. Downregulation of MEF2D expression was coupled to (i) a downregulation of CDKN1A/p21 levels (see Fig. S2F in the supplemental material), (ii) an improvement of the percentage of cells in S phase (see Fig. S2G in the supplemental material), and (iii) an overall increase of cell proliferation (see Fig. S2H in the supplemental material). Finally, the tested MEF2 target genes were all downregulated (see Fig. S2I in the supplemental material).

Small molecules targeting SKP2 show interesting anticancer properties (26). We tested a SKP2 inhibitor for the ability to restrain proliferation of BJ/TERT/p53DN cells, and we also analyzed the contribution of MEF2D for transducing such antiproliferative response. Figure 6E illustrates that the SKP2 inhibitor can diminish the proliferation of BJ/TERT/p53DN cells. More importantly, the antiproliferative effect of the SKP2 inhibitor (Fig. 6F) and the upregulation of p21 (Fig. 6G) were consistently reduced in the absence of MEF2D. These results further confirm a key role of MEF2 in transducing antiproliferative signals, possibly through the engagement of p21.

**CDKN1A is a key element in the antiproliferative activity of MEF2.** To elucidate the contribution of CDKN1A to the antiproliferative signaling of MEF2s, we silenced its expression in BJ/TERT and BJ/TERT/p53DN cells expressing the inducible MEF2 or its mutant with a deletion in the DNA binding domain (Fig. 7A).

When CDKN1A was downregulated, the antiproliferative effect and the inhibition of DNA synthesis elicited by MEF2 upregulation were almost entirely abrogated (Fig. 7B and C).

Changes in CDKN1A levels following MEF2 perturbations could reflect either a direct involvement of MEF2s in regulating its transcription or an indirect role, through the regulation of other TFs, such as KLF4 (27) and KLF2 (28). To clarify these possibilities, we scrutinized the genomic region around the *CDKN1A* transcription start site for the presence of MEF2-binding consensus sequences. Figure 7D schematizes the organization of the *CDKN1A* genomic region and highlights the presence of 6 putative MEF2-binding sequences in the promoter and in the first




| Cdks inhibitors | Ratio in cells expressing HDAC4-GFP versus GFP | p-value |
|---|---|---|
| *Cdkn1a* (p21) | 0,6141 | 2,7677E-02 |
| *Cdkn1b* (p27) | 1,1379 | 3,2716E-01 |
| *Cdkn1c* (p57) | 0.8345 | 8,8199E-01 |
| *Cdkn2a* (p16) | 1,0349 | 6,7010E-01 |
| *Cdkn2b* (p15) | 1,1075 | 4,8153E-01 |
| *Cdkn2c* (p18) | 0,9935 | 9,6692E-01 |
| *Cdkn2d* (p19) | 1,2106 | 1,9732E-01 |
| *Cdkn3* (Cip2) | 0,9801 | 9,1779E-01 |

**FIG 5** MEF2s affect NIH 3T3 fibroblasts proliferation by inducing CDKN1A expression. (A) NIH 3T3 cells expressing the two transgenes and treated with 4-OHT were grown for the indicated times. Data are means ± SD ($n = 3$). (B) Forty-eight hours after seeding, quantification of BrdU positivity of NIH 3T3 cells expressing the indicated transgenes and treated with 4-OHT was performed. Data are means and SD ($n = 5$). (C) Cell cycle profile of NIH 3T3 cells expressing the indicated transgenes and treated with 4-OHT. Analysis was performed 48 h after seeding. Data are means and SD ($n = 4$). (D) NIH 3T3 cells expressing the two transgenes were grown for the indicated times. Data are means ± SD ($n = 3$). (E) Forty-eight hours after seeding, quantification of BrdU positivity of NIH 3T3 cells expressing the indicated transgenes was performed. Data are means and SD ($n = 5$). (F) Cell cycle profile of NIH 3T3 cells expressing the indicated transgenes. Analysis was performed 48 h after seeding. Data are means and SD ($n = 4$). (G) Cell cycle profile of NIH 3T3 cells expressing the indicated transgenes, serum starved (time zero) or at different times after serum addition. SKP2DD was used as a positive control for unrestricted proliferation. Data are means and SD ($n = 3$). (H) mRNA induction ($n$-fold) of the indicated CDK inhibitors, obtained by comparing their levels of expression in NIH 3T3 cells expressing HDAC4-GFP and those expressing GFP as a control (7). (I) mRNA expression levels of MEF2 target genes (*Klf2*, *Klf4*, and *RhoB*) and *Cdkn1a* in NIH 3T3 cells expressing the indicated transgenes and collected 36 h after the seeding. *Gapdh* was used as a control. Data are means and SD ($n = 3$). (J) Immunoblot analysis of p21/CDKN1A levels in NIH 3T3 cells expressing the indicated transgenes. Anti-VP16 and anti-FLAG antibodies were used to reveal the expression of the transgenes. p120 was used as the loading control. (K) mRNA expression levels of *Cdkn1a* in serum-starved NIH 3T3 cells stimulated with 10% FCS as indicated. *Gapdh* was used as a control. Data are means and SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

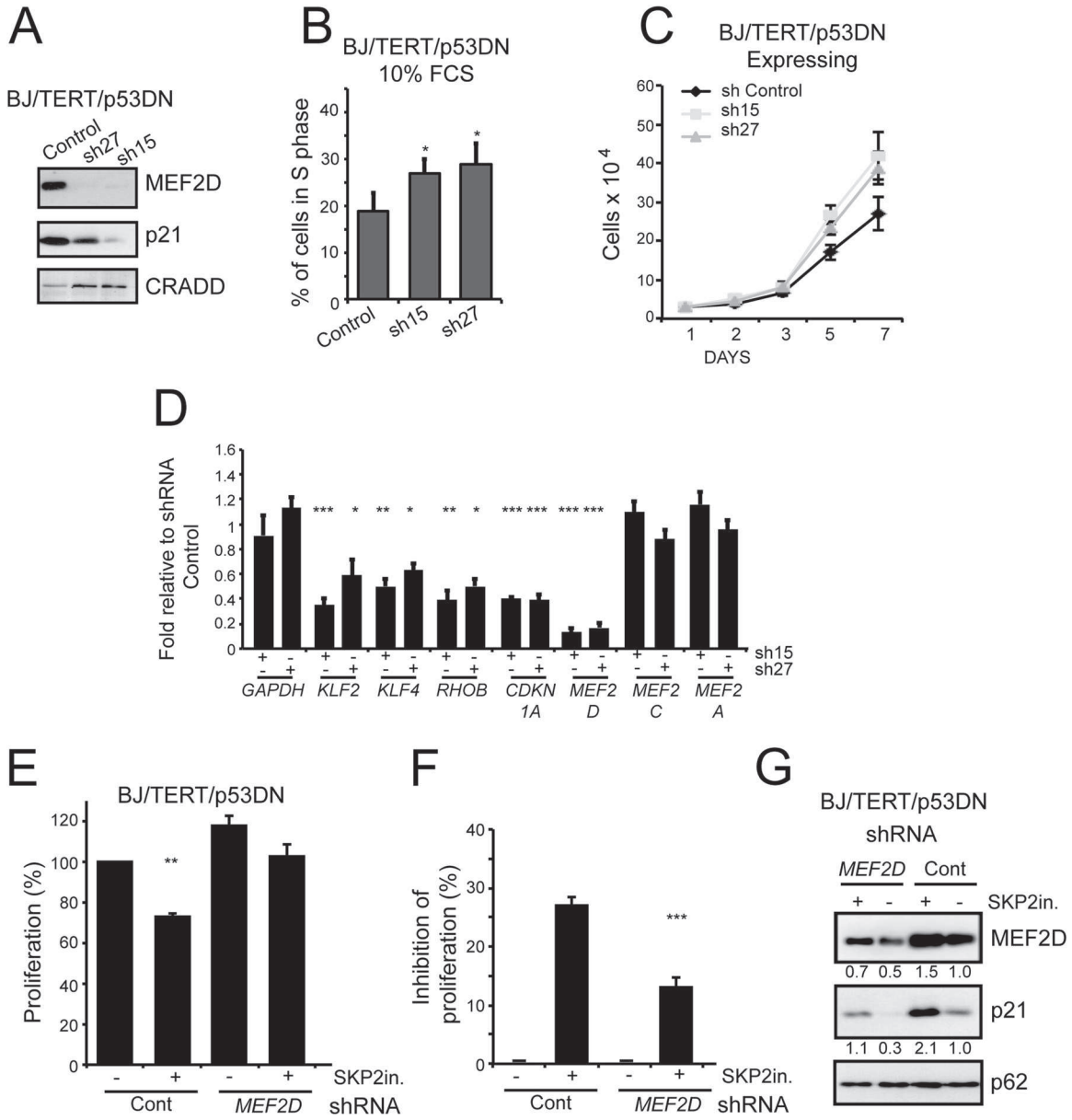



**FIG 6** MEF2s affect proliferation of human fibroblasts by inducing CDKN1A expression. (A) Immunoblot analysis of p21/CDKN1A in BJ/TERT/p53DN cells silenced for MEF2D expression using two different shRNAs (sh15 and sh27). The efficiency of the downregulation was proved with an anti-MEF2D antibody. CRADD was used as the loading control. (B) Quantification of BrdU positivity of BJ/TERT/p53DN silenced (sh15 and sh27) for MEF2D or not silenced (shCT). Analyses were performed 48 h after seeding. Data are means and SD ($n = 4$). (C) BJ/TERT/p53DN cells expressing the indicated shRNAs were grown for the indicated times. Data are means ± SD ($n = 3$). (D) mRNA expression levels of MEF2 target genes (*KLF2*, *KLF4*, and *RHOB*) and of *CDKN1A* in BJ/TERT/p53DN cells in which *MEF2D* expression was downregulated using two different shRNAs, as indicated. Data are means and SD ($n = 3$). (E) Quantification of the proliferation rate of BJ/TERT/p53DN cells silenced for *MEF2D* or not silenced and treated for 24 h with the SKP2 inhibitor or left untreated, as indicated. Data are means and SD ($n = 3$). (F) Proliferation inhibition in BJ/TERT/p53DN cells knocked down for *MEF2D* or not knocked down and treated with SKP2 inhibitor or left untreated, as indicated. Data are relative to those for untreated cells and presented as means and SD ($n = 3$). (G) Immunoblot and densitometric analysis of MEF2D and p21/CDKN1A levels in BJ/TERT/p53DN cells treated as for panel E. p62 (nucleoporin) was used as the loading control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

intron of *CDKN1A* gene. ChIP experiments using two different anti-MEF2C antibodies indicate that MEF2C can interact with the *CDKN1A* genomic region, and the highest enrichment was obtained for the MEF2 consensus sequence at kb +2.1 from the transcription start site (TSS) (Fig. 7E). This enrichment was comparable to the positive control, the region containing the MEF2-binding sequence of the *RhoB* promoter. Similar results were observed when the ChIP experiments were performed using an antibody against MEF2D. We verified that MEF2D also binds the same genomic region in cells expressing mutated p53 (Fig. 7E).

The genomic region of *CDKN1A* bound by MEF2 was previously characterized by the ENCODE project as enriched in histone H3 lysine 27 acetylation (H3K27ac) and histone H3 lysine 4 monomethylation (H3K4me1) (29), two markers of active en-

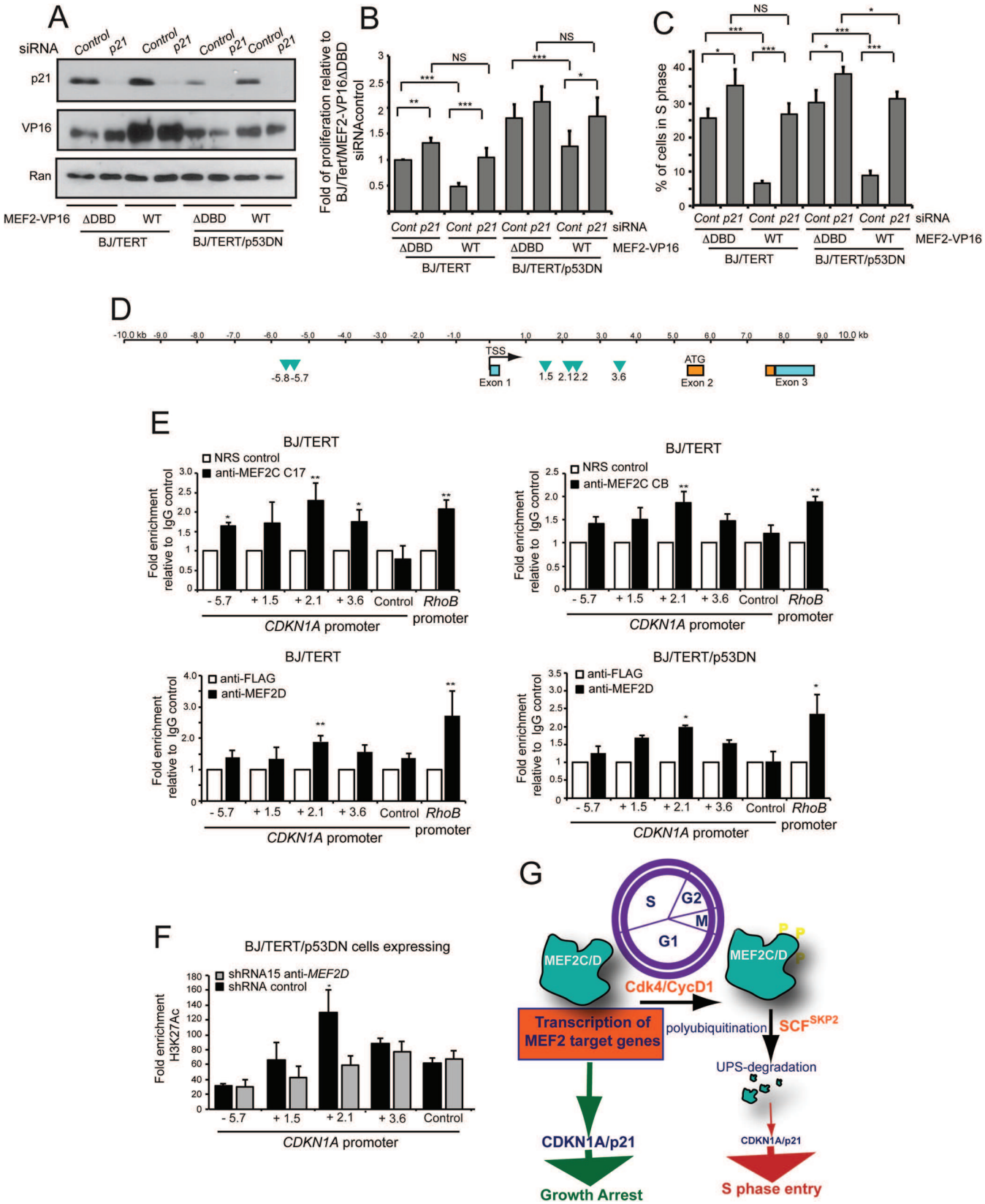



**FIG 7** MEF2D antiproliferative effects rely mainly on its direct regulation of CDKN1A transcription. (A) Immunoblot analysis of p21/CDKN1A in BJ/TERT and BJ/TERT/p53DN cells expressing the indicated transgenes, treated with 4-OHT, and silenced for the p21/CDKN1A gene or not silenced, as indicated. Anti-VP16 and anti-Ran antibodies were used, respectively, to reveal the expression of the transgenes and as the loading control. (B) Quantification of the proliferation rate of the indicated cell lines relative to BJ/TERT/MEF2ΔDBD cells treated for 36 h with siRNA against the p21/CDKN1A gene or the siRNA

hancers (30). These data suggest that MEF2 might actively regulate p21 transcription by binding to an active enhancer and could itself recruit other cofactors, such as the acetyltransferase p300 (31), which in turn cooperate to maintain the open chromatin state. To confirm this hypothesis, we performed ChIP experiment using anti-H3K27Ac antibody. Figure 7F illustrates that the highest enrichment for H3K27 acetylation can be observed around kb +2.1 from the TSS in the same region where MEF2C and MEF2D binding was observed. Furthermore, in cells with downregulated MEF2D, the acetylation of H3K27 was clearly reduced specifically at kb +2.1 from the TSS.

## DISCUSSION

MEF2s are pleiotropic TFs, which influence different genetic programs in relation to specific posttranslational modifications (PTMs) and the associations with other transcription factors, coactivators, or repressors (1). The contribution of MEF2s to several differentiation processes is well known. MEF2s supervise differentiation of myoblasts (32), cardiomyoblasts (33), osteoblasts (24), neuronal cells (10, 34), B lymphocytes, and monocytes (35). Mice with *Mef2* genes knocked out display phenotypes compatible with defects in these differentiative programs at an advanced stage, suggesting that MEF2s regulate the final steps of these processes (1, 24, 33, 35). Furthermore, in a wide variety of differentiated cells, MEF2s are also engaged to modulate adaptive responses (1, 36, 37).

In this work, we investigated the still-obscure role of MEF2s in the regulation of the cell cycle. Using human and murine fibroblasts as cellular models, we have demonstrated the reciprocal influence of the cell cycle machinery on MEF2 activities and of MEF2s on cell cycle progression. We have discovered that, in addition to previously documented engagements of MEF2s in governing the early transcriptional response to serum (immediate early genes) (13, 14, 15), MEF2 transcriptional activity is regulated at supplementary steps during the cell cycle.

By simultaneously monitoring the mRNAs of the MEF2 target genes and the levels of MEF2C and MEF2D proteins, we noticed the following. (i) During growth arrest ($G_0$), mRNA upregulation of three MEF2 target genes (*KLF2*, *KLF4*, and *RHOB*) is tightly coupled to the stabilization of MEF2C and MEF2D proteins. (ii) Following growth factor stimulation of serum-starved cells ($G_0/G_1$ transition), the expression of MEF2 target genes is rapidly and transiently upregulated. This rapid induction of the MEF2 target genes may depend on specific PTMs induced by serum, potentiating MEF2 activity (18, 38). (iii) As cells progress toward the $G_1$/S phases, MEF2 protein levels drop. Again, the mRNA levels of *KLF2*, *KLF4*, and *RHOB* decrease in parallel. The UPS is responsible for this timing-regulated degradation of MEF2C and MEF2D. Overall, the influence of the cell cycle on the half-lives of MEF2C and MEF2D justifies their accumulation under nonproliferative/quiescent conditions.

Phosphorylation plays a key role for targeting MEF2C and MEF2D to the UPS. We provide evidence that phosphorylation of serine residues 98 and 110 is fundamental for mediating the interaction with the F-box protein SKP2 and the subsequent polyubiquitylation-mediated degradation of the TFs. Since residual MEF2 polyubiquitylation was also observed in the presence of the double phosphodead mutant (S98A/S110A) (Fig. 4I), it is possible that additional Ser/Thr residues or different E3 ligases influence MEF2 stability during the cell cycle. Interestingly, a contribution of the same serine residues to the regulation of MEF2C stability was previously proposed (39).

SKP2 is a positive regulator of cell cycle progression and an oncogenic protein that targets tumor suppressor proteins for degradation (19). Hence, MEF2s polyubiquitylation lies at the core of the machinery controlling cell cycle progression: the $SCF^{SKP2}$ (SKP1–CUL1–F-box protein) superenzyme. *In vitro* phosphorylation assays using specific inhibitors and recombinant enzymes suggest that cyclin D1-CDK4 but not cyclin E-CDK2 can phosphorylate serines 98 and 110 of MEF2D. Retinoblastoma tumor suppressor protein RB1 and its family members are key substrates of CDK4/CDK6 D-type cyclins complexes for promoting $G_1$-S transition (40). Interestingly, a catalogue of new substrates of these kinases has been generated. Although more precise information about amino acid involvement is not available, in accordance with our discovery, MEF2D was listed in the catalogue as a CDK4/CDK6 substrate (41).

When we experimentally affected MEF2 levels and activities, the overt evoked phenotype was a reduction of cell proliferation. The importance of the growth-suppressive activity of MEF2s stems also from the diminished antiproliferative impact of the SKP2 inhibitor, when MEF2D expression was downregulated by specific shRNAs.

We have discovered that an important element of the antiproliferative pathway engaged by MEF2s is CDKN1A (Fig. 7G). In agreement with our observations, a recent study demonstrated that ectopically expressed MEF2D can upregulate p21 expression (42). *CDKN1A* transcription fluctuates during the cell cycle, accumulating in $G_0$ and showing a peak after serum stimulation (43). In human fibroblasts, this pattern is strictly dependent on the presence of MEF2D (see Fig. S3 in the supplemental material). We have shown that MEF2C and MEF2D bind a genomic region within the first intron, at kb +2.1 from the TSS of the *CDKN1A* gene, a region characterized by an open chromatin status (29). We have also provided evidence that MEF2D is important for favoring H3K27 acetylation, a well-known marker of active/open chromatin, within the *CDKN1A* gene, in proximity to its binding site. This epigenetic modification may be governed through the engagement of p300/CBP (31), a well-known MEF2 partner (44, 45, 46).

MEF2D is critical for the upregulation of the immediate early genes response to serum. However, at least in BJ/hTERT/p53DN

control. Data are means and SD ($n = 3$). (C) Quantification of BrdU positivity of the indicated cell lines, treated as for panel B. Data are means and SD ($n = 4$). (D) Representation of the *CDKN1A* gene structure and its promoter region 10 kb upstream and downstream from the transcription start site (TSS). The putative MEF2 binding sites are highlighted (green). The coding (orange) and the noncoding (light blue) exons and the ATG leader are also indicated. (E) ChIP of BJ/TERT and BJ/TERT/p53DN cells. Chromatin was immunoprecipitated using two distinct antibodies against MEF2C and one against MEF2D. Normal rabbit serum (NRS) and anti-Flag antibody were used as relative controls. The *RHOB* promoter was used as a positive control, and an internal region (kb +4.7 from the TSS) of the *CDKN1A* gene was used as a negative control. Data are means and SD ($n = 3$). (F) ChIP of BJ/TERT/p53DN cells in which *MEF2D* was knocked down with shRNA 15. The H3K27 acetylation status of the putative MEF2 binding sites on *CDKN1A* promoter is shown. Chromatin was immunoprecipitated with antibodies against acetylated H3K27 (H3K27ac), and normal rabbit serum (NRS) was used as a relative control. Data are means and SD ($n = 3$). (G) Model representing the cell cycle-mediated regulation of MEF2 levels and MEF2 feedback activity on cell cycle progression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

cells, a reduction of MEF2D activity and the consequent upregulation of MEF2 target genes during $G_0/G_1$ transition did not affect entry into S phase (see Fig. S3 in the supplemental material). Certainly, we cannot exclude the possibility that in other cellular contexts, this early activation makes an important contribution to cell cycle progression.

A double role of MEF2 during different phases of the cell cycle could explain the apparent conflicting results about MEF2s' antiproliferative and proproliferative activities found in the literature (14, 15, 47). In particular, MEF2C has been reported to be required for B cell proliferation and survival after BCR stimulation but not after Toll-like receptor stimulation (12); in contrast, MEF2A decreases the proliferation and the migration rates of vascular smooth muscle cells (VSMCs) (48).

A tight and ordered control of the progression through the cell cycle ensures a harmonic regulation of cell proliferation. Progression through the different phases of the cell cycle is orchestrated by the activity of different TFs, which operate under the influence of the cell cycle machinery and allow the ordered (hierarchical) transcription of cell cycle-regulated genes (49). Our studies add MEF2s to the list of the transcriptional regulators, which are an integral part of the machinery that controls the cell cycle.

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## REFERENCES

1. **Potthoff MJ, Olson EN.** 2007. MEF2: a central regulator of diverse developmental programs. Development **134:**4131–4140. http://dx.doi.org/10.1242/dev.008367.
2. **Yu YT.** 1996. Distinct domains of myocyte enhancer binding factor-2A determining nuclear localization and cell type-specific transcriptional activity. J Biol Chem **271:**24675–24683.
3. **Wales S, Hashemi S, Blais AMJ.** 2014. Global MEF2 target gene analysis in cardiac and skeletal muscle reveals novel regulation of DUSP6 by p38MAPK-MEF2 signaling. Nucleic Acids Res **42:**11349–11362. http://dx.doi.org/10.1093/nar/gku813.
4. **Sandmann T, Jensen LJ, Jakobsen JS, Karzynski MM, Eichenlaub MP, Bork P, Furlong EEM.** 2006. A temporal map of transcription factor activity: Mef2 directly regulates target genes at all stages of muscle development. Dev Cell **10:**797–807. http://dx.doi.org/10.1016/j.devcel.2006.04.009.
5. **Shalizi A, Gaudillière B, Yuan Z, Stegmüller J, Shirogane T, Ge Q, Tan Y, Schulman B, Harper JW, Bonni A.** 2006. A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. Science **311:**1012–1017. http://dx.doi.org/10.1126/science.1122513.
6. **Clocchiatti A, Di Giorgio E, Ingrao S, Meyer-Almes F-J, Tripodo C, Brancolini C.** 2013. Class IIa HDACs repressive activities on MEF2-depedent transcription are associated with poor prognosis of $ER^+$ breast tumors. FASEB J **27:**942–954. http://dx.doi.org/10.1096/fj.12-209346.
7. **Di Giorgio E, Clocchiatti A, Piccinin S, Sgorbissa A, Viviani G, Peruzzo P, Romeo S, Rossi S, Dei Tos AP, Maestro R, Brancolini C.** 2013. MEF2 is a converging hub for histone deacetylase 4 and phosphatidylinositol 3-kinase/Akt-induced transformation. Mol Cell Biol **33:**4473–4491. http://dx.doi.org/10.1128/MCB.01050-13.
8. **Barneda-Zahonero B, Román-González L, Collazo O, Rafati H, Islam ABMMK, Bussmann LH, di Tullio A, De Andres L, Graf T, López-Bigas N, Mahmoudi T, Parra M.** 2013. HDAC7 is a repressor of myeloid genes whose downregulation is required for transdifferentiation of pre-B cells into macrophages. PLoS Genet **9:**e1003503. http://dx.doi.org/10.1371/journal.pgen.1003503.
9. **Lyons GE, Micales BK, Schwarz J, Martin JF, Olson EN.** 1995. Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. J Neurosci **15:**5727–5738.
10. **Li H, Radford JC, Ragusa MJ, Shea KL, McKercher SR, Zaremba JD, Soussou W, Nie Z, Kang Y-J, Nakanishi N, Okamoto S, Roberts AJ, Schwarz JJ, Lipton SA.** 2008. Transcription factor MEF2C influences neural stem/progenitor cell differentiation and maturation in vivo. Proc Natl Acad Sci U S A **105:**9397–9402. http://dx.doi.org/10.1073/pnas.0802876105.
11. **Liu N, Nelson BR, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN.** 2014. Requirement of MEF2A, C, and D for skeletal muscle regeneration. Proc Natl Acad Sci U S A **111:**4109–4114. http://dx.doi.org/10.1073/pnas.1401732111.
12. **Wilker PR, Kohyama M, Sandau MM, Albring JC, Nakagawa O, Schwarz JJ, Murphy KM.** 2008. Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation. Nat Immunol **9:**603–612. http://dx.doi.org/10.1038/ni.1609.
13. **Suzuki E, Guo K, Kolman M, Yu YT, Walsh K.** 1995. Serum induction of MEF2/RSRF expression in vascular myocytes is mediated at the level of translation. Mol Cell Biol **15:**3415–3423.
14. **Han TH, Prywes R.** 1995. Regulatory role of MEF2D in serum induction of the c-jun promoter. Mol Cell Biol **15:**2907–2915.
15. **Kato Y, Kravchenko VV, Tapping RI, Han J, Ulevitch RJ, Lee JD.** 1997. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. EMBO J **16:**7054–7066. http://dx.doi.org/10.1093/emboj/16.23.7054.
16. **Mendoza-Maldonado R, Paolinelli R, Galbiati L, Giadrossi S, Giacca M.** 2010. Interaction of the retinoblastoma protein with orc1 and its recruitment to human origins of DNA replication. PLoS One **5:**e13720. http://dx.doi.org/10.1371/journal.pone.0013720.
17. **Paroni G, Mizzau M, Henderson C, Del Sal G, Schneider C, Brancolini C.** 2004. Caspase-dependent regulation of histone deacetylase 4 nuclear-cytoplasmic shuttling promotes apoptosis. Mol Biol Cell **15:**2804–2818. http://dx.doi.org/10.1091/mbc.E03-08-0624.
18. **Henderson CJ, Aleo E, Fontanini A, Maestro R, Paroni G, Brancolini C.** 2005. Caspase activation and apoptosis in response to proteasome inhibitors. Cell Death Differ **12:**1240–1254. http://dx.doi.org/10.1038/sj.cdd.4401729.
19. **Frescas D, Pagano M.** 2008. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. Nat Rev Cancer **8:**438–449. http://dx.doi.org/10.1038/nrc2396.
20. **Carrano AC, Eytan E, Hershko A, Pagano M.** 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol **1:**193–199. http://dx.doi.org/10.1038/12013.
21. **Marinissen MJ, Chiariello M, Pallante M, Gutkind JS.** 1999. A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun promoter: a role for c-Jun NH2-terminal kinase, p38s, and extracellular signal-regulated kinase 5. Mol Cell Biol **19:**4289–4301.
22. **Liu W, Wu G, Li W, Lobur D, Wan Y.** 2007. Cdh1-anaphase-promoting complex targets Skp2 for destruction in transforming growth factor beta-induced growth inhibition. Mol Cell Biol **27:**2967–2979. http://dx.doi.org/10.1128/MCB.01830-06.
23. **Yu ZK, Gervais JL, Zhang H.** 1998. Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. Proc Natl Acad Sci U S A **95:**11324–11329. http://dx.doi.org/10.1073/pnas.95.19.11324.
24. **Arnold MA, Kim Y, Czubryt MP, Phan D, McAnally J, Qi X, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN.** 2007. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. Dev Cell **12:**377–389. http://dx.doi.org/10.1016/j.devcel.2007.02.004.
25. **Bertoli C, Skotheim JM, de Bruin RA.** 2013. Control of cell-cycle transcription during $G_1$ and S phases. Nat Rev Mol Cell Biol **14:**518–528. http://dx.doi.org/10.1038/nrm3629.
26. **Chan CH, Morrow JK, Li CF, Gao Y, Jin G, Moten A, Stagg LJ, Ladbury JE, Cai Z, Xu D, Logothetis CJ, Hung MC, Zhang S, Lin HK.** 2013. Pharmacological inactivation of Skp2 SCF ubiquitin ligase restricts cancer stem cell traits and cancer progression. Cell **154:**556–568. http://dx.doi.org/10.1016/j.cell.2013.06.048.
27. **Chen X, Johns DC, Geiman DE, Marban E, Dang DT, Hamlin G, Sun R, Yang VW.** 2001. Krüppel-like factor 4 (gut-enriched Krüppel-like factor) inhibits cell proliferation by blocking $G_1$/S progression of the cell-cycle. J Biol Chem **276:**30423–30428. http://dx.doi.org/10.1074/jbc.M101194200.

28. **Wu J, Lingrel JB.** 2004. KLF2 inhibits Jurkat T leukemia cell growth via up-regulation of cyclin-dependent kinase inhibitor p21WAF1/CIP1. Oncogene **23:**8088–8096. http://dx.doi.org/10.1038/sj.onc.1207996.
29. **Dunham I, Kundaje A, Aldred SF, et al.** 2012. An integrated encyclopedia of DNA elements in the human genome. Nature **489:**57–74. http://dx.doi.org/10.1038/nature11247.
30. **Zentner GE, Tesar PJ, Scacheri PC.** 2011. Epigenetic signatures distinguish multiple classes of enhancers with distinct cellular functions. Genome Res **21:**1273–1283. http://dx.doi.org/10.1101/gr.122382.111.
31. **Zheng M, Zhu J, Lu T, Liu L, Sun H, Liu Z, Tian J.** 2013. P300-mediated histone acetylation is essential for the regulation of gata4 and MEF2C by BMP2 in H9c2 cells. Cardiovasc Toxicol **13:**316–322. http://dx.doi.org/10.1007/s12012-013-9212-4.
32. **McDermott JC, Cardoso MC, Yu YT, Andres V, Leifer D, Krainc D, Lipton SA, Nadal-Ginard B.** 1993. hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. Mol Cell Biol **13:**2564–2577.
33. **Karamboulas C, Dakubo GD, Liu J, De Repentigny Y, Yutzey K, Wallace VA, Kothary R, Skerjanc IS.** 2006. Disruption of MEF2 activity in cardiomyoblasts inhibits cardiomyogenesis. J Cell Sci **119:**4315–4321. http://dx.doi.org/10.1242/jcs.03186.
34. **Barbosa AC, Kim M-S, Ertunc M, Adachi M, Nelson ED, McAnally J, Richardson JA, Kavalali ET, Monteggia LM, Bassel-Duby R, Olson EN.** 2008. MEF2C, a transcription factor that facilitates learning and memory by negative regulation of synapse numbers and function. Proc Natl Acad Sci U S A **105:**9391–9396. http://dx.doi.org/10.1073/pnas.0802679105.
35. **Canté-Barrett K, Pieters R, Meijerink JPP.** 2014. Myocyte enhancer factor 2C in hematopoiesis and leukemia. Oncogene **33:**403–410. http://dx.doi.org/10.1038/onc.2013.56.
36. **Clark RI, Tan SWS, Péan CB, Roostalu U, Vivancos V, Bronda K, Pilátová M, Fu J, Walker DW, Berdeaux R, Geissmann F, Dionne MS.** 2013. XMEF2 is an in vivo immune-metabolic switch. Cell **155:**435–447. http://dx.doi.org/10.1016/j.cell.2013.09.007.
37. **Chan SF, Sances S, Brill LM, Okamoto S-I, Zaidi R, McKercher SR, Akhtar MW, Nakanishi N, Lipton SA.** 2014. ATM-dependent phosphorylation of MEF2D promotes neuronal survival after DNA damage. J Neurosci **34:**4640–4653. http://dx.doi.org/10.1523/JNEUROSCI.2510-12.2014.
38. **Cavanaugh JE, Ham J, Hetman M, Poser S, Yan C, Xia Z.** 2001. Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity, and cAMP in neurons. J Neurosci **21:**434–443.
39. **Magli A, Angelelli C, Ganassi M, Baruffaldi F, Matafora V, Battini R, Bachi A, Messina G, Rustighi A, Del Sal G, Ferrari S, Molinari S.** 2010. Proline isomerase pin1 represses terminal differentiation and myocyte enhancer factor 2C function in skeletal muscle cells. J Biol Chem **285:**34518–34527. http://dx.doi.org/10.1074/jbc.M110.104133.
40. **Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC.** 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through $G_1$. Cell **98:**859–869. http://dx.doi.org/10.1016/S0092-8674(00)81519-6.
41. **Anders L, Ke N, Hydbring P, Choi YJ, Widlund HR, Chick JM, Zhai H, Vidal M, Gygi SP, Braun P, Sicinski P.** 2011. A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. Cancer Cell **20:**620–634. http://dx.doi.org/10.1016/j.ccr.2011.10.001.
42. **Zhang M, Truscott J, Davie J.** 2013. Loss of MEF2D expression inhibits differentiation and contributes to oncogenesis in rhabdomyosarcoma cells. Mol Cancer **12:**150. http://dx.doi.org/10.1186/1476-4598-12-150.
43. **Li Y, Jenkins CW, Nichols MA, Xiong Y.** 1994. Cell-cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. Oncogene **9:**2261–2268.
44. **Eckner R, Yao TP, Oldread E, Livingston DM.** 1996. Interaction and functional collaboration of p300/CBP and bHLH proteins in muscle and B-cell differentiation. Genes Dev **10:**2478–2490. http://dx.doi.org/10.1101/gad.10.19.2478.
45. **Slepak TI, Webster KA, Zang J, Prentice H, O'Dowd A, Hicks MN, Bishopric NH.** 2001. Control of cardiac-specific transcription by p300 through myocyte enhancer factor-2D. J Biol Chem **276:**7575–7585. http://dx.doi.org/10.1074/jbc.M004625200.
46. **Molinari S, Relaix F, Lemonnier M, Kirschbaum B, Schäfer B, Buckingham M.** 2004. A novel complex regulates cardiac actin gene expression through interaction of Emb, a class VI POU domain protein, MEF2D, and the histone transacetylase p300. Mol Cell Biol **24:**2944–2957. http://dx.doi.org/10.1128/MCB.24.7.2944-2957.2004.
47. **Yamamoto T, Ebisuya M, Ashida F, Okamoto K, Yonehara S, Nishida E.** 2006. Continuous ERK activation downregulates antiproliferative genes throughout $G_1$ phase to allow cell-cycle progression. Curr Biol **16:**1171–1182. http://dx.doi.org/10.1016/j.cub.2006.04.044.
48. **Zhao W, Zhao SP, Peng DQ.** 2012. The effects of myocyte enhancer factor 2A gene on the proliferation, migration and phenotype of vascular smooth muscle cells. Cell Biochem Funct **30:**108–113. http://dx.doi.org/10.1002/cbf.1823.
49. **Skotheim JM, Di Talia S, Siggia ED, Cross FR.** 2008. Positive feedback of $G_1$ cyclins ensures coherent cell-cycle entry. Nature **454:**291–296. http://dx.doi.org/10.1038/nature07118.

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RESEARCH ARTICLE

# Selective class IIa HDAC inhibitors: myth or reality

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**Abstract** The prospect of intervening, through the use of a specific molecule, with a cellular alteration responsible for a disease, is a fundamental ambition of biomedical science. Epigenetic-based therapies appear as a remarkable opportunity to impact on several disorders, including cancer. Many efforts have been made to develop small molecules acting as inhibitors of histone deacetylases (HDACs). These enzymes are key targets to reset altered genetic programs and thus to restore normal cellular activities, including drug responsiveness. Several classes of HDAC inhibitors (HDACis) have been generated, characterized and, in certain cases, approved for the use in clinic. A new frontier is the generation of subtype-specific inhibitors, to increase selectivity and to manage general toxicity. Here we will discuss about a set of molecules, which can interfere with the activity of a specific subclass of HDACs: the class IIa.

**Keywords** SAHA · HDAC3 · HDAC4 · HDAC5 · HDAC7 · HDAC9 · MEF2 · p21 · Therapy · Apoptosis · Cell cycle · Anti-cancer · Neurodegeneration · Inflammation

## Introduction

### Why to target HDACs?

Every complex cellular adaptation and behavior is supervised by changes in the transcriptional machinery, which align the gene expression profile of a specific cell type to the general requirements of the organism. The harmonic regulation of genes transcribed in a specific instant is the result of an integrated and complex network of signals that controls the activity of different transcriptional players. Transcription factors (TFs), epigenetic regulators and "structural" proteins, constituting the chromatin are the chief protagonists under the tight influence of the environment. Alterations in the signaling networks or in the transcriptional players are responsible for aberrations in tissue homeostasis and triggering events in several different diseases, from neurodegeneration up to cancer [1, 2]. The opportunity to reset the transcriptional subverted context, with the therapeutic perspective of curing/alleviating diseases, straightway attracted scientist's attention [3, 4].

Perhaps the simplest approach to develop new drugs is the identification of small molecules, acting as inhibitors of an enzymatic activity that is imperative in a specific disease. In the context of gene transcription, post-translational modifications (PTMs) of histones represent realistic targets for the development of epigenetic therapies aimed to amend transcriptional alterations. Acetylation of lysines, placed in histones but also in TFs is an important PTM, exerting both positive (H3K4, 9, 14, 17, 23; H4K5, 8, 12, 16) and negative (in the case of specific TFs) effects on gene expression [5, 6]. Being acetylation reversible and under the scrutiny of different family of enzymes: HATs (histone acetyl transferases) and HDACs (histone deacetylases), it has attracted several interests as a druggable PTM [7]. In particular, during the past decades, many efforts have been made to isolate, synthesize and characterize small molecules targeting HDACs [8]. HDACis are nowadays represented as a considerable fraction of the epigenetic drugs under study and in some circumstances

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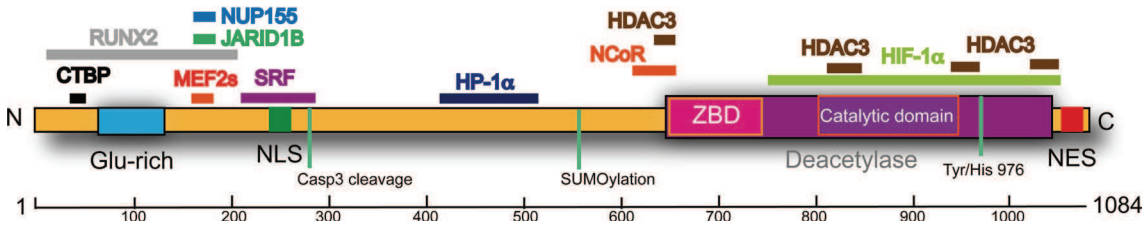



**Fig. 1** Schematic representation of class IIa HDACs highlighting the principal domains. As prototype of class IIa we selected HDAC4. Certain interaction partners, as well as the relative HDAC4 sequences involved, are illustrated

these compounds have been approved for the use in clinic (see below). Importantly, epigenetic drugs in cancer therapy represent an opportunity to revert drug-resistance-associated epigenomes and to prevent or reverse non-responsiveness to anti-cancer drugs [2].

Copious studies on cancer cells' epigenomes have fully justified the rationale of applying HDACis in anti-cancer therapies. Three major intrinsic features of the neoplastic cells could be subject of specific intervention, thanks to HDACis: (1) cancer cells are characterized by an enhanced degree of heterochromatinization compared to normal cells; which makes cancer genomes inaccessible to DNA-damage response enzymes [9]. The treatment of cancer cells with HDACis relaxes chromatin and allows the activation of the DNA-damage response [9]. (2) Several tumor suppressor genes, including some pro-apoptotic genes, are inactivated in cancer cells because of ipo-acetylated promoters [3, 10, 11]. (3) Alterations of the epigenetic machineries embracing HDACs are frequently observed in tumors [12, 13].

Despite the considerable literature debating the use in epigenetic therapies of pan-HDACi and of class I HDACs specific inhibitors [10, 11, 14–22], reviews specifically discussing of molecules acting as inhibitors of class IIa HDACs, are quite rare. In this manuscript we will discuss specifically of them.

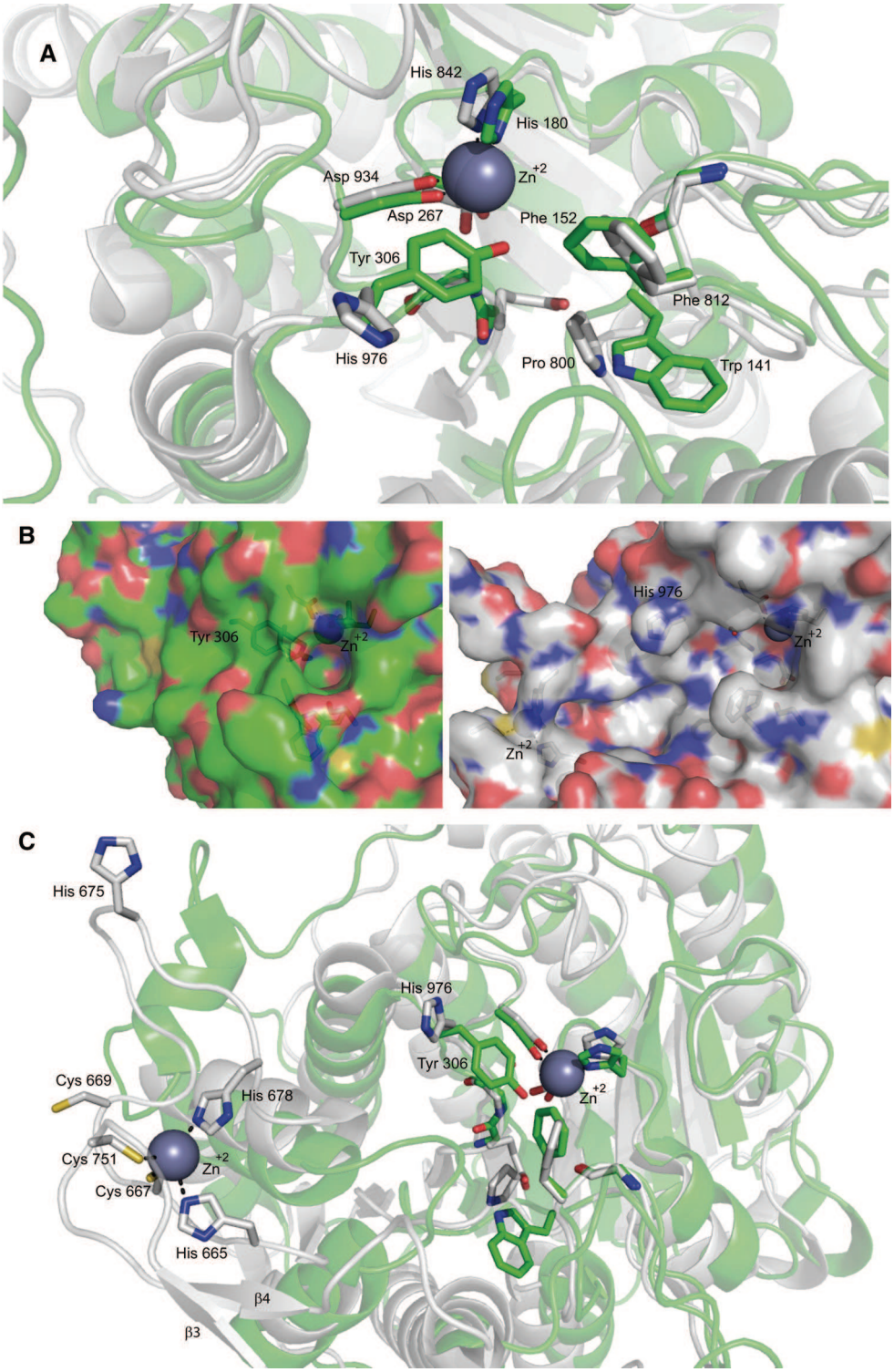
## Class IIa HDACs: to be or not to be a lysine deacetylase

In humans there are 18 HDACs grouped into five different classes according to phylogenesis and sequence homology [7]. Class I HDACs (including HDAC1, 2, 3 and 8), class IIb HDACs (including HDAC6 and 10), class III HDACs or Sirtuins (including all Sirtuins from 1 to 7) and class IV (HDAC11) all displaying enzymatic activities [23]. By contrast, when we discuss about class IIa HDACs (HDAC4, 5, 7 and 9) as histone deacetylases, it should be taken into account that these proteins show an extremely low enzymatic activity against acetylated lysines [24, 25] and are rarely associated with histone tails [26].

Structurally, class IIa HDACs can be divided into two parts: the N- and the C-terminal regions (Fig. 1). The N-terminal regulates the nuclear import and contains a coiled-coil glutamine-rich domain that is peculiar of the family. This region is highly devoted to protein–protein interactions both in terms of homo- and of heterotypic partners. The C-terminal region contains the catalytic "deacetylase" domain and the nuclear export sequence (Fig. 1). These enzymes are under the control of different signaling pathways, which operate through specific PTMs to influence peculiar aspects of the class IIa biology, including the nuclear/cytoplasmic shuttling (for reviews [7, 12, 27, 28]).

The deacetylase domain is made up of approximately 400 residues (aa) arranged into 21 α-helices and 10 β-strands organized in a single domain, structured around a central "catalytic" $Zn^{2+}$ ion [29]. Likewise to class I HDACs, 2 aspartates and an histidine coordinate this $Zn^{2+}$ while 2 other aspartates (Fig. 2a), another histidine, a serine and a leucine coordinate two potassium ions [29–31]. Despite this high similarity, in vertebrates class IIa possess a bigger active site than class I HDACs (Fig. 2b), which impacts on their druggability [26, 29]. The evolution-related event responsible for this structural peculiarity is the mutation of a tyrosine into a histidine, Y967H in HDAC4 [25]. Histidine is sterically less cumbersome and induces the relaxation of the structure. As a consequence, this histidine is far from the central $Zn^{2+}$ and not able to form hydrogen bonds with the intermediate of the enzymatic reaction (Fig. 2a). The intermediate is, therefore, very unstable, thus resulting in an ineffective reaction. Nevertheless, class IIa can efficiently process alternative substrates such as trifluoroacetyl-lysine. Mechanistically, the presence of the trifluoro group should destabilize the amide bond, hence favoring the reaction even in the absence of transition-state stabilization [25].

Importantly, replacing back the His with Tyr generates class IIa HDACs with a catalytic efficiency 1,000-fold higher compared to the wild-type (wt) form [25, 31]. Nonetheless, this mutant does not show enhanced repression respect to the wt, at least in the instance of MEF2-dependent transcription, a well-known class IIa partner [25].




Another distinctive feature of class IIa HDAC catalytic site is the existence of a Zinc Binding Domain (ZBD). This ZBD consists in a β-hairpin surrounded by two antiparallel β-strands, forming a pocket-like structure that accommodates a second "structural" zinc ion [29]. In the case of HDAC4 three cysteines (667, 669, 751) and one histidine

◀**Fig. 2** Representation of class I and class IIa catalytic sites (**a**, **b**) and the zinc binding domain (**c**). **a** Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (*green*) and of class IIa HDAC4 (*white*) catalytic sites. As mentioned in the text the His 976 is rotated away from the active site differently from Tyr 308 in HDAC8. **b** Surface representation of class I HDAC8 (*green*) and class IIa HDAC4 (*white*) catalytic sites. The figure shows the hydrophilic tunnel necessary for the release of the reaction product in HDAC8 (*green*), while in HDAC4 (*white*) the His/Tyr substitution prevents tunnel formation. **c** Superimposition of the inhibitor (TFMK)-bound ribbon structure of class I HDAC8 (*green*) and of class IIa HDAC4 (*white*) catalytic site (*right*) and zinc binding domain of HDAC4 (*left*). β3 and β4 are the two antiparallel β-strands involved in the formation of the pocket-like structure in the zinc binding domain. Importantly, His 665 and His 678 in this inhibitor-bound structure are replaced by Cys 669 and His 675 in the coordination of the zinc ion in the Apo-structure. Unfortunately the crystallization of Apo-HDAC4 was unsuccessful and these differences are deduced from crystallographic studies of the mutant GOF (H976Y) of HDAC4 [31]. The coordinates of the protein structures were retrieved from the protein data bank. Amino acids discussed in the text are labeled and shown in stick representation. The accession codes for the protein structures are: 2VQJ (HDAC4) and 1T69 (HDAC8). Figures are edited using PyMOL Molecular graphics system, Schrödinger, LLC

(675), conserved only among class IIa HDACs, coordinate this $Zn^{2+}$ and made the so-called "core" of the domain [31] (Fig. 2c). Importantly, the inhibitor-bound structure is shown in this figure, where, respect to the Apo-structure, Cys 669 and His 675 replace His 665 and His 678 in the coordination of the $Zn^{2+}$.

This domain is extremely flexible and the oxidation of the cysteines involved in $Zn^{2+}$ coordination (667 and 669 in HDAC4) is sufficient to free the metal, with the consequent opening and deconstruction of the ZBD [31]. Because this domain is head-to-head to the active site (Fig. 2c), it contributes to make the class IIa HDACs' catalytic site more accessible than that of class I HDACs (Fig. 2b) and does not allow the formation of an efficient hydrophilic tunnel necessary for the release of the acetate reaction product [30, 31].

## Old structures and new functions

The enzymatic ineptitude of vertebrates' class IIa deacetylase domain raises several questions and opens the door to different hypothesis. First, they are not completely silenced enzymes. Because class IIa is capable of processing trifluoroacetyl-lysine with high efficiency, still undiscovered new natural substrates could exist [25]. Alternatively, the described enzymatic activity could simply mark a lab finding, without biological implications. Second, as anticipated above, the absence of improved repressive influence in the case of the gain of function His/Tyr substitution in HDAC4, further demonstrates that class IIa HDACs can repress transcription independently from the deacetylase domain [25]. The relevance of the deacetylase-independent repression is testified by MITR, a splice variant of HDAC9 lacking the deacetylase domain [32]. The existence of MITR supports the possibility that the HDAC domain is of little relevance for the functions of class IIa HDACs and may lead to believe that it is an evolutionary heritage intended to being missed. However, since class IIa deacetylase domain has been preserved behind two duplication events occurred during evolution of vertebrates, evolutionists deny the hypothesis that this domain would be subjected to a negative purifying selection [33].

Although there are evidences pointing to deacetylase-independent activities of class IIa, generation of a mouse model in which, mutated versions of this domain can be analyzed in a physiological context will help our understanding. This point is of crucial relevance for the design and development of class IIa inhibitors.

Along with the enzymatic activity, the deacetylase domain can operate as a scaffold for the recruitment of multi-protein complexes containing class I HDAC3 and other co-repressors [31]. HDAC4 interacts with the RD3 domain of N-CoR [24, 34], while HDAC3 binds the SAINT domain [35] and, as a matter of fact, HDAC4 binds N-CoR/SMRT regardless of HDAC3 and only in a second time the deacetylase is recruited [36]. However, the precise order of the sequential molecular interactions driving the assembly of the multi-protein complex is still waiting for a final verification.

When class IIa HDACs are isolated under native conditions, a lysine deacetylase activity can be measured. This activity is due to class I HDACs co-purified with class IIa [24, 37, 38]. The existence of a heterogeneous repressive complex complicates the assessment of effectiveness and specificity of HDACis, when tested on proteins purified from cells or tissues.

A final consideration refers to a fascinating hypothesis, which attributes to class IIa deacetylase domain the function of acetylated lysine reader [26]. In this view, class IIa could act as readers and interpreters of the histone code, thus orchestrating the epigenetic status thanks to their capability of recruiting additional enzymes, such as methylases [39] or deacetylases [24, 36]. A scenario where class IIa HDACs, acting as molecular scaffolds supervise the introduction of different epigenetic markers, onto specific regions of chromatin or in proximity of different acetylated cellular protein. In this context inhibitors of the deacetylase domain could in principle both interfere with the reading activity or, by promoting structural changes, with the possibility of recruiting additional co-repressors.

## Unresolved issues

Biochemically, the enzymatic activity associated to class IIa HDACs could be explained by the recruitment of class I enzymes [24]. Moreover, all the point mutants of the HDAC4 deacetylase domain which, accordingly to Finnin model [40], abrogate its enzymatic activity (H803A, G811A, D838A, D840A, H842A, N845D, D934 N, E973G) demonstrate a perfect correlation between enzymatic activity and the ability to recruit HDAC3 [24]. Classic deacetylase activity is not associated with a cytoplasmic HDAC7 or HDAC4 immunoprecipitated from HEK293 cells and therefore, weakly associated to the mainly nuclear HDAC3 [24, 36]. Similarly, HDAC4 mutants that have lost the ability of binding to N-CoR/SMRT drop the deacetylase activity [24]. Despite in vitro binding experiments prove that the fraction of HDAC3 in complex with HDAC4 is relevant, in vivo HDAC3 preferentially forms homodimers, rather than heterodimers with HDAC4 [41]. Furthermore, the fraction of HDAC4 co-purified with HDAC3 in mammalian cells is extremely low [24, 35, 37].

As aforementioned, another peculiar feature of class IIa deacetylase domain is its sensitivity to redox conditions [31, 42]. Particularly, in HDAC4 the oxidation of cysteines 667 and 669 induces the formation of a disulphide bond that causes the exposition of the NES, the export in the cytoplasm and also the detachment of HDAC3 [31, 42, 43]. This oxidation causes the de-structuration of the HDAC domain because Cys 667 and Cys 669 are directly involved in the "structural" $Zn^{2+}$ coordination and substrate binding [29, 31] (Fig. 2c). These findings show that researchers should be extremely cautious in verifying the redox status when studying class IIa deacetylase domain.

In addition to nuclear roles of class IIa HDACs, recently, a cytoplasmic enzymatic activity has been reported towards non-histone substrates [reviewed in 44]. During muscle denervation HDAC4, which plays a pro-atrophic role in this context [45, 46] can deacetylate and activate MEKK2 [47]. Kinase engagement culminates in AP-1 activation and cytokines production that stimulate muscle remodeling [47]. Interestingly only the wild-type form, capable of shuttling between the nucleus and the cytoplasm and not a nuclear resident mutant of HDAC4 deacetylated MEKK2. Importantly, this activity is independent from HDAC3 and is not shared with HDAC5 [47]. Paradoxically, MEKK2 activation should activate ERK5 and therefore MEF2s, thus pointing to a positive rather than repressive influence of HDAC4 versus MEF2s [48, 49]. A similar cytoplasmic KDAC (lysine deacetylase) activity of class IIa HDACs was reported towards HIF-1α and STAT-1. Also in these circumstances class IIa deacetylase activity seems to be independent from class I HDACs [44].

Another unresolved issue is the requirement of additional factors to exert the full enzymatic activity. Class I HDACs require particular cofactors both for histone and non-histone substrates [35, 41, 50]. For the enzymatic activity of class IIa HDACs towards the synthetic trifluoroacetyl-lysine or against these cytoplasmic partners, any cofactor seems to be dispensable [25].

## The rationale for developing class IIa HDACs inhibitors

HDACis have entered multiple clinical trials principally in virtue of their anti-neoplastic properties [10]. Much more emphasis has been pushed on the identification, synthesis and characterization of class I HDACis. Commonly HDACis show a selective cytotoxicity against tumor cells and weak effects on normal ones [11, 51, 52]. These molecules display cytostatic effects, especially through the induction of p21 and blockage of the cell cycle [53, 54] or by triggering apoptosis via multiple mechanisms [11, 53, 55, 56]. Some HDACis in vivo stimulate also the clearance of tumor cells from the immune system [57, 58] or block angiogenesis [59, 60]. Despite these promising anti-neoplastic properties, entering of HDACis in clinic is slower than expected, principally due to some side effects and toxicity displayed during early-phase clinical trials [14, 61]. In fact, up to now only two HDACis have been approved for the treatment of cutaneous T cell lymphoma: SAHA (Zolinza) in 2006 and Romidepsin/FK-228 in 2009. In 2011 the depsipeptide FK-228 has been further approved for the treatment of peripheral T-cell lymphoma [15]. Considering the recent evidences about a pro-oncogenic potential of class IIa HDACs [12, 37, 38, 62–64] and their impact on epigenetics [65], a stratagem to circumvent the side effects of class I HDACs inhibitors might consist in targeting class IIa HDACs.

Theoretically, targeting class IIa HDACs with specific inhibitors has three major drawbacks:

1. The high similarity of the catalytic site of these proteins to class I HDACs, which makes selective targeting rather difficult to achieve;
2. The formal question about the legitimacy of hitting the catalytic site of proteins that are almost enzymatically inactive against acetylated lysines. About this consideration the work of Bottomley et al. [31] explains how targeting of the catalytic site of class IIa HDACs and in particular the $Zn^{2+}$ atom could impact on the structure of the C-terminus of the proteins, thus compromising their capability to interact with the super complex HDAC3/N-CoR/SMRT. Therefore, targeting class IIa HDAC domain could be an indirect strategy to impact on class I HDACs. By releasing

only class IIa driven deacetylation, a more selected transcriptional re-setting can be achieved, which could favor a drop in toxicity.

3. The methodological approach to measure class IIa HDAC inhibition. Up to now the best-characterized substrate for probing the elusive catalytic activity of vertebrate class IIa histone deacetylases is trifluoroacetyl-lysine [25, 66]. The activity of class I HDACs towards this molecule is indiscernible. Its use as a substrate for the validation of an inhibitor efficiency could exclude all class I HDACs as off-targets. Class IIa HDAC enzymatic activity measured with other methods or with classical substrates (e.g., acetylated H3) or commercial assays, generally based on acetyl-Lys, is extremely low when recombinant proteins are used [24]. Instead, when class IIa are purified from vertebrates the enzymatic activity can be provided by associated class I or IIb enzymes [24, 25, 31, 67]. Therefore, a double check approach should be used to test the potency and specificity of a class IIa HDACis. The potency of the compound should be evaluated by employing trifluoroacetyl-lysine, as a class IIa specific substrate, while its inhibitory activity against other HDAC classes should be excluded using "classical" substrates, such as acetylated lysines. A simplified screening could take advantage from the recently developed trifluoroacetyl-lysine derivative, a trifluoro acetyl-lysine tripeptide named substrate 6, which can be processed by all HDACs, with the exclusion of HDAC10 and 11. This molecule looks like a promising tool for single-run screening aimed to isolate/characterize subtype specific HDACis [68].

## Class IIa inhibitors

Three different peculiarities of class IIa HDACs have been exploited to design specific inhibitors:

a. The catalytic site, and in particular the $Zn^{2+}$ atoms.
b. The nuclear/cytoplasmic shuttling.
c. The N-terminal region and the binding to specific partners, such as the MEF2 family of TFs.

### Targeting the $Zn^{2+}$ binding domain

In accordance to the connecting unit (CU) linker chelator pharmacophore model [16, 69], a classical HDACi is composed of three parts [17]:

1. The MBG (metal binding group or zinc binding group ZBG), which is a group capable of chelating the $Zn^{2+}$ in the catalytic site of HDACs (with the exception of sirtuins).
2. The connecting unit (CU), generally a linker hydrophobic region of five or more carbons, that mimics the acetyl-lysine. It could be linear or aromatic and it perfectly fits to the hydrophobic catalytic site of the targeted HDAC.
3. The CAP hydrophobic domain (usually aromatic) that interacts with aminoacids delimiting the border of the deacetylase catalytic site.

Slight modifications of the described structure impact both on the specificity and potency of the inhibitor.

The availability of the crystal structure of the class IIa deacetylase domain [29, 31] has encouraged the development and synthesis of many hydroxamates stemmed from SAHA, with the purpose of selectively influencing class IIa HDACs. In particular to improve specificity, many efforts have been spent in the modification of the CAP and of the ZBG of SAHA. In principle, the selective targeting of class IIa HDACs would require only some changes in the linker region, to better fit the peculiar catalytic site of class IIa HDACs. A recent study effectively demonstrated that slight modifications only in the linker region of SAHA increase the selectivity towards class IIa and class IIb HDACs [70]. However, the achieved results were not as promising as those obtained after modification of both the CAP and the linker region of SAHA [71]. This double tuning seems to be the better strategy to produce SAHA derivatives specific for class IIa HDACs. In a next future, new generation class IIa HDACis could stem from Tasquinimod (described below) that selectively targets the "structural" and not the "catalytic" $Zn^{2+}$. This peculiarity should increase the specificity because, as discussed above, this "structural zinc" is unique of class IIa HDACs. A summary of the literature data is shown in Fig. 3.

The most characterized of these hydroxamate-like drugs, are:

– MC1568 and MC1575 (Fig. 3, please note that in Fig. 3 we provide for MC1568 the recently reassigned structure [72]) are two class II HDACs inhibitors specific for HDAC4 and HDAC6 [73–76]. They are derivatives of classical class I HDACs inhibitors aroyl-pyrrolyl–hydroxyamides (APHAs), showing selectivity towards class IIa HDACs. The modified linker region provides this selectivity. Compared to the original class I inhibitors, they exhibit a decreased cytotoxic effect [73]. Despite this fact, MC1568 and MC1575 show some cytostatic effects in melanoma cells [76] and in ER + breast cancer cells [74]. The anti-proliferative effect is provoked by a block in the G1 phase of the cell cycle, through the induction of the Cdk inhibitor p21/Cip1/Waf1 [74]. MC1568 efficacy

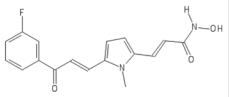
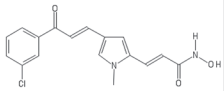
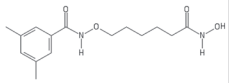
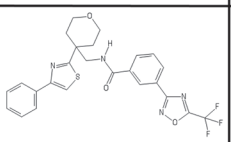
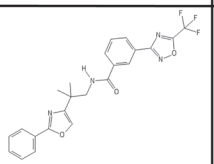
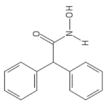
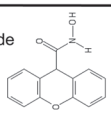
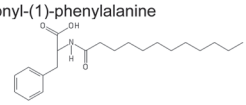
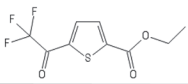
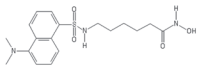
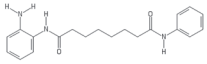
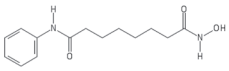
| | | IC50 (μM) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CLASS I | | | | CLASS IIa | | | | CLASS IIb | | CLASS IV | | |
| Name | Structure | HDAC1 | HDAC2 | HDAC3 | HDAC8 | HDAC4 | HDAC5 | HDAC7 | HDAC9 | HDAC6 | HDAC10 | HDAC11 | Substrate | Re. |
| MC1568 |  | 38.72 | NA | NA | NA | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | NA | NA | Acetyl.pep | [73] [75] |
| MC1575 |  | >20 | >20 | >20 | >20 | 5 | NA | NA | NA | NA | NA | NA | Class I Acetyl-H3 Class IIa Trifluoroacetyl Lys | [74] |
| LMK235 |  | 0.320 | 0.881 | NA | 1.278 | 0.0119 | 0.00422 | NA | NA | 0.0557 | NA | 0.852 | Class I ac-H3 Class IIa Boc-Lys trifluoro-acetyl-AMC Class IIb and IV ac-p53 | [71] |
| TMP269 |  | >100 | >100 | >100 | 4.2 | 0.157 | 0.097 | 0.043 | 0.023 | 8,2 | >100 | >100 | Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9 | [26] |
| TMP195 |  | >100 | >100 | >100 | 11.7 | 0.111 | 0.106 | 0.046 | 0.009 | 47,8 | >100 | >100 | Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9 | [26] |
| N-hydroxy-2,2 -diphenylacetamide |  | >10 | 6.06 | NA | 66 | 0.75 | 0.14 | 0.39 | NA | >10 | NA | NA | Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(e-trifluor methylacetyl-AMC HDAC6/8 $^3$H-histone H4 peptide | [80] |
| N-hydroxy-9H -xanthene-9-carboxamide |  | NA | NA | NA | NA | 0.25 | 0.11 | 0.05 | NA | NA | NA | NA | Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC HDAC6/8 $^3$H-histone H4 peptide | [80] |
| N-lauronyl-(1)-phenylalanine |  | >100 | NA | NA | NA | NA | NA | 21 | NA | >100 | NA | NA | Boc-Lys-(Ac)-AMC | [81] |
| Ethyl 5-(trifluoracetyl) thiopene-2-carboxylate |  | 5.7 | NA | 3.5 | NA | 0.32 | NA | NA | NA | 0.55 | NA | NA | Fluor de Lys HDAC1,3 Trfluoroacetamide -Lys HDAC4,6 | [67] |
| Compound 2 |  | 0.95 | 1.38 | 1.12 | 3.98 | 0.33 | 0.40 | 2.56 | NA | 0.13 | 0.42 | 0.48 | Fluor de Lys | [85] |
| BML-210 |  | 37.06 | 22.76 | 5.09 | >300 | NA | NA | >300 | NA | >300 | >300 | NA | Competition binding | [118] |
| SAHA |  | 0.22 | 0.56 | 1.79 | 2.74 | >10 | 1.3 | >10 | >10 | 0.027 | 0.11 | 0.082 | Class I, IIb, IV Fluor de Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC | [80] [85] |

**Fig. 3** Structures and summary of the available literature data on the IC50 for the proposed class IIa inhibitors

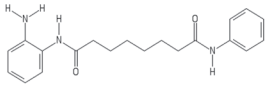
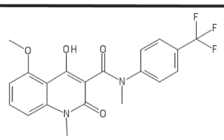
in cancer cells finds rationality in the capability of up-regulating the tumor suppressor Brahma, repressed by HDAC9 [77]. Curiously, MC1568 has been reported stabilizing the HDAC4-MEF2D complex in differentiated C2C12 myoblasts, thus impairing instead of favoring myogenesis [78].

– LMK235 (N-((6-(hydroxyamino)-6-oxoh exyl)oxy)-3,5-dimethylbenzamide) is a hybrid between two

classes of class I HDACis: the hydroxamic acids and the benzamides (Fig. 3) [71]. The specificity towards HDAC4 and HDAC5 is conveyed by the hydrophobic dimethyl substituted phenyl ring, which acts as a CAP group, matching class IIa active site better than class I [71]. This modification makes the molecule less toxic and more suitable for the treatment of some malignances, when compared to class I HDACis. Furthermore, LMK235 is able to re-sensitize cancer cells to cisplatin, better than SAHA [71].

– TMP269 and 195 (Figs. 3, 5) are two recently developed class IIa HDACis in which the classical hydroxamic $Zn^{2+}$ binding domain is substituted by a trifluoromethyloxadiazolyl group (TFMO) [26] that highly resembles the trifluoromethylketone (TFMK) adopted by Bottomley and colleagues in their biochemical study of the ZBD [31]. The ring structure of the TFMO group increases its stability with respect to the highly unstable TFMK series of compounds [79]. Moreover, this TFMO moiety, differently from hydroxamate, acts as a non-chelating metal binding group, which interacts with the "catalytic" $Zn^{2+}$, through weak electrostatic interactions. As a consequence, the TFMO series has fewer off-targets compared to hydroxamates. Augmented selectivity is indirectly proved by gene expression profile studies in (PHA)-activated human peripheral blood mononuclear cells (PBMC) (Fig. 5). In these cells SAHA modulates the expression of 4,556 genes, whereas TMP195 regulates only 76 genes [26]. Curiously this finding is in accordance to what was observed in fibroblasts, where HDAC4 directly modulate only 76 genes [38]. To better characterize the transcriptome profile induced by their TFMO series of compounds, Lobera and colleagues purified T cells (CD3+), B cells (CD19+) and monocytes (CD14+) from the PHA-stimulated PBMC population and separately treated the three subpopulations with TMP195. T and B cells turned out to be very low sensitive to TMP195 (17 and 36 genes regulated, respectively); on the contrary the effect of the compound on monocytes was impressive (587 genes) and was not due to an increase in the expression of class IIa HDACs in these cells compared to the other two cell types. In particular the inhibitor interfered with monocytes to macrophages M-CSF (macrophage colony-stimulating factor)-induced differentiation. These findings candidate class IIa HDACs as druggable targets for immunological diseases [18, 71].
– *N*-hydroxy-2,2-diphenylacetamide and *N*-hydroxy-9H-xanthene-9-carboxamide (respectively, compound 6 and 13 in the original manuscript) are two diphenylmethylene hydroxamic acids characterized by Besterman group as class IIa HDACs specific inhibitors active in the μM range [80]. Both molecules exhibit a certain degree of symmetry and the second compound could be considered as the rigidification of the diphenyl moiety of the first (Fig. 3). This modification increases the specificity of the molecule towards HDAC7 [80].
– *N*-lauroyl-(l)-phenylalanine is a class IIa HDACi active in the μM range (Fig. 3) [81]. It was identified during a screening of a commercial available library of compounds. The specificity was scored not merely by classical measurements of HDAC activity but also through a fluorescence assay, which exploits the competition between a fluorescent substrate and the putative inhibitor for each purified HDAC [81]. This molecule shows anti-tumoral properties against ER+ breast cancer cells and can influence the expression of some MEF2-target genes (Fig. 5) [37].
– Ethyl 5-(trifluoroacetyl)thiophene-2-carboxylate [67] is the founder of a class of compounds, the trifluoroacetylthiophenes, that targets class II HDACs (class IIa and HDAC6) with some specificity. It was identified during a screening of a commercially available library of compounds using both the wt and the GOF mutant of HDAC4 as targets. It is a tripartite molecule characterized by: (i) a trifluoromethyl ketone group that chelates the active site zinc in a bidentate manner, (ii) the central thiophene ring that fits perfectly to class IIa active site and (iii) the amide group that interacts with the surrounding residues. The chemistry and the trifunctional nature of this compound justify its specificity.
– Tasquinimod (Fig. 4) is a promising drug for the treatment of advanced castration resistant prostate cancers [82, 83]. It acts by perturbing the tumor microenvironment. Differently from the aforementioned molecules it was not rationally designed or screened to target HDACs. Nevertheless, this carboxamide is able to enter the ZBD of HDAC4, keeping it in the inactive form and thus reorganizing the HDAC4 catalytic site. Tasquinimod-induced structural changes are causative of N-CoR/SMRT/HDAC3 displacement [43]. This finding is surprisingly considering the pronounced steric hindrance of the molecule, which is profoundly different from all SAHA derivatives. However, by virtue of its selective targeting of the "structural" $Zn^{2+}$", Tasquinimod molecular backbone could substitute SAHA as starting model for the development of specific inhibitors. From a molecular point of view the inactivation of HDAC4 prevents HIF-1α deacetylation, thus inducing its destabilization. Clinically, in hypoxic conditions the activation of HIF-1α transcriptional program stimulates the differentiation of tumor infiltrating myeloid derived suppressor cells into tumor-associated macrophage,

**Fig. 4** Structure and binding interference properties of BML210 and Tasquinimod, two compounds capable of altering interaction of class IIa HDACs with their partners

| Name | Structure | DISPLACEMENT FROM PROTEIN BINDING IC50 (μM) | | Assay | Re. |
|---|---|---|---|---|---|
| | | HDAC4 - MEF2 | HDAC4 - N-CoR | | |
| BML210 |  | 5 | NA | Two-Hybrid in HeLa cells | [95] |
| Tasquinimod |  | NA | <1 | Co-Ip in 293 cells | [43] |

which secrete pro-angiogenic factors [84]. Authors, therefore, proposed Tasquinimod as an anti-angiogenetic drug, which anti-cancer efficacy is being evaluated in pre-clinical models [43].

These last three molecules are considered unconventional inhibitors because, even though characterized by a tripartite motif, they are not SAHA derivatives.

## Targeting the nuclear-cytoplasmic shuttling

In 2011, Brown group made the first attempt of blocking class II HDACs in the cytoplasm [85]. Starting from the structure of SAHA, they generated a couple of molecules by substituting the amino-phenyl group with a fluorescent dansyl group. This modification increases the specificity for class II HDACs in spite of a loss of reactivity against class I HDACs. If used in the μM range, the most effective molecule of the series, named compound 2 (Fig. 3), increases the fraction of cytoplasmic HDAC4 in prostate cancer cells PC3. The authors suggested that since the inhibitor accumulates in the cytoplasm, it binds HDAC4, thus impeding the interaction with importin-1α. As a consequence, the inhibitor increases the fraction of cells in the G1 phase of the cell cycle, the levels of p21/Cip1/Waf1, of acetylated H3 and tubulin. The increase of tubulin acetylation is probably due to the inhibition of HDAC6 [86] and seems to be unrelated to the suppression of class IIa [85].

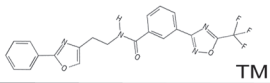
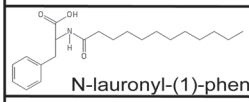
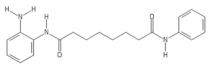
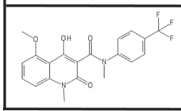
It must be underlined that the IC50 values of these new inhibitors have been estimated by measuring the enzymatic activities of HDACs purified from mammalian cells, using the Fluor–de-Lys substrate [85]. Therefore, in the case of class IIa HDACs, it must be intended as indirect, deriving principally from the associated class I HDACs.

The strategy of interfering with class IIa HDACs nuclear accumulation could be attractive in oncology, as increasing evidences demonstrate that nuclear resident class IIa can display oncogenic functions [37, 38], but it might also present some drawbacks. First of all, class IIa HDACs possess also cytoplasmic functions [reviewed in 44], which could be amplified after inhibition of their nuclear import. Moreover, the cytoplasmic accumulation of class IIa HDACs is sometimes an indirect still uncertain effect of class I inhibition. For example the class I/II inhibitor LBH589, which is a SAHA derivate, confines HDAC4 in the cytoplasm in irradiated non-small cell lung cancer cells [87]. Considering all these drawbacks, the nucleus/cytoplasmic shuttling of class IIa HDACs seems to be the less druggable feature of these proteins.

## Class IIa HDACs N-terminus, which allows their interaction with some partners, such as MEF2 family of TFs

As discussed above, class IIa HDACs' N-terminal region (Fig. 1) mediates the interaction with multiple partners and contains a glutamine-rich domain (with the exception of HDAC7) that allows homo- and heterodimerization among the different class IIa members [12, 88]. The best-characterized class IIa transcriptional partners are the MEF2s proteins [49, 89]. Several of the biological functions attributed to class IIa HDACs are the results of the MEF2s transcriptional repression [27, 37, 38]. The phenotype of the single knock-out of class IIa HDACs could be explained as the effect of MEF2 over-activation in bone (HDAC4), heart (HDAC5/9) and cardiovascular system (HDAC7), in relation to the district in which the single HDACs are more abundant [90–92]. Hence, the design of an inhibitor that displaces class IIa HDACs from MEF2s could be a good approach to selectively interfere with this specific repressive exploit. A limitation to this strategy concerns the promiscuity of the class IIa HDACs sequence required for this interaction (aa 166-184 in HDAC4). In fact, this stretch of amino acids is also involved in the interaction with additional partners, among which, the nucleoporin Nup155 [93] and the demethylase JARID1B [94] (Fig. 1). An alternative plan to influence the MEF2-

**Fig. 5** Summary of the available literature data on the effect of class IIa HDACs inhibitors on MEF2s-dependent transcription. MEF2s are the foremost characterized transcriptional partners of class IIa HDACs. Hence, an effect of these inhibitors on the expression of MEF2s target genes is an important read-out of their activity

| Compound | Cells | Treatment | Up-reg. MEF2-targets | Experiment | Re. |
|---|---|---|---|---|---|
|  TMP195 | PBMCs PHA-stimulated | 3μM 60 hrs | *ASB2,CCL1,ATP1B2* | DNA Microarray | [26] |
|  N-lauronyl-(1)-phenylalanine | MCF7 | 100μM 48 hrs | *KLF2, RHOB, NR4A1 KLF3, MARK1, GADD45γ* | qRT PCR | [37] |
|  BML-210 | SK-UT-1 SK-LMA-1 DMR | 10μM 36 hrs | *KLF2, RHOB, NR4A1* | qRT PCR | [38] |
|  Tasquinimod | LNCap | 50μM 24 hrs | *IRS1, DTNA, ARRDC3 LHX4, KCNG1, ISL2* | DNA Microarray | [82] |

HDAC axis could be targeting the region of MEF2s that interacts with class IIa HDACs. Using this approach, BML-210 (Figs. 3, 4, 5), a weak class I HDAC benzamide inhibitor, was found to interact through its aminophenyl-group with the hydrophobic residues of MEF2s (aa 66-69) thus displacing class IIa HDACs [95]. Using the crystal structure of the HDAC9–MEF2B complex as a guide [96], authors generated a panel of more powerful BML-210 derivatives. In the next future it will be important to further improve the specificity of these compounds to exclude residual targeting of class I HDACs.

## Conclusions and perspectives

The identification of molecules that could reset the transcriptional profile in neoplastic cells has raised many hopes for new anti-cancer therapies [97]. Unfortunately today this goal has been only partially reached. Nevertheless an epigenetic therapy against cancer is still subject of intense research. A new impetus in this field was given by the discovery of the demethylases [98, 99] and the synthesis of their specific inhibitors [100]. A more niche-research concerns class IIa HDACs and their selective inhibitors, which are hypothesized to be less powerful than pan-HDACis but more specific. However, these studies are still in their infancy and the applicability of class IIa HDACis in clinic requires still intense laboratory characterization. Additional experiments and data are mandatory to characterize and understand the contribution of these molecules to epigenetic changes in vivo. Up to now, information about the impact of class IIa HDACis on RNA non-coding world and the role of class IIa HDACs in stemness maintenance are very limited [101]. In parallel the efforts trying to design, isolate and characterize new compounds, acting as epigenetic regulators must persist. In addition, a robust in vitro pre-clinical characterization of molecules already available is needed to define: their molecular mechanism of action, their ideal context of utilization and off-targets effects. All these efforts are justified by the benefits that drug-induced genetic reprogramming could exert on different diseases.

Certainly anti-cancer therapy is the first and most important scope. Nevertheless, the involvement of class IIa HDACs in the regulation of Glut4 [102–105], of the NF-kB pathway [106, 107] and of many neuronal activities [108–111] could stimulate studies about the employment of class IIa HDACis for the treatment of diseases other than cancer, such as diabetes [112], neurodegenerative disorders [113, 114] and inflammatory diseases [26, 115–118]. There are opportunities out there; we just have to find out what is the best compound for each specific application.

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## References

1. Gräff J, Tsai L-H (2013) Histone acetylation: molecular mnemonics on the chromatin. Nat Rev Neurosci 14:97–111. doi:10.1038/nrn3427
2. Helin K, Dhanak D (2013) Chromatin proteins and modifications as drug targets. Nature 502:480–488. doi:10.1038/nature12751
3. Hatziapostolou M, Iliopoulos D (2011) Epigenetic aberrations during oncogenesis. Cell Mol Life Sci 68:1681–1702. doi:10.1007/s00018-010-0624-z
4. Dawson MA, Kouzarides T (2012) Cancer epigenetics: from mechanism to therapy. Cell 150:12–27. doi:10.1016/j.cell.2012.06.013
5. Wang Z, Zang C, Cui K et al (2009) Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell 138:1019–1031. doi:10.1016/j.cell.2009.06.049
6. Mihaylova MM, Vasquez DS, Ravnskjaer K et al (2011) Class IIa histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. Cell 145:607–621. doi:10.1016/j.cell.2011.03.043
7. Yang X-J, Seto E (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol 9:206–218. doi:10.1038/nrm2346

8. Delcuve G, Khan D, Davie J (2012) Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin Epigenenetics 4:5. doi:10.1186/1868-7083-4-5
9. Di Micco R, Sulli G, Dobreva M et al (2011) Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat Cell Biol 13:292–302. doi:10.1038/ncb2170
10. Bolden JE, Peart MJ, Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 5:769–784. doi:10.1038/nrd2133
11. Henderson C, Brancolini C (2003) Apoptotic pathways activated by histone deacetylase inhibitors: implications for the drug-resistant phenotype. Drug Resist Updat 6:247–256. doi:10.1016/S1368-7646(03)00067-0
12. Clocchiatti A, Florean C, Brancolini C (2011) Class IIa HDACs: from important roles in differentiation to possible implications in tumourigenesis. J Cell Mol Med 15:1833–1846. doi:10.1111/j.1582-4934.2011.01321.x
13. Aygün O, Mehta S, Grewal SIS (2013) HDAC-mediated suppression of histone turnover promotes epigenetic stability of heterochromatin. Nat Struct Mol Biol 20:547–554. doi:10.1038/nsmb.2565
14. Prince HM, Bishton MJ, Harrison SJ (2009) Clinical studies of histone deacetylase inhibitors. Clin Cancer Res 15:3958–3969. doi:10.1158/1078-0432.CCR-08-2785
15. Balasubramanian S, Verner E, Buggy JJ (2009) Isoform-specific histone deacetylase inhibitors: the next step? Cancer Lett 280:211–221. doi:10.1016/j.canlet.2009.02.013
16. Bertrand P (2010) Inside HDAC with HDAC inhibitors. Eur J Med Chem 45:2095–2116. doi:10.1016/j.ejmech.2010.02.030
17. Giannini G, Cabri W, Fattorusso C, Rodriquez M (2012) Histone deacetylase inhibitors in the treatment of cancer: overview and perspectives. Future Med Chem 4:1439–1460. doi:10.4155/fmc.12.80
18. Thaler F, Mercurio C (2014) Towards selective inhibition of histone deacetylase isoforms: what has been achieved, where we are and what will be next. Chem Med Chem 9:523–536. doi:10.1002/cmdc.201300413
19. Dekker FJ, van den Bosch T, Martin NI (2014) Small molecule inhibitors of histone acetyltransferases and deacetylases are potential drugs for inflammatory diseases. Drug Discov Today 19:654–660. doi:10.1016/j.drudis.2013.11.012
20. Dokmanovic M, Clarke C, Marks PA (2007) Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 5:981–989. doi:10.1158/1541-7786.MCR-07-0324
21. West AC, Johnstone RW (2014) New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 124:30–39. doi:10.1172/JCI69738
22. Zhang J, Zhong Q (2014) Histone deacetylase inhibitors and cell death. Cell Mol Life Sci. doi:10.1007/s00018-014-1656-6
23. Haberland M, Montgomery RL, Olson EN (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet 10:32–42. doi:10.1038/nrg2485
24. Fischle W, Dequiedt F, Hendzel MJ et al (2002) Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR. Mol Cell 9:45–57. doi:10.1016/S1097-2765(01)00429-4
25. Lahm A, Paolini C, Pallaoro M et al (2007) Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. Proc Natl Acad Sci USA 104:17335–17340. doi:10.1073/pnas.0706487104
26. Lobera M, Madauss KP, Pohlhaus DT et al (2013) Selective class IIa histone deacetylase inhibition via a nonchelating zinc-binding group. Nat Chem Biol 9:319–325. doi:10.1038/nchembio.1223
27. Martin M, Kettmann R, Dequiedt F (2007) Class IIa histone deacetylases: regulating the regulators. Oncogene 26:5450–5467. doi:10.1038/sj.onc.1210613
28. Wang Z, Qin G, Zhao TC (2014) HDAC4: mechanism of regulation and biological functions. Epigenomics 6:139–150. doi:10.2217/epi.13.73
29. Schuetz A, Min J, Allali-Hassani A et al (2008) Human HDAC7 harbors a class IIa histone deacetylase-specific zinc binding motif and cryptic deacetylase activity. J Biol Chem 283:11355–11363. doi:10.1074/jbc.M707362200
30. Vannini A, Volpari C, Filocamo G et al (2004) Crystal structure of a eukaryotic zinc-dependent histone deacetylase, human HDAC8, complexed with a hydroxamic acid inhibitor. Proc Natl Acad Sci USA 101:15064–15069. doi:10.1073/pnas.0404603101
31. Bottomley MJ, Lo Surdo P, Di Giovine P et al (2008) Structural and functional analysis of the human HDAC4 catalytic domain reveals a regulatory structural zinc-binding domain. J Biol Chem 283:26694–26704. doi:10.1074/jbc.M803514200
32. Zhou X, Marks PA, Rifkind RA, Richon VM (2001) Cloning and characterization of a histone deacetylase, HDAC9. Proc Natl Acad Sci USA 98:10572–10577. doi:10.1073/pnas.191375098
33. Gregoretti IV, Lee YM, Goodson HV (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol 338:17–31. doi:10.1016/j.jmb.2004.02.006
34. Huang EY, Zhang J, Miska EA et al (2000) Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. Genes Dev 14:45–54. doi:10.1101/gad.14.1.45
35. Guenther MG, Barak O, Lazar MA (2001) The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol Cell Biol 21:6091–6101. doi:10.1128/MCB.21.18.6091-6101.2001
36. Fischle W, Dequiedt F, Fillion M et al (2001) Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J Biol Chem 276:35826–35835. doi:10.1074/jbc.M104935200
37. Clocchiatti A, Di Giorgio E, Ingrao S et al (2013) Class IIa HDACs repressive activities on MEF2-depedent transcription are associated with poor prognosis of $ER^+$ breast tumors. FASEB J 27:942–954. doi:10.1096/fj.12-209346
38. Di Giorgio E, Clocchiatti A, Piccinin S et al (2013) MEF2 is a converging hub for HDAC4 and PI3K/Akt-induced transformation. Mol Cell Biol 33:4473–4491. doi:10.1128/MCB.01050-13
39. Zhang C, McKinsey T, Olson E (2002) Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. Mol Cell Biol 22:7302–7312. doi:10.1128/MCB.22.20.7302
40. Finnin MS, Donigian JR, Cohen A et al (1999) Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 401:188–193. doi:10.1038/43710
41. Yang W-M, Tsai S-C, Wen Y-D et al (2002) Functional domains of histone deacetylase-3. J Biol Chem 277:9447–9454. doi:10.1074/jbc.M105993200
42. Ago T, Liu T, Zhai P et al (2008) A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. Cell 133:978–993. doi:10.1016/j.cell.2008.04.041
43. Isaacs JT, Antony L, Dalrymple SL et al (2013) Tasquinimod is an allosteric modulator of HDAC4 survival signaling within the compromised cancer microenvironment. Cancer Res 73:1386–1399. doi:10.1158/0008-5472.CAN-12-2730

44. Clocchiatti A, Di Giorgio E, Demarchi F, Brancolini C (2013) Beside the MEF2 axis: unconventional functions of HDAC4. Cell Signal 25:269–276. doi:10.1016/j.cellsig.2012.10.002
45. Cohen TJ, Barrientos T, Hartman ZC et al (2009) The deacetylase HDAC4 controls myocyte enhancing factor-2-dependent structural gene expression in response to neural activity. FASEB J 23:99–106. doi:10.1096/fj.08-115931
46. Moresi V, Williams AH, Meadows E et al (2010) Myogenin and class II HDACs control neurogenic muscle atrophy by inducing E3 ubiquitin ligases. Cell 143:35–45. doi:10.1016/j.cell.2010.09.004
47. Choi MC, Cohen TJ, Barrientos T et al (2012) A direct HDAC4-MAP kinase crosstalk activates muscle atrophy program. Mol Cell 47:122–132. doi:10.1016/j.molcel.2012.04.025
48. Liu L, Cavanaugh JE, Wang Y et al (2003) ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. Proc Natl Acad Sci USA 100:8532–8537. doi:10.1073/pnas.1332804100
49. Grégoire S, Tremblay AM, Xiao L et al (2006) Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. J Biol Chem 281:4423–4433. doi:10.1074/jbc.M509471200
50. Grégoire S, Xiao L, Nie J et al (2007) Histone deacetylase 3 interacts with and deacetylates myocyte enhancer factor 2. Mol Cell Biol 27:1280–1295. doi:10.1128/MCB.00882-06
51. Burgess A, Ruefli A, Beamish H et al (2004) Histone deacetylase inhibitors specifically kill nonproliferating tumour cells. Oncogene 23:6693–6701. doi:10.1038/sj.onc.1207893
52. Bolden JE, Shi W, Jankowski K et al (2013) HDAC inhibitors induce tumor-cell-selective pro-apoptotic transcriptional responses. Cell Death Dis 4:e519. doi:10.1038/cddis.2013.9
53. Vrana JA, Decker RH, Johnson CR et al (1999) Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. Oncogene 18:7016–7025. doi:10.1038/sj.onc.1203176
54. Richon VM, Sandhoff TW, Rifkind RA, Marks PA (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 97:10014–10019. doi:10.1073/pnas.180316197
55. Gammoh N, Lam D, Puente C et al (2012) Role of autophagy in histone deacetylase inhibitor-induced apoptotic and nonapoptotic cell death. Proc Natl Acad Sci 109:6561–6565. doi:10.1073/pnas.1204429109
56. Henderson C, Mizzau M, Paroni G et al (2003) Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). J Biol Chem 278:12579–12589. doi:10.1074/jbc.M213093200
57. Maeda T, Towatari M, Kosugi H, Saito H (2000) Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. Blood 96:3847–3856
58. Magner WJ, Kazim AL, Stewart C et al (2000) Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. J Immunol 165:7017–7024
59. Rossig L, Li H, Fisslthaler B et al (2002) Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. Circ Res 91:837–844. doi:10.1161/01.RES.0000037983.07158.B1
60. Deroanne CF, Bonjean K, Servotte S et al (2002) Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. Oncogene 21:427–436. doi:10.1038/sj.onc.1205108
61. Lane AA, Chabner BA (2009) Histone deacetylase inhibitors in cancer therapy. J Clin Oncol 27:5459–5468. doi:10.1200/JCO.2009.22.1291
62. Wilson AJ, Byun D-S, Nasser S et al (2008) HDAC4 promotes growth of colon cancer cells via repression of p21. Mol Biol Cell 19:4062–4075. doi:10.1091/mbc.E08-02-0139
63. Mottet D, Pirotte S, Lamour V et al (2009) HDAC4 represses p21(WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism. Oncogene 28:243–256. doi:10.1038/onc.2008.371
64. Rad R, Rad L, Wang W et al (2010) PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. Science 330:1104–1107. doi:10.1126/science.1193004
65. Hohl M, Wagner M, Reil J et al (2013) HDAC4 controls histone methylation in response to elevated cardiac load. J Clin Invest 123:1359–1370. doi:10.1172/JCI61084
66. Jones P, Altamura S, De Francesco R et al (2008) Probing the elusive catalytic activity of vertebrate class IIa histone deacetylases. Bioorg Med Chem Lett 18:1814–1819. doi:10.1016/j.bmcl.2008.02.025
67. Jones P, Bottomley MJ, Carfí A et al (2008) 2-Trifluoroacetylthiophenes, a novel series of potent and selective class II histone deacetylase inhibitors. Bioorg Med Chem Lett 18:3456–3461. doi:10.1016/j.bmcl.2008.02.026
68. Bradner JE, West N, Grachan ML et al (2010) Chemical phylogenetics of histone deacetylases. Nat Chem Biol 6:238–243. doi:10.1038/nchembio.313
69. Sternson SM, Wong JC, Grozinger CM, Schreiber SL (2001) Synthesis of 7200 small molecules based on a substructural analysis of the histone deacetylase inhibitors trichostatin and trapoxin. Org Lett 3:4239–4242. doi:10.1021/ol016915f
70. Henkes LM, Haus P, Jäger F et al (2012) Synthesis and biochemical analysis of 2,2,3,3,4,4,5,5,6,6,7,7-dodecafluoro-*N*-hydroxy-octanediamides as inhibitors of human histone deacetylases. Bioorg Med Chem 20:985–995. doi:10.1016/j.bmc.2011.11.041
71. Marek L, Hamacher A, Hansen FK et al (2013) Histone deacetylase (HDAC) inhibitors with a novel connecting unit linker region reveal a selectivity profile for HDAC4 and HDAC5 with improved activity against chemoresistant cancer cells. J Med Chem 56:427–436. doi:10.1021/jm301254q
72. Fleming CL, Ashton TD, Gaur V et al (2014) Improved synthesis and structural reassignment of MC1568: a class IIa selective HDAC inhibitor. J Med Chem 57:1132–1135. doi:10.1021/jm401945k
73. Mai A, Massa S, Pezzi R et al (2005) Class II (IIa)-selective histone deacetylase inhibitors. 1. Synthesis and biological evaluation of novel (aryloxopropenyl)pyrrolyl hydroxyamides. J Med Chem 48:3344–3353. doi:10.1021/jm049002a
74. Duong V, Bret C, Altucci L et al (2008) Specific activity of class II histone deacetylases in human breast cancer cells. Mol Cancer Res 6:1908–1919. doi:10.1158/1541-7786.MCR-08-0299
75. Nebbioso A, Dell'Aversana C, Bugge A et al (2010) HDACs class II-selective inhibition alters nuclear receptor-dependent differentiation. J Mol Endocrinol 45:219–228. doi:10.1677/JME-10-0043
76. Venza I, Visalli M, Oteri R et al (2013) Class II-specific histone deacetylase inhibitors MC1568 and MC1575 suppress IL-8 expression in human melanoma cells. Pigment Cell Melanoma Res 26:193–204. doi:10.1111/pcmr.12049
77. Kahali B, Gramling SJ, Marquez SB et al (2014) Identifying targets for the restoration and reactivation of BRM. Oncogene 33:653–664. doi:10.1038/onc.2012.613
78. Nebbioso A, Manzo F, Miceli M et al (2009) Selective class II HDAC inhibitors impair myogenesis by modulating the stability

and activity of HDAC-MEF2 complexes. EMBO Rep 10:776–782. doi:10.1038/embor.2009.88
79. Ontoria JM, Altamura S, Di Marco A, Ferrigno F, Laufer R, Muraglia E, Palumbi MC, Rowley M, Scarpelli R, Schultz-Fademrecht C, Serafini S, Steinkühler CJP (2009) Identification of novel, selective, and stable inhibitors of class II histone deacetylases. Validation studies of the inhibition of the enzymatic activity of HDAC4 by small molecules as a novel approach for cancer therapy. J Med Chem 52:6782–6789. doi:10.1021/jm900555u
80. Tessier P, Smil DV, Wahhab A et al (2009) Diphenylmethylene hydroxamic acids as selective class IIa histone deacetylase inhibitors. Bioorg Med Chem Lett 19:5684–5688. doi:10.1016/j.bmcl.2009.08.010
81. Haus P, Korbus M, Schröder M, Meyer-Almes F-J (2011) Identification of selective class II histone deacetylase inhibitors using a novel dual-parameter binding assay based on fluorescence anisotropy and lifetime. J Biomol Screen 16:1206–1216. doi:10.1177/1087057111424605
82. Olsson A, Björk A, Vallon-Christersson J et al (2010) Tasquinimod (ABR-215050), a quinoline-3-carboxamide anti-angiogenic agent, modulates the expression of thrombospondin-1 in human prostate tumors. Mol Cancer 9:107. doi:10.1186/1476-4598-9-107
83. Dalrymple SL, Becker RE, Zhou H et al (2012) Tasquinimod prevents the angiogenic rebound induced by fractionated radiation resulting in an enhanced therapeutic response of prostate cancer xenografts. Prostate 72:638–648. doi:10.1002/pros.21467
84. Corzo CA, Condamine T, Lu L et al (2010) HIF-1α regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. J Exp Med 207:2439–2453. doi:10.1084/jem.20100587
85. Kong Y, Jung M, Wang K et al (2011) Histone deacetylase cytoplasmic trapping by a novel fluorescent HDAC inhibitor. Mol Cancer Ther 10:1591–1599. doi:10.1158/1535-7163.MCT-10-0779
86. Zhang Y, Li N, Caron C et al (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. EMBO J 22:1168–1179. doi:10.1093/emboj/cdg115
87. Geng L, Cuneo KC, Fu A et al (2006) Histone deacetylase (HDAC) inhibitor LBH589 increases duration of gamma-H2AX foci and confines HDAC4 to the cytoplasm in irradiated non-small cell lung cancer. Cancer Res 66:11298–11304. doi:10.1158/0008-5472.CAN-06-0049
88. Guo L, Han A, Bates DL et al (2007) Crystal structure of a conserved N-terminal domain of histone deacetylase 4 reveals functional insights into glutamine-rich domains. Proc Natl Acad Sci USA 104:4297–4302. doi:10.1073/pnas.0608041104
89. Miska EA, Karlsson C, Langley E et al (1999) HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J 18:5099–5107. doi:10.1093/emboj/18.18.5099
90. Vega RB, Matsuda K, Oh J et al (2004) Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell 119:555–566. doi:10.1016/j.cell.2004.10.024
91. Chang S, McKinsey TA, Zhang CL et al (2004) Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol Cell Biol 24:8467–8476. doi:10.1128/MCB.24.19.8467-8476.2004
92. Chang S, Young BD, Li S et al (2006) Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. Cell 126:321–334. doi:10.1016/j.cell.2006.05.040
93. Kehat I, Accornero F, Aronow BJ, Molkentin JD (2011) Modulation of chromatin position and gene expression by HDAC4 interaction with nucleoporins. J Cell Biol 193:21–29. doi:10.1083/jcb.201101046
94. Barrett A, Santangelo S, Tan K et al (2007) Breast cancer associated transcriptional repressor PLU-1/JARID1B interacts directly with histone deacetylases. Int J Cancer 121:265–275. doi:10.1002/ijc.22673
95. Jayathilaka N, Han A, Gaffney KJ et al (2012) Inhibition of the function of class IIa HDACs by blocking their interaction with MEF2. Nucleic Acids Res 40:5378–5388. doi:10.1093/nar/gks189
96. Han A, He J, Wu Y et al (2005) Mechanism of recruitment of class II histone deacetylases by myocyte enhancer factor-2. J Mol Biol 345:91–102. doi:10.1016/j.jmb.2004.10.033
97. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 6:38–51. doi:10.1038/nrc1779
98. Shi Y, Lan F, Matson C et al (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119:941–953. doi:10.1016/j.cell.2004.12.012
99. Metzger E, Wissmann M, Yin N et al (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437:436–439. doi:10.1038/nature04020
100. Hoffmann I, Roatsch M, Schmitt ML et al (2012) The role of histone demethylases in cancer therapy. Mol Oncol 6:683–703. doi:10.1016/j.molonc.2012.07.004
101. Zhuang Q, Qing X, Ying Y et al (2013) Class IIa histone deacetylases and myocyte enhancer factor 2 proteins regulate the mesenchymal-to-epithelial transition of somatic cell reprogramming. J Biol Chem 288:12022–12031. doi:10.1074/jbc.M113.460766
102. McGee SL, van Denderen BJW, Howlett KF et al (2008) AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes 57:860–867. doi:10.2337/db07-0843
103. Sparling DP, Griesel BA, Weems J, Olson AL (2008) GLUT4 enhancer factor (GEF) interacts with MEF2A and HDAC5 to regulate the GLUT4 promoter in adipocytes. J Biol Chem 283:7429–7437. doi:10.1074/jbc.M800481200
104. Weems J, Olson AL (2011) Class II histone deacetylases limit GLUT4 gene expression during adipocyte differentiation. J Biol Chem 286:460–468. doi:10.1074/jbc.M110.157107
105. Raichur S, Hooi Teh S, Ohwaki K et al (2012) Histone deacetylase 5 regulates glucose uptake and insulin action in muscle cells. J Mol Endocrinol 49:203–211. doi:10.1530/JME-12-0095
106. Kumar A, Lin Z, SenBanerjee S, Jain MK (2005) Tumor necrosis factor alpha-mediated reduction of KLF2 is due to inhibition of MEF2 by NF-kappaB and histone deacetylases. Mol Cell Biol 25:5893–5903. doi:10.1128/MCB.25.14.5893-5903.2005
107. Baek Y-S, Haas S, Hackstein H et al (2009) Identification of novel transcriptional regulators involved in macrophage differentiation and activation in U937 cells. BMC Immunol 10:18. doi:10.1186/1471-2172-10-18
108. Bolger TA, Zhao X, Cohen TJ et al (2007) The neurodegenerative disease protein ataxin-1 antagonizes the neuronal survival function of myocyte enhancer factor-2. J Biol Chem 282:29186–29192. doi:10.1074/jbc.M704182200
109. Morrison BE, Majdzadeh N, D'Mello SR (2007) Histone deacetylases: focus on the nervous system. Cell Mol Life Sci 64:2258–2269. doi:10.1007/s00018-007-7035-9
110. West AE (2012) Regulated shuttling of the histone deacetylase HDAC5 to the nucleus may put a brake on cocaine addiction. Neuron 73:1–3. doi:10.1016/j.neuron.2011.12.016
111. Li J, Chen J, Ricupero CL et al (2012) Nuclear accumulation of HDAC4 in ATM deficiency promotes neurodegeneration in ataxia telangiectasia. Nat Med 18:783–790. doi:10.1038/nm.2709

112. Hara N, Alkanani AK, Dinarello CA, Zipris D (2014) Histone deacetylase inhibitor suppresses virus-induced proinflammatory responses and type 1 diabetes. J Mol Med 92:93–102. doi:10.1007/s00109-013-1078-1
113. Herman D, Jenssen K, Burnett R et al (2006) Histone deacetylase inhibitors reverse gene silencing in Friedreich's ataxia. Nat Chem Biol 2:551–558. doi:10.1038/nchembio815
114. Burli RW, Luckhurst CA, Aziz O et al (2013) Design, synthesis, and biological evaluation of potent and selective class IIa histone deacetylase (HDAC) inhibitors as a potential therapy for Huntington's disease. J Med Chem 56:9934–9954. doi:10.1021/jm4011884
115. Tao R, de Zoeten EF, Ozkaynak E et al (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med 13:1299–1307. doi:10.1038/nm1652
116. Wang L, de Zoeten EF, Greene MI, Hancock WW (2009) Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3+ regulatory T cells. Nat Rev Drug Discov 8:969–981. doi:10.1038/nrd3031
117. de Zoeten EF, Wang L, Sai H et al (2010) Inhibition of HDAC9 increases T regulatory cell function and prevents colitis in mice. Gastroenterology 138:583–594. doi:10.1053/j.gastro.2009.10.037
118. Bantscheff M, Hopf C, Savitski MM et al (2011) Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. Nat Biotechnol 29:255–265. doi:10.1038/nbt.175