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Crucial role of reversible phosphorylation in the mechanisms governing the biological functions of class IIa Histone Deacetylases

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Summary:
Regulation of class IIa histone deacetylases (HDACs) phosphorylation is crucial because it provides the opportunity to control important developmental processes associated with these key enzymes. Indeed, the transcriptional repressor activity of class IIa HDAC is controlled via their phosphorylation-dependent nucleo-cytoplasmic shuttling. While a lot of efforts have been directed towards the identification of the inactivating kinases that phosphorylate class IIa HDACs, the identity of the antagonist phosphatase remained an open question. During this work, we found that protein phosphatase 2A (PP2A) is responsible for dephosphorylating the class IIa HDACs member HDAC7, thereby regulating its subcellular localization and repressor activity. In order to validate our model, functional consequences of these findings was illustrated during the two main biological processes involving HDAC7, i.e. T-cells apoptosis during negative selection and endothelial cells angiogenic activities during vascular network formation. Cellular PP2A represents a large population of trimeric holoenzymes containing a variable regulatory subunit, whose identity has a crucial role in determining the specificity of PP2A catalytic activity. In an effort to characterize the regulation of HDAC7 dephopshorylation, we identified the relevant PP2A holoenzyme regulating HDAC7 function during vasculogenesis and we found that, among diverse regulatory subunit isoforms, PP2A-Bα uniquely regulates endothelial cell angiogenic properties. PP2A-Bα silencing using small interfering RNAs results in a significant inhibition of endothelial cell tube formation and migration. These results establish PP2A, and more precisely the Bα containg PP2A holoenzyme, as an essential element in the regulation of the class IIa HDACs HDAC7 and unravel a first developmental function for the PP2A regulatory subunit Bα in the genesis of blood vessels.

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Résumé:
La régulation de la phosphorylation des histone désacétylases (ou HDACs) de classe IIa est un processus crucial car il permet le contrôle des programmes développementaux majeurs associés à ces enzymes clé. En effet, l'activité répressive des HDACs de classe IIa est contrôlée via une translocation nucléo-cytoplasmique dépendant de leur phosphorylation. Tandis que beaucoup d'efforts ont été menés afin identifier les kinases phosphorylant les HDACs de classe IIa, la recherche de l'identité de la phosphatase antagoniste est restée marginale. Lors de ce travail, nous avons identifié la protéine phosphatase 2A (PP2A) en tant que phosphatase spécifique de HDAC7, un membre des HDACs de classe IIa. En déphosphorylant HDAC7, PP2A contrôle également sa localisation subcellulaire, ainsi que son activité répressive. Afin de valider notre modèle, les conséquences fonctionnelles de la régulation de HDAC7 par PP2A ont été illustrées dans les deux contextes biologiques majeurs impliquant HDAC7, c'est-à-dire le processus d'apoptose des thymocytes lors de la sélection négative et le processus de formation du réseau de vascularisation. La famille de phosphatases PP2A est constituée d'une vaste population d'enzymes hétérotrimériques, comprenant une sous-unité régulatrice variable dont l'identité détermine la spécificité de l'activité catalytique PP2A. Afin de mieux comprendre la régulation de la déphosphorylation de HDAC7, nous

avons identifié l'holoenzyme PP2A précise responsable de la régulation des fonctions de HDAC7 lors du processus de vasculogenèse. Nous avons en effet découvert que parmi de nombreuses isoformes de la sous-unité régulatrice de PP2A, la sous-unité Bα est la seule à contrôler les propriétés angiogéniques des cellules endothéliales. L'inhibition de PP2A Bα par utilisation d'ARN interférents mène à une diminution significative de la capacité des cellules endothéliales à former des vaisseaux et à migrer. Ces résultats établissent PP2A, et plus précisément l'holoenzyme PP2A contenant la sous-unité régulatrice Bα, en tant qu'éléments essentiels de la régulation de HDAC7 et associe, pour la première fois, un rôle développemental à la sous-unité Bα de PP2A durant la formation des vaisseaux sanguins.

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Chapitre 1: Introduction

Unravelling the mechanisms by which eukaryotic cells regulate gene expression is a fundamental issue in biology. Indeed, precise transcriptional regulation controls virtually all known biological processes governing cell growth, development, differentiation and survival. Some decade of stimulating studies has highlighted the importance of chromatin, the dynamic packaging structure of genomic DNA, in the events leading to either gene activation or gene silencing, or, in other words, leading to regulation of the "on-off" status of genes. The central structural elements of chromatin are specific proteins called histones. Significantly, their amino-terminal tail is subject to a diverse array of posttranslational modifications that largely impact on chromatin structure and accessibility, thereby playing an important regulatory role in the different cellular functions involving DNA, namely replication, transcription, recombination and repair. Among the various histone covalent modifications which combine together to constitute a "code" read by transcriptional regulators, lysine acetylation is certainly one of the most studied. This particular interest is mainly due to the correlation observed between the local acetylation status of chromatin in the vicinity of a promoter and its transcriptional activity: lysine hyperacetylation by histone acetyltransferases (or HATs) is favourable to transcription, whereas hypoacetylation by the opposing action of histone deacetylases (HDACs) is transcriptionaly unfavourable.

Hence, by leading to local closed conformation of chromatin, HDACs act as transcriptional repressors. Eighteen human HDACs have been identified to date. They are divided into different classes based on biochemical, structural and phyllogenetic criteria. The sub-class IIa HDACs which are HDAC4, 5, 7 and 9, was the focus of this work. This key family of transcriptional regulators share several similarities on sequence, domain organization and mechanism of regulation. In this introducing part, we first present a summary of our knowledge on class IIa HDACs, specifically on their inventive efficient signal-controlled regulation and on the functional relevance of these enzymes. Indeed, genetic inactivation studies in mice have established class IIa HDACs as key transcriptional regulators of multiple crucial developmental and differentiation programs.

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1.1. Class IIa Histone Deacetylases

1.1 Class IIa Histone Deacetylases: Regulating the Regulators

I) Summary

In the last decade, the identification of enzymes that regulate acetylation of histones and non-histone proteins has revealed the key role of dynamic acetylation and deacetylation in various cellular processes. Mammalian histone deacetylases (HDACs), which catalyze the removal of acetyl-groups from lysine residues, are grouped into three classes, on the basis of similarity to yeast counterparts. An abundance of experimental evidence has established class IIa HDACs as crucial transcriptional regulators of various developmental and differentiation processes. In the past five years, a tremendous effort has been dedicated to characterizing the regulation of these enzymes. In this introduction, we summarize the latest discoveries in the field and discuss the molecular and structural determinants of class IIa HDACs regulation. Finally, we emphasize that comprehension of the mechanisms underlying class IIa HDAC functions is essential for potential therapeutic applications.

II) Introduction

Protein N^{ε}-acetylation consists of the transfer of an acetyl moiety from an acetyl-coenzyme A (CoA) to the ε-amino group of a lysine residue. This modification was first identified on histones in the 1960s by Allfrey et al. (1964) who proposed that it could "influence RNA synthesis." Forty years later, it has become clear that lysine acetylation can target many cellular proteins in addition to histones. Similarly to phosphorylation, methylation or ubiquitination, lysine acetylation belongs to the panel of post-translational modifications used by the cell to alter the specific properties of a given protein (Kouzarides, 2000; Yang, 2005; Yang & Gregoire, 2005). Lysine acetylation is a dynamic, reversible and tightly regulated protein modification. *In vivo*, acetylation is controlled by the antagonistic activities of acetyltransferases and deacetylases. Because acetylation was originally characterized on histones, lysine acetyltransferases and deacetylases are known as histone acetyltranferases (HATs) and histone deacetylases (HDACs).

Eukaryotic HDACs are divided into two groups based on the specificities of their catalytic mechanism. Group I HDACs are zinc-dependent amidohydrolases. Eleven subtypes have been identified and named chronologically HDAC1 to HDAC11. While they share some degree of homology in their catalytic domain, group I HDACs are further subdivided into class I and class II enzymes on the basis of phylogenetic and structural criteria (Gregoretti et al., 2004). HDAC1, the founding member of class I, shows a high degree of sequence similarity with the yeast transcriptional regulator Rpd3 (Rundlett et al., 1996; Taunton et al., 1996). Together with HDAC1, four additional human Rpd3 orthologs, HDAC2, -3, -8 and -11 make up the mammalian class I HDAC family (Marks et al., 2003). Class II HDACs (HDAC4,-5,-6,-7,-9 and -10) are defined based on their sequence homology with Hda1in *Saccharomyces cerevisiae* (Fischer et al., 2002; Fischle et al., 2001; Fischle et al., 1999; Grozinger et al., 1999; Guardiola & Yao, 2002; Kao et al., 2002; Kao et al., 2000b; Miska et al., 1999; Petrie et al., 2003; Tong et al., 2002; Verdel & Khochbin, 1999; Wang et al., 1999a; Zhou et al., 2001). The Hda1-like sequences of class II HDACs correspond to their catalytic domain. These enzymes also contain additional domains that allow for further subdivision into class IIa (HDAC4, -5, -7 and -9) and class IIb (HDAC6 and -10). Recently, analysis of the silent information regulator (Sir2) protein in yeast and its homologues in higher eukaryotes (SirT1-7) has revealed a nicotinamide adenine dinucleotide NAD^+-dependent HDAC activity. Because of the obvious differences in their catalytic mechanism and their unrelated sequences, SirT proteins constitute the second group of mammalian HDACs often referred as Class III enzymes (Haigis & Guarente, 2006).

This review is restricted to class IIa HDACs and will focus on the latest developments in the understanding of their regulation and biology. For more general information on HDACs, we respectfully refer the reader to previous excellent reviews from our colleagues (Bertos et al., 2001; Grozinger & Schreiber, 2002; de Ruijter et al., 2003; Legube & Trouche, 2003; Marks et al., 2003; Thiagalingam et al., 2003; Verdin et al., 2003; Yang & Seto, 2003; Blander & Guarente, 2004; Marmorstein, 2004; North & Verdin, 2004; Sengupta & Seto, 2004; Ekwall, 2005; Glozak et al., 2005; Nusinzon & Horvath, 2005; Yang & Gregoire, 2005).

III.a) Structure of class IIa HDACs: the C-terminal deacetylase domain

HDAC4, -5, -7 and -9 are defined by their particular bipartite structure (Figure 1A). The so-called HDAC domain is limited to a 400-450 amino acid region located at their C-terminus. This region, which shows the highest homology to yeast Hda1 (52, 53, 51 and 51% similarity for HDAC4, -5, -7 and -9 respectively) is highly conserved amongst class IIa members (around 80% sequence similarity). Structural analysis of FB188 HDAH, a bacterial HDAC-like protein with significant homology to the catalytic domain of class II HDACs has revealed that while the canonical fold of their catalytic pocket is very similar, there are several important differences between class I and class II HDACs that mainly concern the entrance region of the active site cavity and the outer charge transfer relay system (Finnin et al., 1999; Nielsen et al., 2005; Somoza et al., 2004; Vannini et al., 2004). This might explain why, in contrast to class I HDACs, researchers in the field have remained remarkably unsuccessful at obtaining enzymatically active class IIa HDACs in recombinant forms *in vitro* (Hassig et al., 1998; Hu et al., 2000). When successful, these attempts have mainly yielded protein preparations with low specific activity, especially when compared to the corresponding protein analyzed *in vivo* (Wang et al., 1999a). Indeed, when expressed as an isolated subdomain the C-terminal region of class IIa HDACs associates with full-HDAC activity *in vivo* (Fischle et al., 2001; Fischle et al., 2002; Wang et al., 1999a). Therefore, cumbersome transient transfection approaches followed by immunopurification have been required to measure significant deacetylase activity associated with class IIa HDACs (Verdin et al., 2004). This suggests that the enzymatic activity of class IIa members would rely on cellular co-factors or specific posttranslational modifications. Accordingly, there is evidence indicating that HDAC activity of class IIa members is dependent on an HDAC3/N-CoR/SMRT complex (Fischle et al., 2001; Fischle et al., 2002). Related to this, HDAC4 was shown to be sumoylated at lysine 559 (Kirsh et al., 2002). Because a K559R mutation abolished HDAC4-associated HDAC activity, the authors concluded that sumoylation was necessary for full enzymatic activity. However, binding of the K559R HDAC4 mutant to the N-CoR/SMRT/HDAC3 complex was not investigated in the study. Therefore, it cannot be formally excluded that the effect on the enzymatic activity was due to an overall alteration of the protein structure resulting in an inability to associate with the active HDAC3 complex. All these remaining questions illustrate the fact that further efforts should be dedicated to understanding how the activity of class IIa HDACs is controlled *in vivo*.

III.b) Structure of class IIa HDACs: the N-terminal adaptor domain

In addition to their Hda1-like domain, class IIa HDACs are comprised of a 450 to 600 amino acid extension at their N-terminus. Overall, the sequence homology between the members of the family in this region is much lower (30-45%) than that of the HDAC domain. Nevertheless, this region contains conserved amino acid motifs that are specialized for binding an array of proteins. Most of these are DNA-binding transcription factors, for which class IIa HDACs serve as transcriptional corepressors (Table 1). By far the best characterized of such interactions is the association with the MEF2 transcription factors which occurs via a 17 amino acid motif conserved in all class IIa HDACs (Dequiedt et al., 2003; Han et al., 2005; Kao et al., 2001; Lu et al., 2000b; Verdel & Khochbin, 1999; Wang et al., 1999a). Repression of MEF2-targeted promoters via recruitment of class IIa-associated HDAC activity has been extensively documented (Lemercier et al., 2000; Lu et al., 2000b; Miska et al., 1999; Wang et al., 1999a) and plays a key role in the biological functions of these enzymes (see below). Some interactions that take place in the N-terminal region of class IIa HDACs mediate the recruitment of their deacetylase activity into higher-order complexes, together with multiple other enzymes. Two examples of such interactions are interactions with COOH-terminal binding protein (CtBP) and Heterochromatin P1 (HP1). A 100 amino-acid motif has been implicated in the interaction of HDAC4 and HDAC5 with HP1α and associated histone methyl transferase (HMTase) SUV39H1 (Zhang et al., 2002b). The functional significance of the association between class IIa HDACs and HMTases remains unclear. However, considering that lysine deacetylation is a prerequisite to methylation, two attractive models emerge. First, this interaction would allow the recruitment of a deacetylase/methyltransferase complex that would be important for the establishment and maintenance of transcriptional silencing at promoters to which class IIa HDACs are recruited by DNA-binding transcription factors (Zhang et al., 2002b). Alternatively, when bound to methylated histones, the HP1/HMTase complex could propagate a repressive chromatin state through class IIa HDAC-mediated deacetylation and subsequent methylation of adjacent nucleosomal histone tails. On a similar vein, the N-terminus of HDAC4, -5 and -9 comprises an interaction motif for the transcriptional corepressor CtBP (Dressel et al., 2001; Zhang et al., 2001a). Since CtBP is part of a higher-order complex containing chromatin modifying enzymes such as class I HDACs and HMTs

(Shi et al., 2003), it is tempting to speculate that class IIa HDACs might use CtBP to recruit multiple enzymatic activities to their target promoters.

The adaptor domain of class IIa HDACs also contains motifs specifically involved in their regulation. Three clusters of arginine/lysine residues are found between aa 251-272 of HDAC4 and aa 264-285 of HDAC5. Mutational analyses confirmed the ability of these sequences to act as an autonomous nuclear import signal and enlightened their importance in the subcellular localization of HDAC4 and HDAC5 (McKinsey et al., 2000a; Wang & Yang, 2001). Despite a lack of experimental evidence, it is likely that this NLS also mediates the observed interaction between HDAC4 and importinα (Grozinger & Schreiber, 2000). These motifs are fairly well conserved in HDAC7 and HDAC9, althoug it has not been formally demonstrated, it is logical to assume that the corresponding regions constitute authentic nuclear import signals for these class IIa members.

A puzzling feature of the N-terminal adaptor domain of class IIa HDACs is its ability to inhibit transcription when tethered to a heterologous promoter, independently of the C-terminal catalytic domain (Sparrow et al., 1999; Wang et al., 1999a; Zhou et al., 2000a). This property has been assigned to a glutamine-rich domain (aa. 69-155 of HDAC4 that contains 26 glutamine residues) which organizes into a single α-helix and would be involved in homo- and/or hetero-oligomeric protein-protein interactions (Guo et al., 2007; Kirsh et al., 2002). Curiously, this region, which is conserved in HDAC4, -5 and -9 is not present in HDAC7. To date, very few experiments have been conducted to understand how the coiled-coil domain of HDAC4,-5 and -9 might repress transcription. Resolution of this question must await the identification of the cellular factors interacting specifically with this region.

The adapter domain of class IIa HDACs contains specific residues that are subjected to various post-translational modifications, such as proteolytic cleavage (Bakin & Jung, 2004; Liu et al., 2004; Paroni et al., 2004), ubiquitination (Li et al., 2004), sumoylation (Kirsh et al., 2002; Petrie et al., 2003) and most importantly phosphorylation. In response to various stimuli, a set of serine residues in the adapter domain of class IIa HDACs is phosphorylated, creating docking sites for the 14-3-3 proteins (Grozinger & Schreiber, 2000; Wang et al., 2000). Association with 14-3-3 induces nuclear export and cytoplasmic accumulation of class IIa HDACs with concomitant derepression of their target promoters (Grozinger & Schreiber, 2000; Kao et al., 2000b; Liu et al., 2005; Wang et al., 2000). This nuclear export mechanism allows for signal-dependent activation of class IIa HDAC target genes and has proven to be crucial for their regulation (see below). The 14-3-3 proteins are

a highly conserved family of proteins that have important roles in a wide range of cell signaling pathways (Dougherty & Morrison, 2004; Mackintosh, 2004). They bind to specific phosphoserine- or phosphothreonine-containing motifs within target proteins that generally match with either RxxpSxP (mode I) or RxxxpSxP (mode II) (Muslin et al., 1996). Their rigid structure and dimeric nature allow them to act as intra- and intermolecular adaptors and to alter specific properties of their target proteins (Yaffe, 2002). Mutational analyses identified two conserved 14-3-3 interacting motifs in HDAC5 and HDAC9 (centered on Ser^{259} and Ser^{497} in HDAC5 and Ser^{220} and Ser^{451} in HDAC9). Simultaneous mutations of these serine residues into alanines totally abolished interaction between HDAC5/9 and 14-3-3 (McKinsey et al., 2000b; Zhang et al., 2002a). Unexpectedly, the corresponding serine-to-alanine double mutants of HDAC4 and HDAC7 retained residual 14-3-3 binding (Wang et al., 2000; Kao et al., 2001; McKinsey et al., 2000). This observation led to the identification of Ser^{632} in HDAC4 and Ser^{449} in HDAC7 as additional 14-3-3 binding sites (Dequiedt et al., 2003; Grozinger & Schreiber, 2000; Kao et al., 2001; McKinsey et al., 2000a; Wang et al., 2000). Surprisingly, the corresponding motif is also conserved in HDAC5 and -9 (Figure 1B). In addition, we recently identified a cryptic 14-3-3 binding site (Ser^{181}) in HDAC7 (Dequiedt et al., 2006; Dequiedt et al., 2005). This site was overlooked in previous mutational analyses because its phosphorylation seems dependent on prior phosphorylation of the most N-terminal serine residue, Ser^{155}. Whereas Ser^{181} is uniquely present in HDAC7, sequence analyses disclosed other putative cryptic 14-3-3 motifs in the adaptor domain of HDAC4, -5 and -9 (Figure 1B). The functionality and possible biological relevance of these additional sites should be investigated comprehensively.

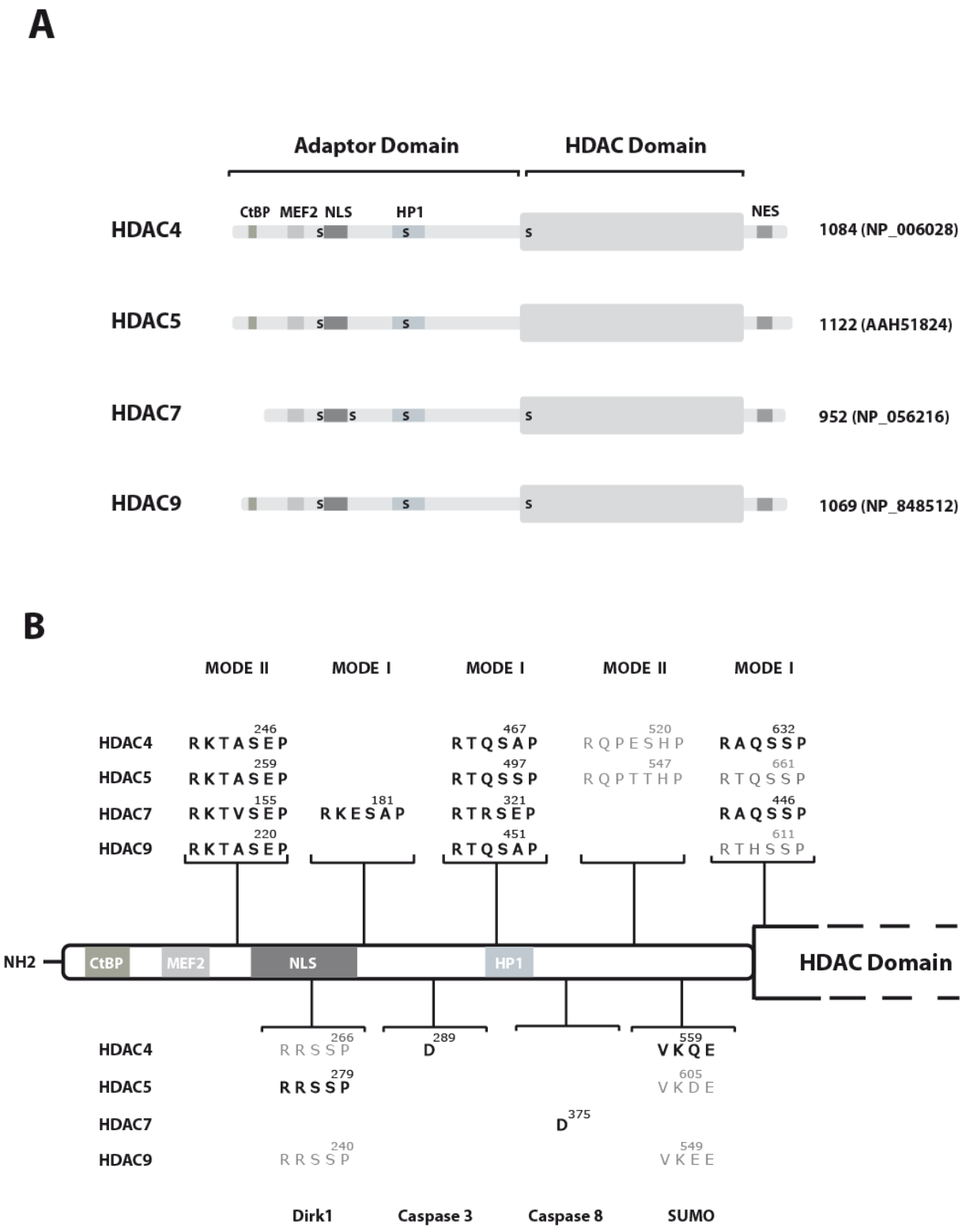


Figure 1: Domain organization of human class IIa HDACs. (A) Schematic representation of HDAC4, HDAC5, HDAC7 and HDAC9. For each protein, the longest isoform is shown, with the total number of residues and the corresponding database accession number. The CtBP, MEF2 and HP1-binding regions along with the NES and NLS are depicted as solid boxes. 14-

3-3 binding sites are indicated with an S. NES, Nuclear export signal; NLS, nuclear localization signal. (B) Identification of post-translational modifications sites in the adaptor domain of class IIa HDACs. Sequence corresponding to the N-terminal regions of HDAC4, -5, -7 and -9 were examined for the presence of 14-3-3-binding motifs (mode I and mode II), Dirk1 phosphorylation target sequence, sumoylation consensus and caspase 3 and 8 cleavage sites. For each identified motif, the position of the critical residue is indicated. Motifs are written in bold when the corresponding post-translation modification was demonstrated experimentally.

Partner	Function	Reference(s)
MEF2	DNA anchoring transcriptional factor	(Dressel et al., 2001; Kao et al., 2001; Lemercier et al., 2000; Lu et al., 2000a; Lu et al., 2000b; Miska et al., 1999; Sparrow et al., 1999; Verdel & Khochbin, 1999; Wang et al., 1999a)
NF-AT3C	DNA-anchoring transcriptional factor	(Dai et al., 2005)
DnaJ (Mrj)	bridging co-factor	(Dai et al., 2005)
Runx 2/3	DNA-anchoring transcriptional factor	(Vega et al., 2004b)
GATA 1/2	DNA-anchoring transcriptional factor	(Ozawa et al., 2001; Watamoto et al., 2003)
FOXP3	DNA-anchoring transcriptional factor	(Li et al., 2007)
Nkx2-5	DNA-anchoring transcriptional factor	(Song et al., 2006)
CAMTA2	bridging co-factor	(Song et al., 2006)
SRF	DNA-anchoring transcriptional factor	(Davis et al., 2003)
Myocardin	bridging co-factor	(Xing et al., 2006a)
NRSF	DNA-anchoring transcriptional factor	(Nakagawa et al., 2006)
BCl6	DNA-anchoring transcriptional factor	(Lemercier et al., 2002)
BCor	BCL6 co-repressor	(Huynh et al., 2000)
BCor-L1	BCoR homolog co-repressor	(Pagan et al., 2007)
PLZF	DNA-anchoring transcriptional factor	(Lemercier et al., 2002)
TR2	nuclear hormone receptor	(Franco et al., 2003)
LXR	nuclear hormone receptor	(Ghisletti et al., 2007)
NCoR	transcriptional co-repressor of nuclear hormone receptor	(Fischle et al., 2001; Fischle et al., 2002; Huang et al., 2000; Kao et al., 2000a)
SMRT	transcriptional co-repressors of nuclear hormone receptor	(Fischle et al., 2001; Fischle et al., 2002; Huang et al., 2000; Kao et al., 2000a)
mSin3A	transcriptional co-repressor adaptator	(Kao et al., 2000a)
RIP140	transcriptional co-repressors of nuclear hormone receptor	(Castet et al., 2004)
REA	transcriptional co-repressors of nuclear hormone receptor	(Kurtev et al., 2004)
ARR19	transcriptional co-repressors of nuclear hormone receptor	(Jeong et al., 2004)
ET-A	G protein-coupled endothelin receptor	(Lee et al., 2001)
ANCO1	ankyrin repeat-containing transcriptional co-repressors of nuclear hormone receptor	(Zhang et al., 2004)
RFXANK	ankyrin repeat-containing co-factor	(McKinsey et al., 2006; Wang et al., 2005)
ANKRA	ankyrin repeat-containing protein	(McKinsey et al., 2006; Wang et al., 2005)
CREB2	DNA anchoring transcriptional factor	(Guan et al., 2002)

NF-Y	DNA anchoring transcriptional factor	(Basile et al., 2006; Imbriano et al., 2005)
TEL	transcriptional co-repressor	(Petrie et al., 2003)
CtBP	transcriptional co-repressor	(Zhang et al., 2001a)
HP1	adaptator protein associated with HMTase	(Zhang et al., 2002b)
TIP60	histone acetyltransferase	(Lee et al., 2001; Li et al., 2007; Xiao et al., 2003)
IκBα	NF-κB inhibitor, transcriptional repressor	(Aguilera et al., 2004)
53BP1	DNA damage response protein	(Kao et al., 2003)
HIF1α	transcription factor	(Kato et al., 2004; Qian et al., 2006)
ICP0	immediate-early protein of HSV-1 virus	(Lomonte et al., 2004)
EBNA2/EBNALP	Epstein-Barr nuclear antigen and Epstein-Barr nuclear antigen leader protein, transcriptional co-activators	(Bryant & Farrell, 2002; Portal et al., 2006)
α-actinin 1/4	actin-binding protein	(Chakraborty et al., 2006)
14-3-3	chaperone proteins	(Grozinger & Schreiber, 2000; Kao et al., 2001; McKinsey et al., 2000a; Wang et al., 2000; Zhang et al., 2001b)
Importinα	nuclear import receptor	(Grozinger & Schreiber, 2000)
Calmodulin	calcium-binding protein	(Berger et al., 2003; Youn et al., 2000)
Ubc9	SUMO E2 conjugating enzyme	(Zhao et al., 2005)
CaMKI	protein kinase	(Backs et al., 2006)
CaMKII	protein kinase	(Backs et al., 2006)
PKD1	protein kinase	(Dequiedt et al., 2005; Parra et al., 2005; Vega et al., 2004a)
MARK2/3	protein kinase	(Dequiedt et al., 2006)
ERK1/2	protein kinase	(Zhou et al., 2000b)
PP1/MYPTI	protein phosphatase	(Parra et al., 2007)
PP2A	protein phosphatase	(Martin et al., 2008)

Table 1. Interaction partners of class IIa HDACs

IV) Functions of class IIa HDACs

Co-repressors of transcription

Class IIa HDACs are thought to act as transcriptional corepressors by deacetylating nucleosomal histones. Since these enzymes do not bind directly to DNA, the current model posits that their deacetylase activity is recruited to specific promoters by sequence-specific DNA binding proteins. Interactions with distinct transcription factors would thus dictate targeting specificity of class IIa HDACs. The canonical example of this model is illustrated by the interaction between class IIa HDACs and MEF2 transcription factors.

Historically, MEF2 was the first cellular partner identified for class IIa HDACs (Lemercier et al., 2000; Lu et al., 2000b; Miska et al., 1999; Sparrow et al., 1999; Wang et al.,

1999a). The MEF2 family consists of four MADS-box transcription factors (MEF2A, -B, -C and -D) involved in numerous signal-dependent pathways of cellular differentiation (Black & Olson, 1998). Members of the MEF2 family can act as repressors or activators of transcription depending on the mutually exclusive recruitment of specific chromatin modifying factors (McKinsey et al., 2001a; Youn et al., 2000). MEF2 associates with class IIa HDACs through a region located at the junction of the MADS/MEF2 domain which does not overlap with the DNA binding region of MEF2 (Lu et al., 2000b). This and other observations are compatible with a simple model in which, through interaction with the N-terminus of class IIa HDACs, DNA-bound MEF2 would recruits the HDAC activity associated with their C-termini to deacetylate local chromatin and repress transcription. This would establish class IIa HDACs as general transcriptional repressors of the multitude of promoters that are under the control of MEF2 transcription factors (McKinsey et al., 2002a). Accordingly, the ability of class IIa HDACs to act as potent inhibitors of MEF2-dependent transcription has been extensively documented (Dressel et al., 2001; Lemercier et al., 2000; Lu et al., 2000b; Miska et al., 1999; Sparrow et al., 1999; Wang et al., 1999a). Several experimental observations suggest that class IIa HDACs might control MEF2 transcriptional activity through additional mechanisms. MITR, a splice variant of HDAC9 that comprises only the N-terminal adaptor domain efficiently impairs MEF2-dependent transcription (Zhang et al., 2001b; Zhou et al., 2000a). Therefore, in addition to the HDAC activity associated with their C-terminus, class IIa HDACs might also repress MEF2 transcriptional activity through association of their N-terminal domain with co-repressors such as HP1 or CtBP (Zhang et al., 2002a; Zhang et al., 2001a; Zhang et al., 2001b). Recently, HDAC4 and -5 were shown to promote sumoylation of MEF2 which results in inhibition of its transcriptional activity (Gregoire et al., 2006; Gregoire & Yang, 2005; Zhao et al., 2005). As opposed to the original model, these results illustrate the multiple levels of regulation of MEF2 by class IIa HDACs and emphasize the need for further work to achieve comprehensive understanding of these mechanisms (Figure 2).

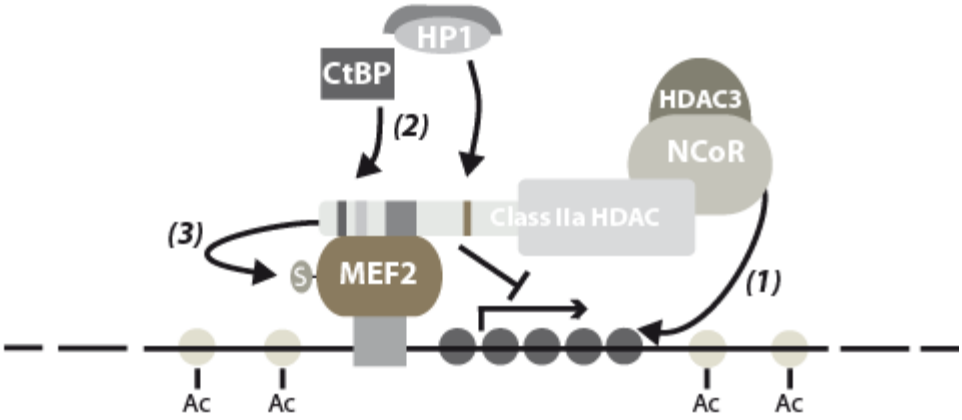


Figure 2: Cartoon depicting various mechanisms involved in the repression of MEF2-dependent transcription by class IIa HDACs. Via their N-terminal adaptor domain, class IIa HDACs interact with the MADS domain of DNA-bound MEF2. At MEF2-targeted promoters, class IIa HDACs establish a repressed chromatin state by recruiting the N-CoR/HDAC3 complex through their C-terminus (1) and co-repressors such as HP1 or CtBP via their N-terminus (2). In addition, class IIa HDACs may also inhibit MEF2 transcriptional activity by promoting its sumoylation (3).

Members of the NFAT family of transcription factors play prominent roles as regulators of calcium-inducible gene expression in diverse cell types (Hogan et al., 2003). A recent study indicated that class IIa HDACs interact with NF-AT3c to repress its transcriptional activity (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 co-factor, the chaperone mammalian relative of DnaJ (Mrj). However, the biological relevance underlying NFAT transcriptional repression through Mrj and class IIa HDACs remains obscure.

Members of the Runx family of transcription factors have important functions in several developmental programs (Otto et al., 2003). Runx1 is involved in hematopietic development and mice deficient in Runx2 display alterations in bone development (Komori et al., 1997; Otto et al., 1997). In the case of Runx3, recent studies have suggested pleitropic functions ranging from immune cell differentiation to tumour development (Puig-Kroger & Corbi, 2006). Several lines of evidence implicate class IIa HDACs as corepressors of Runx-mediated transcription. A recent study reported that HDAC4 associates with Runx2 via its N-terminal

adaptor domain and inhibits its ability to activate transcription (Vega et al., 2004b). Accordingly, reduced histone acetylation of a Runx2 target promoter was observed following overexpression of HDAC4. However, rather than the classical model in which Runx2 would tether HDAC4 to DNA to deacetylate nucleosomal histones, the authors of the study provided evidence that HDAC4 interferes with the ability of Runx2 to bind to its target promoter. In preosteoblasts, HDAC4 and 5 regulate Runx2 activity to control osteoblast differentiation. In this case, the repression of Runx2 activity may rely on the recruitment of class IIa HDACs to Runx2 target promoters through interaction with Smad3 (Kang et al., 2005). Finally, HDAC4 and -5 were reported to directly deacetylate Runx2 and Runx3 which would lead to their ubiquitin-mediated degradation and repression of their transactivation activity (Jeon et al., 2006; Jin et al., 2004). Interestingly, the model that emerges from these observations is dramatically different from what has been established for the regulation of MEF2. While in some instance, class IIa HDACs would act as corepressors of Runx-dependent transcription by promoting local deacetylation of histones, they may also inhibit Runx activity by hindering its DNA binding abilities and by promoting its deacetylation-mediated degradation. Deacetylation of Runx2 by HDAC4/5 reinforces the accumulating evidence suggesting that class IIa HDACs might also target non-histone proteins for deacetylation.

During the past few years, class IIa HDACs have been shown to repress an increasing number of transcriptional regulators through direct or indirect interaction. Interestingly, many of these are important regulators of key genetic programs during development, such as members of the Gata (Ozawa et al., 2001; Watamoto et al., 2003), Forkhead (Li et al., 2007), NK-2 homeodomain-containing (Song et al., 2006) and signal transducer and activator of transcription (STAT) (Xiao et al., 2003) families of transcription factors.

Unfortunately, for the majority, these interactions remain as yet mechanistically and functionally poorly characterized. Future studies will hopefully shed some light on these interesting issues.

Class IIa HDACs are also candidates for cooperation and regulation of the members of the nuclear hormone receptor family. As a general mechanism, the conserved zinc finger DNA-binding domain of nuclear receptors shows ability to interact with HDAC4 (Franco et al., 2003). At least in the case of TR2, this interaction is of functional consequence as the recruitment of HDAC4 leads to a reduction of acetylated histone associated with a TR2-related promoter. More recently, a study identified HDAC7 as a novel corepressor of the androgen receptor (AR) (Karvonen et al., 2006). Class IIa HDACs also interact with RIP140, REA and ARR19, three transcriptional regulators recruited by the nuclear estrogen receptor

for the first two, and by the androgen receptor for the latter respectively (Castet et al., 2004; Jeong et al., 2004; Kurtev et al., 2004).

Interestingly, among the proteins interacting with class IIa HDACs, several contain ankyrin-repeat regions, indicating that similar structural determinants mediate association of class IIa HDACs with these transcription factors. Hence, class IIa HDACs interact with and repress the transcriptional activity of RFXANK, a positive regulator of MHC II genes expression (McKinsey et al., 2006; Wang et al., 2005), of ANCO 1, a nuclear receptor cofactor (Zhang et al., 2004), of B-CoR and B-CoR L1 (Huynh et al., 2000; Pagan et al., 2007), and CAMTA (Song et al., 2006). Class IIa HDACs also interact with ANKRA, a protein related to RFANX, but the functional implications of this interaction are unknown (McKinsey et al., 2006; Wang et al., 2005).

Atypical roles

As transcriptional coactivators of HIF-1α--Recent findings show that general HDAC inhibition may not only lead to gene activation but also to gene repression (Glaser et al., 2003; Nusinzon & Horvath, 2005). Supporting this in the case of class IIa HDACs, the C-terminus of HDAC4 and -7 but not HDAC5 have been shown to bind to hypoxia-inducible factor 1α (HIF-1α), a transcription factor that controls expression of genes responsive to low oxygen tension. Surprisingly, association with HDAC7 leads to increased HIF-1α transcriptional activity under hypoxic conditions (Kato et al., 2004). The exact mechanism underlying the transcriptional activation of HIF1-α by some members of class IIa HDACs remained unclear until recently, when it was reported that inhibition of HDAC4 by small interfering RNA had a positive effect on HIF-1α acetylation which correlated with reduced stability (Qian et al., 2006).

As SUMO E3 ligases--In addition to being sumoylated, HDAC4, and potentially other class IIa members can act as SUMO E3 ligases, a property dependent on their N-terminal domain (Gregoire et al., 2006; Gregoire & Yang, 2005; Zhao et al., 2005). Interestingly, MEF2 was identified as a relevant target for this novel class IIa-associated enzymatic activity. HDAC4 promoted sumoylation of MEF2 which inhibits its transcriptional activity. However, whether this effect is indirect or results from an intrinsic SUMO-ligase activity of HDAC4 is still matter of debate (Gregoire et al., 2006).

HDAC4 as a component of the DNA damage response pathway--Given the recent interaction unravelled between HDAC4 and the repair factor 53BP1, HDAC4 was though to be a

component of the DNA damage response (Kao et al., 2003). Indeed, in response to DNA damage, HDAC4 is recruited to the same dots, or repair foci, together with 53BP1. Moreover, silencing of HDAC4 abrogates DNA damage-induced G2 delay and increases radiosensitivity. Because drugs that inhibit transcription do not reverse these effects, the role of HDAC4 in DNA damage response is likely not restricted to transcriptional repression. Two recent studies shed new light on this mechanism by showing the DNA damage dependent-recruitment of HDAC4 by a repressor complex containing P53 and NF-Y, a complex that is involved in the repression of G2/M promoters (Basile et al., 2006; Imbriano et al., 2005).

HDAC7 as a mitochondrial protein implicated in programmed cell death--Sequence analysis identified a mitochondrial targeting motif in the N-terminal domain of human HDAC7 (Bakin & Jung, 2004). This motif targets HDAC7 to the mitochondria where it is N-terminally processed and can be located in the mitochondrial inner membrane space. The fact that HDAC7, like other pro-apoptotic mitochondrial factors, relocalizes to the cytoplasm in response to apoptosis points to a possible role in the initiation of programmed cell death. However, no additional experimental data is currently available to strengthen the significance of mitochondrial HDAC7 in the process of apoptosis.

V) Regulation of class IIa HDACs

Sub-cellular distribution

As expected from their ability to deacetylate histones, class IIa HDACs are found in the nucleus of most cell lines. However, depending on cell lines examined, a significant portion of the molecules can also be found in the cytoplasm, suggesting that these enzymes may shuttle between the nucleus and the cytoplasm (Fischle et al., 2001; Miska et al., 1999). Early confocal microscopy experiments with Leptomycin B and recently using the fluorescence loss in photobleaching (FLIP) technology confirmed that class IIa HDACs are subject to CRM1-dependent nuclear export (Dequiedt et al., 2006; Grozinger & Schreiber, 2000; Kao et al., 2001; Miska et al., 1999; Wang et al., 2000). Cytoplasmic accumulation of class IIa HDACs renders them unable to impact on transcription since it sequesters them away from their histone substrates and renders them enzymatically inactive as HDACs (Fischle et al., 2001; Fischle et al., 2002). The nucleo-cytoplasmic distribution of Class IIa HDACs is controlled by two separate domains: a nuclear localization signal (NLS) present in the N-terminal adaptor domain of all class IIa HDACs and a nuclear export signal (NES) in the C-terminal part

(McKinsey et al., 2001b; Wang & Yang, 2001). This property of class IIa HDACs has attracted a lot of attention in the past few years and has emerged as a major mechanism in the regulation of these enzymes.

Re-localization of class IIa HDACs to more specific sub-cellular compartments or structures could also participate in the regulation of class IIa HDACs. It was proposed that mitochondrial targeting and processing of HDAC7 may act as an irreversible intracellular reservoir to sequester HDAC7, that would otherwise be available for nucleo-cytoplasmic trafficking (Bakin & Jung, 2004). Class IIa HDACs can localize into specific sub-nuclear structures and HDAC5 has been found to form dot-like nuclear structures, termed matrix associated deacetylase bodies (MAD bodies) (Downes et al., 2000; McKinsey et al., 2000b; Miska et al., 1999; Wang et al., 1999a). In response to DNA damage, HDAC4 is recruited to nuclear repair foci, together with the DNA damage response protein 53BP1 (Kao et al., 2003). Additionally, ICP0, a protein encoded by the HSV-1 virus, has been shown to co-localize with and to promote the nuclear redistribution, in ring-shaped structures, of class IIa HDACs (Lomonte et al., 2004).

Phosphorylation of 14-3-3 binding sites

Several lines of evidence suggest that phosphorylation of their 14-3-3 sites regulates subcellular localization of class IIa HDACs. Inhibition of class IIa HDAC phosphorylation, by protein kinase inhibitors or serine-to-alanine mutation of the 14-3-3 consensus sites, leads to their nuclear accumulation. In contrast, activation of phosphorylation by overexpression of protein kinases leads to cytoplasmic accumulation of class IIa HDACs.

To date, four families of class IIa HDAC kinases have been identified (Figure 3). Historically, members of the Ca^{2+}/calmodulin-dependent kinase family (CaMK), specifically CaMKI and IV, were the first kinases shown to promote nuclear export of class IIa HDACs. Subsequent studies have confirmed the role of these kinases in signal-dependent activation of class IIa HDAC target genes (Chawla et al., 2003; Davis et al., 2003; Kao et al., 2001; Linseman et al., 2003; McKinsey et al., 2000a; McKinsey et al., 2000b). Recently, CaMKII was shown to specifically phosphorylate HDAC4, promote its cytoplasmic accumulation and lead to hypertrophic growth in cardiomyocytes (Backs et al., 2006). In contrast, observations supporting a biologically relevant role for CaMKI/IV in class IIa HDAC phosphorylation remain equivocal and argue that CaMK might not be the sole kinases responsible for regulation of class IIa HDAC nuclear export (Chawla et al., 2003; Dequiedt et al., 2005; Vega

et al., 2004a; Zhang et al., 2002a). Recently, protein kinase D (PKD), a downstream effector in PKC signaling, was shown to directly phosphorylate class IIa HDACs on their 14-3-3 binding sites and induce their cytoplasmic accumulation. Experimental evidence supports a role for the PKD-class IIa HDAC axis in T-cell apoptosis (Dequiedt et al., 2005; Parra et al., 2005), cardiac hypertrophy (Vega et al., 2004a) and B-cell receptor signalling (Matthews et al., 2006). Whether PKD is also involved in other class IIa HDAC-regulated biological processes remains unknown. In addition to CaMK and PKD, two members of the microtubule affinity-regulating kinase (MARK)/Par-1 family, MARK2 and MARK3, were identified as potent class IIa HDAC kinases and were shown to induce their cytoplasmic accumulation (Chang et al., 2005; Dequiedt et al., 2006). Interestingly, CaMK, PKD and MARK fall within the CaMK super family and possess related catalytic domains (McKinsey, 2007). While constitutively active in most cell lines (Dequiedt et al., 2006), MARK, via its upstream kinase LKB1, is also considered a stress-responsive kinase. Indeed, the LKB1/MARK pathway is activated by high osmolarity (Wang et al., 2007) or focal cerebral ischemia (Schneider et al., 2004). The identification of MARK as a class IIa HDAC kinase thus suggests that class IIa HDACs may be implicated in these stress signalling processes. The recent identification of AMPK/SNF1-like kinases as a new family of class IIa HDAC kinases further supports this idea. In *Caenorhabditis elegans*, KIN-29 was shown to phosphorylate the class IIa HDAC, HDA-4 *in vitro* and antagonize its repression of chemoreceptor gene expression in sensory neurons (van der Linden et al., 2007). In addition, salt-inducible kinase (SIK1), a mammalian KIN-29 homolog, phosphorylates two 14-3-3 binding sites of HDAC5, thereby alleviating its repression of MEF2-targeted genes in skeletal muscle (Berdeaux et al., 2007).

The existence of multiple families of kinases targeting class IIa HDACs, each with multiple isoforms, reflects the numerous biological roles of these transcriptional repressors. This diversity allows class IIa HDACs to respond to different signaling pathways, with each signaling pathway providing a functional relevance for a given kinase. Some kinases have been shown to act preferentially on specific members of the class IIa family, which emphasizes the importance that distinct class IIa HDACs may have in specific genetic program (Backs et al., 2006; Chawla et al., 2003; Liu et al., 2005). CaMKII, for example, is able to drive HDAC4, but not HDAC5, out of the nucleus, probably because HDAC4 possesses a non-conserved CaMKII binding site (Backs et al., 2006).

Some class IIa HDAC kinases may display preferences toward specific 14-3-3 sites. *In vitro*, PKD is much less effective at phosphorylating Ser^{259} than Ser^{498} of HDAC5 (Huynh & McKinsey, 2006). In contrast, MARK2 and -3 were shown to uniquely phosphorylate Ser^{155}

but not other 14-3-3 sites of HDAC7 (Dequiedt et al., 2006). CaMKI and -II target different sites in HDAC4 (Backs et al., 2006). These differences suggest that phosphorylation of each 14-3-3 site may have a specific impact on the regulation of class IIa HDACs and that multisite phosphorylation by distinct protein kinases may constitute a tightly regulated mechanism to induce expression of specific target genes in response to specific signals (Figure 3).

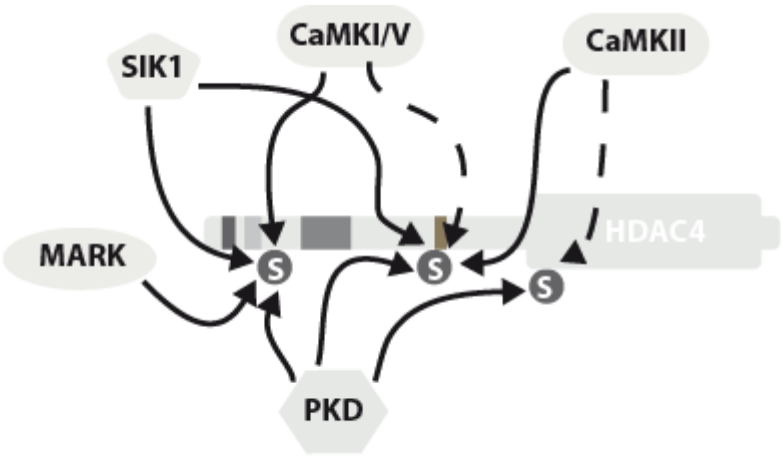


Figure 3: The 14-3-3 binding sites of Class IIa HDACs are phosphorylated by members of multiple families of protein kinases. Phosphorylation of HDAC4 is illustrated as an example as it is the only class IIa member phosphorylated by CaMKII. CaMKI and CaMKIV show a moderate preference for the most N-terminal site, whereas CaMKII exhibits some specificity for the internal site. PKD can phosphorylate indiscriminately the three 14-3-3 binding sites. In contrast, MARK selectively targets the most upstream serine residue. The two most N-terminal sites are optimal recognition motifs for SIK1 and are efficiently phosphorylated by this kinase.

Dephosphorylation at the 14-3-3 binding sites

It is logical to envision the phosphorylation-dependent regulation of class IIa HDACs as a reversible mechanism. In this context, relocalization of class IIa HDACs to the nucleus and reinstatement of their transcriptional inhibition would be expected to occur through dephosphorylation by a cellular phosphatase (Figure 4). Supporting this idea, Calyculin A, an inhibitor of protein phosphatases PP1 and PP2A, has been shown to promote cytoplasmic

retention of HDAC4 and to reduce its interaction with importin α (Grozinger & Schreiber, 2000).

Until recently, the identity of such an activating class IIa HDAC phosphatase was still an open question. A very recent study showed that, few hours after its signal-dependent nuclear export, HDAC7 becomes dephosphorylated and re-enters the nucleus. Myosin phosphatase, containing PP1β and myosin phosphatase targeting subunit 1 (MYPT1), was identified as an HDAC7-associated complex. This complex seems able to dephosphorylate the 14-3-3 binding sites of HDAC7 and promote its nuclear localization after signal-dependent export (Parra et al., 2007). In addition, results from our laboratory reveal that another cellular phosphatase, PP2A, stably associates with the N-terminal domain of class IIa HDACs. Our observations demonstrate that PP2A constitutively dephosphorylates class IIa HDACs *in vivo* and regulates their subcellular localization and repressor activity, thereby controlling their biological functions (Martin et al., 2008). The identification of multiple class IIa HDAC phosphatases may signify that class IIa HDACs dephosphorylation is regulated by different signaling pathways and would allow for flexible control of class IIa HDAC functions. Sequential or coordinated actions of the various protein kinases and phosphatases on the N-terminus of class IIa HDACs would constitute a tightly regulated mechanism to rapidly, appropriately and reversibly induce expression of specific target genes in response to specific signals.

Association with 14-3-3 and other cellular proteins

Association with 14-3-3 proteins soon appeared as a critical factor in the regulation of class IIa HDAC subcellular localization (Grozinger & Schreiber, 2000; Kao et al., 2001; McKinsey et al., 2000b; Wang et al., 2000). As observed for most 14-3-3 binding partners (Muslin et al., 1996), this association is dependent on the phosphorylation of interacting motifs within class IIa HDACs. 14-3-3 proteins are thought to regulate the subcellular localization of class IIa HDACs by modulating the function of their NES and NLS. First, binding of a 14-3-3 dimer would mask the N-terminal NLS and prevent class IIa HDAC recognition by importin α (Grozinger & Schreiber, 2000). Alternatively, binding of 14-3-3 proteins could unmask the C-terminal NES of class IIa HDACs or provide a NES in *trans*, facilitating nuclear export (McKinsey et al., 2000b). It is thus well-established that signal-dependent phosphorylation of the conserved serine residues and subsequent 14-3-3 binding are critical in the control of the subcellular localization of class IIa HDACs. However, it is still difficult to establish the

precise sequence of events involved in this mechanism. Whether binding of 14-3-3 precedes and/or promotes nuclear export or whether it takes place in the cytoplasm and prevents nuclear import of class IIa HDACs remains unclear. Clues about this issue came from experiments using HDAC5 constructs harboring inactivated NES. These mutants are unable to exit the nucleus despite intact phosphorylation 14-3-3 binding sites. Importantly, these mutants cannot efficiently inhibit muscle differentiation, indicating that release from their targeting DNA-binding factor is the crucial step in the relief of their transcriptional repression (Lu et al., 2000a; McKinsey et al., 2000a). In this context, 14-3-3-dependent nuclear export of class IIa HDACs may serve as a supporting mechanism to ensure maximal activation of their target genes (McKinsey et al., 2000a). Interestingly, the fact that 14-3-3 proteins are also regulated by phosphorylation may provide an additional level of regulation of class IIa HDAC nucleo-cytoplasmic shuttling (Ellis et al., 2003; Fu et al., 2000; Shen et al., 2003; Tzivion & Avruch, 2002).

In addition to 14-3-3 proteins, the subcellular localization and repressor activity of class IIa HDACs are controlled by additional cellular factors. The nuclear export of class IIa HDACs is inhibited by Leptomycin B, which suggests that the export receptor CRM1 may interact with the leucine-rich nuclear-export signal (NES) found in class IIa HDACs (Harrison et al., 2004; Wang & Yang, 2001). Whereas no CRM1-class IIa HDACs association has ever been formally documented, overexpression of CRM1 promotes cytoplasmic accumulation of HDAC7 (Gao et al., 2006).

Overexpression of MEF2 was reported to promote nuclear localization of class IIa HDACs. While direct interaction between both proteins was required for nuclear targeting of class IIa HDACs, no experiment was conducted to explain the underlying mechanism (Borghi et al., 2001; Chan et al., 2003; Miska et al., 1999; Wang & Yang, 2001).

Signaling

Multiple signaling pathways impact on class IIa HDAC localization, the effects of which can most of the time, be attributed to the activation of one or more class IIa HDACs protein kinases. In agreement with this, class IIa HDAC mutants in their phosphorylatable serine residues remain nuclear and are thus effective blockers of related developmental programs.

Agonists that stimulate PKD, through PKC-dependent or –independent mechanisms, induce class IIa HDAC phosphorylation and nuclear export. This is the case for the PKC activator PMA, for the B- and T-cell receptors and for several other cell surface receptors,

such as the serotonin receptor and the G protein coupled receptors (GPCR) ETR, α_1-AR and PGF2α. Signaling via Rho-GTPases, mediators of GPCR signaling, and via phospholipase C, a mediator that lies downstream of α_1-AR, also activates PKD and triggers phosphorylation-dependent nuclear export of HDAC5 (Chang et al., 2005; Dequiedt et al., 2005; Harrison et al., 2006). Some of these cell surface receptors also activate calcium fluxes and thus part of their effects could be mediated through activation of CaMKs (Backs et al., 2006). It has been shown that cardiac hypertrophic stimuli, such as pressure overload or expression of activated calcineurin, activate a cardiac kinase(s) that phosphorylates the regulatory serine residues within HDAC5 and HDAC9. However, the identity of this kinase remains unknown (Chang et al., 2004; Zhang et al., 2002a).

Neuronal receptors involved in synaptic activity also regulate class IIa HDACs localization in a way that depends on the phosphorylatable serine residues. Whereas neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5 was induced through both L-type calcium channels and synaptic NMDA receptors, differences in the activation thresholds for HDAC4 and HDAC5 nuclear export were observed (Chawla et al., 2003). Another neuronal signaling pathway converges on class IIa HDACs. In cultured cerebellar granules, HDAC4 accumulates in the nucleus in response to death stimuli. Interestingly, this translocation can be prevented by a survival factor, the neurotrophin BDNF. Treatment with a CaMK inhibitor promoted HDAC5 nuclear translocation, even with BDNF treatment, suggesting that CaMK may be a downstream effector of BDNF in this signaling pathway. Accordingly, the BDNF receptor can activate phospholipase C, which can then produce a rise in intracellular calcium and activate CaMK (Bolger & Yao, 2005). Of note, phospholipase C can also contribute to activate PKD. HDAC5 was reported to undergo similar nuclear translocation in response to low potassium, or to CaMK inhibition (Linseman et al., 2003).

Besides the above examples, very few signals have been reported to induce nuclear import of class IIa HDACs. Heat shock stimulation induced HDAC4 nuclear translocation (Dai et al., 2005), hypoxic conditions, in the presence of HIF1-α, drive HDAC7 to the nucleus (Kato et al., 2004) and hormone occupancy of the androgen receptor induces nuclear transfer of HDAC7 and -4 (Halkidou et al., 2004; Karvonen et al., 2006). The mechanism by which these signals lead to class IIa HDACs nuclear accumulation is not known and undoubtedly deserves further investigation.

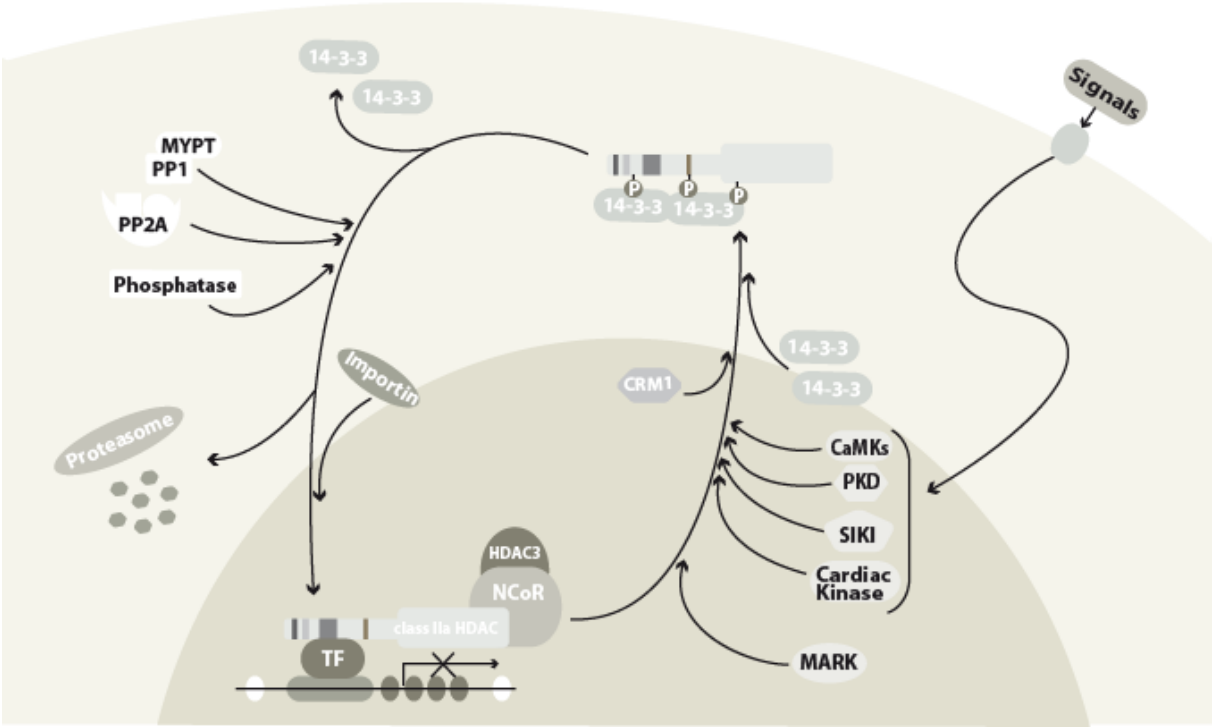


Figure 4: Model of class IIa HDAC regulation. The transcriptional repressor activity of class IIa HDACs is controlled through phosphorylation-dependent nucleo-cytoplasmic relocalization. In response to extra- or intra-cellular signals, PKD, CaMK, SIK1 or MARK phosphorylate specific regulatory serine residues in the adaptor domain of class IIa HDACs. Phosphorylation of class IIa HDACs is associated with displacement from their target promoters, association with 14-3-3 proteins and nuclear export through a CRM1-dependent pathway. This depletes the nuclear compartment of class IIa HDACs and allows for expression of a specific set of target genes. Import of class IIa HDACs to the nucleus and reinstatement of their transcriptional inhibition is achieved through dissociation from 14-3-3 proteins (by a still unknown mechanism) and dephosphorylation by cellular phosphatase such as PP2A or Myosin phosphatase. In the cytoplasm, dephosphorylated class IIa HDACs may be more sensitive to Ubiquitin-mediated proteolytic degradation.

Other posttranslational modifications

In addition to their 14-3-3 binding sites, other class IIa HDAC residues are subject to phosphorylation. Dyrk1B phosphorylates HDAC5 at a serine residue shared by HDAC4 and HDAC9, but not by HDAC7. Phosphorylation by Dyrk1 reduces their nuclear accumulation and leads to MEF2 activation (Deng et al., 2005). This residue does not correspond to a 14-3-

3 binding site and its mutation does not prevent CaMK-induced nuclear export (McKinsey et al., 2000a), but it lies within the nuclear localization region of class IIa HDACs. In contrast, oncogenic Ras 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., 2000b). However, no experiment demonstrated direct phosphorylation of HDAC4 by ERK1/2 nor identified the target residue(s).

HDAC4 and -9 are subject to sumoylation, at a lysine residue also conserved in HDAC5 (Kirsh et al., 2002; Petrie et al., 2003; Tatham et al., 2001). SUMO is believed to alter the interaction properties of its target, often affecting its localization within the cell (Seeler & Dejean, 2001). The functional relevance of class IIa HDAC sumoylation is still unclear, but sumoylation abolished HDAC4-associated HDAC activity (Kirsh et al., 2002). Interestingly, indirect evidence suggests that class IIa HDAC sumoylation might be coordinated with phosphorylation. Indeed, by a still-unknown mechanism, two proteins involved in the process of sumoylation, the SENP "SUMO remover" and PIAS, an E3 ligase, promote phosphorylation of 14-3-3 binding sites of HDAC5 (Chang et al., 2005). In addition, HDAC4 and HDAC7 are cleaved by caspases. In both cases, cleavage separates the N-terminal NLS from the C-terminal NES. Whereas the caspase-generated N-terminal fragment of HDAC4 retains repressor ability (Liu et al., 2004; Paroni et al., 2004), cleavage of HDAC7 abolishes its transcription repressor activity (F. Scott and F. Dequiedt, unpublished observations).

VI) Biological functions of class IIa HDACs

The existence of multiple regulatory pathways converging on class IIa HDACs emphasizes the importance of these enzymes in various biological processes. In the past 5 years, genetic inactivation studies in mice conducted by the Olson laboratory have been instrumental in elucidating the biological functions of these enzymes. These studies have revealed the key role of class IIa members in several important developmental and differentiation processes. Surprisingly, despite the large number of transcriptional regulators targeted by class IIa HDACs, most of their functions seem to involve the transcriptional repression of MEF2 transcription factors. In the following sections, we will review the latest insights into the biological functions of these fascinating enzymes.

Chondrocytes hypertrophy

HDAC4-null mice die during the perinatal period due to severe growth retardations and numerous skeletal abnormalities that result from excessive hypertrophic chondrocyte differentiation and inadequate endochondral ossification (Vega et al., 2004b). This remarkable phenotype was originally attributed to the ability of HDAC4 to repress Runt-related transcription factor-2 (Runx2), a well-recognized positive regulator of chondrocyte hypertrophy (Komori et al., 1997; Otto et al., 1997). Indeed, HDAC4 physically associates with Runx2 and inhibits its transcriptional activities. In addition, the phenotypic abnormalities of HDAC4 KO mice are strikingly reminiscent of those observed in mice with ectopic expression of Runx2 in prehypertrophic chondrocytes (Takeda et al., 2001; Ueta et al., 2001). The precise mechanism by which HDAC4 inhibits Runx2-mediated transcription remains obscure. Original observations suggested that repression of Runx2 by HDAC4 might occur independently of its HDAC catalytic activity. Instead, HDAC4 was thought to impede on Runx2 DNA binding by direct association with its Runt domain (Vega et al., 2004b). However this model failed to explain why HDAC4 mutants consisting of the C-terminal catalytic domain of HDAC4, and thus lacking the Runx2 binding region, retain significant repressive activity. The recent observation that HDAC4 and HDAC5 deacetylate Runx2 and Runx3 provides an explanation for these apparent discrepancies (Jeon et al., 2006; Jin et al., 2004). In addition to preventing Runx2 from binding to DNA, HDAC4 may deacetylate Runx2 and promote its ubiquitin-mediated degradation. This double-targeting of Runx2 by HDAC4 would thus solely explain its key role as a negative regulator of chondrocyte hypertrophy.

This apparently satisfying model was recently challenged by a study published by the same authors and which unravelled the unexpected role of MEF2C in chondrocyte hypertrophy (Arnold et al., 2007). This study reports that homo- or even heterozygous mutation of *Mef2c* is associated with severe skeleton defects resulting from reduced chondrocyte hypertrophy and ossification of endochondral bones. Given the preponderance of the class IIa HDACs-MEF2 axis in several developmental programs (figure 5, see below), this unexpected finding raises the possibility that bone defects associated with deletion of *Hdac4* could, at least partly, result from hyperactivation of MEF2C. Supporting this hypothesis, a genetic antagonism exists between HDAC4 and MEF2: the excessive endochondral ossification observed in HDAC4 null mice is partially abolished by deletion of one *Mef2c* allele. Conversely, inactivation of *Hdac4* in the presence of a heterozygous *Mef2c* allele

partially restored normal endochondral ossification. Interestingly, expression of Runx2 was greatly diminished in the endochondral cartilage of *Mef2c* mutant mice, indicating that some of the defects associated with *Mef2c* deficiency may be mediated by reduced Runx2 activity. By controlling key transcriptional regulators of chondrocyte hypertrophy, it is thus clear that HDAC4 plays a central role in the control of bone development. Interestingly, Runx2 is involved at multiple stages of bone development, such as osteoblast differentiation (Komori, 2008). Using in vitro models, HDAC7 was recently identified as a negative regulator of osteoblast differentiation through its Runx2 co-repressor function (Jensen et al., 2008). In addition, HDAC4 and HDAC5 have been shown to participate in the repression of Runx2 by Smad3 during osteoblast differentiation (Kang et al., 2005). While HDAC4 and -5 are expressed at significant levels in mesenchymal cells and osteoblasts, no evidence for HDAC7 expression in this cell lineage has been reported. In addition, the severe skeletal defects associated with HDAC4 deficiency were not observed in other class IIa HDACs mutant mice and could imply a specific role for HDAC4 as a negative regulator of bone development. A more careful examination of bone development in mice lacking class IIa HDACs should unravel any functional overlap that may exist between these proteins.

Myogenesis

-Skeletal Muscle differentiation

Formation of skeletal muscle involves commitment of multipotential mesodermal precursor cells to the muscle lineage and their proliferation as myoblasts. Upon mitogens withdrawal, proliferating myoblasts exit the cell cycle and differentiate into multinucleated muscle fibers. The myogenic process results from a specific genetic program, with activation of hundreds of muscle-specific genes and repression of genes associated with cell proliferation. MEF2 has long been known as a key transcriptional regulator of skeletal muscle differentiation. Logically, the functional association between MEF2 family members and class IIa HDACs was originally tested in the context of myogenesis (Lemercier et al., 2000; Lu et al., 2000b; Miska et al., 1999; Wang et al., 1999a). *In vitro*, class IIa HDACs negatively regulate muscle differentiation through association with MEF2 and repression of its target genes (Dressel et al., 2001; Haberland et al., 2007; Lu et al., 2000b). In addition, the inhibitory action of class IIa HDACs can be overcome by myogenic signals that disrupt MEF2–HDAC interactions and stimulate nuclear export of these transcriptional repressors during muscle differentiation (McKinsey et al., 2002b). Surprisingly, normal overall skeletal muscle differentiation was

maintained in mice mutated in each individual class IIa HDAC. This apparent discrepancy might result from the partial functional overlap that may exist between class IIa HDACs members. However, several observations indicate that the functional redundancy observed between class IIa HDACs *in vitro* may not exist *in vivo*. Indeed, class IIa members show distinct subcellular localizations in muscle cells. HDAC4 is mainly cytoplasmic in undifferentiated myoblasts and accumulates in the nucleus upon differentiation into myotubes, (Miska et al., 2001). In contrast, HDAC5 and HDAC7 relocate from the nucleus to the cytoplasm as myoblasts differentiate into myotubes (Dressel et al., 2001; McKinsey et al., 2000a). These findings suggest that class IIa HDACs respond differently to physiologic stimuli and could thus have distinct roles during skeletal muscle differentiation.

- *Skeletal muscle remodelling*

Skeletal muscle of adult vertebrates consists of type I and type II myofibers, which differ with respect to size, metabolism and contractile function. Slow-twitch or type I myofibers exhibit an oxidative metabolism and are resistant to fatigue, while in contrast, fast-twitch or type II fibers use glycolytic metabolism, fatigue rapidly and are involved in rapid bursts of activity. Numerous stimuli modulate skeletal muscle phenotype and induce switch from one specialized myofiber type to the other. MEF2 is preferentially activated in slow, oxidative myofibers (Wu et al., 2000a) and activates the slow myofiber gene program (Potthoff et al., 2007). Logically, class IIa HDACs were recently implicated in the regulation of myofiber identity. In slow or oxidative fibers, class IIa HDACs are selectively degraded through a proteasome-dependent pathway. This specific degradation of class IIa HDACs alleviates their repression over MEF2, and probably other unidentified transcription factors allowing the induction of a slow skeletal muscle specific gene program. Mice lacking individual class IIa HDACs in their skeletal muscle did not display abnormalities in fiber-type switching. However deletion of any combination of 4 alleles of *Hdac4*, *-5*, or *-9* results in enhanced slow-fiber gene expression and increased percentage of slow myofibers, revealing functional redundancy between class IIa members. Interestingly, overexpression of constitutively active forms of PKD and CaMKIV, in adult glycolytic fibers of transgenic mice results in an increase in the number of slow fibers and enhances muscle oxidative capacity (Kim et al., 2008a; Wu et al., 2002). Because previous studies have established that these kinases stimulate MEF2 activity by promoting the phosphorylation and export of class II HDACs from the nucleus (Martin et al., 2007), these observations further support a role for class IIa HDACs in fiber type specification.

In response to variations in environmental and functional demands, skeletal muscle adapt by remodelling the biochemical, morphological, and physiological states of individual myofibers (Bassel-Duby & Olson, 2006). These changes involve activation of intracellular signalling pathways and consequent genetic reprogramming, resulting in alterations of muscle mass, contractile properties, and metabolic states. Numerous stimuli, such as exercise, electrical stimulation or microgravity can induce muscle remodelling. Neuron-induced electrical activity has been known for a long time to induce specific transcriptional reprogramming of muscle and several neural activity-responsive genes have been identified. A typical example is the nicotinic acetylcholine receptors (AChRs), whose expression is highly sensitive to muscle innervation. AChR expression relies on the basic helix-loop-helix (bHLH) myogenic transcription factor myogenin. After innervation, neuron-induced electrical activity induces active transcriptional repression of myogenin throughout the muscle, which coincides with decreased extra-synaptic AChR expression levels. Recently, class IIa HDACs have been directly implicated in the regulation of myogenin expression. In innervated muscle, MITR, the non catalytic isoform of HDAC9, is highly expressed and represses transcription of myogenin through inhibition of MEF2. Conversely, denervation induces dramatic transcriptional downregulation of MITR, which correlates with subsequent myogenin and AChR expression (Mejat et al., 2005a). Interestingly, the observation that a MITR mutant protein lacking the MEF2-interacting domain retains 50% inhibitory activity on the expression of AChR and myogenin suggests that MITR could have additional targets besides MEF2. HDAC9-null mice do not show any obvious perturbation of skeletal muscle function under normal conditions but were sensitized to denervation. This sensitized phenotype is reminiscent of the cardiac phenotype observed in HDAC5 and HDAC9- null mice and establishes class IIa HDACs as general stress sensors and integrators.

Besides MEF2, Dash2, a Dachschund related transcriptional co-repressor whose expression is decreased upon denervation, was reported to inhibit myogenin expression in innervated muscle (Tang & Goldman, 2006). In contrast to MITR/HDAC9, HDAC4 expression is highly induced in response to denervation, suggesting that both class IIa HDACs could have opposite effects in denervation-induced nAChR expression (Cohen et al., 2007). This model is supported by the observation that neuronal input also induces nuclear accumulation of HDAC4 (Liu et al., 2005). In the nucleus, HDAC4 associates with the Dach2 promoter and thus activates the myogenin/AChR cascade through active down-regulation of Dach2. Unexpectedly, the opposite regulations of HDAC4 and MITR by neuronal activity lead to the same transcriptional outcome: induction of myogenin/AChR expression by

denervation. The most interesting corollary to these observations is that these two HDAC4 and MITR must impede on the neuronal activity-dependent transcriptional reprogramming of muscle through non-redundant, complementary pathways. Whether similar combinatorial regulation by multiple class IIa members also exists for other transcriptional programs involving class IIa HDACs needs to be investigated.

- Cardiac muscle development

Heart formation is initiated from the mesoderm in vertebrate embryos. The first stage of cardiomyogenesis involves the formation of cardiomyoblasts, which express a distinct subset of transcription factors, including MEF2C, the homeobox transcription factor Nkx2-5 and members of the cardiac GATA subfamily (Zaffran & Frasch, 2002). These factors activate the expression of cardiac-muscle-specific genes to form the differentiated cardiomyocytes. Overexpression of HDAC4, or inhibition of the inactivating class IIa HDAC kinase CaMK, was shown to inhibit the transition from mesoderm to cardiomyoblast during cardiomyogenesis in P19 cells (Karamboulas et al., 2006b). Surprisingly, mice mutated for individual class IIa HDACs do not exhibit obvious abnormalities of the hearth. In contrast, HDAC5/9 double mutant mice are prone to perinatal death from ventricular septum defects and thin ventricular walls, which typically originate from abnormalities in growth and maturation of cardiomyocytes(Chang et al., 2004). Whereas the molecular mechanisms underlying the role of class IIa HDACs in these processes remain obscure, studies in mice and *Drosophila* have established the importance of MEF2 in the control of cardiac muscle differentiation (Bour et al., 1995; Karamboulas et al., 2006a; Lilly et al., 1995; Lin et al., 1997; Ranganayakulu et al., 1995). It is thus likely that at least part of the cardiomyogenesis defects associated with class IIa HDACs inactivation arise from superactivation of MEF2 transcriptional activity. However, it is also possible that class IIa HDACs directly or indirectly represses other transcription factors that are important for cardiomyogenesis, such as SRF, CAMTA, Nkx2-5, myocardin and GATA factors (Davis et al., 2003; Han et al., 2006; Long et al., 2007; Song et al., 2006).

- Cardiac hypertrophy

Cardiac hypertrophy, which is defined as an increase in cardiomyocyte size and reactivation of a specific fetal cardiac gene program, is an adaptive response of the heart to a variety of stress stimuli. Stress-induced hypertrophy may initially normalize ventricular wall stress, but excessive hypertrophic growth of the heart frequently leads to maladaptive changes that

ultimately weakens cardiac performance and can lead to heart failure (Backs & Olson, 2006; Hill & Olson, 2008).

Transcripts for every class IIa HDACs, including the splicing HDAC9 variant MITR are found at high levels in mouse heart (Zhang et al., 2002a). In addition, ectopic expression of constitutively repressive mutants of HDAC4, -5 and -9 (i.e. mutants insensitive to signal-responsive class IIa HDAC kinases) suppress hypertrophy of primary cardiomyocytes and expression of fetal cardiac genes *in vitro* (Backs et al., 2006; Vega et al., 2004a; Zhang et al., 2002a) . Gene-inactivation studies in mice have provided definite evidence of the importance of class IIa HDACs as signal-responsive suppressors of postnatal cardiac growth. HDAC5 or HDAC9-KO mice exhibit no evidence of cardiac abnormalities during the early post-natal period. However, within their first year, these mutant mice develop spontaneous cardiac hypertrophy, which appears to result form hypersensitivity to age-related cardiac stress. HDAC5 and HDAC9 mutant mice also show exacerbated hypertophic response to pressure overload or constitutive activation of calcineurin (Chang et al., 2004; Zhang et al., 2002a). The remarkably similar cardiac phenotypes observed in HDAC5 or HDAC9 mutant mice suggest that these two class IIa members play redundant roles in the control of the same pathological hypertrophy signalling pathways that lead to cardiac hypertrophy. Unfortunately, mice lacking either HDAC4 or HDAC7 are not viable, which has precluded from investigating the potential involvement of these class IIa members in cardiac hypertrophy (Chang et al., 2006; Vega et al., 2004b). Conditional gene deletion studies will be needed to test whether HDAC4 and HDAC7 play functional roles similar to that of HDAC5 and HDAC9 in the cardiac pathologic response.

MEF2, and in particular MEF2D plays an important role as an integrator and mediator of stress-dependent remodelling of the adult heart (Kim et al., 2008b; Lu et al., 2000a; Nadruz et al., 2003). Indeed, *Mefd2d*-null mice display impaired cardiac hypertrophic response to pressure overload and adrenergic signalling (Kim et al., 2008b). Hypertrophic cardiac growth in HDAC5 or HDAC9 mutant mice correlates with superactivation of MEF2, suggesting that MEF2 is the critical target of class IIa HDACs in specific signalling pathways leading to cardiac hypertrophy. Indeed, while inactivation of *Mef2d* alleles renders mice resistant to cardiac remodelling in response to β-adrenergic stimulation, HDAC5 or HDAC9-null mice respond normally to isoproterenol stimulation (Chang et al., 2004; Kim et al., 2008b; Takahashi et al., 1999). This interesting observation could indicate that only specific signalling pathways of cardiac hypertophy involve class IIa HDAC-mediated regulation of MEF2. On the other hand, the insensitivity of mice lacking either HDAC5 or HDAC9 to

adrenergic stimuli could suggest a functional redundancy with other class IIa members in this particular pathway. Identification of the precise role and specific physiological relevant targets of each class IIa HDAC in cardiac hypertrophy will help solving these important issues.

Neuronal survival

HDAC4 and HDAC5 mRNA are highly abundant in the brain suggesting a role for these class IIa members in neurons (Grozinger et al., 1999). HDAC4 is predominantly cytoplasmic in cerebellar granule neurons (CGNs), and signals promoting its nuclear translocation or expression of constitutively nuclear mutants correlates with apoptosis (Bolger & Yao, 2005). Similarly, HDAC5 is mainly cytoplasmic in CGNs cultured under depolarizing conditions. Switching CGNs to non-depolarizing medium, which induces cytoplasm-to-nucleus translocation of HDAC4 and -5, correlates with induction of apoptosis (Linseman et al., 2003). MEF2 is a well-established survival factor in neurons (Mao et al., 1999) and as expected, induction of apoptosis by HDAC4 and -5 correlates with repression of MEF2 transcriptional activity (Bolger & Yao, 2005; Linseman et al., 2003). However, whether HDAC4 and/or HDAC5 target any other transcription factor besides MEF2 remains to be explored. Interestingly, HDAC4 appears to have a more predominant role in neuronal cell death, as siRNA-mediated inhibition of HDAC4 efficiently suppress neuronal death, arguing against functional redundancy between HDAC4 and HDAC5 (Bolger & Yao, 2005). In addition, HDAC5 knock-down mice showed no obvious brain defects, whereas mice lacking HDAC4 have abnormal shaped brains and exencephaly, probably resulting form precocious ossification of the brain (Vega et al., 2004b). However, more careful examination of HDAC4-null mice revealed delay in the formation of folia, suggesting a pro-survival rather than a pro-apoptotic role for HDAC4 (Majdzadeh et al., 2008; Vega et al., 2004b). Indeed, a recent study reported that HDAC4 prevents low-potassium induced neuronal cell death, through inhibition of activity of cyclin-dependent kinase-1 (CDK1) (Grozinger & Schreiber, 2000; Kao et al., 2001; Lu et al., 2000a; Wang et al., 2000). Supporting their results, the authors described higher CDK1 activity in the brain of HDAC4-/- mice (Majdzadeh et al., 2008). The reasons for these contradictory results remain unclear and should instigate further studies about the role of class IIa HDACs in neurons.

Immune cells

After entering the thymus, precursor T-cells differentiate into mature lymphocytes following a complex and highly regulated process (Ciofani & Zuniga-Pflucker, 2007). Complete maturation of developing thymocytes implies that they successfully pass a series of developmental checkpoints. After complete rearrangement of their T-cell receptor (TCR) chains, thymocytes become $CD4^+/CD8^+$, or double-positive (DP) thymocytes and undergo negative and positive selection. During these processes, their TCR is tested for its ability to interact with self-peptide bound to major histocompatibility complex (MHC) molecules of the thymic antigen-presenting cells (APCs). Positive selection will only allow further differentiation of thymocytes carrying a functional TCR. Potentially autoreactive thymocytes bearing TCR with strong affinity to MHC-self peptide complexes are deleted by the apoptotic process of negative selection. The different developmental fates of maturing thymocytes are dictated by the integration of various signals and the translation of these signals into changes in gene expression.

In human, HDAC7 is highly expressed in thymus, heart and lung. Within the thymus, we have shown that HDAC7 is transiently and predominantly expressed in CD4/CD8 DP thymocytes (Dequiedt et al., 2003). Consistent with this, HDAC7 associates with MEF2D and represses the expression of a series of MEF2-target genes involved in negative and positive selections, such as the pro-apoptotic orphan nuclear receptor *Nur77* (Dequiedt et al., 2003; Kasler & Verdin, 2007). The release of HDAC7-mediated repression is achieved through a complex signalling cascade. Engagement of the TCR by MHC-self peptides results in dissociation of HDAC7-MEF2D complexes, phosphorylation and cytoplasmic accumulation of HDAC7 which ultimately leads to de-repression of MEF2-target genes(Dequiedt et al., 2005). Interestingly, microarray-based analysis has revealed that HDAC7 could regulate both MEF2-dependent and independent genes (Kasler & Verdin, 2007). However, how HDAC7 regulates transcription independently of MEF2 was not investigated. Amongst the MEF2-HDAC7 regulated genes in thymocytes, this study also identified HDAC5, which could suggest a role for this class IIa member during T-cell differentiation.

In B cells, signalling from the B-cell antigen receptor has been shown to regulate HDAC5 and HDAC7 phosphorylation, localization and repressive function (Matthews et al., 2006). Even if chemical inhibition of HDACs supports a role for HDACs in B-cell differentiation and survival, the transcriptional targets and functional significance of these findings remain currently unknown.

Vascularisation

Early studies using general HDACs inhibitors suggested the possible implication of zinc-dependent HDACs in the transcriptional control of endothelial gene expression and vascular development (Kim et al., 2001; Kwon et al., 2002). However, until recently, definite experimental evidence about the function of class I and class II HDACs in these processes was lacking.

In situ hybridization studies revealed specific expression of HDAC7 in the vascular endothelium during mouse embryogenesis. Consistent with this, inactivation of the HDAC7 gene in a ubiquitous or endothelial specific manner had dramatic consequences on vascular integrity, with mutant mice dying *in utero* from blood vessel dilatations, ruptures and haemorrhages. In addition, siRNA-mediated inactivation of HDAC7 in human umbilical vein endothelial cells (HUVECs) prevents formation of capillary-like structures *in vitro* (Chang et al., 2006). Microarray analysis revealed that HDAC7 regulates the expression of several genes encoding extracellular matrix and adhesion proteins, among which the secreted matrix metalloprotease 10 (MMP-10). Regulation of *MMP-10* expression by HDAC7 relies on its association with MEF2C, a factor implicated in blood vessel development and vascular integrity (Lin et al., 1998), and constitutes yet another example in which the class IIa HDACs-MEF2 axis controls an important genetic developmental program. Inappropriate expression of MMP10 in HDAC7-null mice would be expected to disrupt endothelial cell-cell adhesion and could thus explain the defects associated with HDAC7 inactivation. However, a more recent study has brought some new light on the role of HDAC7 during angiogenesis and vascularisation. Using a complete set of *in vitro* assays, Mottet and colleagues have shown that the inability of HDAC7-silenced HUVECs to assemble into tube-like structures *in vitro* could result from alterations of their motility(Mottet et al., 2007). In addition, HDAC7 silencing correlates with increased expression of PDGF-B, a regulator of migration and tubulogenesis of endothelial cells (De Marchis et al., 2002). Even though the molecular mechanisms underlying the transcriptional control of PDGF-B by HDAC7 are unknown, these findings raise the appealing possibility that HDAC7 might regulate multiple stages during vasculature formation. Interestingly, HDAC7 silencing was also associated with altered morphology of the cells, which could also impact on their angiogenic properties (Holderfield & Hughes, 2008). Considering its potential impact on multiple aspects of vasculogenesis, identification of HDAC7 target genes has become a necessary step toward a better understanding of vascular development.

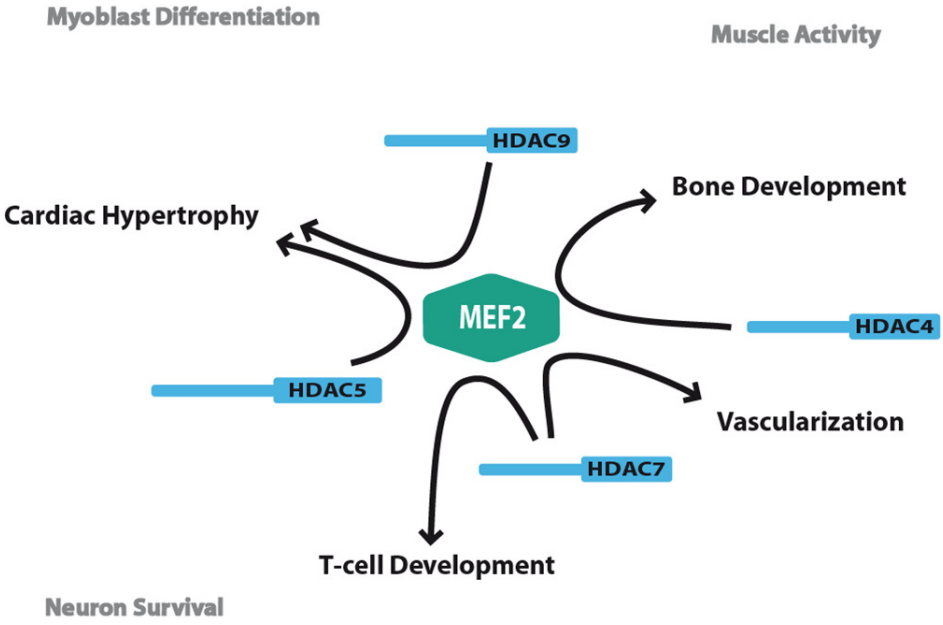


Figure 5: Central role of MEF2 in the biological functions of class IIa HDACs.Gene targeting studies in mice have revealed tissue specific functions for each member of the class IIa HDACs. HDAC4-null mice display inappropriate chondrocyte hypertrophy whereas mice lacking HDAC7 die from cardiovascular defects and mutant mice for HDAC5 and HDAC9 develop spontaneous cardiac hypertrophy. Surprisingly, the known biological functions of class IIa HDACs have been associated with repression of the MEF2 family of transcription factors. A similar class IIa HDACs-MEF2 axis could be involved in cardiac muscle development, skeletal muscle differentiation and remodelling and T-cell apoptosis.

VII) Therapeutic implications

During the past few years, the dedicated efforts of the Olson laboratory have been instrumental in establishing some important biological functions of class IIa HDACs *in vivo*. Strikingly, all these seemingly unrelated processes share the common characteristic of depending on the tight control of MEF2 transcriptional activity by class IIa HDACs. The fact that key processes such as formation of skeletal muscle, cardiac hypertophy, bone development, T-cell differentiation and neuronal survival, are controlled by class IIa HDACs suggests possibilities for therapeutical intervention in numerous human pathologies. Endothelial cell dysfunction is associated with several vascular diseases, such as arteriosclerosis (Verma et al., 2004), stroke and aneurysms (Kadoglou & Liapis, 2004), as

well as tumoral angiogenesis and metastasis (Ranieri & Gasparini, 2001). Dysregulation of growth plate chondrogenesis can result in dwarfism and skeletal abnormalities (Mundlos & Olsen, 1997). Similarly, defects in negative selection can led to autoimmune and lymphoproliferative syndromes (Sarvetnick & Ohashi, 2003; Siggs et al., 2006). It is now essential to establish whether alterations of the class IIa HDAC-MEF2 axis occur in these human pathologies. Genetic alterations of MEF2 family members have been linked to cardiovascular diseases (Visvikis-Siest & Marteau, 2006; Wang, 2005) and acute lymphoblastic leukemia (ALL) (Prima et al., 2005; Yuki et al., 2004). Similarly, alterations of MEF2-transcriptional activity have been implicated in neurodegenerative disorders (Camins et al., 2006) and cardiac hypertrophy (Czubryt & Olson, 2004).

Therapeutic intervention may be envisioned at a variety of points along the MEF2-HDAC pathway, the first one being modulation of class IIa HDAC enzymatic activity. In recent years, molecules inhibiting deacetylase activity of HDACs have generated a lot of interest as potential anti-tumors agents. This led to the development of a pletora of HDAC inhibitors that are now being tested in clinical trials for cancer and lymphoproliferative disorders (Bolden et al., 2006). Now that class IIa HDACs have been established as key modulators in several important developmental processes, the use of such inhibitors may also be considered to modulate the physiological pathways controlled by these enzymes. For instance, HDAC inhibitors may be used to promote bone formation in dwarfism associated with several skeletal dysplasias, or prevent vascularization during tumor metastasis. One caveat of this option is the lack of specificity of the known HDAC inhibitors toward the various human HDACs. Most of the inhibitors against zinc-dependent enzymes to date, target class I and class II HDACs rather non-selectively. Despite extensive efforts, development of class IIa HDAC-specific inhibitors has remained unsuccessful and thus specific targeting of each class IIa HDAC isoform may prove to be a hard goal to reach.

The current model predicts that suppression of class IIa HDAC nuclear export will prevent pathologic expression of MEF2-regulated genes. This could be achieved by preventing signal-induced phosphorylation of class IIa HDACs. In this context, the use of small molecule inhibitors targeting the known class IIa HDAC kinases is an attractive approach. However, this option could be complicated by the functional redundancy that exists between the various families (e.g., CaMKs to PKDs to Marks to SIK1) and between the members of the same family (e.g., PKD1 to PKD2 to PKD3, Mark2 to Mark3, CaMKI to CaMKII to CaMKIV). Nevertheless, the recent finding that HDAC4 is uniquely phosphorylated by CaMKII suggests that there might be some specificity in the regulation of

class IIa HDACs by phosphorylation (Backs et al., 2006). Because class IIa HDAC kinases take part in other important cellular functions, it will be important to test potential side effects of these compounds.

Any alteration of the equilibrium between the activities of cognate phosphatases and kinases would likely affect the subcellular distribution and thus biological functions of class IIa HDACs. Our most recent findings indicate that interaction with 14-3-3 proteins protects class IIa HDACs from dephosphorylation (Martin et al., 2008). Identification of drug-like molecules that displace this interaction would promote access of the phosphatases and consequently tip the balance towards hypophosphorylation and increased repression of MEF2-dependent transcriptional activity. Because 14-3-3, PP2A and myosin phosphatase are all involved in important biological functions, it is essential that the inhibitor be highly specific for the 14-3-3/class IIa HDACs interaction.

VIII) Conclusions and perspective

In the past few years, much has been learned about class IIa HDACs. Multiple interacting partners, involved in the association of class IIa HDACs with multi-enzymatic complexes, targeting to specific promoters or subcellular localization have been identified. Studies in transgenic and knock-out mice have given fundamental insights into their regulation and biological functions. Despite these significant advances several essential questions remain unanswered. The growing list of interacting partners suggests numerous putative transcriptional targets for class IIa HDACs. However, to date, the biologically relevant targets of class IIa HDACs have remained, for the great part, MEF2-dependent promoters. Large scale studies should be undertaken to identify MEF2-independent genes that could be regulated by class IIa HDACs. With the view to developing specific inhibitors for therapeutic purposes, it will be important to fully elucidate the mechanism, regulation and specificity of their deacetylase activity. Whereas the cytoplasmic localization of class IIa HDACs is thought to participate in their inactivation, it could also suggest a specific role for class IIa HDACs in the cytoplasm. This interesting issue deserves to be addressed. Finally, further analysis of class IIa HDAC regulation will not only improve our understanding of several fundamental processes for which they are key regulators, but will also provide invaluable new therapeutic perspectives for numerous human pathologies.

1.2. Protein Phosphatase 2A

As detailed above, several reports emphasize the crucial role of class IIa HDACs phosphorylation in their signal-dependent regulation. Consequently, a lot of efforts have been directed in the past few years towards the identification of specific kinases that phosphorylate class IIa HDACs. But protein phosphorylation is a dynamic and reversible process controlled by protein kinases and protein phosphatases. On our way to a better understanding of class IIa HDACs regulation, we ended up studying a new regulator of class IIa HDACs activity: the Proteine Phosphatase 2A (PP2A). In this second part of the introduction, the recent advances in the structure and regulation of this enzyme are reviewed.

1.2 Recent insights into Protein Phosphatase 2A structure and regulation: the reasons why PP2A is no longer considered as a lazy passive housekeeping enzyme

I) Summary

Although intracellular signal transduction is often portrayed as a protein kinase "domino effect", the counterbalancing function of phopshatases, and thus the control of phosphatase activity, is equally relevant to proper regulation of cellular function. Protein Phosphatase 2A (PP2A) is a widely expressed family of protein phosphatases made of a core dimer, composed of a catalytic (C) and a structural (A) subunit, in association with a third variable regulatory (B) subunit. Although viewed as a constitutive housekeeping enzyme in the past, PP2A is a highly regulated phosphatase and is emerging as an important regulator of multiple cellular processes involving protein phosphorylation. The regulation of PP2A is mainly accomplished by the identity of the regulatory B subunit, which determines substrate specificity, subcellular localization and catalytic activity of the PP2A holoenzyme. In agreement with this, recent findings on the structure and post-translational modifications of PP2A emphasize the importance of PP2A holoenzyme composition in its regulation and pleiotropic activities.

II) Introduction

Reversible protein phosphorylation is an important regulatory mechanism that controls the activities of a myriad of proteins and is thus involved in virtually every major physiological process. In the past, most of the attention was focused primarily on protein kinases and on their regulation, mainly because phosphatases were then viewed as simple housekeeping enzymes. But advances in the understanding of protein phosphatases make now clear that these enzymes are precisely regulated and are as important as kinases in the regulation of cellular processes involving protein phosphorylation.

Protein phosphatase 2A (PP2A) is a very abundant -it accounts for as much as 1% of total cellular proteins-, ubiquitous and remarkably conserved enzyme. A large and still-

growing number of PP2A substrates have been identified, which makes PP2A an important player in the regulation of a plethora of cellular processes.

Following, will be presented the recent advances in the structure and regulation of this fascinating enzyme.

III) Classification

While proteins can be phosphorylated on nine amino acids, serine, threonine and tyrosine phosphorylation are by far the most predominant in eukaryotic cells. The enzymes that dephosphorylate these three amino acids are classified into four groups on the basis of specific catalytic signatures/domain sequences and substrate preference. Among the 150 individual members of the protein phosphatases superfamily, more than two thirds belong to the protein tyrosine phosphatase family (PTP), which dephosphorylates phosphotyrosine and, in some cases also phosphoserine and phosphothreonine. The majority of the remaining enzymes are specific for phosphorylated serine and threonine residues and are divided into two families (Cohen, 2002; Moorhead et al., 2009): the phosphoprotein phosphatases (PPP) and the Mg2+ or Mn2+-dependent protein phosphatases (PPM). Recently, serine and tyrosine-phosphatases with an aspartic acid signature (DXDXT/V) have been identified that include the FCP/SCP [TFIIF (transcription initiation factor IIF)-associating component of CTD (C-terminal domain) phosphatase/small CTD phosphatase] and HAD (haloacid dehalogenase) family of enzymes (Moorhead et al., 2009).

The serine-threonine phosphatases share the common property of relying on the nucleophilic attack of the phosphorus atom by a metal-activated water molecule for their catalytic mechanism (Barford, 1996). The PPM family of phosphatases is mainly represented by the protein phosphatase type 2C (PP2C) whereas the PPP family is most diverse and contains 5 subfamilies. The PPP1 subfamily includes PP1 and the PPP2/4/6 subfamily comprises PP2A, PP4 and PP6. The PPP3 subfamily contains the Ca2+-activated PP2B. Two other minor families exists termed PPP5 and PPP7 which respectively comprises PP5 and PP7.

IV) Structure of PP2A

The native forms of PP2A holoenzymes are predominantly heterotrimers in which a core dimer, $PP2A_D$, made of a structural A subunit (also known as PR65) and a catalytic C subunit,

$PP2A_C$, is associated with a third variable regulatory B-type subunit. In addition to the classical PP2A heterotrimer, studies demonstrated that independent $PP2A_D$ core dimers are found within cells (Janssens & Goris, 2001; Kremmer et al., 1997). In addition, some specific PP2A dimers, in which the A subunit is replaced by the α4 protein have been recently identified (Yang et al., 2007).

The mammalian catalytic C subunit has two isoforms (α and β) which are 97% identical, ubiquitously expressed, highly conserved. While $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ seem to be interchangeable *in vitro* (Zhou et al., 2003), studies in mice suggested that both isoforms are not functionally redundant *in vivo* (Gotz & Schild, 2003; Gotz et al., 1998). Within the PP2A holoenzyme, the A subunit functions as a scaffold for the recruitment of the C and B-type subunits as well as additional proteins. The structural A subunit also exists in two isoforms, α and β, which are widely expressed and 86% identical in primary sequence (Hemmings et al., 1990). Interestingly, each A isoform shows differential ability to interact with B-type and C subunits (Zhou et al., 2003).

By far, the most variable subunit of the PP2A holoenzyme is the B-type subunit. To date, about 20 different isoforms have been described that are encoded by distinct genes or result from alternative splicing of a single gene. The mammalian B-type subunits are classified into three subfamilies, called PR55/B, PR61/B′ and PR72/B″ (table 1). Each B-type subunit can potentially combine with any of the two isoforms of both the A and C subunits, generating over 75 potential trimeric PP2A holoenzymes (Janssens & Goris, 2001; Janssens et al., 2008). This multiple combinatorial association is central to the mechanisms that regulate PP2A activity and ensure the pleiotropic roles of this important enzyme (Li & Virshup, 2002; Ruediger et al., 1992a).

The structure of the PP2A holoenzyme has long remained elusive. The first structural information came from the isolated PR65/A scaffolding subunit, which consists entirely of 15 tandemly repeated motifs known as HEAT (huntingtin-elongation-A subunit of PP2A -TOR). Canonical HEAT motifs consist of two helixes which form a helical hairpin. In PR65/A, 15 HEAT motifs stack together to form an elongated , horseshoe-shaped molecule with a continuous hydrophobic core (Groves et al., 1999; Hemmings et al., 1990; Walter et al., 1989). However, it was more than 15 years later that the crystal structures of a $PP2A_D$ and a $PP2A_{T61\gamma1}$ holoenzymes were solved (Cho & Xu, 2007; Xing et al., 2006b; Xu et al., 2006). The structural analysis of the PP2A core dimer showed that the catalytic subunit contains two catalytic metal ions at the active site and adopts a globular structure with an α/β fold, typical of the serine/threonine phosphoprotein phosphatase (PPP) family of phosphatases (Barford,

1996; Xing et al., 2006b). Consistent with previous mutagenesis studies (Ruediger et al., 1992b), structural data also revealed that the scaffolding subunit binds to the catalytic subunit via the intra-repeat loops of one end of its HEAT-repeats. Interestingly, these studies also pointed to a remarkable conformational flexibility of the A subunit, which undergoes pronounced conformational changes when incorporated into the PP2A core enzyme.

Name	**Gene #**	**Synonyms**
C subunit		
PP2A cataltytic subunit α	PPP2CA	PP2A-α, PP2A-Cα
PP2A cataltytic subunit β	PPP2CB	
A subunit		
PP2A structural subunit α	PPP2R1A	65kD regulatory subunit, PP2A-Aα, PR65α, R1-α
PP2A structural subunit β	PPP2R1B	
B subunit		
PP2A regulatory B subunit α	PPP2R2A	B55α, PR55α, R2α, 55kD regulatory subunit
PP2A regulatory B subunit β	PPP2R2B	
PP2A regulatory B subunit γ	PPP2R2C	
PP2A regulatory B subunit δ	PPP2R2D	
PP2A regulatory B' subunit α	PPP2R5A	B56α, PR61α, R5α, 56kD regulatory subunit
PP2A regulatory B' subunit β	PPP2R5B	
PP2A regulatory B' subunit γ	PPP2R5C	
PP2A regulatory B' subunit δ	PPP2R5D	
PP2A regulatory B' subunit ε	PPP2R5E	
PP2A regulatory B" α	PPP2R3A	B72/130, PR72/130, R3α, 72/130kD regulatory subunit
PP2A regulatory B" β	PPP2R3B	B70/48, PR70/48, R3β, 70/48kD regulatory subunit
PP2A regulatory B" γ	PPP2R3C	G5PR

Table 1. Nomenclature and corresponding gene names of PP2A various subunits

The crystal structure of a trimeric PP2A holoenzyme containing a regulatory PR61/B'$_\gamma$ subunit was reported independently by two laboratories (Cho & Xu, 2007; Xu et al., 2006). These studies revealed that, despite lacking canonical HEAT motifs, the PR61/B′$_\gamma$ subunit harbors a superhelical structure similar to that of A, with an apparent curvature that forms HEAT-like repeat motifs. In the PP2A$_{T61\gamma}$ trimer, the horseshoe-shaped A subunit undergoes additional conformational rearrangements, which brings the amino and carboxyl termini in close proximity. The C subunit and the convex side of the PR61/B' pseudo HEAT bind to the intra-repeat loops of HEAT repeats 2-7 and 11-15 respectively of the scaffold A subunit. PR61/B′$_\gamma$ also makes extensive discrete contacts with the C subunit by itself. In particular, the

C-terminal tail of the C subunit docks on the interface of the A and B-type subunits, where it could regulate the recruitment of the B-type subunit.

Crystal structure analysis gave valuable insights on how B-type subunits could regulate PP2A substrate specificities. Indeed, while the active site pocket of the PP2A catalytic subunit appears accessible to substrate, the binding of the PR61/B' subunit in the holoenzyme markedly changes the physicochemical environment near the active site and limits the accessible surface to the active site and provides novel potential substrate binding surfaces.

Crystallisation data also provided structural basis for PP2A regulation by post-translational modifications of the catalytic subunit. Methylation of the C-terminus of $PP2A_C$ selectively affects the assembly of PP2A trimers *in vivo* (see below). Nevertheless, *in vitro* holoenzyme formation is independent of $PP2A_C$ methylation since a C-terminal truncated mutant or an unmethylated catalytic subunit can still stably form a $PP2A_{T55}$ or $PP2A_{T61}$ trimeric complex (Ikehara et al., 2007; Xu et al., 2006). In addition to methylation, tyrosine phosphorylation is another modification of $PP2A_C$ C-terminal tail that regulates PP2A activity. Structural data indicate that a hydrogen bond forms between the side chain of the targeted Tyr307 residue and a carbonyl group in the peptide backbone of PR61/B′. Tyrosine phosphorylation would therefore be detrimental to the assembly of PP2A holoenzyme containing a PR61/B' subunit. Direct interaction between phosphorylated tyrosine and the active site within the catalytic subunit could also explain why tyrosine phosphorylation of $PP2A_C$ inhibits PP2A activity (Cho & Xu, 2007).

More recently, a study reported the crystal structure of a PP2A holoenzyme containing another family of regulatory subunit: the PR55/B_α family member (Xu et al., 2008). The sequence similarity between the various subfamilies of the regulatory subunits is very low, and in agreement with this, the structure of PR55/B_α subunit differs from the PR61/B' helical structure. Instead, the PR55/B_α subunit forms a seven-bladed β propeller, with each blade comprising four antiparallel β strands. In addition to the propeller core, PR55/B_α also contains additional secondary structure elements located above the top face which contribute to the formation of a putative substrate-binding groove in close proximity to the active site of the C subunit of PP2A. As observed for PR61/B', the regulatory PR55/B_α subunit recognizes the amino-terminal HEAT repeats of the A subunit. In contrast, PR55/B_α makes few interactions with the catalytic subunit, compared to the PR61/B' subunit, which leads to a relatively loose holoenzyme. The structural observations further suggest that the PR55/B_α subunit may form a relatively stable complex with the isolated A subunit, but do not seem to support the notion that the C subunit is required for interaction between the A and B-type subunits. Due to the

distinct structure of the PR55/B and PR61/B' structural subunits, the conformation of the scaffold A subunit is different in the $PP2A_{T55}$ holoenzyme compared to the $PP2A_{T61}$. The intrinsic conformational plasticity of the A subunit might therefore be important in order to interact with structurally different regulatory subunits (Xu et al., 2008). Indeed, the third regulatory subunit family PR72/B″ is predicted to adopt yet a different structure and contains two calcium binding EF hands (Janssens et al., 2003).

The recent characterisations of the structures of the PP2A holoenzyme are of prime interest because they constitute a new basis to improve the understanding of some aspects of PP2A assembly, function and regulation.

IV) Regulation

PP2A has been historically regarded as a relatively non-specific and unregulated enzyme. This allegation is in direct contradiction with the discrepancy that exists between the relatively small number of Ser/Thr phosphatases and the plethora of proteins that are reversibly phosphorylated on serine or threonine residues. It is now clear that PP2A, and the other protein phosphatases, are subjected to finely tuned control mechanisms that allow cells to adequately orchestrate changes in protein phosphorylation during virtually every cellular process (figure 1).

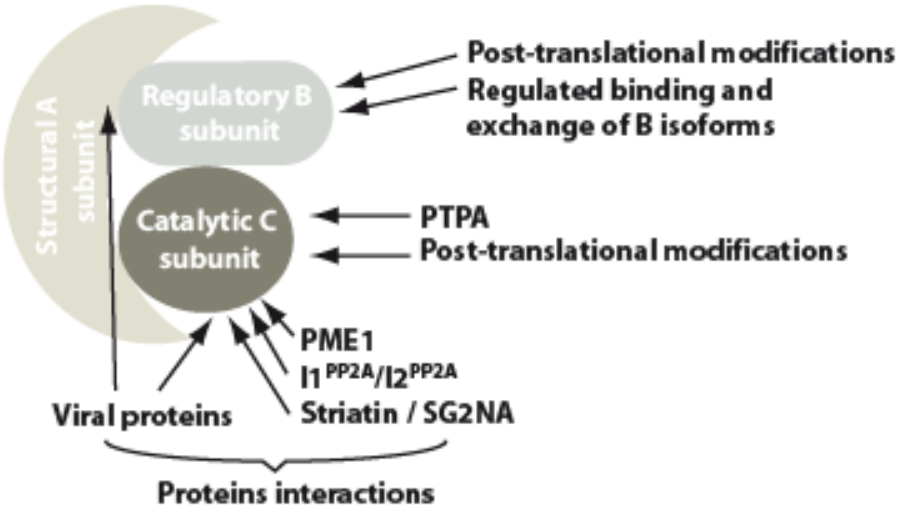


Figure 1. Summary of PP2A principal mechanisms of regulation

Holoenzyme composition

The composition of the holoenzyme is the most impacting determinant in the regulation of PP2A pleiotropic functions. It is now well recognized that the identity of the variable B-type subunit incorporated in the holoenzyme has specific consequences on PP2A activity. In accordance with structural data, binding of specific B-type subunit modulates the catalytic activity of PP2A *in vitro* (Sontag, 2001) and probably *in vivo*. In addition, the nature of the B-type subunit also influences substrate selectivity (Agostinis et al., 1992; Agostinis et al., 1990; Agostinis et al., 1987; Cegielska et al., 1994; Imaoka et al., 1983; Mayer-Jaekel & Hemmings, 1994; Mumby et al., 1987; Sontag et al., 1996). Lastly, subunit composition impacts PP2A localisation within the cell by targeting the phosphatase to specific subcellular compartments (Sontag, 2001).

Despite the lack of definite experimental evidence, recent data have lead to the model that the composition of the PP2A holoenzyme is not static *in vivo* and interconversions by dynamic exchange of regulatory subunits may represent a mechanism by which cells can quickly adapt to cellular demand at a given time. The observation that various viral proteins can replace specific regulatory subunits within PP2A holoenzyme *in vivo* provides a proof-of-principle that exchange between PP2A subunits is possible. In addition, B-type subunits can compete for binding to the $PP2A_D$ core complex *in vitro* (Kamibayashi et al., 1995) and suggest that the same phenomenon occurs within the cell. As detailed below, PP2A is subjected to diverse post-translational modifications which can have various impacts on B-type subunit binding. In this context, regulated specific post-translational modifications represent an attractive mechanism for controlling PP2A B-type subunit exchange. Alone or in combination, these post-translational modifications may constitute a "PP2A code" that dictates the formation of a specific holoenzyme or promotes the exchange between two subunits (Janssens et al., 2008).

Binding partners

The number of proteins interacting with PP2A is large and still-growing. These proteins can interact with one or more subunits and sometimes associate with a specific PP2A holoenzyme. PP2A partners play critical roles in its function and regulation. For instance, some interactors have been shown to target PP2A to specific cellular domains and regulatory functions (Ito et al., 2000; Kawabe et al., 1997; Sontag et al., 1995; Sontag et al., 1999; Takahashi et al., 1999; Turowski et al., 1999; Voorhoeve et al., 1999; Yan et al., 2000).

Moreover, PP2A is part of multi-molecular signalling complexes through its binding to specific kinases (Lebrin et al., 1999; Westphal et al., 1998; Westphal et al., 1999) or scaffolding proteins (Kikuchi, 1999).

Among the vast array of PP2A partners are multiple viral proteins, such as the polyoma small t and middle T, as well as with the small DNA tumour viruses simian virus 40 small t (SV40 ST) (Arroyo & Hahn, 2005; Janssens & Goris, 2001; Janssens et al., 2005). By directly binding to PP2A these viral antigens inhibit its phosphatase activity (Cayla et al., 1993; Kamibayashi et al., 1994; Scheidtmann et al., 1991; Yang et al., 1991) and /or displace the B-type subunit from the holoenzyme (Chen et al., 2004; Mumby & Walter, 1991; Pallas et al., 1990). This impairs the prevailing cellular functions of PP2A and might explain the transforming activities of these viral proteins.

Structure of the SV40 ST/PP2A complex was recently solved and provides the basis to explain PP2A inhibition by viral proteins. One domain of SV40 ST is in a position to directly interact with the PP2A catalytic C subunit, near its active site. Therefore, it is likely that binding of SV40 ST alters PP2A phosphatase activity through direct competition with substrate for access to the catalytic site. In addition, two distinct SV40 ST domains interact with a specific region of the structural A subunit that is also recognized by PR55/B and PR61B'. This observation could explain the competition that exists between SV40 ST and regulatory subunits for binding to the core enzyme (Chen et al., 2007; Cho et al., 2007). However, SV40 ST has surprising little affinity for PP2A and does not efficiently displace PR55/B, PR61/B' or PR72/B" from their respective holoenzymes *in vitro* (Chen et al., 2007; Cho et al., 2007). It is thus likely that modulation of PP2A holoenzyme assembly through displacement of structural subunits is only a minor contributor in the inhibition of PP2A activity by SV40 ST.

The $PP2A_D$ core dimer also forms stable complexes with two calmodulin-binding scaffolding proteins, Striatin and the S/G2 nuclear autoantigen (SG2NA), which suggests that species of PP2A could be recruited to Ca2+-dependent signal transduction cascades (Moreno et al., 2000). Striatin and SG2NA share some homology with PR61/B' isoforms and have sometimes been considered as a fourth regulatory subunit family. These PP2A interactors illustrate the fact that the distinction between a bona fide regulatory subunit and a binding partner is sometimes difficult. A proposition would be to consider a protein as a regulatory subunit only if it contains the canonincal A subunit-binding domain conserved in the existing regulatory subunits (Janssens et al., 2008).

Two intracellular heat stable inhibitors of PP2A, named $I1^{PP2A}$/Phap and $I2^{PP2A}$/SET have been identified. Both proteins inhibit specifically all holoenzyme forms of PP2A, probably by binding to the catalytic subunit (Li et al., 1996a; Li et al., 1996b).

Post-translational modifications

The catalytic subunit of the phosphoprotein phosphatase (PPP) family members is very conserved both in sequence and structure. The most distinctive feature of this subunit consists in a unique C-terminal tail which extends away from the globular structure and is crucially located at the interface between the two other subunits (Cho & Xu, 2007; Xing et al., 2006b; Xu et al., 2006). Consistent with an important functional role for this domain, a highly conserved Thr304-Pro-Asp-Tyr-Phe-Leu309 motif is heavily post-translationally modified by methylation, tyrosine and threonine phosphorylation. These modifications are crucial for PP2A regulation and holoenzyme formation.

Leu309 residue is subjected to carboxymethylation by the S-adenosylmethionine-dependent LCMT1 (leucine carboxyl methyltransferase 1) (De Baere et al., 1999; Lee & Stock, 1993). The reverse demethylation is achieved through the action of a specific phosphatase methylesterase, PME-1 (Lee et al., 1996). Carboxymethylation of $PP2A_C$ has been directly implicated in the regulation of PP2A holoenzyme assembly. Indeed, several studies have shown that methylation enhances the affinity of the PP2A core enzyme for some but not all regulatory subunits. More specifically, C-terminal $PP2A_C$ methylation seems to selectively affect the assembly of PP2A trimers containing a PR55/B subunit (Bryant et al., 1999; Gentry et al., 2005; Longin et al., 2007; Nunbhakdi-Craig et al., 2007; Ogris et al., 1997; Tolstykh et al., 2000; Wei et al., 2001; Wu et al., 2000b; Yu et al., 2001). In contrast, methylation of the C subunit seems to have little impact on the recruitment of other regulatory subunits (Gentry et al., 2005; Longin et al., 2007; Nunbhakdi-Craig et al., 2007; Wei et al., 2001). One indication of this selectivity relies on the observation that $PP2A_{T61}$ and $PP2A_{T72}$ can recruit a mixture of methylated and demethylated $PP2A_C$, whereas $PP2A_{T55}$ exclusively associates with methylated $PP2A_C$. Recent insights on PP2A structure suggest a plausible mechanism for how methylation could affect PP2A holoenzyme assembly. Indeed, crystal structure of a $PP2A_{T61}$ heterotrimeric PP2A holoenzyme has shown that the C-terminal $PP2A_C$ residue Leu309 does not mediate direct contact with the Aα or PR61/$B'_{\gamma 1}$ subunits but is located in a highly negatively charged environment formed by the side chains of Glu62, Asp63, Glu64 and Glu101 of the A subunit (Cho and Xu, 2007). Although methylation is not

strictly required for $PP2A_{T61\gamma1}$ assembly, neutralization of the $PP2A_C$ C-terminal negative charge by carboxymethylation would promote docking of the tail in this area and, therefore, binding of $PR61/B'_{\gamma1}$ to $PP2A_D$. Methylation of the catalytic subunit is thus a crucial determinant in PP2A holoenzyme composition and could participate in the regulation of its diverse functions *in vivo*. Surprisingly, several studies argue that methylation of the C subunit is not required for the *in vitro* assembly of PP2A holoenzymes involving the PR55/B and PR61/B′ regulatory subunits (Ikehara et al., 2007; Xu et al., 2006). It is important to note that most of *in vitro* studies use an inactive form of $PP2A_C$ harbouring a mutation which is known to alter the affinity of $PP2A_C$ for interacting partners (Janssens et al., 2008). Nonetheless, methylation may facilitate the assembly of the holoenzyme through enhanced binding affinity between the PP2A core enzyme and the regulatory subunit, this slight advantage being sufficient to tip the balance for holoenzyme assembly in cells but not *in vitro*. $PP2A_C$ carboxy-methylation in cells could also promote assembly of PP2A holoenzymes by recruiting assembly factors or by targeting the catalytic subunit to a specific cellular compartment where the assembly takes place.

Due to its importance in PP2A selective composition, regulation of $PP2A_C$ carboxy-methylation attracted a lot of attention these past few years. Methylation of $PP2A_C$ changes during cell cycle, suggesting a critical role in cell-cycle regulation (Janssens & Goris, 2001; Lee & Pallas, 2007). In addition, differences in subcellular localizations of LCMT1 and PME-1 suggest that methylation and demethylation might be spatially controlled (Longin et al., 2008). Interestingly, structure of PME-1 in complex with PP2A reveals that PME-1 directly binds to the active site of $PP2A_C$, what is supposed to lead to the eviction of the metal ions required for the catalytic activity of PP2A (Longin et al., 2004; Xing et al., 2008). These findings indicate that, in addition to removing the methyl group from Leu309, PME-1 could directly control the phosphatase activity of PP2A. The interaction also results in the activation of PME-1 by structural rearrangement, which ensures the specificity of the methylesterase activity towards PP2A (Xing et al., 2008).

In addition to methylation at Leu309, the $PP2A_C$ tail is also subjected to phosphorylation on Tyr307 and possibly on Thr304. Tyr307 phosphorylation seems to have two striking consequences. First, it could inhibit the interaction of $PP2A_C$ with PR61/B' (Longin et al., 2007; Nunbhakdi-Craig et al., 2007) by annihilating an hydrogen bound between Tyr307 of the catalytic subunit and the carbonyl group of Val257 in the peptide backbone of the $PR61/B'_{\gamma1}$ subunit (Cho & Xu, 2007). On the other hand, Tyr307 could indirectly affect the assembly of the PP2A holoenzyme containing PR55/B by preventing

methylation of Leu309. Indeed, it has been suggested that Tyr307 phosphorylation might impair access to the LCMT1 cavity (Longin et al., 2007; Nunbhakdi-Craig et al., 2007; Ogris et al., 1997; Yu et al., 2001).

It should be emphasized that the above observations result from mutagenesis analysis and need to be physiologically confirmed. Mutagenesis studies have also pointed out a role for threonine phosphorylation in B-type subunit selection. Phosphorylation of Thr304 induces the selective inhibition of PR55/B subunit recruitment (Gentry et al., 2005; Longin et al., 2007; Nunbhakdi-Craig et al., 2007; Ogris et al., 1997; Wei et al., 2001) without affecting Leu309 methylation (Longin et al., 2007; Yu et al., 2001).

Evidence suggests that regulatory subunits, and in particular PR61/B' could also be subjected to phosphorylation. Phosphorylation of PR61/B′ could have opposing effects depending on the physiological context. While phosphorylation of a conserved Ser/Pro motif by extracellular signal-regulated kinase ERK would promote dissociation of PR61/B′ from the catalytic subunit (Cho & Xu, 2007; Letourneux et al., 2006), phosphorylation of Ser37 by Chk1 enhances holoenzyme formation (Margolis et al., 2006).

It is now well-admitted that post-translational modifications of PP2A subunits have important roles in various aspects of holoenzyme regulation. Particularly, each B-type subunit is associated with a combination of specific post-translational modifications on $PP2A_C$. Leu309 methylation specifically favors formation of PR55/B subunit-containing PP2A holoenzyme. In contrast, Tyr307 phosphorylation is defavourable to association with PR55/B and PR61/B' and Thr304 selectively inhibits incorporation of PR55/B. This has lead to the notion of a "PP2A code" on the C-terminal tail that dictates the formation of specific PP2A holoenzymes (Janssens et al., 2008).

Substrate specificity

The reversible protein phosphorylation on proline-directed Ser/Thr motifs (Ser/Thr-Pro) is a key regulatory mechanism for the control of various cellular processes. Pro can exist in two conformations, *cis* and *trans*, in this motif. PP2A is considered as a major Pro-directed phosphatase which dephosphorylates phospho-Ser/Thr-Pro substrates. Studies of several PP2A substrates including Tau, Cdc25C, Myc and Raf1 substrates have lead to the hypothesis that a *trans* configuration of the proline residue adjacent to the phosphorylated residue is more favourable to dephosphorylation by PP2A. Pin1 is a peptidyl-prolyl isomerase (PPIase) which catalyses *cis-to-trans* isomerisation of specific pSer/Thr-Pro motifs. Studies have suggested

that isomerisation of the Ser-Pro bound by Pin1 would be required to promote dephosphorylation of substrates by PP2A (Dougherty et al., 2005; Stukenberg & Kirschner, 2001; Yeh et al., 2004; Zhou et al., 2000c). Nevertheless, some observation that specific PP2A holoenzyme (especially $PP2A_{T55}$) could dephosphorylate these motifs without Pin1 requirement are not compatible with this model (Agostinis et al., 1992; Mayer-Jaekel et al., 1994)

On the other hand, PP2A itself seems subjected to proline isomerization. Indeed, PTPA (phosphotyrosyl phosphatase activator, newly renamed phosphatase two A phosphatase activator) can activate the classical Ser-Thr phosphatase activity of a native inactive PP2A form (Longin et al., 2004) through an isomerase activity. Isomerization induces a conformational change in PP2A which correlates with its activation (Jordens et al., 2006; Leulliot et al., 2006).

V) Conclusions

Genetic deletion of PP2A catalytic subunit is lethal in yeast (Sneddon et al., 1990), demonstrating the prevailing place of PP2A in homeostasis. In accordance with this and early observations (Bialojan & Takai, 1988), dysregulation of PP2A-regulated signalling pathways can contribute to cancer (Arroyo & Hahn, 2005; Eichhorn et al., 2009; Janssens et al., 2005). Initial understanding of PP2A as a tumour suppressor was mainly based on the tumor-promoting activities of okadaic acid, the most famous naturally occurring PP2A inhibitor. But this loss of function approach does not discriminate between specific holoenzyme contribution and it now appears that the description of PP2A as a tumour suppressor is oversymplistic and needs more investigation (Eichhorn et al., 2009). Moreover, studies employing general inhibitory strategy, like okadaic acid, have pointed to a role for PP2A in multiple pathologies besides cancer. In this context, in order to improve our knowledge of this clinically relevant target protein, it seems important to dissect PP2A-controlled signalling pathways and, to achieve this, to precisely delineate specific cellular functions and context of each holoenzyme. Further studies on the precise role of individual PP2A B-type regulatory subunits within these signalling cascades is thus a challenging question for the future.

Chapitre 2: Objectives

Class IIa HDACs (HDAC4, -5, -7 and -9) act as transcriptional modulators of specific genetic programs associated with several key developmental processes. The transcriptional repressor activity of class IIa HDACs is controlled via phosphorylation-dependent nucleo-cytoplasmic shuttling. Phosphorylation of conserved serine residues in their N-terminal domain triggers association with 14-3-3 proteins, which overcomes the repressor activity of class IIa HDACs by eliciting their sequestration in the cytoplasm and making them unavailable for their cognate transcription factors and corepressors. Because regulation of class IIa HDAC phosphorylation provides the opportunity to control their associated developmental processes, a great deal of effort has been invested in identifying the inactivating kinases targeting class IIa HDACs 14-3-3 motifs. Nevertheless, it is logical to envision the phosphorylation-dependent regulation of class IIa HDACs as a reversible mechanism. Surprisingly, the identity of the antagonist phosphatase is still an open question.

In this context, the aim of this work is to identify and characterize such an activating class IIa HDACs specific phosphatase, using HDAC7 member as a paradigm. In order to validate its relevance, we also intend to assess the influence of the putative phosphatase on class IIa HDACs subcellular localization and repressive activity.

To achieve this, an *in vitro* dephosphorylation assay in presence of specific inhibitors will first allow us to find out the specific phosphatase targeting HDAC7. Interaction between the presumed phosphatase and its HDAC7 substrate will also be confirmed by immunoprecipitation experiments. The role of this phosphatase as a regulator of HDAC7 phosphorylation, subcellular localisation and transcriptional repressor activity will be analysed following its inhibition by specific chemical inhibitor and RNA interference. Afterwards, functional assays will be managed in order to illustrate the functional consequences of dephosphorylation-associated HDAC7 regulation during its two main related biological processes, i.e. T-cells apoptosis and endothelial cells angiogenic activity.

Finally, we will set up additional assays in order to further characterize the identified phosphatase and its regulation. Indeed, the control of phosphatase activity is equally relevant as kinase regulation to proper regulation of cellular function. Accordingly, protein phosphatases are now emerging as precisely regulated enzymes. Given the fact that recent knock-out mice studies have revealed an important biological role for HDAC7 during vasculogenesis at some stages of embryogenesis, we aim to study the HDAC7 phosphatase

regulation during this particular process. Many protein phosphatases are heteroligomeres, the composition of which determining the specificity of the holoenzyme catalytic activity. Using RNA interference coupled to functional assays measuring endothelial cells angiogenic abilities, we will determine the precise composition of the phosphatase that contributes to HDAC7 regulation during vascular network.

Chapitre 3: Results

3.1. Constitutive, selective and hierarchical phosphorylation of HDAC7

As stated in the introductory section, class IIa HDACs have been established as key regulators of several important developmental processes. In this context, study of the precise control of class IIa HDACs activity through the regulation of their phosphorylation could provide significant progresses in the understanding of biological functions involving class IIa HDACs. During our thesis, we tackled this complex problem and identified new molecular mechanisms involved in the regulation of these important enzymes.

In this section, we report the observation that class IIa HDACs are subjected to constitutive phosphorylation and identified EMK and C-TAK1, two members of the microtubule affinity-regulating kinase (MARK)/Par-1 family, as regulators of this process.

3.1. A New Role for hPar-1 kinases, EMK and C-TAK1 in Regulating Localization and Activity of Class IIa Histone Deacetylases

I) Summary

Class IIa histone deacetylases (HDACs) are found both in the cytoplasm and in the nucleus where they repress genes involved in several major developmental programs. In response to specific signals, the repressive activity of class IIa HDACs is neutralized through their phosphorylation on multiple N-terminal serine residues and 14-3-3-mediated nuclear exclusion. Here, we demonstrate that class IIa HDACs are subjected to signal-independent nuclear export that relies on their constitutive phosphorylation. We identify EMK and C-TAK1, two members of the MARK/Par-1 family, as regulators of this process. We further show that EMK and C-TAK1 phosphorylate class IIa HDACs on one of their multiple 14-3-3 binding sites and alter their subcellular localization and repressive function. Using HDAC7 as a paradigm, we extend these findings by demonstrating that signal-independent phosphorylation of class IIa HDACs conforms to a hierarchical pattern in which phosphorylation of the MARK/Par-1 site is a prerequisite for the phosphorylation of the other 14-3-3 sites. We propose that this multisite hierarchical phosphorylation by a variety of kinases allows for sophisticated regulation of class IIa HDACs function.

II) Introduction

Deacetylation of histones by histone deacetylases (HDACs) results in a compact chromatin structure that occludes the transcriptional machinery from accessing DNA and as such, HDACs mostly function as transcriptional repressors (Ng & Bird, 2000; Thiel et al., 2004). The 18 human HDACs identified to date are grouped into four distinct classes, with members of class II further subdivided into two subclasses, IIa and IIb (Yang & Gregoire, 2005). The class IIa HDACs (HDAC4, -5, -7 and -9) have a modular structure, comprising a conserved catalytic region at their C-terminus and an 'adapter' N-terminal domain that plays a central role in their regulation. First, this region contains conserved amino acid motifs that are specialized for binding an array of transcription factors. As an example, a

short motif is implicated in interactions with members of the MEF2 family and repression of MEF2-targeted promoters via recruitment of class IIa-associated HDAC activity has been extensively documented (Dequiedt et al., 2003; Lemercier et al., 2000; Lu et al., 2000a; McKinsey et al., 2000b; Miska et al., 1999; Wang et al., 1999a; Zhang et al., 2002a).

The adapter domain of class IIa HDACs is also subject to various post-translational modifications such as proteolytic cleavage (Bakin & Jung, 2004; Liu et al., 2004; Paroni et al., 2004), ubiquitination (Li et al., 2004), sumoylation (Kirsh et al., 2002; Petrie et al., 2003) and most importantly phosphorylation. Phosphorylation has recently emerged as the primary mechanism in the regulation of class IIa HDACs-mediated repression (Yang & Gregoire, 2005). In response to various stimuli, a number of serine residues in the adapter domain of class IIa HDACs are phosphorylated and become docking sites for 14-3-3 proteins. Association with 14-3-3 induces CMR1-dependent nuclear export and cytoplasmic accumulation of class IIa HDACs with concomitant derepression of their target promoters (Grozinger & Schreiber, 2000; Kao et al., 2001; Lu et al., 2000a; Wang et al., 2000). This nuclear export mechanism allows for signal-dependent activation of class IIa HDACs target genes and has proven to be crucial for various developmental programs such as muscle differentiation (McKinsey et al., 2000a) and activity (Mejat et al., 2005a), cardiac hypertrophy (Chang et al., 2004; Zhang et al., 2002a), T-cell apoptosis (Dequiedt et al., 2003), bone development (Vega et al., 2004b) and neuron survival (Bolger & Yao, 2005; Linseman et al., 2003).

Different signaling pathways converge on the signal-responsive serine residues of class IIa HDACs. It is now well established that members of Ca^{2+}/calmodulin-dependent kinases (CaMKs) promote nuclear export of class IIa HDACs (Chawla et al., 2003; Davis et al., 2003; Kao et al., 2001; Linseman et al., 2003; McKinsey et al., 2000a; McKinsey et al., 2000b). Recently, we and others have reported that specific stimuli can induce nuclear export of class IIa HDACs through a Ca^{2+}-independent mechanism involving Protein Kinase C (PKC). Protein Kinase D (PKD, also known as PKCμ), a downstream effector in PKC signaling, was indeed shown to directly phosphorylate HDAC5 and HDAC7 on the serine residues that control their nucleocytoplasmic trafficking (Dequiedt et al., 2005; Parra et al., 2005; Vega et al., 2004a). In addition, several reports suggest that other protein kinases might also be involved in signal-dependent class IIa HDACs phosphorylation and subcellular localization (Zhang et al., 2002a; Zhao et al., 2001). More interestingly, recent observations indicate that subcellular localization of class IIa HDACs might also be

constitutively regulated in a signal-independent manner (Bolger & Yao, 2005; Liu et al., 2005).

In this study, we identified the hPar-1/MARK (Microtubule Affinity-Regulating Kinase) kinases, EMK and C-TAK1 as constitutively active kinases regulating class IIa HDACs subcellular trafficking. Both kinases directly phosphorylate class IIa HDACs on their N-terminal adapter domain, promoting their nuclear export and leading to derepression of MEF2-dependent transcription. Unexpectedly, we found that, among the multiple conserved residues previously involved in nucleocytoplasmic shuttling of class IIa HDACs, MARK/Par-1 kinases specifically target a unique site. More importantly, phosphorylation of this site is a prerequisite for subsequent phosphorylation at other serine residues. These results support a model of hierarchical class IIa HDACs phosphorylation and establish a new role for MARK/Par-1 kinases in the control of gene expression.

III) Materials and Methods

Plasmids, Antibodies and Chemicals

C-terminal green fluorescence (GFP) fusion proteins of human HDACs have been described elsewhere (Fischle et al., 2001; Fischle et al., 2002). GST-fusion proteins of the N- (aa 1-490) and C-terminus (aa 490-915) of HDAC7 and the N-terminus (aa 1-661) of HDAC4 have been described in (Dequiedt et al., 2005; Fischle et al., 2002). GST-S155, GST-S181, GST-S321 and GST-S449 constructs contain respectively amino acids 130-180, 156-216, 267-345 and 396-490 of HDAC7, cloned into pGEX4T1 (Pharmacia) (Dequiedt et al., 2005). GST-HDAC4 and GST-HDAC5 correspond to amino-acids 221-272 and 234-285 of HDAC4 and HDAC5 respectively. Serine-to-alanine and leucine-to-alanine substitutions were introduced by PCR and mutations were verified by direct DNA sequencing according to standard methods. GST-Cdc25C and GST-Cdc25CS216A fusion proteins are referenced in (Bachmann et al., 2004; Peng et al., 1998). Expression vectors for active EMK and C-TAK1 have been described (Ogg et al., 1994; Peng et al., 1998). The Nur77 Luciferase construct has been described elsewhere (Dequiedt et al., 2003).

Anti-FLAG, anti-panPKC, anti-pan14-3-3, anti-c-jun, anti-HDAC7, anti-HDAC1, anti-Actin and anti-Tubulin antibodies were purchased from Santa Cruz Biotechonology (Santa Cruz, CA). Anti-C-TAK1, anti-RSK1 and RSK2, anti-MSK1 and MSK2 were obtained from Upstate Biotech (Lake Placid, NY). Monoclonal antibody against EMK has

been described in (Hurov et al., 2001). Polyclonal antibodies against phosphorylated Ser^{155} and phosphorylated Ser^{181} were generated by 21st Century Biochemicals (Marlboro, MA). Rabbits were immunized with the KLH-linked peptides HFPLRKTVpS_{155}EPNLKLRYKP and KNPLLRKEpS_{181}APPSLRRRP respectively and sera were collected and purified according to the company's procedures.

Staurosporine (Alexis Biochemical Corp., Lausen, Switzerland) and Leptomycin B (Merck Biosciences Inc, Darmstadt, Germany) were used at 1 μM and 10 ng/ml respectively.

Cell Culture

The following cell lines were used in these experiments: Cos7, African green monkey, SV40-transformed kidney cells (ATCC CRL-1651); HeLa, human cervical epitheloid carcinoma (ATCC CCL-2.1); HEK293, transformed human kidney (ATCC CRL-1573) and Do11.10 T-cell hybridomas (White et al., 1983). Cell lines were grown in recommended medium (DMEM for HeLa, HEK293 and Cos7 and RPMI 1640 for Do11.10 cells respectively) supplemented with 10% fetal bovine serum (FBS), 2mM Glutamine and 50U/ml of streptomycin/penicillin at 37°C in a humidified incubator.

RNA interference (RNAi)

Functionally validated Stealth™ RNAi Duopacks, each containing two non-overlapping Stealth™ RNAi molecules (duplex 1 and duplex 2) directed against C-TAK1 or EMK, and corresponding Stealth™ RNAi Negative Control were purchased from Invitrogen (Invitrogen, CA). HeLa cells were plated to achieve 60 to 80% confluence at the time of transfection. GFP-HDAC7 (for immunofluorescence analysis) or FLAG-HDAC7 (for western blotting analysis) were transfected along with either pooled siRNA duplexes 1 for EMK and C-TAK1 (50 nM of each duplex) or control siRNA using Lipofectamine™ 2000 according manufacturer's instructions. Twenty-four hours after this first transfection, cells were transfected with pooled siRNA duplexes 2 for each kinases, or control siRNA. Cells were then left to recover in complete medium for an additional 36-48 h before being processed for immunofluorescence or western blotting analysis as described below.

Confocal microscopy

For steady-state immunofluorescence experiments, GFP constructs were transiently transfected with standard calcium phosphate method (for Cos7 and 293 cells) or with

Lipofectamine™ 2000 (HeLa cells) according to the manufacturer's instructions (Invitrogen, CA). Localization of the fluorescent proteins was assessed on fixed cells (for Cos7 and HeLa) or live cells (for HEK293) by confocal microscopy (Axivert 200 with LSM 510; Carl Zeiss Microscopy). When indicated, the average percentage of cells showing nuclear exclusion of the GFP-tagged protein was assessed by examining at least 3 independent fields each containing more than 50 cells. Imaging of living Cos7 cells was performed after transfection with FUGENE 6 (Roche Diagnostics, Basel, Switzerland) on a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany) equipped with a 488-nm argon laser. Cells were grown and transfected in MatTek glass-bottomed dishes (MatTek, Ashland, MA). Forty-eight hours after transfection cells were maintained in DMEM supplemented with 30 mM HEPES, pH 7.0. During the measurements the medium was kept at 37°C in an atmosphere containing 5% CO_2 using a LSM 510 Incubator S (Carl Zeiss). Quantitative analysis of the nuclear and cytoplasmic fluorescence intensity was performed on images of the midsection of living cells. The midsection was first determined by a Z-stack. The nuclear/cytoplasmic ratio of fluorescence intensity was quantified using the Image J public domain Java image processing program (http://rsb.info.nih.gov/ij/download.htm). For quantification, the fluorescence intensity in the cytoplasmic or nuclear compartment was determined in a 0.5 x 0.5-µm square that was centered in the nucleus or cytoplasm, respectively (2 values per cell). Results are the means of the fluorescence intensity determined in 20 cells expressing the GFP-fusion HDAC7 proteins. The relative nuclear fluorescence intensity was calculated using the following equation: Finuclear: Finuc/n/Ficyt/n. Finuc is the fluorescence intensity in the nucleus, Ficyt is the relative fluorescence intensity in the cytoplasm, and n is the number of cells examined.

For Fluorescence Loss in Photobleaching (FLIP) experiments, a time series of images was recorded live was collected as described above. The bleach rate for each series of images was calculated and used to correct the fluorescence intensities of the images. For qualitative FLIP analysis, cells imaged using the 488-nm line of an argon laser of a Zeiss LSM510 META confocal microscope operating at 40% laser power and 5% transmission (imaging intensity). Four imaging scans of a single cell were performed. Then, the cytoplasm was chosen as region of interest by the LSM 510 Meta software and selectively bleached four times with 100% transmission (bleaching intensity) 30 s each, followed by imaging scans at the times indicated in the figure. At least 10 data sets were analyzed for

each time point. The background corrected nuclear fluorescence at the time before the initial photobleaching was set as 100%.

GST Fusion Proteins: Expression, Purification and Pull-downs

HDACs portions and human EMK and C-TAK1 were purified as GST-fusion proteins in BL21 RIP (Stratagene) according to protocols described elsewhere (Dequiedt et al., 2005). For IVK assays, purified GST-hEMK and GST-hC-TAK1 were first eluted from the beads by incubation in 25 mM reduced glutathione, 50 mM Tris-HCl pH 8. Recombinant, active GST-PKD1 was produced and purified from mammalian cells as described previously (Vertommen et al., 2000).

Pull-down reactions and immunoaffinity purifications form cell extracts were performed exactly as described (Dequiedt et al., 2003; Dequiedt et al., 2005).

SDS-PAGE and Western Blotting

SDS-PAGE and western blot analysis were performed according to standard procedures. (Sambrook et al., 1989) Western blots were developed with the ECL detection kit (Amersham Pharmacia Biotech).

In vitro kinase (IVK) assay and in-gel kinase (IGK) assay

Purified GST-fusion proteins were incubated with a dilution of recombinant active protein kinase (0.5 µg/ml) in 30 µl of phosphorylation mix containing 10 µM ATP with 10µCi [γ-^{32}P]ATP, 25 mM Tris, pH 7.5 and 10 mM $MgCl_2$. After 30 min at 30 °C, reactions were terminated by adding an equal amount of 2× SDS-PAGE sample buffer, resolved by SDS-PAGE analysis and phosphorylated proteins were visualized by autoradiography.

IGK assays protocol was a slightly modified version of (Shi et al., 2002). Briefly, pull-down reactions were resolved by standard SDS-PAGE analysis with no exogenous substrate incorporation in the gel matrix. After electrophoresis, the gel was soaked in 20% 2-propanol in 50 mM Tris/HCl, pH 8.0 and then in buffer A [50 mM Tris/HCl with 5 mM dithiothreitol (DTT)] to remove SDS. Resolved proteins were denatured by incubating the gel for 1 h in a solution of 6 M guanidine-HCl in buffer A and then renaturated in buffer A containing 0.04% Tween-20 overnight at 4°C. The gel was pre-incubated in the phosphorylation buffer (see above) omitting the [γ-^{32}P]ATP. 10 µM of ATP with 100 µCi [γ-^{32}P] ATP were then added and the phosphorylation was allowed to proceed for 1 h at 30°C. Finally, the gel was washed in 5 % (w/v) trichloroacetic acid plus 1 % (w/v) sodium

pyrophosphate, dried and analyzed by autoradiography to detect autophosphorylated proteins.

Reporter assays

Transient transfections of Do11.10 cells using the DEAE-Dextran method and Dual Luciferase (Promega) reporter assays have described before (Dequiedt et al., 2003; Dequiedt et al., 2005).

Metabolic labeling and phosphorylation site analysis

For in vivo phosphorylation site mapping, Do11.10 cells or transfected HEK293 cells were incubated for 3 h at 37°C in phosphate-free DMEM containing 500 µCi of [32P] orthophosphate per ml. Cells were then washed twice in ice-cold phosphate-buffered saline (PBS) and lyzed in IPLS buffer (Fischle et al., 2002). Labeled proteins were purified by immunoaffinity. In vitro phosphorylation reactions of GST-HDAC7Nter by purified GST-hC-TAK1, GST-EMK or GST-PKD were performed following the IGK protocol described above.

Purified proteins were resolved by SDS-PAGE analysis and coomassie staining. Phosphorylated proteins were in-gel digested with trypsin. The phosphorylated peptides were separated by high pressure liquid chromatography (HPLC) on a Thermo Hypercarb column (2.1 mm x 15 cm) in an acetonitrile gradient in 0.1 % (v/v) trifluoroacetic acid (solvent A). Elution was performed with the following gradient program: 5-100% solvent B (70% (v/v) acetonitrile in solvent A) over 100 min at a flow rate of 200 µl/min generated by a model 1100 Agilent HPLC system. Radioactive peaks were detected by Cerenkov counting, dried down under vacuum, redissolved in 5 µl of 50% (v/v) acetonitrile/0.3% (v/v) acetic acid and analyzed by nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) in a LCQ Deca XP Plus ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). Spectra were taken in full MS and zoom scan mode to determine parent ion monoisotopic masses and their charge state. Phosphopeptides were identified in MS/MS mode as by the loss of H_3PO_4 (98 Da) under low collision-induced dissociation energy and the phosphorylated residue was pinpointed in MS^3 mode.

IV) Results

Class IIa HDACs are constitutively phosphorylated and bound to 14-3-3 proteins

Phosphorylation-dependent nuclear exclusion of class IIa HDACs is thought to be mediated by at least two signal-responsive families of kinases, the CaMKs and PKDs. However, accumulating evidence suggests that class IIa HDACs could also be phosphorylated in the absence of external stimulus and implies the existence of additional HDACs kinases distinct from CaMKs or PKD (Bolger & Yao, 2005; Kao et al., 2001; Liu et al., 2005).

As a step toward the identification of these new class IIa HDACs kinases, we first investigated whether endogenous HDAC7 could be phosphorylated in normal growing conditions. In vivo, HDAC7 is predominantly expressed in double positive thymocytes (Dequiedt et al., 2003). Logically, we found that amongst numerous cell lines tested, T-cell hybridomas express high levels of the HDAC7 protein (data not shown). Do11.10 T-cell hybridoma cells were thus metabolically labeled with inorganic ^{32}P and endogenous HDAC7 was recovered by immunoprecipitation. As shown in Figure 1A, HDAC7 appeared as a phosphorylated protein after SDS-PAGE analysis and autoradiography. Interestingly, phosphorylation of endogenous HDAC7 was almost completely abolished by treatment with staurosporine, a general serine/threonine kinase inhibitor (Fig. 1A, Stauro. +).

According to the current model, phosphorylation of specific serine residues in class IIa HDACs creates docking sites for 14-3-3 proteins. To confirm the above observations, we thus examined the interaction between HDAC7 and 14-3-3 proteins in Do11.10 cells. Endogenous HDAC7 (Fig. 1B, left panel, IP: α-HDAC7) or ectopically expressed Flag-tagged HDAC7 (Fig. 1B, right panel, IP: α-FLAG) were immunoprecipitated and analyzed for association with endogenous 14-3-3 proteins. Western blot analysis revealed a constitutive association between endogenous 14-3-3 proteins and endogenous or FLAG-HDAC7 (Fig. 1B, α-14-3-3). Interestingly, treatment with staurosporine resulted in a drastic reduction in the interaction between HDAC7 and 14-3-3 proteins. Similar observations were made with HDAC4 and -5 in HEK293 cells (Fig. S1). These results thus suggest that class IIa HDACs exists as phosphoproteins in unstimulated cells and are constitutively associated with 14-3-3 proteins, in a phosphorylation-dependent manner.

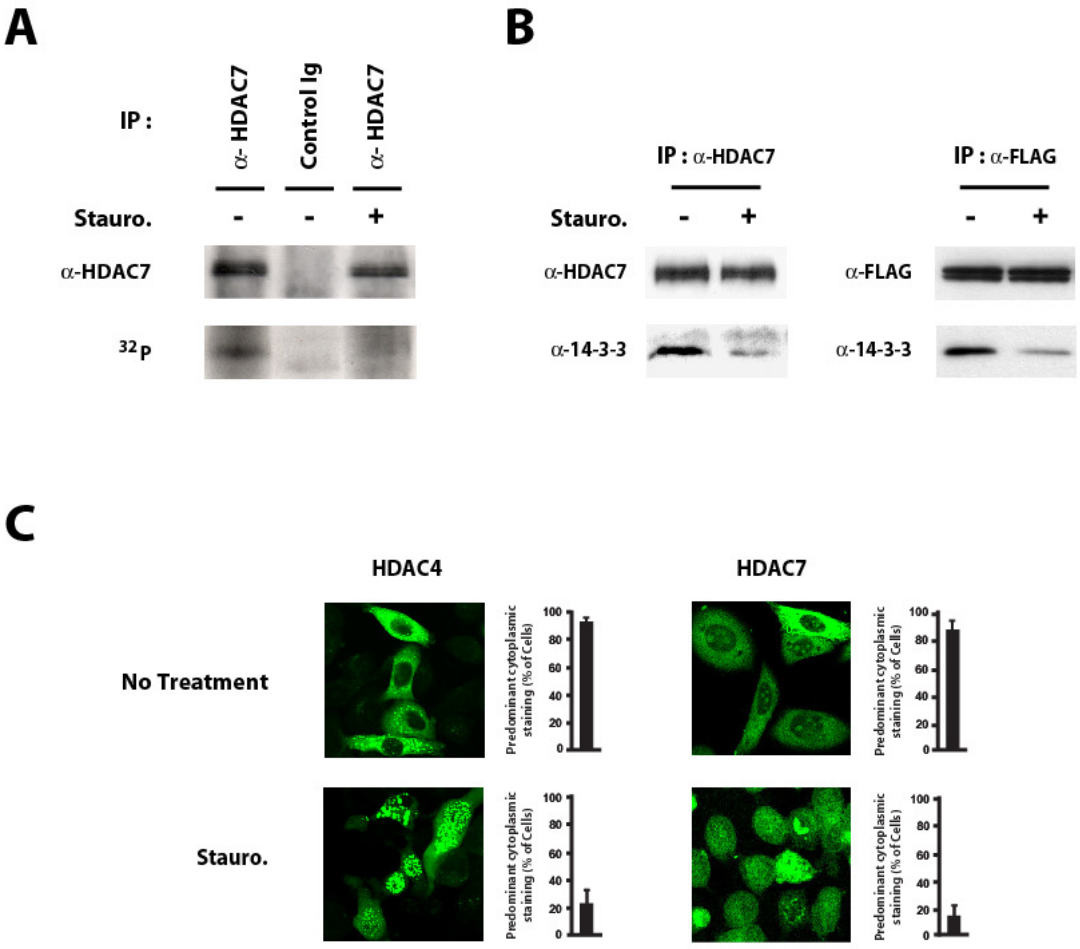


Figure 1. Class IIa HDACs are constitutively phosphorylated, bound to 14-3-3 proteins and subjected to phosphorylation-dependent nucleo-cytoplasmic shuttling.

(A) Do11.10 cells were labeled with [^{32}P] orthophosphate and subsequently treated with Staurosporine (+) or left untreated (-). Endogenous HDAC7 was immunoprecipitated and analyzed by SDS-PAGE followed by western blotting (α-HDAC7) or autoradiography (^{32}P).

(B) DO11.10 cells, transduced with FLAG-tagged HDAC7 (right panel) or not (left panel) were left untreated or treated with Staurosporine for 1 hr. Endogenous (left panel) or ectopically expressed HDAC7 (right panel) was immunoprecipitated from cell lysates using the indicated antibodies. Immunoprecipitated material was then subjected to western blotting analysis with antibodies directed against endogenous HDAC7 (α-HDAC7), the FLAG epitope (α-FLAG) or 14-3-3 proteins (α-14-3-3).

(C) HeLa cells expressing GFP-HDAC4 or GFP-HDAC7 were either left untreated (No Treatment) or treated with Staurosporine (Stauro.). After 1 h of treatment, the subcellular distribution of GFP-HDACs was determined using confocal microscopy. Bar histograms represent the mean percentages of cells showing predominant cytoplasmic localization of GFP-HDACs in each condition.

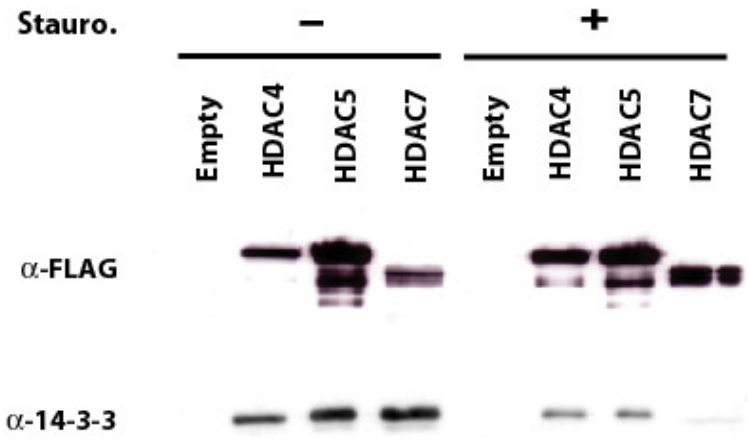


Figure S1. HEK293 cells were transiently transfected with expression vectors encoding FLAG-tagged versions of HDAC4, -5 and -7 or the empty vector as control. Forty-eight hours post-transfection, cells were treated with Staurosporine or left untreated. Recombinant proteins were immunoprecipitated with an anti-FLAG antiserum and analyzed by Western blotting with antibodies directed against the FLAG epitope (α-FLAG) and 14-3-3 family members (α-14-3-3).

HDAC7 constitutively shuttles from the nucleus to the cytoplasm

Phosphorylation of class IIa HDACs and association with 14-3-3 proteins controls their distribution between the nucleus and the cytoplasm. We thus examined the steady-state subcellular localization of GFP-HDAC4 and -HDAC7 in HeLa cells (Fig. 1C). In normal growth conditions, the vast majority of cells showed predominantly cytoplasmic localization of both HDAC4 and -7 (Fig. 1C, No Treatment). In contrast, under hypophosphorylating conditions induced by staurosporine, both HDACs accumulated in the nucleus (Fig. 1C, Stauro.).

It is now well known that class IIa HDACs show differential subcellular localization depending on the cell line examined (Fischle et al., 2001; Kao et al., 2001; Wang et al., 2000). This suggests that the machinery controlling nuclear export of class IIa HDACs is differently effective in different cell types. To generalize the above observations, we examined the effect of staurosporine in Cos7 cells, where ectopically expressed class IIa HDACs have been shown to localize primarily in the nucleus (Fischle et al., 2001; McKinsey et al., 2000a; McKinsey et al., 2001b). In the majority of the transfected cells, GFP-HDAC7 was for the most part, found in the nucleus, with only a small fraction of the protein present in the cytoplasm. However, in about 25 % of the cells, HDAC7 was predominantly detected in the cytoplasm (Fig. 2A, No treatment). Similarly to HeLa cells, treatment with staurosporine was associated with significant nuclear retention of HDAC7. Indeed, after 1h of treatment, the protein was almost exclusively localized in the nucleus and the proportion of cells with predominant cytoplasmic staining dropped to less than 5% (Fig. 2A, Stauro.). Of note, staurosporine also increased nuclear retention of GFP-HDAC7 in HEK293 cells, where it distributes equally between the nucleus and the cytoplasm in untreated cells (Fig.S2). Our observations so far clearly unraveled basal phosphorylation and 14-3-3 binding of class IIa HDACs and point toward a constitutive, phosphorylation-dependent, nuclear efflux of these enzymes in various normally growing cell lines. To challenge this model, we examined the intracellular mobility of HDAC7 in Cos7 cells, which appear to be the least potent in exporting class IIa HDACs from the nucleus to the cytoplasm. GFP-HDAC7 was thus expressed in Cos7 cells and its nuclear efflux was examined on live cells using the FLIP technology. For this purpose, the cytoplasm of transfected cells was selectively and repeatedly bleached and loss of fluorescence was monitored in the nucleus. FLIP experiments revealed a substantial loss of nuclear fluorescence of GFP-HDAC7 in living Cos7 cells after bleaching of the cytoplasm (Fig. 2B). This loss of nuclear fluorescence was completely blocked by Leptomycin B, an inhibitor of CRM1-dependent nuclear export (Fig. 2B, + LMB). Quantification of the data confirmed these observations and showed a 50% decrease in nuclear fluorescence of GFP-HDAC7wt, 25 minutes after cytoplasmic bleaching (Fig. 2C, HDAC7wt). In accordance with the steady-state localization data (Fig. 2A), staurosporine treatment totally abolished constitutive nuclear export of HDAC7 (Fig. 2B and 2C, HDAC7wt+Stauro). A similar effect was observed when the four 14-3-3 binding sites of HDAC7 [Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449} see (Dequiedt et al., 2005)] were mutated to alanines (Fig. 2B and 2C, HDAC7ΔP). These results clearly demonstrate a constitutive efflux of HDAC7 from the nucleus to the

cytoplasm in normal growing conditions. In addition, they are consistent with a model in which the dynamic nuclear export of class IIa HDACs is dependent on the constitutive phosphorylation of their conserved 14-3-3 binding sites. This thus implies the existence of additional class IIa HDACs kinases.

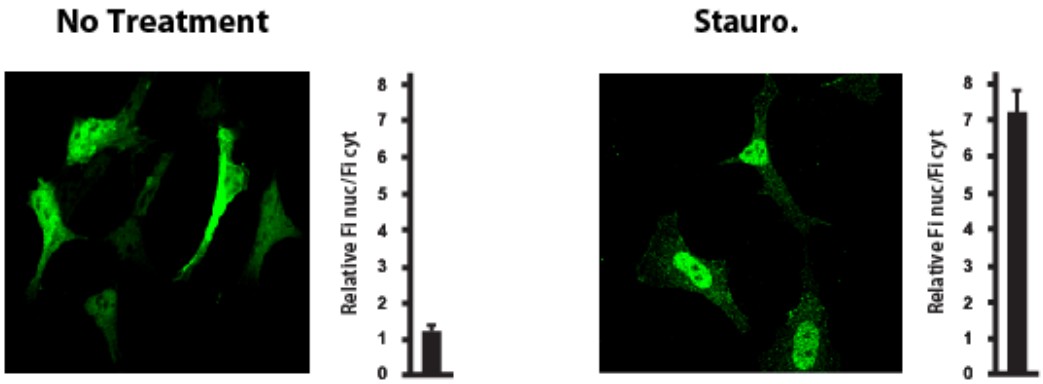


Figure S2. GFP-HDAC7 was transfected into HEK293 cells. Forty-eight hours post-transfection, cells were left untreated (No Treatment) or treated with Staurosporine (Stauro.) for 1 h before the localization of HDAC7 was examined by confocal microscopy.

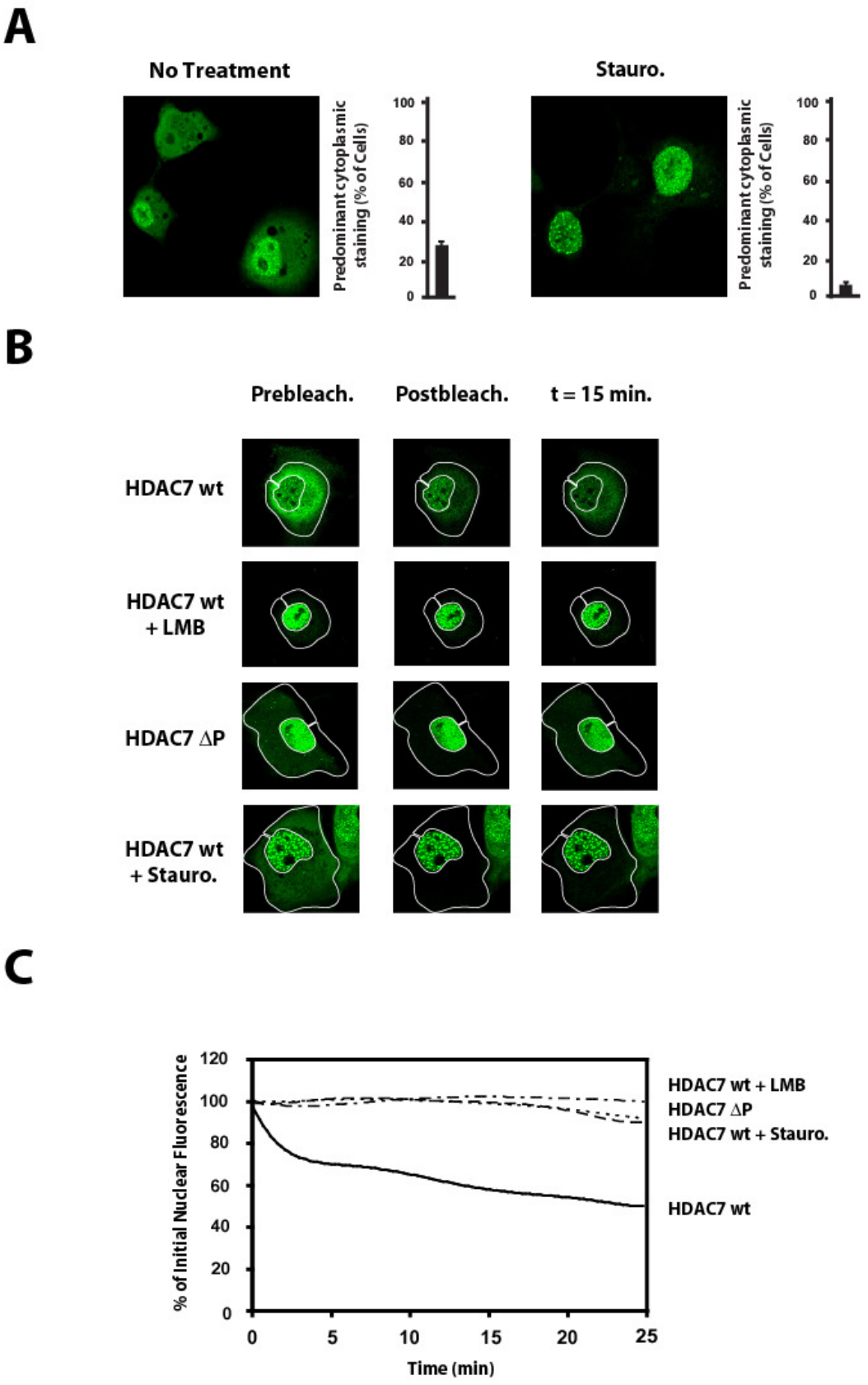


Figure 2. Class IIa HDACs are subjected to phosphorylation-dependent nucleo-cytoplasmic efflux.

(A) Recombinant GFP-HDAC7 was transfected into Cos7 cells. Forty-eight hours post-transfection, cells were left untreated (No Treatment) or treated with Staurosporine (Stauro.) for 1 h before the localization of HDAC7 was examined by confocal microscopy.

(B) Cos7 cells were transfected with constructs expressing GFP-fusion proteins corresponding to HDAC7 (HDAC7wt) or a mutant of HDAC7 in which the residues Ser^{155}, Ser^{181}, Ser^{321}, and Ser^{449} were mutated to alanines (HDAC7ΔP). Forty-eight hours after transfection, cells were left untreated or treated with Staurosporine (Stauro.) or leptomycin B (LMB) as indicated. The cytoplasm of transfected cells was repeatedly bleached, and the loss of fluorescence in the nuclear region was assessed by confocal microscopy.

(C) Relative loss of fluorescence in the nuclear region was performed as described in *Materials and Methods*. FLIP data are represented as non linear fit curves with the following symbols : HDAC7wt (▬), HDAC7wt+Stauro. (▬ ▬), HDAC7ΔP (▬▬), HDAC7wt+LMB (▬ ▪).

An 85 kDa autophosphorylating kinase associates with the N-terminus of Class IIa HDACs

In an attempt to identify the kinases responsible for the constitutive nuclear export of class IIa HDACs, we used the N-terminus of HDAC7, which contains the four previously identified phosphorylatable serines (Dequiedt et al., 2005), in a GST pull-down assay. The material pulled-down from unstimulated 293 cell extracts was analyzed by an in-gel kinase (IGK) assay to determine the molecular weight(s) of associated cellular kinase(s). Bands of variable intensity were observed in the input lane, revealing the presence of several constitutively active kinases in the lysate. One band, with an apparent molecular weight of approximately 85 kDa was specifically detected in association with GST-HDAC7Nter (Fig. 3A). Identical results were obtained with extracts from Cos7, NIH3T3, HeLa, Do11.10 and DPK cells (data not shown).

We then expressed the HDAC7 sequences surrounding each phosphorylatable serine residue as GST-fusion proteins (respectively GST-S155, GST-S181, GST-S321 and GST-S449) and examined their ability to recruit the 85 kDa kinase in a pull-down assay. Surprisingly, only the fusion protein corresponding to Ser^{155} specifically associated with the 85 kDa kinase (Fig. 3B). In addition, when Ser^{155} was mutated into alanine, association with the constitutively active kinase was greatly impaired, thus indicating that the interaction

specifically involves Ser^{155} (Fig. 3C). To generalize our findings, we tested other class IIa HDACs for their ability to recruit the same 85 kDa kinase activity. Pull-down assays were carried out with GST-fusion proteins corresponding to regions of HDAC4 and HDAC5 centered on Ser^{246} and Ser^{259} respectively, the residues corresponding to Ser^{155} of HDAC7. IGK assays revealed that an autophosphorylating kinase with a similar molecular weight of approximately 85 kDa was highly enriched in the material associated with GST-HDAC4, GST-HDAC5 and GST-HDAC7, but not with GST alone (Fig. 3D). These results show that members of class IIa HDACs can associate with a similar, if not identical 85 kDa autophosphorylating kinase that is constitutively active in numerous mammalian cell lines.

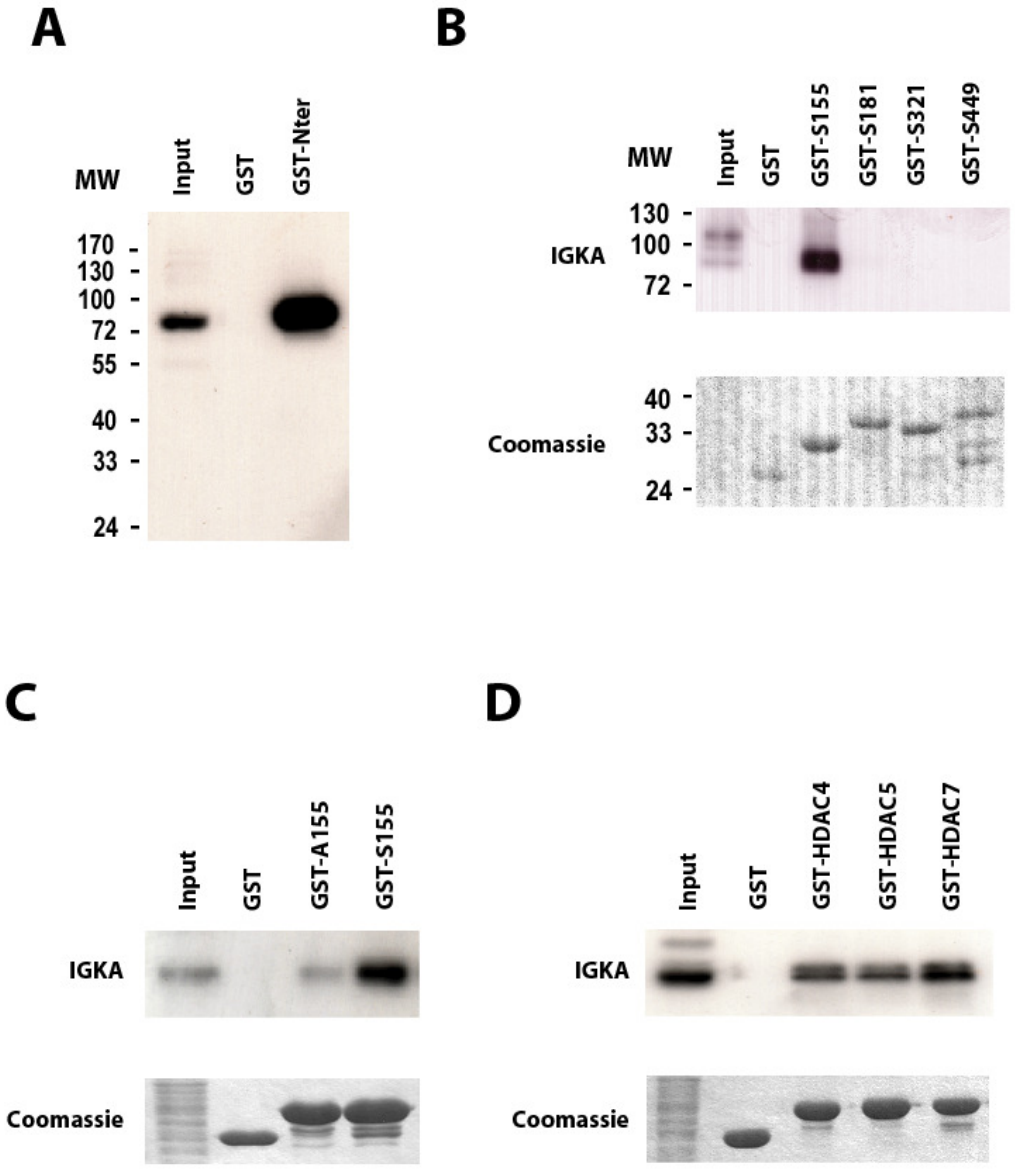


Figure 3. The N-terminus of Class IIa HDACs associates with an 85 kDa autophosphorylating kinase

(A) The N-terminus of HDAC7 was expressed as a GST-fusion protein (GST-Nter) and incubated with total cellular extracts from unstimulated, normally growing HEK293 cells. A control reaction was performed in parallel with the GST protein alone. Associated autophosphorylating kinase activities were revealed by IGK assay and autoradiography. The input lane corresponds to 10% of the lysate engaged in the pull-down.

(B) GST-fusion proteins corresponding to the sequences surrounding the 4 phosphorylatable serine residues in HDAC7 (GST-S155, GST-S181, GST-S321 and GST-S449) were used in independent pull-down reactions with lysates from HEK293 cells. Autophosphorylating protein kinase activities in the associated material were detected by IGK assay. The gel was stained with coomassie, dried and analyzed by autoradiography. The lane marked "input" equals 10% of the cell lysate used in the reaction.

(C) A GST-fusion protein corresponding to the sequences around Ser^{155} of HDAC7 (GST-S155) was incubated with HEK293 cell extracts in a pull-down assay. A mutant fusion-protein harboring a serine to alanine substitution (GST-A155) was used as control. Pull-down reactions were resolved by SDS-PAGE and analyzed by IGK assay. The gel was stained with coomassie, dried and autophosphorylating protein kinase activity was visualized by autoradiography. Input amounts to 10% of the cell lysate used in each reaction.

(D) GST-fusion proteins corresponding to Ser^{246} of HDAC4, Ser^{259} of HDAC5 and Ser^{155} of HDAC7 (GST-HDAC4, GST-HDAC5 and GST-HDAC7 respectively) were incubated with extracts from HEK293 cells. A control reaction was performed with the GST protein alone. Pull-down reactions were analyzed by IGK and autoradiography to visualized autophosphorylating kinase activities. The input lane corresponds to 10% of the material used in each reaction.

The 85 kDa autophosphorylating kinase activity includes hPar-1 kinases, C-TAK1 and EMK

The apparent molecular weight observed in IGK assays precludes the autophosphorylating kinase(s) from being a member of the CaMK or PKD families. To identify this new class IIa HDACs-associated kinase, we screened the human kinome for constitutively active

serine/threonine protein kinases with apparent molecular weights between 80 and 100 kDa (Manning et al., 2002) and showing autophosphorylation in an IGK assay. This search identified members of the PKC, RSK, MARK/Par-1 and MSK families. We then tested if any of these kinases could interact with Ser^{155} of HDAC7. Pull-down assays were carried out with GST-S155 or GST-A155 and GST as control and analyzed by sequential western blotting with antibodies directed against PKC family members, RSK1/2, the MARK/Par-1 kinase C-TAK1 and MSK1/2. Among these, GST-S155 specifically associated with endogenous C-TAK1 (Fig. 4A). More importantly, mutation of Ser^{155} into alanine greatly reduced the amount of bound C-TAK1. Because these results strongly suggested that C-TAK1 could be the 85 kDa autophosphorylating kinase described above, we then tested the ability of C-TAK1 to associate with other class IIa members. As expected, GST-fusion proteins corresponding to Ser^{246} and Ser^{259} of HDAC4 and HDAC5 respectively were also able to specifically recruit endogenous C-TAK1 in pull-down assays (Fig. 4B).

Other MARK/Par-1 family members include the two closely related hPar-1c and hPar-1b/EMK that phosphorylate microtubules associated proteins (MAPs) MAP2, MAP4 and Tau (Drewes et al., 1997). We next investigated if EMK could participate in the 85 kDa kinase activity described above. Pull-down reactions prepared with GST-S155 and GST-A155 were then tested for the presence of EMK. Western blot analysis revealed that while endogenous EMK associated with GST-S155, the interaction was greatly impaired by mutation of Ser^{155} into alanine (Fig. 4C).

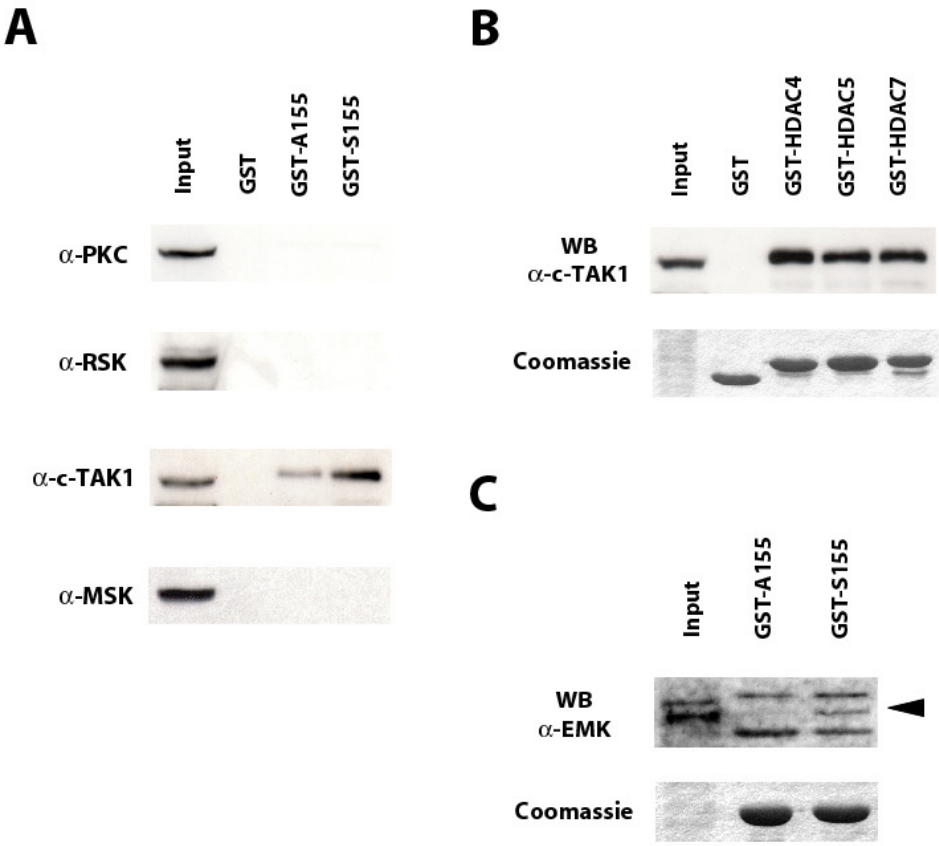


Figure 4. MARK/Par-1 kinases, C-TAK1 and EMK associate with the N-terminus of HDAC7.

(A) GST-fusion proteins corresponding to Ser^{155} of HDAC7 (GST-S155), to the serine-to-alanine mutant (GST-A155) or to GST alone were incubated with HEK293 cell extracts in a pull-down assay. Reactions were resolved by SDS-PAGE and analyzed for the presence of endogenous PKC family members (α-PKC), RSK1 and RSK2 (α-RSK), C-TAK1 (α-C-TAK1) and MSK1 and MSK2 (α-MSK) by western blotting.

(B) Binding of endogenous C-TAK1 to GST-fusion proteins corresponding to Ser^{246} of HDAC4, Ser^{259} of HDAC5 and Ser^{155} of HDAC7 (GST-HDAC4, GST-HDAC5 and GST-HDAC7 respectively) after a pull-down assay was analyzed by western blotting or coomassie staining for loading control.

(C) Pull-down reactions described in (A) were analyzed by SDS-PAGE followed by western blotting for detection of associated endogenous EMK or by coomassie staining for control loading. Input equals 10% of the cell extract used in each reaction. The arrow indicates the band corresponding to EMK in the pull-down reactions. Other bands result from cross-reactivity of the anti-EMK antiserum with bacterial proteins co-purifying with the GST-fusion proteins.

C-TAK1 phosphorylates serine 155 of HDAC7 in vitro

We next asked whether C-TAK1 could directly phosphorylate the N-terminus of HDAC7, which contains the four phosphorylatable serines involved in nucleo-cytoplasmic shuttling of HDAC7 (i.e. Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449}). For this purpose, the N- or C-terminal domains of HDAC7 were expressed as GST-fusion proteins and incubated with purified recombinant C-TAK1 in an in vitro kinase (IVK) assay. Since C-TAK1 has been shown to phosphorylate Cdc25C on serine 216 (Ogg et al., 1994; Peng et al., 1998), we used GST-Cdc25C wt and GST-Cdc25C S216A as controls. By comparison with GST-Cdc25C wt, the N-terminus of HDAC7 was very efficiently phosphorylated by recombinant C-TAK1 (Fig. 5A, GST-HDAC7Nter and GST-Cdc25Cwt). In contrast, C-TAK1 was unable to phosphorylate the C-terminus of HDAC7 or the Cdc25C S216A mutant (Fig. 5A, GST-HDAC7Cter and GST-Cdc25CS216A respectively).

Hydrophobic residues at -5, +1 and +5, as well as an arginine at the -3 position relative to the phosphorylated serine seem to be crucial for optimal phosphorylation by C-TAK1 (Muller et al., 2003). Except for the presence of a hydrophobic residue at the +5 and +1 positions, the four phosphorylation sites previously identified in the N-terminus of HDAC7 match this consensus phosphorylation motif (Fig. 5B). These observations suggest that C-TAK1 could directly phosphorylate HDAC7 and identify Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449} as putative target sites for C-TAK1. To test this hypothesis, GST fusion proteins corresponding to each serine residue (respectively GST-S155, GST-S181, GST-S321 and GST-S449) were evaluated as potential substrate for C-TAK1 in an IVK assay. Surprisingly, only Ser^{155} was efficiently phosphorylated by recombinant C-TAK1 (Fig. 5C).

EMK and C-TAK1 display site preference among the four serine residues of HDAC7

To confirm and extend these observations, we performed an exhaustive analysis of C-TAK1 target sites in the N-terminus of HDAC7. This region of HDAC7 was incubated with $\gamma[^{32}P]$ ATP and recombinant C-TAK1 in vitro. A control reaction was performed in parallel with PKD, which phosphorylates Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449} in vitro (Dequiedt et al., 2005). Labeled proteins were digested with trypsin and the resulting peptides were separated by high pressure liquid chromatography (HPLC). Radioactive fractions were then analyzed by mass spectrometry to identify the phosphorylated residue(s). Labeling with PKD led to four major radioactive peaks (Fig. 5D, PKD), which corresponded to the formerly identified Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449}. In contrast, after phosphorylation with C-TAK1, the HPLC profile exhibited a single major phosphorylation peak (Fig. 5D, C-TAK1). Mass

spectrometry analysis showed that this peak corresponded to phosphorylated Ser^{155}. In contrast to PKD, which targets all four serine residues implicated in the nuclear-cytoplasmic shuttling of HDAC7, these results demonstrate that C-TAK1 specifically phosphorylates Ser^{155} (Fig. 5C and 5D).

Our results showed that EMK can associate with the N-terminal Ser^{155} of HDAC7 (Fig. 4C). In addition, sequences around this serine residue, which are conserved in all class IIa HDACs (Ser^{246}, Ser^{259}, Ser^{155} and Ser^{220} of HDAC4, -5, -7 and -9 respectively), match with the KxGS motif phosphorylated by EMK in Tau (Drewes et al., 1997). We therefore investigated whether EMK, similarly to C-TAK1, could phosphorylate Ser^{155} of HDAC7. As expected, the entire N-terminal domain of HDAC7, which contains all four 14-3-3 binding sites, was very efficiently phosphorylated by purified C-TAK1 or EMK in IVK assays (Fig. 5E, GST-Nter). More importantly, when the sole Ser^{155} was mutated to alanine, phosphorylation by both kinases was totally abolished (Fig. 5E, GST-NterS155A). Taken together, these results demonstrate the specificity of C-TAK1 and EMK for Ser^{155} over the three other serine residues previously involved in the nuclear cytoplasmic shuttling of HDAC7.

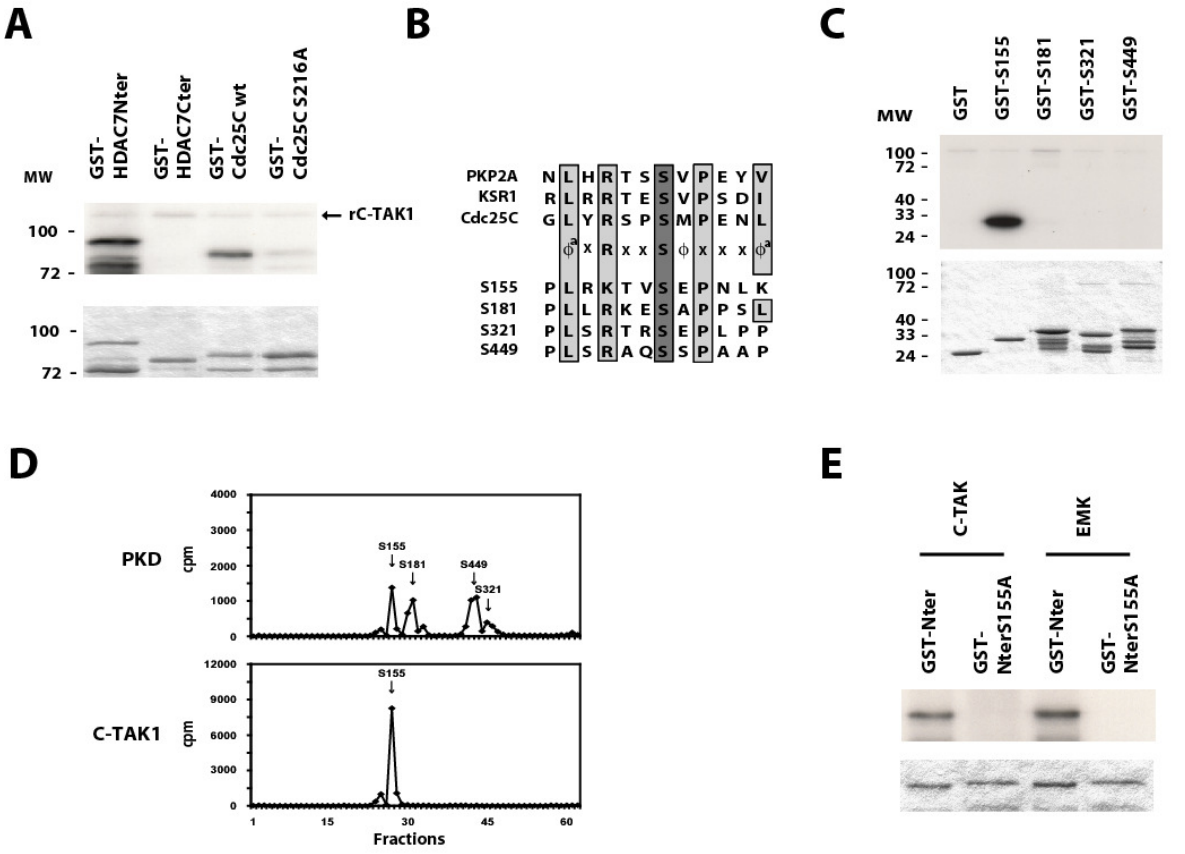


Figure 5. C-TAK1 and EMK specifically phosphorylates Ser^{155} of HDAC7 in vitro

(A) The C- and N- terminal domains of HDAC7, and wild-type or S216A mutant Cdc25C were produced as GST-fusion proteins (GST-HDAC7Cter, GST-HDAC7Nter, GST-Cdc25Cwt and GST-Cdc25CS216A respectively). Equal amounts of purified recombinant proteins were used in IVK assays with recombinant active C-TAK1. IVK reactions were analyzed by SDS-PAGE and coomassie staining (lower panel) prior to autoradiography (upper panel). The arrow indicates the signal resulting form autophosphorylated C-TAK1 (rC-TAK1).

(B) Sequences around the 4 phosphorylatable serine residues in HDAC7 match with the canonical C-TAK1 phosphorylation motif.

(C) GST-fusion proteins corresponding to the sequences surrounding Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449} of HDAC7 were used as substrates in independent IVK assays with active recombinant C-TAK1. Reactions were resolved by SDS-PAGE and gel was stained with coomassie (lower panel), dried and analyzed by autoradiography (upper panel).

(D) The N-terminus of HDAC7 was expressed as a GST fusion protein and used as a substrate for recombinant PKD or C-TAK1 in IVK assays. Labeled proteins were resolved by SDS-PAGE and digested with trypsin. The resulting peptides were then separated by HPLC. Positions of the peptides containing each phosphorylatable serine are indicated on the radioactivity profile (as confirmed by mass spectrometry).

(E) GST-fusion proteins corresponding to the N-terminal domain of HDAC7 (GST-Nter) or to the same region with a serine to alanine substitution at position Ser^{155} (GST-NterS155A) were phosphorylated in vitro by EMK or C-TAK1. IVK reactions were analyzed by SDS-PAGE and coomassie staining (lower panel) prior to autoradiography (upper panel).

HDAC7 is phosphorylated on Ser^{155} in vivo

We next examined the phosphorylation status of Ser^{155} on endogenous HDAC7 in vivo. For this purpose, we first developed an antibody that specifically recognizes the phosphorylated form of HDAC7 Ser^{155} (see Fig. S3). Total extracts from Do.11.10 T-cell hybridomas, which express high levels of endogenous HDAC7, were examined by western blotting with the phosphospecific antibody for Ser^{155}. As shown in Figure 6A, strong basal phosphorylation of HDAC7 was consistently observed at Ser^{155} in normally growing cells

(Fig. 6A, α-pS155). More importantly, Ser^{155} phosphorylation was totally lost when cells were treated with staurosporine.

Because sequences around Ser^{155} of HDAC7 are highly conserved in other members of the class IIa, the phospho-specific antibody against Ser^{155} also recognizes the corresponding phosphorylated serines in HDAC4 and -5 (Ser^{246} and Ser^{259} respectively). To generalize our observations to other members of the class IIa, FLAG-tagged versions of HDAC4, -5 and -7 were transiently expressed in HEK293 cells and examined by western blotting using the phospho-specific antibody (Fig. 6B, α-pS155). Confirming our observations on endogenous HDAC7, all three class IIa HDACs showed basal phosphorylation of their respective serine residue, which was significantly reduced upon treatment with staurosporine.

The above findings suggest that phosphorylation of Ser^{155} in HDAC7 (or Ser^{246} and Ser^{259} in HDAC4 and -5 respectively) might be important for constitutive nuclear export. To test this hypothesis, we fractionated extracts from HEK293 cells transiently transfected with FLAG-tagged HDAC4 or HDAC7, into nuclear and cytoplasmic fractions. Confirming our immunofluorescence data (see Fig. S2), comparable amounts of HDAC4- or HDAC7-FLAG were found in both fractions (Fig. 6C). However, western blot analysis with the phosphospecific antibody revealed that HDAC7 phosphorylated at Ser^{155} and HDAC4 phosphorylated at Ser^{246} were highly enriched in the cytoplasm (Fig. 6C). To confirm and extend these observations, we performed a similar experiment in HeLa and Cos7 cells expressing FLAG-tagged HDAC7. In accordance with the immunofluorescence data (Fig. 1 and 2), HDAC7 localized primarily in the cytoplasm of HeLa cells, where it is phosphorylated on Ser^{155} (Fig. 6D, HeLa). In contrast, HDAC7 was found almost exclusively in the nucleus of Cos7 cells and no phosphorylation of Ser^{155} could be detected (Fig. 6D, Cos7). Taken together, these results establish a strong correlation between phosphorylation of Ser^{155} and cytoplasmic localization of HDAC7.

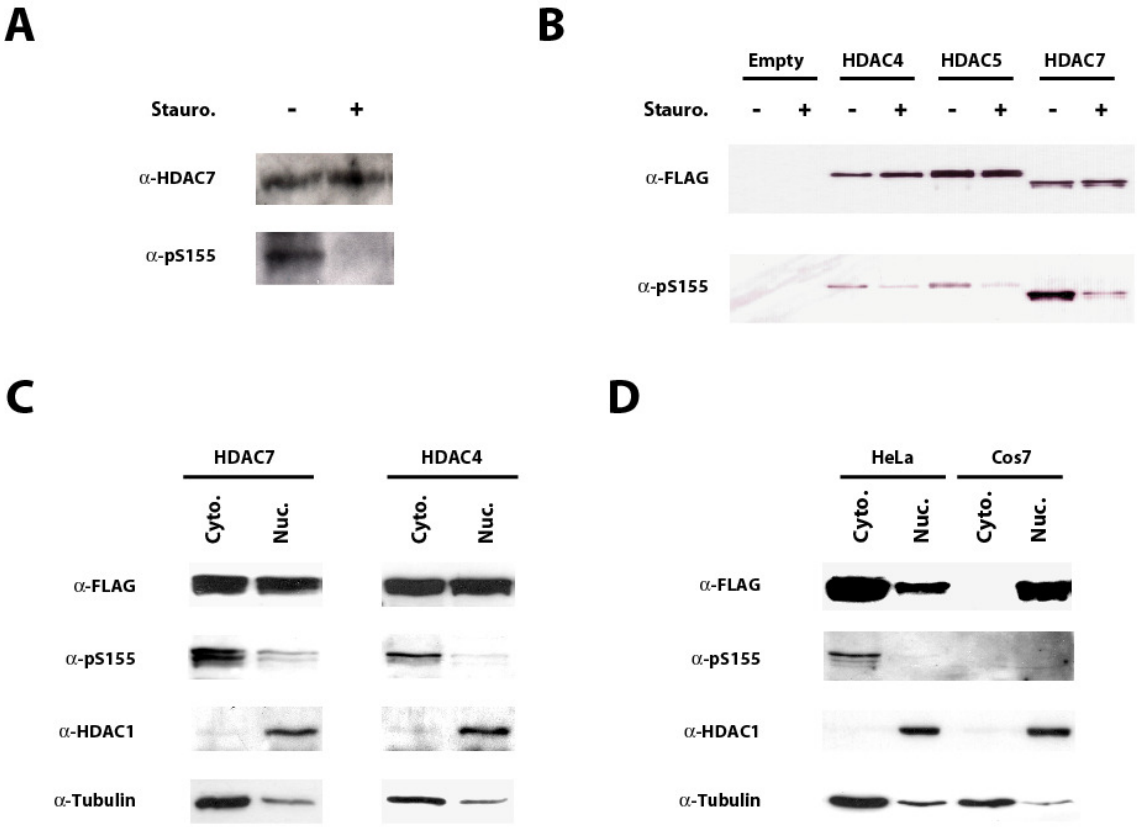


Figure 6. HDAC7 is phosphorylated on Ser^{155} in vivo

(A) Total cell lysates were prepared from Do11.10 cells which were first treated with Staurosporine (+) for 1 hour or left untreated (-). Phosphorylation of endogenous HDAC7 was detected by western blotting with the antibody specific for phosphorylated Ser^{155} (α-pS155). As loading control, the same membrane was stripped and immunoblotted with an antibody against endogenous HDAC7 (α-HDAC7).

(B) HEK293 cells were transiently transfected with expression vectors encoding FLAG-tagged versions of wild-type HDAC4, -5 and 7 or the empty vector (Empty). Total cellular extracts were analyzed by SDS-PAGE and subjected to immunoblotting using antibodies directed either against the FLAG epitope (α-FLAG) and phosphorylated Ser^{155} (α-pS155).

(C) Nuclear (Nuc.) and cytoplasmic (Cyto.) extracts were prepared from HEK293 cells transiently expressing FLAG-tagged HDAC7 or HDAC4. Both extracts were analyzed by western blotting with antibodies against the FLAG epitope (α-FLAG) and phosphorylated Ser^{155} (α-pHDACs). As control, extracts were also analyzed

by western blotting with antisera against the cytoplasmic protein Tubulin (α-Tubulin) and the nuclear protein HDAC1 (α-HDAC1).

(D) Nuclear (Nuc.) and cytoplasmic (Cyto.) extracts were prepared from HeLa or Cos7 cells transiently expressing FLAG-tagged HDAC7. Equal protein amounts from each extracts were analyzed by western blotting with antisera against the FLAG epitope (α-FLAG) and phosphorylated Ser^{155} (α-pS155). As control, extracts were also analyzed by western blotting with antibody against the cytoplasmic protein Tubulin (α-Tubulin) and the nuclear protein HDAC1 (α-HDAC1).

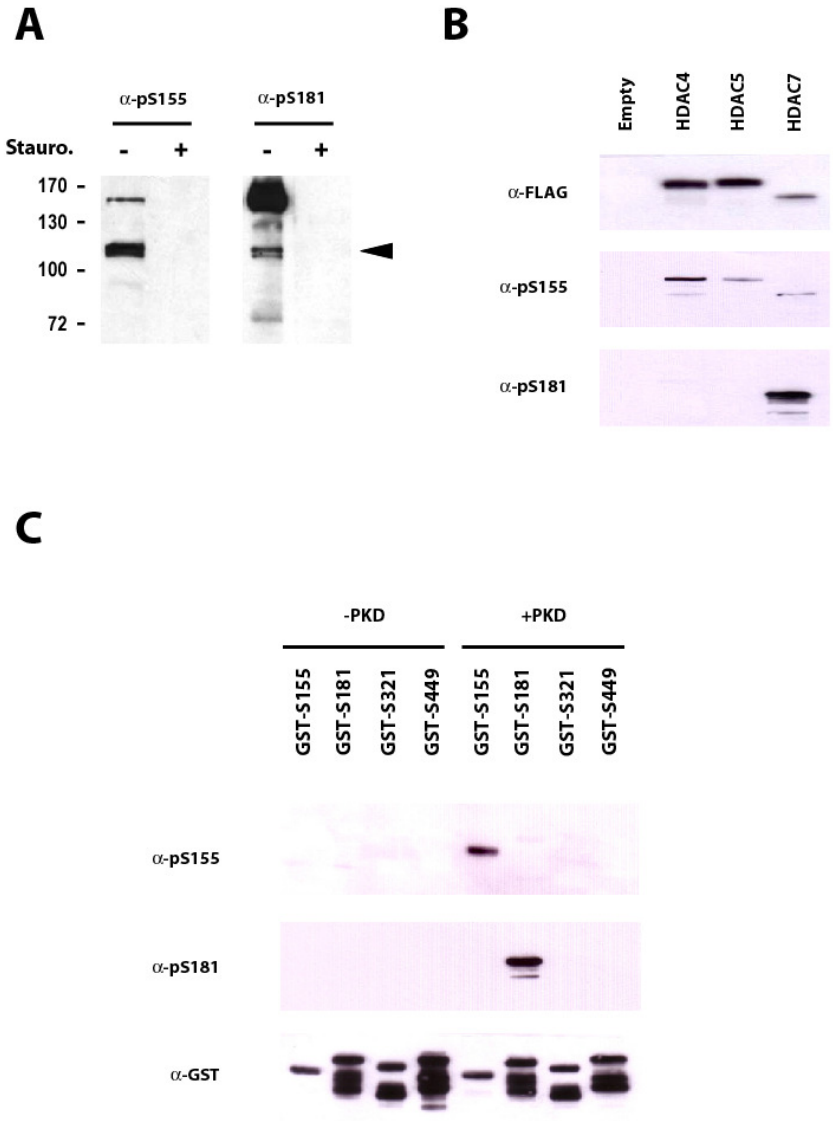


Figure S3.

(A) Total cell lysates were prepared from Do11.10 cells which were first treated with Staurosporine (+) for 1 hour or left untreated (-). Cell extracts were resolved by SDS-PAGE and analyzed by western blotting with antibodies specific for phosphorylated Ser^{155} (α-pS155) or phosphorylated Ser^{181} (α-pS155). The specific signal corresponding to HDAC7 phosphorylated at Ser^{155} or Ser^{181} is indicated by an arrow. The signal located at 160 kDa is due to cross reactivity of antibodies with an unknown phosphorylated cellular protein.

(B) HEK293 cells were transiently transfected with expression vectors encoding FLAG-tagged versions of HDAC4, -5 and -7 or the empty vector as control. Forty-eight hours post-transfection, cells were lyzed and recombinant proteins were immunoprecipitated with an anti-FLAG antiserum. Immunoprecipitated material was analyzed by Western blotting with antibodies directed against the FLAG epitope (α-FLAG) and with antibodies specific for phosphorylated Ser^{155} (α-pS155) or phosphorylated Ser^{181} (α-pS181). Because sequences around Ser^{155} of HDAC7 are highly conserved in other members of the class IIa, the phospho-specific antibody against Ser^{155} also recognizes the corresponding phosphorylated serines in HDAC4 and -5 (Ser^{246} and Ser^{259} respectively). In contrast, Ser^{181} is not conserved in other class IIa members, and as such, the α-pS181 is highly specific for HDAC7.

(C) HDAC7 sequences surrounding each putative PKD site were expressed as GST fusion proteins (respectively GST-S158, GST-S181, GST-S321 and GST-S449, see (Dequiedt et al., 2005)). Purified GST-fusion proteins were incubated with (+) or without (-) a dilution of recombinant active PKD1 in an IVKA, in the presence of cold ATP. Phosphorylated proteins were analyzed by SDS-PAGE and visualized by western blotting with antibodies specific for phosphorylated Ser^{155} (α-pS155) or phosphorylated Ser^{181} (α-pS181). Membranes were stripped and then probed with anti-GST to assess for equal loading. This experiment shows that both antisera are very specific for the corresponding phosphorylated serine and display no cross-reactivity against the non-phosphorylated GST-constructs.

EMK and C-TAK1 alter nuclear export of Class IIa HDACs and regulate their repressive activity

Our results so far strongly suggest that in the absence of extracellular stimuli, EMK and C-TAK1 control class IIa HDACs localization by phosphorylating their most N-terminal 14-3-3 binding site (i. e., Ser^{246}, Ser^{259} and Ser^{155} in HDAC4, -5 and -7 respectively). In Cos7 cells, class IIa HDACs are mainly found in the nucleus, probably because the mechanisms controlling their nuclear export of class IIa HDACs are poorly efficient in these cells. To test our model, we examined the steady-state localization of HDAC7 in the presence of overexpressed EMK or C-TAK1 in Cos7 cells. As observed before, HDAC7 was mainly found in the nucleus of Cos7 cells when expressed alone (Fig. 7A). In contrast, co-expression of EMK and C-TAK1 induced dramatic cytoplasmic accumulation of HDAC7. However, we did not observed convincing co-localization of HDAC7 with either MARK members in the cytosol (Fig. 7A, merged). To generalize these observations, we tested the effect of EMK and C-TAK1 on the subcellular localization of HDAC4. By comparison with HDAC7, HDAC4 was even more sensitive to cytoplasmic retention by MARK kinases, with approximately 80% of cells showing predominant cytoplasmic staining (Fig. 7B). We next tested whether cytoplasmic accumulation of class IIa HDACs induced by MARK/Par-1 kinases resulted directly from an increase in their nuclear export. FLIP experiments revealed a remarkable increase in the nucleocytoplasmic efflux of HDAC7 by both EMK and C-TAK1 resulting in less than 10% of the initial fluorescence left in the nucleus after 25 minutes (Fig. 7C, HDAC7+EMK, HDAC7+C-TAK1).

In T-cells, we have shown that HDAC7 associates with MEF2D to repress the Nur77 promoter and that this inhibitory action is relieved by TCR-signaling which induces HDAC7 phosphorylation and cytoplasmic relocalization (Dequiedt et al., 2003). As EMK and C-TAK1 promote nuclear export of class IIa HDACs, both kinases would be expected to overcome the inhibitory activity of HDAC7 over the Nur77 promoter, even in the absence of TCR-signaling. To address this question, we used the isolated Nur77 promoter in reporter assays. As expected, the transcriptional activity of the Nur77 promoter was strongly inhibited by HDAC7 (Fig. 7D). Overexpression of EMK or C-TAK1 totally reversed the repressive effect of wild-type HDAC7. Of note, EMK increased the transcriptional activity of Nur77 up to two fold above levels observed in the absence of HDAC7.

To further assess the functional consequences of class IIa HDACs phosphorylation by MARK/Par-1 kinases we examined the ability of EMK and C-TAK1 to activate c-jun

expression, another class IIa HDACs-repressed gene (Wang et al., 1999a).For this purpose, EMK and C-TAK1 were independently expressed in Cos7 cells and levels of endogenous c-jun were examined by western blotting. As expected, ectopic expression of EMK or C-TAK1 was associated with a marked increase in c-jun levels (Fig. 7E, α-c-jun).

Taken together, these data demonstrate that MARK/Par-1 kinases EMK and C-TAK1 are physiologically relevant kinases for class IIa HDACs and strongly support a novel role for these kinases in gene regulation.

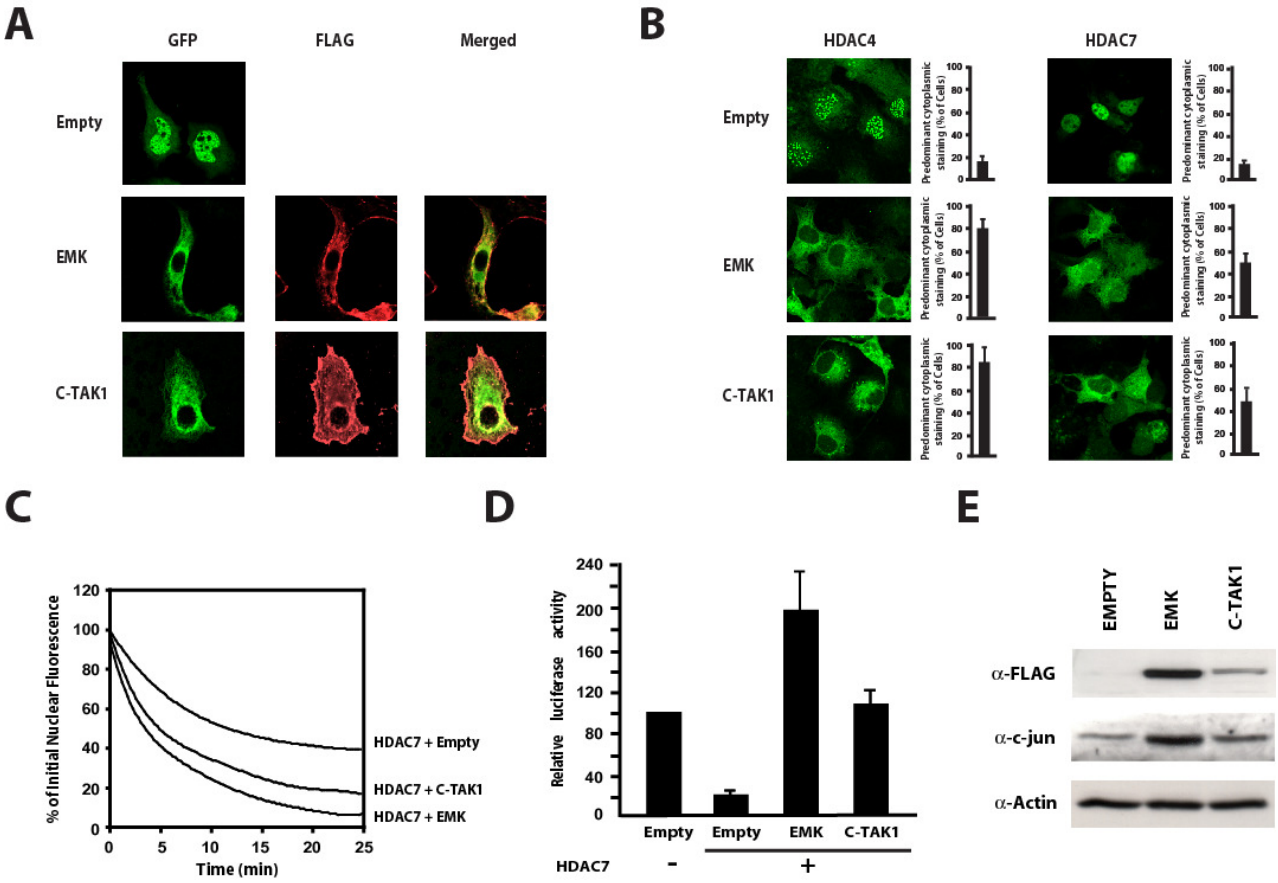


Figure 7. EMK and C-TAK1 control nuclear export of Class IIa HDACs and regulate their repressive activity.

(A) Cos7 cells were transfected with expression vectors for GFP-HDAC7 and FLAG-tagged EMK or C-TAK1. The intracellular localization of HDAC7 was detected by direct immunoflorescence (GFP) while MARKs were revealed by indirect immunoflorescence using an AlexaFlour-568 labeled anti-FLAG antibody (FLAG).

(B) Cos7 cells were transfected with expression vectors for GFP-HDAC4 or GFP-HDAC7 and constructs for EMK or C-TAK1. The subcellular distribution of the GFP-fused HDACs proteins was examined by confocal immunofluorescence microscopy. Bar histograms represent the mean percentages of cells showing predominant cytoplasmic staining.

(C) A construct encoding GFP-HDAC7 was transfected into Cos7 cells, either with expressing vectors for EMK, C-TAK1 or a control plasmid (Empty). The cytoplasm of GFP-positive cells was repeatedly bleached and FLIP measurements were performed as described in *Materials and Methods*. Analyses of the FLIP data from three independent experiments are shown as fit curves.

(D) Do11.10 cells were transiently transfected with a luciferase reporter plasmid driven by the Nur77 promoter and the expression plasmids for the indicated proteins. Luciferase activities are presented relative to the basal luciferase activity of the reporter. Results are from 5 independent experiments each performed in triplicate.

(E) Total cellular lysates were prepared from Cos7 transiently expressing FLAG-tagged versions of EMK and C-TAK1. Cell lysates were examined by western blotting with antisera for c-jun, FLAG, and actin.

EMK and C-TAK1 regulate phosphorylation and cytoplasmic localization of class IIa HDACs in the absence of extracellular stimuli

We have previously shown that PKD efficiently phosphorylates Ser^{155} of HDAC7, even when the leucine residue at position -5 (Leu^{150}) is substituted with an alanine (Dequiedt et al., 2005). Interestingly, all C-TAK1 substrates identified so far have a leucine at position -5 of the targeted serine (Fig. 5B). While testing the importance of this leucine residue in EMK and C-TAK1 target recognition, we found out that the substitution of HDAC7 Leu^{150} to alanine totally abolished phosphorylation of Ser^{155} by both kinases (Fig. S4).

Based on these results, we generated a HDAC7 mutant specifically deficient for phosphorylation by EMK/C-TAK1 where Leu^{150} was mutated to alanine (HDAC7L150A). We examined the in vivo phosphorylation of Ser^{155} in the context of this mutant using the phosphospecific antibody. As observed above (Fig. 6), HDAC7wt showed strong basal phosphorylation of Ser^{155} and robust association with 14-3-3 proteins (Fig. 8A). Interestingly, the L150A mutation totally inhibited phosphorylation of Ser^{155}. In addition, the same mutation also reduced association with 14-3-3 proteins.

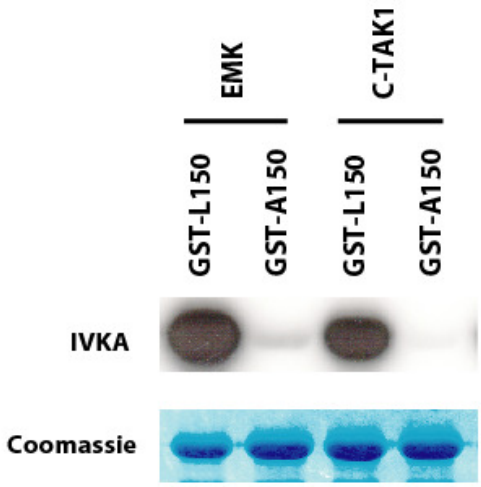


Figure S4. A GST-fusion protein corresponding to the sequences centered on Ser[155] of HDAC7 (GST-L150) was used as substrate in IVK assays with recombinant EMK or C-TAK1. A mutant-fusion protein where Leu[150] was substituted with alanine was tested in parallel. Reactions were resolved by SDS-PAGE and gel was stained with coomassie, dried and analyzed by autoradiography.

Since the HDAC7L150A mutant is not phosphorylated on Ser[155], it should consequently be impaired in its ability to exit the nucleus. To verify this hypothesis, we examined the subcellular localization of the L150A mutant in HeLa cells, where HDAC7 is very efficiently exported from the nucleus. Indeed, as shown above (Fig. 1C), wild-type HDAC7 was cytoplasmic in the majority of normally growing HeLa cells (Fig. 8B, HDAC7wt). By contrast, only 30% of HeLa cells expressing the L150AHDAC7 mutant showed predominant cytoplasmic staining (Fig. 8B, HDAC7L150A). Interestingly, FLIP experiments revealed a clear difference between the abilities of wild-type HDAC7 and the L150A mutant to exit the nucleus. For wild-type HDAC7, half of the nuclear fluorescence was lost in about 10 min after bleaching the cytoplasm, and 35% of the initial nuclear fluorescence was left after 25 min (Fig. 8C, HDAC7wt). In contrast, the loss in nuclear fluorescence was much slower for HDAC7L150A, which reached a plateau of ~65% of its initial value after 25 min (Fig. 8C, HDAC7L150A). These experiments show that the L150A HDAC7 mutant is greatly impaired in its ability to exit the nucleus, which results in an altered steady-state subcellular localization. These results thus strongly suggest that EMK and/or C-TAK1 specifically target Ser[155] of HDAC7 to control its nuclear export.

To establish this hypothesis more firmly, we used RNA interference (RNAi) to inhibit endogenous C-TAK1 and EMK activities in HeLa cells. A combination of small-interfering

RNAs (siRNAs) directed against both C-TAK1 and EMK reduced the endogenous levels of both kinases (Fig. 8D, left panel). Coincident with this reduction, we observed a substantial decrease in the phosphorylation of Ser^{155} in HDAC7 (Fig. 8D, right panel). As expected, knockdown of EMK and C-TAK1 also altered subcellular distribution of HDAC7. Indeed, when HeLa cells were co-transfected with GFP-HDAC7 and siRNAs against EMK and C-TAK, the proportion of cells showing a predominant cytoplasmic staining decreased significantly (Fig. 8E).

Altogether, these data strongly suggest that MARK/Par-1 kinases EMK and C-TAK1 phosphorylate class IIa HDACs on their most upstream 14-3-3 binding site and regulate their constitutive nuclear export in vivo.

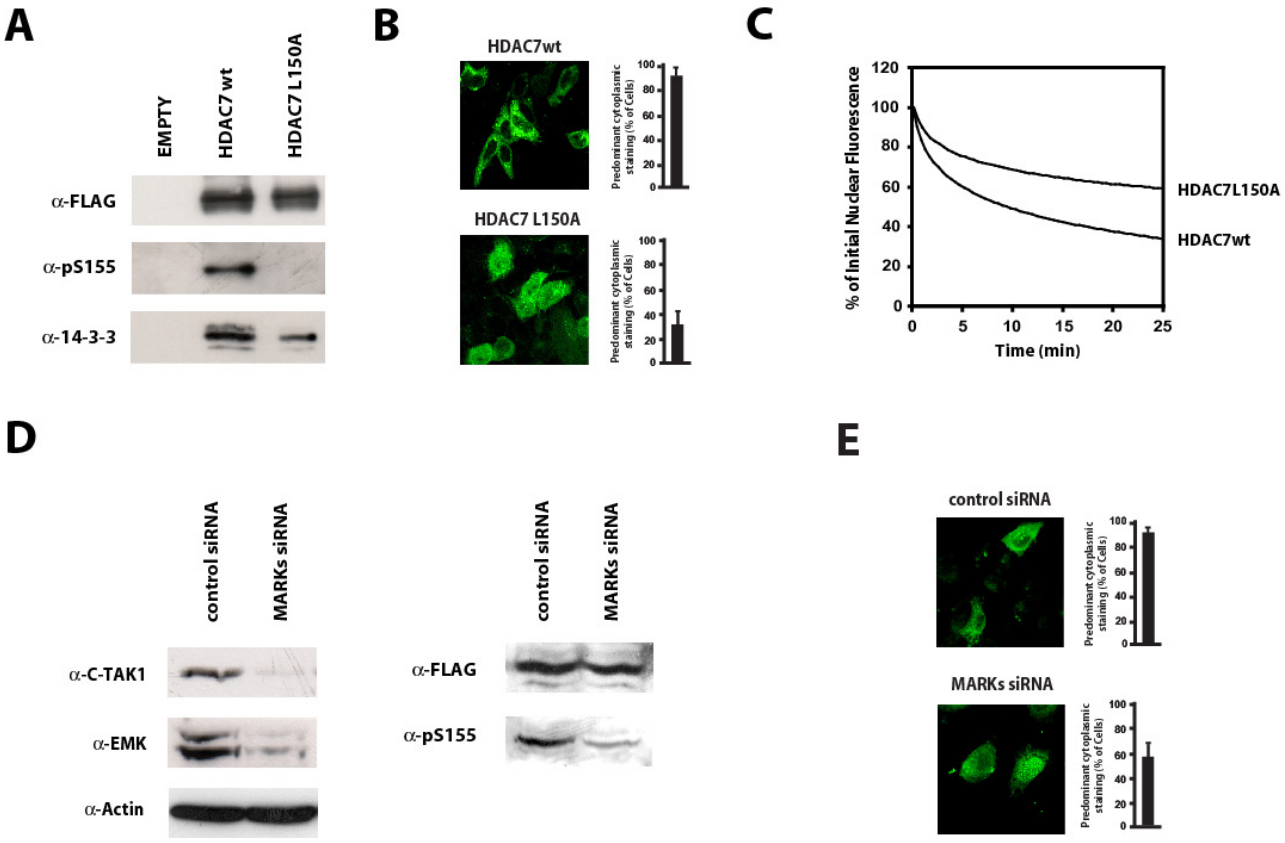


Figure 8. EMK and c-TAK1 phosphorylate Ser155 of HDAC7 in vivo

(A) FLAG-tagged versions of wild type HDAC7 (HDAC7wt) or the L150A HDAC7 mutant (HDAC7L150A) were transiently expressed in HEK293 cells, immunoprecipitated and examined by western blot analysis with antibodies directed against the FLAG epitope (α-FLAG), 14-3-3 family members (α-14-3-3) or HDAC7 phosphorylated at Ser^{155} (α-pS155).

(B) HeLa cells were transfected with constructs encoding GFP-fusion proteins of wild-type HDAC7 (HDAC7wt) or the L150A HDAC7 mutant (HDAC7L150A). Subcellular localization of the fluorescent proteins was observed by confocal microscopy.

(C) Cos7 cells were transfected with constructs encoding GFP-tagged wild-type or L150A HDAC7. Cytoplamsic photobleaching and FLIP measurements were performed as described in *Materials and Methods*. Graphical analyses of the FLIP data from at least three independent experiments are shown as fit curves.

(D) HeLa cells were transfected with FLAG-tagged HDAC7, along with pooled siRNA against EMK and C-TAK1 or with control siRNA. The next day, cells were transfected with a mix of siRNAs targeting an alternative sequence both in EMK and C-TAK1 or with control siRNA (see *Materials and Methods*). Seventy-two hours after the initial transfection, cell were harvested and lyzed. Whole cell lyzates were analyzed by western blotting with antibodies against EMK, C-TAK1, Actin, the FLAG epitope or phosphorylated Ser155.

(E) HeLa cells were transfected with an expression vector for GFP-HDAC7 along with pools of siRNAs directed against two different sequences of both EMK and C-TAK1 or control siRNA, as described in (D). Sixty hours after the initial transfection, localization of GFP-HDAC7 was examined by confocal immunofluorescence microscopy. Bar histograms represent the mean percentages of cells showing predominant cytoplasmic staining.

14-3-3 binding sites are hierarchically phosphorylated in HDAC7

The biological significance behind the specific constitutive phosphorylation of HDAC7 Ser^{155} (and corresponding HDAC4 Ser^{246}, HDAC5 Ser^{259} and HDAC9 Ser^{220}) by MARK/Par-1 kinases remained elusive. To address the role of Ser^{155} phosphorylation in the signal-independent nuclear efflux of HDAC7, we examined the dynamic nuclear export of an HDAC7 protein where Ser^{155} was mutated into alanine (HDAC7S155A). Results from FLIP analysis were compared with data obtained with wild-type HDAC7 and the HDAC7ΔP mutant. Surprisingly, mutation of Ser^{155} alone had an effect comparable to mutating simultaneously the four serine residues and almost completely abolished constitutive nuclear export, as demonstrated by a constant post-bleach relative nuclear fluorescence in HDAC7S155A expressing cells (Fig. 9A). This observation demonstrates

that Ser^{155} plays a dominant role in HDAC7 constitutive phosphorylation, association with 14-3-3 and nuclear export.

To further address the importance of Ser^{155}, we examined the phosphorylation pattern of the HDAC7S155A mutant protein in vivo. For this purpose, wild-type and S155A mutant HDAC7 proteins were metabolically labeled with [^{32}P] orthophosphate, affinity-purified and trypsin-digested for HPLC analysis. As shown in Figure 9B, wild-type HDAC7 was found to be constitutively phosphorylated on five major sites, among which the four serine residues previously implicated in sub-cellular trafficking of HDAC7, i.e., Ser^{155}, Ser^{181}, Ser^{321} and S^{449} were identified. Very unexpectedly, the S155A mutant exhibited a drastically different phosphorylation pattern, as the alanine mutation at Ser^{155} also resulted in a complete loss of phosphorylation at Ser^{181} (Fig. 9B, HDAC7S155A).

We next tested whether phosphorylation of Ser^{181} could also be dependent on the two other 14-3-3 sites, Ser^{321} or Ser^{449}. HDAC7 mutants, where each of the phosphorylatable serine residue was independently mutated to alanine (HDAC7S155A, HDACS181A, HDAC7S321A and HDAC7S449A) were thus examined by western blot with antibodies specific for the phosphorylated forms of Ser^{155} and Ser^{181} (Fig. 9C, see also Fig. S3 for α-pS181 characterization). As expected, phosphorylation of Ser^{155} was detected for all constructs except for HDAC7S155A. Basal phosphorylation of Ser^{181} was undetectable after staurosporine treatment (data not shown) or in the HDAC7S181A mutant, confirming the specificity of the phospho-Ser^{181} antibody (Fig. 9C, HDAC7S181A, α-pS181). In accordance with the HPLC data, phosphorylation of Ser^{181} was totally abolished when Ser^{155} was mutated into alanine (Fig. 9C, HDAC7S155A, α-pS181). Phosphorylation of Ser^{181} was uniquely dependent on Ser^{155} as it was unaltered by the S321A or S449A mutations. These observations thus confirm that the presence and/or the phosphorylation of Ser^{155} are required for subsequent phosphorylation of Ser^{181} in vivo.

To discriminate between both hypotheses, we examined the phosphorylation levels of Ser^{181} in the HDAC7L150A mutant, where Ser^{155} is present but poorly phosphorylated (Fig. 8A) both by western blotting and HPLC analysis (Fig. 9D and 9E, respectively). Interestingly, the L150A mutant also showed a concomitant reduction of Ser^{181} phosphorylation (Fig. 9D and 9E). These results demonstrate that basal phosphorylation of Ser^{155} is required for phosphorylation of Ser^{181} and raised the exciting possibility that class IIa HDACs may be regulated through a process of hierarchical phosphorylation.

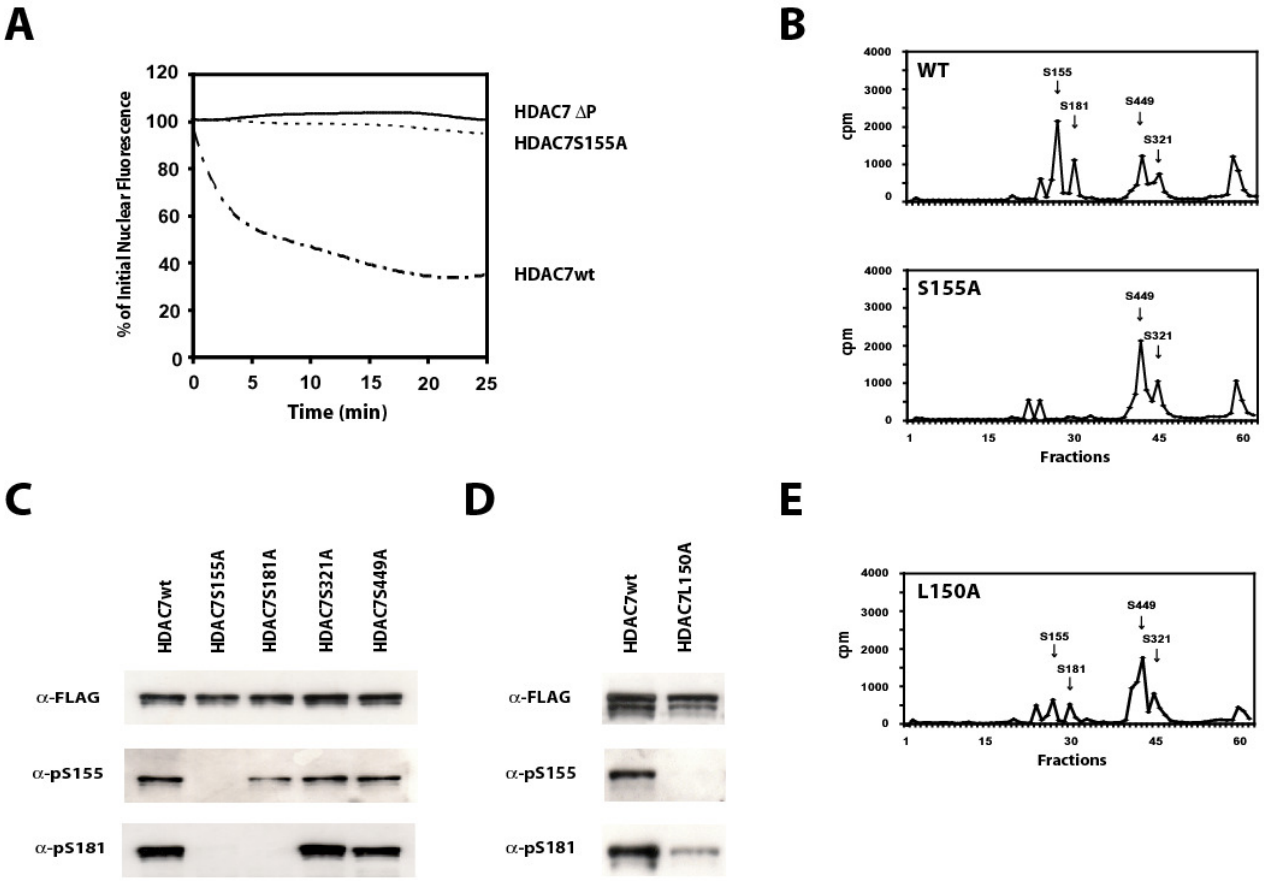


Figure 9. 14-3-3 binding sites in HDAC7 are hierarchically phosphorylated.

(A) Cos7 cells were transfected with constructs expressing GFP-fusion proteins corresponding to wild-type HDAC7 (HDAC7wt), a mutant of HDAC7 in which the residues Ser^{155} was mutated to alanine alone (HDAC7S155A) or together with Ser^{181}, Ser^{321}, and Ser^{449} (HDAC7ΔP). Forty-eight hours post-transfection, the cytoplasm of fluorescent cells was bleached and relative loss of fluorescence in the nuclear region was measured, as described in *Materials and Methods*. FLIP data are represented as non linear fit curves with the following symbols : HDAC7wt (▬ ▬), HDAC7S155A (▬▬▬) and HDAC7ΔP (▬).

(B) HEK293 cells expressing wild-type or S155A HDAC7 were labeled in vivo with [^{32}P] orthophosphate. The labeled proteins were purified by immunoaffinity, digested with trypsin and examined by HPLC analysis as described in *Materials and Methods*.

(C) Expression constructs for FLAG-tagged wild-type HDAC7 (HDAC7wt), or mutant of HDAC7 in which the residues Ser^{155}, Ser^{181}, Ser^{321}, or Ser^{449} were mutated to alanine independently (HDAC7S155A, HDAC7S181A,

HDAC7S321A and HDAC7S449A respectively) or together (HDAC7ΔP) were transiently expressed in HEK293 cells. Cell extracts were examined by western blotting with antibodies directed against the FLAG epitope (α-FLAG), the phosphorylated forms of Ser^{155} (α-pS155) or Ser^{181} (α-pS181).

(D) HEK293 cells were transiently transfected with expression vectors encoding FLAG-tagged versions of wild-type HDAC7 (HDAC7wt) or the L150A mutant (HDAC7L150A). Total cellular extracts were analyzed by SDS-PAGE and subjected to immunoblotting using antibodies directed either against the FLAG epitope (α-FLAG), or HDAC7 phosphorylated at Ser^{155} (α-pS155) or Ser^{181} (α-pS181).

(E) HEK293 cells expressing the L150A HDAC7 mutant were labeled in vivo with [^{32}P] orthophosphate. The labeled protein was purified by immunoaffinity, digested with trypsin and examined by HPLC analysis as described in *Materials and Methods*.

V) Discussion

Signal-dependent cytoplasmic relocalization of class IIa HDACs is achieved through phosphorylation of multiple serine residues by members of the CaMK and PKD families. In addition, some recent observations suggest that the subcellular localization of class IIa HDACs might also be constitutively regulated in a signal-independent manner (Bolger & Yao, 2005; Kao et al., 2001; Wang et al., 2000). Our study identifies EMK and C-TAK1, two members of the MARK/Par-1 family, as new kinases interacting with the N-terminal adapter domain of class IIa HDACs. We have extended this finding by showing that EMK and C-TAK1 phosphorylate class IIa HDACs on one of their multiple 14-3-3 binding sites and alter their subcellular localization and repressive function in normally growing cells. Our study identified a new biological function for Par-1 kinases, as we provide direct experimental evidence that they can play a role in regulating gene transcription. An intriguing question that remains to be addressed is whether class IIa HDACs could play noncanonical roles in any Par-1 functions, such as regulating cell polarity, microtubules dynamic and stability, cell cycle progression and intracellular signaling (Tassan & Le Goff, 2004).

Par-1 kinases substrate specificity

The human MARK/Par-1 protein kinases include hPar-1a/C-TAK1 (MARK3/p78), hPar-1b/EMK (MARK2), hPar-1c (MARK1), and hPar-1d (MARK4/MARKL1). EMK and hPar-1c were originally identified based on their ability to phosphorylate tau and related MAPs proteins MAP2 and MAP4 on their homologous KXGS motif (Drewes et al., 1997). Recently, hPar-1d was also shown to phosphorylate the same KXGS motif (Trinczek et al., 2004). Strikingly, the KTVS motif around Ser^{155} of HDAC7 (conserved in other class IIa HDACs as KTAS) is very homologous to the KXGS consensus for hPar-1b, -c and -d. Although it remains to be formally tested, it is logical to speculate that, in addition to EMK and C-TAK1, hPar-1c and hPar-1d could also phosphorylate class IIa HDACs.

Aside from other family members, C-TAK1/Par-1a exhibits specific substrate requirements. An extensive mutational analysis has defined ϕ^{a}XRXXSϕXXXϕ^{a} (where ϕ and ϕ^{a} are respectively a hydrophobic and a hydrophobic aliphatic residue) as its optimal substrate phosphorylation motif (Muller et al., 2003). From this study, the arginine residue at the -3 position relative to the phosphorylated serine, as well as hydrophobic amino acids at the +1 and +5 positions were proven to be essential for efficient phosphorylation by C-TAK1. Surprisingly, none of these crucial amino acids is found around the serine residue phosphorylated by Par-1 kinases in class IIa HDACs (Ser^{246}, Ser^{259}, Ser^{155} and Ser^{220} of HDAC4, -5, -7 and -9 respectively, see Fig. 5B). Moreover, because they contain a hydrophobic residue at the +5 position, motifs around other phosphorylatable serines of HDAC7, Ser^{181}, Ser^{321} and Ser^{449} (and corresponding residues in other class IIa HDACs) are very homologous to the optimal C-TAK1 consensus. Nonetheless, C-TAK1 does not phosphorylate (see Fig. 5C and 5D) nor interact with (data not shown) any of these serine residues in vitro.

Previously identified substrates of C-TAK1 include the Cdc25C phosphatase (Ogg et al., 1994; Peng et al., 1998), the tyrosine phosphatase PTPH1 (Zhang et al., 1997), the mitogen-activated protein kinase (MAPK) scaffolding protein KSR1 and plakophilin 2 (PKP2) (Muller et al., 2001; Muller et al., 2003). Strikingly, for all known substrates of C-TAK1, the phosphorylated residue generates a 14-3-3 binding site. The identification of class IIa HDACs as C-TAK1 substrates thus provide additional evidence that C-TAK1 may function as a master regulator in the subcellular distribution of several proteins with diverse cellular functions. Identification of additional endogenous substrates for C-TAK1 should confirm this hypothesis.

Convergence of multiple families of protein kinases on class IIa HDACs

The 14-3-3 binding sites of Class IIa HDACs are efficiently phosphorylated by members of multiple families of protein kinases. In response to Ca^{2+} signaling, CaMKI, II and IV have proven to be able to induce nuclear exclusion and cytoplasmic accumulation of class IIa HDACs in various cell types (Yang & Gregoire, 2005). Similarly, PKC signaling leads to the activation of PKD1, which directly phosphorylates the multiple signal-responsive serine residues on class IIa HDACs (Dequiedt et al., 2005; Vega et al., 2004a). We have accumulated new evidence that PKD2 and PKD3, the two other PKD family members can also do so (Seufferlein and Dequiedt, unpublished observations). Here, we show that EMK and C-TAK1, two members of the MARK/Par-1 family can target one of the 14-3-3 binding sites in the N-terminus of HDAC4 and -7 (and presumably other class IIa members). It thus appears that the adapter domain of class IIa HDACs is targeted by multiple members of multiple protein kinase families. This has been illustrated in an elegant expression screen which was published while this manuscript was in preparation (Chang et al., 2005). Looking for modulators of HDAC5 phosphorylation, the authors identified multiple protein kinases, including MARK2.

Experimentally, the convergence of multiple protein kinase families on the adapter domain of class IIa HDACs makes it difficult to fortify conclusions with convincing loss-of-function data. Knockdown of either EMK or C-TAK1 alone had no impact on the phosphorylation of Ser^{155} in HDAC7 (Data not shown). As shown in Fig. 9D, only simultaneous knockdown of both EMK and C-TAK1 led to a reduced Ser^{155} phosphorylation. The existence of multiple class IIa HDACs kinases confirms that these enzymes are regulated by different signaling pathways and emphasizes the importance that class IIa HDACs may have in various genetic programs. In addition, it also enlightens the fact that the functional relevance of each proposed HDAC kinase should be envisioned in a cellular/signaling context-dependent manner.

Multisite and hierarchical phosphorylation of class IIa HDACs

Multisite phosphorylation often offers a sophisticated means to regulate protein functions. In most cases, each site impacts differently on a specific property of the protein, such as stability, cellular localization, catalytic activity, etc... The N-terminus of class IIa HDACs contains multiple phosphorylation sites; with HDAC7 having four identified phosphorylatable serine residues within a 300 amino-acid region. Prior to the current study, these sites were thought to be phosphorylated indistinguishably by the same protein kinases

in response to the same signals and equally important for class IIa HDACs nuclear export. In this context, why class IIa HDACs would have multiple phosphorylation sites in their N-terminus remained obscure. Here, we establish the uniqueness and prominence of the most upstream phosphorylatable serine residue (Ser^{246}, Ser^{259} and Ser^{155} in HDAC4, 5 and 7 respectively). First, we demonstrate that this site is uniquely and specifically phosphorylated by hPar-1 kinases in a constitutive manner. In addition, we establish the primary role of this residue in the constitutive nuclear export of class IIa HDACs. In exploring the functional relevance of these observations for HDAC7, we unexpectedly discovered that basal phosphorylation of Ser^{155} was a prerequisite to the phosphorylation of Ser^{181}. For the first time, these findings unravel that class IIa HDACs undergo hierarchical phosphorylation of their 14-3-3 binding sites. Although it remains to be formally tested, it is tempting to speculate that similar hierarchical phosphorylations exist for other sites on HDAC7, and for other members of the class IIa family.

Par-1 kinases are constitutively active enzymes, and accordingly, we observed constitutive phosphorylation of the most upstream phosphorylatable serine residue in class IIa HDACs. In this context, phosphorylation of the Par-1 target site has two important functions. First, it recruits 14-3-3 proteins and regulates the nucleo-cytoplasmic fluxes of the class IIa HDACs in the absence of any extracellular signaling. Second, phosphorylation of this particular site in HDAC7 allows subsequent phosphorylation of another 14-3-3 binding site, Ser^{181}, which is also important for nuclear efflux (data not shown). These findings thus confer a central role to the most upstream phosphorylatable serine residue of class IIa HDACs. The precise mechanism by which phosphorylation of this residue would promote phosphorylation at other sites of class IIa HDACs remains unknown. Incorporation of a phosphate group at this site could alter conformation of class IIa HDACs and render other serine residues accessible. Alternatively, phosphorylation of Ser^{155} could also create a docking site for other still unknown kinases that require a priming phosphorylation in order to phosphorylate their substrate (e.g. GSK3). Finally, binding of 14-3-3 proteins at this serine residue, subsequently to its phosphorylation, could induce drastic conformational changes and have a similar effect. Mutants of class IIa HDACs that can still be phosphorylated at their most upstream serine residue, but deficient in 14-3-3 recruitment should allow to discriminate between these options.

In this study, we provide multiple evidences that Ser^{155} phosphorylation is crucial for HDAC7 nuclear efflux. First, we show that phosphorylation of Ser^{155} coincides with cytoplasmic localization (Fig. 6C and 6D). In addition, mutation of Ser^{155} to alanine has an

effect comparable to mutating the four serine residues simultaneously (Fig. 9A). However, phosphorylation of Ser^{155} has no impact on the phosphorylation of Ser^{321} or Ser^{449} of HDAC7 (Fig. 9B). These observations thus suggest that Ser^{155}/Ser^{181} phosphorylation is necessary for nuclear export of HDAC7. Interestingly, HDAC7 nuclear localization signal (NLS) spans over amino acids 160-168, exactly between Ser^{155} and Ser^{181}. One speculative model would be that Ser^{155} would function as a 'gatekeeper' (Yaffe, 2002), whose constitutive phosphorylation is necessary for binding of a 14-3-3 dimer, but may not be sufficient for nuclear export. By a still unknown mechanism, phosphorylation of Ser^{155} would favor subsequent phosphorylation of Ser^{181}, by a signal-responsive kinase or a still unidentified constitutively active kinase, depending on the cellular context. Dual phosphorylation of Ser^{155}/Ser^{181} would simultaneously engage both monomeric subunits of a 14-3-3 dimer, which would prevent recognition of the NLS by importin α. Whether masking of the NLS by a 14-3-3 dimer would be sufficient for nuclear export or would necessitate signal-mediated phosphorylation of Ser^{321} and/or Ser^{449} remains unclear.

Our study provides experimental evidence that the multiple phosphorylation sites in class IIa HDACs display specific properties. In this context, combinatorial phosphorylation of these enzymes by multiple kinases would allow for a flexible and sophisticated control of their functions. Sequential or/and coordinated actions of the various protein kinases on the N-terminus of class IIa HDACs would constitute a tightly regulated mechanism to rapidly, adequately and reversibly induce expression of specific target genes in response to specific signals. Although the pieces are starting to fall into place, more efforts are required to fully understand class IIa HDACs regulation by multisite phosphorylation

3.2. Regulation of HDAC7 by PP2A

Our above results clearly establish the importance of phosphorylation by constitutively active protein kinases in the regulation of class IIa HDACs. Because phosphorylation is known as a reversible process, we hypothesized that protein phosphatase, the antagonistic counterpart of protein kinases might be equally important in regulating class IIa HDACs. The results presented in the following section corroborate our hypothesis and allowed us to identify and characterize the specific protein phosphatase targeting class IIa HDACs.

3.2 Protein Phosphatase 2A Controls the Activity of Histone Deacetylase 7 During T-Cell Apoptosis and Angiogenesis

I) Summary

Class IIa histone deacetylases (HDACs) act as key transcriptional regulators in several important developmental programs. Their activities are controlled via phosphorylation-dependent nucleocytoplasmic shuttling. Phosphorylation of conserved serine residues triggers association with 14-3-3 proteins and cytoplasmic relocalization of class IIa HDACs, which leads to the derepression of their target genes. While a lot of effort has been made towards the identification of the inactivating kinases that phosphorylate class IIa HDAC 14-3-3 motifs, the existence of an antagonistic protein phosphatase remains elusive. Here, we identify PP2A as a phosphatase responsible for dephosphorylating the 14-3-3 binding sites in class IIa HDACs. Interestingly, dephosphorylation of class IIa HDACs by PP2A is prevented by competitive association of 14-3-3 proteins. Using both okadaic acid treatment and RNA interference, we demonstrate that PP2A constitutively dephosphorylates the class IIa member HDAC7 to control its biological functions as a regulator of T-cell apoptosis and endothelial cell biology. This study unravels a dynamic interplay between 14-3-3s, protein kinases and PP2A and provides a new model for the regulation of class IIa HDACs.

II) Introduction

Deacetylation of histones by histone deacetylases (HDACs) results in a compact chromatin structure that imposes specific restrictions on the transcriptional machinery. Based on structural and biochemical characteristics, the 18 human HDACs fall into four distinct classes, with members of the class II further divided into two subclasses, IIa and IIb (Martin et al., 2007). Class IIa HDACs (HDAC4, -5, -7 and -9) are regulated by phosphorylation-dependent nuclear export. Several canonical binding motifs for 14-3-3 proteins are found in the N-terminal adapter domain of all class IIa HDAC members. When phosphorylated on specific serine residues, these consensus motifs recruit 14-3-3 proteins. Association with 14-3-3 overcomes the repressor activity of class IIa HDACs by eliciting their sequestration in the

cytoplasm and making them unavailable for their cognate transcription factors and corepressors.

Class IIa HDACs act as transcriptional modulators of specific genetic programs associated with several important developmental processes (Martin et al., 2007). In humans, HDAC7 is transiently and predominantly expressed in CD4/CD8 double positive thymocytes, where it represses the expression of *nur77*, a pro-apoptotic gene involved in negative selection (Dequiedt et al., 2003). Recently, *in situ* hybridization unraveled expression of HDAC7 in developing vascular endothelium of mouse embryo. Consistent with this, inactivation of the HDAC7 gene led to embryonic lethality resulting from blood vessel dilatations, rupture and hemorrhages. The vascular defects associated with HDAC7 deficiency in mouse was attributed to the upregulation of matrix metalloprotease 10 (MMP-10), a secreted proteinase that degrades the extracellular matrix (Chang et al., 2006).

Modulation of class IIa HDAC subcellular distribution by phosphorylation provides the opportunity to control these important developmental processes. While a great deal of effort has been invested in identifying the kinases targeting class IIa HDAC 14-3-3 motifs (Chang et al., 2005; Chawla et al., 2003; Davis et al., 2003; Dequiedt et al., 2006; Dequiedt et al., 2005; Kao et al., 2001; Linseman et al., 2003; Matthews et al., 2006; McKinsey et al., 2000a; McKinsey et al., 2000b), the identity of antagonistic phosphatase(s) remains elusive.

III) Materials and methods

Plasmids, Antibodies and Chemicals

FLAG-, GFP- or GST-fusion proteins of human HDACs have been described elsewhere (Dequiedt et al., 2006; Dequiedt et al., 2005). The His-fusion protein of HDAC7 (His-Nter-HDAC7) corresponds to aa 1 to 490 of HDAC7. GST-14-3-3 and 14-4-3-Myc fusions correspond to isoform zeta of human 14-3-3. Minimal wild-type and mutant MEF2 reporter constructs (pMEF2wt-Luc and pMEF2mt-Luc) were as described (Dequiedt et al., 2003). The MMP10-reporter construct was a kind gift of Dr. E.N. Olson (Chang et al., 2006).

Anti-HDAC7 antibodies were from Santa Cruz Biotechonology (Santa Cruz, CA) and Abcam (Cambridge, UK). Anti-14-3-3s, anti-PP2A-A, anti-PP2B, anti-PP4 and anti-Myc antibodies were purchased from Santa Cruz Biotechonology and anti-Flag antibodies were from Sigma. Monoclonal and polyclonal antibodies against the catalytic subunit of PP2A (PP2A-C) and PP1α were from Millipore Bioscience. Polyclonal antibodies against

phosphorylated Ser^{155} and phosphorylated Ser^{181} were described elsewhere (Dequiedt et al., 2006). The R18 peptide was purchased from Biomol International, LP (Plymouth Meeting, PA). Cyclosporine A, Calyculin A and FK506 were obtained from Alexis Biochemical Corp (Lausen, Switzerland). Okadaïc acid was from Calbiochem.

Immunoprecipitation, SDS-PAGE and Western Blotting

Total cell lysates from HEK293T or Do11.10 cells were prepared in IPLS buffer and immunoprecipitations were carried out as described in (Fischle et al., 2002). SDS-PAGE and Western blot analysis were performed according to standard procedures.

GST and His fusion proteins: expression, purification

GST fusion proteins were purified according to protocols described elsewhere (Dequiedt et al., 2006). His-N-ter-HDAC7 was purified by using a Cobalt-based immobilized metal affinity chromatography resin (BD Biosciences Clontech, Mississauga, Ontario, Canada).

Reporter assays

Reporter assays in Do11.10 cells were performed with Dual Luciferase reporter assay (Promega) following the protocol described in (Dequiedt et al., 2003). Twenty-four hours after siRNA transfection, HUVEC cells were transfected with the indicated plasmids and processed for Dual Luciferase reporter assays twenty four hours later, following the procedure described in (Potente et al., 2005). All transfections were performed in duplicate and are represented as the mean of at least three independent experiments.

Cell Culture, transfections and viral transduction

Cell lines were obtained from the American Type Culture Collection and grown in the recommended medium. DO11.10 cells were transfected by the DEAE-dextran/chloroquine method. HEK293T cells were transfected by the standard calcium phosphate precipitation method. Plasmid transfections in HUVECs were performed using the Targefect F2 and Virofect reagents (Targeting Systems) according to the manufacturers' protocols. Polyclonal GFP- or FLAG-HDAC7-expressing cell lines were obtained by infecting Do11.10 cells with corresponding MSCV recombinant retroviruses as described previously (Dequiedt et al., 2003).For *in vivo* phosphatase inhibition experiments, OA and FK506 were added in the culture medium at 20 nM and 600 nM respectively, for 5h.

Phosphorylation of GST and His fusion proteins

The 14-3-3 binding sites of purified GST- or HIS-fusion HDAC4, -5 and -7 proteins were phosphorylated with recombinant active PKD1, EMK or c-TAK1 in the presence of [γ-^{32}P]ATP, as described in (Dequiedt et al., 2006; Dequiedt et al., 2005).

In vitro recombinant HDAC dephosphorylation assay

Aliquots of PKD1-phosphorylated GST-HDAC7 were incubated in Do11.10 cell lysates prepared in phosphatase buffer (50 mM Tris-Cl, 150 mM NaCl, 0.25% Nonidet P-40, Proteases inhibitors) for 1h at 30°C. When appropriate, dephosphorylation reactions were supplemented with 0.5 µM calyculin A, 2 µM Cyclosporine A, 400 nM FK506, or indicated concentrations of okadaic acid. The PP2A-dephosphorylation assay was performed at 30°C, for 30 min in the phosphatase buffer containing 0.2 U of purified PP2A A/C heterodimer (for Fig. 1C and 2B) or A/C/B55α heterotrimer (for Fig. 5B). For protection experiments by 14-3-3s, recombinant GST-14-3-3ζ or GST alone were added to the reaction mixture and left to interact with the phosphorylated His-Nter-HDAC7 for 10 min at 4°C before adding purified PP2A/C dimer. Dephosphorylation reactions were terminated by adding an equal amount of 2xSDS-PAGE sample buffer and boiling for 5 minutes. Phosphorylation was then assessed by SDS-PAGE and autoradiography or in-gel trypsin digest and HPLC analysis (see below).

In vitro Flag-HDAC dephosphorylation assay

Lysates from HEK293T cells expressing Flag-tagged HDAC7 were prepared in phosphatase assay buffer and incubated at 30°C for 30 min, with or without prior addition of the R18 peptide (25µM). Dephosphorylation of HDAC7 was analyzed by SDS-PAGE and Western blotting analysis using the phosphospecific antibodies.

RNA interference (RNAi)

Two functionally validated On Target Plus siRNA molecules directed against the α or β isoforms of PP2A catalytic subunit, or the corresponding Non-targeting control siRNA were purchased from Dharmacon (Lafayette, CO). HUVECs and HeLa cells were transfected with pooled siRNA (50 nM each) against PP2A-Cα and PP2A-Cβ or control siRNA using the GeneTrans II reagent (MoBiTec) and Lipofectamine (Invitrogen), respectively.

Immunofluorescence

Localization of the fluorescent proteins was assessed on fixed cells by confocal microscopy (Axiovert 200 with LSM 510; Carl Zeiss Microscopy). The average percentage of cells showing predominant cytoplasmic localization of the GFP-tagged protein was assessed by examining at least 3 independent fields each containing more than 50 cells. Visualization of the cell nuclei was achieved by staining of DNA with using Draq5 (Biostatus limited, UK) or Topro (Molecular Probes).

RT-PCR analysis

Primers sequences and amplification conditions for specific genes are available upon request.

HDAC7 polyclonal cell lines and apoptosis

These assays were carried out according to protocols described in our previous studies (Dequiedt et al., 2003; Dequiedt et al., 2005).

Matrigel *in vitro* angiogenesis assays

Twenty-four hours after transfection with the indicated siRNA, HUVECs were transfected with HDAC7-expression vectors and processed for the Matrigel assays 24 hours later. Assays were performed exactly as described in (Potente et al., 2005).

Metabolic labeling and phosphorylation site analysis

In vivo radiolabelling and immunoaffinity purification of the HDAC7-Flag proteins were performed exactly as described in (Dequiedt et al., 2006).

IV) Results

PP2A dephosphorylates the 14-3-3 sites of the class IIa HDAC7 in vitro

To identify putative class IIa HDAC phosphatases, the N-terminal domain (aa 1-490) of HDAC7 was radiolabeled with protein kinase D (PKD), which specifically phosphorylates the four previously identified 14-3-3 binding sites, *i.e.* Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449} (Dequiedt et al., 2005) and incubated with total cell lysate in a buffer compatible with phosphatase activities. In these conditions, we observed a time-dependent decrease in HDAC7 phosphorylation, which unravels the existence of a cellular protein phosphatase capable of

dephosphorylating the 14-3-3 sites in HDAC7 (Fig 1A). This phosphatase activity, was inhibited by Calyculin A (CA), an inhibitor of PP1 and PP2A, but not by the potent PP2B inhibitors, Cyclosporine A (CsA) and FK506 (Fig. S1). In addition, okadaïc acid (OA), an inhibitor with substantial preference for PP2A (IC_{50} = 0.1 nM) over PP1 *in vitro* (IC_{50} = 10 nM) totally inhibited dephosphorylation of the HDAC7 14-3-3 sites when used at 10 nM (Fig 1B). These observations clearly suggest that PP2A or a PP2A-like enzyme is involved in HDAC7 dephosphorylation.

We next tested the ability of PP2A to directly dephosphorylate the 14-3-3 binding sites in HDAC7. As shown in Figure 1C, we observed a time-dependent dephosphorylation of HDAC7 on incubation with purified PP2A. To determine whether all four 14-3-3 binding motifs in HDAC7 were similarly targeted by PP2A, aliquots of the dephosphorylation reaction were taken at various time points and phosphorylation of Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449} was followed by high pressure liquid chromatography (HPLC) analysis (Dequiedt et al., 2006). A time-dependent reduction of phosphorylation was observed for each serine residue (Fig. 1D). Interestingly, the relative decrease was comparable for each site. These results confirm that PP2A is capable of dephosphorylating HDAC7 *in vitro* and also demonstrate that PP2A dephosphorylation occurs without any preference amongst the four phosphorylation sites.

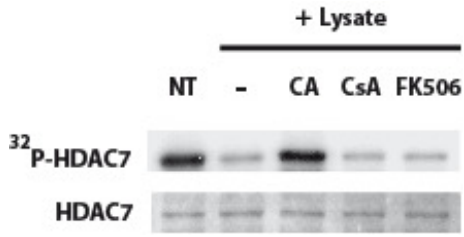


Figure S1: Dephosphorylation of ^{32}P-HDAC7 by total cellular extract was performed for 1h in phosphatase assay buffer supplemented or not (-) with 0.5 µM CA, 2 µM CsA or 400 nM FK506. Residual phosphorylation was assessed as described in (A) by comparison with a control reaction performed in the absence of cell extract (NT).

PP2A associates with HDAC7 in vivo

In cells, PP2A holoenzymes comprise a heterodimeric core complex, made of a catalytic (C) and scaffold (A) subunits. To confirm that HDAC7 is targeted by PP2A *in vivo*, we first examined its ability to associate with the core PP2A-A/C complex. Because 14-3-3 proteins and PP2A would be expected to target the same phosphorylated residues in HDAC7, we

hypothesized that 14-3-3 binding would hinder access of PP2A to HDAC7. To test this, coimmunoprecipitation experiments between HDAC7 and PP2A were performed in the presence of R18, a peptide that has been previously shown to displace 14-3-3 from their target proteins (Wang et al., 1999b). In the absence of R18, coimmunoprecipitation experiments revealed a weak but specific association of HDAC7 with endogenous catalytic (PP2A-C) and structural (PP2A-A) PP2A subunits. In contrast, 14-3-3 proteins were found to robustly interact with HDAC7 (Fig. 1E). As expected, the presence of R18 in the lysate dissociated endogenous 14-3-3 from HDAC7. Concomitant to the displacement of 14-3-3, we observed a spectacular increase in the amount of endogenous PP2A-A/C associated with HDAC7. These results show that PP2A and 14-3-3 competitively interacts with HDAC7 *in vivo*. Importantly, the interaction between HDAC7 and the core PP2A enzyme is highly specific, since none of the other serine/threonine phosphatases examined, such as PP1α, PP2B or PP4 was found associated with HDAC7, even in the presence of the R18 peptide (Fig. 1E).

To validate the biological relevance of the above observations, association of endogenous PP2A and HDAC7 proteins was investigated. Immunoprecipitates of the PP2A catalytic core were prepared from Do11.10 cells, a T-cell hybridoma cell line that specifically expresses high levels of HDAC7 (Dequiedt et al., 2003). As shown in Figure 1F, endogenous HDAC7 was weakly but readily detected in the PP2A-C immunoprecipitate. However, interaction between PP2A and endogenous HDAC7 was greatly enhanced in the presence of the R18 peptide. Similarly, when endogenous HDAC7 was immunoprecipitated in the presence of the R18 peptide, endogenous PP2A was efficiently coimmunoprecipitated. These observations are consistent with the idea that 14-3-3 and PP2A compete for the same phosphorylation sites in HDAC7.

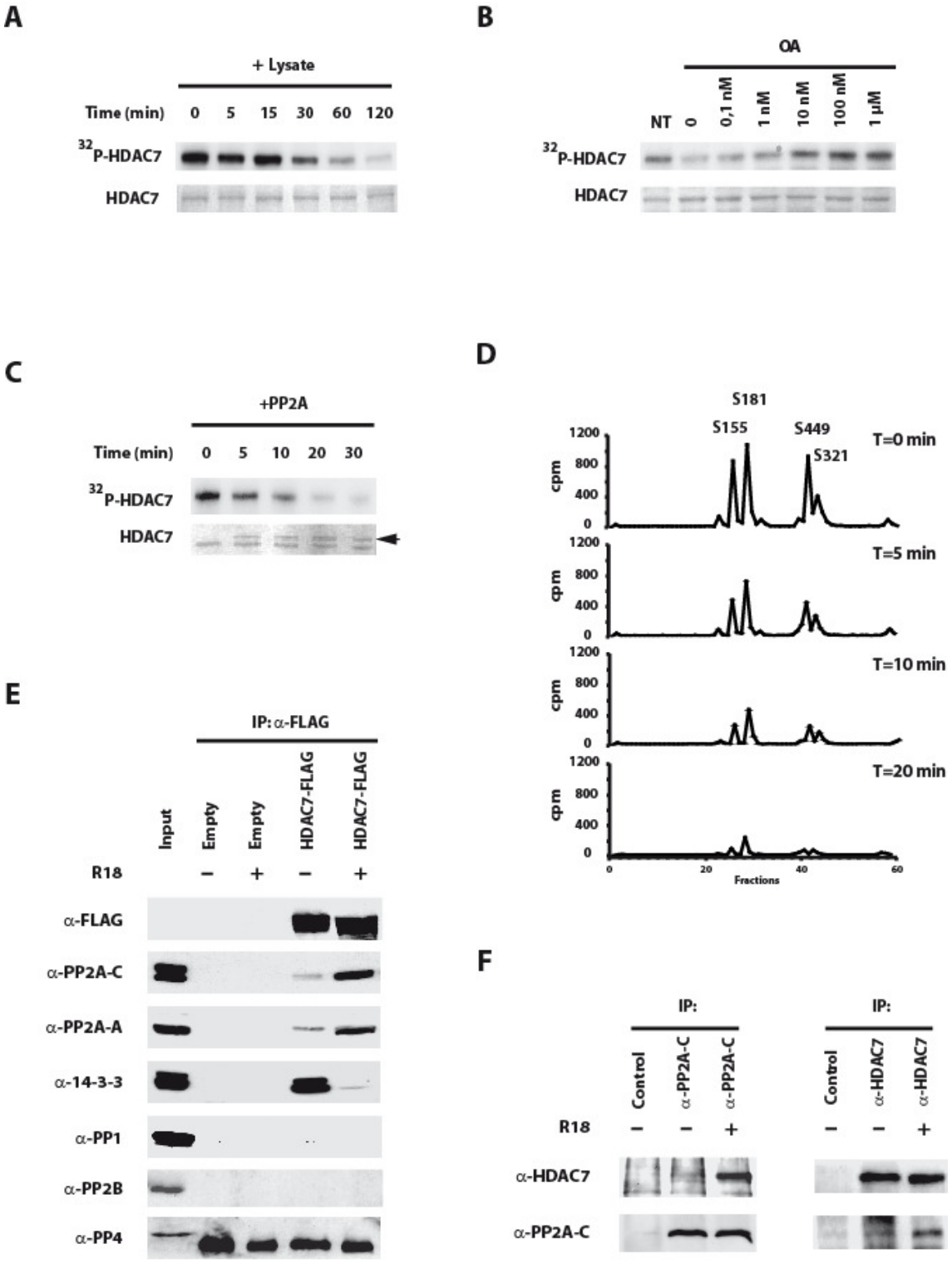


Figure 1. PP2A dephosphorylates the 14-3-3 binding sites in HDAC7 *in vitro*.

(A) The 14-3-3 binding sites of HDAC7 were phosphorylated by recombinant PKD in the presence of radioactive ATP. The radiolabeled protein (^{32}P-HDAC7) was incubated at 30°C, with total cellular extracts. At the indicated times, aliquots were

analyzed by SDS-PAGE and dephosphorylation was assessed by autoradiography (upper panel) and Coomassie Blue (lower panel).

(B) The ^{32}P-labelled HDAC7 protein was incubated for 1h at 30°C without (NT) or with cell extracts containing increasing concentrations of OA. Residual phosphorylation of HDAC7 was assessed as described in (A) by comparison with a control reaction performed in the absence of cell extract (NT).

(C) and (D) The ^{32}P-labelled HDAC7 protein was incubated with purified $PP2A_D$. Time-dependent dephosphorylation was assessed by SDS-PAGE (C) or HPLC analysis (D) as described in (Dequiedt et al., 2006). Arrow indicates an unidentified protein present in the PP2A preparation.

(E) Flag-HDAC7 was immunoprecipitated from HEK293 cells in the absence (-) or presence (+) of R18 peptide (25µM) and analyzed by immunoblotting with the indicated antibodies. Control immunoprecipitation was performed in parallel from cells transfected with the empty expression vector (Empty)

(F) Endogenous PP2A (left panel) or HDAC7 (right panel) were immunoprecipitated from Do11.10 cells in the presence (+) or absence (-) of the R18 peptide. In each case, a control immunoprecipitation reaction was performed with a corresponding isotype control antibody (Control). Immune complexes were analyzed by western blotting with the indicated antibodies.

14-3-3 proteins protect HDAC7 from dephosphorylation by PP2A in vitro

Based on the above observations, we reasoned that binding of 14-3-3 proteins might prevent PP2A from gaining access to and dephosphorylating HDAC7. To test this, total lysate from HDAC7-expressing cells was incubated at 30°C in a buffer compatible with dephosphorylation and immunoblotted with antibodies specific for phosphorylated Ser^{155} and Ser^{181}(Dequiedt et al., 2006). No significant dephosphorylation of either serine residues was observed under these conditions (Fig. 2A). By contrast, Ser^{155} and Ser^{181} were completely dephosphorylated when 14-3-3s were forced to dissociate by addition of the R18 peptide.

To more firmly establish our findings, in vitro dephosphorylation of HDAC7 by PP2A was performed in the presence of recombinant GST-14-3-3ζ. While dephosphorylation of HDAC7 by PP2A was nearly total in the presence of GST, addition of GST-14-3-3ζ completely prevented HDAC7 dephosphorylation (Fig. 2B).

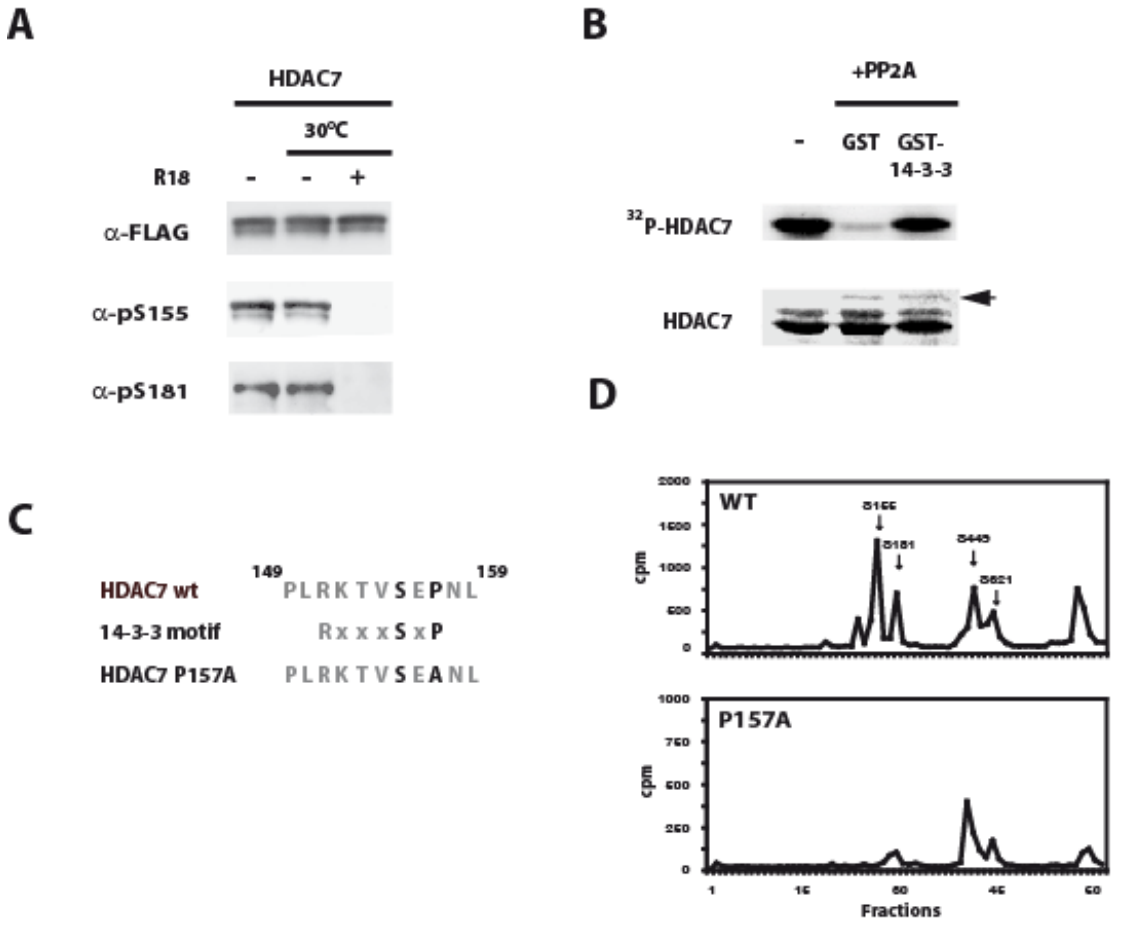


Figure 2. 14-3-3 proteins prevent dephosphorylation of HDAC7 by PP2A

(A) Lysates from HEK293 cells expressing Flag-tagged HDAC7 were prepared in phosphatase assay buffer and incubated at 30°C for 30 min in the absence or presence of the R18 peptide. Dephosphorylation of HDAC7 was analyzed by western blotting with antibodies against phosphorylated-Ser^{155} (α-pS155) and –Ser^{181} (α-pS181).

(B) A His-fusion protein corresponding to the N-terminal domain of HDAC7 was subjected to *in vitro* dephosphorylation by purified $PP2A_D$. When indicated, GST-14-3-3ζ or GST alone were incubated with $PP2A_D$. Dephosphorylation was assessed after SDS-PAGE, Coomassie Blue staining (lower panel) and autoradiography (upper panel). An unidentified protein present in the PP2A preparation is indicated by an arrow.

(C) Sequences around Ser^{155} in HDAC7 match with the canonical mode II 14-3-3 binding motif.

(D) Wild-type or P157A mutant versions of Flag-HDAC7 were metabolically labeled *in vivo* with [^{32}P] orthophosphate, immunoprecipitated and their phosphorylation profile was established by HPLC analysis. Labeled peaks containing each phosphorylation site serine residue are indicated (as confirmed by mass spectrometry).

14-3-3s regulate phosphorylation of HDAC7 by preventing dephosphorylation by PP2A in vivo.

In 14-3-3 target motifs, the proline residue at position +2 relative to the phosphorylated serine is crucial for recognition (Yaffe et al., 1997). To investigate whether a defect in 14-3-3 binding would translate into increased dephosphorylation of HDAC7 *in vivo*, we generated a mutant of HDAC7 in which Pro^{157} was changed to alanine (Fig. 2C, HDAC7P157A). While this mutation did not impair Ser^{155} phosphorylation by the known HDAC7 kinases (Supporting information, Fig. S2), it greatly reduced the interaction of 14-3-3 with Ser^{155}, both *in vitro* and *in vivo* (Supporting information Fig. S3). To examine the *in vivo* phosphorylation of Ser^{155} in the absence of 14-3-3 binding, wild-type and P157A mutant HDAC7 proteins were metabolically labeled with [^{32}P] orthophosphate and affinity purified. The phosphorylation status of each serine residue was then analyzed by HPLC analysis. As expected, the inability of sequences around Ser^{155} to maintain stable interaction with 14-3-3 in the P157A HDAC7 mutant resulted in complete loss of Ser^{155} phosphorylation *in vivo* (Fig. 2D). By contrast, phosphorylation of Ser^{321} and Ser^{449} remained unaffected. Lack of Ser^{155} phosphorylation was also associated with reduced phosphorylation at Ser^{181}, confirming the hierarchical phosphorylation of these two sites in HDAC7 (Dequiedt et al., 2006). Importantly, treatment with OA restored phosphorylation of the P157A HDAC7 mutant protein (Supporting information, Fig. S4). These observations demonstrate that the reduced phosphorylation of the P157A HDAC7 mutant is mainly due to its increased susceptibility to dephosphorylation by PP2A, but not to its inability to be phosphorylated by class IIa HDAC kinases. Altogether, these results strongly support the model in which association with 14-3-3s prevents dephosphorylation of HDAC7 by PP2A.

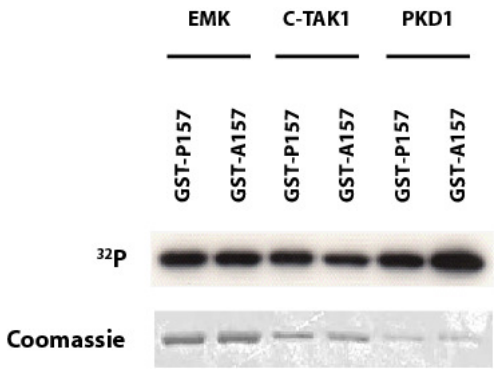


Figure S2: Class IIa HDACs kinases phosphorylate Ser[155] in the context of the P157A HDAC7 mutant. Sequences around Ser[155] from HDAC7wt and HDAC7P157A were expressed as GST-fusion proteins (respectively GST-P157 and GST-A157) and used in an *in vitro* phosphorylation assay with known HDAC7 kinases, i.e., EMK, C-TAK1 and PKD1. Proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining (lower panel). Phosphorylation was assessed by autoradiography (upper panel).

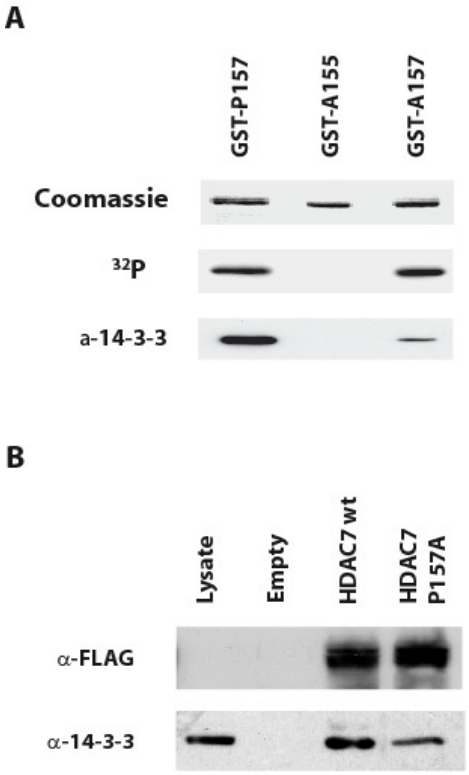


Figure S3. The P157A mutation impairs binding of 14-3-3 to Ser^{155} in HDAC7 P157A. (A) GST-fusion proteins corresponding to the sequences around Ser^{155} of HDAC7wt or HDAC7P157A (GST-P157 and GST-A157 respectively) were phosphorylated *in vitro* with PKD1 and [^{32}P] ATP as described in *Materials and Methods*. A mutant fusion protein, where Ser^{155} was mutated into alanine was used as control (GST-A155). Phosphorylated fusion proteins were used in pull-down assays with total extracts from HEK293 cells expressing Myc-tagged 14-3-3ζ. Pull-down reactions were analyzed by SDS-PAGE, Coomassie Blue staining (Coomassie), autoradiography (^{32}P) and western blotting with an anti-Myc antibody (α-Myc). Despite comparable levels of Ser^{155} phosphorylation in both fusion proteins (GST-P157 and GST-A157, ^{32}P), GST-A157 was greatly impaired in its ability to associate with 14-3-3 compared to GST-P157 (GST-P157 and GST-A157, α-Myc). As a control, GST-A155 was not phosphorylated by PKD nor interacted with 14-3-3 proteins. (B) FLAG-tagged wild-type HDAC7 (wt) or the P157A mutant (P157A) were immunoprecipitated from HEK293 cells, and examined by immunoblotting with the indicated antibodies. Control immunoprecipitation was performed in parallel from cells transfected with the empty expression vector (Empty).

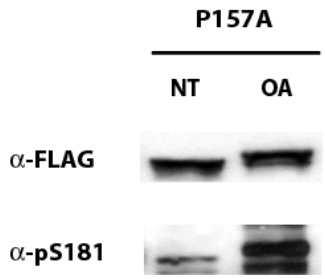


Figure S4. Inhibition of PP2A activity leads to hyperphosphorylation of the HDAC7P157A mutant. Cos7 cells were transfected with a Flag-tagged HDAC7 mutant protein harboring the P157A substitution and treated or not with 20 nM OA (OA and NT respectively) for 5 hours. Phosphorylation of HDAC7P157A was examined in total cell lysates by western blot analysis with the antibody directed against phosphorylated-Ser^{181} (α-pS181). As loading control, the same membrane was stripped and immunoblotted with an antibody against the Flag epitope (α-Flag).

PP2A inhibition increases in vivo phosphorylation and cytoplasmic accumulation of HDAC7

To establish that PP2A acts as a physiological HDAC7 phosphatase *in vivo*, Do11.10 cells were treated with concentrations of OA previously shown to inhibit PP2A but not other OA-sensitive phosphatases, such as PP1 (Dounay & Forsyth, 2002). As illustrated for Ser^{181}, treatment with OA dramatically increased the basal phosphorylation of 14-3-3 motifs on endogenous HDAC7 (Fig. 3A). By contrast, treatment with the PP2B-inhibitor FK506 had no effect on the phosphorylation of HDAC7.

Phosphorylation-dependent association of class IIa HDACs with 14-3-3 controls their distribution between the nucleus and the cytoplasm. Supporting the hypothesis that PP2A dephosphorylates the 14-3-3 binding sites of HDAC7 *in vivo*, treatment with OA induced relocalization of HDAC7 from the nucleus to the cytoplasm of Do.11.10 cells (Fig. 3B). Interestingly, OA had no effect on the cellular distribution of a phosphorylation/shuttling-deficient mutant of HDAC7, in which the four phosphorylatable serine residues are mutated into alanine (HDAC7ΔP,(Dequiedt et al., 2006)). These data demonstrate that inhibition of PP2A activity is associated with hyperphosphorylation and cytoplasmic sequestration of HDAC7.

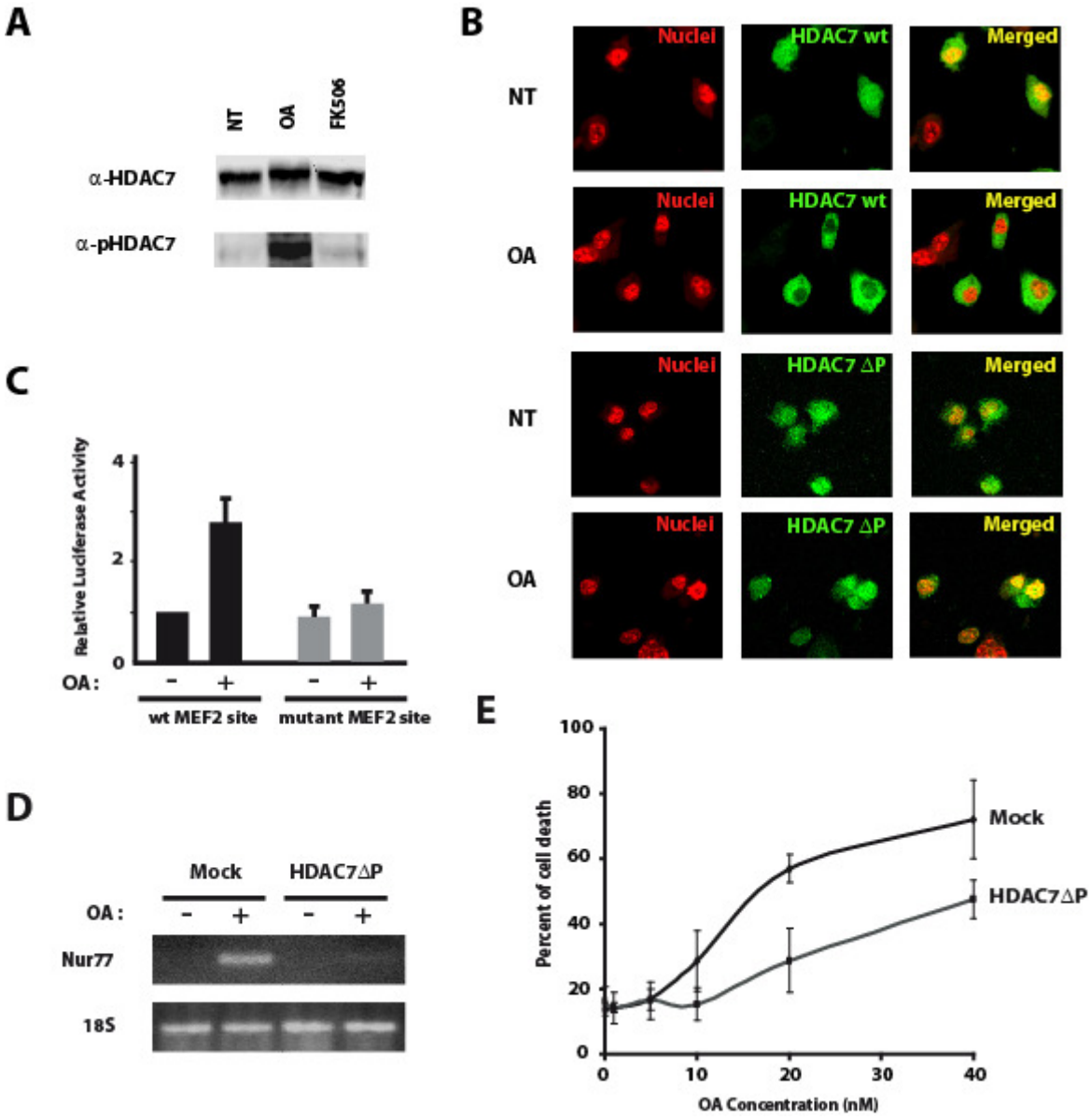


Figure 3. PP2A participates in the regulation of *Nur77*-mediated apoptosis in T-cell hybridomas by HDAC7.

(A) Do11.10 cells were left untreated (NT) or were treated with OA (20 nM) or FK506 (600 nM) for 5 h. Phosphorylation of endogenous HDAC7 was then detected by Western blotting with antibody specific for phosphorylated Ser^{181} (α-pS181).

(B) Do11.10 cells stably expressing wild-type or ΔP mutant HDAC7 proteins fused to GFP were left untreated (NT) or treated with OA (20 nM) for 5 h. Subcellular localization of HDAC7 proteins was examined by confocal immunofluorescence microscopy (Green). Cell nuclei were visualized by Draq5 stain (Red).

(C) Do11.10 cells were transfected with a luciferase reporter plasmid driven by a multimerized wild-type or mutant MEF2-binding consensus and treated with OA for 10 h (+) or left untreated (-). Luciferase activities are presented relative to the activity of the wild-type reporter in untreated cells. Values are the mean of four independent experiments.

(D) Do11.10 cells transduced with a HDAC7ΔP-FLAG expressing retroviral construct or the empty vector as control (Mock) were left untreated or treated with OA for 10h. Expression of *Nur77* was examined by RT-PCR analysis. Amplification of 18S ribosomal RNA was used as an internal control.

(E) Do11.10 cells described in (D) were treated with increasing amounts of OA for 24 h. Curves illustrate the mean apoptotic rates from 8 experiments performed from two independently established sets of polyclonal cell lines.

PP2A controls the repressor activity of HDAC7

In thymocytes, HDAC7 associates with MEF2D to repress the *Nur77* promoter. This inhibitory action is relieved by phosphorylation-dependent cytoplasmic relocalisation of HDAC7 (Dequiedt et al., 2003). As PP2A inhibition favors cytoplasmic accumulation of HDAC7, we reasoned that it should concomitantly release MEF2D from HDAC7-mediated transcriptional repression. To verify this assumption, endogenous MEF2D activity was assessed in Do11.10 cells with a reporter construct containing multimerized MEF2D-binding sites. As expected, treatment with OA induced a significant increase in endogenous MEF2D-dependent transcriptional activity (Fig. 3C). The above observations were next confirmed on a MEF2D-regulated cellular gene. Indeed, treatment with OA was associated with derepression of the *Nur77* promoter (Fig. S5) and significant increase in levels of *Nur77* mRNA (Fig. 3D). Importantly, OA-mediated activation of *Nur77* expression was completely abolished in cells expressing the HDAC7ΔP mutant, which lacks the phosphorylatable serine residues and thus remains in the nucleus to repress *Nur77* expression.

Taken together, these data demonstrate that PP2A contributes to HDAC7-mediated transcriptional repression by controlling its phosphorylation and subcellular localisation.

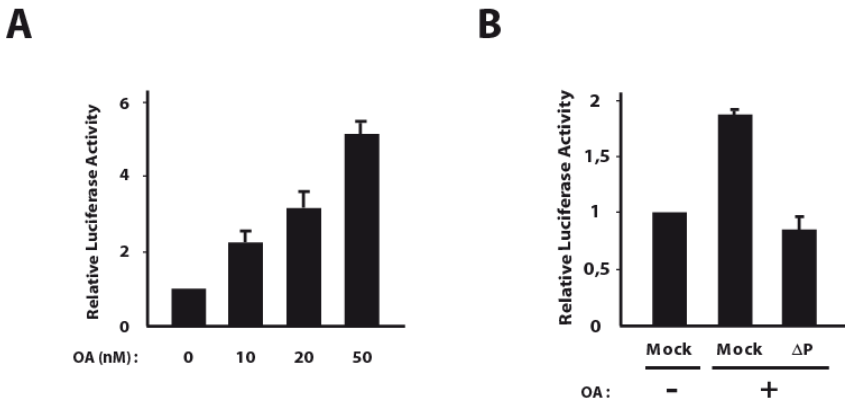


Figure S5. Inhibition of PP2A activity is associated with upregulation of Nur77 transcription. (A) Do11.10 cells transfected with a luciferase reporter plasmid driven by the *Nur77* promoter (pNur77-Luc, (2)) were treated with increasing concentrations of OA for 10 h. Luciferase activities are presented relative to the activity of the reporter in untreated cells. The results are means from 3 independent experiments each performed in triplicate. (B) Do11.10 cells were transfected with a luciferase reporter plasmid driven by a Nur77-responsive promoter whose activity is a direct reflection of the endogenous level and activity of the Nur77 protein (11). When indicated, an expression plasmid for the phosphorylation-deficient HDAC7ΔP mutant (ΔP) or the corresponding empty vector (Mock) were transfected with the reporter construct.Two days after transfection, cells were treated with 20 nM OA for 10 h (+) or left untreated (-). Luciferase activities are presented relative to the activity of the reporter in untreated cells. Values are the mean of four independent experiments, each performed in triplicate.

PP2A regulates the biological functions of HDAC7

By modulating the expression of *Nur77*, HDAC7 is a key regulator of apoptosis in developing thymocytes (Dequiedt et al., 2003). Given the role of PP2A in controlling the repressive function of HDAC7, we hypothesized that inhibition of PP2A activity should trigger *Nur77*-associated apoptotic programs. Consistent with this hypothesis, OA dose-dependently induced apoptosis in Do11.10 cells (Fig. 3E). Importantly, OA-induced apoptosis was reduced by overexpressing the HDAC7ΔP mutant. These observations indicate that OA treatment induces T-cell hybridoma apoptosis by a mechanism that, at least partly, involves cytoplasmic sequestration of HDAC7 and derepression of the pro-apoptotic *Nur77* gene.

Suppression of HDAC7 expression in human umbilical vein endothelial cells (HUVECs) is associated with upregulation of *MMP10* expression and impairs the ability of endothelial cells to form a vascular-like network (Chang et al., 2006). To further establish the functional significance of our findings, we used small interfering RNA (siRNA) to inhibit expression of PP2A catalytic subunits in endothelial cells (HUVECs). RT-PCR and western blot analysis confirmed the efficient and specific knock-down of both PP2A-C isoforms after siRNA treatment (Supporting information, Fig. S6). Supporting a role for PP2A in the regulation of HDAC7, silencing of endogenous PP2A-C expression in HUVECs coincided with increased phosphorylation of the 14-3-3 sites in endogenous HDAC7 (Fig. 4A). Accordingly, loss of PP2A also had a striking effect on the subcellular localization of HDAC7. Indeed, whereas HDAC7 was exclusively nuclear in control HUVECs, it accumulated in the cytoplasm of PP2A-deficient cells (Fig. 4B).

As observed for T-cell hybridomas, we predicted that suppression of PP2A activity in HUVECs would lead to the functional inactivation of HDAC7 and recapitulate the defects associated with HDAC7-deficiency (Chang et al., 2006). To verify this, we first examined the transcriptional activity of *MMP10*, a canonical HDAC7 target gene in HUVECs. As expected, transfection of siRNA against PP2A-C was associated with a dramatic upregulation of *MMP10* expression (Fig. 4C). Importantly, activation of the *MMP10* promoter following PP2A knock-down was totally inhibited by overexpression of the HDAC7ΔP mutant (Fig. 4D). In an *in vitro* model of angiogenesis, HUVECs grown on Matrigel spontaneously form a primitive vascular network. As reported by others (Chang et al., 2006), HDAC7 plays a crucial role in this process since HUVECs failed to organize into capillary-like structures when transfected with HDAC7 siRNA (Fig. 4E). Consistent with our model, specific inhibition of endogenous PP2A-C expression by siRNA reduced capillary tube formation of HUVECs (Fig. 4E and 4F). Strikingly, overexpression of the phosphorylation-deficient HDAC7ΔP mutant counteracted the effects of PP2A knockdown and totally restored the angiogenic ability of PP2A-silenced HUVEC cells. These data demonstrate that PP2A participates in HDAC7 functions in endothelial cells and strongly support the conclusion that PP2A is a physiologically relevant HDAC7 phosphatase.

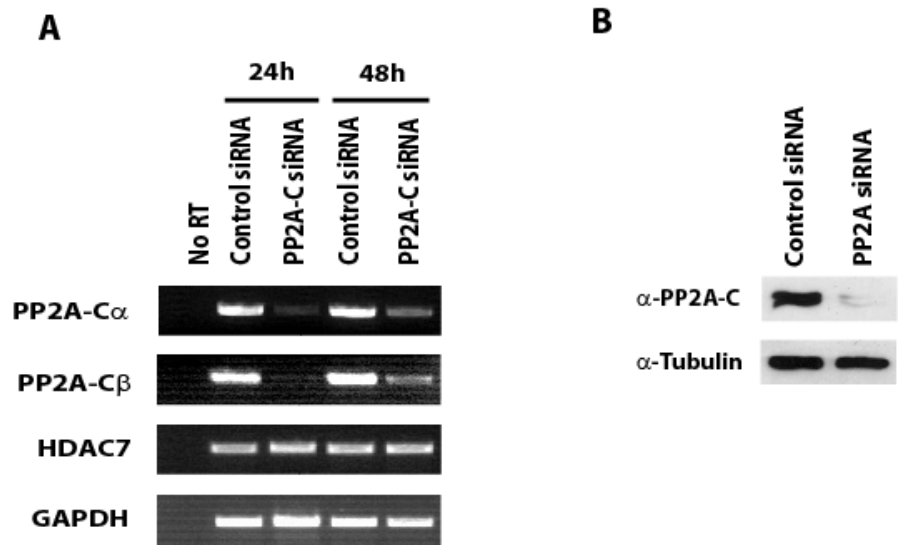


Figure S6. Inhibition of PP2A levels by siRNA.(A) HUVECs were co-transfected with siRNA targeting the α or β isoforms of PP2A catalytic subunit. A non-targeting siRNA was used as control. Total RNA was isolated at 24 or 48 hours post-transfection and expression of the α and β PP2A-C isoforms (PP2A-Cα and PP2A-Cβ respectively) and HDAC7 were assessed by RT-PCR. Expression of GAPDH was used to normalize mRNA amounts in each sample. The data shown are representative of two independent experiments. (B) Levels of PP2A-C were examined by western blotting at 36 hours post-transfection.

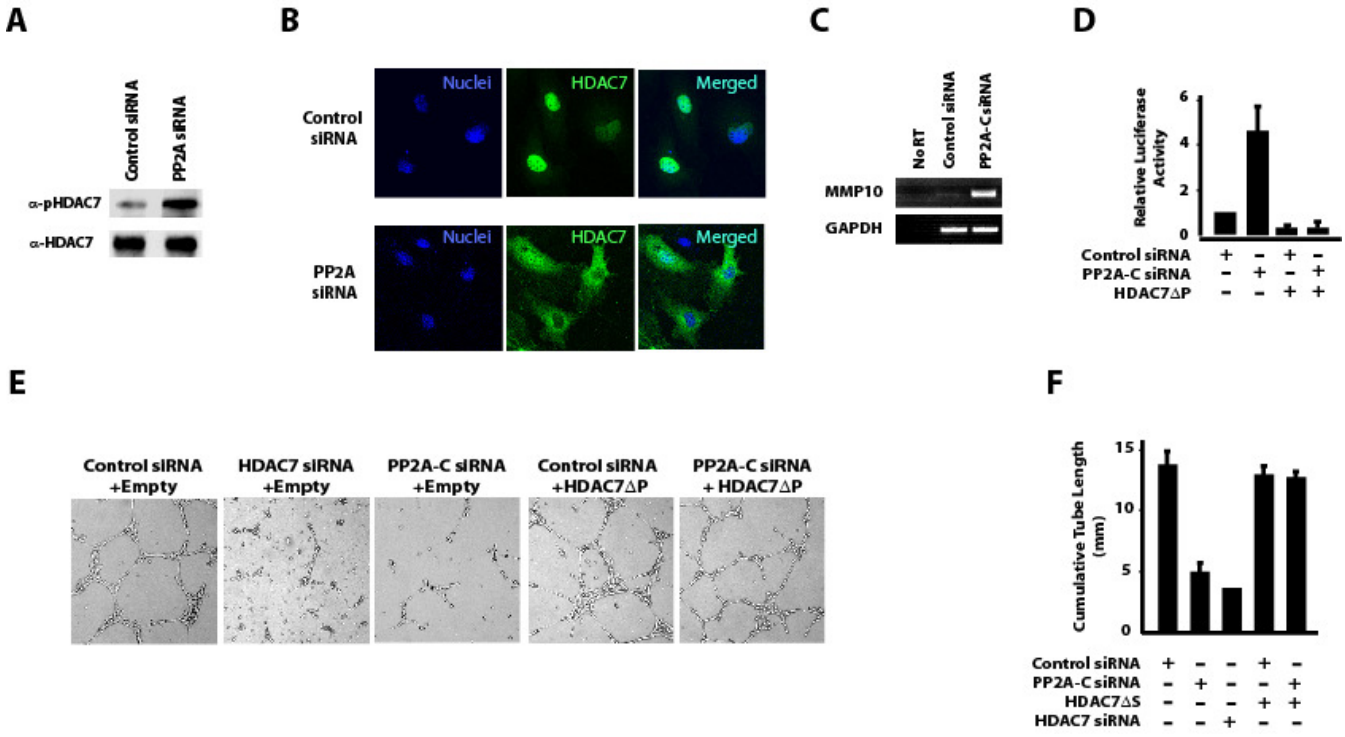


Figure 4. PP2A controls the pro-angiogenic activity of HDAC7 in HUVECs.

(A) Endogenous HDAC7 was immunoprecipitated from HUVEC transfected with combination of siRNA against the α and β isoforms of PP2A catalytic subunit and analyzed by western blotting with an antibody specific for phosphorylated Ser^{181} (α-pS181).

(B) Twenty-four hours after siRNA treatment as described in (A), HUVECs were transfected with a plasmid coding for a GFP-HDAC7. Subcellular localisation of HDAC7 (Green) was examined by confocal microscopy. Cell nuclei were visualized by Topro counterstain (Blue).

(C) HUVECs cells were transfected as in (A). Twenty-four hours post-transfection, expression of *MMP10* was analyzed by RT-PCR.

(D) Twenty-four hours after transfection with the indicated siRNA, cells were transfected with the MMP10-reporter construct, along with an expression vector for HDAC7ΔP. The activity of the MMP10-promoter was assessed by luciferase assay and is shown relative to its basal activity, measured in control siRNA-treated

cells and in the absence of HDAC7ΔP. Results are means + standard deviation (SD) from four independent experiments.

(E) HUVECs were transfected with indicated siRNAs. Twenty-four hours after siRNA treatment, cells were transfected with a HDAC7ΔP-expressing vector or the corresponding empty vector as control (Empty) and processed for the Matrigel assay. Micrographs of one representative experiment out of four are shown.

(F) Cumulative length of capillary-like structures was measured by light microscopy after 24 hours in four independent experiments as described in (F). Data are presented as means + SD.

PP2A controls the subcellular localisation of class IIa HDACs

We next wanted to investigate whether PP2A might also regulate other class IIa members. We first examined the ability of HDAC4 and HDAC5 to interact with endogenous PP2A *in vivo*. As observed for HDAC7, HDAC4 and -5 associated specifically with a PP2A core complex, containing the catalytic (PP2A-C) and structural (PP2A-A) subunits (Fig. 5A). In addition, purified PP2A readily dephosphorylated the 14-3-3 sites in HDAC4 and -5 in an *in vitro* dephosphorylation assay (Fig. 5B). To further support the hypothesis that PP2A has the ability to regulate all class IIa members *in vivo*, we examined the effect of PP2A depletion on class IIa HDACs subcellular localization. In HeLa cells treated with control siRNA, class IIa HDACs mainly localize in the nuclear compartment (Fig. 5C and 5D). In contrast, cells treated with siRNA specific for the catalytic subunit of PP2A exhibited a dramatically altered subcellular localization of class IIa HDACs, with HDAC4, -5 and -7 accumulating almost exclusively in the cytoplasm of most cells.

Altogether, these data strongly support the hypothesis that dephosphorylation by PP2A is an important regulatory mechanism common to all members of the class IIa HDAC family.

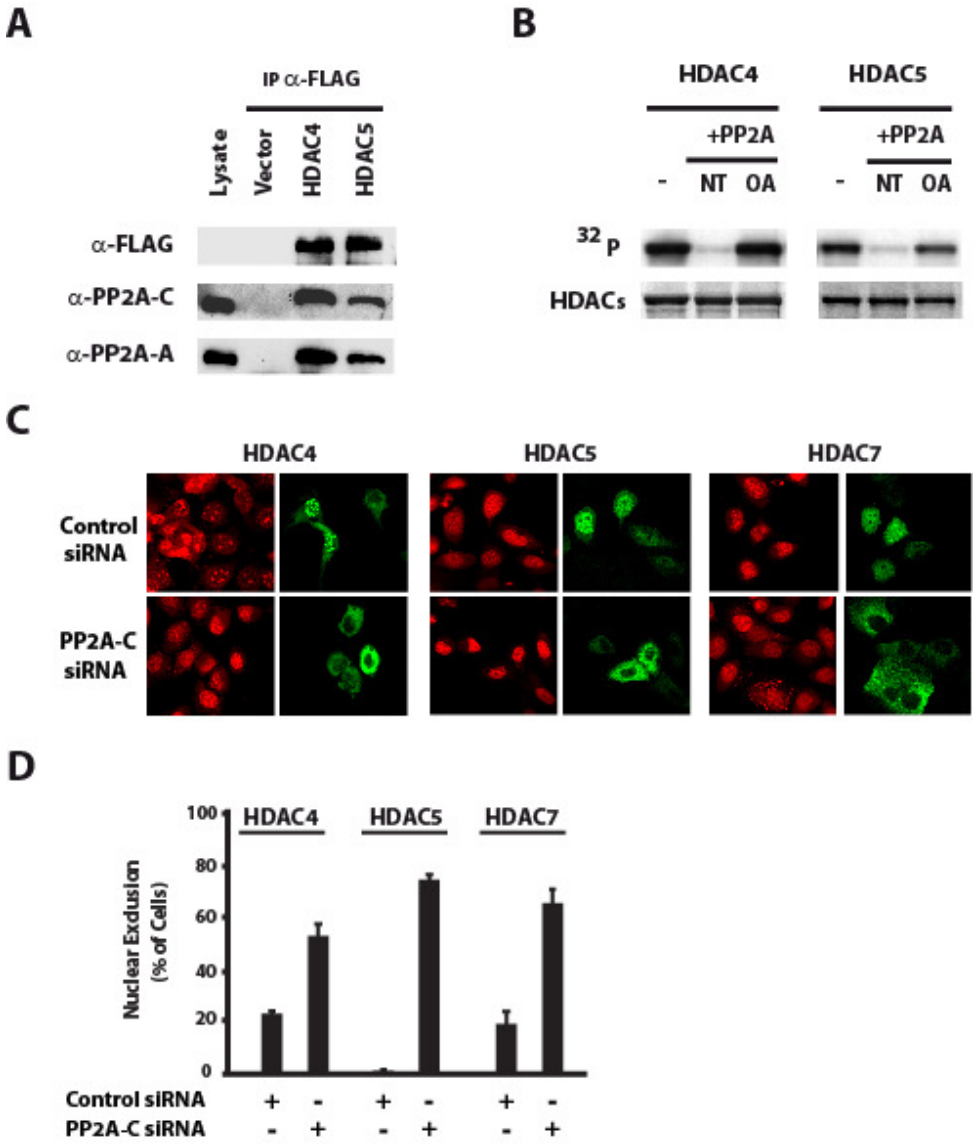


Figure 5. Regulation by PP2A is a common feature of class IIa HDACs

(A) Flag-tagged HDAC4 and -5 were immunoprecipitated from HEK293 cells and analyzed by immunoblotting with the indicated antibodies.

(B) Dephosphorylation of the N-terminal domains of HDAC4, or -5 was performed in the absence (-) or presence of a purified PP2A trimer (+PP2A). Where indicated OA was added to the reaction mixture. Residual phosphorylation was assessed after SDS-PAGE, Coomassie Blue staining (lower panel) and autoradiography (upper panel).

(C) A combination of siRNA against the α and β isoforms of PP2A catalytic subunit was transfected into HeLa cells, along with expression vectors for GFP-HDAC4, -5 or -7. A non-targeting siRNA was used as control. Subcellular localization of GFP-HDACs was examined by confocal microscopy (Green). Cell nuclei were visualized by Draq5 stain (Red).

(D) Bar histograms representing the mean percentages of cells showing predominant cytoplasmic localization of GFP-HDACs. Results are from 3 independent experiments as described in (C).

V) Discussion

The net phosphorylation of a given regulatory site results from the controlled balance between protein kinase and phosphatase activities. In this study, we focused on the canonical class IIa member HDAC7 and tested the possibility that, protein phosphatases might be involved in class IIa HDAC regulation to counterbalance their inactivation by related kinases. Our observations clearly show that PP2A controls HDAC7 phosphorylation, subcellular localization and repressive function.

In a recent study, we demonstrated basal phosphorylation of class IIa HDACs in normally growing cells (Dequiedt et al., 2006). Several of the observations reported here now unravel their constitutive dephosphorylation by PP2A. First, we show that PP2A is associated with class IIa members in normally growing cells. Second, in the absence of any of the conventional extracellular signals that activate the known class IIa HDAC kinases, inhibition of PP2A activity by OA or knock-down of PP2A-C expression by RNAi leads to hyperphosphorylation and nuclear export of class IIa HDACs. Thus, a fraction (if not all) of these enzymes seems to undergo constitutive phosphorylation/dephosphorylation cycles in unstimulated cells, as a result of a dynamic equilibrium between the antagonist activities of PP2A and constitutively active kinases. Interestingly, this suggests that there might be a constant flow of class IIa HDACs between the nucleus and the cytoplasm. The biological significance of why these enzymes would have to be constitutively cycling between both cellular compartments remains unknown. Because the phosphorylatable serine residues in class IIa HDACs represent 14-3-3 binding sites, 14-3-3 proteins were thought to be directly responsible for the nucleo-cytoplasmic shuttling of class IIa HDACs. In this study, we unexpectedly identify a new role for 14-3-3 proteins in the phosphorylation and nucleo-cytoplasmic shuttling of class IIa HDACs that might challenge this model. Since PP2A and 14-3-3 target the same serine residues, we propose that masking of the phosphoserines by 14-3-3 binding impedes phosphatase access to class IIa HDACs. Consistent with this idea, we show that 14-3-3 binding prevents dephosphorylation of HDAC7 by PP2A *in vitro* and *in vivo*. Further studies must now be directed at better understanding how PP2A

dephosphorylates class IIa HDACs. While it is clear that interaction with 14-3-3 proteins protects class IIa HDACs from PP2A-mediated dephosphorylation, what helps PP2A gain access to the phosphorylated 14-3-3 binding sites remains to be elucidated.

During the preparation of this manuscript, a study was published reporting that protein phosphatase 1β (PP1β) and myosin phosphatase targeting subunit 1 (MYPT1), two subunits of the mysosin phosphatase complex, co-purify with Flag-HDAC7 (Parra et al., 2007). Based on functional data, the authors of the study proposed that HDAC7 phosphorylation and its activity as a transcriptional repressor would be regulated by myosin phosphatase. Independently of the technical reasons that may explain why their study and ours led to the identification of two different HDAC7 phosphatases, they also found that OA treatment was associated with increased phosphorylation and cytoplasmic localization of HDAC7. However, myosin phosphatase is insensitive to low concentrations of OA (Ito et al., 2004; Lontay et al., 2005). Our data clearly show that association with 14-3-3 prevents access and dephosphorylation of HDAC7 by PP2A, thus supporting a model in which PP2A specifically and directly targets the 14-3-3 motifs in HDAC7. In contrast, interaction of HDAC7 with myosin phosphatase did not require prior displacement of 14-3-3. Therefore, it is not clear whether MYPT1/PP1β would impact HDAC7 phosphorylation directly or via an indirect mechanism such as, (i) activation of a class IIa HDAC kinase or (ii) enhanced protection of HDAC7 by 14-3-3. On the other hand, there is no need for the two reports to be conflicting. Indeed, many HDAC7 kinases have been identified to date and it is conceivable that more than one phosphatase could dephosphorylate HDAC7. The existence of various, time or localization-specific, HDAC7 phosphatases would provide the cells with the adaptability that they require in order to respond adequately and efficiently to the key differentiation processes associated with this important enzyme.

The results of the present study identify PP2A as an important regulator of endothelial angiogenic functions and T-cell apoptosis by controlling the subcellular localization and repressive function of HDAC7. Interestingly, PP2A has been directly implicated in these processes (Lacroix et al., 2002; Michell et al., 2001; Nebl et al., 1998; Urbich et al., 2002; Woetmann et al., 1999). Our results also strongly indicate that dephosphorylation by PP2A may be a common feature amongst class IIa HDACs (Fig. 5). In the future, it will thus be essential to verify whether PP2A is indeed implicated in the various developmental programs that are controlled by other class IIa HDACs, such as cardiac growth (Chang et al., 2004; Zhang et al., 2002a), muscle differentiation (McKinsey et al., 2000a) and activity (Mejat et al., 2005b) and bone formation (Vega et al., 2004b).

3.3. PP2A-Bα in angiogenesis

In the second chapter, we identified the PP2A as the first phosphatase involved in the regulation of class IIa HDACs. The existence of HDAC7 specific protein kinases and protein phosphatases highlights the reversibility of HDAC7 regulation and involves the precise regulation of each of these activities by biological context specific cellular factors. In order to respond to its numerous functions, PP2A has evolved finely tuned control mechanisms. PP2A composition is in great part responsible for the regulation of its pleiotropic functions. The identity of the PP2A variable B-type regulatory subunits has a crucial role in the control of PP2A activity. In this context, we decided to investigate the precise composition of the PP2A holoenzyme targeting class IIa HDACs member HDAC7 in the particular context of angiogenesis.

3.3 Identification of the first developmental function for PP2A Bα regulatory subunit: Bα regulates migration-related angiogenesis through the control of class IIa HDAC7 activity

I) Summary

The major Ser/Thr phosphatase PP2A is involved in the regulation of many cellular processes, including regulation of different signal transduction pathways. Cellular PP2A represents a large population of trimeric holoenzymes, comprising conserved catalytic and structural subunits along with a variable regulatory subunit. The identity of this third subunit has a crucial role in the control of PP2A activity, by determining substrate specificity, subcellular localisation and catalytic activity of the holoenzyme. Nevertheless, few studies have uncovered the relevant PP2A holoenzyme for a particular pathway. In an effort to identify a role for PP2A regulatory subunit in the development of blood vessels, we found that among diverse regulatory subunit isoforms, PP2A-Bα uniquely regulates endothelial cell angiogenic properties. PP2A-Bα silencing using small interfering RNAs results in a significant inhibition of endothelial cell tube formation and migration. In addition, microarray studies unravel a functional interplay between the PP2A regulatory subunit Bα and the class IIa HDAC7 in the regulation of endothelial cell angiogenic functions. Moreover, we show that PP2A-Bα suppression impairs HDAC7 precisely regulated sub-cellular localisation during VEGF stimulation. Thus, our results suggest an important role for PP2A regulatory subunit Bα in the regulation of angiogenesis.

II) Introduction

Reversible protein phosphorylation is a key intracellular mechanism for altering the biological activity of a myriad of regulatory and structural proteins and is thus involved in virtually every major physiological process. Although protein kinases have been historically considered as the undisputed guardians of intracellular signal transduction, the counterbalancing function of phosphatases, and thus the control of phosphatase activity, is equally relevant to proper regulation of cellular functions. Protein phosphatase 2A (PP2A) is a remarkably conserved, ubiquitous and very abundant serine/threonine phosphatase. By dephosphorylating a plethora of cellular proteins, PP2A is involved in the regulation of a wide

array of signaling cascade (Arroyo & Hahn, 2005; Janssens & Goris, 2001; Sontag, 2001; Virshup, 2000). Nevertheless, potential roles for PP2A in developmental programs remain undisclosed.

To accomodate its numerous functions, PP2A has evolved finely tuned control mechanisms, with holoenzyme composition being the most impacting factor in PP2A regulation. In cells, most of PP2A activity exists as heterotrimers, in which a core dimer, $PP2A_D$, made of a structural A and a catalytic C subunit, is associated with a third variable regulatory B-type subunit. In opposition to the well conserved A and C subunits, various families of regulatory B subunit, each giving rise to several isoforms, have been described. Each B subunit can potentially combine with any of the two isoforms of both the A and C subunits, generating over 75 potential distinct trimeric PP2A holoenzymes (Janssens & Goris, 2001; Janssens et al., 2008). It is well recognized that B-type subunit identity has a crucial role in the control of PP2A activity, by determining substrate specificity, subcellular localisation and catalytic activity of the PP2A holoenzyme (Agostinis et al., 1992; Agostinis et al., 1990; Agostinis et al., 1987; Cegielska et al., 1994; Imaoka et al., 1983; Mayer-Jaekel et al., 1994; Mumby et al., 1987; Sontag, 2001; Sontag et al., 1996). Due to the non-selectivity of loss of function approaches, most PP2A studies do not discriminate between specific holoenzyme contributions. Very few biological roles could thus be precisely attributed to any specific PP2A regulatory subunit.

Angiogenesis, the process of new blood-vessel formation and growth, is crucial for normal vascular development and homeostasis: organ growth in the embryo and repair of wounded tissue in the adult. In response to angiogenic stimuli, endothelial cells proliferate, migrate and coalesce to form primitive vascular networks that undergo remodeling to give rise to mature blood vessels (Risau, 1997). Imbalance in these processes contributes to the pathogenesis of numerous disorders (Carmeliet, 2005). Despite the importance of angiogenesis regulation, few data exist about the involvement of the dominating enzyme PP2A in this process (Gabel et al., 1999; Schmidt et al., 2006; Urbich et al., 2002). We report here that, specific silencing of PP2A regulatory subunit Bα uniquely impairs endothelial cells angiogenic capacities in a Matrigel assay, which supports a key role for this PP2A regulatory subunit in the vascular endothelium. In addition, our data indicate that Bα regulates cell migration, a vasculogenesis-associated process in endothelial cells. Microarray analysis reveals that PP2A-Bα controls the expression of several genes involved in angiogenesis and unravels a strong correlation between Bα-regulated vascular signaling and the class IIa HDAC member HDAC7. Localisation studies further reveal the involvement of PP2A-Bα in the

VEGF-dependent regulation of HDAC7 subcellular localisation, which controls the activity of this transcriptional repressor. Taken together, these results establish for the first time the functional role of a specific PP2A regulatory subunit in a major developmental program, i.e. angiogenesis.

III) Methods

Plasmids, Antibodies and Chemicals

Constructs coding for C-terminal FLAG- or N-terminal GFP-tagged versions of human HDAC7 have been described elsewhere (Dequiedt et al., 2003). GST-fusion protein of the N terminus of HDAC7 was constructed by inserting aa 1 to 490 of HDAC7 into the vector pGEX-4T2 (Invitrogen, Carlsbad, CA). The HA-fusion protein of PP2A Bα was a kind gift of Dr D. Virshup.

Anti-HA antibodies were purchased from Santa Cruz Biotechonology (Santa Cruz, CA) and anti-Flag and anti-actin antibodies were from Sigma. The monoclonal Bα antibody was a generous gift from Dr E. Ogris.

The PP2A trimer was de novo purified from rabbit skeletal muscle and was a kind gift of Dr J. Goris. Draq5 was from Biostatus.

Cell Culture and transfections

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Lonza and grown at 37°C in endothelial basal medium (EBM) supplemented with hydrocortisone (1µg/ml), bovine brain extract (12µg/ml), gentamicin (50µg/ml), amphotericin B (50ng/ml) epidermal growth factor (10 ng/ml) (Lonza) and 10% FCS (Perbio). Plasmids and siRNA transfections in HUVECs were performed using respectively the JetPEI-HUVEC (Polyplus) and the GeneTrans II (MoBiTec) reagents according to the manufacturers' protocols. For *in vivo* localisation experiments, VEGF was added in the culture medium at 50 nM for the indicated time.

Previously to scratch-wound and localisation experiments, HUVECs were starved overnight into respectively 2% and 0.5% serum containing medium.

HEK293 cells were grown in recommended medium (DMEM, lonza) supplemented with 10% fetal bovine serum (FBS), 2mM Glutamine and 50U/ml of streptomycin/penicillin and tansfected with standard calcium phosphate method.

Proliferation and Propidium Iodure assays

Thirty hours after transfection, HUVEC cells were trypsinized and counted (for proliferation assay) or washed, permeabilized in a buffer containing 0.1% citrate de Sodium, 0.1% Triton-X, and 20 µg/ml RNase, and incubated with 40µg/ml of Propidium Iodure (PI). Cell apoptosis was assessed by flow cytometry using a FACScan cytometer (Becton Dickinson).

GST fusion proteins: expression, purification and phosphorylation

GST fusion proteins were expressed in BL21 RIP (Invitrogen) and purified by using a glutathione affinity chromatography resin, according to the procedures described by the manufacturer (BD Biosciences Clontech, Mississauga, Ontario, Canada). The 14-3-3 binding sites of purified GST-HDAC7 proteins were phosphorylated with recombinant active PKD1 (0.5 µg/ml) in the presence of [γ-^{32}P]ATP, as described in (Dequiedt et al., 2005).

SDS-PAGE and Western Blotting

SDS-PAGE and Western blot analysis were performed according to standard procedures and developed with the ECL detection kit (GE Healthcare Bio-Sciences, Uppsala, Sweden).

In vitro recombinant HDAC dephosphorylation assay

Aliquots of PKD1-phosphorylated GST-HDAC7 were incubated in a phosphatase buffer containing 0.2 U of PP2A A/C/B55α heterotrimer for 30 min at 30°C. When indicated, dephosphorylation reactions were inhibited with 20 nM OA. Dephosphorylation reactions were terminated by adding an equal amount of 2x SDS-PAGE sample buffer and boiling for 5 minutes. Phosphorylation was then assessed by SDS-PAGE and autoradiography.

Immunoprecipitation

Total cell lysates were prepared from HEK 293 cells in IPLS buffer and Flag-tagged protein immunoprecipitation was carried out overnight at 4°C with M2-agarose (Sigma-Aldrich, Inc) antibody (15µl/ml). Immunoprecipitates were washed 3 x in IPLS buffer, 2 x in IPHS buffer, 2 x in RIPA buffer and 2 x in IPLS buffer before being processed for Western blot analysis.

RNA interference (RNAi)

Two unrelated siRNA molecule directed against the α isoform of PP2A B regulatory subunit, or the corresponding Non-targeting control siRNA were designed using the on-line siRNA Selection Progam at Whitehead Institute for Biomedical Research and purchased from (Eurogenntec). Sequences of Bα and Bα bis are available on demand.

Immunofluorescence

For immunofluorescence experiments, GFP-HDAC7 expression constructs were ectopically expressed in HUVECs cells. Twenty-four hours after plasmid transfection, HUVECs were further transfected with indicated siRNA. VEGF treatment was started another twenty-four hours later. Localization of the fluorescent proteins was assessed on fixed cells by confocal microscopy (Axiovert 200 with LSM 510; Carl Zeiss Microscopy). The average percentage of cells showing predominant cytoplasmic localization of the GFP-tagged protein was assessed by examining at least 6 independent fields each containing more than 30 cells.

RT-PCR analysis

Total RNA was extracted from control- or Bα siRNA-treated HUVECs using Trizol reagent (Invitrogen) and used as a template for reverse transcription with random hexamer primers using the Transcriptor reverse transcriptase kit (Roche). Primers sequences and amplification conditions for specific genes are available upon request.

Tube formation assays

Forty-eight hours after transfection with the indicated siRNA, HUVECs were processed for the Matrigel assays. In summary, 5 x 10^4 cells were cultured in a 24-well plate coated with 100 µl Matrigel Basement Membrane Matrix (BD Biosciences). Tube length was quantified after 12h hours by measuring the cumulative tube length in 5 random microscopic fields with the program WCIF imageJ.

Scratch-wound assays

Confluent HUVECs monolayers were scraped 48 hours after siRNA transfection using a sterile P200 tip to create a cell free zone. VEGF was then added in the culture medium. Two different fields of each wound were photographed: immediately after injury and 21 hours later. Quantification of cell migration was made by measuring the mean width of each wound throughout the cell free zone using the program WCIF imageJ.

Microarray

Gene expression profiling of siRNA-transfected HUVECs (n=3) was performed using the gene chip expression assay (HG-U133A) as previously described (Hofmann et al., 2001). The protocol for sample preparation and microarray processing was carried out according to the standard Affymetrix GeneChip protocol (Affymetrix). Data were analysed with GeneSpring software (Agilent Technologies).

IV) Results

All PP2A B and B' subunits are expressed in HUVECs.

To investigate the role of PP2A B-type subunits in the regulation of angiogenesis, we first assess the expression of the various isoforms of the best characterized families of PP2A regulatory subunits, i.e. B (B55) and B' (B56). Indeed, several PP2A regulatory subunits show restricted tissue expression (Sontag, 2001). Members of the B and B' family of regulatory subunits are encoded by four and five distinct genes respectively: Bα, -β, -δ, -γ and B'α, -β, -δ, -γ, -ε. Reverse-Transcription PCR (RT-PCR) analysis showed that all examined B and B' isoforms are expressed in human umbilical vein endothelial cells (HUVECs), although at different levels (figure 1A). Of note, Bβ, Bγ and B'β are hardly detectable, which is consistent with the fact that these isoforms are considered brain-specific (Mayer et al., 1991; McCright & Virshup, 1995; Zolnierowicz et al., 1994).

Then, in order to explore the potential role of the various B and B' PP2A regulatory subunits in endothelial cells function, we inhibited their expression independently using small interfering RNA (siRNA). We first assessed the consequence of B-type subunits suppression on HUVECs viability and proliferation. Inhibition of Bβ, B'δ and B'ε led to a significant increase in cell apoptosis (figure 1B). As expected, deletion of Bβ, B'δ and B'ε correlated with reduced proliferation (figure 1C). In contrast, siRNA-mediated knock-down of Bα, Bδ, Bγ, B'α, B'β and B'γ had no significant effect on cell apoptosis and proliferation. The efficiency and the specificity of our siRNA-mediated approach were then assessed by RT-PCR. Because of high cell death HUVECs knocked-down for Bβ, B'δ and B'ε were excluded from this analysis. As shown in figure 1D, siRNA designed against Bα, Bδ, Bγ, B'α, B'β and B'γ specifically and efficiently reduced expression of the corresponding isoform. This observation is particularly important considering the high degree of homology that exists between the B and B' isoforms.

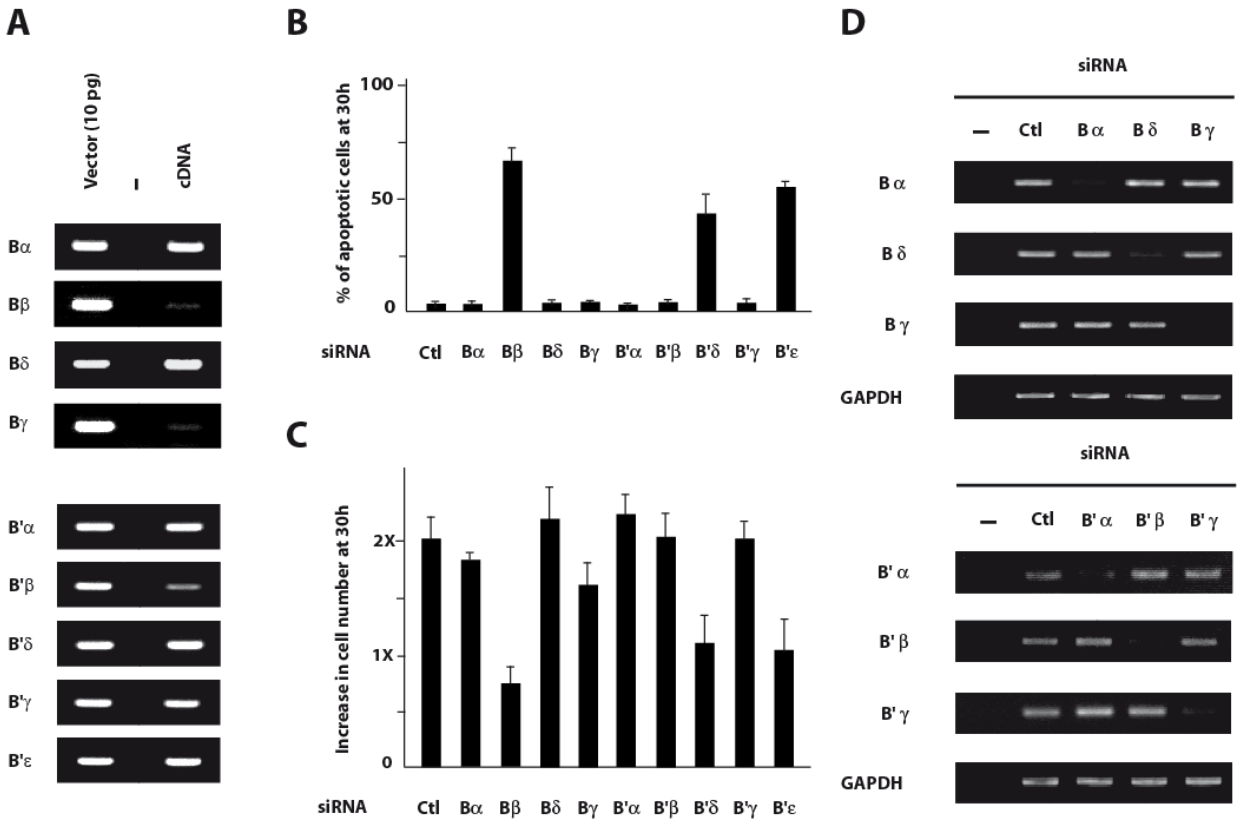


Figure 1. Knock-down of PP2A regulatory subunits isoforms in HUVECs.

(D) RNA expression of various PP2A regulatory subunits isoforms in HUVECs was assessed by Reverse-Transcription PCR (RT-PCR). As a control of PCR reactions efficiency, amplifications were also performed on 10 pg of an expression vector coding for the corresponding isoform (vector).

(E) Thirty hours after siRNA transfection, HUVECs were analyzed for cell death by Propidium Iodure labelling. Percentage of apoptotic cells was assessed by flow cytometry.

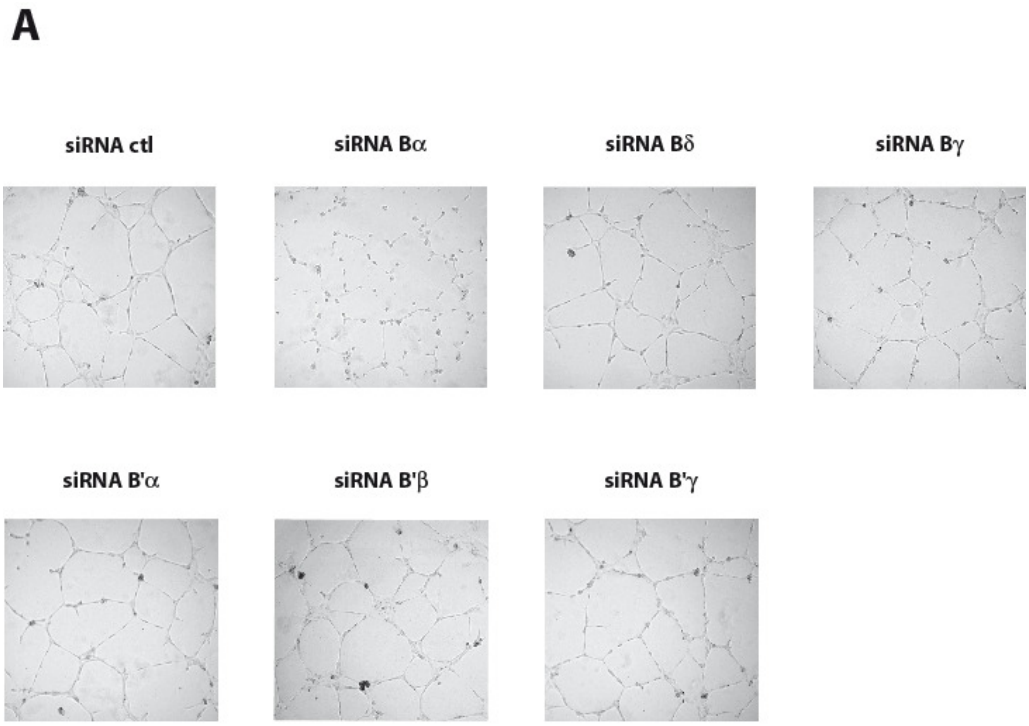
(F) Thirty hours after siRNA transfection, HUVECs were harvested and counted. Increase in cell number was calculated as the ratio between cell number before and thirty hours after transfection.

(G) HUVECs were transfected with siRNA targeting the indicated PP2A regulatory subunits isoforms, or a non-related siRNA control. Thirty-six hours later, the effects of gene silencing were assessed by RT-PCR. Expression of GAPDH was used to normalize mRNA amounts in each sample.

PP2A regulatory subunit Bα is necessary for endothelial tube formation.

The impact of PP2A regulatory subunits suppression on endothelial cells angiogenic activity, was assessed in vitro in a tubulogenesis assay (Matrigel assay). To achieve this, HUVECs transfected with siRNA against PP2A B-type subunits were seeded into a matrix-rich basement and their ability to form three-dimensional capillary-like structures was assessed by measuring outgrown capillary tube length. As clearly illustrated in Figure 2A and 2B) Bα was the only subunit whose silencing prevented the ability of endothelial cells to organize into a primitive vascular netwok (figures 2A and 2B).

To control for the specificity of this approach, we generated an alternate siRNA targeting an unrelated Bα sequence (Bα bis siRNA). Western blotting and RT-PCR confirmed the efficient suppression of Bα following transfection with both siRNA, knock-down by the former being more effective (figure 3A). Importantly, the alternative siRNA gave comparable results in term of tube formation in the Matrigel assay (figures 3B and 3C). These results demonstrate that endogenous PP2A-Bα is functionally important for the tubulogenic activity of endothelial cells.



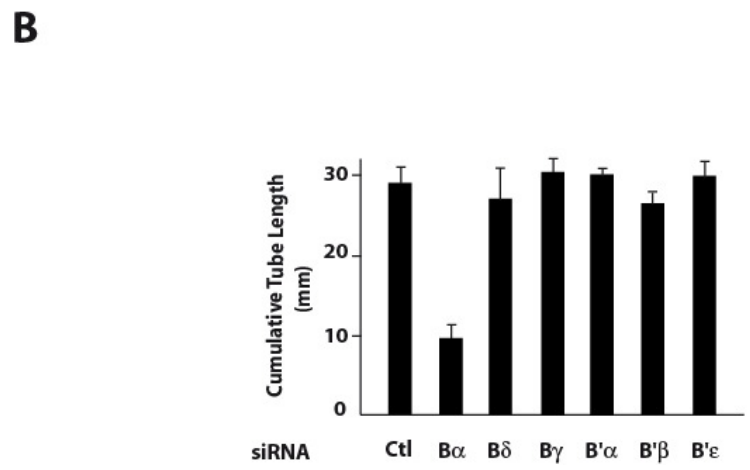


Figure 2. PP2A-Bα controls angiogenesis in endothelial cells.

(A) HUVECs were transfected with siRNA against PP2A regulatory subunits or control siRNA. Cells were then cultured for 48 hours before being seeded on Matrigel. Micrographs of one representative experiment out of five are shown.

(B) Cumulative length of capillary-like structures was measured by light microscopy in five different fields.

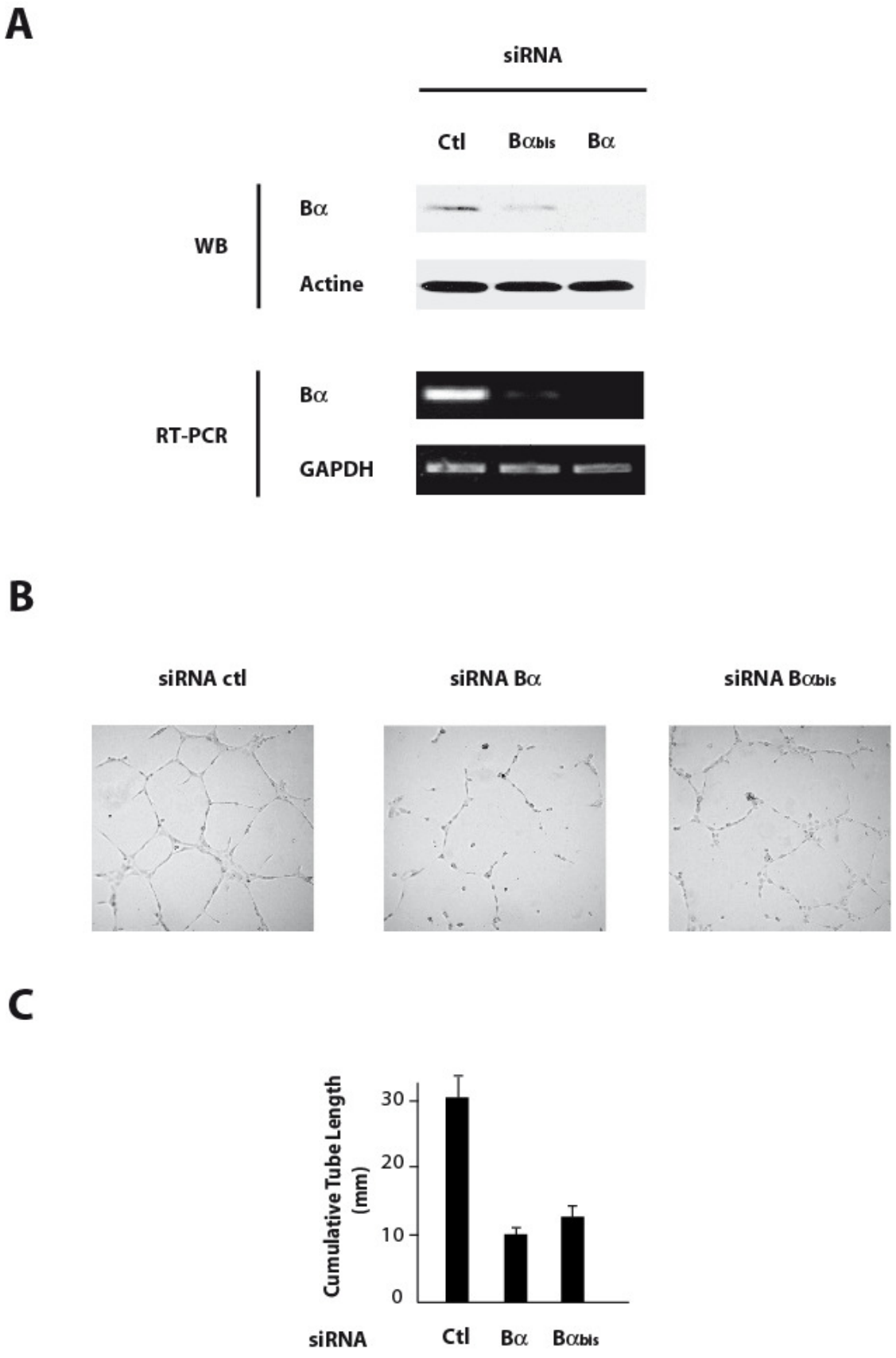


Figure 3. Silencing of PP2A-Bα inhibits tube formation.

(A) HUVECs were transfected with two different siRNAs against PP2A-Bα or the non-targeting control siRNA. Cell lysates isolated at 48 hours post-transfection were subjected to Western blotting (WB) using antibodies against Bα and actin as a loading control. In parallel, total RNA was isolated and expression of PP2A-Bα was assessed by RT-PCR. GAPDH was used as loading control.

(B) Cells transfected as described in A were seeded on a gelled basement membrane matrix (Matrigel). Micrographs of one representative experiment out of four are shown.

(C) Cumulative length of capillary-like structures was measured by light microscopy in four independent experiments.

PP2A regulatory subunit Bα silencing inhibits endothelial cell migration.

To further characterize the role of PP2A-Bα in angiogenesis, we next investigated whether the failure detected in the Matrigel assay could be related to a defect on cell migration, an event involved in the process of angiogenesis. For this purpose, the impact of Bα silencing on cell migration was explored using the scratch-wound assay. In this assay, the migratory potential of endothelial cells is evaluated by measuring their ability to close a wound created in a cell monolayer (Liang et al., 2007). Of note, cell proliferation and survival also participate in the overall angiogenic activity of endothelial cells. However, Bα inactivation does not appear to induce any alteration of HUVECs viability (figures 1B and 1C).

We decided to assess HUVECs migration in response to VEGF (Vascular Endothelial Growth Factor), a key angiogenic stimulus implicated in nearly all aspects of vascular endothelial cell biology. As shown in figure 4, VEGF-induced wound healing was significantly reduced in Bα (and Bα bis) siRNA treated cells. Indeed, after 21 hours only 25% of the scratch area was covered by Bα-deficient cells, whereas 80% of the wound surface was closed by control siRNA-transfected cells (figure 4B). This result indicates that PP2A-Bα contributes to endothelial cells angiogenic activity by promoting cell migration in response to VEGF, a key step in angiogenesis.

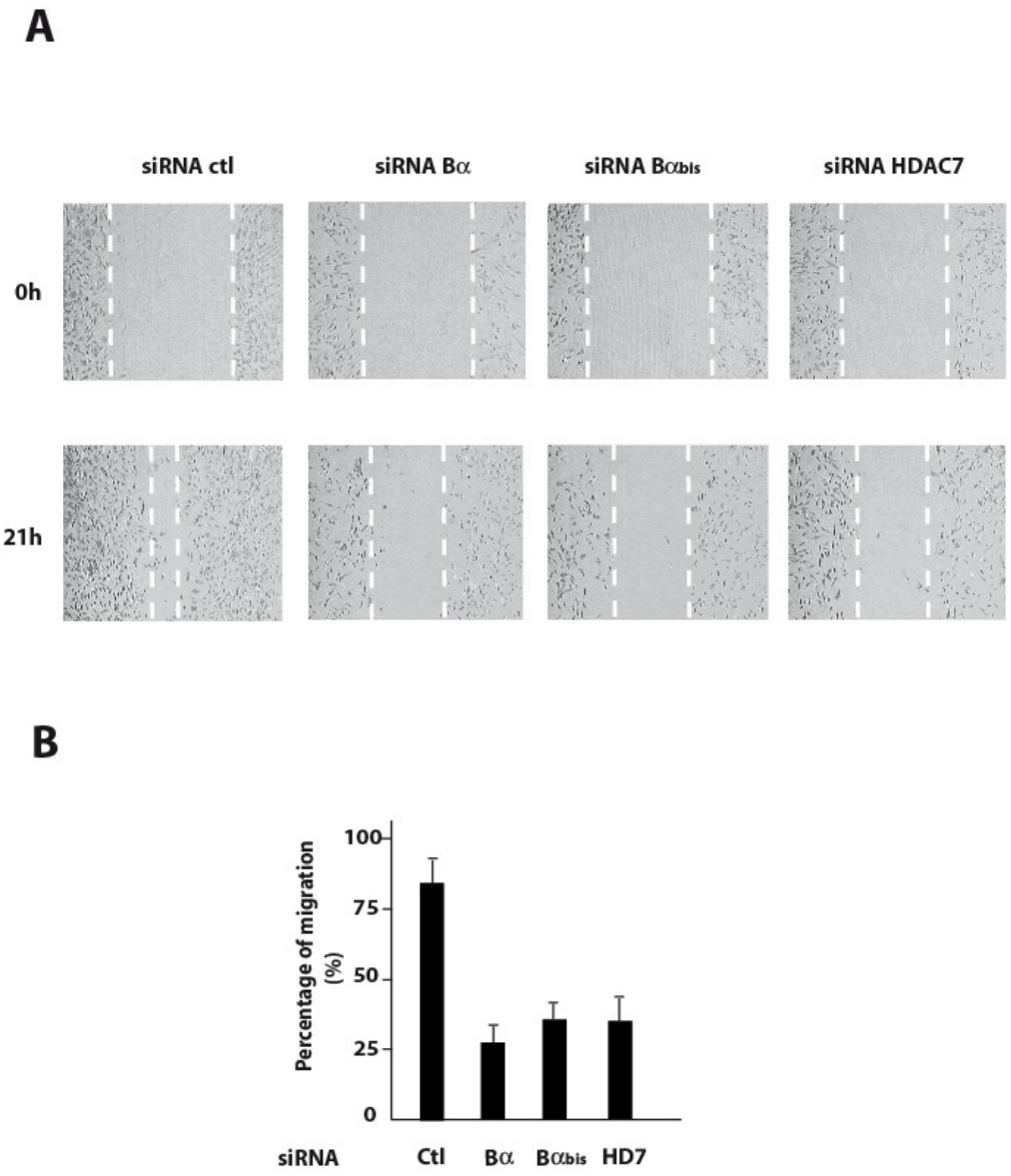


Figure 4. Supression of PP2A-Bα activity results in HUVECs migration inhibition.

(F) HUVECs transcfected with indicated siRNA were subjected to migration assay in response to VEGF as described in the Methods section. Micrographs of one representative experiment are shown.

(G) Quantitative analysis was performed by scoring the initial wound size (0h) at 100% and calculating the average percentage of wound closure after 21 hours. Results were representative of four independent experiments.

PP2A-Bα- and HDAC7- regulated vascular pathways are interrelated

Strikingly, vascular defects associated with PP2A Bα suppression are reminiscent of those observed in HDAC7 deficient cells. HDAC7, a class IIa histone deacetylase, is a

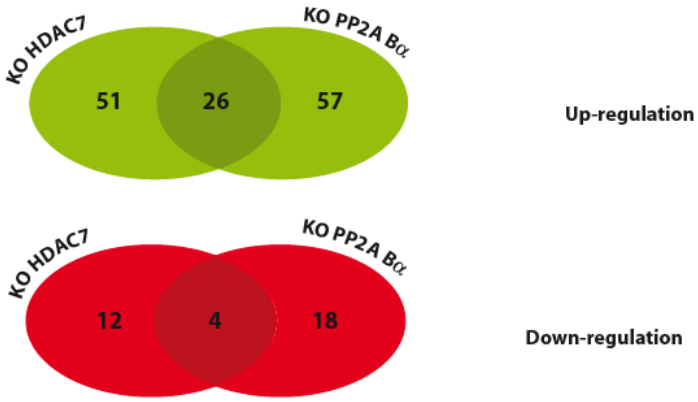
transcriptional repressor which was shown to be crucial for vascular integrity maintenance, its suppression impairing endothelial cell tube formation activity in a Matrigel assay (Chang et al., 2006; Martin et al., 2008). In addition, a study using a gene knock-down strategy targeting various HDACs recently revealed that HDAC7 silencing impairs endothelial cell migration (Mottet et al., 2007). We indeed confirmed that HDAC7 inactivation is associated with migration defects in HUVECs (figure 4, siRNA HDAC7). Moreover, we have shown that PP2A catalytic activity participates in endothelial cell biology by controlling HDAC7 transcriptional activity (Martin et al., 2008). Given this piece of information, we hypothesised that the effects of PP2A-Bα silencing on HUVECs angiogenic properties could be mediated by the deregulation of HDAC7.

To ascertain a functional relationship between PP2A Bα and HDAC7, we performed a comparative transcriptomic analysis of PP2A Bα- and HDAC7-regulated genes in endothelial cells using the DNA microarray technology. Total RNA was isolated from HUVECs transfected with PP2A-Bα-, HDAC7-, or control siRNA and analysed with the Affymetrix gene chip expression assay (Hofmann et al., 2001). We then established HUVECs transcriptional changes associated with PP2A-Bα or HDAC7 depletion comparatively to the control cells. In an effort to get the most robust data, we focused on genes whose RNA level changed by more than 3 fold compared to the control. Using this criterion, we found that only 51 and 18 genes showed respectively up- and down-regulation in PP2A Bα-deficient cells. Strikingly, the expression of a comparable number of genes (i.e. 57 and 12 respectively) was similarly altered in HDAC7-null cells. Very interestingly, we found a lot of redundancy between both data sets: indeed, almost half of the genes upregulated in the absence of PP2A Bα were found amongst the genes up-regulated following HDAC7 knock-down (figure 5A). This result demonstrates that a great portion of the PP2A Bα-regulated genes are also under the control of the transcriptional repressor HDAC7. Such a strinking observation firmly establishes a functional link between PP2A-Bα and HDAC7.

Amongst the common set of genes similarly regulated by PP2A-Bα and HDAC7 in HUVECs, careful analysis revealed numerous genes previously established as key actors of multiple vascular functions, particularly migration (Figure 5B). As an illustrative example, E-selectin is an inducible cell-adhesion molecule on endothelial cells, which plays a role in hematopoietic stem cells homing, induces angiogenesis in the rat cornea and stimulates chemotaxis and tube formation in endothelial cells (Koch et al., 1995; Kumar et al., 2003;

Mazo et al., 1998). This observation is in total concordance with a critical role of HDAC7 and PP2A Bα in the regulation of migration-related vasculogenesis.

A



B

Common	Genbank	Description	Function
ELAM1	NM_000450	Selectin E, endothelial adhesion molecule 1	Adhesion molecules mediating migration
ICAM1	AI608725	Intracellular adhesion molecule-1	Adhesion molecules mediating migration
ETB, ETRB	NM_003991	Endothelin receptor type B	Receptor of a pro-angiogenic factor
KIAA0777	NM_021069	Arg/Abl-interacting protein ArgBP2	Adaptator protein regulating adhesion and migration
ICE, IL1BC	U13699	Caspase 1, interleukin 1β convertase	Regulators of IL-1, mediator between inflammation
IL1R3, IL-1RACP	NM_002182	Interleukin 1 receptor accessory protein	and angiogenesis
MCP1	S69738	Small inducible cytokine, monocyte chemotactic protein 1	Chemotactic factor
CIAP2, HIAP1	U37546	Baculoviral IAP repeat-containing 3	Cell survival factor, focal adhesion factor

Figure 5. Transcriptome analysis of PP2A-Bα and HDAC7 regulated genes.

(A) Total RNA from control-, Bα- and HDAC7 siRNA-transfected HUVECs was isolated 36 hours after transfection and gene expression profile was assessed with the Affimetrix gene chip expression assay. Venn diagrams show the number of genes whose RNA level changed by more than three fold up (upper panel) or down (lower panel) in Bα-, HDAC7- or Bα- and HDAC7- (intersection) silenced cells compared to the control.

(B) Selected angiogenesis related genes that were up-regulated in both Bα- and HDAC7- knock-down HUVECs.

PP2A-Bα is an HDAC7 phosphatase

Results from our microarray analysis suggest that PP2A-Bα and HDAC7 belong to the same transcriptional regulatory network. The transcriptional activity of class IIa HDACs is regulated via a complex phospho-dependent nucleocytoplasmic shuttling process. When phosphorylated on conserved serine residues located in their amino-terminal adaptator domain

class IIa HDACs translocate from the nucleus to the cytoplasm, which prevents them from impacting on the transcription of their target genes (Martin et al., 2007). HDAC7 has recently been identified as a key regulator of vasculogenesis associated transcriptional programs (Chang et al., 2006; Mottet et al., 2007). In a prior study, we have demonstrated that cellular PP2A catalytic activity is required to maintain HDAC7 dephosphorylation, nuclear localization and repressive function in HUVECs (Martin et al., 2008). Based on these considerations, we reasoned that PP2A regulatory subunit Bα could impact on endothelial cells angiogenic capacities by participating in HDAC7 regulation through dephosphorylation.

To validate this hypothesis, we first verified that HDAC7 is a *bona fide* substrate for a PP2A holoenzyme containing a Bα regulatory subunit. To achieve this, PP2A A/C/Bα heterotrimer was purified from cells and tested for its capacity to directly dephosphorylate HDAC7 *in vitro*. As shown in figure 6A, the N-terminal domain of HDAC7 specifically radiolabeled *in vitro* on the four previously identified phosphorylation sites (figure 6A, -) was readily dephosphorylated upon incubation with the PP2A Bα-holoenzyme in an okadaic acid (a PP2A inhibitor)-dependent manner (figure 6A, NT and OA). Because this result strongly suggests that Bα containing PP2A holoenzyme can act as a HDAC7 phosphatase, we tested the ability of Bα to associate with HDAC7. Co-immunoprecipitation experiments revealed that PP2A-Bα regulatory subunit indeed robustly associates with HDAC7 (figure 6B).

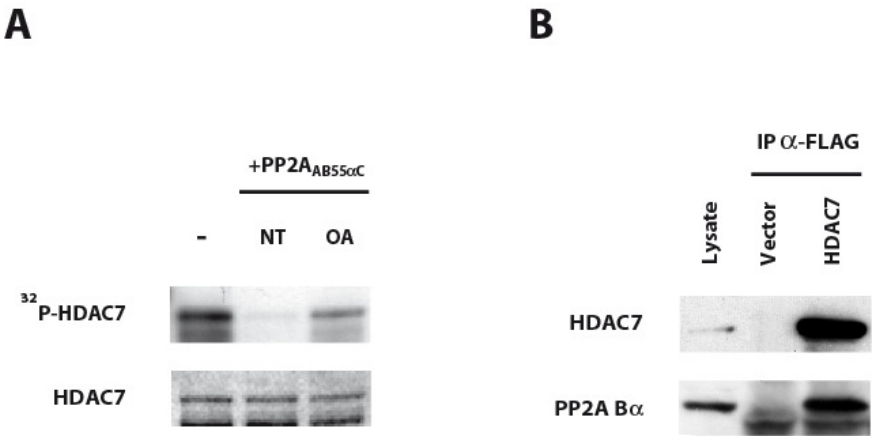


Figure 6. Bα containing PP2A holoenzyme dephosphorylates and interacts with HDAC7.

(A) The N-terminus of HDAC7 was specifically phosphorylated on its four phosphorylation sites (i.e. Ser^{155}, Ser^{181}, Ser^{321} and Ser^{449}) by recombinant PKD in the presence of radioactive ATP. The radiolabeled protein (^{32}P-HDAC7) was

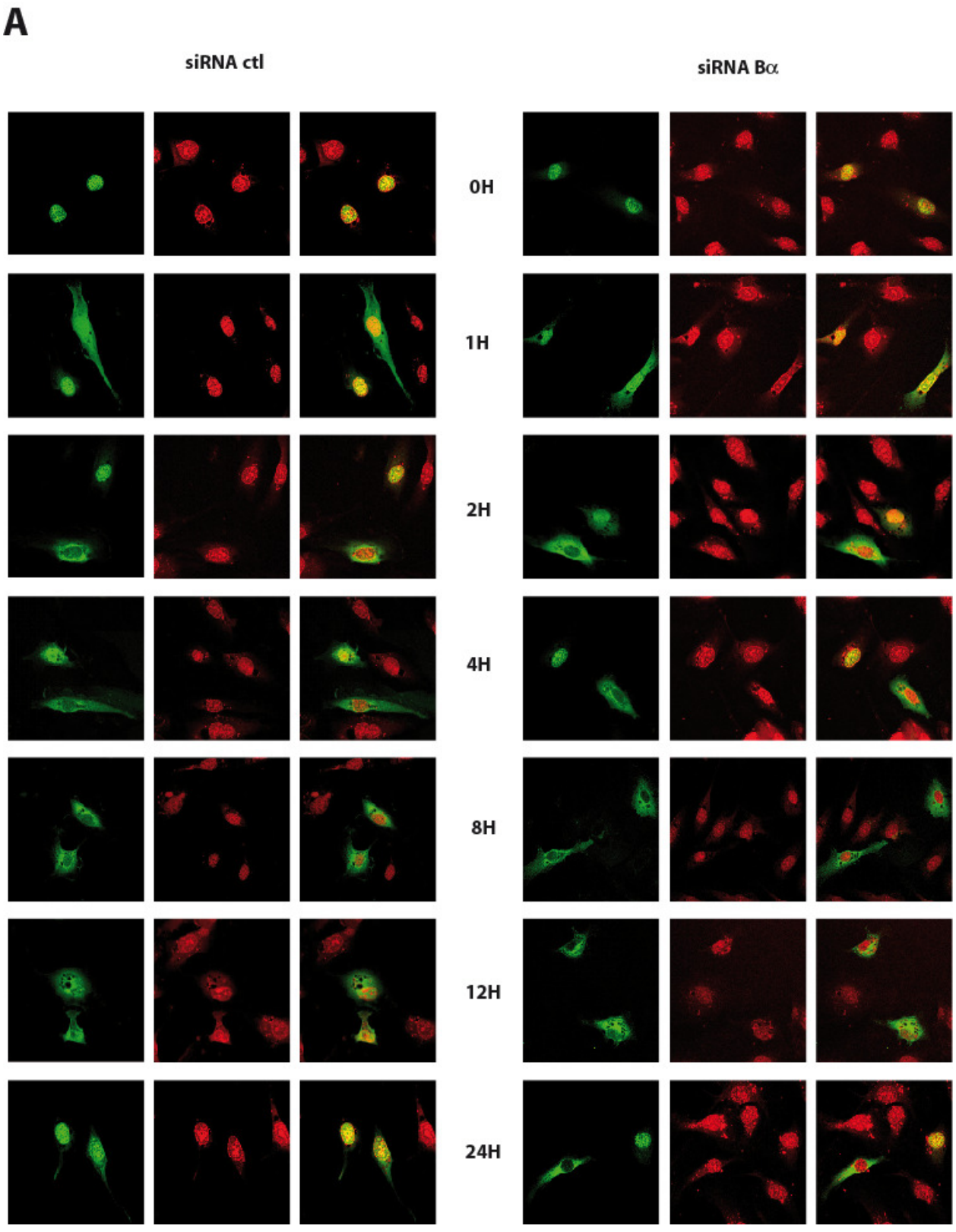
incubated at 30°C for 30 min in phosphatase assay buffer, in the absence (-) or presence of a PP2A heterotrimer (A/C/B55α) (+$PP2A_{AB55\alpha C}$). Where indicated OA (20 nM) was added to the reaction mixture. Proteins were visualized by Coomassie Blue staining (lower panel) and phosphorylation was assessed by autoradiography (upper panel).

(B) Flag-tagged HDAC7 was immunoprecipitated from MYC-tagged PP2A-Bα expressing HEK293 cells. Immune complexes were analyzed by Western blotting with specific antibodies against the MYC (PP2A Bα) or the Flag (HDAC7) epitope. Total cell lysate was included as a control to identify the position of the PP2A subunit.

PP2A-Bα inactivation prevents HDAC7 nuclear relocalisation in a VEGF signaling context

Many signaling cascades impact on class IIa HDACs phosphorylation, localisation and repressive function (Martin et al., 2007). In the vascular environment, HDAC7 has been shown to respond to VEGF signaling, one of the prevaling pro-angiogenic factor. Specifically, through Protein Kinase D activation, VEGF induces HDAC7 phosphorylation and nuclear exclusion. This cytoplasmic localisation is transient: HDAC7 accumulates back in the nucleus few hours after its VEGF-mediated export and reinstates its repressive activity over its target genes (Ha et al., 2008; Wang et al., 2008). This temporary HDAC7 inactivation is thought to be crucial to its functions and involves its dephosphorylation. Our results demonstrate an important role for Bα in VEGF-induced migration of endothelial cells (figure 4). We thus asked whether PP2A-Bα could be involved in HDAC7 nuclear relocalisation in the particular context of VEGF signaling. To verify this, we examined the effect of PP2A-Bα silencing on HDAC7 subcellular localization during VEGF-stimulation. As previously reported (Ha et al., 2008; Wang et al., 2008), HDAC7 is primarily localized in the nucleus of the majority of serum-starved HUVECs. In contrast, the number of cells showing nuclear export and cytoplasmic relocalization of HDAC7 gradually increases upon VEGF treatment, reaching a maximum at 4-8 hours after VEGF stimulation, with ~50% of the cells showing complete exclusion of HDAC7 from the nucleus. Upon longer treatments, HDAC7 gradually relocalizes to the nucleus and becomes almost totally nuclear again at 24h (figure 7, siRNA ctl). Interestingly, significantly different obsevations were made in siRNA Bα-transfected HUVECs. The extent and kinetics of VEGF-induced nuclear export of HDAC7 in Bα-knocked down cells was very similar to those of control cells. However, the expected reinstatement of HDAC7 nuclear localisation was not observed in Bα deficient cells, Indeed,

in the absence of PP2A-Bα, HDAC7 remained mainly cytoplasmic, even at 24h post-VEGF treatment (figure 7, siRNA Bα). In agreement with our model, these observations demonstrate that PP2A regulatory subunit Bα participates in HDAC7 nuclear re-entry after VEGF stimulation.


A
siRNA ctl
siRNA Bα
0H
1H
2H
4H
8H
12H
24H

B

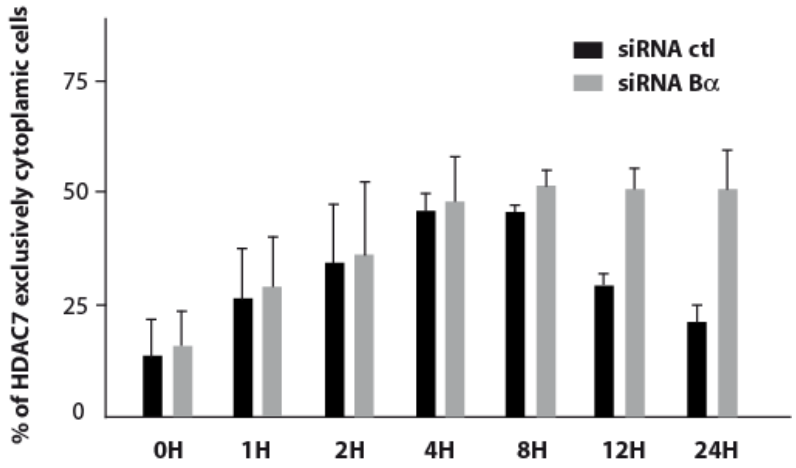


Figure 7. Bα inhibition prevents HDAC7 nuclear relocalisation after VEGF stimulation

(A) An expression vector for a GFP-fusion protein of HDAC7 was transfected into HUVECs. Twenty-four hours after transfection, cells were transfected with siRNA against PP2A-Bα. A non-targeting siRNA was used as control. Cells were cultured for an additional day before the subcellular localisation of HDAC7 proteins (in green) was examined after VEGF treatment by confocal microscopy. Nuclei were stained with Draq 5 (in red).

(B) Bar histograms represent the mean percentages of cells showing predominant cytoplasmic staining.

V) Discussion

The abundant protein phosphatase PP2A is a trimeric enzyme, made of a core enzyme, comprising a structural and a catalytic subunit, that is associated with a third variable regulatory B-type subunit (Janssens & Goris, 2001). About 20 different isoforms have been described for the B subunit so far. This diversity in the regulatory component generates a large collection of distinct holoenzymes, which accounts for the pleiotropic functions of PP2A (Li & Virshup, 2002; Ruediger et al., 1992a). Despite the significant number of regulatory roles assigned to PP2A, the precise roles of individual PP2A regulatory subunits in the signaling pathways involving this key enzyme remain unknown. The B (B55) family of PP2A regulatory subunits is diverse, with each member displaying distinct tissue expression, developmental expression, and subcellular localization patterns (Sontag, 2001). The

widespread Bα isoform is the predominant member of this family and has been involved in microtubules assembly in Alzheimer disease (Gong et al., 1994), virus-induced apoptosis (Van Hoof & Goris, 2003), the RAS-RAF-MAP kinase signaling pathways (Adams et al., 2005; Ory et al., 2003), and the TGF-β/Activin/Nodal signaling (Batut et al., 2008).

In the present study we report that the PP2A regulatory subunit Bα is a crucial element in the regulation of endothelial angiogenic functions during blood vessel formation. We show that Bα is expressed in human vascular endothelial cells and that its specific silencing abolishes the property of cells to assemble into a primitive vascular network in a Matrigel assay. In addition, PP2A-Bα knock-down led to a significant deregulation in endothelial cells migration, a key step involved in angiogenesis. Notably, none of the other PP2A regulatory subunit tested had similar effect on tube formation, neither on migration (data not shown). Altogether, these data indicated a specific function of Bα during vascularisation.

Little is known about the implication of PP2A in the vascular system. Global inhibition of PP2A by okadaic acid restricts endothelial cell motility (Gabel et al., 1999) and induces ERK1/2-kinase phosphorylation, an important signaling event in vascular morphogenesis (Schmidt et al., 2006). More described is the finding that PP2A dephosphorylates and inactivates the endothelial isoform of nitric oxide synthase (eNOS), as well as its specific activating kinase Akt (Greif et al., 2002; Miao et al., 2008; Urbich et al., 2002). Through the synthesis of nitric oxide, eNOS is a key mediator of vascular homeostasis and regulates cytoskeletal rearrangements leading to endothelial cells migration (Morales-Ruiz et al., 2000; Walford & Loscalzo, 2003; Ziche & Morbidelli, 2000). Therefore, it will be interesting to find out if the regulatory subunit Bα could be involved in PP2A mediated-eNOS dephosphorylation, which might explain its importance in endothelial cells angiogenic properties.

Nevertheless, our results provide strong evidence for a critical role of PP2A-Bα in a transcriptional control of vascularisation. Indeed, in order to delineate the pathway(s) by which PP2A-Bα impacts on endothelial cells angiogenic functions, we set up a genome wide-analysis of its potential regulated genes. Our study identifies the first set of Bα target genes, which, consistent with its role in angiogenesis, includes multiple angiogenesis related genes. Among these, we identified endothelin receptor B (ET-B) as a gene targeted for repression by Bα. This receptor has been shown to modulates endothelial cell proliferation and migration (Cruz et al., 2001; Morbidelli et al., 1995; Salani et al., 2000). Although a proangiogenic role has long been attributed for ET-B (Salani et al., 2000), alternative study have shown conflicting results and described a putative proangiogenic property of endothelin antagonists

in tumor progression (Egidy et al., 2000). Thus, the angiogenic role of endothelin receptors *in vivo* remains to be clarified. However, an intriguing issue arising from our data is that most of angiogenic factors found to be up-regulated by PP2A-Bα silencing are described as stimulating effectors of angiogenesis. This seems to be in contradiction with the dramatic impaired vascular phenotype associated with Bα knock-down observed in our functional assays. Nevertheless, the vision of a clear-cut distinction between pro and anti angiogenic factors seems to be oversimplistic, as illustrated above with ET-B. Formation of the vasculature is a complex process requiring an overall dynamic balance between multiple gene products. Hence, the dysregulation of a "pro-angiogenic" factor, like its excessive production, could be harmful for the angiogenic process.

Close inspection of Bα-regulated genes list revealed that many of these are also induced by the omnipotent angiogenic factor VEGF. This holds true for genes such as E-selectin, MCP-1, ICAM-1 (Minami et al., 2004) and a member of the CIAP2 family (Tran et al., 1999). Through the activation of a number of genes, VEGF signaling plays crucial roles in angiogenesis by regulating endothelial cell proliferation, migration, and survival. Recently, VEGF has been shown to regulate transcription partly by signaling through the regulation of the transcriptional activity of the class IIa HDAC7 (Ha et al., 2008; Wang et al., 2008). In the light of our previous results showing that PP2A catalytic activity contributes to the regulation of HDAC7 repressive functions during vascular process (Martin et al., 2008), we speculated that HDAC7 could be a molecular target for PP2A-Bα in endothelial cells. Indeed, our results show that a Bα containing PP2A holoenzyme binds to and dephosphorylates HDAC7. More importantly, deletion of PP2A-Bα in HUVECs is heavily reminiscent of HDAC7 knock-down, as it prevents endothelial cell migration, tubulogenesis and leads to up-regulation of a large set of common genes. This functional interdependency is further underscored by the fact that localisation experiments demonstrate that PP2A-Bα is involved in the dynamic wave of nucleocytoplasmic HDAC7 shuttling in response to VEGF. Indeed, we show that Bα silencing prevents HDAC7 nuclear re-entry, an event thought to be crucial to its transcriptional-related functions. Taken together, these results point to HDAC7 as a transcriptional effector in the Bα-dependent angiogenic signaling pathway.

The cardiovascular system is the first functional organ system to assemble during vertebrate development. It provides oxygen, nutrients and hormones to organs and maintains tissue homeostasis, and is thus essential for embryo survival and life from early stages to adult. The process of new vessel formation, called angiogenesis, consists of the complex coordination of diverse events, like endothelial cells activation, proliferation, migration, tube

formation and branching. This process requires finely tuned time- and signal-dependent regulation. In addition to its prime importance during embryonic development and normal cellular response to diverse physiological factors, angiogenesis plays crucial roles in carcinogenesis, particularly during metastases dissemination (Bellacosa et al., 2005; Ergun et al., 2006; Kerr, 2004; Roskoski, 2007). Even though numerous effectors of these processes have been largely described, the molecular genetic and epigenetic mechanisms involved remain largely unknown. Here, we integrate a novel important player, the PP2A regulatory subunit Bα, in the regulation of angiogenesis. This raises interesting new prospects for the development of new strategies to control physiological and pathological angiogenesis.

Chapitre 4: Conclusion and future prospects

Class IIa HDACs are master regulators of various developmental programs such as T-cell ontogeny, cardiac growth, muscle differentiation and activity, bone formation, neuron survival and blood vessel development. Therefore, developmental and signal-dependent regulation of class IIa HDACs repressive activity is crucial for the integrity of all these biological processes. This regulation is achieved through class IIa HDACs signal-dependent subcellular relocalization that relies on the phosphorylation of 14-3-3 binding sites located in their N-terminal adaptor domain. Association with 14-3-3s promotes relocalization of class IIa HDACs from the nucleus to the cytoplasm which is associated with derepression of their target promoters. This model of class IIa HDACs regulation that was prevailing when we started this work is illustrated in Figure 1A.

Regulation of class IIa HDAC activity should be envisonned as a reversible mechanism.In this case, re-establishment of class IIa HDACs transcriptional inhibition would be expected to occur through dephosphorylation of the 14-3-3 binding sites and translocation from the cytoplasm to the nucleus. In accordance with this, we identified PP2A as the specific HDAC7 regulating phosphatase. Indeed, we provide robust evidence that PP2A directly dephosphorylates four specific serine residues in HDAC7 and thus controls its localisation and repressive functions. In order to validate our model, we also illustrated the functional consequences of HDAC7 regulation by PP2A in two major biological contexts involving HDAC7, i.e. thymocytes apoptosis and blood vessel formation. In addition, we unexpectedly identify a new role for 14-3-3 proteins in class IIa HDACs phosphorylation and nucleo-cytoplasmic shuttling. Our results strongly suggest that interaction with 14-3-3 proteins protects class IIa HDACs from PP2A-mediated dephosphorylation and prevents their nuclear re-entry. Indeed, deficiency in 14-3-3 binding increased the propensity of HDAC7 to be dephosphorylated.

Very interestingly, this latter finding connects with the results we detailed in the first chapter of this work, in which we show that phosphorylation of HDAC7 serine 181 is dependent on prior phosphorylation of serine 155. Based on the prevailing model of 14-3-3 binding their targets as dimers, we speculated that, in the absence of serine 155 phosphorylation, the affinity of the single phosphoserine 181 is too weak to promote stable interaction with a 14-3-3 dimer. Due to the absence of 14-3-3 binding, ser 181 is not protected and is thus easily dephosphorylated by PP2A.

Altogether these results unravel a new dynamic interplay between 14-3-3, protein kinases and PP2A in the regulation of class IIa HDACs function, and could be integrated into the regulation scheme of these enzymes, as showed in figure 1B.

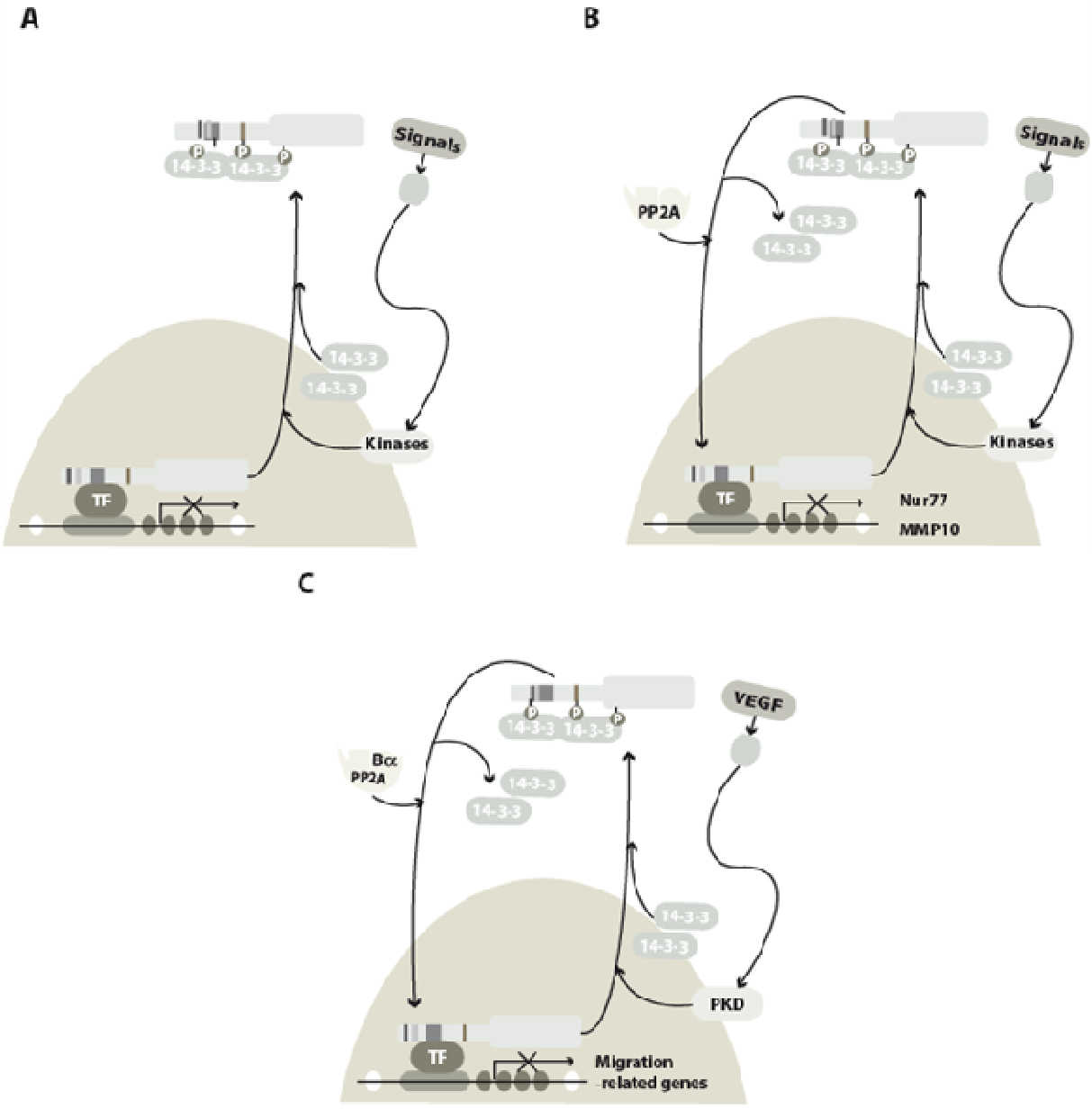


Figure 1

Cartoons depicting models of class IIa HDACs regulation: model prevailing at the beginning of this work (A), completed by our results (B) and in the particular context of angiogenesis (C).

Although our findings render this model more complete and sophisticated, several question marks remain. It is well documented that association with 14-3-3 impacts on class IIa HDACs localization. Nevertheless, as detailed in the introductory section devoted to class IIa HDACs, the exact mechanism by which these chaperone proteins induced class IIa HDACs cytoplasmic accumulation, as well as the precise subcellular site where association between 14-3-3 and class IIa occurs remains controversial. In addition to emphasize the importance of 14-3-3 proteins in the regulation class IIa HDACs localization, our results bring new opportunities to solve some of these issues. We show that 14-3-3 proteins regulate class IIa HDACs nuclear import by preventing their dephosphorylation and thus function as cytoplasmic anchors for these proteins. Based on this, we can speculate a model in which signal-dependent phosphorylation of nuclear class IIa HDACs leads to their nuclear export. Once in the cytoplasm, binding of 14-3-3 to class IIa HDACs protects them from PP2A dephosphorylation and thus prevents their nuclear import. Whether or not binding of 14-3-3 takes place in the nucleus -these ubiquitous proteins could be found in the nucleus as well as in the cytoplasm- and promotes nuclear export, association of class IIa HDACs with 14-3-3 culminates in the cytoplasmique retention of the HDACs. In accordance with the hypothesis of a prevailing cytoplasmic role for 14-3-3, two recent studies showed that association with 14-3-3 is dispensable for class IIa HDACs nuclear export (Gao et al., 2006), but decrease their nuclear import (Nishino et al., 2008).

In this context, nuclear re-entry of cytoplasmic class IIa HDACs through their dephosphorylation by PP2A requires their prior dissociation from 14-3-3. Nevertheless, what molecular event helps PP2A gain access to the phosphorylated 14-3-3 binding sites *in vivo* is still unknown and will be an attractive challenging question for the future. 14-3-3 removal could be achieved by PP2A itself or another competitor, as this is the case for prohibitin, a ubiquitous protein that displace 14-3-3 from phosphorylated Raf (Rajalingam & Rudel, 2005). In another attractive example, 14-3-3 release from the cdc25 phosphatase is accomplished by the creation of a 14-3-3 "reservoir", consisting of phosphorylated intermediate filament proteins, including vimentin and keratin, that bind 14-3-3 with high-affinity and sequester them (Margolis et al., 2006). It is also interesting to note that 14-3-3 dimers could be subjected to phosphorylation, which disrupts the dimer and decrease affinity for its target (Woodcock et al., 2003).

Most studies reported that relocalization of class IIa HDACs is achieved through signal-dependent phosphorylation by signal-responsive protein kinases. Evidence reported in this

work unravels constitutive class IIa HDACs phosphorylation and dephosphorylation by hPar-1 kinases and PP2A respectively. We indeed show that class IIa HDACs are constitutively associated with both enzymes in normally growing cells and that inhibition of their activity leads to a modification of HDAC7 phosphorylation and localization in unstimulated cells. Thus, a fraction of class IIa HDACs seems to undergo signal-independent phosphorylation/dephosphorylation cycles. Constitutive phosphorylation of class IIa HDACs by h-Par1 kinases is specific for the most upstream phosphorylatable HDAC7 site, serine 155. Constitutive phosphorylation of serine 155 could serve as a prerequisite, for example to pre-bind a 14-3-3 dimer, in order to allow HDAC7 to respond more rapidly to its specific signals. Our *in vitro* assay demonstrates that PP2A dephosphorylation occurs without any preference amongst the four phosphorylation sites of HDAC7. However, additional experiments could be helpful to confirm this lack of specificity *in vivo*.

Any alteration of the equilibrium between class IIa HDACs specific kinases, phosphatase and 14-3-3 proteins would affect the subcellular distribution and thus biological function of class IIa HDACs. It should be noted that, in addition to be constitutively active in most cell lines, the h-Par1 member MARK kinase is also considered as a stress-responsive kinase (Schneider et al., 2004; Wang et al., 2007). Our results unravel new opportunities for to regulate class IIa HDACs localization at multiple levels. As observed for class IIa HDAC kinases, the catalytic activity of the specific class IIa HDACs targeting phosphatase might itself be regulated. In addition, the accessibility of the phosphorylatable class IIa HDAC residues, through 14-3-3 binding, would be a major determinant in the phosphorylation/dephosphorylation process.

PP2A composition is in great part responsible for the regulation of its pleiotropic functions. In cells, most of PP2A activity exists as heterotrimers, comprising a catalytic C, a structural A and a regulatory B subunit. In opposition to the well conserved A and C subunits, various families of the regulatory B subunit, each giving rise to several isoforms, have been described. The identity of the variable B-type subunits has a crucial role in the control of PP2A activity by determining substrate specificity, cellular localisation and catalytic activity of the PP2A holoenzyme. In the last part of our work, we identified the precise holoenzyme, i.e. the Bα regulatory subunit containing PP2A holoenzyme, which regulates HDAC7 function in angiogenesis. In addition to improve our understanding of class IIa HDACs regulation (figure 1C, discussed below), our results associate for the first time a specific PP2A regulatory subunit with a major developmental program, i.e. the establishement of the vasculature.

In order to confirm the role of PP2A Bα subunit during vascular network formation, it will be interesting to validate our results in an animal model using genetic inactivation. Zebrafish possess similar anatomical and physiological characteristics as vertebrates and its specific embryonic development makes developmental processes studies easier. For these reasons, Zebrafish is considered as a powerful model to study mechanisms controlling angiogenesis (Norrby, 2006; Ny et al., 2006; Stoletov & Klemke, 2008). Hence, studies of potential Zebrafish vascular development defects resulting from PP2A-Bα inactivation would be of great interest.

Our results specifically implicate Bα subunit in endothelial cells VEGF response. VEGF is a member of a family of ligand proteins that has been identified as the most potent and predominant mediator of angiogenesis, during embryonic development as well as in the adults. In addition to demonstrate its involvement in endothelial cells migration in response to VEGF, we showed that PP2A regulatory subunit Bα participates in HDAC7 nuclear re-entry after VEGF stimulation, an event thought to be crucial for HDAC7 functions in angiogenesis. Consistent with the view of HDAC7 as a transcriptional effector in the Bα-dependent angiogenic signaling pathway, our microarray analysis shown a large redundancy between genes regulated by both proteins and hence revealed a strong functional relationship between PP2A Bα and HDAC7 in endothelial cells. Moreover, many of these similarly regulated genes are known as VEGF responsive genes.

In the light of these results, we could integrate PP2A Bα in the model of VEGF signalling to HDAC7 during angiogenesis. As shown in figure 1C, VEGF signalling triggers PKD activation, and maybe other class IIa HDAC kinases. For instance, inhibition of CamKII has been shown to reduce VEGF-induced transcriptional activity in endothelial cells and VEGF has been shown to activate this kinase in vascular smooth muscle (Chandra & Angle, 2005; Maiti et al., 2008). Phosphorylation promotes HDAC7 nuclear export and concomitant derepression of a set of target genes, including several migration-related genes that we found in our microarray analysis. VEGF-mediated transcriptional activation is transient and thus HDAC7 would be expected to go back to the nucleus to blunt the transcriptional activity of VEGF target genes. Our results strongly suggest that this is accomplished through dephosphorylation of HDAC7 by a specific Bα containing PP2A holoenzyme. The fact that canonical HDAC7 target genes, i.e. MMP10 and Nur77 genes were not among the list of

genes activated by PP2A Bα or HDAC7 depletion is quite surprising, but preliminary results prompt us to speculate that the high threshold we used in the microarray experiment is responsible for this observation. Indeed, qPCR analysis revealed that both genes were upregulated in HDAC7- and Bα-depleated cells (data not shown).

This dynamic and time-dependent regulation of HDAC7 requires precise regulation. In this context, it is possible that, in order to ensure rapid and maximal HDAC7 phosphorylation, VEGF not only activates HDAC7 specific phosphorylation but also represses its dephosphorylation. To achieve this, VEGF signalling would control both kinases (PKD) and phosphatases (PP2A) activities, which would ultimately lead to a dynamic and precise regulation of HDAC7 phosphorylation. Future experiments should be undertaken in order to validate this hypothesis and follow the evolution of Bα associated phosphatase activity during VEGF stimulation. A first experiment would be to examine the evolution of Bα expression level, in terms of mRNA and protein, following VEGF treatment. Additionally, an *in vitro* dephosphorylation assay would allow the measurement of Bα associated PP2A activity during the same VEGf stimulation. In addition to influencing holoenzyme specific activity through their identity, PP2A regulatory subunit could also indirectly regulate this activity. For instance, it has been demonstrated that the catalytic subunit methylation status influences its specific association with Bα regulatory subunit (Bryant et al., 1999; Longin et al., 2008; Ogris et al., 1997; Tolstykh et al., 2000; Wu et al., 2000b; Yu et al., 2001). In another example, B' subunit phopshorylation involves its integration into an active holoenzyme (Margolis et al., 2006). Use of bidimensionnal electrophoresis would highlight potential Bα post-translational modifications in response to VEGF, which could be identified using mass spectrometry.

In addition to its importance during embryonic development and normal cellular response to diverse physiological factors, angiogenesis plays a crucial role in carcinogenesis, particularly during metastases dissemination. Even though numerous effectors of this process have been largely described, identification of PP2A-Bα, and PP2A-Bα-related HDAC7 regulation as important players in angiogenesis could hold promises in term of development of new strategies to control pathological angiogenesis.

Chapitre 5: Bibliography

5.1. My bibliography

I) Publications

1. Dequiedt F., **Martin M.,** Von Blume J., Vertommen D., Lecomte E., Mari N., Heinen M.F., Bachman M., Twizere J.C., Huang M.C., Rider M.H., Piwnica-Worms H., Seufferlein T., Kettmann R. (2005). 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 (19):** 7086-7102.

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4. Martin M., Kettmann R., Dequiedt F. Class II HDACs: conducting differentiation and development. *International Journal of Developmental Biology :* in press

5. Martin M., Kettmann R., Dequiedt F.
Recent insights into Proteine Phosphatase 2A structure and regulation: the reasons why PP2A is no longer considered as a lazy passive housekeeping enzyme. Submitted to BASE

6. Martin M., Bruyr J., Potente M., Demoitié P., Kettmann R., Dequiedt F. Identification of a unique developmental function for PP2A Bα regulatory subunit: Bα regulates migration-related angiogenesis through the control of class IIa HDAC7 activity. In preparation

II) Meeting abstracts

1. MARK/Par-1 kinases, EMK and C-tak1 regulate localization and activity of class IIa HDACs through hierarchical phosphorylation
Cell Signaling world 2006, Luxembourg, January 2006
Poster presentation

2.Protein phosphatase 2A controls the subcellular distribution and biological activity of class IIa histone deacetylase.
Phosphorylation, signaling and disease, Cold Spring Harbor New York, May 2007
Poster presentation

3.Protein phosphatase 2A controls the subcellular distribution and biological activity of HDAC7.

FASEB summer research conference on Protein Phosphatase, Snowmass Colorado, July 2008

Oral presentation

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