PP2A Regulates HDAC4 Nuclear Import

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Different signal-regulated serine/threonine kinases phosphorylate class II histone deacetylases (HDACs) to promote nuclear export, cytosolic accumulation, and activation of gene transcription. However, little is known about mechanisms operating in the opposite direction, which, possibly through phosphatases, should promote class II HDACs nuclear entry and subsequent gene repression. Here we show that HDAC4 forms a complex with the PP2A holoenzyme C α , A α , B/PR55 α . In vitro and in vivo binding studies demonstrate that the N-terminus of HDAC4 interacts with the catalytic subunit of PP2A. HDAC4 is dephosphorylated by PP2A and experiments using okadaic acid or RNA interference have revealed that PP2A controls HDAC4 nuclear import. Moreover, we identified serine 298 as a putative phosphorylation site important for HDAC4 nuclear import. The HDAC4 mutant mimicking phosphorylation of serine 298 is defective in nuclear import. Mutation of serine 298 to alanine partially rescues the defect in HDAC4 nuclear import observed in cells with down-regulated PP2A. These observations suggest that PP2A, via the dephosphorylation of multiple serines including the 14-3-3 binding sites and serine 298, controls HDAC4 nuclear import.

INTRODUCTION

Epigenetic modifications of chromatin modulate changes in gene expression in response to a plethora of signals. Covalent posttranslational modifications of histones alter chromatin structure in an orchestrated manner to control gene activation or repression (Berger, 2007).

Acetylation of specific lysines present within the N-terminal extensions of the core histones is an important switch in the control of gene transcription (de Ruijter *et al.*, 2003). Histone acetylation is under the tight control of two opposite classes of enzymes: histone deacetylases (HDACs) and histone acetyl transferases (HATs; Berger, 2007). HDACs and HATs represent the yin and yang elements that regulate gene repression and activation (Henderson and Brancolini, 2003).

HDACs, by removing acetyl groups at the ϵ -amino groups of lysine residues of histones, are chief mediators of epigenetic gene silencing. HDACs belong to a heterogeneous family of over a dozen members, which are grouped into distinct subfamilies by sequence similarities and structural features (Verdin *et al.*, 2003). The class IIa subfamily includes HDAC-4, -5, -7, and -9. These deacetylases share an N-terminal extension that confers responsiveness to calcium signals and a catalytic C-terminal region. The N-terminal portion also mediates interactions with transcription factors and contains a conserved glutamine-rich domain that can repress transcription independently of the C-terminal cata-

lytic domain (Guo *et al.*, 2007). The increasing list of transcription factors and corepressors that has been shown to be class IIa HDACs partners suggests a role of these deacetylases in a wide range of cellular activities (Yang and Gregoire, 2005).

Class IIa HDACs shuttle between the nucleus and the cytoplasm due to a nuclear localization signal (NLS) located at the N-terminus and a nuclear export sequence (NES) present at the C-terminus. Many signals operate on class IIa HDACs by controlling their subcellular localization (McKinsey *et al.*, 2001; Wang and Yang, 2001). A set of conserved serine residues is the critical determinant of HDAC localization. These serines, once phosphorylated, become attachment sites for 14-3-3 chaperone proteins, which escort HDACs into the cytoplasm and relieve transcriptional repression (Grozinger and Schreiber, 2000; Wang *et al.*, 2000). Hence, HDAC phosphorylation is the signal-responsive modification that couples extracellular events to chromatin remodelling (McKinsey *et al.*, 2000; Miska *et al.*, 2001). Phosphorylation of the 14-3-3 binding sites, nuclear export of HDACs, and repressional relief are promoted by a remarkable number of kinases including PKD, CaMKs, PKCs, and (MARK)-Par-1 kinases (Zhou *et al.*, 2000; Chang *et al.*, 2005; Backs *et al.*, 2006; Dequiedt *et al.*, 2006; Berdeux *et al.*, 2007).

Among the different class IIa members, HDAC4 nuclear/cytoplasmic shuttling is also regulated by proteolysis during apoptosis. Cleavage of HDAC4 by caspases generates an N-terminal fragment that contains the NLS and accumulates in the nucleus, where it represses transcription and induces cell death (Liu *et al.*, 2004; Paroni *et al.*, 2004).

Genetic studies have proved that class IIa HDACs play an important role in tissue-specific growth and development. In particular, mice lacking HDAC4 die perinatally because of abnormal chondrocyte hypertrophy that results in ectopic and premature ossification of endochondrial bones (Vega *et*

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al., 2004). In humans, genome-wide studies have identified alterations and mutations of HDAC4 in melanoma and breast cancer (Sjoberg *et al.*, 2006; Stark and Hayward, 2007). Further studies have suggested a role for HDAC4 in DNA repair, apoptosis, and differentiation (Bolger and Yao, 2005; Yang and Gregorie, 2005; Geng *et al.*, 2006).

Despite the involvement of HDAC4 in many different cellular responses, few data are available on the mechanisms regulating its nuclear entry and how its nuclear import is linked to signaling pathways requiring HDAC4-dependent repression.

In the present study, by identifying and characterizing new cytosolic partners for HDAC4, we have discovered that the serine/threonine protein phosphatase 2A (PP2A) interacts with and dephosphorylates HDAC4. Experiments using okadaic acid (OA) and RNA interference (RNAi) demonstrated that PP2A operates as a regulator of HDAC4 nuclear import. Moreover, we identified serine 298 as a novel important target for such control. Caspase cleavage of HDAC4 at the Asp 289 residue releases the deacetylase from PP2A control, thus reinforcing the nuclear accumulation of the N-terminal fragment devoted to proapoptotic activities. In conclusion, we propose that nuclear import of HDAC4 is subjected to intense regulation and that multiple signals, triggering different posttranslational modifications of HDAC4, operate in a coordinated or antagonistic manner to control gene expression.

MATERIALS AND METHODS

Plasmid Constructs

The HDAC4-Flag Tet-On expression vector pTRE-HDAC4-F was obtained by digesting pHDAC4-F with XbaI followed by Klenow fill-in and HindIII partial digestion. The resulting HDAC4-Flag encoding fragment was inserted into the HindIII and EcoRV restricted pTRE2hyg plasmid (BD Biosciences Clontech, San Jose, CA), in which the second HindIII site had been previously removed by digestion, Klenow fill-in, and self-ligation. pFLAG-CMV5 constructs expressing HDAC4 and its deletion mutants were previously described (Paroni *et al.*, 2004, 2007). HDAC4-GFP point mutants were generated by *in vitro* mutagenesis using the Gene-Taylor kit (Invitrogen, Carlsbad, CA) and full-length pEGFPN1-HDAC4 as template.

The following primers were used: primer S266D FW, 5'-AAGTGGC-CGAAAGACGGAGCGACCCCTGTTACG-3'; primer S266D RV, 5'-GCTC-CGTCTTTCGGCCACTTCTGCTTACG-3'; primer S298D FW, 5'-CGT-GCAGCAGCGCCCGAGCGACGCCAGCTC-3'; primer S298D RV, 5'-GCCTGG GCGCTGCTGCACGCGAGTCTGT-3'; primer S302D FW, 5'-CCCCAGGTCGGGACCCGACGCCCAACAACAG-3'; primer S302D RV, 5'-GCTGGGTCCGGAGCCTGGGCGCTGCTGCA-3'; primer S339D FW, 5'-GA CTTGTGGCAGGAGGCGACGCCCTCCACT-3'; and primer S339D RV, 5'-GCCTTCTCGTGGCCACAAGTCTGTGCGCCAA-3'.

All constructs generated were sequenced to check for introduced mutations, deletions, and the translating fidelity of the inserted PCR fragments.

Western Blotting

Proteins obtained after an SDS denaturing lysis and sonication were separated by SDS-PAGE and finally transferred to a 0.2- μ m pore-sized nitrocellulose membrane. Membranes were incubated with the following antibodies: anti-HDAC4 (Paroni *et al.*, 2004), anti-FLAG M2 (Sigma, St. Louis, MO), anti-tubulin (Paroni *et al.*, 2004), anti-PP2A/C subunit (Upstate, Lake Placid, NY), anti-PP2A/A subunit (Upstate), anti-Smac (Henderson *et al.*, 2005). Blots were then rinsed three times with Blotto/Tween 20 and incubated with peroxidase-conjugated goat anti-rabbit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or goat anti-mouse (Euroclone, Milan, Italy) for 1 h at room temperature. Blots were then washed three times in Blotto/Tween 20, rinsed in phosphate-buffered saline, and developed with Super Signal West Pico, as recommended by the vendor (Pierce, Rockford, IL).

In Vitro Binding Assays, Immunoprecipitation, and In Vitro Dephosphorylation

Glutathione S-transferase (GST) and GST fusion proteins were prepared as described previously (Paroni *et al.*, 2002). Radiolabeled HDAC4 and its deletion mutants were generated using TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI). GST fusion proteins immobilized onto glutathione-Sepharose beads were incubated with 200 μ l of binding buffer (20 mM

HEPES, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μ M each chymostatin, leupeptin, antipain, and pepstatin) at 4°C for 3 h in the presence of the appropriate amounts of the ³⁵S-labeled proteins. The beads were separated by brief centrifugation and washed four times with washing buffer containing 50 mM Tris HCl, pH 7.5, 150 mM NaCl, and 0.5% Triton X-100. Beads were boiled in SDS sample buffer, and proteins were resolved by 12% SDS-PAGE.

Immunoprecipitations were performed as previously described (Fontanini *et al.*, 2005). Briefly, IMR90-E1A cells were transfected with the indicated constructs and allowed to grow for 24 h before lysis with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100. Lysates were incubated with the indicated antibodies overnight. One hour after protein A bead addition, beads were washed extensively with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100, and samples were resolved by SDS-PAGE and analyzed by immunoblot.

In Vitro Phosphorylation and Dephosphorylation Assays

In vitro phosphorylation was performed as previously described (Brancolini and Schneider, 1994). Briefly, IMR90-E1A cells grown in plastic tissue culture dishes (15-cm diameter) were treated with OA (50 nM) or left untreated for 12 h, harvested with a rubber scraper, and sedimented by centrifugation at 1000 rpm for 5 min. After two washes, pellets were resuspended in 200 μ l of kinase/lysis buffer containing 150 mM NaCl, 0.5% NP-40, 2 mM PMSF, 20 mM triethanolamine (TEA), pH 7.5, 20 mM MgCl₂, 2 mM MnCl₂, 50 mM NaF, 1 mM Na₃VO₄, Microcystine-LR, and 10 nM OA.

The *in vitro* kinase assay was performed using 80 μ l of cellular lysates and ~3 μ g each of GST-HDAC4/241–601 fusion protein or GST alone, 20 μ M rATP, and 10 μ Ci of [γ -³²P]ATP (GE Healthcare Waukesha, WI; 3000 Ci/mmol, 110 TBq/mmol) for 30 min at 30°C. The GST or GST-HDAC4 fusion proteins were purified by incubation of cellular lysates with glutathione-Sepharose beads at 4°C for 30 min. After six washes in washing buffer, the samples were released by boiling for 3 min in SDS-PAGE sample buffer. Samples were then analyzed by SDS-PAGE followed by Coomassie staining and autoradiography.

For *in vitro* dephosphorylation assays, HDAC4 was either immunoprecipitated from E1A cells with an anti-HDAC4 antibody (Paroni *et al.*, 2004) or immunoprecipitated from HeLa cells with an anti-FLAG antibody (Sigma) or were GST-pulled down, when used as a recombinant fragment (aa 241–601) in fusion with GST. The phosphatase reaction was performed in 20 mM MOPS, pH 7.5, 0.1 mM MnCl₂, 1 mM dithiothreitol (DTT), 50 μ M leupeptin, and 0.1 mg/ml serum albumin for 45 min at 30°C using 0.3 U of recombinant PP2A/C (Upstate Biotechnology). Reactions were terminated by boiling in SDS buffer, and then phospho- and total HDAC4-Flag or HDAC4 were analyzed by Western blot with anti-Flag, anti-HDAC4, or anti-phospho-Ser/Thr antibodies, respectively (Zymed, South San Francisco, CA). Densitometric scanning of HDAC4 bands was performed using LAS 3000 Luminescence Image Analyzer (Fuji, Tokyo, Japan). The ratios between phospho-HDAC4/total HDAC4 values were calculated and plotted, assigning a value of 100 to the untreated sample.

Cell Culture, Transfection, siRNA Experiments and Fluorescent Microscopy

U2OS cell lines expressing HDAC4-GFP or inducible HDAC4 HDAC4 Δ C (aa 1–289)-GFP upon ponasterone treatment were generated using the pInd/pVgRXR system (Invitrogen). Ratjadone C (Alexis, San Diego, CA) and OA (LC Laboratories, Woburn, MA) were used at the final concentration of 10 ng/ml and 1 μ M, respectively. The 293 EBNA1 Tet-ON cell clone was used as a recipient to generate HDAC4-Flag Tet-ON stable transfectants. The parental clone had been generated by stable transfection of HEK293 EBNA1 cells with a constitutive bicistronic expression vector encoding both the rTA2^{S2}-S2 Tet activator and the rTS Tet repressor (Lamartina *et al.*, 2003).

Stealth RNAi were purchased from Invitrogen: PP2A/C RNAi (base pairs PP2A, FW 5'-GCAAGAGGUUCGAUGUCCAGUACU-3'; PP2A, RV 5'-AGUAAUCUGGACAUCCUCCUUGC-3'), SMAC stealth siRNA (Invitrogen) was described previously (Henderson *et al.*, 2005). Cells were transfected 24 h after plating by adding the OptiMem medium containing Lipofectamine (Invitrogen) plus the stealth RNAi oligos.

Cells were maintained in modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1 $\times$ penicillin-streptomycin (100 $\times$ stock; Invitrogen), 1 $\times$ L-glutamine (100 $\times$ stock; Invitrogen), 250 μ g/ml G418 (geneticin, Invitrogen), and 0.5 μ g/ml puromycin (Sigma Aldrich; complete DMEM).

For subcellular localization studies, cells were fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were washed with PBS, 0.1 M glycine, pH 7.5, and were subjected to Hoechst 33258 staining. Cells were examined with a laser scanning microscope (Leica TCS SP, Heidelberg, Germany) equipped with a 488–534 λ argon laser.

HDAC4-Flag Expression and Immunopurification

After the 24-h induction with 1 μ g/ml doxocycline, HDAC4-Flag Tet-ON cells were collected in ice-cold PBS, homogenized, and sonicated in lysis buffer (20 mM HEPES, pH 7.9, 0.25 mM EDTA, 10% glycerol, 300 mM NaCl,

0.5% NP40) containing 1 mM PMSF, and Complete protease inhibitor mix (Roche Diagnostics, Mannheim, Germany), followed by 1-h incubation at 4°C. Soluble whole cell extracts were obtained by centrifugation at 12000 rpm in a SS34 rotor for 30 min at 4°C. HDAC4-Flag protein complexes were immunoprecipitated on an anti-Flag M2 affinity gel and eluted in 50 mM HEPES, pH 7.4, 5% glycerol, 0.01% Triton X-100, and 100 mM NaCl in the presence of 100 µg/ml 3× Flag peptide. Mock samples were prepared from parental cells using the same procedure. HDAC4-Flag concentration was determined by Coomassie-stained SDS-PAGE gels, using a reference protein for quantification. For 2D gel analysis, 800 µl of HDAC4-Flag complex sample (15 µg of HDAC4-Flag) or mock sample was subjected to protein desalting/concentration using the 2D Clean-Up Kit (GE Healthcare). The protein pellets were resuspended in 50 µl of urea buffer (30 mM Tris, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS), and added with an equal volume of 2× sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 50 µl Pharmalyte 3-10) before loading them on the first dimension gel.

2D Electrophoresis

Mock and HDAC4-Flag complex samples were cup-loaded in 3–10 NL 18-cm immobilized pH gradient (IPG) strips (GE Healthcare). Isoelectric focusing was done on a IPGphor (GE Healthcare) for a total of 40,000 Vh, at 20°C (300 V for 3 h, ramp to 1000 V in 6 h, ramp to 8000 V in 3 h, and 8000 V for up to 4 h). The current limit was 50 µA/strip. For cysteine reduction and alkylation, the focused strips were equilibrated in equilibration buffer (100 mM Tris, pH 8.0, 30% [vol/vol] glycerol, 2% [wt/vol] SDS, 6 M urea) containing 0.5% (wt/vol) DTT for 15 min, and an additional 15 min with equilibration buffer containing 4.5% (wt/vol) iodoacetamide. IPG strips were placed on top of a 12.5% SDS gel and the second-dimension electrophoresis was performed in a Peltier-cooled Ettan Dalt 12 electrophoresis unit (GE Healthcare) at 2 W/gel, for 16 h at 25°C. For phosphorylated proteins selective staining, the gels were removed from the plates and fixed overnight in 10% trichloroacetic acid/50% methanol, then washed four times for 15 min each with water, and placed for 4 h in ProQ Diamond solution (Molecular Probes, Eugene, OR). The gels were left in destaining solution (50 mM sodium acetate, pH 4.0, 20% acetonitrile) for 4 h and the ProQ Diamond image was obtained by scanning at 532/580-nm wavelengths (excitation and emission) with a Typhoon 9410 (GE Healthcare). After scanning, the gels were placed for 12 h in Sypro Ruby solution (Molecular Probes) for total protein stain. Afterward, the gels were washed with 10% MeOH, 7% acetic acid three times for 30 min each and scanned at 480/633-nm wavelengths with the same scanner.

In Gel Digestion, Mass Spectrometry, and Database Search

Proteins of interest were excised from the gel and placed in a 96-well plate using ProExcision spot picker from Perkin Elmer-Cetus Instruments (Shelton, CT). Excised spots were digested with Multiprobe liquid handler (Perkin Elmer-Cetus Instruments). Gel plugs were first washed with 100 mM ammonium bicarbonate for 5 min, 100 mM ammonium bicarbonate/ACN 50/50 for 5 min, and pure ACN for other 5 min. Fifty microliters of 12 ng/µl trypsin (Promega) were added to each gel piece, and after 45 min at 4°C, the trypsin solution was replaced with 50 mM ammonium bicarbonate for 12 h at 37°C. Tryptic peptides were then extracted from the gel with 5% formic acid and dried by vacuum centrifugation. Peptides were resuspended in 0.5% acetic acid and analyzed by LC-IT-MS/MS. An LQ DECA XP-Plus ion trap mass spectrometer (Thermo Electron, San José, CA) equipped with an in-house-built microelectrospray ion source coupled with a Surveyor HPLC (Thermo Electron) was used. Samples were loaded in an in-house packed pre-column (C18 resin, 5-µm particle size) placed before a C18 column packed in the sprayer. Peptides were separated and eluted from the column with a 0.5% acetic acid/acetonitrile gradient (from 0.5% acetic acid/acetonitrile 98/2–0.5% acetic acid/acetonitrile 50/50 in 30 min, flow rate 1 µL/min). Mass spectrometry (MS) and MS/MS spectra acquired in a data-dependent manner were searched against the SWISS-PROT database using the TurboSequest software provided by the manufacturer.

RESULTS

Mapping the Cytoplasmic Binding Partners of HDAC4

In an effort to identify novel cytoplasmic HDAC4 regulators, we searched for proteins that copurified with HDAC4 under native conditions. To this aim, a stable HEK293 Tet-ON cell clone expressing Flag-tagged HDAC4 in a doxocycline-inducible manner was isolated and used as a source of HDAC4 protein complexes. Ectopically expressed HDAC4 localized preferentially to the cytoplasm of these cells, similar to endogenous HDAC4 (not shown). To identify the HDAC4 protein interactors, the HDAC4-complex was immunopurified on anti-Flag beads from native cell extracts.

Figure 1. 2D gel of the HDAC4-Flag protein complex immunopurified in native conditions from HEK293 whole cell lysate. (A) Sypro Ruby stain and MS identification of the proteins coimmunopurified with HDAC4-Flag. Highlighted are all the proteins interacting specifically with cytosolic HDAC4-FLAG but not with the anti-FLAG antibody. Of particular interest was the identification of the PP2A complex (the scaffold and regulatory subunits A and B and the catalytic subunit C). Some HDAC4-Flag proteolytic degradation during cell lysis was observed. (B) Expanded view of Sypro Ruby and ProQ Diamond images of HDAC4-Flag showing the high number of different phosphorylated isoforms. The unique unphosphorylated HDAC4 isoform is indicated by an arrow.

To identify nonspecific binders, a mock sample from parental cell extracts was also prepared. The HDAC4-Flag and mock samples were resolved by 2D gel electrophoresis, and the protein spots corresponding to HDAC4-specific interactors were identified by MS (Figure 1A). Among those, we found known HDAC4 protein partners including the different 14-3-3 isoforms (γ , τ , ϵ , and η) and calcium/calmodulin-dependent protein kinase II δ (CaMKII δ). In addition, a number of new HDAC4 interactors were identified. They are represented by cytoskeletal and mitotic spindle components (α - and β -tubulins, α -spectrin, and kinesin-like protein 1/KIF11), protein folding and processing components (Hsp70, GR78), and the N-CoR corepressor complex component TBLR1. Of note, we have also isolated α -actinin 4, previously identified as a class IIa associated protein (Chakraborty *et al.*, 2006).

Of particular relevance for uncovering potential novel mechanisms regulating HDAC4 phosphorylation status, nuclear-cytoplasmic shuttling, and biological activities was the finding of its association with PP2A holoenzyme, composed of the catalytic subunit (C α), the scaffold subunit (A/PR65 α), and the regulatory subunit (B/PR55 α).

Interestingly, HDAC4 showed a multispot distribution compatible with different posttranslationally modified isoforms. In fact, all HDAC4 spots, with the exception of the one with the highest isoelectric point, corresponded to different phosphorylated species (Figure 1B). Putative phospho-acceptor sites were mainly Ser and Thr, because HDAC4-Flag failed to react with anti-phospho-Tyr antibody.

To further confirm the binding between HDAC4 and PP2A and to map the domains involved in the interaction, we performed GST-fusion protein pulldown experiments. Recombinant PP2A/C α and PP2A/65 α fused to GST were incubated with in vitro-translated HDAC4 wild-type (wt) or with

HDAC4 N- and C-terminal fragments representing the products of the caspase cleavage (Figure 2A). Figure 2B illustrates that the amino-terminal region of HDAC4 specifically interacts *in vitro* with the catalytic subunit of PP2A (PP2A/C α).

Within the N-terminal region of HDAC4 lies the transcription factors docking site responsible for binding various HDAC4 partners, among which the best characterized is MEF2C (Yang and Gregoire, 2005). Hence, we decided to map in more detail the segment of HDAC4 required for the interaction with PP2A/C α .

Various deletions of the N-terminal segment including the transcription factors docking site were generated (Figure 2A). All the different HDAC4 fragments were *in vitro* translated and incubated with GST alone or GST fused to PP2A/C α , PP2A/65 α , or MEF2C for comparison (Figure 2B). From these experiments we can assume that 1) MEF2C and PP2A/C α bind HDAC4 with similar efficiencies, thus confirming that PP2A is a relevant HDAC4 partner, and 2) MEF2C and PP2A/C α bind the same domain within the N-terminal region of HDAC4.

Figure 3. In vitro dephosphorylation of HDAC4-Flag by recombinant and endogenous HDAC4-associated PP2A. (A) Western blot showing total cell lysates prepared from E1A, SH-SY5Y, and C2C12 cells and analyzed with antibodies against HDAC4 and tubulin. E1A cells were treated with OA (1 μ M) for indicated times. SH-SY5Y and C2C12 were treated with OA (1 μ M) for 2 and 4 h, respectively. (B) HDAC4 immunoprecipitated from E1A cells left untreated or treated with OA were incubated with recombinant PP2A. Immunocomplexes were probed with the same anti-HDAC4 antibody. A fraction of the lysates without immunoprecipitation was used as input control (Total L). (C) A representative Western blot is shown developed with anti-phospho Ser/Thr antibodies (top), or anti-Flag antibodies (bottom). In lane 2, immunopurified HDAC4-Flag was incubated with recombinant PP2A, and it was treated for 60 min at 37°C with 100 nM okadaic acid in lane 3. (D) Phospho-HDAC4 quantification by densitometric scanning of the Western blot shown in C. Phospho-HDAC4 values were normalized for total HDAC4 values and expressed in arbitrary units assigning a value of 100 to the untreated sample. (E) In vitro phosphorylation/dephosphorylation assays. Cellular extracts from E1A cells left untreated or treated with OA (50 nM for 12 h) were used to perform in vitro phosphorylation of recombinant GST-HDAC4 fragment 241-601 or GST in the presence of γ ATP. After in vitro phosphorylation, recombinant proteins were incubated with PP2A as described in *Material and Methods*. (F) Western blot showing total cell lysates prepared from E1A cells, treated with OA (1 μ M), ratjadone (10 ng/ml), or with ratjadone before OA treatment and analyzed with antibodies against HDAC4 and tubulin.

We also confirmed that HDAC4 interacts with PP2A/C α through its N-terminal region in living cells. FLAG-tagged wt HDAC4, HDAC4/ Δ N, and HDAC4/ Δ C were expressed in E1A cells, and immunoprecipitations were performed using anti-FLAG or anti-PP2A/C antibodies. After SDS-PAGE electrophoresis, immunoblotting using anti-PP2A/C, anti-FLAG or anti-PR/65 α antibodies revealed the relative partners. Figure 2, C and D, show that a complex between HDAC4 and PP2A could be detected *in vivo* when immunoprecipitations were performed with the anti-FLAG or with the anti-PP2A/C antibodies. Similar to the *in vitro* studies, the N-terminal region of HDAC4 is also required for *in vivo* binding of PP2A.

Finally, we explored whether histone deacetylase inhibitors could influence the interaction between PP2A and HDAC4. Our studies indicate that HDAC4 also constitutes a complex with PP2A in the presence of the histone deacetylase inhibitor trichostatin A (data not shown).

PP2A Can Modulate HDAC4 Phosphorylation

To understand the molecular implications of the described interaction, we investigated whether PP2A could modulate HDAC4 phosphorylation. As a first step, we investigated the

Figure 4. OA impairs the nuclear import of HDAC4. (A) HDAC4-GFP expression was induced in U2OS cells after the addition of ponasterone A. Next, cells were treated with OA (1 μ M), with ratjadone (10 ng/ml), with both, or were left untreated as indicated. Fluorescent microscopy was used to visualize the subcellular localization of HDAC4-GFP. (B) Quantitative analysis of the experiments showed in A. Approximately 300 cells, from three independent experiments, were scored. Data represent arithmetic means $\pm$ SD.

effect of the PP2A inhibitor, OA, on HDAC4 phosphorylation. Electrophoretic mobility shift of HDAC4 was previously observed under conditions promoting its hyperphosphorylation *in vivo* (Grozinger and Schreiber, 2000); hence, immunoblotting was used to visualize the electrophoretic mobility of HDAC4 in lysates obtained from E1A cells treated with OA. Figure 3A shows that, in the presence of OA, migration of HDAC4 was clearly retarded in a time-dependent manner, presumably due to hyperphosphorylation. The mobility shift was also observed in SH-SY5Y neuroblastoma cells and, even though less pronounced, in the C2C12 cell line. These findings might reflect the participation of both PP1 and PP2A in dephosphorylation of HDAC4.

To demonstrate that the OA-dependent electrophoretic mobility shift of HDAC4 was indeed due to hyperphosphorylation and that PP2A can dephosphorylate HDAC4, we immunoprecipitated HDAC4 from OA-treated and untreated cells and the immunocomplexes were incubated with recombinant PP2A. The OA-dependent mobility shift of HDAC4 was abrogated after incubation with PP2A, thus confirming its reliance on hyperphosphorylation (Figure 3B). It is important to note that PP2A could also trigger a slight shift in the electrophoretic mobility of HDAC4 when it was added to the immunocomplex isolated from untreated cells.

To confirm that OA and PP2A modulate HDAC4 phosphorylation, we took advantage of the stable HEK293 Tet-ON cell clone. Because HDAC4 is highly phosphorylated at multiple Ser/Thr sites in this cell line (see Figure 1B), we tested the ability of recombinant PP2A to dephosphorylate immunopurified HDAC4-Flag in the absence of a pretreatment with OA. We used an anti-phospho Ser/

Thr specific antibody to measure HDAC4 phosphorylation levels.

As shown in Figure 3, C and D, HDAC4 phosphorylation levels decreased about twofold in the presence of the phosphatase, thus confirming that HDAC4 is a direct PP2A substrate. In addition, treatment of HDAC4-Flag with OA in the absence of recombinant PP2A further increased the phosphorylation status of the protein. This finding suggests that endogenous PP2A, coimmunoprecipitated with HDAC4, is enzymatically active and able to partially antagonize the action of kinases such as CaMKII δ that are present in the complex, as verified by the MS analysis (Figure 1A).

To further prove that PP2A triggers HDAC4 dephosphorylation, we used an *in vitro* phosphorylation assay. An HDAC4 fragment (aa 241–601) fused to GST was phosphorylated *in vitro* using cellular lysates from E1A cells treated with OA or left untreated. After phosphorylation, GST-HDAC4 fusion was incubated with recombinant PP2A. Results showed in Figure 3E confirm that OA promotes HDAC4 hyperphosphorylation and that PP2A can dephosphorylate HDAC4.

Finally, we explored whether nuclear accumulation of PP2A was associated with the mobility shift induced by OA treatment. E1A cells were treated with ratjadone to block nuclear export, with OA, or with ratjadone before OA treatment. Immunofluorescence studies confirmed that ratjadone induces nuclear accumulation of HDAC4 (data not shown). The immunoblot in Figure 3F demonstrates that pretreatment with ratjadone abolishes the mobility shift of HDAC4 induced by OA. This result suggests that the kinase/s responsible for HDAC4 hyperphosphorylation, in response to OA treatment, are localized in the cytoplasm.

Figure 5. PP2A is required for efficient nuclear import of HDAC4. (A) Western blot showing total cell lysates prepared from U2OS cells transfected with PP2A/C or Smac RNAi oligos and treated with ponasterone A for the indicated time to induce HDAC4-GFP expression. PP2A/C, Smac, and tubulin expression was evaluated by probing with the specific antibodies. (B) In U2OS cells silenced for PP2A/C or Smac, HDAC4-GFP expression was induced by ponasterone A for the indicated time. Next, cells were left untreated or treated with ratjadone to block nuclear export. Fluorescent microscopy images illustrate the subcellular localization of HDAC4-GFP. (C) Quantitative analysis of the experiments showed in (B). Approximately 300 cells from three independent experiments were scored. Data represent arithmetic means $\pm$ SD. (D) Western blot showing total cell lysates prepared from E1A cells transfected with PP2A/C or Smac RNAi oligos together with pEGFP-HDAC4 as described in (E). PP2A/C, Smac, and tubulin expression was evaluated by probing with the specific antibodies. (E) E1A cells transfected with pEGFP-HDAC4 and the indicated RNAi oligos after 44 h were further grown for 1 h in the presence of ratjadone (10 ng/ml). Fluorescent microscopy images illustrate the subcellular localization of HDAC4-GFP. (F) Quantitative analysis of the experiments showed in D. Approximately 300 cells, from three independent experiments, were scored. Data represent arithmetic means $\pm$ SD.

Okadaic Acid Controls HDAC4 Nuclear Import

HDAC4 possesses intrinsic nuclear import and nuclear export signals pivotal for the nuclear-cytoplasmic shuttling. Multiple serine/threonine kinases control HDAC4 nuclear-cytoplasmic trafficking through phosphorylation of critical serine residues (Yang and Gregoire, 2005). By interacting with and dephosphorylating HDAC4, PP2A could play a major role in the signaling network that controls HDAC4 subcellular localization. To investigate this hypothesis, we used an osteosarcoma cell line (U2OS) expressing HDAC4 fused to GFP under the control of a ponasterone-dependent promoter.

On treatment with OA, U2OS cells lose adherence and tend to detach from the culture dish into the medium (data not shown). For this reason, cells were subjected to cytospin before fixation. To ensure that cell detachment and cytospin did not alter HDAC4 localization, untreated cells were trypsinized and subjected to the same procedure.

Ratjadone, but not OA treatment, promoted the nuclear accumulation of HDAC4 (Figure 4). Most importantly, pretreatment with OA abolished the nuclear accumulation of HDAC4 elicited by ratjadone. These experiments demonstrate that OA blocks the nuclear import of HDAC4 and suggest that PP2A may control HDAC4 nuclear import.

PP2A Activity Is Required for HDAC4 Nuclear Import

OA inhibits many protein phosphatases, including protein phosphatase 1 (PP1) and PP2A. To more precisely establish the role of PP2A in the control of HDAC4 nuclear import, we

down-regulated the expression of the PP2A catalytic subunit (PP2A/C) by RNAi. U2OS cells were transfected with the RNAi specific for PP2A/C or with RNAi directed against the mitochondrial protein Smac, as a control. After transfection, HDAC4-GFP expression was induced by ponasterone and cells were treated with ratjadone to block nuclear export. The results shown in Figure 5A demonstrate that both PP2A/C and Smac were suppressed by RNAi, and more importantly, only the down-regulation of PP2A hampered the nuclear import of HDAC4 (Figure 5, B and C).

In U2OS cells, the import of HDAC4 was partially inhibited by the down-regulation of PP2A/C. The partial effect could be caused by incomplete silencing of PP2A/C or incomplete transfection of the siRNA, which is never complete.

To further confirm the role of PP2A, we decided to use E1A cells to transiently express HDAC4-GFP together with the RNAi for Smac and PP2A/C. Here again, Smac and PP2A/C expression levels were reduced by the specific RNAi (Figure 5D), whereas nuclear accumulation of HDAC4 in ratjadone treated cells was clearly impaired only by the RNAi specific for PP2A/C (Figures 5, E and F). To exclude the possibility that PP2A down-regulation indirectly affected nuclear import of HDAC4 by interfering with the nuclear import machinery, we evaluated the nuclear import of GFP fused to a NLS (Paroni *et al.*, 2007). PP2A down-regulation did not influence the nuclear import of NLS-GFP (Figure 6, D–F, and data not shown).

These data clearly prove that PP2A activity controls the nuclear import of HDAC4.

Figure 6. Caspase cleavage unbinds HDAC4 from the PP2A-dependent control of nuclear import. (A) Western blot showing total cell lysates prepared from U2OS cells transfected with PP2A/C or Smac RNAi oligos and treated with ponasterone A for the indicated time to induce HDAC4/ Δ C-GFP expression. PP2A/C, Smac, and tubulin expression was evaluated by probing with the specific antibodies. (B) In U2OS cells silenced for PP2A/C or Smac, HDAC4/ Δ C-GFP expression was induced by ponasterone A for the indicated time. Next, cells were left untreated or treated with ratjadone to block nuclear export. Fluorescent microscopy images illustrate the subcellular localization of HDAC4/ Δ C-GFP. (C) Quantitative analysis of the experiments showed in (B). Approximately 300 cells from three independent experiments were scored. Data represent arithmetic means $\pm$ SD. (D) Representative Western blot showing total cell lysates prepared from E1A cells transfected with PP2A/C or Smac RNAi oligos together with pEGFP-HDAC4/ Δ C. PP2A/C, Smac, and tubulin expression was evaluated by probing with the specific antibodies. (E) E1A cells transfected with pEGFP-HDAC4/ Δ C or pEGFP-HDAC4/TM, or pEGFP-NLS-GFP, and the indicated RNAi oligos after 44 h were further grown for 1 h in the presence of ratjadone (10 ng/ml). Fluorescent microscopy images illustrate the subcellular localization of the indicated GFP fusions. (F) Quantitative analysis of the experiments showed in D. Approximately 300 cells from three independent experiments were scored. Data represent arithmetic means $\pm$ SD.

The Caspase-cleaved Form of HDAC4 Is Constitutively Imported in the Nucleus in a PP2A-independent Manner

During apoptosis, HDAC4 is cleaved at Asp 289 by caspase-3 (Paroni *et al.*, 2004). The cleavage separates the N-terminal region containing the NLS from the C-terminal encoding the NES, thus favoring the nuclear accumulation of the N-terminal fragment and induction of apoptosis.

To evaluate the role of PP2A in the control of the nuclear import of the caspase-cleaved HDAC4, we generated U2OS cells expressing HDAC4/ Δ C (1–289) under ponasterone control. Next, as previously described for HDAC4 wt, cells were transfected with RNAi for PP2A/C or Smac, and after ponasterone addition, cells were incubated with ratjadone as indicated (Figure 6B). HDAC4/ Δ C nuclear import is unaffected by PP2A/C down-regulation. This result indicates that caspase-3 liberates HDAC4 from PP2A control.

The same results were obtained in E1A cells after transient transfection of HDAC4/ Δ C-GFP and silencing of PP2A/C (Figure 6D). Fluorescent microscopy and the quantitative analysis illustrated in Figure 6, E and F, confirmed that nuclear import of the caspase-generated fragment takes place independently of PP2A activity.

Phosphorylation of serines 246, 467, and 632 creates the binding sites for 14-3-3 proteins, which promote HDAC4 nuclear export and impair its nuclear import (Wang and Yang, 2001; Backs *et al.*, 2006). To explore whether additional serines, other than the 14-3-3 binding sites, which are implicated in the regulation of HDAC4 nuclear import, we analyzed the subcellular localization of the HDAC4 mutant (HDAC4/TM) in which serines 246, 467, and 632 were replaced with alanines (Paroni *et al.*, 2007).

E1A cells were transiently transfected with HDAC4/TM-GFP and RNAi for PP2A/C, or Smac as a control. As expected, fluorescent microscopy and the quantitative analysis illustrated in Figure 6, E and F, revealed that in the absence of ratjadone treatment, HDAC4/TM was largely nuclear in Smac silenced cells (compare Figure 5, E and F, with 6, E and F). Moreover, these figures also demonstrate that nuclear import of HDAC4/TM is partially impaired by the presence of the siRNA for PP2A. In fact, a cytoplasmic accumulation of the TM mutant in cells with down-regulated PP2A is apparent. This accumulation remarks that further serine/threonine residues, other than the 14-3-3 binding sites, can modulate HDAC4 nuclear import.

This result confirms that dephosphorylation of the 14-3-3 binding sites is important for the nuclear import of HDAC4, but it also suggests that additional phosphorylation events are involved in this control.

Serine 298 Plays a Critical Role in the Regulation of HDAC4 Nuclear Import

Our data suggest that PP2A could dephosphorylate critical serine/threonine residues in HDAC4, which are important to promote or impede its nuclear import. Recently, a role for CaMKII in blocking HDAC4 nuclear import has been suggested (Backs *et al.*, 2006), thus confirming that nuclear import of HDAC4 is also regulated by phosphorylation-dependent signals. Three serines (246, 467, 632) are critical targets, once phosphorylated, of the 14-3-3 proteins. Nuclear import of the HDAC4/TM was partially impaired by PP2A/C down-regulation (Figures 6, D–F). Moreover, OA treatment also produced an electrophoretic mobility shift in the case of HDAC4/TM (data not shown), and finally, the 2D gel analysis indicated that HDAC4 is phosphorylated on multiple residues.

Overall, this body of evidence implies that additional phosphorylation sites should be present in HDAC4, which could be target of PP2A to allow nuclear import.

Phosphorylation site prediction using bioinformatics tools has been useful to guide the identification of kinases' amino acid targets *in vivo*. To identify putative phosphoserine and phosphothreonine residues that could modulate nuclear import, the HDAC4 sequence was interrogated with NetPhos (www.cbs.dtu.dk/services/NetPhos/; Blom *et al.*, 1999) and ScanSite (scansite.mit.edu/; Obenauer *et al.*, 2003). Because PP2A binds the N-terminal region of HDAC4 and the caspase-cleaved form of HDAC4 shows unrestrained nuclear import, we rationalized that the putative critical phospho-residues should be located distal to the caspase-cleavage site. Hence, we focused our attention on the region of HDAC4 (aa 251–340), which comprises the NLS and the caspase-cleavage motif.

Both software programs predicted four serines to be a target of kinases within the selected region (Figure 7A). Serine 266 lies within the NLS, whereas serines 298, 302, and 339 are located after the caspase cleavage site (aa 289).

To verify the contribution of the predicted phosphoserines in the regulation of HDAC4 nuclear import, these residues were mutated to aspartic acid to mimic serine phosphorylation.

The different mutants were expressed in E1A cells as fusion proteins with GFP and the subcellular localization analyzed by fluorescent microscopy. All the generated S/D mutants accumulated in the cytosol similar to the wt (Figure 7, B and C).

When nuclear export was inhibited by ratjadone, mutants S266D, S302D, and S339D accumulated in the nucleus, similar to wt HDAC4. In contrast, under the same conditions, mutant S298D remained consistently confined to the cytoplasm and it did not efficiently accumulate in the nuclei like the wt form (Figures 7, B and C). Interestingly, serine 298 is conserved between human and chicken HDAC4 and among other class II family members with the exception of HDAC7 (Figure 7A).

We also confirmed the subcellular localization of the generated mutants in U2OS cells. The quantitative analysis represented in Figure 7D confirmed that the mutant S298D was unable to accumulate in the nucleus in response to ratjadone. Curiously, in U2OS, a limited effect on the efficiency of nuclear import was also observed in the case of the S302D mutant.

To confirm the role of serine 298 in the control of HDAC4 nuclear import, this residue was mutated to alanine in order to mimic a constitutive dephosphorylation. Next, the S298A mutant was expressed in E1A cells in which PP2A/C was down-regulated by the specific RNAi, and the localization of the mutant was observed in untreated and ratjadone treated cells in comparison with the wt protein (Figure 7F).

PP2A/C and Smac were efficiently down-regulated by the transfection of the specific RNAi (Figure 7E). In untreated cells silenced for Smac, the S298A mutant showed a cytosolic localization like the wt protein, and upon inhibition of nuclear export, it accumulated in the nucleus, again similarly to the wt. Conversely, in cells silenced for PP2A/C the S298A mutant accumulated in the nucleus more efficiently than the wt protein upon ratjadone treatment (Figure 7F). However, although less S298A mutant remained in the cytosol compared with the wt form, its import was incomplete, thus suggesting that phosphorylation of additional serine/threonines, such as the 14-3-3 binding sites, could still result in a certain amount of HDAC4 in the cytoplasm.

Figure 7. Identification of serine 298 as a critical residue in the control of HDAC4 nuclear import. (A) Sequence alignment of class IIa HDACs covering the region of HDAC4 region screened for the presence of Ser/Thr phosphorylation consensus sites. The putative

In summary, these results indicate that serine 298 plays an important role in the control of HDAC4 nuclear import.

MEF2C-dependent Nuclear Import of the S298D Mutant

HDAC4 nuclear accumulation is promoted by MEF2C (Borghi *et al.*, 2001). Thus, to study the effect of serine 298 on HDAC4 nuclear import in a ratjadone-independent context, we decided to investigate the subcellular localization of the S298D mutant when coexpressed with MEF2C. As a control, we generated HDAC4 lacking the NLS (aa 251–272; Wang and Yang, 2001). GFP fusions of HDAC4 wt, the S298D mutant, and Δ NLS were transfected in E1A cells alone, or in combination with MEF2C. For comparison, cells in which HDAC4 constructs were expressed alone were incubated with ratjadone.

As described above, the wt protein efficiently accumulated in the nucleus after inhibition of the nuclear export, whereas the S298D mutant was largely defective. Interestingly, we noted that blocking the nuclear export promoted, in many cells the accumulation into the nucleus of the Δ NLS mutant (Figures 8, A and C), thus indicating the existence of alternative mechanisms assisting HDAC4 nuclear import independently from the NLS.

As previously observed (Borghi *et al.*, 2001), MEF2C powerfully promoted the nuclear accumulation of HDAC4 wt (Figure 8, B and C). The S298D mutant, when coexpressed with MEF2C, was also imported quite efficiently into the nucleus (Figures 8, B and C). It is evident that the S298D mutant compared with the wt shows some defects in the nuclear import in the presence of MEF2C; however, in contrast to the ratjadone treatment, nuclear import of S298D was restored by MEF2C. Surprisingly, MEF2C promoted the nuclear import of the Δ NLS mutant with less efficiency, when compared with the S298D mutant. These experiments demonstrated that the S298D and Δ NLS mutants showed peculiar impairments in nuclear accumulation in response to specific signals, such as ratjadone treatment or MEF2C coexpression. Overall, these results suggest the existence of multiple mechanisms controlling the nuclear import of HDAC4.

Finally, we decided to explore the repressive activity of the S298D mutant on a MEF2C-dependent promoter. Figure 8D shows that the S298D mutant was less repressive on MEF2C transcriptional activity compared with the wt. This

result implies that PP2A could influence HDAC4-repressive activity.

DISCUSSION

Gene expression plasticity allows eukaryotic cells to rapidly respond to developmental and environmental inputs. HDACs, which are important modulators of gene expression, are subject to intense regulation by environmental signals. Serine/threonine phosphorylation elicited by different kinases controls HDACs functions at multiple levels. Phosphorylation can control HDAC enzymatic activities directly or indirectly by the achievement of subcellular relocalization of the HDACs (Verdin *et al.*, 2003, Yang and Gregoire, 2005; Berger, 2007). The phosphorylation state of any protein represents a balance of the actions of specific protein kinases and protein phosphatases. Recently, it was discovered that different HDACs make complexes with and are regulated by various protein phosphatases (Galasinski *et al.*, 2002; Brush *et al.*, 2004; Zhang *et al.*, 2005; Parra *et al.*, 2007). In this article we have demonstrated for the first time that HDAC4 forms a complex with PP2A. Proteomic mapping of the cytosolic HDAC4-partners, coimmunoprecipitation experiments, *in vitro* binding assays, and functional studies all confirmed the interaction between HDAC4 and PP2A.

PP2A is one of the major serine/threonine phosphatases implicated in the regulation of a broad range of cellular processes including cell cycle, DNA repair, apoptosis, membrane trafficking, and adhesion (Sontag, 2001; Janssens *et al.*, 2005). PP2A is a multiprotein complex formed by a catalytic subunit PP2A/C of 36 kDa, a structural/regulatory subunit PR65 or the 65-kDa A subunit, and a third regulatory subunit known as the B subunit. Each of these subunits is encoded by several distinct genes to form various holoenzymes once they are assembled (Janssens *et al.*, 2005). We have shown that in 293 cells, HDAC4 establishes a complex mainly with the PP2A holoenzyme α , $A\alpha$, B/PR55 α .

Proteins can associate with PP2A through different mechanisms. Certain PP2A partners interact with the free catalytic subunit, others interact with the regulatory subunits or with the oligomeric complex (Janssens and Goris, 2001; Janssens *et al.*, 2005). Our data indicate that HDAC4 associates with the oligomeric form of PP2A through the direct binding of the catalytic subunit PP2A/C. However, we cannot exclude that the B/PR55 α subunit also contributes to the binding. Indeed, both the PP2A/C and B55 δ regulatory subunits have been shown to take part in the interaction with securin (Gil-Bernabé *et al.*, 2006).

In some cases, the interaction with specific partners results in a negative regulation of PP2A activity (Janssens *et al.*, 2005). Although we have not determined the effect of HDAC4 on the specific activity of the phosphatase, our observations suggest that HDAC4 does not block PP2A enzymatic activity. Conversely, data presented herein strongly indicate that HDAC4 behaves as a PP2A substrate and that the phosphatase promotes HDAC4 nuclear import.

The following body of evidence supports these ideas: 1) OA and RNAi for PP2A/C both interfere with HDAC4 nuclear import; 2) the S298D mutant is impaired in nuclear import; 3) the S298A mutant shows a higher tendency to accumulate in the nucleus when PP2A expression is down-regulated; and 4) recombinant PP2A can dephosphorylate HDAC4 *in vitro*.

HDAC4 subcellular localization is under the intense control of different kinases devoted to excluding the deacetylase from the nucleus to promote transcription (Zhou *et al.*, 2000;

Figure 7 (cont). phosphorylation sites and the relative kinases are marked in bold. (B) Confocal images illustrating the subcellular localization of HDAC4 wt and of the full-length HDAC4 mutated in the indicated serines when expressed in E1A cells, as fusion with GFP, using the pEGFPN1 vector. Ratjadone was used to block the nuclear export. (C) Quantitative analysis of the experiments showed in B. Approximately 300 cells from three independent experiments were scored. Data represent arithmetic means $\pm$ SD. (D) Quantitative analysis of the subcellular localization of HDAC4 wt and the indicated mutants in U2OS cells, as fusion with GFP, using the pEGFPN1 vector. Approximately 300 cells from three independent experiments were scored. Data represent arithmetic means $\pm$ SD. (E) Representative Western blot showing total cell lysates prepared from E1A cells transfected with PP2A/C or Smac RNAi oligos together with pEGFP-HDAC4. PP2A/C, Smac, and tubulin expression was evaluated by probing with the specific antibodies. (F) E1A cells transfected with PP2A/C or Smac RNAi oligos together with pEGFP-HDAC4 or pEGFP-HDAC4/S298A, after 44 h were further grown for 1 h in the presence of ratjadone (10 ng/ml). Approximately 300 cells from four independent experiments were scored by fluorescent microscopy for HDAC4-GFP localization. Data represent arithmetic means $\pm$ SD.

Figure 8. Analysis of the HDAC4 S298D mutant nuclear. (A) Confocal images illustrating the subcellular localization of HDAC4, wt, Δ NLS, and S298D mutant when expressed in E1A cells. Ratjadone was used to block nuclear export. (B) Confocal images illustrating the subcellular localization of MEF2C and of HDAC4, wt, Δ NLS, or S298D mutant when coexpressed with MEF2C in E1A cells. Cells were fixed and stained with mouse anti-HA antibodies to visualize MEF2C. A representative of two independent experiments is shown. (C) Quantitative analysis of the experiments showed in A and B. Approximately 200 cells from three independent experiments were scored. Data represent arithmetic means $\pm$ SD. (D) IMR90-E1A cells were transfected with the 3X-MEF2-Luc luciferase reporter (1 μ g), the transfection control *Renilla* luciferase reporter pRL-CMV (20 ng), pcDNA3.1-HA-MEF2C (1 μ g), and with 10 or 100 ng of pEGFP expressing HDAC4 wt or the S298D mutant. pEGFP alone, 100 ng, was used as a reference. Cells were lysed 24 h after transfection. Data represent arithmetic means $\pm$ SD of three independent experiments.

Chang *et al.*, 2005; Backs *et al.*, 2006; Dequiedt *et al.*, 2006; Berdeaux *et al.*, 2007). Therefore, it would be expected that phosphatases promote nuclear accumulation of HDAC4 and transcriptional repression.

Proteins destined for transport into the nucleus contain amino acid targeting sequences called nuclear NLSs. NLSs mediate passage of cargo proteins into the nucleus, conferring recognition by specific members of the IMP importins superfamily, in which multiple α - and β -forms are known. Nuclear protein import is mediated either by one of a number of IMP β s or by a heterodimer of IMP α / β 1, where NLS recognition occurs through IMP α interaction (Poon and Jans, 2005; Lange *et al.*, 2007). Residues 251–272 of HDAC4 constitute a functional arginine/lysine-rich NLS showing a tripartite organization (Wang and Yang, 2001), which can interact *in vivo* with IMP α (Grozinger and Schreiber, 2000).

Phosphorylation of specific residues is a common strategy used to influence IMPs binding to the NLS and as a consequence nuclear import (Poon and Jans, 2005; Lange *et al.*,

2007). Depending on its subcellular localization, CaMKII can either induce nuclear export or block nuclear import of HDAC4. Phosphorylation of serines 246, 467, and 632 creates the 14-3-3 binding sites necessary for nuclear export and may also block nuclear import (Backs *et al.*, 2006). In fact, when FLAG-tagged HDAC4 was isolated from cells treated with the PP1 and PP2A inhibitor calyculin, the amount of coimmunoprecipitated IMP α was dramatically reduced, whereas binding to 14-3-3 was augmented (Grozinger and Schreiber, 2000).

In the current model, 14-3-3 blockade of the HDAC4 NLS might be important to inhibit its nuclear import (Bridges and Moorhead, 2004). In this study, we have confirmed that HDAC4 nuclear import is controlled by phosphorylation, and we provide evidence for novel regulatory mechanisms that act through PP2A on serine 298.

Why does serine 298 play such a pivotal role in the regulation of HDAC4 nuclear import? Different hypotheses can be formulated. Dephosphorylation/phosphorylation at

serine 298 could enhance/repress IMP binding to the NLS either dependently or independently of 14-3-3 proteins. However, in contrast with serine 246, which is the more plausible candidate for such regulation, serine 298 does not lie in close proximity to the NLS. Alternatively, phosphorylation at serine 298 could mediate the interaction with cytosolic anchoring proteins or reduce the binding of factors promoting its nuclear import.

Indeed, some data indicate that regulation of HDAC4 nuclear import could be more complex. HDAC4 can also accumulate in the nucleus in the absence of the intrinsic NLS. Hence, it is possible that more than one mechanism supports HDAC4 nuclear import. The existence of multiple import pathways may ensure a more sophisticated regulation of HDAC4 suppressive activities.

It is important to note that caspase processing of HDAC4 at Asp 289 could release the deacetylase from PP2A control on serine 298 (Paroni *et al.*, 2004). This cleavage would impinge on the nuclear accumulation of the HDAC4 N-terminal fragment which contains the NLS and is also devoted to proapoptotic activities.

In conclusion, we have identified PP2A as a new HDAC4 interaction partner, which controls the nuclear import of the deacetylase. It is evident that regulation of nuclear import could be only a single aspect of a versatile interplay between PP2A and HDAC4. For example, the deacetylase could act as a platform to tether the phosphatase in close proximity to different HDAC4 interactors in order to promote their dephosphorylation (Canettieri *et al.*, 2003). Previous studies have suggested that this holds true for calcineurin, also known as protein phosphatase 2B. HDAC4 can interact with calcineurin. It has been hypothesized that MEF2 activation, which includes dephosphorylation of serine 444 and inhibition of sumoylation on lysine 439, as induced by calcineurin, is permitted by the recruitment of the phosphatase to MEF2, through HDAC4 (Gregoire *et al.*, 2006). To this end, our preliminary data indicate that a complex between HDAC4 and PP2A could also exist in the nucleus. This complex, through the dephosphorylation of the 14-3-3 binding sites, could also be important for inhibiting HDAC4 export into the cytosol.

Further investigations of the interplay between HDAC4 and PP2A should shed light not only on how developmental and environmental signals control gene expression plasticity in eukaryotic cells, but also on how alterations in PP2A and HDAC4, which are evident in certain tumors (Sjoberg *et al.*, 2006; Stark and Hayward, 2007), could affect gene expression during cancer development.

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