Protein phosphatase 2A controls the activity of histone deacetylase 7 during T cell apoptosis and angiogenesis

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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. Although a lot of effort has been made toward 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 functions. This study unravels a dynamic interplay among 14-3-3s, protein kinases, and PP2A and provides a model for the regulation of class IIa HDACs.

chromatin | shuttling | endothelial cells | thymocytes | 14-3-3

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 (1). 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 (1). In humans, HDAC7 is transiently and predominantly expressed in CD4/CD8 double positive thymocytes, where it represses the expression of *nur77*, a proapoptotic gene involved in negative selection (2). Recently, *in situ* hybridization unraveled expression of HDAC7 in the vascular endothelium of the developing 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 were attributed to the up-regulation of matrix metalloproteinase 10 (MMP-10), a secreted proteinase that degrades the extracellular matrix (3).

Modulation of class IIa HDAC subcellular distribution by phosphorylation provides the opportunity to control these important developmental processes. Although a great deal of effort has been invested in identifying the kinases targeting class IIa HDAC 14-3-3 motifs (4–13), the identity of antagonistic phosphatase(s) remains elusive.

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 (amino acids 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} (8), 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. 1*A*). This phosphatase activity was inhibited by calyculin A, an inhibitor of PP1 and PP2A, but not by the potent PP2B inhibitors cyclosporine A and FK506 [supporting information (SI) Fig. 6]. In addition, okadaic 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. 1*B*). 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 Fig. 1*C*, 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 HPLC analysis (7). A time-dependent reduction of phosphorylation was observed for each serine residue (Fig. 1*D*). 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 dephosphoryla-

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The authors declare no conflict of interest.

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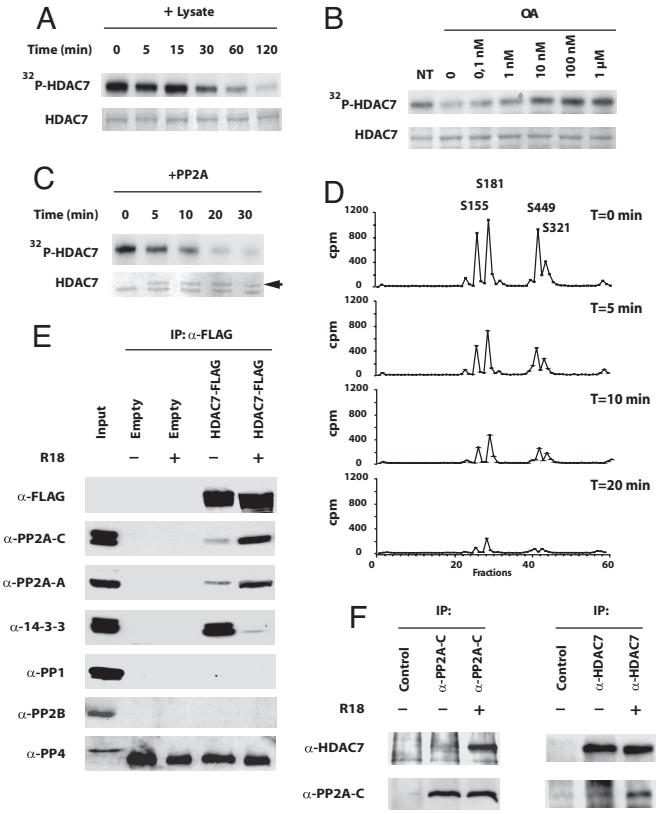

Fig. 1. PP2A dephosphorylates the 14-3-3 binding sites in HDAC7 *in vitro*. (*A*) The 14-3-3 binding sites of HDAC7 were phosphorylated by recombinant PKD in the presence of radioactive ATP. The radiolabeled protein (^{32}P-HDAC7) was incubated at 30°C with total cellular extracts. At the indicated times, aliquots were analyzed by SDS/PAGE and dephosphorylation was assessed by autoradiography (*Upper*) and Coomassie blue (*Lower*). (*B*) The ^{32}P-labeled HDAC7 protein was incubated for 1 h at 30°C without (NT) or with cell extracts containing increasing concentrations of OA. Residual phosphorylation of HDAC7 was assessed as described in *A*. (*C* and *D*) The ^{32}P-labeled HDAC7 protein was incubated with purified PP2A. Time-dependent dephosphorylation was assessed by SDS/PAGE (*C*) or HPLC analysis (*D*) as described in ref. 7. The 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 (25 μM) and analyzed by immunoblotting with the indicated antibodies. (*F*) Endogenous PP2A (*Left*) or HDAC7 (*Right*) was 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.

tion occurs without any preference among the four phosphorylation sites.

PP2A Associates with HDAC7 *in Vivo*. In cells, PP2A holoenzymes comprise a heterodimeric core complex, made of 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 (14). 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. 1*E*). 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 interact with HDAC7 *in vivo*. Importantly, the interaction between HDAC7 and the core PP2A enzyme is highly specific, because 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. 1*E*).

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 (2). As shown in Fig. 1*F*, 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.

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} (7). No significant dephosphorylation of either serine residues was observed under these conditions (Fig. 2*A*). 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. Whereas dephosphorylation of HDAC7 by PP2A was nearly total in the presence of GST, addition of GST-14-3-3ζ completely prevented HDAC7 dephosphorylation (Fig. 2*B*).

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 (15). 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. 2*C*, HDAC7 P157A). Although this mutation did not impair Ser^{155} phosphorylation by the known HDAC7 kinases (SI Fig. 7), it greatly reduced the interaction of 14-3-3 with Ser^{155} both *in vitro* and *in vivo* (SI Fig. 8). 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. 2*D*). 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 (7). Importantly, treatment with OA restored phosphorylation of the P157A HDAC7 mutant protein (SI Fig. 9). 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

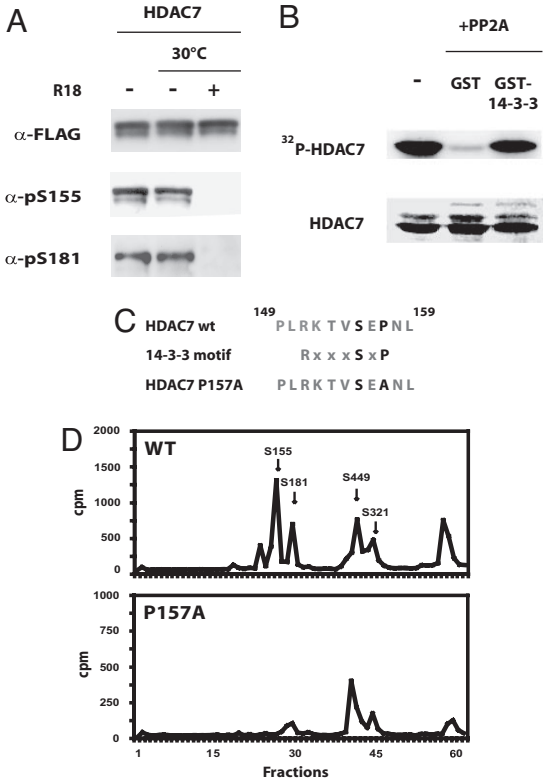

Fig. 2. 14-3-3 proteins prevent dephosphorylation of HDAC7 by PP2A. (*A*) Lysates from HEK293 cells expressing Flag-tagged HDAC7 were prepared in phosphatase assay buffer and incubated at 30°C for 30 min in the absence or presence of the R18 peptide. Dephosphorylation of HDAC7 was analyzed by Western 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. When indicated, GST-14-3-3ζ or GST alone was incubated with PP2A. Dephosphorylation was assessed after SDS/PAGE, Coomassie blue staining (*Lower*), and autoradiography (*Upper*). (*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 and immunoprecipitated, and their phosphorylation profile was established by HPLC analysis. Labeled peaks containing each phosphorylation site serine residue are indicated.

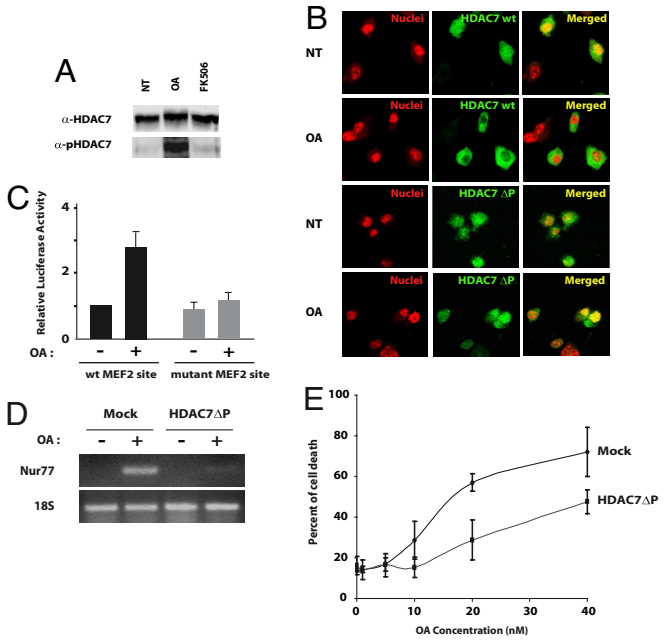

Fig. 3. PP2A participates in the regulation of *Nur77*-mediated apoptosis in T cell hybridomas by HDAC7. (*A*) Do11.10 cells were left untreated (NT) or were treated with OA (20 nM) or FK506 (600 nM) for 5 h. Phosphorylation of endogenous HDAC7 was then detected by Western blotting with antibody specific 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 (−). 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 10 h. 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 eight experiments performed from two independently established sets of polyclonal cell lines.

results strongly support the model in which association with 14-3-3s prevents dephosphorylation of HDAC7 by PP2A.

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 (16). As illustrated for Ser^{181}, treatment with OA dramatically increased the basal phosphorylation of 14-3-3 motifs on endogenous HDAC7 (Fig. 3*A*). 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. 3*B*). 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 (7)]. These data demonstrate that inhibition of PP2A activity is associated with hyperphosphorylation and cytoplasmic sequestration of HDAC7.

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 relocalization of HDAC7 (2). Because 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. 3*C*). The above observations were next confirmed on a MEF2D-regulated cellular gene. Indeed, treatment with OA was associated with derepression of the *Nur77* promoter (SI Fig. 10) and a significant increase in levels of *Nur77* mRNA (Fig. 3*D*). 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

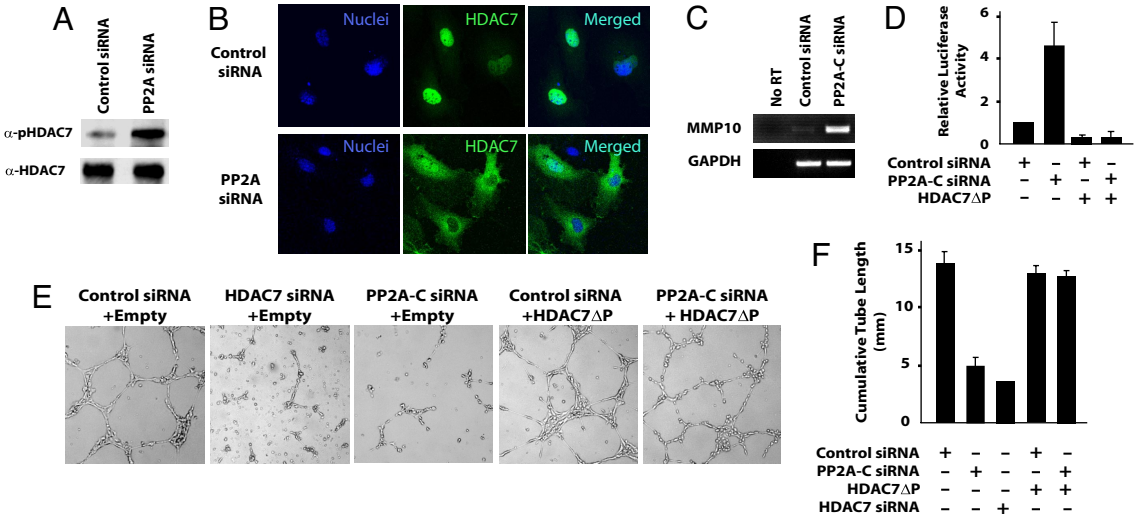

Fig. 4. PP2A controls the angiogenic activity of HDAC7 in HUVECs. (*A*) Endogenous HDAC7 was immunoprecipitated from HUVEC transfected with siRNA against PP2A catalytic subunit and analyzed by Western blotting with an antibody specific for phosphorylated Ser[181] (α-pS181). (*B*) Twenty-four hours after siRNA treatment as described in *A*, HUVECs were transfected with a plasmid coding for a GFP-HDAC7. Subcellular localization of HDAC7 (green) was examined by confocal microscopy. Cell nuclei were visualized by Topro counterstain (blue). (*C*) HUVECs were transfected as in *A*. Twenty-four hours after 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. Results are means + 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 of four are shown. (*F*) Cumulative length of capillary-like structures was measured by light microscopy after 24 h in four independent experiments as described in *E*. Data are presented as means + SD.

HDAC7-mediated transcriptional repression by controlling its phosphorylation and subcellular localization.

PP2A Regulates the Biological Functions of HDAC7. By modulating the expression of *Nur77*, HDAC7 is a key regulator of apoptosis in developing thymocytes (2). 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. 3*E*). 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 proapoptotic *Nur77* gene.

Suppression of HDAC7 expression in human umbilical vein endothelial cells (HUVECs) is associated with up-regulation of *MMP10* expression and impairs the ability of endothelial cells to form a vascular-like network (3). To further establish the functional significance of our findings, we used siRNA to inhibit expression of PP2A catalytic subunits in endothelial cells (HUVECs). RT-PCR and Western blot analysis confirmed the efficient and specific knockdown of both PP2A-C isoforms after siRNA treatment (SI Fig. 11). 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. 4*A*). 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. 4*B*).

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 (3). 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 up-regulation of *MMP10* expression (Fig. 4*C*). Importantly, activation of the *MMP10* promoter after PP2A knockdown was totally inhibited by overexpression of the HDAC7ΔP mutant (Fig. 4*D*). In an *in vitro* model of angiogenesis, HUVECs grown on Matrigel spontaneously form a primitive vascular-like network. As reported by others (3), HDAC7 plays a crucial role in this process, because HUVECs failed to organize into capillary-like structures when transfected with HDAC7 siRNA (Fig. 4*E*). Consistent with our model, specific inhibition of endogenous PP2A-C expression by siRNA reduced capillary tube formation of HUVECs (Fig. 4 *E* and *F*). Strikingly, overexpression of the phosphorylation-deficient HDAC7ΔP mutant counteracted the effects of PP2A knockdown and totally restored the angiogenic activity 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.

PP2A Controls the Subcellular Localization 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. 5*A*). In addition, purified PP2A readily dephosphorylated the 14-3-3 sites in HDAC4 and -5 in an *in vitro* dephosphorylation assay (Fig. 5*B*). 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 HDAC subcellular localization. In HeLa cells treated with control siRNA, class IIa HDACs mainly localize in the nuclear compartment (Fig. 5 *C* and *D*). 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.

Discussion

In a recent study we demonstrated basal phosphorylation of class IIa HDACs in normally growing cells (7). Several of the observa-

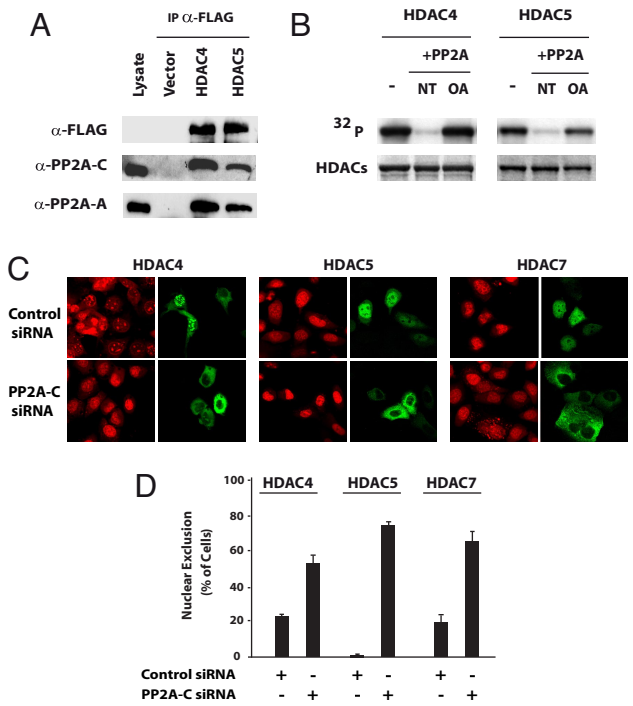

Fig. 5. Regulation by PP2A is a common feature of class IIa HDACs. (*A*) Flag-tagged HDAC4 and -5 were immunoprecipitated from HEK293 cells and analyzed by immunoblotting with the indicated antibodies. (*B*) Dephosphorylation of the N-terminal domains of HDAC4 or -5 was performed in the absence (−) or presence of purified PP2A (+PP2A). Where indicated OA was added to the reaction mixture. Residual phosphorylation was assessed after SDS/PAGE, Coomassie blue staining (*Lower*), and autoradiography (*Upper*). (*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 nontargeting 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 three independent experiments as described in *C*.

tions 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 knockdown 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 nucleocytoplasmic shuttling of class IIa HDACs. In this study we unexpectedly identify a role for 14-3-3 proteins in the phosphorylation and nucleocytoplasmic shuttling of class IIa HDACs that might challenge this model. Because 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. Although 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 article, a study was published reporting that protein phosphatase 1β (PP1β) and myosin phosphatase targeting subunit 1 (MYPT1), two subunits of the myosin phosphatase complex, copurify with Flag-HDAC7 (17). 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. Independent 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 (18, 19). 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 affect HDAC7 phosphorylation directly or via an indirect mechanism such as (*i*) inactivation of a class IIa HDAC kinase or (*ii*) impaired 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 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 (20–24). Our results also strongly indicate that dephosphorylation by PP2A may be a common feature among 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 (25, 26), muscle differentiation (12) and activity (27), and bone formation (28).

Materials and Methods

Plasmids, Antibodies, and Chemicals. Descriptions of the plasmids, antibodies, and chemicals are provided in *SI Text*.

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 ref. 29. SDS/PAGE and Western blot analysis were performed according to standard procedures.

GST and His Fusion Proteins: Expression and Purification. GST fusion proteins were purified according to protocols described elsewhere (7). His-N-ter-HDAC7 was purified by using a Cobalt-based immobilized metal affinity chromatography resin (BD Biosciences Clontech).

Reporter Assays. Reporter assays in Do11.10 cells were performed with Dual Luciferase reporter assay (Promega) following the protocol described in ref. 2. Twenty-four hours after siRNA transfection, HUVEC cells were transfected with the indicated plasmids and processed for Dual Luciferase reporter assays 24 h later, following the procedure described in ref. 30. All transfections were performed in duplicate, and data presented are 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 by 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 (2).

For *in vivo* phosphatase inhibition experiments, OA and FK506 were added in the culture medium at 20 nM and 600 nM, respectively, for 5 h.

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 refs. 7 and 8.

***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 1 h 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 units of purified PP2A A/C heterodimer (for Figs. 1 *D* and *E* and 3*B*) or A/C/B55α heterotrimer (for Fig. 5*B*). For protection experiments by 14-3-3s, recombinant GST-14-3-3ζ or GST alone was 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 2$\times$ SDS/PAGE sample buffer and boiling for 5 min. 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 (25 μM). Dephosphorylation of HDAC7 was analyzed by SDS/PAGE and Western blotting analysis using the phosphospecific antibodies.

RNAi. Two functionally validated On Target Plus siRNA molecules directed against the α or β isoforms of PP2A catalytic subunit, or the corresponding nontargeting control siRNA were purchased from Dharmacon. 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 three independent fields each containing $>$50 cells. Visualization of the cell nuclei was achieved by staining of DNA with Draq5 (Biostatus) 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, Metabolic Labeling, and Phosphorylation Site Analysis. These assays were carried out according to protocols described in our previous studies (2, 7, 8).

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 h later. Assays were performed exactly as described in ref. 30.

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