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mHDA1/HDAC5 Histone Deacetylase Interacts with and Represses MEF2A Transcriptional Activity*

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Abstract

Recently we identified a new family of histone deacetylases in higher eukaryotes related to yeast HDA1 and showed their differentiation-dependent expression. Data presented here indicate that HDAC5 (previously named mHDA1), one member of this family, might be a potent regulator of cell differentiation by interacting specifically with determinant transcription factors. We found that HDAC5 was able to interact *in vivo* and *in vitro* with MEF2A, a MADS box transcription factor, and to strongly inhibit its transcriptional activity. Surprisingly, this repression was independent of HDAC5 deacetylase domain. The N-terminal non-deacetylase domain of HDAC5 was able to ensure an efficient repression of MEF2A-dependent transcription. We then mapped protein domains involved in the HDAC5-MEF2A interaction and showed that MADS box/MEF2-domain region of MEF2A interacts specifically with a limited region in the N-terminal part of HDAC5 which also possesses a distinct repressor domain. These data show that two independent class II histone deacetylases HDAC4 and HDAC5 are able to interact with members of the MEF2 transcription factor family and regulate their transcriptional activity, thus suggesting a critical role for these deacetylases in the control of cell proliferation/differentiation.

Introduction

Acetylation of chromatin proteins and transcription factors is part of a complex signaling system that is largely involved in the control of gene expression (1). Thus far, the specific involvement of histone acetyltransferases and deacetylases in the control of individual gene expression has been clearly established (2, 3). One major role of these enzymes is the control of cell differentiation in response to specific signals. Evidence exists for the participation of the RPD3-related members in this process (4). Recently, however, a new family of higher eukaryotic histone deacetylases, distinct from the already characterized RPD3-related members, has been identified (5-7). These enzymes are related to yeast HDA1 histone deacetylase and within the cloned members, two show sequence homology and the same domain organization and are called HDAC4 and HDAC5 (5, 6). Despite this homology, HDAC4 and HDAC5 are probably capable of exerting distinct functions, since immunoprecipitation experiments showed that in cells they can be associated with different partners (6). A member, named mHDA2/HDAC6, shows unique features within deacetylases, in that it possesses two HDA1 homology domains (5, 6). In contrast to the RPD3-related members, the expression of these genes is not ubiquitous. HDAC5 and mHDA2/HDAC6 expression is activated upon cell differentiation (5). These observations suggest that members of the class II histone deacetylases may play a specific role in the regulation of cell differentiation. Looking for potential partners of HDAC5, we obtained evidence of interaction

between HDAC5 and MEF2 transcription factors. We therefore focused our efforts on investigating this issue. The MEF2 family of transcription factors belongs to the large family of MADS box transcriptional regulators present from yeast to humans (8). Besides their established role in myogenesis (9), MEF2 family members have been implicated in gene activation in response to mitogenic signaling (10, 11; for review, see Ref. 9). Very recently, it has been shown that one member of the class II histone deacetylase, HDAC4, can interact with two different members of MEF2 family, MEF2A (12) and MEF2C (13). Here we show that HDAC5, a distinct member of class II deacetylase, is also able to interact specifically with the MEF2A transcription factor and to repress its transcriptional activator capacity. Data presented here and the fact that the induction of various differentiation programs was found to be associated with an up-regulation of HDAC5 gene expression (5), suggested that HDAC5 might be a general regulator of cell differentiation. We discuss here the possibility that HDAC5 might control the early stages of various differentiation programs by inhibiting the precocious activity of determinant transcription factors.

Materials and Methods

DNA Constructs-- MEF2-Luc, L8G5-Luc, and L8-Luc reporter plasmids were generated from MEF2-CAT¹ (14), L8G5-CAT, and L8-CAT (15), by replacing the CAT reporter gene by luciferase (respective promoter regions were cloned in the pGL2 basic vector, Promega). PMT2-MEF2A (14), pcDNA-mHDA2/HDAC6 (5), and LexA-VP16 (15) expression vectors have been described before. Deletions in HDAC5 were obtained by polymerase chain reaction. The resulting DNA fragments were cloned in pcDNA3.1 (Invitrogen) in-frame with a N-terminal HA-tag. GAL4 DNA-binding domain fusion protein constructs were generated by polymerase chain reaction. DNA fragments coding for HDAC5 amino acids 123-673 and 673-1113 were cloned in pcDNA-GAL4 DB, in-frame with the DNA-binding domain (amino acids 1-147) of GAL4. Mouse HDAC4 and human MITR cDNAs have been obtained by polymerase chain reaction from mouse embryo and human skeletal muscle cDNA libraries, respectively (Marathon ready cDNA, CLONTECH).

Transfection-- HeLa cells were seeded in 6-well dishes (10^5 cells/well) and transfected 2 days later with Exgen 500 (Euromedex) according to the supplier's protocol. In each transfection, DNA amount was kept constant with pcDNA3 empty vector. Cell extracts were prepared 24 h post-transfection. Luciferase and β -galactosidase activities were measured using the "Luciferase Assay System" (Promega) and the Luminescent β -Gal Detection kit (CLONTECH), respectively.

In Vitro Interaction-- Glutathione *S*-transferase (GST) pull-down assays were performed as described (16). GST fusion proteins were produced in BL21 *Escherichia coli* transformed with pGEX-5X-3 plasmid (Amersham Pharmacia Biotech), encoding GST alone, or GST fused to various domains of MEF2A protein. ³⁵S-Labeled proteins were produced in rabbit reticulocyte lysate from pcDNA plasmids using the TNT transcription/translation kit (Promega) and [³⁵S]methionine (Amersham Pharmacia Biotech).

Co-immunoprecipitation, Immunofluorescence, and Western Blot-- HeLa cells were transfected with Exgen500 as described above using 5 μ g of pMT2-Mef2A or pMT2 vector and 5 μ g of pcDNA-HDAC5 (HA tagged) or pcDNA empty plasmid per 100-mm plate. 24 h post-transfection, cells were washed in cold phosphate-buffered saline and lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride for 20 min on ice. After centrifugation ($12,000 \times g$, 5 min, 4 °C) extracts were diluted

with 1 volume of lysis buffer without Nonidet P-40 and incubated with 4 μ g of polyclonal anti-MEF2 antibody for 1 h at 4 °C. 30- μ l aliquots of protein G-Sepharose were added and the incubation was continued for 2 h. Precipitates were washed in lysis buffer containing 0.25% Nonidet P-40 and finally re-suspended in SDS-polyacrylamide gel electrophoresis loading buffer. Western blot analysis was performed using standard procedures and immunocomplexes were detected by chemiluminescence (ECL+, Amersham Pharmacia Biotech). Immunofluorescence was performed as described (17). The following primary antibodies have been used: anti-MEF2 (C-21, Santa Cruz), anti-HA (3F10, Roche Molecular Biochemicals), and anti-HDAC5 (rabbit polyclonal antibodies raised against a peptide localized at the C-terminal region of the protein).

Results

MEF2A Transcription Factor and HDAC5 Are Found in a Complex in Vivo-- In our previous work we reported the domain organization of HDAC5 histone deacetylase (5). The histone deacetylase domain of this protein was found to be located at the C-terminal half of the protein and the N-terminal non-deacetylase domain did not show any significant homology with sequences present in the data base. In order to have insight into the function of HDAC5, we periodically searched the data bank for new sequences homologous to the N-terminal domain of HDAC5. This approach allowed us to identify a *Xenopus* cDNA presenting significant sequence homology with the non-deacetylase N-terminal domain of HDAC5 (accession number, Z97214, the putative protein encoded by this cDNA is now known as MITR, Ref. 18). The GenBank sequence information presented MITR as a potential partner of *Xenopus* MEF2 homologues. The sequence homology between HDAC5 and MITR suggested that HDAC5 could also be a partner of MEF2 transcription factors. In order to investigate this possibility, we first expressed HDAC5 and MEF2A in HeLa cells and examined the formation of complexes bearing both proteins *in vivo* by co-immunoprecipitation. Extracts obtained from cells transfected with HA-tagged HDAC5, MEF2A, and both expression vectors were immunoprecipitated with anti-MEF2 antibodies. The presence of MEF2A and HA-HDAC5 in immunoprecipitated material was then monitored using anti-HA and anti-MEF2 antibodies. Fig. 1 shows that anti-MEF2 antibodies are able to co-immunoprecipitate efficiently HDAC5 when both proteins are expressed in cells (Fig. 1, *anti-HA panel, far right*). The endogenous MEF2 in HeLa cells can also interact with HDAC5 since ectopically expressed HDAC5 was also found associated with endogenous MEF2, in the absence of exogenous MEF2A expression (Fig. 1). It is interesting to note that HeLa cells transfected with HDAC5-expression vector produced two HA-tagged HDAC5 proteins (Fig. 1, *input panel*) and within these two proteins only the full-length one could interact with MEF2A.

HDAC5 Interacts Directly with MEF2A-- In order to investigate the possibility of direct interaction between HDAC5 and MEF2A, we prepared bacterially expressed fusion proteins containing GST fused to different regions of MEF2A and examined the interaction of these proteins with ³⁵S-labeled full-length HDAC5. Fig. 2A shows that a GST fusion protein harboring the N-terminal half of MEF2A is able to interact efficiently with HDAC5 (Fig. 2A, GST-MEF2A 1-243), while the C-terminal half and the middle part of the protein did not recognize HDAC5 (Fig. 2A, GST-MEF2A 244-507 and GST MEF2A 87-243). This observation suggested that the region encompassing MADS box and the so-called MEF2 domain, has the ability to interact with HDAC5. To know whether MADS box or MEF2 domain or both are involved in the interaction with HDAC5, we fused both or each of these domains to GST and analyzed the interaction with HDAC5 as above. These experiments

showed that MADS box/MEF domain is sufficient to direct an efficient interaction with HDAC5 (Fig. 2A, GST MEF2A 1-86). However, the MADS box alone was found to be able to interact weakly with HDAC5 (Fig. 2A, GST MEF2A 1-57) while the MEF2 domain was not able to do so (Fig. 2A, GST MEF2A 58-86). The specificity of this interaction was shown by considering the capacity of MEF2A N-terminal region to interact with two other deacetylases, HDAC1 and mHDA2/HDAC6. In this experiment we also used HDAC4 and MITR as known partners of MEF2. Data show in Fig. 2B confirmed the interaction of these proteins with MEF2A as it has been reported very recently (12, 13, 18). However, no interaction between MEF2A and HDAC1 or mHDA2/HDAC6 was observed in these conditions (Fig. 2B), showing the specific interaction between HDAC5 and MEF2A in our assays.

A Limited Region in the N-terminal Non-deacetylase Domain of HDAC5 Is Involved in the Interaction with MEF2A-- To determine regions in HDAC5 involved in the interaction with MEF2A, expression vectors encoding HDAC5 deletion mutants were prepared and used to obtain corresponding ³⁵S-labeled proteins. These proteins were incubated with a GST-MEF2A MADS box/MEF2 domain fusion protein and a pull-down was performed. A truncated HDAC5 molecule lacking the first 123 amino acids was capable of interacting with MEF2A, while the removal of additional 57 amino acids completely abolished the binding (Fig. 2C, compare HDAC5 123-1113 and 180-1113 proteins). This observation showed that the region of 57 amino acids, located between amino acids 123 and 180 plays a major role in interaction with MEF2A. In order to confirm these findings, we expressed ³⁵S-labeled polypeptides corresponding to the 123-292 and 123-673 regions of HDAC5 and found that both interact efficiently with MEF2A (Fig. 2C, HDAC5 123-292 and HDAC5 123-673, respectively). In these assays the deacetylase domain of HDAC5 could not interact with MEF2A (Fig. 2C, HDAC5 674-1113). These experiments showed that a limited region of HDAC5 encompassing amino acids 123-180 plays an essential role in the direct interaction with MEF2A.

HDAC5-MEF2A Interaction Represses the Transactivator Capacity of MEF2A-- A transient transfection assay was designed to evaluate the role of HDAC5-MEF2A interaction on the transcriptional activity of MEF2A. The reporter system used consisted of the minimal promoter region of myosin heavy chain gene flanked by two MEF2-binding sites (14), cloned upstream of a luciferase gene. In HeLa cells, the expression of MEF2A led to a 20-fold activation of reporter gene expression (Fig. 3A). Co-expression of MEF2A and HDAC5 showed a very efficient inhibition of the activator capacity of MEF2A. Indeed, the use of as little as 1 or 5 ng of HDAC5 expression vector in these co-transfection assays, repressed significantly MEF2A activity (Fig. 3A). We then showed that the repression of MEF2A activity by HDAC5 was as efficient as that observed by HDAC4 (12). However, the capacity to repress MEF2A was not shared by all members of class II histone deacetylases, since the expression of the third member of this family (mHDA2/HDAC6) did not alter MEF2A activity (Fig. 3B).

We then tried to show whether HDAC5-MEF2A interaction is necessary to repress MEF2A activity. HeLa cells were co-transfected with the MEF2 responsive reporter, a MEF2A expression vector and vectors expressing different deletion mutants of HDAC5. As shown before, the expression of full-length HDAC5 repressed efficiently MEF2A activity (Fig. 3D, 1-1113 construct). Interestingly, this repression was found to be independent of the

deacetylase domain. Indeed a truncated HDAC5 bearing only the non-deacetylase domain of the protein was almost as efficient in repressing the MEF2A transcriptional activity as the full-length HDAC5 (Fig. 3D, compare 1-1113 and 123-673 constructs). This repression was found to be dependent on MEF2A-HDAC5 interaction, since a deletion mutant lacking the N-terminal region, defined to be the site of interaction with MEF2A, was absolutely inefficient in repression (Fig. 3D, 180-1113 construct). However, HDAC5/MEF2A interaction alone was not sufficient to repress transcription since two constructs expressing truncated versions of the N-terminal non-deacetylase domain of HDAC5, able to interact with MEF2A *in vitro*, were unable to repress transcription (Fig. 3D, 123-292 and Δ 241-292 constructs, respectively). This experiment showed that the N-terminal region of HDAC5 possesses in addition to the MEF2-binding domain, a distinct repression domain. This region is localized C-terminal to the MEF2-binding domain and covers a region that encompasses amino acids 241-292 of HDAC5. Indeed, the deletion of this region abolished HDAC5 repressive activity. However, amino acids located C-terminal to this region are also important for this repressive activity, since the 123-292 version of HDAC5 were unable to repress transcription. The expression of the deacetylase domain alone did not show any repressive activity (Fig. 3D, 674-1113 construct). Western blots presented in Fig. 3, C and E, show that the vectors used in these experiments express efficiently all the expected proteins. However, it appeared that most of the constructs used, besides the expected protein (Fig. 3E, *arrows*), produced at least another protein. We could show that these products are not a result of degradation but are generated after the splicing of a fraction of the messenger transcribed from the plasmid. Indeed the use of different antibodies and the cloning of a mRNA generated after transfection, showed that the full-length mRNA transcribed from the plasmid can undergo splicing and generate variants (not shown and see Fig. 5B).

HDAC5 Possesses Multiple Transcriptional Repression Domains-- Since the deacetylase domain of HDAC5 was found to be unnecessary to repress MEF2A transcriptional activity, one could question the ability of this domain to repress transcription. In order to investigate this issue, we targeted the HDAC5 deacetylase domain into a promoter containing GAL4-binding sites. The reporter system used contained eight copies of the binding sites for LexA immediately adjacent to five copies of the binding site for GAL4 (15), all cloned upstream of a luciferase gene. In the presence of LexA-VP16 fusion co-activator and the GAL4 DNA-binding domain alone (GAL4-DB), this reporter was activated to high levels of expression (Fig. 4, *GAL4-DB*). An expression vector was prepared expressing a fusion protein containing the deacetylase domain of HDAC5 (amino acids 674-1113) fused to the GAL4 DNA-binding domain. Co-expression of LexA-VP16 and GAL4-HDAC5 showed that the HDAC5 deacetylase domain could inhibit by 10-fold the transcriptional activity of LexA-VP16 (Fig. 4, *HDAC5 674-1113* construct). We showed also that the non-deacetylase domain of HDAC5 possesses an independent repression domain capable of repressing MEF2A transcriptional activity (Fig. 3D). To confirm this finding we also fused HDAC5 N-terminal non-deacetylase domain (amino acids 123-673) to the GAL4 DNA-binding domain. The expression of this fusion protein together with LexA-VP16 led also to an efficient repression of LexA-VP16 transcriptional activity (Fig. 4, *HDAC5 123-673* construct). In order to show the specificity of this repressive activity, we prepared an expression vector encoding GAL4 DNA-binding domain fused to the MEF2-binding domain of HDAC5 (amino acid 123-292). This region of HDAC5 possesses the ability to interact with MEF2A (Fig. 2C) but does not repress its transcriptional activity (Fig. 3D). Therefore, it should not be able to repress the VP16-dependent transcriptional activation. As expected, the expression of this protein did not affect the ability of LexA-VP16 to direct an efficient transcription of the reporter gene (Fig. 4,

These data showed that the HDAC5 deacetylase domain, although unnecessary to repress MEF2A transcriptional activity, is an active repressor domain. They also confirmed that besides the deacetylase domain, HDAC5 possesses another repression domain located in its N-terminal region.

HDAC4 and HDAC5 Are Two Distinct Proteins-- The present report and those published recently indicate that HDAC4 and HDAC5 are able to interact with members of MEF2 transcription factors. These two members of class II deacetylases, although sharing similar domain organization and sequence homology, may be able to ensure different functions in cells, as they have been found to be associated with distinct partners (6). In order to obtain more evidence of functional differences between these two deacetylases, we transfected HeLa cells with HA-tagged HDAC4 and HDAC5 expression vectors and examined the cellular distribution of these proteins using an anti-HA antibody. In cells transfected with HDAC4 expression vector a significant proportion of cells showed foci of HDAC4 accumulation in the nucleus (Fig. 5A, *top left*), as it has been previously shown (7, 12) while none of the cells transfected with HA-HDAC5 expression vectors showed this particular pattern of expression (Fig. 5A, *top right*). In the majority of these cells, a homogenous distribution of HA-HDAC5 was observed in the nucleus (Fig. 5A, *HDAC5 panel*). Moreover, Western blot analysis of cells transfected with HA-HDAC5 expression vectors showed the expression of two major forms of the protein, one presenting the expected size and the other was found to have a molecular mass around 83 kDa (Fig. 5B, *HDAC5 panel*, α -HA, *arrows*). The use of an antibody raised against the very C-terminal region of HDAC5 revealed the same bands (Fig. 5B, *HDAC5 panel*, α -HDAC5, *arrows*). This experiment showed that the smaller band is not the result of proteolysis but rather is an altered form of the protein, possessing both the N-terminal HA-tag and the C-terminal epitope. This observation suggests that a fraction of the messenger produced from the vector undergo splicing giving rise to HDAC5 variants. Indeed, we cloned and sequenced a cDNA produced after transfection and confirmed the above hypothesis (not shown). Interestingly, the smaller HDAC5-related protein is not able to interact with MEF2A *in vivo* (Fig. 1, compare, *input* and *IP α -MEF2*). It appeared therefore that transfected cells are capable of generating at least one form of HDAC5 that is unable to interact with MEF2A transcription factor. We are currently analyzing the presence of such HDAC5 variants produced from the endogenous HDAC5 gene. Western blot analysis of cell transfected with HA-HDAC4 showed the presence of a single band at the expected size. These data show that HDAC4 and HDAC5, although both interacting with MEF2A and modulating its activity, are different proteins and are probably involved in distinct regulatory pathways.

Discussion

Within class II histone deacetylases, HDAC4 and HDAC5 encoded by two independent genes, show similar domain organization and sequence homology (6). However, the analysis of associated proteins showed that in cells these deacetylases might enter functionally distinct complexes. Indeed, HDAC4, but not HDAC5 was found associated with RbAp48 (6). Data presented here further support this idea and show that when expressed in cells, HDAC4 and HDAC5 present very different patterns of nuclear localization. Moreover, an altered form of HDAC5, unable to interact with MEF2A could be generated in cells after transfection. Differentially spliced forms of HDAC5 have been already observed in different tissues and cell lines (5).² All these observations suggest that HDAC4 and HDAC5 exert distinct

functions in cells and are probably involved in different regulatory pathways. Our data showed, however, that HDAC5, like HDAC4, is capable of interacting specifically with the MEF2A transcription factor. This interaction completely abolished the transcriptional activity of MEF2A. We were surprised to find that this repression was independent of the deacetylase activity of HDAC5. Indeed, the N-terminal region of HDAC5 was found to interact with MEF2A and repress its transcriptional activity. Moreover, we found that MEF2A interaction and transcriptional repression are ensured by distinct regions of HDAC5 N-terminal non-deacetylase domain. The repression domain in this region of HDAC5 might recruit cellular histone deacetylases to ensure its repressive activity. Indeed, it has been shown that MITR, which shows significant sequence homology with the non-deacetylase domain of HDAC5 can interact with HDAC1 (18) and moreover, both HDAC4 and HDAC5 were found to be associated with HDAC3 (6). One may question the role of the histone deacetylase domain of HDAC5 in the activity of this protein. We showed here that this domain is fully functional in repressing transcription when it is targeted to a promoter via the GAL4 DNA-binding domain. We believe therefore that HDAC5 is a multifunctional repressor; it is capable of a repressive activity, independent of or dependent on its own deacetylase domain. However, one should keep in mind that the deacetylase activity of HDAC5 may be required to repress transcriptional activity of endogenous MEF2A-responsive genes. MEF2A, and other transcription factors, may target HDAC5 into chromatin where the deacetylase domain could participate in a chromatin remodeling activity. Moreover, HDAC5 like RPD3-related members, may enter distinct multifunctional complexes via "adaptors." Indeed, evidence does exist that HDAC4 can form a complex when expressed in HeLa cells (7). The way HDAC5 and HDAC4 are used in the regulation of gene expression appears therefore to be similar to that of HDAC1 and HDAC2. Indeed, these RPD3-related deacetylases are capable of interacting directly with transcription factors, *i.e.* YY1, Sp1, etc. (19, 20) and/or they may enter various complexes via interaction with molecules such as Sin3. Sin3 seems to play a pivotal role in targeting RPD3-related HDACs into various and distinct complexes (4). However, Sin3 was not found associated with HDAC4 and HDAC5 (6) indicating that other molecules may accomplish a Sin3-like function in targeting HDAC5 and HDAC4 into different complexes. Interestingly, HDAC4, HDAC5, and a new member of the class II histone-deacetylase, HDAC7, were found to interact directly with corepressors N-CoR and SMRT (21, 22), confirming the fact that these new deacetylases can target various complexes in a Sin3-independent fashion. The fact that RbAp48 was found to form a complex specifically with HDAC4 (6) provides some hints concerning the nature of complexes containing this deacetylase. Indeed, RbAp48, besides its involvement in NRD and Sin3 complexes (23, 24), where HDAC4 was not found (6), is associated with chromatin assembly machinery (25, 26). A role for HDAC4 in histone metabolism is therefore possible. The inhibition of MEF2A and MEF2C by HDAC4 (12, 13), as well as that of MEF2A by HDAC5 reported here, indicate the possible involvement of these proteins in various regulatory pathways. Since MEF2 transcription factors regulate the activity of numerous muscle-specific genes and play an important role in myogenesis, HDAC4/HDAC5 could be considered as potential regulators of myogenesis. However, a different role for these two proteins in myogenic differentiation could be envisaged since we have observed spliced forms of HDAC5 messenger (but not HDAC4) in myoblasts.³ Moreover, since MEF2 members are also involved in the regulation of cell proliferation, one might also propose a connection between HDAC4/HDAC5 activity and MEF2 function in this process. Previously, we showed that the expression of HDAC5 was up-regulated during a variety of differentiation programs (5). A careful examination of the timing of HDAC5 expression during the induced differentiation of murine erythroleukemia cells, showed a transient activation of HDAC5 gene expression (5). This accumulation occurs between 6 and 12 h of induction and is followed by decay in mRNA

content. This period precedes the commitment and the induction of globin gene expression (17). The same transient induction of HDAC5 gene expression was observed during the *in vitro* differentiation of myoblasts to myotubes.³ It is therefore tempting to think that HDAC5 plays a role in the pre-commitment period of various differentiation programs and functions as an early and transient inhibitor of specific transcription factors.

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Footnotes

[‡]Contributed equally to the results of this work.

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² C. Lemerrier, A. Verdel, B. Galloo, S. Curtet, M.-P. Brocard, and S. Khochbin, unpublished observations.

³ C. Lemerrier, A. Verdel, B. Galloo, S. Curtet, M.-P. Brocard, and S. Khochbin, unpublished data.

Abbreviations

The abbreviations used are: CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase.

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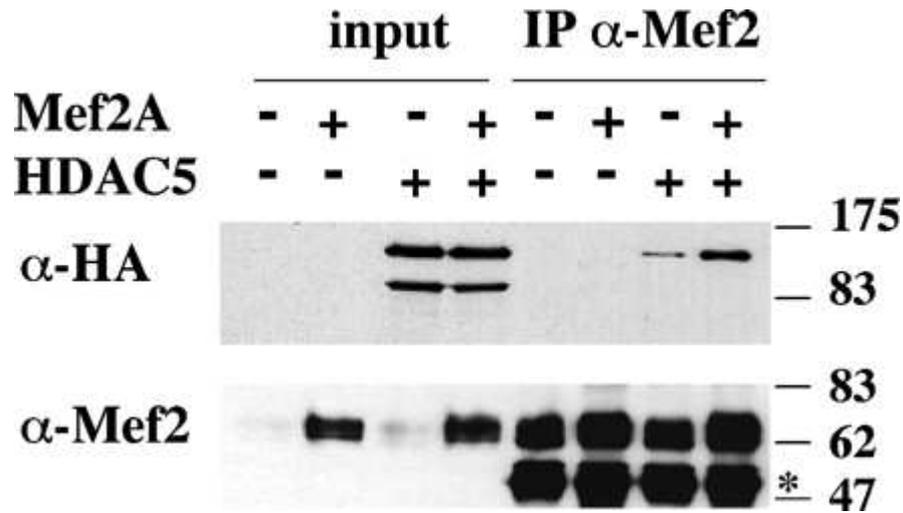


Fig. 1. HDAC5 and MEF2A are found in a complex *in vivo*. HeLa cells were transfected with 5 μ g of either HA-tagged HDAC5 or MEF2A or both expression vectors. 24 h post-transfection cell extracts were prepared and subjected to immunoprecipitation using an anti-MEF2 antibody (*IP panel*). The immunoprecipitated material was then used to obtain a Western blot and the presence of HA-HDAC5 and MEF2 was monitored using anti-HA and anti-MEF2 antibodies (α -HA and α -Mef2, respectively). A fraction of the extracts was used to control the presence of the proteins of interest in the extracts before immunoprecipitation (*input panel*). Asterisk in the lower panel indicates the Ig band.

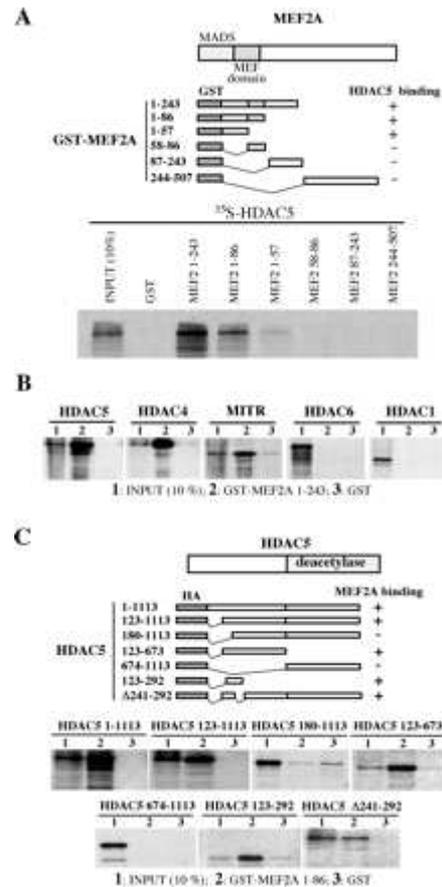


Fig. 2. Direct interaction between MEF2A and HDAC5. *A*, mapping of MEF2A-HDAC5 interacting domains. The indicated regions of MEF2A (schemes in *upper panel*) were fused to GST and bacterially expressed fusion proteins immobilized on glutathione beads were incubated with ^{35}S -labeled HDAC5. After pull-down, bound material was subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. *B*, MEF2A interacts specifically with HDAC5, HDAC4, and MITR but not with HDAC1 and HDAC6. Indicated ^{35}S -labeled proteins were incubated with GST-MEF2A (1-243 fragment) immobilized on glutathione beads and subjected to pull-down and analyzed as above. *C*, a limited region of the N-terminal non-deacetylase domain of HDAC5 is involved in interaction with MEF2A. GST-MEF2A (*MADS-box*+*MEF2 domain*, 1-86) was used in a pull-down experiment as above using full-length ^{35}S -labeled HDAC5 (1-1113) or indicated deletion mutants (*upper panel*).

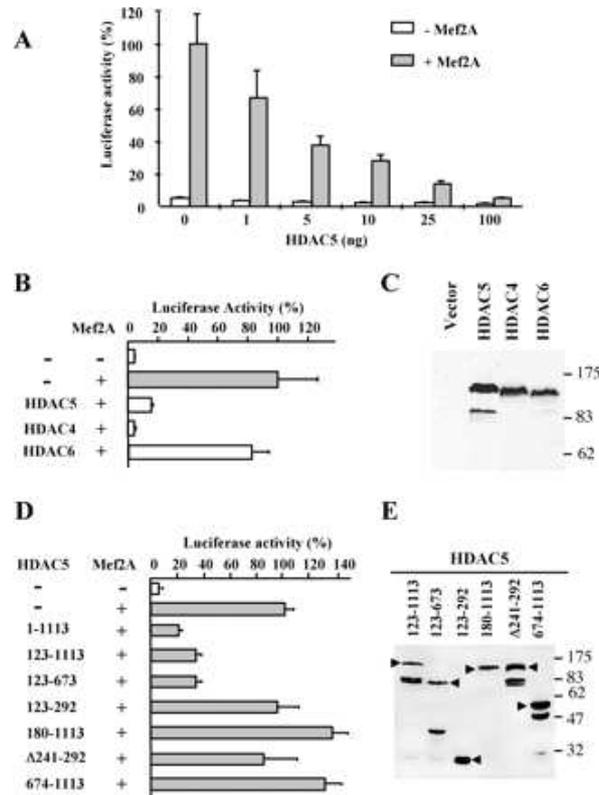


Fig. 3. Repression of MEF2A transcriptional activity by HDAC5. *A*, HDAC5 represses the transcriptional activity of MEF2A. One μg of a plasmid containing a luciferase reporter gene cloned downstream of myosin heavy chain minimal promoter flanked by two MEF2-binding sites was used to transfect HeLa cells. Co-transfections were carried out with 200 ng of a MEF2A expression vector alone (*lane 0*) or together with the indicated amounts of HDAC5 expression vectors. 100 ng of a β -galactosidase expression vector were also used in each transfection. 24 h post-transfection, luciferase activity was measured and normalized with respect to that of β -galactosidase. 100% represents mean values of three independent experiments performed in the absence of HDAC5 expression, standard deviations are indicated. *B*, HDAC5 is able to repress specifically MEF2A transcriptional activity. A transfection experiment was set up using the MEF2-responsive luciferase reporter gene, MEF2A expression vector (200 ng) without (-) or with (+) 20 ng of the indicated expression vectors. Luciferase and β -galactosidase activities were measured and normalized as above. *C*, HeLa cells were transfected with 10 μg of expression vectors encoding the indicated HA-tagged proteins and a Western blot was obtained and probed with an anti-HA antibody to show the expression of the proteins. *D*, HDAC5-dependent repression of MEF2 activity depends on HDAC5-MEF2A interaction. The MEF2-responsive luciferase reporter gene was used to transfect cells together with MEF2A expression vector, without (-) or with (+) 20 ng of a vector expressing the indicated deletion mutants of HDAC5. *Numbers* (amino acids), refer to the regions of HDAC5 analyzed (see also Fig. 2C). Δ 241-292, represents a HDAC5 mutant bearing a deletion in the indicated region. 674-1113 construct expresses the deacetylase domain of HDAC5. Luciferase and β -galactosidase activities were measured, normalized, and presented as above. *E*, a Western blot obtained and analyzed as in *B* shows the expression of HA-tagged HDAC5 mutants. *Arrowheads* indicate the position of the expected full-length proteins.

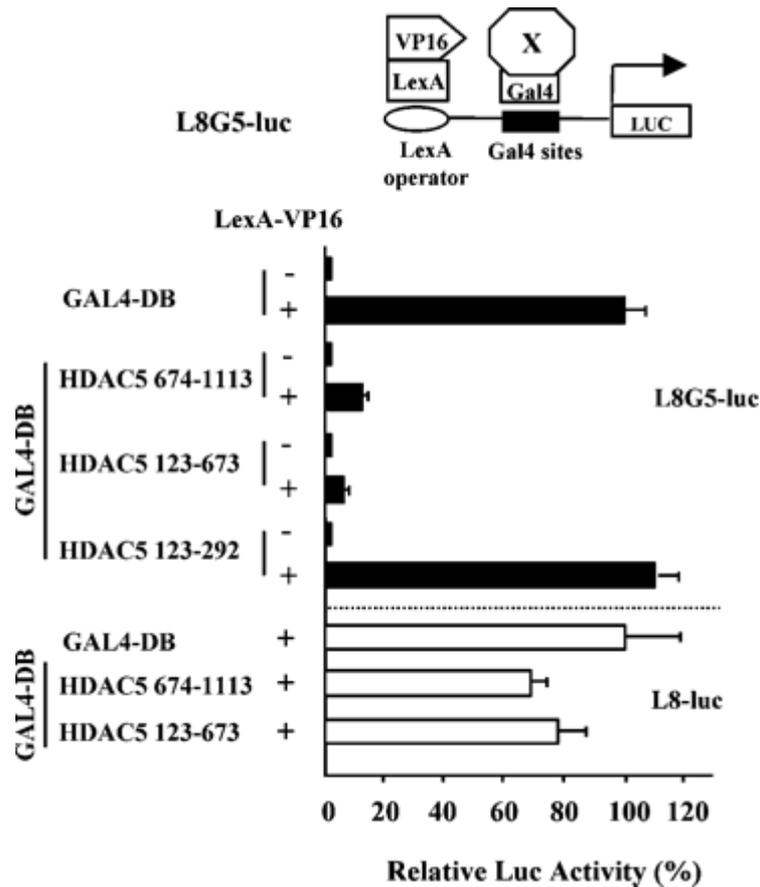


Fig. 4. HDAC5 possesses two distinct repressor domains. HeLa cells were transfected with 400 ng of L8G5-luc reporter (scheme) and 50 ng of vectors expressing either the GAL4 DNA-binding domain (*GAL4-DB*) or the indicated regions of HDAC5 fused to GAL4 DNA-binding domain, in the absence (-) or presence (+) of 100 ng of Lex-A VP16 expression vector (*black histograms*). In the control experiment, cells were transfected with the a reporter gene lacking the GAL4-binding sites (L8-Luc) and expression vectors encoding the Lex-A VP16 activator as well as the indicated GAL4-HDAC5 fusion proteins (*open histograms*). Luciferase and β -galactosidase activities were measured, normalized, and presented as described in the legend to Fig. 3.

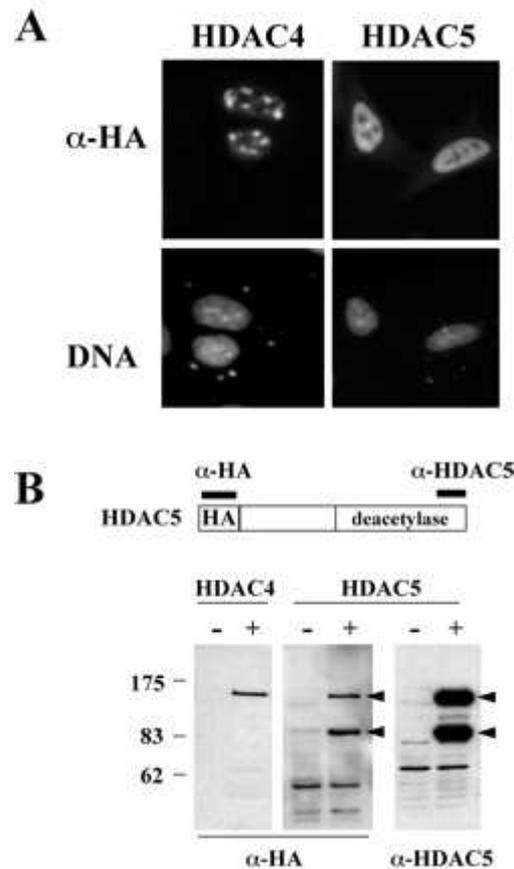


Fig. 5. HDAC4 and HDAC5 are two different proteins. *A*, different distribution of HDAC4 and HDAC5 in HeLa nuclei after transfection. HeLa cells were transfected with 4 μ g of expression vectors encoding HA-tagged HDAC4 and HDAC5. 24 h post-transfection cells were fixed and subjected to immunofluorescence detection of proteins using an anti-HA antibody (α -HA panel). DNA panels show nuclei of cells expressing HDAC4 and HDAC5 stained with DNA-specific dye Hoechst 33258. *B*, HDAC5 encoding vector expresses two different forms of HDAC5. HeLa cells were transfected with either HA-HDAC4 or HA-HDAC5 expression vectors (+) or the corresponding empty vectors (-). Extracts prepared from these cells were subjected to Western blot analysis using an anti-HA antibody (α -HA panel) or antibodies raised against the C-terminal region of HDAC5 (α -HDAC5 panel). Note that the two HDAC5-related bands are revealed by both anti-HA anti-HDAC5 antibodies (arrowheads).