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HDAC4 deacetylase associates with and represses the MEF2 transcription factor

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The acetylation state of histones can influence transcription. Acetylation, carried out by acetyltransferases such as CBP/p300 and P/CAF, is commonly associated with transcriptional stimulation, whereas deacetylation, mediated by the three known human deacetylases HDAC1, 2 and 3, causes transcriptional repression. The known human deacetylases represent a single family and are homologues of the yeast RPD3 deacetylase. Here we identify and characterize HDAC4, a representative of a new human histone deacetylase family, which is homologous to the yeast HDA1 deacetylase. We show that HDAC4, unlike other deacetylases, shuttles between the nucleus and the cytoplasm in a process involving active nuclear export. In the nucleus, HDAC4 associates with the myocyte enhancer factor MEF2A. Binding of HDAC4 to MEF2A results in the repression of MEF2A transcriptional activation, a function that requires the deacetylase domain of HDAC4. These results identify MEF2A as a nuclear target for HDAC4-mediated repression and suggests that compartmentalization may be a novel mechanism for controlling the nuclear activity of this new family of deacetylases.

Keywords: HDAC4/HDA1/histone deacetylase/MEF2/nuclear export

Introduction

Chromatin, which is thought to be repressive for transcription as compared with naked DNA, is the *in vivo* template for transcription. DNA-binding transcriptional activators therefore not only have to recruit the basal transcriptional machinery, but also modify the chromatin template in such a way that transcription factors have access to DNA and transcriptional initiation is facilitated. One way of modifying the chromatin template is at the level of nucleosomes. The N-terminal tails of nucleosomal histones have been known for a long time to be acetylated differentially. Histones acetylated at specific residues are associated with transcriptionally active regions of the genome, whereas hypoacetylated histones are found in transcriptionally silent regions (Grunstein, 1997; Struhl, 1998). Recently, transcriptional activators have been shown to recruit chromatin-modifying acetyltransferases such as CBP/p300 or P/CAF to promoters of specific genes in order to facilitate transcription. In addition, transcription factors themselves are acetylated and their activity has been demonstrated to be regulated by acetylation (Kouzarides, 1999). In contrast, transcriptional repressors have been shown to recruit histone deacetylase activity-containing complexes in order to repress transcription (Kouzarides, 1999). Thus, acetyltransferases and deacetylases play a major role in the regulation of transcription *in vivo*.

In humans there is only a single family of histone deacetylases known to date, consisting of HDAC1, HDAC2 and HDAC3 (Taunton *et al.*, 1996; Yang *et al.*, 1996; Dangond *et al.*, 1998; Emiliani *et al.*, 1998). All three are closely related to the yeast transcriptional regulator RPD3 (Vidal and Gaber, 1991). In yeast two histone deacetylases RPD3 and HDA1 have been characterized so far and a further three open reading frames, HOS1, HOS2 and HOS3, share strong sequence similarity with histone deacetylases (Rundlett *et al.*, 1996). The RPD3/HDAC1 histone deacetylases have been found in several large protein complexes, all of which contain subunits that are conserved between yeast and humans (Pazin and Kadonaga, 1997). These complexes are recruited to promoters via transcriptional repressors in humans (e.g. Mad/Max, RB and nuclear hormone receptors; Pazin and Kadonaga, 1997; Brehm, 1999) and in yeast (e.g. UME6; Kadosh and Struhl, 1997). Yeast HDA1 was found to be in a separate complex from RPD3 (Rundlett *et al.*, 1996). However, no factors recruiting HDA1 to a promoter have been identified so far. Recently, two putative HDA1 homologues have been identified in mouse, whose function remains to be determined (Verdel and Khochbin, 1999).

The human myocyte enhancer factor 2 (MEF2) transcription factors belong to the MADS-box family of DNA binding transcription factors, which were originally identified through their homology to SRF (Pollock, 1991). MEF2 proteins are conserved between *Drosophila melanogaster*, *Caenorhabditis elegans* and vertebrates. In vertebrates there are four *mef2* genes, *mef2a–d* (Olson *et al.*, 1995). MEF2 proteins share an additional region of homology just C-terminal of the MADS box, termed the MEF2 domain. MEF2 proteins bind to DNA as homo- or heterodimers. They bind to and co-operate with basic helix–loop–helix (bHLH) transcription factors like MyoD (Black *et al.*, 1998). MEF2 binding sites have been identified in many muscle-specific genes, which also have been shown to be activated by MEF2 proteins (Gossett *et al.*, 1989; Cserjesi and Olson, 1991). Several studies, including genetic analysis in mouse and *Drosophila*, demonstrate a role for MEF2 factors in both smooth and skeletal muscle differentiation (Lilly *et al.*, 1995; Lin *et al.*, 1997). However, in adult tissues only the expression

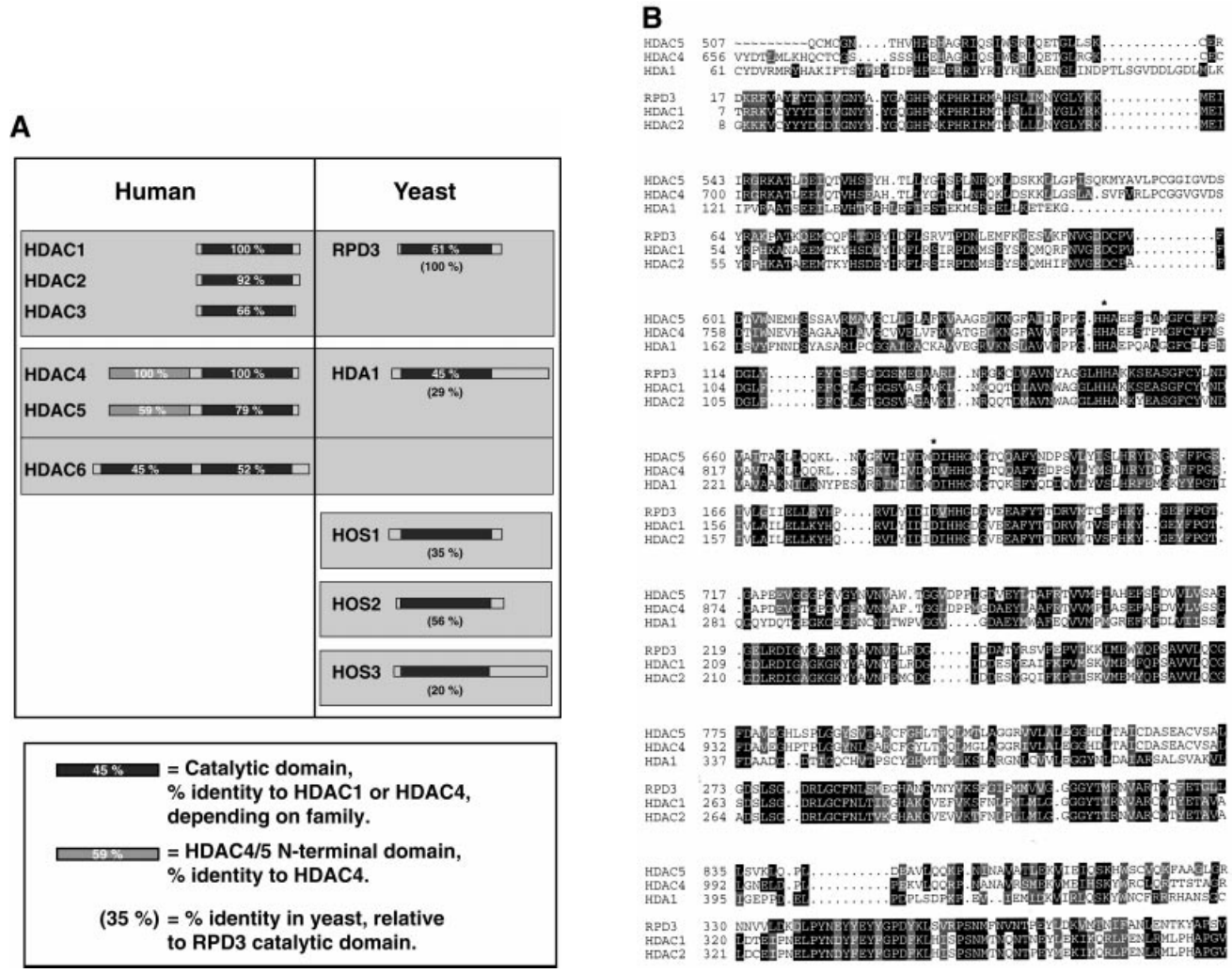

Fig. 1. (**A**) Schematic representation of the relationship between the different families of human and *Saccharomyces cerevisiae* histone deacetylases. Percentages refer to the amino acid sequence identities in the putative catalytic domains as calculated by the GAP program of the GCG package. HDAC1, -2, -3 and RPD3 are part of a conserved family. HDAC4, HDAC5, HDAC6 and HDA1 form a distinct family. The two putative pairs of homologues HDAC1/RPD3 and HDAC4/HDA1 are more similar to each other (61 and 45%, respectively) than RPD3 and HDA1 (29%). Human proteins sharing high sequence similarity with HOS1, HOS2 or HOS3 have not been identified so far. (**B**) An alignment of the putative catalytic domains of HDAC1, HDAC2, RPD3, HDA1, HDAC4 and HDAC5 was created using the PILEUP program from the GCG package. Identical and related amino acids are shaded. The conserved residues that were targeted by site-directed mutagenesis (HDAC4 D840N, H803A) are highlighted by an asterix.

of MEF2C is restricted, whereas the other genes are expressed ubiquitously. Thus, MEF2 proteins may have additional functions apart from their involvement in muscle differentiation. Indeed, MEF2 proteins have been implicated in the serum-dependent regulation of the c-*jun* promoter (Han and Prywes, 1995; Clarke *et al.*, 1998).

In this study we characterize the founding member of a new family of human histone deacetylases, HDAC4, with similarity to the yeast histone deacetylase HDA1. HDAC4 has histone deacetylase activity that is trichostatin A (TSA) sensitive and is abolished by a single point mutation in the catalytic domain. HDAC4 localizes to both the nucleus and the cytoplasm and this compartmentalization is regulated by active protein export from the nucleus. HDAC4 deacetylase activity represses transcription of a high basal promoter. We identify the transcription factor MEF2A as a target for HDAC4 activity in the nucleus. MEF2A co-immunoprecipitates and co-localizes with HDAC4 *in vivo*. MEF2-dependent transcription is silenced by HDAC4 deacetylase activity. Together, these data suggest a novel mode of regulation of deacetylase activity, via compartmentalization, and identify MEF2A as a nuclear target of the new deacetylase HDAC4.

Results

HDAC4 is a founding member of a new human histone deacetylase family

The mammalian and yeast deacetylases belong to a superfamily of evolutionarily conserved enzymes, which includes bacterial amidohydrolases and acetoin utilization proteins (Leipe and Landsman, 1997). The bacterial enzymes represent a conservation of structure rather than function. We searched the DDBJ/EMBL/GenBank database for sequences that show homology with the catalytic domain of the deacetylases and identified two novel, related human proteins. These two human proteins, which

we designate HDAC4 and HDAC5, form part of a new human deacetylase family for two reasons: first, they are more homologous to the yeast HDA1 deacetylase than the known human deacetylases HDAC1, HDAC2 and HDAC3 (Figure 1A and B), and secondly, they contain a large and highly conserved N-terminal sequence that is not found in the other human HDAC proteins or in the yeast HDA1 deacetylase. Thus, HDAC4 and HDAC5 represent members of a new family of human deacetylases, which are more related to the yeast HDA1 protein, whereas HDAC1, HDAC2 and HDAC3 are part of a separate family related to the yeast RPD3 deacetylase. The sequence of HDAC5 was initially described as an antigen in human colon cancer and named NY-CO-9 (Scanlan, 1998). The cloning of an HDAC5/NY-CO-9 homologue in mouse has recently been reported (Verdel and Khochbin, 1999) and a mouse EST for HDAC4 has been deposited in the DBEST database.

A separate human deacetylase, designated HDAC6, which contains two putative catalytic domains, was also identified in our search. Both domains share higher sequence similarity to HDA1 than to the other yeast deacetylases. However, given that HDAC6 does not have the characteristic N-terminal extension found in HDAC4 and HDAC5, this protein is likely to represent an HDA1 subfamily. HDAC6 does not have an obvious counterpart in yeast, but does have a counterpart in *C.elegans* and mouse (Verdel and Khochbin, 1999).

HDAC4 possesses histone deacetylase activity which can repress transcription

We first set out to establish if one of the new family members, namely HDAC4, associates with deacetylase activity. To this end, we expressed in 293T cells a Myc-tagged version of HDAC4 or two point mutants (D840N, H803A), carrying a mutation in the potential deacetylase catalytic domain. Both D840 and H803 are highly conserved residues in the histone deacetylase superfamily (Figure 1B), and mutation of the analogous residues in HDAC1 (D176 and H141, respectively) have been shown to abolish catalytic activity (Hassig *et al.*, 1998). Importantly, HDAC1 H141A retains the ability to associate with members of the HDAC1 deacetylase complex such as RbA-p48 and mSin3A, suggesting that the mutant is structurally intact. Figure 2A shows that precipitation of Myc-HDAC4 with an anti-Myc antibody co-precipitates large amounts of histone deacetylase activity. Both point mutations severely compromise enzyme function. Equal expression of the wild-type and mutant protein was verified via Western blotting (data not shown). Both deacetylase domain mutants are able to interact with proteins *in vivo* (see below, Figure 5B) suggesting that the overall structure of the protein is maintained. Furthermore, the analogy with HDAC1 H141A suggests that HDAC4 H803A is only compromised in its catalytic activity. Using the same immunoprecipitation assay we show in Figure 2B that deacetylation by HDAC4 is sensitive to TSA, a specific histone deacetylase inhibitor. Together, these data and the homology with other histone deacetylases suggest that HDAC4 has intrinsic deacetylase activity, although we cannot exclude the possibility that a factor co-purifying from the lysates contributes to this activity. Such an activity may be responsible for the residual activity seen with the mutants (Figure 2A). This formal proof is lacking for all deacetylases described as so far no recombinant enzymes have been purified from *Escherichia coli* in an active state.

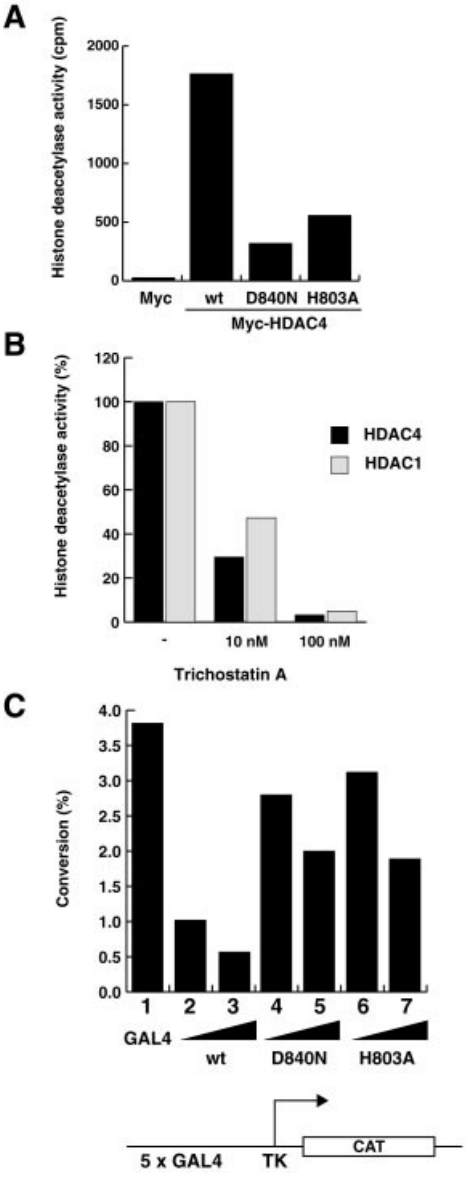

Fig. 2. HDAC4 is a histone deacetylase and represses transcription. (**A**) pcDNA3.1-A-Myc/His, pcHDAC4-Myc, pcHDAC4-Myc(D840N) and pcHDAC4-Myc(H803A) were transfected into 293T cells and lysates immunoprecipitated using the anti-Myc antibody. Precipitates were subjected to histone deacetylase assay as indicated. (**B**) 293T cells were transfected with pcHDAC1-Myc and pcHDAC4-Myc, and immunoprecipitates were prepared as in (A). Immunoprecipitates were divided and incubated with different concentrations of TSA prior to deacetylase assays. Activities are represented as percentage of activity in the absence of TSA. (**C**) HeLa cells were transfected with 1 μg of 5×GAL4TK-CAT and either 500 ng of pcGAL4 or pcGAL4–HDAC4, pcGAL4–HDAC4(D840N) and pcGAL4–HDAC4(H803A) (25 and 150 ng). Total GAL4 concentration was equalized to 500 ng. All of the GAL4 constructs tested were expressed at equal levels and had no significant effect on a control promoter, 0×GAL4TK-CAT, which lacks GAL4 sites (data not shown).

Histone deacetylase activity has been shown to be repressive for transcription. To establish whether HDAC4 has a repressive potential, a GAL4–HDAC4 fusion was

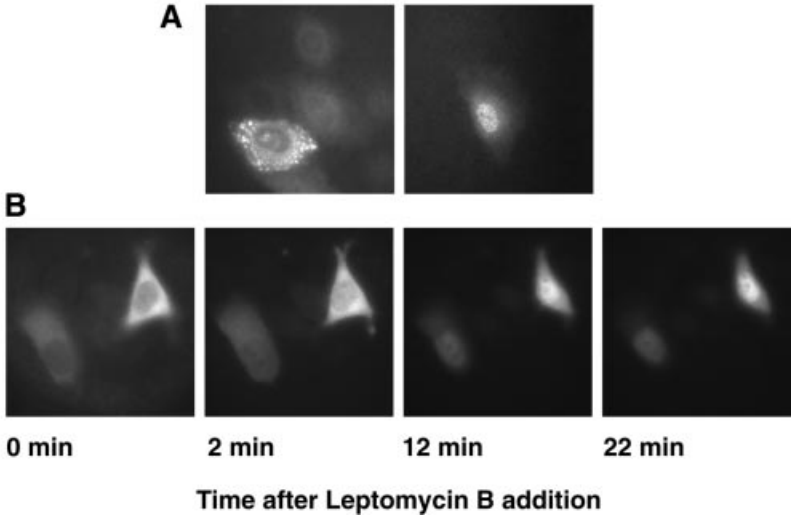

Fig. 3. HDAC4 shuttles between the nucleus and the cytoplasm. (**A**) pcHDAC4–GFP was microinjected into HeLa cells. Four hours after injection, living cells were visualized using fluorescence microscopy. (**B**) HeLa cells were injected with pcHDAC4(118–1084)–GFP and after 4 h 20 nM of leptomycin B (LMB) was added to the culture medium of the living cells. GFP–HDAC4 fluorescence was followed by time-lapse microscopy.

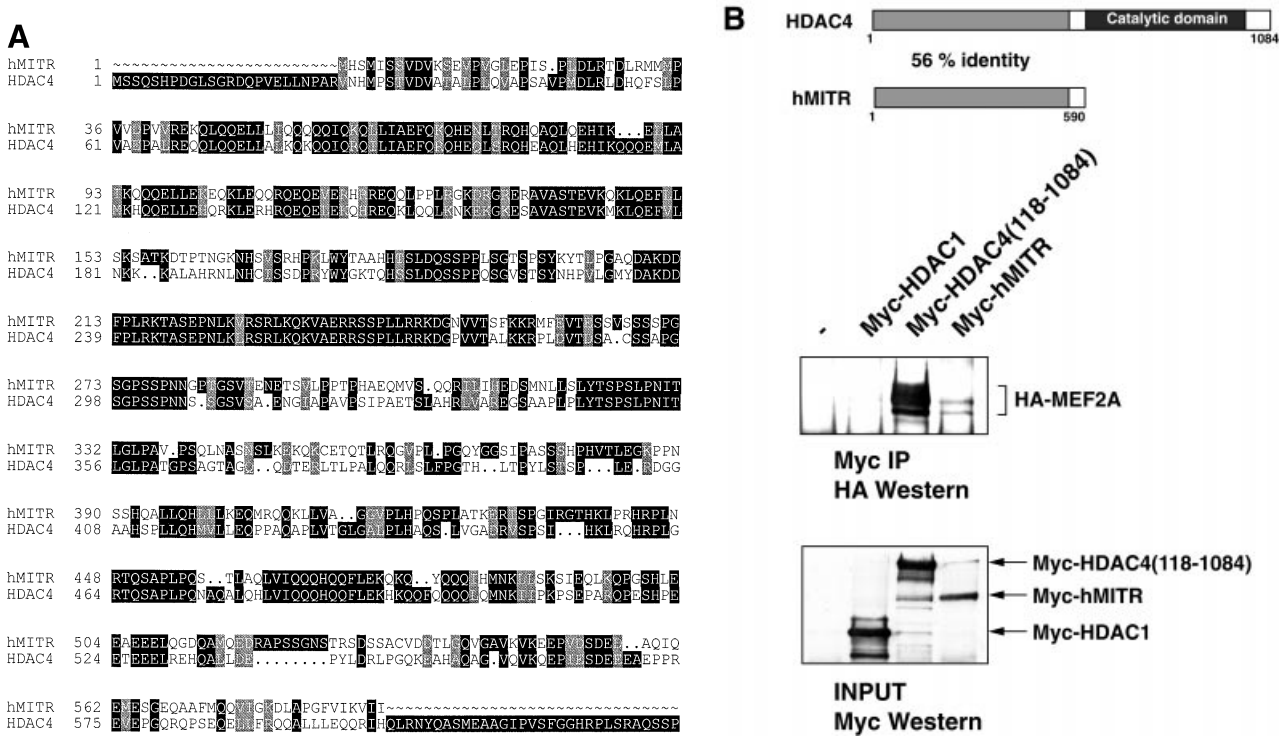

Fig. 4. HDAC4 and hMITR interact with the transcription factor MEF2A. (**A**) Alignment of the N-terminus of HDAC4 with the corresponding region of hMITR using PILEUP from the GCG package. Identical and related amino acids are shaded. (**B**) HDAC4 and hMITR co-immunoprecipitate. Diagram of the structure of HDAC4 and hMITR. hMITR lacks a deacetylase domain. The N-terminal extension of HDAC4 is 56% identical to a corresponding region in hMITR. 293T cells were transfected with HA–MEF2A and either pcDNA3.1-A-Myc/His, pcHDAC1-Myc, pcHDAC4(118–1084)-Myc or pchMITR-Myc. Lysates were immunoprecipitated using an anti-Myc antibody and precipitates were subjected to SDS–PAGE followed by Western blotting using an anti-HA antibody. Expression levels were verified through Western blotting using appropriate antibodies. The corresponding anti-Myc Western blot from lysate is shown.

tested for its ability to repress a high basal level promoter that is driven by the viral thymidine kinase promoter and bears GAL4 binding sites. Figure 2C shows that GAL4–HDAC4 can repress this promoter in a dose-dependent manner (Figure 2C, lanes 1–3). This repression is sensitive to TSA (data not shown, and Figure 7) and both mutations in the HDAC4 catalytic domain (D840N, H803A) severely compromise but do not abolish this repression (Figure 2C, lanes 4–6). These results suggest that repression is mediated, at least partly, by the deacetylase activity of HDAC4. The fact that both mutants have residual repression capacity suggests that a second repression mechanism, which may or may not involve associated deacetylase activities, is operational. The GAL4–HDAC4 fusion had no effect on a promoter that did not carry GAL4 binding sites (data not shown).

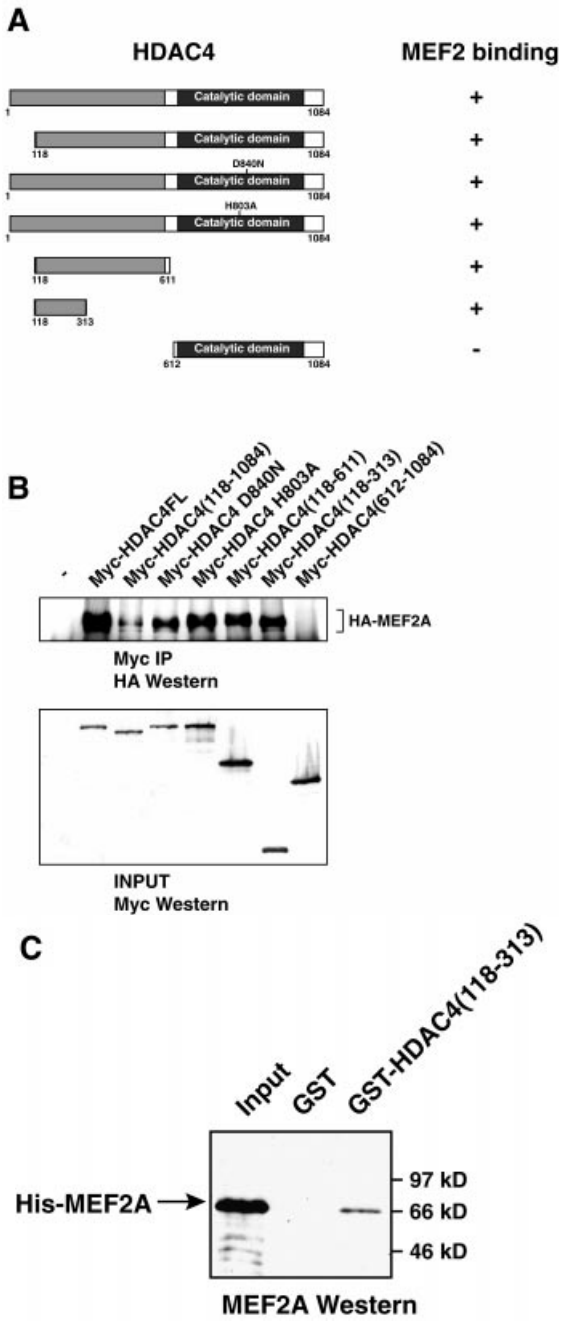

Fig. 5. The N-terminus of HDAC4 interacts with MEF2A both *in vivo* and *in vitro*. (**A**) Diagram of the mapping of MEF2A binding on HDAC4. (**B**) HeLa cells were transfected with HA–MEF2A and Myc-tagged HDAC4 deletion constructs as indicated. Lysates were immunoprecipitated using an anti-Myc antibody and precipitates were subjected to SDS–PAGE followed by Western blotting using an anti-HA antibody. Expression of Myc-tagged fusions is shown by Western blotting. (**C**) Recombinant purified GST and GST–HDAC4(118–313), pre-bound to glutathione beads, were incubated with recombinant purified His–MEF2A. Bound material was subjected to SDS–PAGE and Western analysis using MEF2A-specific serum. Fifty per cent of His–MEF2A input was loaded.

HDAC4 shuttles between nucleus and cytoplasm

If HDAC4 is a transcriptional repressor protein then it would be expected to localize to the nucleus. To establish the localization of HDAC4 in the cell, we fused the green fluorescent protein (GFP) to HDAC4 and microinjected the fusion into HeLa cells. Surprisingly, a large number of the cells expressed GFP–HDAC4 in the cytoplasm, but a substantial proportion contained purely nuclear HDAC4 (Figure 3A). GFP has the advantage of allowing the study of living cells. However, to exclude the possibility of GFP affecting the localization of the GFP–HDAC4 fusion protein, we also analysed the localization of a Myc-tagged HDAC4 protein using immunofluorescence experiments. We found the anti–Myc antibody staining to be identical to the GFP labelling (data not shown).

The fact that HDAC4 showed either cytoplasmic or nuclear localization suggested to us the possibility of nucleo-cytoplasmic shuttling. We therefore asked if HDAC4 was being actively exported into the cytoplasm. To establish this we used a drug, leptomycin B, which inhibits nuclear export. Figure 3B shows that within minutes of the addition of leptomycin B, cytoplasmically localized GFP–HDAC4 relocates to the nucleus. These results indicate that in HeLa cells, HDAC4 is actively exported to the cytoplasm, but in some cells this export was interrupted to allow nuclear localization of HDAC4.

HDAC4 associates with MEF2A in vivo

A recently identified *Xenopus laevis* protein, MITR, shows extensive sequence similarity to the N-terminus of HDAC4 but lacks the C-terminal catalytic domain of the HDAC4 deacetylase. The human equivalent of MITR, hMITR, shows 56% identity to the N-terminal extension of HDAC4 (Figure 4A and B). Given that *Xenopus* MITR interacts with *Xenopus* MEF2A and MEF2D transcription factors (Sparrow *et al*., 1999) we considered the possibility that HDAC4 may be targeted to promoters via an association with MEF2A. We therefore asked whether HDAC4 and MEF2A interact *in vivo*. 293T cells expressing a HA-tagged MEF2A along with Myc-tagged versions of HDAC1, HDAC4 and hMITR, were lysed, immunoprecipitated with an anti-Myc antibody and Western blotted with a HA-tag-specific antibody. Figure 4B shows that hMITR and HDAC4 can interact with MEF2A. This result suggests that the N-terminus of HDAC4, which is homologous to hMITR, is required for the interaction with MEF2A. In agreement with this idea, Figure 4B shows that the HDAC1 deacetylase, which lacks the region of homology between HDAC4 and hMITR, does not associate with MEF2A. This result highlights the specificity of targeting for deacetylases, since under similar conditions HDAC1 is capable of interacting with the retinoblastoma protein (Brehm *et al.*, 1998; Luo *et al*., 1998; Magnaghi Jaulin *et al.*, 1998).

In order to identify the region of HDAC4 that interacts with MEF2A we performed a similar co-immunoprecipitation experiment in HeLa cells. Several Myc-tagged deletions of HDAC4, including the full-length protein and mutants, were expressed together with HA–MEF2A. As indicated by the homology with hMITR, residues 118–313 of HDAC4 are sufficient for the interaction with MEF2A (Figure 5A and B). The C-terminus of HDAC4 is not required for the interaction and mutations in the catalytic domain of HDAC4 (D840N, H803A) do not abolish the interaction.

We next asked if the interaction between the N-terminus of HDAC4 and MEF2A is direct. To this end both HDAC4 and MEF2A were produced as recombinant proteins in *E.coli*. Figure 5C shows that GST–HDAC4(118–313)

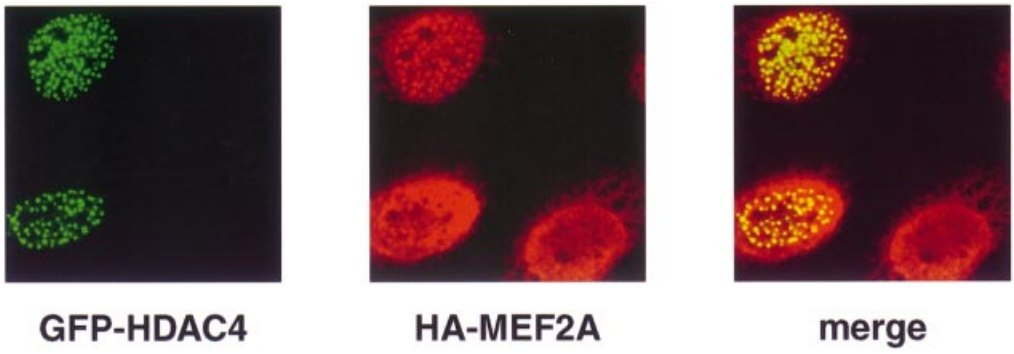

Fig. 6. HDAC4 and MEF2A colocalize in the nucleus. HeLa cells were microinjected with equal amounts of pcHDAC4(118–1084)–GFP and HA–MEF2A expression constructs. Four hours after injection cells were fixed in methanol/acetone and stained with anti-HA antibodies. Cells were analysed by confocal microscopy. HA–MEF2A immunostaining is in red, GFP–HDAC4 in green.

binds specifically to His-tagged MEF2A as compared with a GST control in a GST-pulldown assay, suggesting that indeed the two proteins can associate directly.

To further verify the interaction between HDAC4 and MEF2A *in vivo* we tried to establish if they colocalize in the nuclei of HeLa cells using confocal microscopy. Figure 6 shows that GFP–HDAC4 localizes to specific areas in the nucleus. A similar dotted localization can be seen when a HA–MEF2A fusion is expressed and monitored by immunofluorescence using a HA-specific antibody. The co-localization of HDAC4 and MEF2A is clearly observed when the two confocal images are merged. Although it is unclear what the dotted areas in the nucleus represent, these data confirm the co-immunoprecipitation data and demonstrate that HDAC4 and MEF2A can associate within the nucleus. A further demonstration of the interaction is the fact that co-expression of HA–MEF2A with GFP–HDAC4 increases the proportion of cells containing nuclear HDAC4 (data not shown).

HDAC4 negatively regulates MEF2A-dependent transcription

Given that HDAC4 has the potential to repress transcription (Figure 2C), we next tested whether HDAC4 can repress the ability of MEF2A to act as a transcriptional activator. HeLa cells have abundant MEF2 site binding activity, which is composed predominantly of MEF2A and MEF2D (Dodou *et al.*, 1995; Ornatsky, 1996). Figure 7A shows that a luciferase reporter driven by MEF2 sites is active in HeLa cells (lane 1). This MEF2-driven reporter is repressed when wild-type HDAC4 is introduced into HeLa cells (lane 2). However, when point mutants of the HDAC4 deacetylase domain (D840N, H803A) are used, this repression is reduced (lanes 3 and 4). Furthermore, this HDAC4-dependent repression is not seen when the cells are pre-treated with TSA (lanes 5–8). The effects seen with endogenous MEF2 site binding activities becomes even more pronounced when exogenous MEF2 is introduced into HeLa cells. Figure 7B shows that the MEF2 reporter can be further activated by exogenous MEF2A (lanes 1 and 2) and this activation can be completely repressed by HDAC4 (lane 3). The enzyme dead mutants D840N and H803A are less efficient at repressing the MEF2 promoter (lanes 4 and 5). HDAC4 represses MEF2A activity >6-fold (lanes 2 and 3), but in the presence of TSA HDAC4 represses only by 50% (lanes 7 and 8). Collectively, these results suggest that the intrinsic deacetylase activity of HDAC4 is important for its ability to repress MEF2A, but that additional repressive mechanisms are operational. Similar conclusions were reached from the repressive capacity of the GAL4–HDAC4 fusion (Figure 2C).

Discussion

Here we introduce HDAC4 as a founding member of a new family of human deacetylases. So far, all the known deacetylases can be classified into a single group, which is closely related to the yeast protein RPD3. The three mammalian deacetylases, HDAC1, HDAC2 and HDAC3 share several properties and two of them, HDAC1 and HDAC2, are found in the same complex in the cell (Hassig *et al.*, 1998). HDAC4, on the other hand, is a putative homologue of the yeast HDA1 deacetylase. Since HDA1 and RPD3 are found in distinct physical complexes it seems likely that, as in yeast, HDAC4 and the closely related protein HDAC5, may be part of a human complex distinct from HDAC1 and HDAC2. In addition, given that RPD3 and HDA1 regulate a distinct set of genes in yeast (Rundlett *et al.*, 1998), it may well be the case that HDAC4 has separate functions compared with the RPD3-related human deacetylases characterized to date. This study identifies several novel features of HDAC4, which are consistent with this idea.

The first feature of HDAC4 that has not been recognized previously for any of the RPD3 related deacetylases is its nucleo-cytoplasmic shuttling. Exclusion of HDAC4 from the nucleus via active nuclear export suggests that the nuclear role of HDAC4 may be required only in a restricted time frame during the cell cycle or during differentiation processes. The signal that allows localization of HDAC4 in the nucleus is unknown. However, the process of compartmentalization clearly provides a very efficient way of selectively regulating the nuclear deacetylase activity of one enzyme but not another. Such a difference in compartmentalization of enzyme activities suggests that the targets for the deacetylase activity of HDAC4 may be different from that of HDAC1, HDAC2 and HDAC3. Indeed, as discussed below, some evidence for this is provided by the binding of HDAC4 to specific promoter-bound factors.

The second feature of HDAC4 that sets it apart from the other known deacetylases, is its association with a specific transcription factor, MEF2A, possibly using

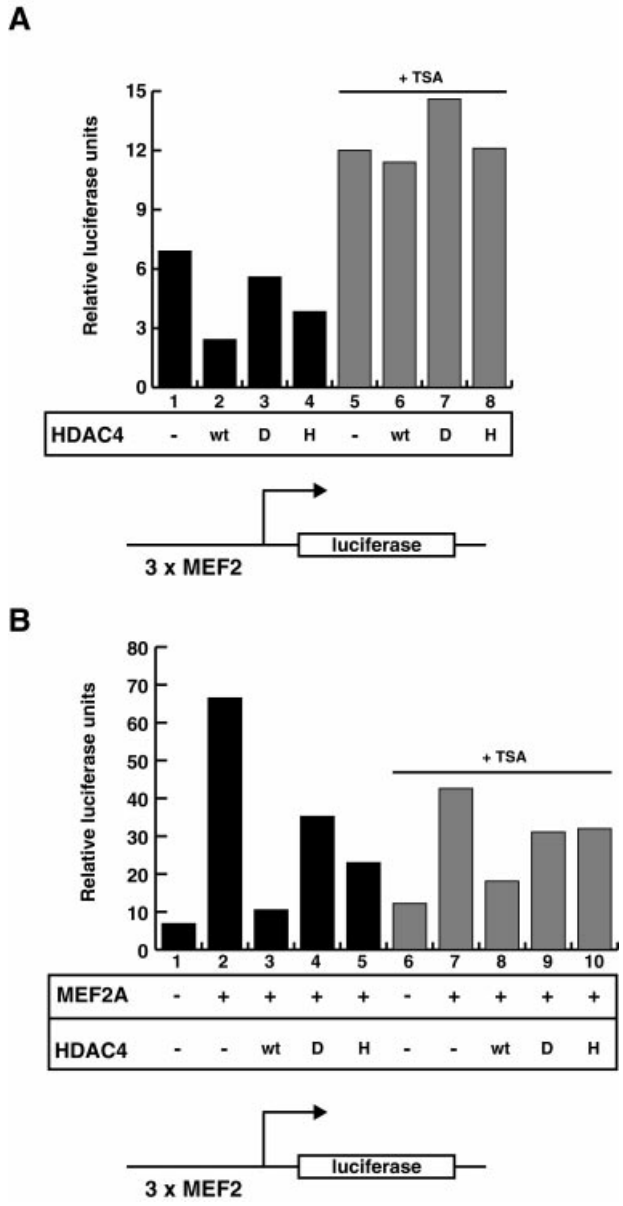

Fig. 7. HDAC4 represses MEF2A-dependent transcription. (**A**) HeLa cells were transfected with 3 μg of a 3×MEF2A–luciferase construct (lane 1–8) and 1.5 μg of pcHDAC4-Myc ('wt' lane 2), pcHDAC4-Myc(D840N) ('D' lane 3) or pcHDAC4-Myc(H803A) ('H' lane 4). In parallel, TSA was added at 330 nM 16 h after transfection (lanes 5–8). (**B**) HeLa cells were transfected with 3 μg of a 3×MEF2A–luciferase construct (lanes 1–10). HA–MEF2A (3 μg) was added in lanes 2–5 and 7–10. In addition, HDAC4 expression plasmids were included as described for (A). TSA was included in lanes 6–10. Expression of transfected proteins was verified using Western blotting (data not shown).

sequences in its unique N-terminal extension. A member of the mammalian RPD3 family, HDAC1, which does not contain these sequences, does not associate with MEF2A (Figure 4B). In contrast, all members of the mammalian RDP3 family bind the E2F transcription factor via an association with the retinoblastoma tumour suppressor protein (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi Jaulin *et al.*, 1998; data not shown). These results provide some early indication that at least some of the transcription factors that target HDAC4 to the promoter may be different from the ones that target the previously known deacetylases. Discrimination between promoter-bound transcription factors may well result in the regulation of a distinct set of promoters by the two different families of deacetylases. In yeast this has been shown to be the case: RPD3 is required for the deacetylation of the promoters of the *INO1, IME2 and SPO13* genes, whereas HDA1 has no effect on those promoters (Rundlett *et al.*, 1998).

The MEF2 transcription factor has been implicated in the regulation of myogenic promoters and in the regulation of the c-*jun* promoter. In all these cases MEF2 has been shown to be a positive factor. The activity of MEF2 is stimulated by the direct association with acetyltransferases such as CBP/p300, an indication that acetylation is involved in the activating role of MEF2 (Eckner *et al.*, 1996; Sartorelli *et al.*, 1997). The association of MEF2A with HDAC4 suggests that this deacetylase may be acting as a co-repressor, switching off MEF2 activity when it is no longer required on a promoter. In this scenario, acetyltransferases and deacetylases may bind successively to regulate MEF2 activity. An alternative scenario is that the MEF2A–HDAC4 complex may function uniquely as a repressor complex only on a subset of promoters. These may be as yet unidentified genes whose activity is selectively silenced by MEF2A–HDAC4, but which are not under the positive control of MEF2. There are other examples of transcription factors whose activity is regulated positively by acetyltransferases and negatively regulated by deacetylases, namely E2F (Trouche *et al.*, 1996; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi Jaulin *et al.*, 1998) and Pit1 (Xu *et al.*, 1998). In the case of E2F, promoters exist that contain either positively or negatively acting E2F binding sites, suggesting that deacetylase targeted to E2F via RB is not required on all promoters.

The MEF2 factors have been shown to be essential for muscle differentiation in various systems, and MEF2 is thought of as a factor with tissue-specific function. A tissue-specific role for MEF2 is partly supported by its expression pattern. Although MEF2 activity is present in various tissues, at least some factors, e.g. mouse MEF2C, are restricted to muscle brain and spleen (Martin *et al.*, 1993). Interestingly, MITR expression in the developing frog embryo also shows a muscle-specific expression pattern (Sparrow *et al.*, 1999). Furthermore, during the preparation of this manuscript, the cloning of full-length HDAC4 was reported and expression was found to be highest in skeletal muscle and brain (Grozinger *et al.*, 1999). These observations suggest that HDAC4 might play a role in MEF2-dependent regulation of differentiation. In the myogenic C2C12 line, MEF2 site DNA-binding activity is present throughout the process of differentiation from myoblasts to myotubes. However, MEF2-regulated genes are activated in a specific temporal pattern during differentiation (Olson *et al.*, 1995). It is tempting to speculate that HDAC4 may contribute to this silencing of MEF2-regulated genes during the process of terminal differentiation. Control of the nuclear distribution of HDAC4 during the differentiation process could provide the signal for the selective silencing of such genes.

Materials and methods

Recombinant DNA

The protein sequences described can be accessed from the DDBJ/EMBL/GenBank databases through the following accession numbers:

AAD29046 (HDAC4), AAD29047 (HDAC5), BAA74924 (HDAC6) and BAA34464 (hMITR). A full-length cDNA for hMITR (KIAA0744) and the cDNA for HDAC4 (KIAA0288, AB006626) were provided by Osamu Ohara and Takahiro Nagase at the Kazusa DNA Research Institute, Laboratory of DNA Technology, Yana 1532-3, Kisarazu, Chiba 292-0812, Japan (Ohara *et al.*, 1997; Nagase *et al.*, 1998). During the preparation of this manuscript, a point mutation in the published HDAC4 cDNA was reported (Grozinger *et al.*, 1999). This mutation was repaired using PCR. HDAC4 cDNA was amplified using PCR and subcloned into pcDNA3 and pcDNA3.1-A-Myc/His (both Invitrogen) to give pcHDAC4 and pcHDAC4-Myc. HDAC4 in this manuscript refers to this repaired, full-length clone. The GAL4 DNA-binding domain (amino acids 1–146) was added N-terminally to pcHDAC4 and inserted into pcDNA3 to give pcGAL4–HDAC4 and pcGAL4 (via subcloning). The Myc/His tag in pcHDAC4 was also replaced by an optimized version of GFP (Zernicka-Goetz *et al.*, 1996) to give pcHDAC4–GFP. HDAC1 and hMITR cDNAs were both amplified using PCR and cloned into pcDNA3.1-A-Myc/His to give pcHDAC1-Myc and pchMITR-Myc. The point mutations D840N and H803A were introduced into pcGAL4HDAC4 and pcHDAC4-Myc via site-directed mutagenesis using the Quickchange kit (Stratagene). Deletion constructs of HDAC4 were cloned using PCR analogous to full-length HDAC4. HDAC4(118–313) was also subcloned into pGEX (Pharmacia). All constructs were verified by DNA sequencing. 5×GAL4TK-CAT and 0×GAL4TK-CAT reporter constructs were both described earlier (Morkel *et al.*, 1997). The 3×MCK–MEF2A–luciferase reporter and HA–MEF2A construct were generous gifts from Mona Nemer. His–MEF2A was a gift from Jiahuai Han.

Cell culture and transfections

HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) with 10% FBS (Gibco-BRL), penicillin, streptomycin, and glutamine (all Gibco-BRL). Cells were grown at 37°C in an atmosphere containing 5% CO_2. Cells were transfected using the $Ca_3(PO_4)_2$ technique as described (Hagemeier *et al.*, 1993a).

Immunoprecipitations

293T cells in culture dishes (15 cm diameter) were transfected with 30 μg of expression vectors. HeLa cells (10-cm dishes) were transfected with 10 μg of DNA. Cells were washed in ice-cold phosphate-buffered saline and lysed in IPH buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) at 4°C for 30 min. Lysates were cleared by centrifugation, diluted five times in IPH buffer containing 0.1% NP-40 and incubated with 5 μg anti-Myc mouse monoclonal antibody (Boehringer Mannheim, Germany) for 1 h. A 50 μl aliquot of a slurry of protein A/G–Sepharose beads (Pharmacia) was added and incubation continued for 2 h with rotation at 4°C. Precipitates were washed six times in ice-cold IPH and either resuspended in loading buffer for SDS–PAGE or used for deacetylase assays.

SDS–PAGE and Western analysis

SDS–PAGE and Western blotting were performed according to standard procedures (Martin *et al.*, 1995). Anti-HA antibody and anti-Myc antibody (both from Boehringer Mannheim, Germany) were used at a concentration of 1 μg/ml. The anti–MEF2A antibody (Santa Cruz) was used at 1 μg/ml.

Histone deacetylase assays

Histone deacetylase assays were carried out essentially as described (Taunton *et al.*, 1996; Brehm *et al.*, 1998) in a volume of 100 μl of IPH buffer containing 250 000 c.p.m. of a tritium-labelled acetylated histone H4 peptide. For inhibition experiments, reaction mixtures were pre-incubated with TSA (Wako BioProducts) for 10 min prior to H4 peptide at 37°C.

Protein expression

GST fusion proteins were expressed in *E.coli* strain BLR using the pGEX (Pharmacia) vector system. Purification of GST fusions from crude bacterial lysates was performed as described previously (Bannister and Kouzarides, 1996). Histidine-tagged proteins were expressed in *E.coli* strain BLR using pET (Novagen) vector systems. Purification was carried out as described (Brehm *et al.*, 1999).

Direct interaction assay

Equal amounts of GST fusions pre-bound to glutathione–Sepharose beads (Pharmacia) were incubated in 200 μl of Z′ buffer (25 mM HEPES pH 7.5, 12.5 mM $MgCl_2$, 150 mM KCl, 20% glycerol, 0.1% NP-40, 1 mM DTT) with 30 μg of BSA for 10 min at room temperature. His-tagged MEF2A was then added to the reactions and incubated at room temperature for 1 h. Beads were spun down and after four washes in NETN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Bound material was resuspended in 1× SDS loading buffer and subjected to SDS–PAGE followed by Western blotting.

Gene reporter assays

HeLa and 293T cells grown in culture dishes (5 cm diameter) were transfected at 40–60% confluency with a total of 10 μg of DNA. Cells were washed 16 h after transfection and incubated for an additional 24 h, either in the presence or absence of TSA (330 nM), before harvesting. Chloramphenicol acetyltransferase and luciferase assays were performed as described previously (Hagemeier *et al.*, 1993b).

Immunofluorescence and confocal microscopy

Cells were fixed and permeabilized in cold methanol/acetone and stained as described previously (Pines and Hunter, 1991). Anti-HA mouse monoclonal antibody (Boehringer Mannheim, Germany) was used at a 1:200 dilution. Cy5-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., USA) was diluted 1:200. Fixed and mounted cells were analysed by confocal microscopy using a Bio-Rad MRC 1024 confocal system on an upright Nikon fluorescence microscope equipped with a 60×, 1.4 NA oil immersion lens. Using the argon ion 488 nm line, cells were scanned using 10% laser power, 2.5× zoom and 10 times Kalman averaging. Images were exported into Adobe Photoshop for processing and printing.

Microinjection, fluorescence microscopy and CCD imaging

Cells were incubated in CO_2-independent medium without phenol red (Gibco-BRL) and incubated at 37°C using a ΔT 0.15 mm dish (Bioptechs, PA). Cells were imaged by time-lapse fluorescence microscopy using a Leica DMIRBE microscope equipped with custom filter sets (Chroma Technology, VE), a PentaMax camera (Princeton Instruments, NJ) and a Lambda 10-2 filter wheel (Sutter, CA) controlled by a PowerWave computer (PowerComputing, TX) running IP Lab Spectrum software (Scanalytics, Inc., VA) as described (Karlsson and Pines, 1998). Images were exported into Adobe Photoshop and printed on a dye-sublimation printer (Tektronix, USA).

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Similar observations for HDAC4 are also reported in Wang,A.H., Bertos,N.R., Vezmar,M., Pelletier,N., Crosato,M., Heng,H.H., Th'ng,J., Han,J. and Yang,X.-J. (1999) HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. *Mol. Cell. Biol*., in press.