MEF2 Is a Converging Hub for Histone Deacetylase 4 and Phosphatidylinositol 3-Kinase/Akt-Induced Transformation

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The MEF2-class IIa histone deacetylase (HDAC) axis operates in several differentiation pathways and in numerous adaptive responses. We show here that nuclear active HDAC4 and HDAC7 display transforming capability. HDAC4 oncogenic potential depends on the repression of a limited set of genes, most of which are MEF2 targets. Genes verified as targets of the MEF2-HDAC axis are also under the influence of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that affects MEF2 protein stability. A signature of MEF2 target genes identified by this study is recurrently repressed in soft tissue sarcomas. Correlation studies depicted two distinct groups of soft tissue sarcomas: one in which MEF2 repression correlates with PTEN downregulation and a second group in which MEF2 repression correlates with HDAC4 levels. Finally, simultaneous pharmacological inhibition of the PI3K/Akt pathway and of MEF2-HDAC interaction shows additive effects on the transcription of MEF2 target genes and on sarcoma cells proliferation. Overall, our work pinpoints an important role of the MEF2-HDAC class IIa axis in tumorigenesis.

Gene transcription is under the influence of complex regulative networks integrating multiple signaling events that end up with the final decision of activating or repressing specific genetic programs. Histone deacetylases (HDACs) play important roles in the regulation of different genetic programs controlling differentiation, survival, tissue homeostasis and metabolism (1, 2). Among the different deacetylases, the class IIa HDACs, including HDAC4, HDAC5, HDAC7, and HDAC9, show a limited enzymatic activity but are equally powerful repressors of transcription by virtue of assembly into multiprotein complexes that recruit other transcriptional corepressor (3–5). Environmental signals control class IIa HDACs activities through different strategies, including regulation of transcription/translation, ubiquitin-dependent degradation, and selective proteolysis (6–11).

A widespread and rapid strategy to modulate class IIa repressive potential is operated through the control of their subcellular localization. These deacetylases shuttle in and out of the nucleus in a phosphorylation-dependent manner. A set of conserved serines, once phosphorylated become docking sites for 14-3-3 chaperone proteins, which escort the deacetylases from the nucleus into the cytoplasm, thus limiting their repressive influence (1, 5, 11–13). In contrast, phosphatases such as PP2A can promote HDAC nuclear import and consequently gene repression (14, 15).

Since class IIa HDACs omit DNA-binding domains, they must bind DNA-binding transcription factors in order to influence gene expression (1, 5, 16–18). Important partners of class IIa HDACs are the transcription factors of the MEF2 family. Genetic studies and the generation of animal models testified to the important role of the MEF2-HDAC axis during development, differentiation, and tissue homeostasis (19).

Molecular pathways that normally ensure proper embryogenesis and tissue maintenance in postembryonic life are subverted during the carcinogenetic process (20). Alterations of the class IIa HDACs and MEF2 transcription factors have been observed in certain cancers (11, 21–24). Overall, the data are scattered and debated, and, more importantly, the impact of the MEF2-

HDAC axis on the tumorigenic process is still undefined. In the present study we addressed the prooncogenic role for class IIa HDACs. Since previous reports correlated HDAC4 with cell proliferation (25–27), we focused in particular on this deacetylase as a model.

MATERIALS AND METHODS

Cell cultures and reagents. NIH 3T3 mouse fibroblasts and human IMR90-E1A cells were grown in Dulbecco modified Eagle medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (all from Lonza). Cells expressing the inducible form of MEF2 were grown in DMEM without phenol (Sigma-Aldrich). BALB/c 3T3 cells were generated from BALB/c primary MEF using the 3T3 protocol (28) and were grown in DMEM supplemented with 10% calf serum. The human leiomyosarcoma cell lines SKUT-1, DMR, and SK-LMS1 were cultivated as previously described (42). For analyses of cell growth, 10⁴ cells were seeded, and the medium was changed every 2 days.

The following chemicals were used (the final concentrations are indicated): 20 µM LY (LY294002; LC laboratories); 2.5 µM MG132, 10 µM BML-210, 1 µM 4-hydroxytamoxifen (4-OHT), 10 µM resazurin, 0.5 mg of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]/ml, and dimethyl sulfoxide (DMSO) (all from Sigma-Aldrich); and leptomycin B (LC Laboratories). The primary antibodies were anti-green fluorescent protein (anti-GFP), anti-HDAC4 (29), antipaxillin, and anti-Ran (BD Transduction Laboratories), anti-VP16 (sc-7545; Santa Cruz), antihemagglutinin (anti-HA; Sigma-Aldrich), antiubiquitin (Covance), anti-nucleoporin p62, anti-RAN, anti-pp120, and anti-MEF2D

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(BD Transduction Laboratories), and anti-Erk, anti-pErk, anti-Akt, anti-Aktp473, anti-MEF2C D80C, and anti-MYC (Cell Signaling).

Plasmid construction, transfection, retroviral infection, and silencing. pEGFPN1 constructs expressing human HDAC4 and its mutants, pcDNA3.1 HA-MEF2C, 3×MEF2-Luc, and pRL-CMV, were previously described (9). All of the cDNAs used were from humans. Cells expressing the different transgenes were generated by retroviral infection as described previously (9). To generate pBABE-Puro-MEF2c-VP16-ER, p-BABE-MEF2cΔDBD-VP16-ER, pWZL-Hygro-MEF2c-VP16-ER, and pWZL-Hygro-MEF2c-ΔDBD-VP16-ER MEF2, the relative cDNAs were subcloned into pBABE-Puro and pWZL-Hygro plasmids using a PCR method and then checked by sequencing. The dominant-negative version of MEF2 encodes for amino acids (aa) 1 to 117. pWZL-HDAC4-TMΔMEF2 was generated in two steps. The N terminus (aa 1 to 166 and aa 184 to 221) was generated by PCR and cloned into pcDNA3+ (EcoRI/BamHI and BamHI/SalI). Finally, fragment 1-221 was subcloned into pWZL-HDAC4-TM-GFP restricted by using Eco-SalI. Silencing of NIH 3T3 and IMR90-E1A were performed with 70 μM small interfering RNA (siRNA; Invitrogen).

Immunofluorescence and immunoblotting. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. The secondary antibodies were Alexa Fluor 488-, 546-, or 633-conjugated anti-mouse and anti-rabbit secondary antibodies (Molecular Probes). Actin was labeled with phalloidin-AF546 (Molecular Probes) or phalloidin-ATTO 665 (Sigma-Aldrich). The cells were imaged with a Leica confocal scanner SP equipped with a 488 λ Ar laser and a 543 to 633 λ HeNe laser.

Cell lysates after SDS-PAGE and immunoblotting were incubated with primary antibodies. Secondary antibodies were obtained from Sigma-Aldrich, and blots were developed with Super Signal West Dura (Pierce). For antibody stripping, blots were incubated for 30 min at 60°C in stripping solution containing 100 mM β-mercaptoethanol.

RNA extraction and quantitative qRT-PCR. Cells were lysed using Tri-Reagent (Molecular Research Center). A total of 1 μg of total RNA was retrotranscribed by using 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) analyses were performed using Bio-Rad CFX96 and SYBR Green technology. The data were analyzed by use of a comparative threshold cycle using β₂-microglobulin, HPRT (hypoxanthine phosphoribosyltransferase), and β-actin as normalizer genes. All reactions were done in triplicate.

Adhesion and random motility measurements. Random motility was assayed by time-lapse video microscopy as previously described (9). To study adhesion and spreading, plates were coated with 10 μg of fibronectin/ml or bovine serum albumin (BSA; Sigma-Aldrich). Cells were seeded at 6×10^4 /ml and, after 10 min, time-lapse analysis was performed. Time-lapse images were analyzed by using Metamorph software (Molecular Devices) and ImageJ. The results are pooled from eight independent experiments.

Soft agar and tumorigenicity assays. Equal volumes of 1.2% agar and DMEM were mixed to generate 0.6% base agar. A total of 10^5 NIH 3T3, BALB/c 3T3, or sarcoma cells expressing the different transgenes were seeded in 0.3% top agar, followed by incubation at 37°C in humidified

conditions. The cells were grown for 15 days, and the culture medium was changed twice per week. Foci were visualized by using MTT staining. For tumorigenicity assays, 400,000 cells expressing the different transgenes were injected subcutaneously into immunocompromised nude mice. In parallel, 100,000 cells of the same cell suspension were plate counted 24 h after plating to check for equal number injection and cell viability. The tumor size was monitored twice per week.

RNA expression array and data analysis. Total RNA was isolated by using RNeasy (Qiagen). RNA samples were labeled according to the standard one-cycle amplification and labeling protocol (Affymetrix, Santa Clara, CA). Labeled cRNAs were hybridized on Affymetrix GeneChip Gene 1.0 ST mouse arrays. Scanning was performed using a GeneChip Scanner 3000 7G (Affymetrix), whereas Microarray Analysis Suite 5.0 software (Affymetrix) was used for preliminary data analysis. One-way analysis of variance was applied to replicates to discard missense gene expression values. We adopted a cutoff of 1.5 for the fold change. Gene set enrichment analysis (GSEA) (30) was used to investigate putative statistical association between genes modulated by HDAC4 and genes perturbed by other signal transduction pathways. The HDAC4 signature was used to interrogate 3,272 curated MSigDB gene sets and 91 data sets available on the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) and coming from DNA microarray experiment on murine fibroblasts. For the analysis, the maximum value of each probe was chosen; the ranking was done according to a signal-to-noise metric, and 1,000 permutations were used to generate the null distribution.

HDAC assay. HDAC assay was performed using a fluorogenic assay kit, the Fluor de Lys-Green HDAC assay (BIOMOL), according to manufacturer's instructions. Briefly, HDAC4 immunoprecipitates were resuspended in the HDAC assay buffer and incubated with Fluor de Lys-Green substrate for 30 min at 37°C. The fluorogenic reaction was triggered by adding developer according to the manufacturer's instructions, and the fluorescence was measured after 15 min and stopped with trichostatin A (TSA). HDAC inhibitor TSA (40 μM) was used as an internal control to measure the background signal. A total of 1.5 μg of anti-HDAC4 and anti-USP33, as a control IgG, were used for immunoprecipitations.

Chromatin immunoprecipitation. For each chromatin immunoprecipitation, 4.5×10^6 cells were used. DNA-protein complexes were cross-linked with 1% formaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS) for 15 min at room temperature. After quenching and two washes in PBS, the cells were collected and then lysed for 10 min with lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40) containing protease inhibitor cocktail (Sigma-Aldrich). The pellets were resuspended in RIPA-100 and sonicated using a Bioruptor UCD-200 (Diagenode) with pulses of 30 s for 15 min, resulting in an average size of ~500 bp for genomic DNA fragments. Samples were precleared and immunoprecipitated overnight with 2 μg of anti-GFP or anti-USP33 antibodies, followed by incubation with protein A blocked with BSA and salmon sperm DNA (1 μg/μl) at 4°C for 2 h. Beads and inputs were treated with proteinase K overnight at 68°C to degrade proteins and reverse cross-linking. Genomic DNA was finally purified with Qiagen QIAquick PCR purification kit and eluted in 65 μl of water.

FIG 1 Morphological changes in cells expressing HDAC4/TM. (A) Confocal pictures of NIH 3T3 cells expressing GFP and the different chimeras. Immunofluorescence analysis was performed to visualize HRasV12. AF546-phalloidin was used to decorate F-actin. Scale bar, 50 μm. (B) Immunoblot assays were performed to visualize the different transgenes. The antibodies used were anti-GFP and HDAC4-GFP, anti-HRas, and anti-Erks as a loading control. (C) qRT-PCR analysis was performed to quantify mRNAs levels of the HDAC4-target gene, *Klf2*. *Gapdh* was used as a control gene. The *Klf2* mRNA levels were relative to GFP-expressing cells. (D) Confocal pictures of cells expressing GFP, GFP-HDAC4/WT, and GFP-HDAC4/TMi2. Immunofluorescence analysis was performed to visualize paxillin subcellular localization. AF546-phalloidin was used to decorate F-actin. Scale bar, 50 μm. (E) NIH 3T3 cells expressing HDAC4/WT, HDAC4/TMi2, or GFP were plated onto BSA- or fibronectin-covered dishes and subjected to time-lapse analysis for the indicated times. The data are presented as the average areas. (F) At 24 h after seeding, NIH 3T3 cells expressing HDAC4/WT, HDAC4/TMi2, or GFP were subjected to time-lapse analysis for 6 h. The data are presented as the average migration rates. (G) qRT-PCR analysis was performed to quantify *Klf2* mRNAs after the transfection of cells expressing HDAC4/TMi2 with siRNA against HDAC4 or control siRNA. *Klf2* mRNA levels were relative to GFP-expressing cells. Immunoblotting was performed with anti-GFP antibodies to prove the siRNA efficiency. (H) Confocal pictures of cells expressing HDAC4/TMi2 transfected with siRNAs against HDAC4 or control siRNA. Immunofluorescence analysis was performed as described in panel D. Scale bar, 50 μm. *, $P < 0.05$; ***, $P < 0.001$.

FIG 2 Transforming ability of HDAC4/TM. (A) NIH 3T3 cells expressing the indicated transgenes were grown in DMEM supplemented with 10% FBS. (B) Growth in soft agar of NIH 3T3 expressing the indicated transgenes, foci were stained with MTT. (C) Quantitative results of colony formation. (D) Analysis of the tumorigenic properties of NIH 3T3 cells expressing the indicated genes when injected into immunocompromised nude mice. HDAC4/TM-expressing cells generate tumors, with nodules becoming palpable ~20 days later compared to HRasV12-transformed cells. Pictures were obtained at week 6. (E) Immunoblot assays were performed to visualize the different transgenes expressed in the BALB/c 3T3 cell lines. The antibodies used were anti-GFP to detect GFP and HDAC4-GFP. Anti-Erks antibody was used as a loading control. (F) Quantitative results of colony formation in soft agar of BALB/c 3T3 cells expressing the indicated transgenes. (G) Analysis of the tumorigenic properties of BALB/c 3T3 cells expressing the indicated genes when injected into immunocompromised nude mice. (H) Confocal pictures of NIH 3T3 cells expressing GFP chimeras of HDAC7-WT and a mutant defective in the four serine binding sites for 14-3-3 proteins (HDAC7-S/A). Scale bar, 50 μ m. (I) Immunoblot assays were performed to visualize the different transgenes expressed in the NIH 3T3 cell lines. The antibodies used were anti-GFP to detect GFP, HDAC7-WT, and HDAC7-S/A. Anti-p62 antibody was used as a loading control. (J) qRT-PCR analysis was performed to quantify mRNAs levels of *Klf2*. *Gapdh* was used as control gene. *Klf2* mRNA levels were relative to GFP-expressing cells. (K) Quantitative results of colony formation in soft agar of NIH 3T3 cells expressing the indicated transgenes. **, $P < 0.01$; ***, $P < 0.001$.

FIG 3 Identification of genes repressed by HDAC4/TM. (A) Diagram representation of the HDAC4/TM target genes. Microarray analyses were performed on GFP- and HDAC4/TM-expressing cells (repressed genes are indicated in red) and in HDAC4/TM cells transfected with control siRNA and the same cells transfected with a siRNA against human HDAC4 (induced genes are indicated in green). (B) Gene ontology (GO) analysis using the PANTHER database was performed to interpret the biological processes under the regulation of the 47 genes repressed by HDAC4. (C) GO analysis using the PANTHER database was performed to classify the 47 genes repressed by HDAC4 in terms of subcellular localization. (D to G) GSEA plots show the enrichment of HDAC4-repressed genes among protein coding genes ranked according to PTEN and TSC2 status and the fold change in LY-treated cells versus control cells. See Materials and Methods for details.

Reporter gene assay. The promoter of *RhoB* (300 bp) was cloned from NIH 3T3 genomic DNA by PCR into the pGL3 plasmid. The following oligonucleotides were used: *RhoB_FW_XhoI*, 5'-ATC CTC GAG CAA TCG GAG CCA AGC TCC GC-3'; and *RhoB_RV_HindIII*, 5'-ATC AAG CTT GAG CTG GCC GGG CGC GGG CA-3'. IMR90-E1A cells were transfected at 30 to 40% confluence with the indicated mammalian expression plasmids. In the LY experiments, cells were collected 12 h after transfection, split into two plates, and treated after 6 h with LY-294002 or DMSO. The luciferase activity was measured and normalized for *Renilla* luciferase activity using the dual-luciferase reporter assay system according to the vendor's instructions (Promega). The empty vectors pEGFP or pUSE were used to normalize the total amounts of transfected DNA.

Immunoprecipitation. Coimmunoprecipitations were performed as previously described (9). Briefly, cells were collected directly from culture dishes into radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.2% SDS, 1% NP-40, 0.5% sodium deoxycholate) and supplemented with protease inhibitors. Lysates were incubated for 5 h with the antibody against green fluorescent protein (GFP). After 1 h of incubation with protein A-beads (GE), several washes were performed. Samples were resolved by SDS-PAGE and analyzed by immunoblotting.

Bioinformatics analysis. To analyze MEF2 target gene expression in human cancers, the presence of a putative MEF2 binding site in the proximal promoters was scored using Cister (zlab.bu.edu/~mfrith/cister.shtml). Among our list of 29 human MEF2 target genes, we selected 25 that have good-quality probes and a proximal MEF2 binding site. For this analysis, 40 human cancer data sets available on GEO were selected according to sample abundance and the platform used (Affymetrix Human Genome U95 version 2 array; Affymetrix Human Genome U133B/Plus2). The data from each DNA microarray experiment were considered separately and were log₂ transformed, normalized at the probe set level, and median centered. In the case of multiple probe sets, we discarded any that could hybridize with other transcripts, in addition to the expected level for >33% of the probes (scored using PLANdbAffy [<http://affymetrix2.bioinf.fbb.msu.ru>] [31] and Genecruiser [<http://genecruiser.broadinstitute.org>]). In the case of missing information about a probe set, we used the class A probe set according to the NetAffy (www.affymetrix.com/analysis/index.affx) classification. We then collapsed the multiple values of each gene by averaging them.

The median values representing this signature in each sample were plotted, resulting in a series of box plots. The significance was calculated considering as positive events the samples in which the median of the 25 MEF2 genes is less than zero and applying a Poisson test of significance. The resulting *P* value was corrected for multiple testing by applying Holm-Bonferroni correction (*P* < 0.05). For correlation analysis, the Spearman rank correlation coefficient and the corresponding statistical significance were calculated using the R package.

Tissue array construction and immunohistochemistry. Paraffin-embedded samples from leiomyosarcoma were available from 26 patients. All cases were histologically and immunohistochemically validated. Multiple tissue cores (three per sample) with a diameter of 1 mm were taken by using a Tissue Arrayer (TMA Master 3dHistech) and arrayed on a recipient block according to standard procedures. Immunohistochemistry for HDAC4 (1:100) was performed by using an automated immunostainer (Autostainer; Dako Cytomation). Antigen retrieval was performed with citrate buffer at pH 6 for HDAC4 and at pH 9 with EnVision FLEX target

retrieval solution (Dako Cytomation), respectively. As a negative control, sections were stained without adding the primary antibody. Slides were independently evaluated by two observers. All tumors were scored for the intensity of signal (scoring range: 0 = no expression; 1 = weak expression; 2 = moderate expression; 3 = strong expression). The presence of sub-cellular localization (i.e., nuclear or cytoplasmic) was recorded as well. Mean intensities and percentages of duplicate cores were used for the final analysis.

Statistics. For experimental data, a Student *t* test was used. A *P* value of <0.05 was chosen as the statistical limit of significance. Unless otherwise indicated, all of the data in the figures are arithmetic means $\pm$ the standard deviations from at least three independent experiments.

RESULTS

Nucleus-localized HDAC4 triggers morphological changes and increased proliferation in NIH 3T3 cells. To investigate the role of HDAC4 in the control of cell growth and proliferation, we engineered NIH 3T3 cells to express GFP-tagged HDAC4/WT or its nucleus-localized version (TM), which is defective in 14-3-3 binding. This mutant, by mimicking the dephosphorylated form (Ser/Ala mutations) of the deacetylase, displays nuclear localization and exhibits increased repressive transcriptional activity (12, 13). NIH 3T3 cells expressing oncogenic HRasV12 or GFP were used as positive and negative controls, respectively. Two independent infections with the TM allele were exploited (TM/i1 and TM/i2).

Immunofluorescence analysis proved that HDAC4/WT is mainly cytosolic (Fig. 1A) and subjected to nuclear/cytoplasmic shuttling (data not shown). In contrast, the TM mutant is predominantly nuclear, with some cells showing pan/cytoplasmic localization. Figure 1B illustrates the expression levels of the different transgenes. In general HDAC4/TM was expressed to less extent compared to the WT.

The repressive influence of HDAC4 was then measured by using an MEF2 target, the transcription factor *Klf2* (24) (Fig. 1C). qRT-PCR experiments demonstrated that in HDAC4 expressing cells *Klf2* mRNA is reduced and, as expected, the TM mutant is a more potent repressor. *Klf2* mRNA levels were also decreased in cells expressing HRasV12.

Morphological inspection of engineered cells revealed that whereas no relevant changes in cell shape were detectable after ectopic expression of the WT allele, the expression of the nuclear allele (both TM/i1 and TM/i2) resulted in the gain of a spindle-like morphology, characterized by reduced size and reduced spreading/adhesion (Fig. 1A and D). Moreover, the nuclear allele promoted an overt reorganization of actin cytoskeleton characterized by the loss of stress fibers and by an increase in membrane ruffles.

To gain more insight into the morphological changes induced by HDAC4/TM we compared the organization of focal adhesions (FA) in the different cell lines. Since TMi1 and TMi2 evidenced the same morphological changes but TMi2 expressed a higher level of

FIG 4 Several HDAC4-repressed genes are MEF2 targets. (A) The mRNA expression levels for 11 HDAC4 target genes and *Gapdh*, as a control, were measured using qRT-PCR in GFP- and HDAC4/TM-expressing cells. Cells were also treated with LY for 12 or 24 h. The mRNA levels were relative to untreated GFP-expressing cells. (B) The mRNA expression levels of 11 HDAC4 target genes were measured using qRT-PCR in GFP- and MEF2DN-expressing cells. (C) Chromatin immunoprecipitation of NIH 3T3 cells overexpressing MEF2-GFP or control Puro. Chromatin was immunoprecipitated with anti-GFP antibody or anti-USP33 (2 μ g) as a control. For each of the genes examined, we compared the fold enrichment over input (1/100) between the proximal (1 to 1,000 bp) and the distal (>3,000 bp) promoters, as indicated. (D) Nucleotide sequence analysis of the human and mouse *RhoB* proximal promoters. The putative MEF2 binding site is underlined. (E) Relative luciferase activity after cotransfection in IMR90-E1A cells of the reporter plasmids p*RhoB-Luc* (−300/−1) and p3 $\times$ MEF2-luc, together with *MEF2C*, as indicated. The *Renilla* luciferase plasmid was used as an internal control. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

FIG 5 Regulation of the MEF2-dependent transcription by the PI3K/Akt pathway. (A) IMR90-E1A cells were transfected with the 3×MEF2-Luc (1 μg), the internal control pRL-CMV (20 ng), pcDNA3.1-HA-MEF2C (1 μg), and 300 ng of pEGFP expressing HDAC4. Cells were treated or not for 24 h with LY. (B) IMR90-E1A cells were transfected as in panel A, together with the indicated HDAC4 mutants. Cells were treated or not for 24 h with LY. (C) IMR90-E1A cells were transfected with the 3×MEF2-Luc (1 μg), the internal control pRL-CMV (20 ng), pcDNA3.1-HA-MEF2C (1 μg), and 1 μg of pUSE vectors expressing Myr-Akt or its catalytically inactive point mutant K179M. (D) IMR90-E1A cells transfected with siRNAs against HDAC4, HDAC5, and HDAC9 or with the same amount of a control siRNA were cotransfected after 12 h with 3×MEF2-Luc (1 μg), the internal control pRL-CMV (20 ng), and eventually pcDNA3.1-HA-MEF2C (1 μg), as indicated. After 12 h, the cells were split into two plates and treated or not for 24 h with LY. (E) qRT-PCR analysis was performed to quantify the mRNA levels of *HDAC4*, *HDAC5*, and *HDAC9* in IMR90-E1A cells, cotransfected with the indicated siRNAs. *GAPDH* was used as a control gene. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the transgene and more efficiently repressed *Klf2* expression, we used TMi2 for the subsequent analysis. In GFP- and HDAC4-expressing cells, paxillin marking FA is localized at the cell periphery, with distinct punctate staining (Fig. 1D). In contrast, in HDAC4/TM cells, a prominent diffuse staining of paxillin is evident, thus confirming the profound changes of actin cytoskeleton and of FA.

To quantify the differences in adhesion/spreading elicited by HDAC4/TM, we analyzed the behavior of the three cell lines when plated onto BSA or fibronectin. Figure 1E demonstrates that

HDAC4/TM cells evidence a restricted spreading under both conditions. Defects in cells spreading could be responsible for the reduced size observed in Fig. 1A/D.

HDAC4 can influence cell motility (9). Since we have observed an insightful rearrangement of actin cytoskeleton in HDAC4/TM cells, we compared the random cell motility of the different cell lines by performing a time-lapse microscopy analysis. Figure 1F shows that HDAC4/WT-expressing cells increase motility to $0.78 \mu\text{m}/\text{min}$ (standard error of the mean [SEM] = 0.026 ; $n = 141$) compared to GFP-expressing cells

FIG 6 The PI3K/Akt pathway influences MEF2 protein stability. (A) HDAC4 was immunoprecipitated from NIH 3T3 cells treated or not for 18 h with LY. The HDAC activity was measured 15 min after the addition of the developer. (B) Confocal pictures of IMR90-E1A cells transfected with pcDNA3.1-HA-MEF2C (1 μ g) and pEGFPN1-HDAC4 (300 ng) and treated or not with LY for 24 h. Immunofluorescence analysis was performed to visualize HDAC4 and MEF2C subcellular localization. Scale bar, 50 μ m. (C) Quantification of endogenous HDAC4 subcellular localization in IMR90-E1A cells after the treatment with LY or DMSO for 24 h. For each experiment, at least 200 cells were counted ($n = 3$). (D) IMR90-E1A cells were transfected with pcDNA3.1-HA-MEF2C (1 μ g), and 300 ng of pEGFP expressing HDAC4, as indicated. After 12 h, cells were harvested, split into two plates and treated with the PI3K inhibitor LY. After 24 h, cellular lysates were generated and subjected to immunoblot analysis using the anti-GFP and the anti-HA antibodies. Nucleoporin (p62) was used as loading control. (E) IMR90-E1A cells were transfected with pcDNA3.1-HA-MEF2C (1 μ g), and 1 μ g of pUSE vectors expressing Myr-Akt or its catalytically inactive point mutant K179M. After 24 h, cellular lysates were generated and subjected to immunoblot analysis with the anti-Akt and the anti-HA antibodies. Nucleoporin (p62) was used as loading control. (F) Immunoblot analysis of MEF2 family members in IMR90-E1A and NIH 3T3 cells treated with LY and the proteasome inhibitor MG132 as indicated. p120 was used as loading control. (G) IMR90-E1A cells were cotransfected with HA-ubiquitin and MEF2C-GFP or GFP. After 24 h, the cells were treated or not for 12 h with LY, followed by 12 h with MG132. GFP fusions were immunoprecipitated with an antibody to GFP and subjected to immunoblotting with an antiubiquitin antibody. After being stripped, the filter was probed with an anti-GFP antibody. Inputs have been included. (H) Immunoblot analysis of MEF2C and MEF2D levels in NIH 3T3 cells expressing the catalytically active PI3K (PI3KCA) treated with MG132 as indicated. Cellular lysates were generated and subjected to immunoblot analysis with the specific antibodies. The Akt phosphorylation levels were also probed. p120 was used as a loading control. (I) mRNA expression levels of selected MEF2-HDAC4 target genes and *Gapdh*, as a control, were measured using qRT-PCR in NIH 3T3 cells expressing PI3KCA or Puro. Samples were normalized to HPRT, GAPDH, and β -actin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(0.56 μ m/min; SEM = 0.02; $n = 136$). This increase is significantly more pronounced in cells expressing HDAC4/TM (1.15 μ m/min; SEM = 0.04; $n = 171$).

To confirm that the observed changes were elicited by HDAC4/

TM, we silenced its expression using a human specific siRNA. The efficiency of silencing was proved by immunoblotting and by augmented *Klf2* expression (Fig. 1G). The downregulation of HDAC4/TM led to a reversion to the morphological changes de-

scribed above. The cells increase spreading, rebuild stress fibers, and reorganize FA (Fig. 1H).

HDAC4/TM induces cell transformation and tumorigenesis.

The altered morphology and the increased motility of cells expressing HDAC4/TM are reminiscent of a transformed phenotype. Moreover, the two cell lines expressing the TM allele have a proliferative potential greater than cells expressing GFP or HDAC4/WT, overcoming the contact inhibition, similarly to HRasV12 transformed cells (Fig. 2A). To assess whether TM-expressing cells acquire parameters of transformation, we investigated their ability to form colonies in soft agar (Fig. 2B and C). Cells expressing HDAC4/TM but not GFP or HDAC4/WT developed large colonies in soft agar, similarly to HRasV12-expressing cells. In summary, HDAC4/TM dismisses contact-dependent inhibition and confers anchorage-independent growth. Finally, HDAC4/TM-engineered NIH 3T3 cells but not GFP or HDAC4/WT cells generated tumors when subcutaneously injected into athymic nude mice (Fig. 2D).

The oncogenic properties of the HDAC4/TM allele were confirmed also in BALB/c 3T3 cells, ruling out the possibility that the observed phenotypes were context dependent (Fig. 2E to G). As expected, HDAC4/WT was largely cytoplasmic, whereas the TM mutant accumulated in the nucleus (data not shown). HDAC4/TM-expressing but not HDAC4/WT- or GFP-expressing BALB/c 3T3 cells were able to grow in soft agar (Fig. 2F). When BALB/c 3T3 cells expressing HDAC4/TM were subcutaneously injected into nude mice, they efficiently generated tumors (Fig. 2G).

To understand whether HDAC4 shares with other class IIa members this prooncogenic activity, we generated NIH 3T3 cells expressing HDAC7/WT or its nucleus-localized version (S/A), which is defective in all four serine binding sites for 14-3-3 proteins (32). Similar to HDAC4/TM, HDAC7-S/A was mostly nuclear (Fig. 2H) and, although expressed to a lower extent compared to the cytosolic HDAC7/WT (Fig. 2I), it exerted a stronger repression on *Klf2* expression (Fig. 2J). HDAC7-S/A cells mimicked the morphological changes observed in HDAC4/TM-expressing cells (data not shown). Moreover, similar to HDAC4/TM, HDAC7-S/A conferred the NIH 3T3 transformed phenotype and anchorage-independent growth capability (Fig. 2K). Overall, our findings suggest that nucleus-resident class IIa HDACs can elicit tumorigenic conversion of immortalized mouse fibroblasts.

Identification of genes under the influence of HDAC4. To identify key mediators of class II HDAC oncogenic properties, the transcriptional expression profiles of HDAC4/TM and HDAC4/GFP were compared. To further corroborate our results, microar-

ray analysis was also performed when HDAC4 expression was silenced in TM cells. In this manner, we identified 47 genes whose expression is both repressed in HDAC4/TM cells, compared to GFP cells, and induced in HDAC4/TM cells after HDAC4 silencing (Fig. 3A).

We next examined the publicly available databases Gene Ontology (www.geneontology.com) and Panther (www.pantherdb.org) to assess the representation of different biological functions among genes repressed by HDAC4/TM (Fig. 3B). The top-ranking GO biological functions were proliferation (18%) and differentiation/development/morphogenesis (13%). Interestingly, the third category was the regulation of transcription/DNA binding, and the top GO subcellular component is the nucleus (22%) (Fig. 3C). These evidences indicate that HDAC4 profoundly reprograms the expression profile and thus the cell fate. Not surprisingly, several transcription factor genes (*Nr4a1*, *Nr4a2*, *Klf2*, *Klf3*, *Klf4*, *Bhlhe41*, *Pbx3*, and *Foxf1a*) can be found among the 47 genes repressed by HDAC4.

To gain insight into the signaling pathways deregulated by HDAC4/TM, we used GSEA (30). We compared our DNA microarray data with gene sets from the 3,272 curated MSigDB data sets. From this analysis we found that the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signature is among the most enriched (data not shown). To confirm this result, we used as data sets 91 DNA microarray experiments available on GEO, including different models of transformation in murine fibroblasts and our gene list as a gene set. The signature of HDAC4 significantly overlaps genes repressed by the PI3K/Akt/mTOR pathway (Fig. 3D to G) through PTEN ablation (33) (normalized enrichment score [NES] = 1.46, $P < 0.005$) or TSC2 inhibition (34) (NES = 1.61, $P < 0.05$). Furthermore, the HDAC4 signature is negatively enriched for gene expression profiles elicited by the inhibition of the PI3K/Akt/mTOR pathway, using the PI3K inhibitor LY (35) (NES = -1.8485, $P < 0.005$), or induced PTEN expression in *Pten*^{-/-} MEFs (33) (NES = -1.5228, $P < 0.05$).

Several genes repressed by HDAC4 are MEF2 targets and are negatively regulated by the PI3K/Akt pathway. To validate the microarray studies, we performed qRT-PCR analysis on a panel of 11 genes of the 47 described above, among which we included some MEF2 targets (*Klf2*, *Klf4*, *Edn1*, and *Nr4a1*) and others not previously associated with MEF2 (*RhoB*, *Nr4a2*, *Trib1*, *Anxa8*, *Irs1*, *Fgf7*, and *Errf1*). *Gapdh* was used as control. Furthermore, GFP and HDAC4/TM cells were also treated with the PI3K inhibitor LY for 12 and 24 h to validate the GSEA. Except for *Fgf7*, *Errf1*, and *Edn1*, the expression of all HDAC4 targets was upregu-

FIG 7 MEF2 transcriptional activation can revert the oncogenic phenotype. (A) Immunoblot analysis of MEF2-VP16-ER levels in NIH 3T3 cells expressing GFP or HDAC4-TM/GFP or control vector (Hygro-Puro). MEF2-VP16-ER-dependent transcription was induced by treating cells with 4-OHT for 24 h. Cellular lysates were generated and subjected to immunoblot analysis with anti-VP16 or anti-GFP antibodies. p62 (nucleoporin) was used as loading control. (B) Confocal pictures showing MEF2-ER-VP16 nuclear accumulation after the induction with 4-OHT in NIH 3T3 HDAC4/TM cells (Hygro) stably expressing MEF2-VP16-ER (Puro). Immunofluorescence analyses to visualize MEF2-VP16-ER subcellular localization were performed with an anti-VP16 antibody. Scale bar, 50 μ m. (C) Confocal pictures of HDAC4/TM cells expressing MEF2-ER-VP16 chimera or its mutant defective in DNA binding MEF2 Δ DBD-ER-VP16 grown in the presence of 4-OHT. Immunofluorescence analysis was performed to visualize HDAC4 and paxillin subcellular localizations. AF546-phalloidin was used to decorate F-actin. Scale bar, 50 μ m. (D) mRNA expression levels of selected MEF2-HDAC4 target genes and *Gapdh*, as a control, were measured by using qRT-PCR in HDAC4/TM cells expressing MEF2-ER-VP16 or the mutant MEF2 Δ DBD-ER-VP16. (E) HDAC4/TM cells were grown in DMEM supplemented with 10% FBS. The day after seeding, 4-OHT was added. (F) Quantitative results of colony formation in soft agar of NIH 3T3 cells expressing GFP or HDAC4/TM and the two MEF2 forms. The day after seeding, 4-OHT was added to culture medium. (G) Immunoblot analysis of MEF2-VP16-ER and MEF2 Δ DBD-VP16-ER levels in NIH 3T3 cells expressing PI3KCA or the control vector (Puro). MEF2-dependent transcription was induced by treating cells with 4-OHT for 24 h. Cellular lysates were generated and subjected to immunoblot analysis with anti-VP16 or the indicated antibodies to monitor PI3K activation. p120 was used as a loading control. (H) Quantitative results of colony formation in soft agar of NIH 3T3 cells expressing Puro or PI3KCA and the two MEF2 forms. The day after seeding, 4-OHT was added to the culture medium. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

lated after inhibition of the PI3K signaling. Interestingly, addition of the PI3K inhibitor reduces but did not abrogate the repressive activity of HDAC4 on these genes (Fig. 4A).

In order to clarify which genes of the panel are MEF2 targets, we generated NIH 3T3 cells expressing MEF2DN, a dominant-negative version of MEF2C (36) fused to GFP. MEF2DN-GFP was expressed at a lower level compared to HDAC4-GFP and less efficiently repressed MEF2-dependent transcription (data not shown). Except for *Errfi1*, all of the selected HDAC4 target genes were also downregulated after the expression of MEF2DN (Fig. 4B). To further attest the identified genes as MEF2 targets, we performed chromatin immunoprecipitation experiments in MEF2C-GFP-overexpressing cells. We selected a set of genes whose expression was influenced by MEF2DN, namely, *Irs1*, *RhoB*, *Klf4*, *Anxa8*, and *Klf2*. All of the selected genes are significantly enriched for MEF2 binding in the proximal promoter (Fig. 4C). Interestingly, several MEF2 targets identified by our study (*Irs1*, *RhoB*, *Klf2*, *Nr4a1*, *Nr4a2*, *Fgf7*, and *Trib1*) have recently been proposed as MEF2 targets in a lymphoblastic cell line by the ENCODE project (37).

Since *RhoB* showed the highest enrichment in the ChIP experiments, we decided to further prove its relationships with MEF2 by cloning its proximal promoter. The MEF2 consensus sequence embedded in the *RhoB* proximal promoter is highlighted in Fig. 4D. Its coexpression, together with MEF2C, dramatically augmented the luciferase activity, used as a reporter gene (Fig. 4F).

The PI3K/Akt pathway represses MEF2 transcriptional activity. The GSEA and the effect of the PI3K inhibitor LY suggest that the PI3K/Akt pathway could be involved in the regulation of genes, which are also under the influence of MEF2/HDAC4 axis. To further prove this relationship, we evaluated the ability of LY to directly influence MEF2-dependent transcription. Human fibroblasts expressing the E1A oncogene were used for these studies because of their high transfection efficiency. Treatment with LY increased MEF2C-dependent transcription but modestly affected the HDAC4 repressive influence (Fig. 5A). Similar results were obtained in NIH 3T3 cells (data not shown). Afterward, we explored the susceptibility of a set of HDAC4 variants to LY treatment. All of the different mutants show a behavior similar to that of the WT, being able to suppress MEF2C-dependent transcription also in the presence of the inhibitor (Fig. 5B). The only exception was the HDAC4 mutant lacking the amino terminus, which is defective for MEF2 binding and thus for repressive activity (8).

The repressive effect of PI3K/Akt signaling pathway on MEF2-dependent transcription was corroborated in IMR90-E1A cells expressing a constitutive active (A) version (Myr-Akt) of Akt (Fig. 5C). In contrast, a dominant-negative (I) form of Akt (K179M) increased the MEF2C-dependent transcriptional activity (Fig. 5C). Similarly to the effect of LY, the repressive influence of HDAC4 was only weakly affected by the coexpression of the Akt variants.

HDAC4 and PI3K/Akt could exert their repressive influence on MEF2-dependent transcription as components of the same pathway or as independent arms of different signaling pathways. To answer this question, we evaluated whether the depletion of HDAC4 and LY showed additive effects on MEF2-dependent transcription. Because of compensatory mechanisms and redundant functions among class IIa HDACs (24), HDAC4, HDAC5, and HDAC9 were simultaneously silenced. Together with the

three siRNAs, the MEF2-Luc reporter was cotransfected. Subsequently, the cells were incubated or not with LY. As a further control, in an additional set of experiments, we ectopically expressed MEF2C.

Transcription from the MEF2 promoter was upregulated 2-fold after PI3K inhibition (Fig. 5D). Silencing of class IIa HDAC4/5/9 increased transcription by ~4-fold. Downregulation of class IIa HDACs in the presence of LY dramatically augmented MEF2-dependent transcription (20-fold). When the experiment was repeated in the presence of ectopic MEF2C, the trend was similar. Silencing of class IIa HDACs and inhibition of the PI3K pathway demonstrated additive effects on MEF2-dependent transcription. Figure 5E shows the siRNA efficiency, as measured by qRT-PCR.

The PI3K/Akt pathway influences MEF2 protein stability. Although we have provided data that the PI3K/Akt pathway can influence MEF2 transcriptional activity, the mechanism involved remains obscure. In order to gain insight into the mechanisms exerted by PI3K/Akt signaling on MEF2s, we analyzed whether LY could influence the deacetylase activity associated with HDAC4 (Fig. 6A) or HDAC4 and MEF2C colocalization (Fig. 6B and C). All of the analyzed parameters were unaffected by LY.

We next compared the levels of MEF2C when expressed in the presence or absence of LY. We also coexpressed HDAC4 to evaluate its effect on MEF2C under these conditions. Immunoblot analysis confirmed that HDAC4 influences the electrophoretic mobility of MEF2C (Fig. 6D) (38). Importantly, LY augmented MEF2C protein levels independently from HDAC4. Similarly, the coexpression of the inactive mutant of Akt sustained MEF2 expression, whereas the active form reduced its level (Fig. 6E).

To confirm that the PI3K/Akt can impact MEF2 stability, we treated IMR90-E1A and NIH 3T3 cells with LY, in the presence or not of the proteasome inhibitor MG132. Extracts were generated, and the protein levels of MEF2C and MEF2D compared. LY and MG132 can augment the levels of the two MEF2 isoforms, and the effect is not additive (Fig. 6F). These data suggest that the PI3K/Akt pathway impinges on MEF2 by controlling its turnover via the ubiquitin-proteasome system. To prove this hypothesis, we evaluated MEF2C polyubiquitination in the presence of LY. Coimmunoprecipitation studies in E1A cells, coexpressing MEF2C-GFP and HA-ubiquitin, demonstrated that the PI3K/Akt pathway is required for the polyubiquitination of MEF2C (Fig. 6G).

To further strengthen the relationships between MEF2 transcription, protein degradation, and the PI3K/Akt pathway, we generated NIH 3T3 cells expressing catalytic active PI3K. The levels of both MEF2C and MEF2D are reduced in cells expressing the PI3KCA compared to control cells, and treatment with the proteasome inhibitor rescued the levels of both MEF2 isoforms (Fig. 6H). In agreement with this observation, the expression levels of several MEF2 targets (*Klf2*, *End1*, *Irs1*, and *RhoB*) were reduced in cells expressing constitutive active PI3K (Fig. 6I).

Activation of MEF2 reverses the oncogenic properties of cells expressing HDAC4/TM and PI3K/CA. In addition to MEF2, HDAC4 can influence other transcription factors and, of the identified 47 genes, some are not MEF2 targets. To understand whether the oncogenic phenotype of cells expressing HDAC4 depends on the repression of the MEF2 genetic program, we decided to reactivate MEF2-dependent transcription in HDAC4-transfected cells. We took advantage from a MEF2-VP16-ER chimera in which the ligand-binding domain of the estrogen receptor (ER)

is fused to the C terminus of the constitutively active MEF2-VP16 fusion protein (39). We also used as a control the MEF2-VP16-ER lacking the DNA-binding domain (Δ DBD aa58-86). Immunoblot analysis of the different transgenes expressed in HDAC4/TM or GFP cells is shown in Fig. 7A. We also monitored the subcellular localization of MEF2-VP16-ER in HDAC4/TM cells to verify its nuclear accumulation after 4-OHT treatment (Fig. 7B). Induction of MEF2-VP16 in HDAC4/TM transformed cells reversed the morphological alterations and promoted stress fiber formation and focal adhesion assembling. In contrast, induction of MEF2 Δ DBD-VP16 was ineffective (Fig. 7C).

Induction of MEF2-VP16 but not of its deletion version (Δ DBD) elicited the upregulation of MEF2 target genes also in cells expressing HDAC4/TM (Fig. 7D). The reactivation of the MEF2 program was sufficient to dramatically limit the proliferative potential of cells expressing HDAC4/TM to a level similar to GFP-expressing cells (Fig. 7E). Finally, growth in soft agar of HDAC4/TM-expressing cells was suppressed as well upon induction of MEF2-VP16 expression (Fig. 7F).

To corroborate the key role of MEF2 in counteracting transformation, we introduced the MEF2-VP16-ER chimera or its DNA-binding deletion version in PI3KCA-transfected cells (Fig. 7G). Also in this case, the induction of MEF2-VP16 but not of its deleted version (Δ DBD) suppressed the ability of the transformed cells to grow in soft agar (Fig. 7H). These results indicate that MEF2 is an important target of the HDAC4 and PI3K transforming activity and suggest that the repression of the MEF2 genetic program is sufficient to confer oncogenic properties to NIH 3T3 cells.

HDAC4/TM defective in MEF2 binding loses its transforming activity. To show that the repression of MEF2 transcription is a key step for HDAC4 transforming activity, we constructed a nuclear mutant of HDAC4 unable to complex MEF2. The MEF2 binding region, which comprises aa 166 to 184, was deleted from HDAC4/TM to produce HDAC4/TM Δ MEF2 (Fig. 8A). NIH 3T3 cells expressing HDAC4/TM and HDAC4/TM Δ MEF2 were next generated. The 166-184 mutant shows a clear nuclear localization (Fig. 8B) and is expressed at levels similar to the HDAC4/TM (Fig. 8C). The repressive activity of HDAC4/TM on *Klf2* expression was abrogated after the removal of aa 166 to 184 (Fig. 8D). A coimmunoprecipitation study confirmed that the binding to MEF2D was impaired in the HDAC4/TM Δ MEF2 mutant (Fig. 8E). Having characterized the properties of this new mutant, we next investigated its transforming ability. Figure 8F testifies that cells expressing HDAC4/TM Δ MEF2 are unable to grow in soft agar in contrast

FIG 8 HDAC4/TM defective in MEF2 binding loses its transforming activity. (A) Scheme of HDAC4/TM highlighting the deacetylase domain and the region involved in MEF2 binding. The deletion mutant generated for the present study is also illustrated. (B) Confocal pictures of NIH 3T3 cells expressing HDAC4/TM-GFP or its deleted version for MEF2 binding. Scale bar, 50 μ m. (C) Immunoblot analysis of HDAC4/TM and HDAC4/TM Δ MEF2 levels in NIH 3T3 cells. Immunoblot analysis was performed with anti-GFP antibodies. p62 (nucleoporin) was used as a loading control. (D) qRT-PCR analysis was performed to quantify mRNAs levels of the HDAC4 target gene, *Klf2*. *Gapdh* was used as a control gene. *Klf2* mRNA levels were relative to GFP-expressing cells. (E) Cellular lysates from NIH 3T3 cells expressing HDAC4/TM and HDAC4/TM Δ MEF2 were immunoprecipitated with an anti-GFP antibody. Immunoblots were performed with anti-MEF2D and anti-GFP antibodies. NRS, normal rabbit serum. (F) Quantitative results of colony formation in soft agar of NIH 3T3 cells expressing the indicated transgenes. ***, $P < 0.001$.

B GSEA on STSs using the MEF2 signature

DESCRIPTION	ACCESSION	NES	P
Normal tissue vs Liposarcoma (dedifferentiated)	GSE2719	1.66	0.016
Normal tissue vs Liposarcoma (myxoid and round cell)	GSE2719	2.52	0.001
Normal tissue vs Liposarcoma (pleomorphic)	GSE2719	1.74	0.026
Normal fat vs Liposarcoma (dedifferentiated)	GSE21122	1.79	0.0079
Normal fat vs Liposarcoma (myxoid and round cell)	GSE21122	2.05	0.001
Normal fat vs Liposarcoma (pleomorphic)	GSE21122	1.92	0.002
Lipoma vs Liposarcoma (myxoid and round cell)	GSE6481	1.96	0.001
STS Grade 1 vs STS Grade 3	GSE12972	1.78	0.003

C STS: Correlations of the expression levels (ρ) between the MEF2 signature and the indicated genes

TUMOR	REF.	ACCESSION	N. SAMPLES	PTEN ρ	P	HDAC4 ρ	P
Liposarcoma	[40]	GSE21122	89	0.48	0.0001	-0.10	0.33
Liposarcoma	[41]	GSE23980	84	0.44	0.001	-0.32	0.046
Leiomyosarcoma	[40]	GSE21122	25	0.38	0.05	-0.30	0.13
Leiomyosarcoma	[41]	GSE23980	57	0.48	0.0002	-0.21	0.12

D PTEN clustered STS: Correlations of the expression levels (ρ) between the MEF2 signature and HDAC4

TUMOR	REF.	ACCESSION	N. SAMPLES	PTEN levels	HDAC4 ρ	P
Liposarcoma	[40]	GSE21122	61	<-0.5	-0.05	0.7
Liposarcoma	[40]	GSE23980	52	<-0.5	-0.28	0.086
Leiomyosarcoma	[40]	GSE21122	17	<-0.5	-0.19	0.44
Leiomyosarcoma	[41]	GSE23980	45	<-0.5	-0.11	0.46
Liposarcoma	[40]	GSE21122	28	>-0.5	-0.56	0.002
Liposarcoma	[41]	GSE23980	32	>-0.5	-0.49	0.006
Leiomyosarcoma	[40]	GSE21122	8	>-0.5	-0.54	0.06
Leiomyosarcoma	[41]	GSE23980	13	>-0.5	-0.68	0.02

to HDAC4/TM cells. In conclusion, these results further support the idea that the repression of MEF2 transcription is essential for HDAC4 transforming activity.

A signature of 25 MEF2 target genes repressed by HDAC4 in NIH 3T3 cells is significantly repressed in human in STS. Data collected thus far suggest that dysfunctions of the MEF2-HDAC4 axis could play a role in tumorigenesis. As a first step for understanding our discovery in the context of human tumors, we decided to explore whether the expression of 25 genes (see Fig. S1A and B in the supplemental material), containing MEF2-binding sites in the proximal promoters and whose expression was repressed by HDAC4, is also repressed in human cancers. The transcriptomes of 14 tumor types coming from 40 DNA microarray GEO data sets were interrogated with this signature. This analysis allowed us to discover that the MEF2 signature was significantly repressed in soft tissue sarcoma (STS), gastric cancer, lymphoblastic leukemia, and metastatic melanoma (Fig. 9A). In particular, STSs turned out to be the tumors scoring the strongest repression of these 25 genes. The downregulation of this MEF2 signature in STSs was also confirmed by means of GSEA. The MEF2 signature resulted significantly enriched in normal tissues compared to tumors, and its repression parallels the progression of tumor malignancy (Fig. 9B).

To further portray the association between STSs and the MEF2 signature, we applied a statistical analysis to determine the correlation values, in terms of expression levels, between the 25 MEF2 targets and genes influencing their expression, including MEF2s, class IIa HDACs, and PTEN, using two data sets (40, 41). Among the MEF2 family members, MEF2C shows the highest expression in STSs. As expected, the MEF2C levels correlate with the levels of the 25 MEF2 target genes ($\rho = 0.35$; $P < 0.05$). This result implies that although MEF2 downregulation can contribute to the repression of MEF2 target gene in STSs, additional mechanisms also exists. In both studies, PTEN was generally repressed, and its repression correlated well with the levels of the 25 MEF2 target genes (Fig. 9C).

In STSs HDAC4 evidenced a heterogeneous pattern of expression (see Fig. S1C in the supplemental material and also Fig. 9D). With the exclusion of the liposarcomas from the Gibault study (Fig. 9C), we failed to observe a significant inverse correlation between MEF2 target genes and HDAC4 expression. Interestingly, when STSs were clustered into two groups based on the level of PTEN expression: negative (< -0.5) or residual (≥ -0.5), HDAC4 levels negatively correlated with the MEF2 signature only in tumors displaying residual PTEN expression (Fig. 9D). The expression of other class IIa members did not correlate with the repression of the 25 MEF2 target genes (data not shown).

Immunohistochemical analysis of a series of 26 human primary leiomyosarcomas revealed a diffuse/pan, although weak reactivity for anti-HDAC4 antibody in 12 cases (Fig. 9E2) and an intense diffuse signal in 5 cases, with prominent nuclear accumu-

lation in 2 cases (Fig. 9E3). Immunohistochemical data are summarized in Fig. S2 in the supplemental material. This result is in line with our *in silico* predictions, evoking a contribution of HDAC4 to the repression of MEF2 transcription only in a subgroup of STSs.

MEF2 negatively impacts on the proliferation of human sarcoma cells. Having discovered a correlation between MEF2 transcriptional activity and STSs, we examined the contribution of MEF2 to the tumorigenic phenotype of a panel of human leiomyosarcoma cell lines (LMS) (42). We initially verified whether, similarly to NIH 3T3 cells, MEF2D and MEF2C levels are under the control of the PI3K/Akt pathway. Experiments with LY and MG123, alone or in combination, indicated that in LMS cells the PI3K/Akt pathway also controls MEF2C and MEF2D protein stability via the proteasome (Fig. 10A). Next, we engineered LMS cells with the MEF2-VP16-ER chimera for inducible MEF2-dependent transcription. The induction of MEF2 was sufficient to reduce the proliferation (Fig. 10B), as well as the anchorage-independent growth, of LMS cell lines (Fig. 10C). Overall, these results support the hypothesis of MEF2 as a tumor suppressor in STSs.

Pharmacological cotargeting of the PI3K/Akt pathway and of the MEF2-HDAC axis in sarcoma cells. To additionally prove the independent and synergistic action of HDAC4 and of the PI3K/Akt pathway on MEF2s and to evaluate the therapeutic perspective of our discovery, we used LY in conjunction with BML-210, a recently defined inhibitor of the interaction between class IIa HDACs and MEF2s (17).

BML-210 discharges the binding between HDAC4 and MEF2D (Fig. 11A) and MEF2 transcriptional activity is augmented in the presence of BML-210 (Fig. 11B). Moreover, both BML-210 and LY inhibit the proliferation of LMS cell lines and, most importantly, the combination of the two drugs shows additive effects in terms of the suppression of proliferation (Fig. 11C), which stems from a delayed cell cycle progression, as shown in Fig. 11D. Moreover, the transcription of the MEF2 target genes *KLF2*, *NR4A1*, and *RHOB* was in general augmented in LMS cells when grown in the presence of both drugs compared to single treatments (Fig. 11E).

DISCUSSION

This study provides unprecedented and compelling evidence of the tumorigenic potential of the MEF2-HDAC axis. Murine fibroblasts engineered to express nuclear active HDAC4 gain a transformed phenotype, including elongated morphology, loss of contact inhibition, anchorage-independent growth, and tumorigenicity in a xenograft assay. Cell transformation as elicited by HDAC4 is accompanied by the repression of a limited number of genes, including several transcription factors. The selective influence of HDAC4 on the transcription of important regulatory nodes can explain the dramatic shift in the proliferative attitude of the cells.

FIG 9 Expression of the MEF2 target genes in human tumors. (A) Box plots depicted in light-blue mark tumors where the MEF2 signature is significantly below zero and with at least the 50% of the values below an arbitrary threshold of -0.5 . Significance was calculated by using the Poisson test (Holm-Bonferroni correction, $P < 0.05$). (B) GSEA on STSs, using the MEF2 signature as a gene set. (C) Expression level correlations between the MEF2 signature and HDAC4 or PTEN in three different types of STSs. Statistically significant correlations are indicated in green, whereas statistically significant inverse correlations are indicated in red. (D) Expression level correlations between MEF2 signature and HDAC4 in different types of STS subdivided into two subclasses according to the expression of PTEN. $\log_2(\text{PTEN})$ of -0.5 was selected as the cutoff to identify the two populations. Statistically significant inverse correlations are shown in red. (E) Immunohistochemical analysis of HDAC4 expression in leiomyosarcoma. HDAC4 showed absent E1 (few positive inflammatory cells are present), low pan/cytoplasmic expression E2, or increased expression and nuclear localization E3.

Although MEF2s are important partners of HDAC4, the literature provide a long list of other proteins able to complex with HDAC4 (18). Hence, whether the dysregulation of the MEF2-HDAC4 axis is accountable for the oncogenic behavior cannot be automatically evoked. Our results indicate that repression of the MEF2 genetic program is crucial for the HDAC4 transforming action. First, most (70%) of the genes repressed by HDAC4 are putative MEF2 targets. Second, the restoration of the MEF2-dependent transcription in cells expressing nuclear HDAC4 reverts the transformed phenotype. Third, loss of MEF2 binding abrogates the transforming capability of nuclear active HDAC4.

The oncogenic potential appears to be shared among class IIa members. An HDAC7 mutant defective for 14-3-3 binding was also able to transform NIH 3T3 cells. Of note, a mutation in serine 155 of HDAC7, a binding site for 14-3-3 proteins, has been recently described in non-Hodgkin lymphoma (43). Although overexpression of the WT forms of HDAC4 and HDAC7 was insufficient for a robust transformation, we cannot exclude that *in vivo* an increase of class IIa HDACs levels might have an impact on MEF2 and on tumor development (11, 24, 44).

Genes identified as targets of the MEF2-HDAC4 axis can also be repressed by PI3K/Akt signaling. HDAC4 and the PI3K/Akt pathway repress MEF2 transcription through independent routes. Although HDAC4 binds MEF2s and possibly generates a repressed state on chromatin (1), the PI3K/Akt signaling promotes polyubiquitination and proteasome-mediated degradation of MEF2s. Previous studies have reported that, in the context of muscle differentiation, the PI3K/Akt pathway could enhance MEF2 transcriptional activity (45, 46). However, the mechanism engaged by Akt is debated, and there are evidences contrasting with the idea of Akt as an activator of MEF2 (47). Most importantly, the positive influence of the PI3K/Akt pathway on MEF2s was not confirmed in cancer cells (45). Analogous to our findings, the phosphorylation-dependent degradation of MEF2C has been previously reported (48).

The subset of MEF2 targets repressed by HDAC4 turned out to be significantly repressed in certain tumors, particularly in STSs, which share with NIH 3T3 the mesenchymal origin, highlighting the relevance of the cell context in HDAC4/MEF2-mediated phenotypes (19). Importantly, reactivation of MEF2 transcription in PI3K-transformed cells and also in human sarcoma cell lines was sufficient to reduce proliferation and to impact on anchorage-independent growth.

In STSs, repression of the MEF2 targets mainly correlates with the downregulation of PTEN, the negative regulator of the PI3K/Akt pathway. Intriguingly, in tumors that retain partial PTEN expression, MEF2 targets are still repressed. In these cases, repression inversely correlates with HDAC4 levels. This suggests that PTEN loss and HDAC4 overexpression could represent two alternative mechanisms for suppressing the MEF2 genetic program in STSs.

FIG 10 Regulation and functions of MEF2s in human sarcoma cells. (A) Immunoblot analysis of MEF2C and MEF2D levels in human sarcoma cell lines treated or not with LY. Cellular lysates were generated and subjected to immunoblot analysis with the indicated antibodies. (B) Human sarcoma cells expressing MEF2-VP16-ER or MEF2ΔDBD-VP16-ER were grown in DMEM supplemented with 10% FBS. The day after seeding, 4-OHT was added to culture medium. (C) Quantitative results of colony formation in soft agar of human sarcoma cells expressing MEF2-VP16-ER or MEF2ΔDBD-VP16-ER. The day after seeding, 4-OHT was added to culture medium. ***, $P < 0.001$.

FIG 11 Pharmacological targeting of MEF2-HDAC axis and PI3K/Akt pathway. (A) Cellular lysates from IMR90-E1A cells treated or not for 36 h with BML-210 were immunoprecipitated with an anti-HDAC4 antibody. Immunoblots were performed with the anti-MEF2D and anti-HDAC4 antibodies. (B) IMR90-E1A cells were transfected as described in Fig. 5A. After 12 h, the cells were treated or not with BML-210 for 36 h. (C) Human sarcoma cells were seeded in 96-well and treated for 48 h with LY and/or BML-210. The proliferative rate was scored by using a resazurin assay. (D) Doubling time (DB) of human sarcoma cells (5×10^4) treated as in panel C. The DB was calculated according to the following formula: $DB = (t_2 - t_1) \cdot [\log_2 / \log(q_2/q_1)]$, where t_2 is time 2, t_1 is time 1, q_1 is the number cells at t_1 , and q_2 is the number of cells at t_2 . (E) mRNA expression levels of selected MEF2-HDAC4 target genes and *Gapdh*, as a control, were measured using qRT-PCR in human sarcoma cells treated for 36 h as in panel C. (F) Model representing the two different actions of PI3K/Akt signaling and of HDAC4 on MEF2-dependent transcription. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The independent action of HDAC4 and of PI3K/Akt on MEF2 was confirmed also by the use of selective inhibitors. Blocking the PI3K/Akt pathway and impeding the interaction between MEF2 and class IIa HDACs produced additive effects on the transcription of MEF2 target genes and much strongly suppressed proliferation in sarcoma cell lines. This observation highlights the importance of targeting both pathways for the development of more efficient therapies for the treatment of STS.

In conclusion, our work suggests a model (Fig. 11F) wherein MEF2 is a converging hub for the transformation promoted by different oncogenic pathways. In this context, MEF2s behave as tumor suppressors, which suggests that the restoration of MEF2 activity could be exploited as a novel therapeutic avenue.

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